The persistence of time: the lifespan of *Bacillus anthracis* spores in environmental reservoirs

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Abstract

Anthrax is a lethal bacterial zoonosis primarily affecting herbivorous wildlife and livestock. Upon host death *Bacillus anthracis* vegetative cells form spores capable of surviving for years in soil. Anthrax transmission requires host exposure to large spore doses. Thus, conditions that facilitate higher spore concentrations or promote spore survival will increase the probability that a pathogen reservoir infects future hosts. We investigated abiotic and pathogen genomic variation in relation to spore concentrations in surface soils (0-1cm depth) at 40 plains zebra *(Equus quagga)* anthrax carcass sites in Namibia. Specifically, how initial spore concentrations and spore survival were affected by seasonality-associated with the timing of host mortality, local soil characteristics, and pathogen genomic variation. Zebras dying of anthrax in wet seasons—the peak season for anthrax in Etosha National Park—had soil spore concentrations 1.36 orders of magnitude higher than those that died in dry seasons. No other variables considered affected spore concentrations, and spore survival rates did not differ among sites. Surface soils at these pathogen reservoirs remained culture positive for a range of 3.8 – 10.4 years after host death. Future research could evaluate if seasonal patterns in spore concentrations are driven by differences in sporulation success or levels of terminal bacteremia.

Keywords: anthrax; bacterial spores; genomics; infectious disease reservoir; transmission hotspots

1. Introduction

For environmentally transmitted pathogens, the environment supports intermediate stages allowing the pathogen to move from an infected host to a susceptible host. Hosts shed pathogen propagules into the environment during the infection or upon host death, depending on the pathogen and its mode of infection. Once released from a host, pathogens are subjected to abiotic and biotic factors which affect their survival and the likelihood of onward transmission to a susceptible host [1]. The environmental life stage of pathogens is poorly understood compared to processes occurring during host infection [2]. Here we investigated the lifespan of pathogen environmental reservoirs for a long-lived, spore forming bacterium, *Bacillus anthracis*, to determine how spore concentrations in soil reservoirs vary based on abiotic factors and pathogen genomic variation.

Abiotic factors such as soil properties, temperature, and humidity can strongly influence the survival and transmission of environmental pathogens. Soil moisture and physiochemical properties of soil such as pH, organic content and texture have been demonstrated to affect survival of bacteria such as *Leptospira* [3]. The survival and dispersal of *Coccidioides immitis*, the soil-borne pathogen that causes valley fever, is influenced by soil moisture and wind [4]. The cholera-causing bacterium, *Vibrio cholerae,* thrives during high rainfall periods [5] when zooplankton that act as vectors bloom [6]. Transmissible gastroenteritis virus and mouse hepatitis virus persist longer on environmental surfaces at low temperatures and are inactivated at higher temperatures [7].

Pathogen traits evolved to promote survival in response to environmental stressors can also facilitate onward transmission. These survival traits may include metabolic, physiological or structural features encoded in the genome, or variation resulting from phenotypic

plasticity. Genomic approaches allow for the screening of thousands of loci simultaneously in many pathogen strains to identify genomic diversity. Pan-genome analyses allow the detection of all genes present among strains of a species and can identify the presence or absence of particular genes across the diversity of strains. This approach permits further in-depth analysis and the identification of genetic markers for detection of interesting phenotypic traits [8]. We thus used whole genome sequencing and pan-genome analyses, coupled with more traditional microbiological techniques for quantifying bacteria, to investigate gene presence or absence patterns with respect to variation in pathogen concentrations in environmental reservoirs over time.

Bacillus anthracis, the etiological agent of anthrax has two life stages, vegetative cells and spores. Vegetative cells rapidly reproduce within the host, releasing toxins that lead to acute septicemia and death [9]. Upon host death vegetative cells are released into the environment together with hemorrhagic fluids, and sporulate upon exposure to oxygen and nutrient deprivation [10]. Spores of *B. anthracis* are highly resistant to adverse environmental conditions and can withstand a variety of stressors such as heat and ultraviolet (UV) radiation [11]. Surveys from *B. anthracis* contaminated soils indicate long-term survival of spores creating enzootic sites [12, 13]. Despite high terminal *B. anthracis* cell counts in host blood, concentrations of spores culturable from soil at carcass sites are significantly lower [14], suggesting only a portion of vegetative cells in the host make it to the spore stage. Sporulation time is affected by environmental conditions, including temperature, humidity, alkalinity and nutrient availability [15, 16]. Once vegetative cells are released into the environment, the window for sporulation is estimated to be within 1-3 days depending on temperature and humidity [17]; 2-5 days on environmental surface based on laboratory experiments [18] or out to 4 days from field observations [19]. Thus, if abiotic conditions are favorable, more spores will be formed during this window of opportunity than if

conditions are unfavorable for sporulation. Once spores are formed, soil properties can alter survival rates, with survival promoted by calcium content and alkalinity [12].

Spore concentrations found in soils at anthrax carcass sites are highly variable among sites and decline over time [20]. While scavengers are expected to alter soil spore concentrations by opening carcasses and releasing infectious blood to the environment, an experimental study found no difference in spore concentrations when vertebrate scavengers were present or excluded from carcasses [19]. Thus, fundamental questions surrounding the environmental reservoir of *B. anthracis* remain unanswered, including what factors affect spore concentrations at pathogen reservoir sites (i.e., anthrax carcass sites) and how long these sites remain infectious. These are critically important questions for understanding disease epidemiology, given that very large spore doses are required for lethal infections [20]. Here we focus on spore concentrations in the soil surface, as the part of the soil most likely to be contacted by mammalian hosts [20].

Research into the factors affecting the survival or suitability of environments for *B. anthracis* typically focus on abiotic factors as mentioned above and not on the genomic variation of the pathogen. Pathogen diversity is rarely considered as a potential source of phenotypic variation for this pathogen, given the highly monomorphic nature of *B. anthracis* [21]. However, recent studies suggest differences in pathogen phenotypes among locations, which could have a genetic component [22, 23]. We leverage a unique 12-year dataset on *B. anthracis* spore concentrations at plains zebra (*Equus quagga*) anthrax carcass sites and whole genome DNA sequencing of an isolate from each site to investigate abiotic factors and pathogen genomic variation affecting spore concentrations and spore survival at anthrax carcass sites.

