

Frequent and seasonally variable sublethal anthrax infections are accompanied by short-lived immunity in an endemic system

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Summary

1. Few studies have examined host-pathogen interactions in wildlife from an immunological perspective, particularly in the context of seasonal and longitudinal dynamics. In addition, though most ecological immunology studies employ serological antibody assays, endpoint titre determination is usually based on subjective criteria and needs to be made more objective.

2. Despite the fact that anthrax is an ancient and emerging zoonotic infectious disease found world-wide, its natural ecology is not well understood. In particular, little is known about the adaptive immune responses of wild herbivore hosts against *Bacillus anthracis*.

3. Working in the natural anthrax system of Etosha National Park, Namibia, we collected 154 serum samples from plains zebra (*Equus quagga*), 21 from springbok (*Antidorcas marsupialis*) and 45 from African elephants (*Loxodonta africana*) over 2–3 years, resampling individuals when possible for seasonal and longitudinal comparisons. We used enzyme-linked immunosorbent assays to measure anti-anthrax antibody titres and developed three increasingly conservative models to determine endpoint titres with more rigorous, objective mensuration.

4. Between 52 and 87% of zebra, 0–15% of springbok and 3–52% of elephants had measurable anti-anthrax antibody titres, depending on the model used. While the ability of elephants and springbok to mount anti-anthrax adaptive immune responses is still equivocal, our results indicate that zebra in ENP often survive sublethal anthrax infections, encounter most *B. anthracis* in the wet season and can partially booster their immunity to *B. anthracis*.

5. Thus, rather than being solely a lethal disease, anthrax often occurs as a sublethal infection in some susceptible hosts. Though we found that adaptive immunity to anthrax wanes rapidly, subsequent and frequent sublethal *B. anthracis* infections cause maturation of anti-anthrax immunity. By triggering host immune responses, these common sublethal infections may act as immunomodulators and affect population dynamics through indirect immunological and co-infection effects.

6. In addition, with our three endpoint titre models, we introduce more mensuration rigour into serological antibody assays, even under the often-restrictive conditions that come with adapting laboratory immunology methods to wild systems. With these methods, we identified significantly more zebras responding immunologically to anthrax than have previous studies using less comprehensive titre analyses.

Key-words: antibody persistence, bacteria, ecological immunology, ELISA, endoparasites, host-parasite interactions, microbiology, microparasites, seroconversion dynamics, serology

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Introduction

Anthrax, a zoonotic bacterial disease, occurs nearly world-wide and threatens human health, biodiversity, economics and agricultural security (Turnbull 2008). While the disease primarily affects herbivores, humans and some carnivores are also susceptible (Turnbull *et al.* 2004). The disease has been known since antiquity, but it has been increasingly reported from new areas, and evidence suggests that outbreaks are increasing in frequency and severity (Clegg 2006; Wafula, Atimedi & Charles 2007) and are spreading to locations and populations previously free of the disease (Clegg *et al.* 2007; Muoria *et al.* 2007). Managing such outbreaks has become a global priority and requires filling in the fundamental gaps in our understanding of natural anthrax ecology. Our research aims to shed light on these ecological patterns from the perspective of host susceptibility.

Etosha National Park (ENP) in Namibia (Fig. 1) experiences regular anthrax outbreaks that were first documented in 1964 (Ebedes 1976) and that have been recorded since 1968 by the Etosha Ecological Institute (EEI) (Turner *et al.* 2013). ENP's open habitats are dominated by plains ungulates, three of which, plains zebra (*Equus quagga*), springbok (*Antidorcas marsupialis*) and blue wildebeest (*Connochaetes taurinus*), experience yearly anthrax outbreaks; these three species are responsible for 97% of anthrax deaths in the park, despite making up only 55% of the park's population of large herbivores (Lindeque & Turnbull 1994). Although sporadic cases of anthrax occur year-round in ENP, outbreaks primarily occur in plains ungulates at the end of the wet season (Fig. 2) (Lindeque 1991), unlike in other systems (e.g. Gainer & Saunders 1989; Hampson *et al.* 2011). Anthrax in ENP affects both sexes similarly, even when adjusted

for population sex ratios (Lindeque 1991; Z. Havarua, unpublished data), and does not appear to involve invertebrate mechanical vectors (Lindeque 1991). Elephants (*Loxodonta africana*) also experience anthrax infections in the park, with increased incidence at the beginning of the wet season (Lindeque 1991) (Fig. 2).

Anthrax is caused by *Bacillus anthracis*, a large, gram-positive bacterium that undergoes vegetative reproduction in infected hosts but otherwise exists in a very hardy, long-lived, infectious spore form in the environment. Most evidence suggests that anthrax can only be transmitted environmentally through the spore form (Hanna & Ireland 1999), usually via ingestion of a large spore dose (Watson & Keir 1994). Within hosts, spores germinate into fast-multiplying vegetative forms that produce three soluble toxic factors, including protective antigen (PA) that complexes with the other two factors and allows them to enter host cells (Mogridge, Cunningham & Collier 2002). The collective actions of these toxins result in the peracute to acute death of susceptible hosts with an overwhelming septicaemia of up to 10^9 bacterial cells per millilitre of blood (Collier & Young 2003).

Humoral immunity, particularly against the PA toxin, plays a very important role in a host's fight against anthrax; several studies have demonstrated that the magnitude of a host's anti-PA IgG antibody titre is correlated with level of protection against the disease (Reuveny *et al.* 2001; Marcus *et al.* 2004). Most carnivores that regularly ingest anthrax carcasses mount strong adaptive immune responses to anthrax that can be naturally boosted with frequent pathogen contact (Turnbull *et al.* 1992a,b; Lembo *et al.* 2011; Bellan *et al.* 2012). Herbivores, however, while capable of forming anti-PA antibodies with vaccination, often require rigorous vaccination booster schedules to counter their initial low immune responses

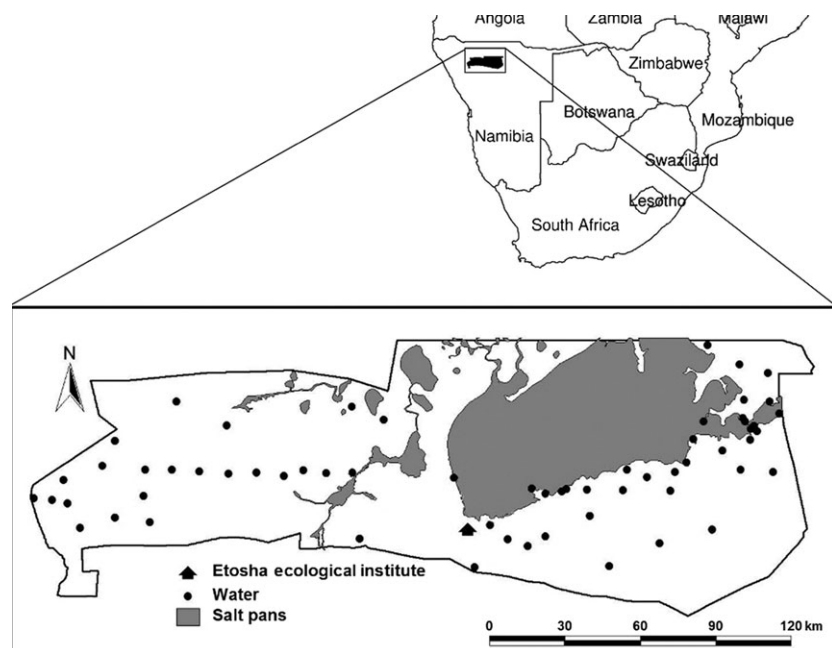


Fig. 1. Etosha National Park in northern Namibia. Most animal sampling occurred in the plains within a 20–60-km radius from the Etosha Ecological Institute. Perennial watering points are marked with black circles.