We investigated if variation in spore concentrations among anthrax carcass sites could be linked to abiotic factors or genomic differences among isolates collected from the anthrax cases

associated with these sites. We hypothesized that the season in which hosts die may affect sporulation success, with higher spore counts detected in soils at carcass sites when hosts die during seasons with warm and humid conditions than during seasons with cool and dry conditions. Although soil conditions have the potential to alter both initial spore concentrations and spore survival rates, the soils across the study area are similar, hence we did not expect to see soil characteristics affecting spore concentrations at this spatial scale.

This study was motivated by an interest in knowing how long spores survive in surface soils at natural anthrax carcass sites in an unmanaged ecosystem with soils that support disease endemicity, and what drives variation in spore concentrations among sites. These questions have yet to be explored in a natural setting and are worth exploring in relation to what is known about this pathogen from controlled, experimental laboratory studies. Furthermore, conducting a long-term study of spore concentrations at pathogen reservoir sites allowed us to calculate the lifespan of these pathogen reservoirs in a natural, endemic ecosystem, filling an important gap in our understanding of the epidemiology of this highly lethal environmentally transmitted disease.

2. Materials and Methods

2.1. Study area and sites

This study was conducted in Etosha National Park, Namibia (hereafter Etosha), a semi-arid savanna ecosystem with endemic anthrax infections occurring annually. Anthrax was first documented in Etosha in 1964, with detailed mortality data kept since 1976 [14], allowing for long term epidemiological and ecological studies of this disease in a natural environment. Anthrax control efforts were conducted in this ecosystem until the early 1980s, when challenges of carcass disposal during a *Loxodonta Africana* (African elephant) outbreak led to a decision to not attempt

141 management of this disease [24]. This recognition of anthrax as part of the natural ecosystem
142 allows for observational research opportunities, such as presented here, that would not be possible
143 in most other locations.

144 This study took place on a mix of grassland and dwarf shrub savanna habitats found
145 southwest of the Etosha salt pan, a high-risk area for anthrax transmission [25]. Our study area
146 covered approximately 350 km^2 located within S 18.984 -19.697 and E 15.699 -16.094. Soils in
147 this area are high in calcium carbonate and moderate to high in alkalinity, salinity and drainage
148 [26, 27]. Central Etosha receives an average of 358 ± 124 mm of rainfall annually, with most rain
149 falling between January and March [28]. *Bacillus anthracis* infections in plains zebra primarily
150 peak during February – April, toward the end of the wet season [27, 29]. This study extends
151 previous research by Turner et al. [20] that investigated exposure risk at anthrax carcass sites
152 compared to water sources. As part of that study, *B. anthracis* spore concentrations in surface soils
153 at 40 zebra anthrax carcass sites were measured annually for up to five years (for additional details
154 on how sites were selected for this study, see Turner et al. [30]). Here, our study investigates spore
155 survival patterns over a 12-year period (2010-2021), extending the published dataset for an
156 additional seven years. All carcass sites were marked with a metal stake to ensure sampling at the
157 same location over time. The 40 sites included 25 sites from anthrax mortalities that occurred in
158 2010, 4 from 2011, 8 from 2012, and 3 from 2013. Of these monitored anthrax cases 34 occurred
159 in February and March (during the hot-wet season) and six occurring from May-August (during
160 the cool-dry season).

161

162 *2.2. Soil sampling and Bacillus anthracis spore counts*

Surface soils are those most likely to be accessible to grazers [20], hence soil samples of 0-1 cm depth and within 1 m of the marked center of the carcass site were collected annually between 2010 and 2021 (one combined sample per site per year). These samples were collected from February-April (coinciding with the seasonal anthrax peak in cases) for the first 8 years and from June-July thereafter. The initial sample for year 0 at a carcass site was collected at varying times after host death (11 – 63 days), allowing time for the carcass to be scavenged and sporulation to occur. Not all sites were sampled in year 0 based on when they were formed, 7/40 sites had the first sample only in year 1 (range 7-12.8 months after death). We quantified *B. anthracis* spore concentrations following standard soil culturing protocols using polymixin-lysozyme-ethylendiaminetetraacetic acid (PLET) agar as the growth medium [9] in serial dilution from 5 g of homogenized soil, with counts adjusted based on soil dry mass, to calculate the number of colony forming units (CFUs) per gram of dry soil (hereafter, spores per gram). Additional details on sampling, culturing and the first five years of culture data are described within Turner et al. [20]; carcass sites and spore count data are available at [31].

2.3. Weather and soil characteristics data

To investigate the effect of the abiotic environment on *B. anthracis* spore concentrations we considered soil characteristics and seasonal variables as a proxy for environmental changes in temperature and humidity that we did not record at individual sites. The seasonal variables included a categorical variable for the season of death, defined as wet season (December - April) or dry season (May to November), and a numeric variable for the week of the year the animal died (1-52 weeks).

185 Soil cores of 10 cm depth located 9 m from the center of each carcass site in four directions
186 were collected and pooled for each site for soil characterization. We sampled 9 m from the site
187 center to determine the background soils and not the effect of the carcass on those soils (the carcass
188 effect on soils was considered in Turner et al. [30]). Samples for soil analyses were collected from
189 each site twice, in 2012-2013 and 2014. Soil analyses including soil texture (categories of loam,
190 sandy loam, and silty loam), soil composition (percentage of sand, silt, clay and organic matter),
191 pH, and soil chemistry (parts per million (ppm)of phosphorus, potassium, magnesium, calcium
192 and sodium). Samples for soil chemistry were analyzed at the Ministry of Agriculture, Water and
193 Forestry Laboratory in Windhoek, Namibia. The replicate measurements of soil properties from
194 the 2012-2013 and 2014 samples were similar [30], and for statistical analyses below, we used the
195 averaged value of the two samples.
196
197 *2.4. Data analysis of spore counts*
198 We investigated factors associated with spore survival over the 12-year study period. We
199 used a linear mixed model with $\log_{10}$ transformed spore counts (+1) per gram of soil as the response
200 variable and the time since host death (in days), and soil characteristics (soil pH, soil chemistry
201 and soil texture) as fixed effects. Since samples were collected at marked sites over time, we
202 included the site ID as a random effect. We investigated the random effect of site identity both on
203 the y-intercept and on the slope of the linear survival trend estimated for each site, to see if site
204 identity only affected the estimated starting concentration of spores (i.e., the y-intercept) or if sites
205 also varied in spore survival rates (i.e., if the slope of spore concentrations over time varied among
206 sites). To obtain the best model, we first determined the best model of fixed effects for both
207 random effect structures independently based on Akaike information criterion (AIC) and R^2

values. We then compared the random effect structure by using a likelihood ratio test between the two final models.

In the spore concentration samples, a count of zero could represent a true zero (there are no spores in the surface soils at the site) or a false zero (spores are present but were not in the sample or were not detected). Since our goal was to see how spore counts decreased over time, we removed false zeros from the dataset prior to the analysis and retained only the first "true zero" to improve estimates of survival trends based on spore counts (for comparison, spore counts over time including all data are shown in Fig. S1). We considered a count of zero as false if a later sample from that site was culture positive. False zeros could be due to sample variation in a subsample collected from a heterogenous site or errors arising from sample processing. We considered a count of zero as the first true negative once the surface soil from a site turned negative and remained negative in subsequent samples. By year 11 of the study, surface soils from all sites were culture negative, a pattern we confirmed in year 12, where again, all sites were culture negative.

We then evaluated if variation in the starting spore concentrations among sites could be attributed to seasonality in the timing of death or soil characteristics at the site. Since the timing of the first soil sample at a carcass site varied and a decrease in spore counts is detectable in the first weeks after death [32], we estimated initial spore concentration for each site using the fitted y-intercepts of the selected linear mixed effect model for spore survival. Using these estimated $\log_{10}$ transformed starting spore concentrations, we fit a linear model on the spore counts with explanatory variables including week of death, season of death (wet or dry), maximum temperature, rainfall, and soil characteristics.

All statistical analyses were conducted using R software version 4.0.2 [33]. Linear models were analyzed with the package lme4 [34]; random effects were tested using the package lmerTest [35]; model selection was done using the package MuMIn [36].

2.5. Genomic data analysis

Genomic DNA was extracted from a single colony of *B. anthracis* isolated from each carcass using Qiagen DNeasy Blood and Tissue kit following the manufacturer's protocol for gram-positive bacterial DNA. Libraries were prepared using the KAPA HyperPrep kit Illumina platform KR0961 – v6.17 following the KAPA protocol adjusting to the following parameters: 10ng input WGS DNA and 11 cycles for amplification. Sequencing was performed on the Illumina HiSeq 4000 platform, with 150-bp paired-end reads (raw reads are available at the National Centre for Biotechnology Information under the BioProject accession number PRJNA914838). De novo assemblies were constructed using the Shovill pipeline v1.1.0 (https://github.com/tseemann/shovill), employing the genome assembler SPAdes v3.14 [37]. Genome assembly quality was assessed using Quast v5.0.2 [38], Checkm v1.1.3 [39] and BUSCO v5.0.0 [40]. Sequence quality baselines were set at 95% completeness, < 5% contamination, < 200 contigs, and N50 > 50,000bp.

Genomes were annotated using Prokka v1.14.6 [41] and the *B. anthracis* pan-genome across the Etosha sample-set was characterized using Roary v8.2.12 [42]. The core genes (those present in 99% to 100% of genomes) and soft-core genes (those present in 95% to < 99% of genomes) were used for the detection of single nucleotide polymorphisms (SNPs) which were extracted using SNP-sites v2.5.1 [43]. These SNPs (14,758) were then used to construct a maximum likelihood (ML) core genome phylogeny using RAxML-NG v1.0.2 [44]. A population

genomic approach was used to identify distinct clusters across the phylogenetic dataset using an extension of the classification scheme described by Bruce et al. [45] with HierBAPS [46]. HierBAPS provides a method for hierarchically clustering DNA sequence data to reveal nested population structure. The phylogenetic relationship among isolates was visualized against initial spore concentrations to examine associations between genotype and phenotype.

Accessory genes were used to assess gene variation amongst isolates and to construct a gene presence/absence matrix visualized using the Interactive Tree of Life software (iTOL) v6 [47]. The accessory genome is important to understand variation amongst genomes of species and thus, their specific lifestyles and evolutionary trajectories [48]. These accessory genes derived from the sample set were further clustered into categories of cloud genes (present in < 15% of genomes) and shell genes (present in 15% to < 95% of genomes). Annotated gene variation in the accessory genes was used as a means to highlight any differences among isolates, in particular, linked to variation in initial spore concentrations. Thus, we focused on sporulation genes. Additionally, each genome assembly was screened for antimicrobial resistance (AMR) genes using the comprehensive antibiotic resistance database (CARD) [49] and virulence factors using the virulence factor database (VFDB) [50] in Abricate v1.0.1 (https://github.com/tseemann/abricate). We used the default parameters for each program. We hypothesized that isolates with additional AMR genes might be detected from sites with higher initial spore concentrations compared to isolates lacking AMR genes.

3. Results

3.1. Variation in spore concentrations

Spore concentrations were variable among sites, with a maximum count across all samples of 1.19 x 10^9 spores/g of soil. *Bacillus anthracis* spores could no longer be cultured from surface soils at these sites by 3.8 – 10.4 years after host death. Sites formed in dry seasons were culture negative an average of 8.0 years after host death (range 3.8 – 9.8 years), while sites formed in wet seasons were culture negative after 9.3 years (range 6.2 – 10.4 years). By 10 years after death, all sites were culture negative, a pattern confirmed in the following year (Fig. 1a).

The season in which the host died (β = -1.39, SE = 4.5 x10^{-1}, p = 0.004) and days after host death (β = -1.30 x10^{-3}, SE = 5.9 x10^{-5}, $p < 0.0001$) were the only significant variables linked to spore counts (Fig. 1; Table 1). As expected, soil properties at sites did not significantly affect spore counts. For the random effects considered, we detected a significant effect of site identity on initial spore concentrations (i.e., y-intercepts) but the likelihood ratio test showed no significant improvement by including random slopes (p = 0.10). Thus, the spore decline rates (i.e., slopes) were similar among sites (Table S1). This suggests that while sites varied in their initial spore counts, the decline in spores over time proceeds at a similar rate among sites.