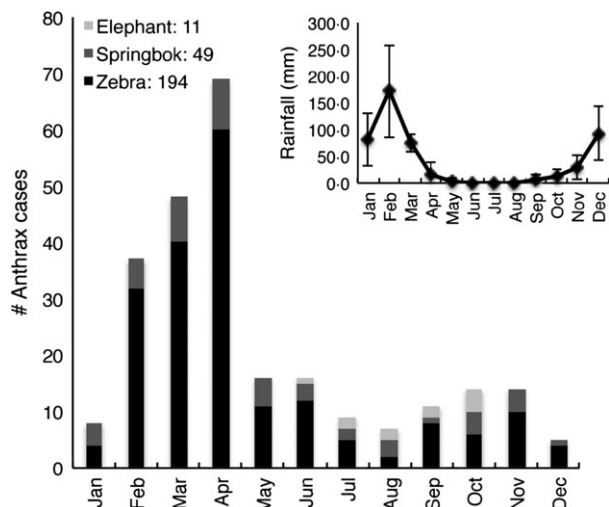


Fig. 2. Total culture-confirmed anthrax cases for elephant, springbok and plains zebra by month for 2008–2010 (years of this study). The graph insert indicates mean monthly rainfall \pm standard deviation in the Okaukuejo region for this same time period.

(Turnbull 2008). The few previous studies of natural immunity against anthrax in wildlife and livestock have indicated that, with the exception of some bovids, many wild herbivores may not develop and maintain measurable anti-anthrax antibody titres at all (Turnbull *et al.* 1992a,b; Turnbull *et al.* 2001; Lembo *et al.* 2011). As measuring anti-PA titres is the most diagnostically reliable way to determine prior exposure to sublethal anthrax infection (Turnbull *et al.* 1992a,b), these studies are interpreted as implying that most herbivores in these endemic disease systems are anthrax naïve and, on exposure to a large dose of *B. anthracis*, contract fulminant infection and die (Watson & Keir 1994).

Given the longevity of the infectious spore form and the lack of definitive evidence for epidemiologically significant vegetation reproduction of *B. anthracis* outside of a vertebrate host (Hanna & Ireland 1999; but see Saile & Koehler 2006 and Dey, Hoffman & Glomski 2012), it remains unclear why sporadic or cyclic outbreaks of the disease should occur rather than a more constant incidence of anthrax cases. Given the endemic nature of anthrax in ENP and the fact that anthrax deaths do occur throughout the year in this system, it appears that animals can come into contact with *B. anthracis* spores in all seasons. This then raises the questions of whether exposure varies with season, host susceptibility changes with season or hosts are able to survive some anthrax infections. We were thus motivated to more closely examine the immune dynamics of anthrax in the wild, using anti-PA antibody titres both to gauge anti-anthrax immune responsiveness in plains zebra, African elephants and springbok in ENP, and as a signature of the incidence of sublethal anthrax infections in this system.

Additionally, we approached the problem of assessing antibody titres using a common immunology assay

protocol (enzyme-linked immunosorbent assay, ELISA) in a more comprehensive and objective way. Serology via ELISA, the 'bread and butter' of ecological immunology studies, can be used to characterize the prevalence of infectious agents in wildlife systems, as well as to measure host immune function. ELISA methods, however, while long established in laboratory studies, are often not as straightforward in wildlife research: positive controls, quantitatively titrated or not, are often impossible to come by; often only a few negative controls can be obtained from zoo collections; and determination of end-point titres is usually not quantitative, with methods for determining titres varying greatly in their subjectivity, sensitivity, specificity and statistical rigour. We have attempted with our present study to address these concerns.

Methods

STUDY AREA AND SPECIES

This study was conducted in Etosha National Park (ENP), a 22 915 km² fenced conservation area in northern Namibia, located between 18°30'S–19°30'S and 14°15'E–17°10'E (Fig. 1). Rainfall in ENP is highly seasonal: the rainy season lasts from November through April, with the greatest rainfall occurring during January and February (Gasaway, Gasaway & Berry 1996; Auer 1997) (Fig. 2). The only perennial water available to the park's wildlife is found in man-made boreholes, or in natural artesian or contact springs (Auer 1997).

Zebra and springbok are the two most abundant plains ungulate species in ENP, with populations of *c.* 13 000 (95% CI rounded to nearest 100: 10 900–15 000) and *c.* 15 600 (95% CI rounded: 13 200–17 900). Elephants have a population of *c.* 2600 (95% CI rounded: 1900–3300) (EEI unpublished data 2005).

ANIMAL CAPTURE AND SAMPLING

Between 2008 and 2010, samples were obtained from all three study species. With a capture team, we immobilized animals through the use of reversible anaesthetics injected remotely via Pneu-Darts (Pneu-Dart Inc., Williamsport, PA, USA). Animals were fitted with VHF (very high frequency) tracking collars (LoxoTrack, Aeroeskoebing, Denmark) or VHF-GPS/GSM (global positioning system/global system for mobile communications) collars (Africa Wildlife Tracking, Pretoria, Republic of South Africa) during the first immobilization to enable resampling of animals over several seasons. Anaesthesia was reversed immediately upon collection of all samples. Animals were first immobilized and sampled on the plains within an *c.* 20 km radius of Okaukuejo (60 km for elephants) (Fig. 1); in subsequent seasons, zebra were sampled between Okaukuejo and 100 km to the east in Halali, whereas springbok and elephants were sampled again in the original area. Only adult animals were sampled for all species. We lost two zebra to predation over the course of our study (both tested negative for anthrax), with no other animal deaths. All animals were safely handled under animal handling protocol AUP R217-0509B (University of California, Berkeley).

A total of 154 serum samples were collected from zebra (10 males, 144 females), 44 from elephants (24 males, 20 females) and 21 from springbok (12 males, nine females). Serum was kept at -20°C for up to 6 months and was thereafter stored at -80°C until analysed. Zebra were sampled over five seasons total, two wet and three dry, with several individuals sampled between two and five times total (Table 1). Springbok were sampled over two seasons, one wet and one dry, and elephants were sampled over four seasons, one wet and three dry, with several animals resampled once (Table 1).