Starting spore concentrations were significantly higher when hosts died in the wet season than in the dry season ($\log_{10}$ mean intercept for wet season: 5.53 spores/g of soil; dry season: 4.17 spores/g of soil); $p < 0.005$; Fig. 1b). The lifespan of spore concentrations detected at anthrax carcass sites was not significantly different for sites formed in dry seasons (predicted mean: 9.2, predicted range 7.9 – 10.6 years) and sites formed in wet seasons (predicted mean 12.2, predicted range 11.4 – 13.2 years).

3.2. Phylogenomic distribution, population structure and pan-genome

297 An isolate of *B. anthracis* from 37/40 anthrax carcass sites was sequenced, from which 36
298 genomes met the baseline criteria for quality assessment (the excluded isolate had 62.31%
299 contamination). The remaining whole genome sequences were assembled and used to construct a
300 maximum likelihood phylogenic tree based on core SNPs. The isolates were highly similar with
301 the exception of isolate WT-233 which clustered on a branch of its own. Based on the *B. anthracis*
302 population structure classification developed by Bruce et al. [45], all isolates grouped into cluster
303 5.4, also known as A.Br.Aust 94; these isolates further clustered into four sub-clusters (Fig 2). The
304 initial concentrations estimated from the random intercept model for each site showed no obvious
305 relationship to pathogen population structure (Fig. 2).

306 The pan-genome of the Etosha isolates consisted of 5,818 genes of which hypothetical
307 proteins accounted for 2,811 genes (see Table S2 for a list of all annotated genes). We identified
308 5,704 core genes (present in all 36 genomes), 14 soft-core genes (present in 35 genomes), 29 shell
309 genes (present in 6 – 34 genomes) and 71 cloud genes (present in 1 – 5 genomes). The core genome
310 comprised 98.28% of the Etosha pan-genome, while the accessory genome comprised 1.72% of
311 the sample-set pan-genome. A total of 100 accessory gene sequences from the sample set were
312 identified of which 77 were annotated as hypothetical proteins (Table 2; Fig. 3). Genes known to
313 code proteins that are essential for sporulation pathways and spore formation (sigma factor, *sig*;
314 sporulation kinase, *kin*, sporulation proteins, *spo*; putative sporulation protein coding genes, *ylmC*
315 & *ytaF*) were present in all 36 isolates.

316 All known virulence factors (13 detected by the VFDB) were present in all genomes and
317 five AMR genes were detected, with each present in all genomes (Table 3). One isolate (WT-233)
318 had multiple gene sequences annotated as the Calmodulin-sensitive adenylate cyclase (*cya*)
319 virulence gene (Fig. 3); future study could evaluate if these differences lead to changes in virulence

during the in-host phase. Interestingly, the carcass site associated with this unique isolate had the lowest initial spore count among sites formed during the wet season (Fig. 2).

4. Discussion

There are critical gaps in our understanding of pathogen life-history in the environmental stage [51]. Determining the abiotic and biotic factors affecting pathogen survival in environmental reservoirs could lead to a better understanding of processes controlling transmission and fitness of pathogenic bacteria and inform disease management strategies. In this study we investigated the effects of variation in soil characteristics, seasonality, and *B. anthracis* genomic diversity on spore concentrations at plains zebra anthrax carcass sites in Namibia. Among these potential factors, the season in which the host died, and the time since host death were the only significant variables linked to concentrations of spores in soils. Initial spore concentrations were on average 1.36 orders of magnitude higher at carcass sites formed in the wet season than at those formed in the dry season. Despite differences in starting concentrations, once these sites were formed, spore survival rates were similar among sites (Fig. 1b).

We hypothesized that the season in which a host died could affect sporulation success, with higher spore counts detected in soils at carcass sites when hosts died during warm and humid conditions than during cool and dry conditions. The observed seasonal pattern in initial spore concentrations could be a result of weather differences during the window of sporulation, with warmer, more humid conditions in the wet season promoting a greater rate of sporulation than is possible during the cooler, drier conditions in the dry season (which would be in agreement with laboratory studies [15, 16]). In addition to weather effects on sporulation rates, the observed seasonal pattern in initial spore concentrations could also be explained by changes in host

susceptibility to anthrax infection [52]. If nutritional stress during the dry season leads to host mortality from relatively smaller pathogen populations than what occurs during the wet season, the resulting spore concentrations could be lower. However, this explanation may be less plausible than the alternative since the observed mortalities occurred relatively early in the dry season, before resource restriction and nutritional stress are likely to have an impact on host immunity. The dry season sample was relatively small (5 sites), although this is not surprising given the wet-season timing of anthrax outbreaks (with cases peaking in February – April) [20]. As such, confirming seasonal patterns in initial spore concentration of *B. anthracis* may not be possible from field studies and may instead be determined through experimental work under controlled conditions. Temperature, humidity, alkalinity and nutrient availability are known to be factors affecting sporulation time in laboratory settings [15, 16]. Confirming seasonal differences in spore concentrations and underlying drivers of these differences could be determined through future research.

In this anthrax-endemic region, *B. anthracis* spores in surface soils were detectable for up to a decade. Given that the spore exposure dose needed for a lethal anthrax infection is high, ranging from $10^5 – 10^6$ spores [20], we speculate that sites formed during the wet season could be more infectious, and infectious for a longer time compared to those formed in the dry season. If the seasonal differences in spore counts detected in this study lead to epidemiological differences in transmission risk among sites, this could reinforce the seasonal patterns observed for this disease. Whether these differences in spore concentrations based on the season of death are epidemiologically relevant or not could be evaluated in future studies.

Notably, we found that the predicted lifespan for spore detection at these sites was several years longer than what was measured (Fig. 1). Spores were no longer detected in surface soils at

366 all sites by 2020, 10 years after the first sites were formed. The 2020 sample was collected
367 following the worst drought on record in this ecosystem. Rainfall during 2019 was so low that
368 grasses in the study area did not grow, changing host movement patterns and anthrax risk [25].
369 The prolonged exposure of the soil surface to UV radiation during this drought may have shortened
370 the lifespan of spores in the surface soils of these sites, a hypothesis we were unable to test with
371 the available data. Spores may still be found below the soil surface at these sites, if rainfall causes
372 spore to leach deeper into the soil [53], however we focused on spores in surface soils with the
373 expectation that these would be most likely to be contacted by herbivorous mammals.