ANTI-PA ELISAS

We adapted the ELISA procedure to measure serum anti-PA antibody titres from Pitt *et al.* 2001; Marcus *et al.* 2004; and Turnbull *et al.* 2008. Each well of a 96-well ELISA plate was coated with PA at a concentration of $0.375\ \mu\text{L}$. Wells were blocked with a mixture of PBS, $0.5\ \text{mL L}^{-1}$ Tween-20, and 10% (w/v) skim milk powder (Oxoid Laboratory Preparations; Basingstoke, Hampshire, UK) (PBSTM).

Serial, twofold dilutions were made (1 : 4–1 : 8192) to the ends of rows in duplicate for all samples and negative controls in PBSTM. We used goat anti-horse IgG heavy and light chain horseradish peroxidase (HRP) conjugate for zebra and rabbit anti-goat IgG heavy and light chain HRP conjugate for springbok (Bethyl Laboratories, Montgomery, TX, USA), similar to methods of previous studies (Turnbull *et al.* 2004, 2008). As there are no domestic species closely related to elephants, Bethyl Laboratories manufactured goat anti-elephant IgG heavy and light chain secondary antibodies for this study. TMB substrate (Kirkegaard & Perry Laboratories; Gaithersburg, MD, USA) was added, and the reaction was stopped after 30 min with 2N sulphuric acid. Well absorbance was read as optical density (OD) at 450 nm on a SpectraMax M2 Microplate Reader using SOFTMAX PRO software v5.3 (Molecular Devices; Sunnyvale, CA, USA).

We obtained six serum samples from plains zebra from the Woodland Park Zoo (Seattle, Washington) and four serum samples from African elephants and seven serum samples from domestic goats (as the phylogenetically closest available proxies for springbok) from the Oakland Zoo (Oakland, CA, USA) for use as negative controls. We discarded three goat samples from

further analysis due to extreme haemolysis in these samples. While the number of negative controls we obtained is small, it is not uncommon for ELISA studies to use one only negative control sample (e.g. Work *et al.* 2000; Reuveny *et al.* 2001), to simply use background absorbance values as control values rather than a dedicated negative control (e.g. Aloni-Grinstein *et al.* 2005), or to use no obvious negative controls at all (e.g. Turnbull *et al.* 1992a,b; Svensson, Sinervo & Comendant 2001). The highest reported number of negative control samples that we have found in previous studies was five (Schwanz *et al.* 2011) and thus our control numbers are reasonable and representative of the typical availability of negative control samples for both laboratory and wildlife work.

Using the preceding ELISA procedure, negative controls were first analysed individually in duplicate, twofold serial dilution titration series (1 : 4–1 : 8192). We chose a middle-responding control zebra as the negative control for all subsequent zebra sample ELISAs; this control essentially represented the mean of the negative control samples. We also accounted for variation between negative controls in subsequent analyses (see Rule 3 below). While all of the elephant negative controls and all goat negative controls were also not significantly different from each other in their respective analyses, we combined all elephant controls and all goat controls in equal parts into separate, mixed samples to be used as subsequent master negative controls; doing so, rather than choosing one random control, is likely more effective for precluding questions about the choice of control comparators.

ELISA ENDPOINT TITRE DETERMINATION

Previous ecological immunology and anthrax serology studies have used a variety of methods for determining ELISA endpoint titres, with varying degrees of subjectivity. While some studies benefit from the availability of quantitative kits for the antibodies in question, most involve the development of novel, more qualitative assays using available materials. Some studies have had access to positive controls and have used these to determine sample endpoint titres in varying ways. However, we were unable to obtain positive controls from vaccinated animals for our assays – a common limitation in wildlife disease immunology

Table 1. Capture seasons and timing for zebra, springbok and elephant. '#New': new individuals and their samples; '#Re-S': animals resampled at least once in that season. #Re-S x2, 3, 4 or 5 are the total numbers of animals for each species resampled 2, 3, 4 or 5 times

Species	Capture Season	Nominal Season	Date (month/year)	#New	# Re-S	# Re-S x2	# Re-S x3	# Re-S x4	# Re-S x5
Zebra	S1	Wet	3-4/08	45	0				
	S2	Dry	10-11/08	14	22				
	S3	Wet	4-5/09	6	29				
	S4	Dry	9-11/09	4	9				
	S5	Dry	8/10	0	25				
	Totals	5			69	85	20	11	12
Springbok	S1	Dry	8-9/09	13	0				
	S2	Wet	4-5/10	0	8				
	Totals	2		13	8	8	0	0	0
Elephant	S1	Dry	10/08	10	0				
	S2	Dry	7-10/08	18	0				
	S3	Wet	3/10	1	1				
	S4	Dry	8/10	5	11				
	Totals	4			34	12	12	0	0

studies – and therefore developed novel endpoint titre determination protocols based solely on more readily available negative control samples from zoo animals.

Endpoint titre determination using only negative controls as comparisons has been fraught with subjectivity with methods varying widely among published research. Studies have determined positive titres in a variety of ways, for example by using subjective, pre-determined OD cut-offs (e.g. Chabot *et al.* 2004); by using two- or threefold differences above negative control or background ODs as cut-offs (e.g. Aloni-Grinstein *et al.* 2005); by determining positive samples as those above mean negative control OD plus two or three standard deviations (e.g. Reuveny *et al.* 2001); or by comparing samples with competitively inhibited titrations and using a pre-determined comparative cut-off (e.g. Turnbull *et al.* 2008). Frey, Di Canzio and Zurakowski (1998) addressed some of these problems by establishing a method for determining ELISA endpoint titres based on the *t*-distribution of negative control readings. Their method increased sensitivity for detecting weak immune responses and established good statistical power even with relatively low numbers (5–30) of negative controls. We expand on these methods here to make them even more relevant to ecological immunology studies, in which there are often much larger amounts of variation than in laboratory studies, even amongst negative controls.

We addressed the following issues:

- 1 Even when different ELISA plates are run concurrently, there is often great enough inter-plate variability that differences between low responders and negative controls may be lost against background variation. This was addressed by running a negative control sample on every plate.
- 2 The degree of binding in ELISA may vary substantially across a range of sera dilutions.
- 3 There can be high background noise particularly when crude antigens are used to coat ELISA plates (Frey, Di Canzio & Zurakowski 1998). Issues 2 and 3 were addressed by running the negative control, in replicate on each plate, in the same serial dilution titration series as is used for the experimental samples; while Frey *et al.* suggested this step be tried in a pilot experiment, we believe that the background noise and variability in ecological samples make it imperative that this step be included at all times, on all plates.
- 4 Negative control sera may only be available in very small amounts.
- 5 Plate ‘real estate’ is limited, and reagents may be as well. Issues 4 and 5 were addressed by examining the titrated response curves of all negative controls (in duplicate) prior to the experimental assays and then either by combining the low-to-middle-responding negative controls into a 1 : 1 pooled negative control mixture or by choosing one middle-responding abundant sample at random for use as the negative control for all subsequent plates. This required only two rows of space out of eight on a 96-well plate.

We then established three rules for endpoint titre determination:

Rule 1: Liberal cut-off. The titre is the highest dilution in the titration series for which the mean sample OD for that dilution is greater than the mean negative control OD titration curve from all plates. This is similar to some cut-off rules used in previous studies (e.g. Reuveny *et al.* 2001; Aloni-Grinstein *et al.* 2005), though is likely more sensitive, but

potentially less specific, than rules using mean negative control OD ± 2 or 3SD and improves by capturing the ‘moving target’ of dilution dynamics.

Rule 2: Medium-conservative cut-off. The within-plate inter-duplicate standard deviation is estimated as:

$$\hat{\sigma}_{\text{dup},d} = \sqrt{\frac{\sum_i \left(\frac{r_{d,i,1} - r_{d,i,2}}{2}\right)^2}{n-1}}$$

where $d = 1 : 2^2, 1 : 2^3, \dots, 1 : 2^{13}$; $r_{d,i,1}$ and $r_{d,i,2}$ are the duplicate optical densities of the i -th sample at dilution d ; and n is the number of samples (indexed by i). The endpoint titre is the last dilution at which

$$\overline{OD}_{s,d} > \overline{OD}_{nc,d} + 1.96 \times \hat{\sigma}_{\text{dup},d}$$

where $\overline{OD}_{s,d}$ is the mean sample OD at a dilution and $\overline{OD}_{nc,d}$ is the mean negative control OD at a dilution buffered by a 95% confidence interval determined by the inter-duplicate error at that dilution, across all samples. We recommend doing these calculations after all experimental samples have been analysed, and recalculating all titres if additional samples are run.

Rule 3: Ultra-conservative cut-off. The inter-negative control standard deviation is estimated as:

$$\hat{\sigma}_{\text{ncnt},d} = \sqrt{\frac{\sum_{i=1}^n (r_{d,i} - \bar{r}_d)^2}{n-1}}$$

where d is as above, $r_{d,1}, \dots, r_{d,n}$ are the optical densities of each of the unique negative control animals run on the negative control-only plate; \bar{r}_d is the mean optical density at dilution d across all negative control samples run on the negative control-only plate; and n is the number of unique negative control animals run on the negative control-only plate (indexed by i). The endpoint titre is the last dilution at which

$$\overline{OD}_{s,d} > \overline{OD}_{nc,d} + 1.96 \times \hat{\sigma}_{\text{ncnt},d}$$

where $\overline{OD}_{s,d}$ is as above and $\overline{OD}_{nc,d}$ is the mean negative control OD at a dilution buffered by a 95% confidence interval determined by the inter-negative control error at that dilution, across all samples. This rule is usually more conservative than Rule 2 due to the fact that variation between ODs of different negative control samples is often greater than variation between ODs of the same sample run in duplicate.

TITRE PATTERNS WITH AGE AND SEASON

Generalized estimating equation (GEE) models were developed using R v2.15.2 and the *geepack* package (Højsgaard, Halekoh & Yan 2006; R Core Development Team 2012) to examine the influences of age, rainfall and capture area on anti-PA titres in resampled zebra. For all models, a working correlation matrix with a first-order autoregressive relationship was used because, while individual immune factors are likely correlated through time, these correlations should decrease between later time points and earlier samplings (Zuur *et al.* 2009).

In the Seasonal model, the presence or absence of an anti-PA titre via Rule 2 was included as the dependent variable, and capture area (Okaukuejo or Halali), animal age in days, and season

type (wet or dry) were included as independent variables. Age was determined to half a year (converted to days) by using the methods of Smuts 1974.

In the Rainfall model, the presence or absence of an anti-PA titre via Rule 2 was included as the dependent variable, and capture area, animal age in days, and cumulative rainfall 1 and 2 months prior to sampling were included as independent variables. This model was developed separately to avoid collinearity between season and rainfall parameters. Cumulative rainfall prior to sampling was determined by summing daily rainfall amounts (in mm) over the 30 or 60 days prior to each individual capture event. Previous ENP studies determined that gastrointestinal helminth infection intensity in zebras is significantly related to rainfall 1 and 2 months prior (Turner 2009). As parasite infection peaks occur only a few weeks prior to anthrax outbreaks in these hosts (W.C. Turner, unpublished data), these measures of rainfall were used here both as finer-scaled representations of season and as potential proxies for other seasonal effects on anthrax exposure. In both Seasonal and Rainfall models, the dependent outcome was anti-PA seropositivity, modelled as a Bernoulli outcome linked to independent variables via a logit link function.

The GEE models were developed by using a backwards stepwise refinement method based on comparing the quasi-likelihood under the independence model criterion (QIC) values between maximal models and models with variables removed (Pan 2001). The QIC is analogous to Akaike's information criterion (AIC) for GEEs, which are not strictly likelihood-based. After stepwise selection of the main terms, interaction terms between the remaining explanatory variables were added, and models were further subjected to backwards stepwise selection. Models were validated by plotting Pearson's residuals against fitted values to look for residual patterns, examining residual histograms to assess the normality of error distributions, and plotting residuals against each explanatory variable to test for homogeneity of error variances (Zuur *et al.* 2009). For the final, best-fit models, Wald chi-squared tests were used to determine the significance of each estimated parameter.

Results

PREVALENCE OF ANTI-PA ANTIBODIES

Prevalence of anti-PA antibodies in the three study species varied with the ELISA endpoint titre rule used (Table 2; Figs 3 and 4). While few springbok and elephants showed positive titres for the more conservative rules, seropositivity prevalence among unique zebras (i.e. resampled

animals were included only once in the aggregate and determined to be positive if they exhibited positive titres at least once) ranged from 52 to 87% across the three endpoint determination rules (Table 2). Due to the lower apparent sensitivity with Rule 1, and because Rule 2 appeared to be sensitive enough to capture a large number of positive zebra titres in our assay while still employing a conservative assessment rubric, we used zebra titres generated with Rule 2 calculations for further analyses.

Prevalences of anti-PA antibodies in unique springbok ranged from 0 to 15% (Table 2), with Rule 1 yielding 1.5 times as many seropositive animals as Rule 2. Prevalence of anti-PA seropositivity in unique elephants ranged from 3 to 52% (Table 2). Given the presence of some seropositive animals via Rule 2, we are confident that at least some of the positive animals via Rule 1 were true positives. While understanding that Rule 1 may also be less specific, we focus further elephant titre analyses on Rule 1 results to allow greater resolution of our examinations of longitudinal titre changes. While we could not compare sex ratios of animals with positive titres for zebra and springbok due to small sample sizes (too few zebra males; too few seropositive springbok), we compared prevalence and magnitude of anti-PA titres in elephant bulls and cows. Prevalence in males (47.8%) and females (47.6%) did not differ, nor did the magnitude of titres between the sexes (Wilcoxon $U = 90$, $N_1 = 11$, $N_2 = 10$, $P = 0.139$).