374 Pan-genome analysis of isolates from the monitored carcass sites did not reveal any
375 obvious connections between genotypic diversity and phenotypic traits related to initial spore
376 concentration or spore survival. Based on the core genome SNPs, the phylogenetic distribution of
377 the isolates grouped into cluster 5.4, of the primary cluster 5 or (A.Br.Aust-94). Cluster 5 is the
378 most diverse cluster of *B. anthracis*, both genetically and geographically, with more SNPs than
379 other clusters and consisting of isolates from all continents in its range [45]. However, on the
380 relatively small spatial scale of this study, genomic diversity was low with the exception of one
381 isolate (WT-233), and the initial spore concentrations did not appear to be affected by genomic
382 factors based on phylogenetic clustering. Isolates were similar in terms of the shared gene
383 sequences detected, with the core genome of the sample-set comprising of 98% of genes. The
384 accessory genome composed of only 2% of the pan-gemone . All genes essential for sporulation
385 were conserved in the core genome, with no variation inthe number of gene annotations among
386 isolates. This finding could change with the addition of more genomes, thus, the pan-genome
387 across the Etosha sample set provides only preliminary findings on gene difference and calls for
388 further work -intopatterns between gene differences and spore persistance. In addition, AMR gene

presence or absence and virulence factors did not vary among isolates. Noting from the example of *Clostridium tetani*, we note that the presence or absence of gene coding sequences does not necessarily translate to the formation of proteins that may affect functional traits [54].

Despite the great similarity among the isolates in our sample, isolate WT-233 was the most genetically diverse in our sample, with an additional three annotations of the *cya* virulence gene present in this isolate. The carcass site associated with this isolate (10-042) was also the site with the lowest initial spore concentration for those formed in the wet season. If this isolate is more virulent than other strains, it may kill its host more quickly, leading to a lower spore concentration in the environment. While this is only a speculation for a single isolate from a single anthrax case, it suggests that there may be more variation in this pathogen and its phenotype than expected from its clonal nature. Additional investigation into how the detected multiple annotations of virulence gene sequences affects infection virulence, terminal bacteremia and the subsequent role of pathogen diversity in epidemiological dynamics may be warranted.

Bacillus anthracis spores can persist in the environment for years, enabling the cycle of transmission to continue. Our study indicates that anthrax carcass sites do not remain hotspots for exposure for extremely long periods of time, since surface soils at these sites only maintained detectable spores for up to a decade and may pose risk of transmission for shorter periods [20]. While these results support our understanding of endemic anthrax systems in which annually occurring cases maintain the circulation of the pathogen in the ecosystem, there remains a gap in our understanding of how anthrax is maintained in ecosystems with decades or longer occurring between outbreaks. Future studies investigating spore dynamics below the soil surface, germination of spores in hosts, rates of replication, and host diversity may shed light on how other parts of the *B. anthracis* life cycle affects spore concentrations in the environmental reservoir.

There also may be more variation in pathogen genetic diversity, sporulation or spore survival rates across larger spatial scales. Given the deadly potential of *B. anthracis* and its classification as a Tier 1 select agent pathogen by the U.S Government (i.e., agents having the potential to pose a severe threat to public health and safety, thus subject to increased regulations) [55], addressing questions of its phenotypic diversity in natural ecosystems is particularly challenging. However, studies of pathogen-environment interactions may be particularly useful in understanding the complexity of anthrax transmission dynamics in different ecological settings across its geographic range.

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571 [56] Consortium U. UniProt: a worldwide hub of protein knowledge. Nucleic acids research
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573

574 Table 1. Linear mixed effects models of *Bacillus anthracis* spore concentrations in carcass site
575 soils from Etosha National Park, Namibia. Results show Akaike information criterion (AIC) values
576 for the full linear mixed model and backwards elimination of variables to reach the best model
577 based on ΔAIC, in the Lmer package in R. Soil variables considered include the percent
578 contribution of sand, silt, organic matter (OM), the concentration of Mg, K, Ca, P, Na and pH.
579 Season is the season of death and Site Age is the age of the site (in days after death) when a sample
580 was collected. Site ID is the site identity and was included as a random variable in all models.

581 Full model: $\log_{10}(CFU+1) \sim$ silt + Mg + K + Ca + pH + P + OM + Na + sand + Site Age + Season
582 + Site ID as random effect; AIC = 1049.776
583 Selected model: $\log_{10}(CFU+1) \sim$ Site Age + Season + site ID as random effect, AIC = 962.628
584

Variable	Order of Elimination	F-Value	p-value	AIC
All				1031.8
K	1	0.001	0.98	1015.8
Ca	2	0.012	0.91	999.6
silt	3	0.044	0.84	993.8
pH	4	0.252	0.62	994
P	5	0.212	0.65	988.9
Mg	6	0.198	0.66	978
OM	7	3.487	0.07	977.1
Na	8	3.373	0.07	965
sand	9	3.840	0.06	**960.4**
Site age	**0**	**486.691**	**<0.0001**	
Season	**0**	**9.499**	**0.004**	

585

586 Table 2. Accessory genes from the Etosha samples that are categorized into shell and cloud genes of *Bacillus anthracis* isolates from

587 Etosha National Park, Namibia.

Gene	Frequency	Annotation	Possible function
Etosha sample-set shell genes (N=29)			
*helD_2**	10	DNA helicase IV	Catalyzes unwinding of DNA
topB_5	34	DNA topoisomerase 3	Releases the supercoiling and torsional tension of DNA
mnaA_1	23	UDP-N-acetylglucosamine 2-epimerase	
mnaA_2	22	UDP-N-acetylglucosamine 2-epimerase	Enzyme that catalyzes the chemical reaction UDP-N-acetyl-D-glucosamine
*mnaA_1**	13	UDP-N-acetylglucosamine 2-epimerase	
*mnaA_2**	10	UDP-N-acetylglucosamine 2-epimerase	
dnaI_1	34	Primosomal protein DnaI	DNA replication
hypothetical proteins (n=22)	6-34		
Etosha sample-set cloud genes (N=71)			
apt_3	1	Adenine phosphoribosyltransferase	Catalyzes a salvage reaction resulting in the formation of
*apt_3**	1	Adenine phosphoribosyltransferase	AMP
cya_1	1	Calmodulin-sensitive adenylate cyclase	
cya_2	1	Calmodulin-sensitive adenylate cyclase	Acts as a calmodulin-dependent adenylyl cyclase by converting ATP to cAMP
cya_3	1	Calmodulin-sensitive adenylate cyclase	
cya_4	1	Calmodulin-sensitive adenylate cyclase	

*cya_2**	1	Calmodulin-sensitive adenylate cyclase	
sepF_1	1	cell division protein SepF	Cell division
dnaA_4	1	Chromosomal replication initiator protein DnaA	Initiation and regulation of chromosomal replication
*dnaA_1**	2	Chromosomal replication initiator protein DnaA	
*topB_5**	1	DNA topoisomerase 3	Releases the supercoiling and torsional tension of DNA
group_86	2	IS3 family transposase ISHaha4	
group_87	1	IS3 family transposase ISHaha4	Involved in the transposition of the insertion sequence (IS)
group_75	1	IS4 family transposase IS231S	
repX_2	1	Plasmid replication protein RepX	Plasmid replication
xerS_1	1	Tyrosine recombinase XerS	DNA binding
55 hypothetical proteins	1-5		

588 Function obtained from UniProt [56] using *Bacillus* species

589 Table 3: Antimicrobial resistance genes and virulence factors detected in the genomes of *Bacillus*
590 *anthracis* genomes from Etosha National Park, Namibia. Antimicrobial resistance genes were
591 detected with comprehensive antibiotic resistance database [49] and virulence factors were
592 detected with the virulence factor database [50] in Abricate (Seeman, available at:
593 https://github.com/tseemann/abricate). All antimicrobial resistance genes and virulence factors
594 were present in all 36 genomes.

Gene	Definition
Antimicrobial resistance genes	
bla1	chromosomal-encoded beta-lactamase which hydrolyzes penicillin
bla2	chromosomal-encoded beta-lactamase which has penicillin cephalosporin and carbapenem-hydrolyzing abilities
fosB	A thiol transferase that leads to the resistance of fosfomycin
mphL	A chromosomally-encoded macrolide phosphotransferases that inactivate 14- and 15-membered macrolides such as erythromycin clarithromycin azithromycin
vanZF	vanZ variant found in the vanF gene cluster
Virulence factors	
bas3109	thiol-activated cytolysin
nheA	non-hemolytic enterotoxin A
nheB	non-hemolytic enterotoxin B
nheC	non-hemolytic enterotoxin C
inhA	immune inhibitor A metalloprotease
capB	involved in Poly-gamma-glutamate synthesis
capC	involved in Poly-gamma-glutamate synthesis
capA	required for Poly-gamma-glutamate transport
dep/capD	gamma-glutamyltranspeptidase necessary for polyglutamate anchoring to peptidoglycan
capE	involved in Poly-gamma-glutamate synthesis
lef	anthrax toxin lethal factor precursor
pagA	anthrax toxin moiety protective antigen
cya	calmodulin sensitive adenylate cyclase edema