CHANGES IN ANTI-PA TITRES OVER TIME

Eleven resampled zebra seroconverted positively (i.e. changed from having no titre to having a measurable titre) and 13 seroconverted negatively (i.e. changed from having a measurable titre to having no measurable titre) during the course of our study (Fig. 5). For zebra sampled four or five times over the 2-year study period, we found that few animals maintained relatively stable titres over several seasons, with the majority of titres increasing or decreasing substantially within each sampling interval (Fig. 5). As our sampling intervals were coarse (between 3 and 8 months for individual recaptures), we were not able to determine exactly when a negatively seroconverting animal lost its measurable titre. However, because both measurable titre increases and decreases occurred frequently

Table 2. Prevalence (%) of antibodies against *Bacillus anthracis* PA toxin in zebra, springbok and elephant as determined by three endpoint titre rules. 'All': all samples tested from that species, including resampled individuals. 'Unique': resampled animals were included only once in the aggregate and determined to be positive if they exhibited positive titres at least once. Numbers in parentheses: number seropositive

Species	N		Rule 1		Rule 2		Rule 3	
	All	Unique	All	Unique	All	Unique	All	Unique
Zebra	154	69	91.6 (141)	86.9 (60)	67.5 (104)	62.3 (43)	54.5 (84)	52.2 (36)
Springbok	21	13	14.3 (3)	15.4 (2)	9.5 (2)	7.7 (1)	0.0	0.0
Elephant	44	33	52.3 (23)	51.5 (17)	2.3 (1)	3.0 (1)	2.3 (1)	3.0 (1)

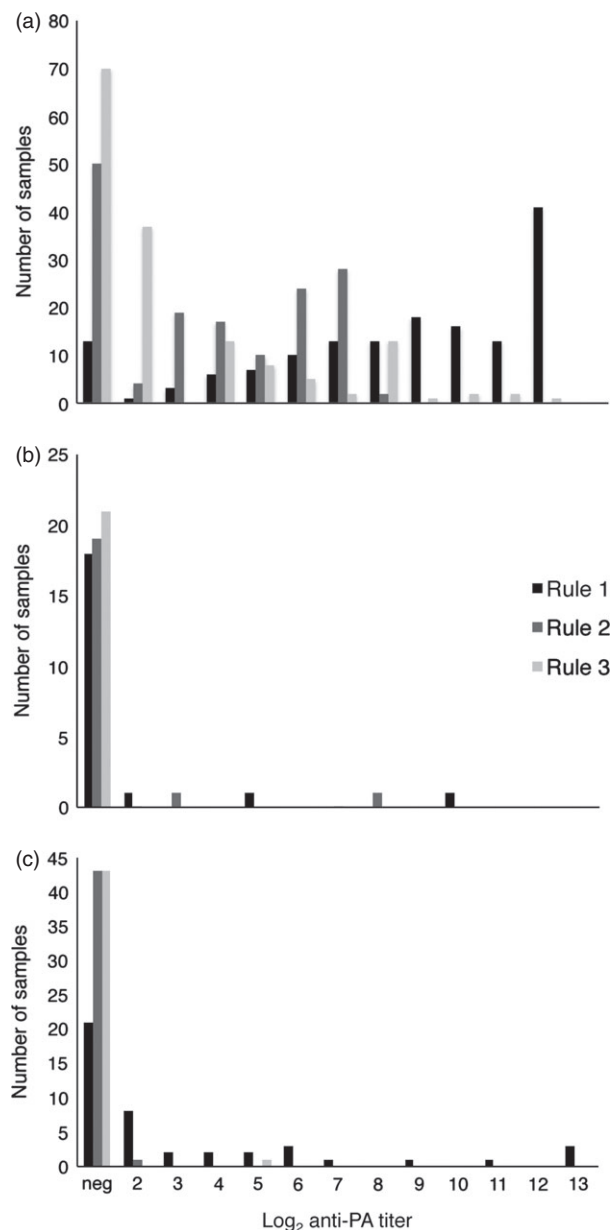


Fig. 3. Antibody endpoint titres to *Bacillus anthracis* PA toxin in (a) plains zebra, (b) springbok and (c) elephant, determined by each of the three titre cut-off rules. Titres are shown as \log_2 of the reciprocal of dilution cut-off for clarity.

and rapidly between our sampling intervals, we were confident that animals did not maintain titres for significantly longer than these time periods without additional *B. anthracis* exposure. Thus, examining animals that seroconverted negatively over the course of one sampling interval, we found that mean time to negative seroconversion was $< 6.3 \pm 1.3$ months (mean \pm SD; $N = 9$), with a range of < 3.5 – 7.4 month.

Of the three seropositive springbok via Rule 1, only one was resampled while also negatively seroconverting from one season to the next, sometime over the course of 7–8 month. Three elephants negatively seroconverted over

a mean time of $< 17.5 \pm 4.6$ month and range of 12.6–21.7 month. Given the low sample size of resampled springbok and elephants, the low number of sampling times and the long sampling intervals, these are obviously coarse estimates of negative seroconversion times for these species.

RELATIONSHIPS BETWEEN ANTI-PA TITRES, SEASON AND AGE IN ZEBRA

The best fitting Seasonal GEE model contained season and age, though only season was significant (odds of having a positive titre estimated to be 0.9998 smaller for every additional day of age, 95% CI (0.9995, 1.00003); Wald statistic = 0.76, $P = 0.382$); the wet season was significantly correlated with higher anti-PA seropositivity (odds ratio 2.31, 95% CI (1.66, 3.21); Wald statistic = 6.52, $P = 0.011$, Δ QIC = 2.00 between models with season and age and with only season), with a seropositivity of 76% and 58% in the wet and dry seasons, respectively (Fig. 6).

The best fitting Rainfall GEE model contained cumulative rainfall 1 month prior to capture, cumulative rainfall 2 months prior to capture (hereafter simply 'Rain1' and 'Rain2') and age. Rain1 was significantly, negatively associated with anti-PA titre (odds of having a positive vs. negative titre are estimated to be 0.964 smaller for every millimetre of extra Rain1, 95% CI (0.951, 0.977); Wald statistic = 7.90, $P = 0.005$), while Rain2 was significantly, positively associated with anti-PA titre (odds ratio 1.016, 95% CI (1.011, 1.022); Wald statistic = 9.53, $P = 0.002$, Δ QIC = 2.00 between models with rainfalls and age and with only rainfalls). Age was not significant in this model (odds ratio 0.9998, 95% CI (0.9996, 1.00003); Wald statistic = 0.73, $P = 0.392$). The best fitting Rainfall model had a slightly improved QIC over the best fitting Seasonal model (Δ QIC = 1.00).

Discussion

HOSTS IN ENP EXPERIENCE SUBLETHAL ANTHRAX INFECTIONS

Our data show that, regardless of which of our rules are used for ELISA endpoint antibody titre calculations, zebra in ENP experience sublethal anthrax infections and survive, bearing the immune signatures of these infections. Using anti-PA titres as a proxy for sublethal anthrax infection occurrence, and given the fact that anthrax is not a chronic disease (Hugh-Jones & De Vos 2002), the cumulative incidence of sublethal anthrax in the ENP zebra population over the 3 years of this study can be estimated to be at least 62% (Table 2). Given that we observed positive and negative seroconversions for several zebra between sampling occasions roughly 6 months apart, many more positive seroconversions were likely missed. Thus, we believe this estimate is biased downward

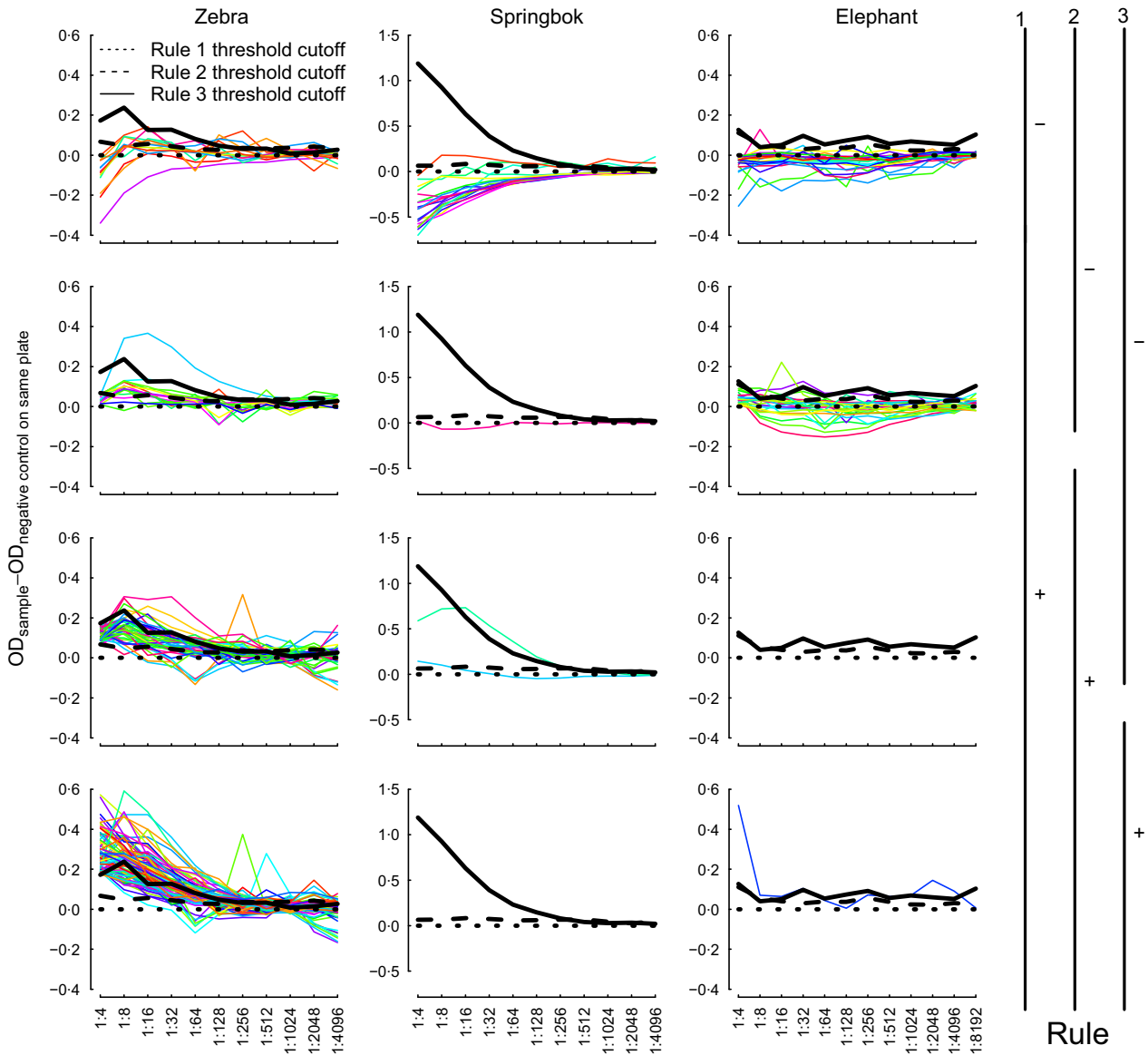


Fig. 4. Optical density titre cut-off rules by species. Coloured lines represent the difference between the optical densities (OD) of a sample Etosha animal (averaged between duplicates) and the OD of the negative control sample (averaged between duplicates) run on the same plate. The thick black lines show the threshold cut-offs for these differences that determined the titre assigned to an animal. Each sample's OD curve appears only once in this figure (i.e. there are 154 coloured lines in the left column representing each unique zebra serum sample). Any animals whose OD curve was never greater than the negative control on its plate are considered seronegative by all three Rules and appear only in the top row. Animals are considered positive by Rules 1, 2 and 3 when their OD curve was greater than the negative control (dotted line), greater than the negative control plus the 1.96 times the standard deviation between duplicate samples (dashed line), or greater than the negative control plus 1.96 times the standard deviation amongst the negative control population (run on separate plates; solid line), respectively. Thus, animals that are positive by Rule 3, the most conservative criterion, appear only in the bottom (fourth) row. The third row shows the animals that are positive by both Rules 1 and 2 but not Rule 3 and, similarly, the second row shows animals that are positive by Rule 1 but not by Rules 2 and 3.

and gives a lower bound for the actual incidence of sublethal anthrax infections in these hosts.

In addition, we found that zebras are more likely to have measurable anti-PA antibody titres in wet seasons compared to dry seasons, perhaps indicating that anthrax exposure rates differ between seasons (see Discussion below). However, when actual amounts of rainfall are taken into account in our system, a more complex picture emerges. While more cumulative rainfall in the 2 months

prior to sampling predicts higher anti-PA antibody titres, more rainfall in the 1 month prior to sampling predicts lower anti-PA antibody titres. This may be at least in part due to a time-lag effect of rainfall, and changes in foraging behaviour and animal movement with changes in rainfall. Additionally, given the strong correlation between rainfall and GI parasite infection intensities in this system, the time-lag between GI parasite peaks and anthrax deaths in the wet season (W. Turner, unpublished data),