595

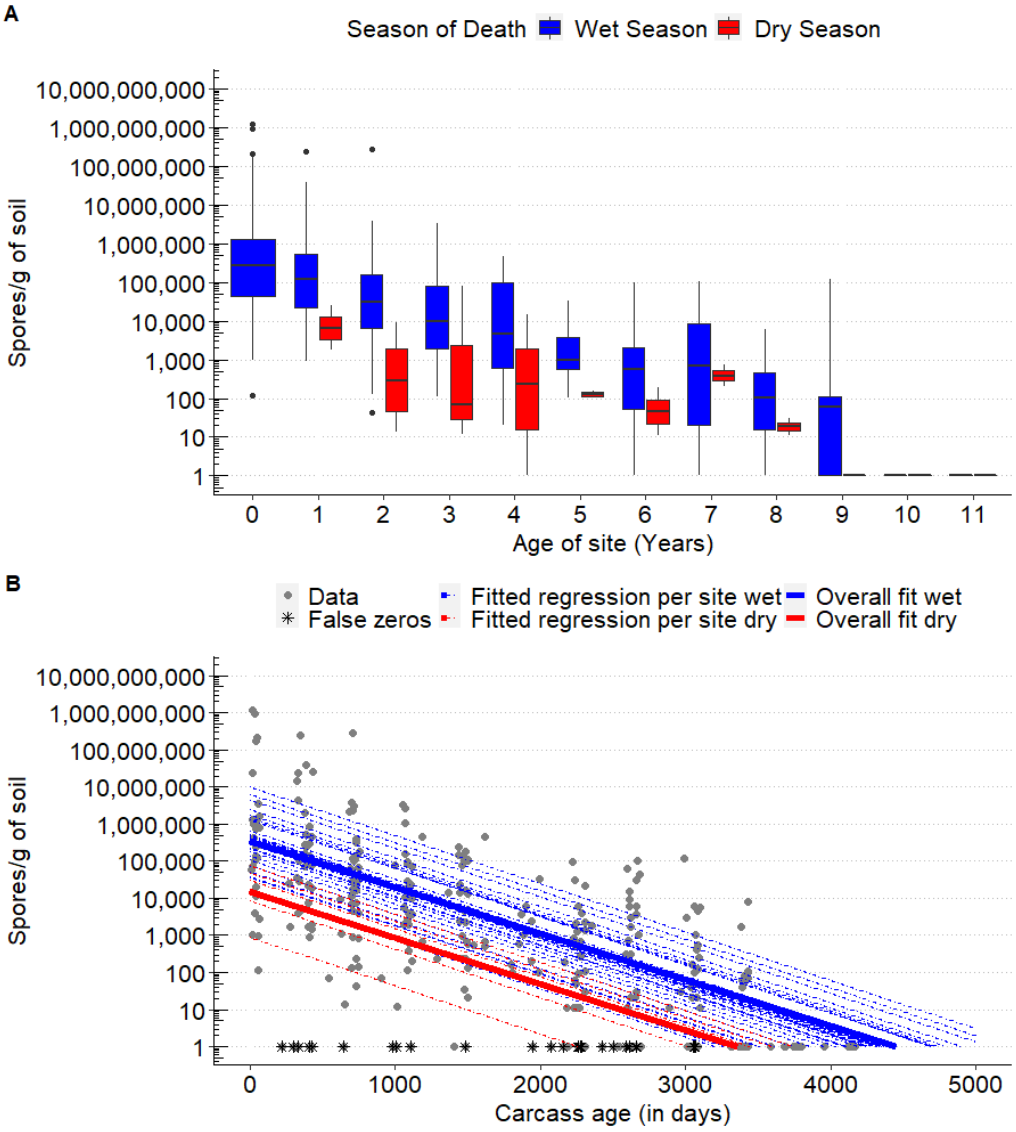

Fig. 1. Decline in *Bacillus anthracis* spore counts over time in surface soils at all monitored anthrax carcass sites in Etosha. A) The variation in spore concentrations over the 12-year dataset (0 is the year of death). Surface soils from all sites were culture-negative for *B. anthracis* by 10 years after death. B) Spore count data (gray dots) from each site fit by a linear mixed model of spore survival over time. The bold lines are the overall fit across sites for dry (red) and wet (blue) seasons respectively. The dashed lines are the fit for each of the 40 individual carcass sites, for sites formed in the wet (blue) or dry (red) season.

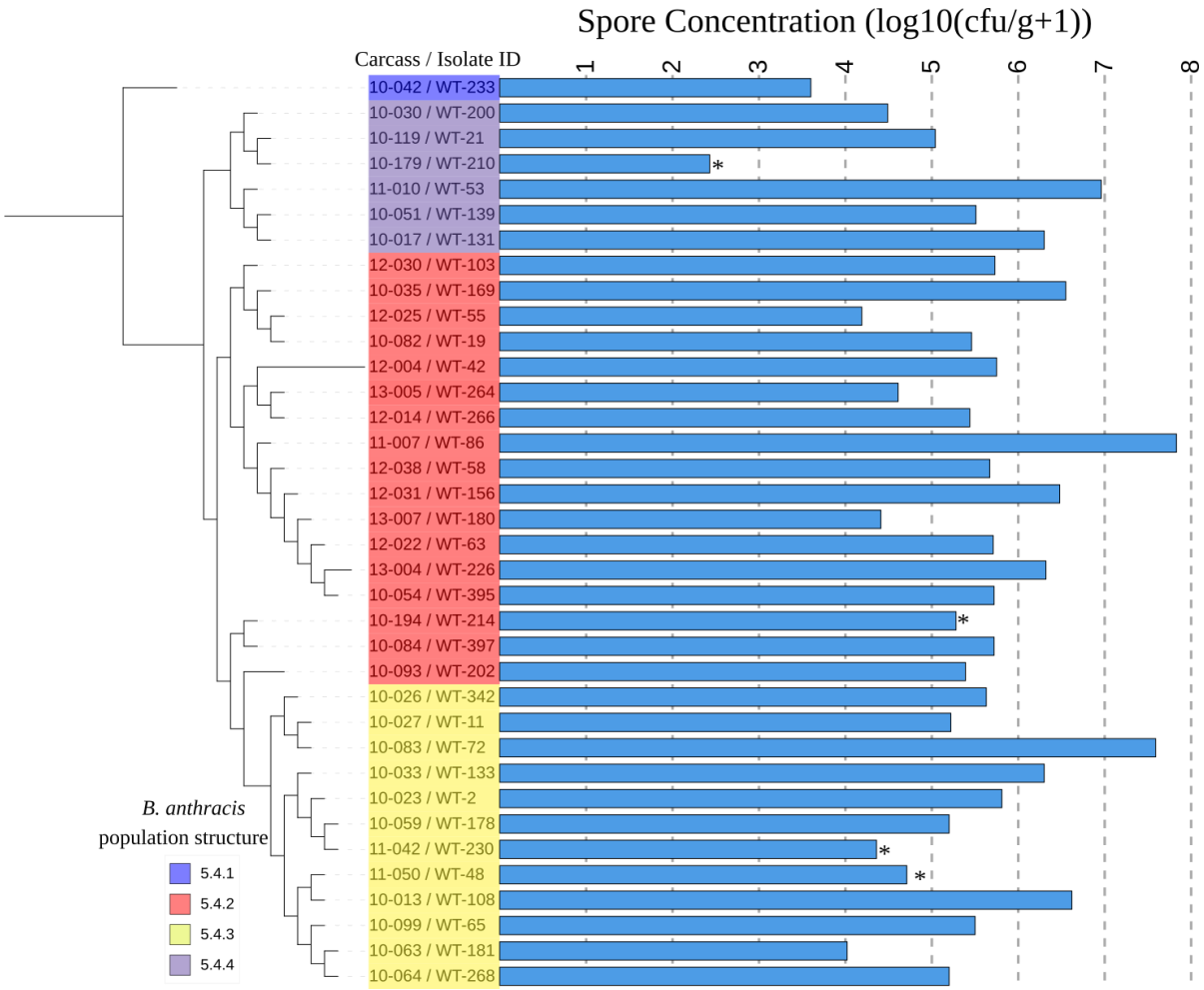

Fig. 2. Maximum likelihood tree of *Bacillus anthracis* isolates from plains zebra (*Equus quagga*) anthrax carcass sites in Etosha National Park, Namibia, and the estimated initial spore concentrations in soils at each carcass site (blue bars) from the y-intercept for each carcass site fit in the linear mixed model (Fig. 1). The population structure of *B. anthracis* isolates was based on 14,759 core chromosomal single nucleotide polymorphisms (SNPs). The cluster designation (5.4.1-5.4.4) was assigned using the program hierBAPS using an extension of the *B. anthracis* classification by [45]. Carcass sites formed in the dry season are marked with *.