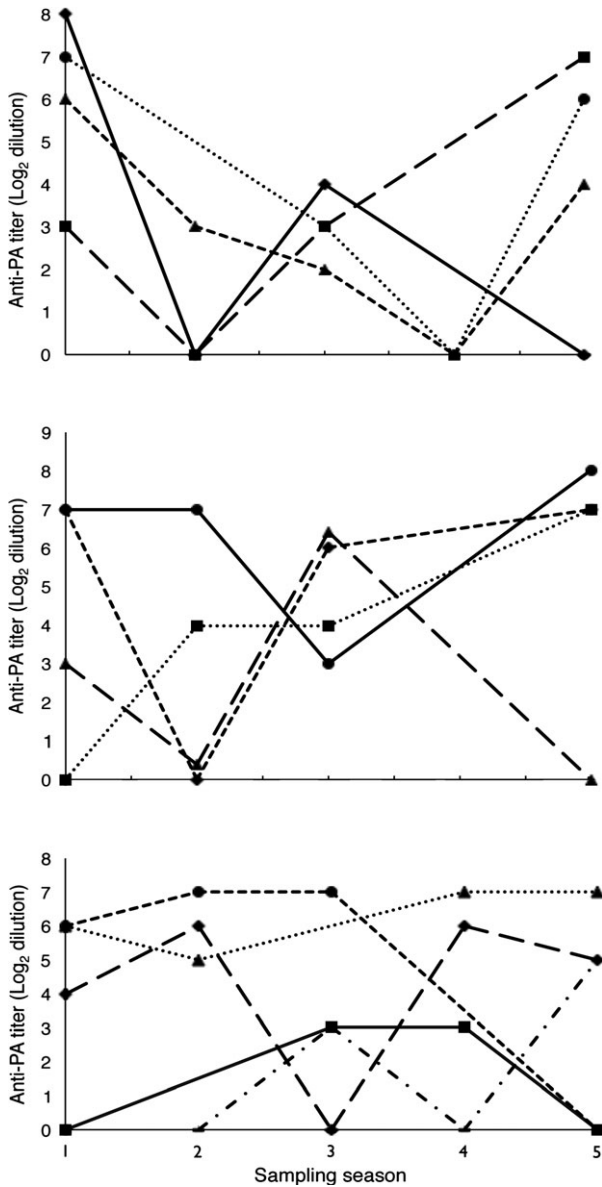


Fig. 5. Change over time of zebra antibody titres to *Bacillus anthracis* PA toxin. Titres are shown as the \log_2 of the reciprocal of the dilution endpoint as determined by Rule 2. Only individuals sampled 4 or 5 times are shown, with each plot showing 4–5 different animals for 13 zebra total across the three plots. Sampling seasons 1, 2, 3 and 4 are roughly 6 months apart from each other, whereas S5 is c. 12 months after S4. Overlapping portions of some plots have been jittered slightly for clarity.

and the fact that GI parasites can be strongly immunomodulatory and predispose hosts to bacterial coinfections (Chen *et al.* 2005), it is likely that there are coinfection and immunomodulatory effects here not being accounted for here.

Our antibody titre results in elephants and springbok are somewhat equivocal; however, the fact that we did see at least one animal of each of these species with a measurable titre indicates that both elephants and springbok are also capable of mounting adaptive immune responses against anthrax and that they experience and survive

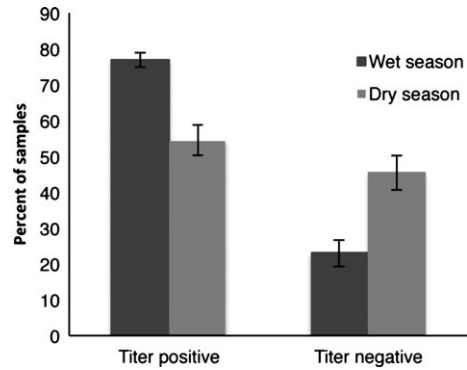


Fig. 6. Percentage (\pm 95% CI) of zebra samples with positive antibody titres or no titres against *Bacillus anthracis* PA toxin, compared between wet and dry seasons.

anthrax infection upon occasion. As approximately half of elephant samples showed measurable anti-PA titres under Rule 1 (Table 2), with some titres being quite high (Figs 3 and 4), it is likely that Rule 2 was simply not sensitive enough in this case to detect all of the true positive titres. As such, it is likely that the cumulative incidence of sublethal anthrax in elephants over the course of this study was somewhere in between that estimated by Rule 2 (3%) and that estimated by Rule 1 (c. 50%).

COMPARISON TO PREVIOUS STUDIES

The results of our study differ from those of the few other wildlife anthrax serology studies. Out of 131 herbivore sera from ENP tested previously, only eight (three springbok, one giraffe, four black rhino) had positive, measurable anti-PA titres, and no zebras ($N = 24$) and no elephants ($N = 12$) tested with positive titres (Turnbull *et al.* 1992a,b). That study, however, used a competitive inhibition ELISA to measure anti-PA antibodies; this requires enough antibody so that there will, at some point in the dilution curves, exist a bound antibody excess compared to soluble antigen. Thus, this assay may not be sensitive enough to detect lower responders. In addition, this assay uses each animal as its own control and comparison, which is not as rigorous as comparing samples to a designated control.

While Lembo *et al.* (2011) found some measurable anti-PA titres in Serengeti buffalo and wildebeest, they found no positive titres in zebras ($N = 85$). Lack of assay sensitivity for low responders may have been an issue; their study used a new, commercially available anti-PA antibody kit (QuickELISA anthrax PA kit immunoassay; Immunetics, Inc., Boston, MA, USA), which, while promising, has yet to be validated for many species (Lembo *et al.* 2011). The real difference in titres, however, likely lies in the fact that anthrax is less ubiquitous in the Serengeti ecosystem than it is in ENP. The Serengeti study only detected 283 suspected (non-culture-confirmed) anthrax positive cases (34 of them zebra) over 14 years, whereas we found 254 culture-confirmed anthrax carcasses

in our three study species (of 270 for all host species) over our 3-year study period (194 of them zebras) (Fig. 2). Thus, the environmental level of *B. anthracis* in the Serengeti ecosystem may be significantly lower than that in ENP, resulting in their herbivores encountering and ingesting significantly fewer *B. anthracis* spores than do ENP herbivores.

SURVIVING ANTHRAX EXPOSURE

A full plasma cell/antibody memory response requires at least 3–6 days to mature (Marcus *et al.* 2004), which is usually too slow to be effective against a fulminant anthrax infection that can subacutely kill the host. Innate immune cells such as macrophages and neutrophils likely play a large role in preventing at least lower doses of anthrax from becoming established in susceptible hosts (Cote, Van Rooijen & Welkos 2006). In addition, evidence from imaging studies indicates that *B. anthracis* spores germinate and establish infections at the initial site of inoculation and do not spread systemically until nearly 1.5–2 days after infection (Glomski *et al.* 2007). This delay may give the immune system enough time to prevent dissemination and fulminant infection.

Adaptive immune mechanisms, including low anti-PA titres prior to memory maturation, may also be protective early in low-dose anthrax infection. PA has been detected on the surfaces of *B. anthracis* spores as early as 1 h into the germination process, and anti-PA antibodies have been found to bind strongly to the surface of spores (Welkos *et al.* 2001; Cote *et al.* 2005). This early antibody binding has direct consequences for the fate of the spore: it increases the rate of macrophage phagocytosis of spores and enhances the rate of intracellular spore germination, which is essential as macrophages can only kill germinating spores (Welkos *et al.* 2001).

MAINTAINING IMMUNITY TO ANTHRAX

While all three study species mounted measurable adaptive immune responses against anthrax toxin, these responses were short-lived. The average time to loss of titre in zebra of *c.* < 6 months is not surprising, given that anthrax vaccines must be boosted at least annually in humans and livestock (Turnbull *et al.* 1992a,b; Turnbull 2008). While our sampling time intervals only allowed us to detect negative seroconversion within an 8–12 month scale for springbok and elephants, the evidence suggests that anti-anthrax adaptive immunity in these hosts may also be short-lived.

While the best models as chosen by QIC included the negative effect of animal age on seropositivity, the effect of age was not statistically significant as determined by Wald confidence intervals. Though this effect was too noisy or too weak to be significant at our sample size, we believe this indicates that animals may be unlikely to maintain a positive titre trajectory as they age due to

low-dose exposures that are too temporally inconsistent in the face of rapidly waning immunity. However, we did find evidence for at least short-term maturation of the anti-anthrax immune response in several animals, indicating the presence of a natural booster effect with subsequent infections and that memory responses are useful and successful in fighting off anthrax (Bellan *et al.* 2012; Figs 5 and 6). Evidence from laboratory studies indicates that a maturing memory response can, in fact, occur even with extremely low initial doses of *B. anthracis* and that these memory responses can be protective even in the face of higher subsequent *B. anthracis* dose challenge (Marcus *et al.* 2004). In that study, some animals vaccinated with moderate doses also showed full survival after a subsequent large *B. anthracis* dose, even though their titres had decreased to non-detectable levels in the months prior to challenge.

These findings may have important implications for wild herbivores becoming infected with low doses of *B. anthracis*; by doing so, they may be effectively ‘vaccinating’ themselves against anthrax, and even low anti-PA titres may be more protective against subsequent infection than has been previously thought. Given the fact that very low infections with *B. anthracis* might be defeated by a host without creating an immune signature (e.g. Aloni-Grinstein *et al.* 2005 had to booster guinea pigs three times with an oral anthrax vaccine to obtain measurable titres), the first anti-PA titre in our study animals may represent multiple previous, undetected infections.

SPECIES DIFFERENCES IN ANTHRAX IMMUNITY

While the species variation in anthrax titre prevalence in this study (Table 2) may be due, in part, to differences in antibody reagents used for each species assay, it also likely reflects some ecological, life history-related, or immunological species differences. Springbok in ENP do experience anthrax outbreaks in the wet season, but at much lower observable numbers than do zebra (Lindeque 1991; Fig. 2). Although lower observed mortality rates could be due in part to a detection bias precipitated by smaller body size and quicker consumption after death, it is likely also due to different infection rates between the two species. Rainfall and vegetation greening effects have previously been found to be important in predicting anthrax outbreaks in a North American anthrax system, though no underlying mechanisms connecting rainfall and outbreaks were postulated in this study (Blackburn & Goodin 2013). Interestingly, a recent study in ENP found that increased rainfall caused ungulates to ingest more soil in the wet season, likely due, at least in part, to loosening of soil around plant roots (Turner *et al.* 2013). However, while soil ingestion increased significantly during the wet season compared to the dry for both zebra and springbok, zebra ingested seven times more soil per day than did springbok. Thus, springbok likely have less overall contact with *B. anthracis* spores than do zebras, likely due to

the predominance of browsing in springbok and grazing in zebras (Gordon & Prins 2008). In addition, we found that zebras are significantly more likely to have a positive anti-PA titre in the wet season compared to the dry season (Fig. 6), indicating that they are likely encountering more *B. anthracis* at this time, perhaps due to soil ingestion. Zebras, as hindgut fermenters, also eat significantly more per body weight than do ruminants such as springbok (Gordon & Prins 2008), which may also increase their spore exposure rate. In addition, zebra are significantly more water-dependent than are the more arid-adapted springbok; zebra in ENP drink from water sources nearly every day (R. Zidon, unpublished data), whereas springbok drink less often and can sometimes obtain all of their water needs from nutritional sources (Nagy & Knight 1994; Auer 1997). While waterborne spread is unlikely to cause fulminant anthrax infection (Durrheim *et al.* 2009), the low concentrations of *B. anthracis* spores found in up to 26% of ENP watering sources may be enough to 'booster' zebras more often than springbok with sublethal anthrax infections (Lindeque & Turnbull 1994).

Recent work by Beyer *et al.* (2012) revealed that zebras, springbok and elephants in ENP are infected with and die from the same strains of anthrax, so outbreak timing differences in elephants are not microbiological in nature. Elephant movement patterns in ENP, however, are quite different from those of the plains herbivores; when the majority of zebras and springbok are congregating in the wet season in the area surrounding Okaukuejo, elephants are very scarce on the Okaukuejo plains (J. W. Kilian, unpublished data). Elephants return to these plains, where the majority of anthrax outbreaks have occurred throughout known ENP history, in the dry season. Recent zebra deaths in this area during the previous season may create potent locally infectious zones (LIZs) of concentrated *B. anthracis*; even if these spore sinks become dispersed over the landscape with time, it is likely that these LIZs remain for at least the next several months for elephants to contact (Lindeque 1991; Bellan *et al.* 2013). As elephants are mostly browsers (Codron *et al.* 2006), they, like springbok, may not ingest *B. anthracis* as much and as often as do zebras, thereby resulting in lower anti-PA titres compared to those in zebras and potentially less protection against subsequent doses. These combined factors may help account for the differences in elephant anti-anthrax titre prevalence, as well as the differences in outbreak timing in this species compared to other ENP hosts.

Similar to zebras, however, elephants are quite water-dependent, with home range sizes determined by water hole density (Young & Van Aarde 2010) and elephants ingesting large quantities of water at a time. Thus, it might be predicted that exposure to low doses of spores in drinking water would also prompt titre development in elephants. The lower seroprevalence in elephants compared to zebras in this study may thus indicate that water exposure does not play an important role in sublethal

anthrax exposure in ENP, or that elephant immune responses to anthrax differ greatly from those of zebras.

Conclusions

In summary, zebra in ENP often become infected with, and survive, *B. anthracis* infection. This is the first study to demonstrate that zebra can mount protective immune responses against *B. anthracis*. In the process, these hosts can build up their immunity to *B. anthracis* over time, at least over the short term. Zebra appear to have a higher exposure to *B. anthracis* in the wet season, though factors other than season alone also likely affect the complex immune dynamics of anthrax in these hosts. Elephants and springbok in ENP also appear to experience sublethal anthrax infections, though at lower rates than do zebras. Though adaptive immunity to anthrax wanes rapidly in all three of our study species, there is evidence that subsequent sublethal *B. anthracis* infections that occur frequently enough can act as immune boosters that result in maturation of the anti-PA response in these hosts. This antibody maturation response also serves as an immune signature indicating that even susceptible hosts in an anthrax-endemic system survive multiple anthrax infections over their lifetimes.

These results show that, rather than being an all-or-nothing disease, anthrax often occurs as a sublethal infection in susceptible herbivorous hosts in an endemic anthrax system. Plains herbivore populations in ENP are not food limited, and it has been hypothesized that, alongside predation, anthrax is a main population-limiting factor in this system (Gasaway, Gasaway & Berry 1996). Indeed, recent evidence suggests that observed mortalities likely severely underestimate true outbreak size in zebras (Bellan *et al.* 2013). Our findings of high rates of sublethal anthrax infections in these hosts support the hypothesis that anthrax in endemic systems plays a larger role in population dynamics than has been previously appreciated.

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Data accessibility

Data available from the *Dryad Digital Repository*: <http://doi.org/10.5061/dryad.73f23>.

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