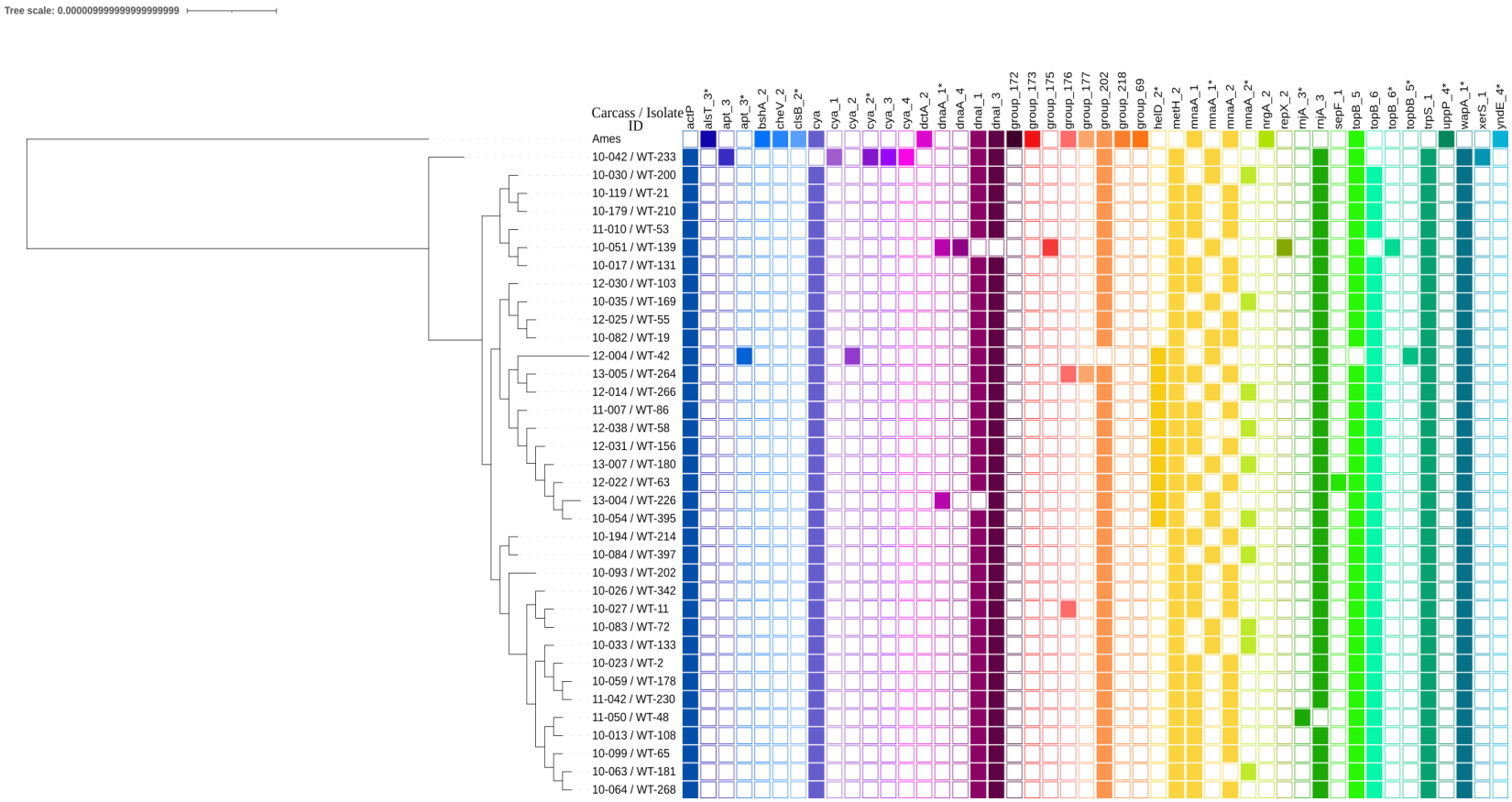

Fig. 3: The presence/absence of annotated accessory genes identified in a pangenome analysis comprising the Ames reference strain and isolates of *Bacillus anthracis* from plains zebra (*Equus quagga*) anthrax carcasses in Etosha National Park, Namibia. The figure excludes hypothetical genes detected in the pangenome analysis. Filled boxes indicate presence of the gene and no fill indicates the absence of a gene. The *B. anthracis* Ames genome was included as a comparison.

** Indicates non-unique gene name families*

620 Table S1: *Bacillus anthracis* starting spore concentrations (i.e., intercept) and decline rate (i.e,
621 slope) derived from the linear mixed model with random intercept and fixed slope of spore/g of
622 soil from anthrax carcass site in Etosha.

ID	Season	intercept	Slope	ID	Season	intercept	Slope
10-020	Wet	4.166	-0.001	12-038	Wet	5.627	-0.001
10-042	Wet	4.252	-0.001	10-069	Wet	5.629	-0.001
10-179	Dry	4.328	-0.001	10-026	Wet	5.657	-0.001
10-063	Wet	4.559	-0.001	10-093	Wet	5.659	-0.001
12-025	Wet	4.577	-0.001	12-004	Wet	5.675	-0.001
13-007	Wet	4.709	-0.001	10-054	Wet	5.690	-0.001
13-005	Wet	4.874	-0.001	12-030	Wet	5.717	-0.001
10-030	Wet	5.028	-0.001	10-023	Wet	5.813	-0.001
10-119	Wet	5.146	-0.001	11-042	Dry	5.871	-0.001
10-027	Wet	5.261	-0.001	11-050	Dry	6.065	-0.001
10-219	Dry	5.313	-0.001	10-033	Wet	6.107	-0.001
10-064	Wet	5.334	-0.001	13-004	Wet	6.122	-0.001
10-059	Wet	5.342	-0.001	10-017	Wet	6.125	-0.001
12-024	Wet	5.353	-0.001	10-035	Wet	6.170	-0.001
10-082	Wet	5.454	-0.001	12-031	Wet	6.246	-0.001
12-022	Wet	5.514	-0.001	10-194	Dry	6.259	-0.001
10-099	Wet	5.534	-0.001	10-013	Wet	6.389	-0.001
12-014	Wet	5.537	-0.001	11-010	Wet	6.642	-0.001
10-051	Wet	5.559	-0.001	10-083	Wet	6.801	-0.001
10-084	Wet	5.594	-0.001	11-007	Wet	6.994	-0.001

623

624 Table S2: Gene_presence_absence.csv

625

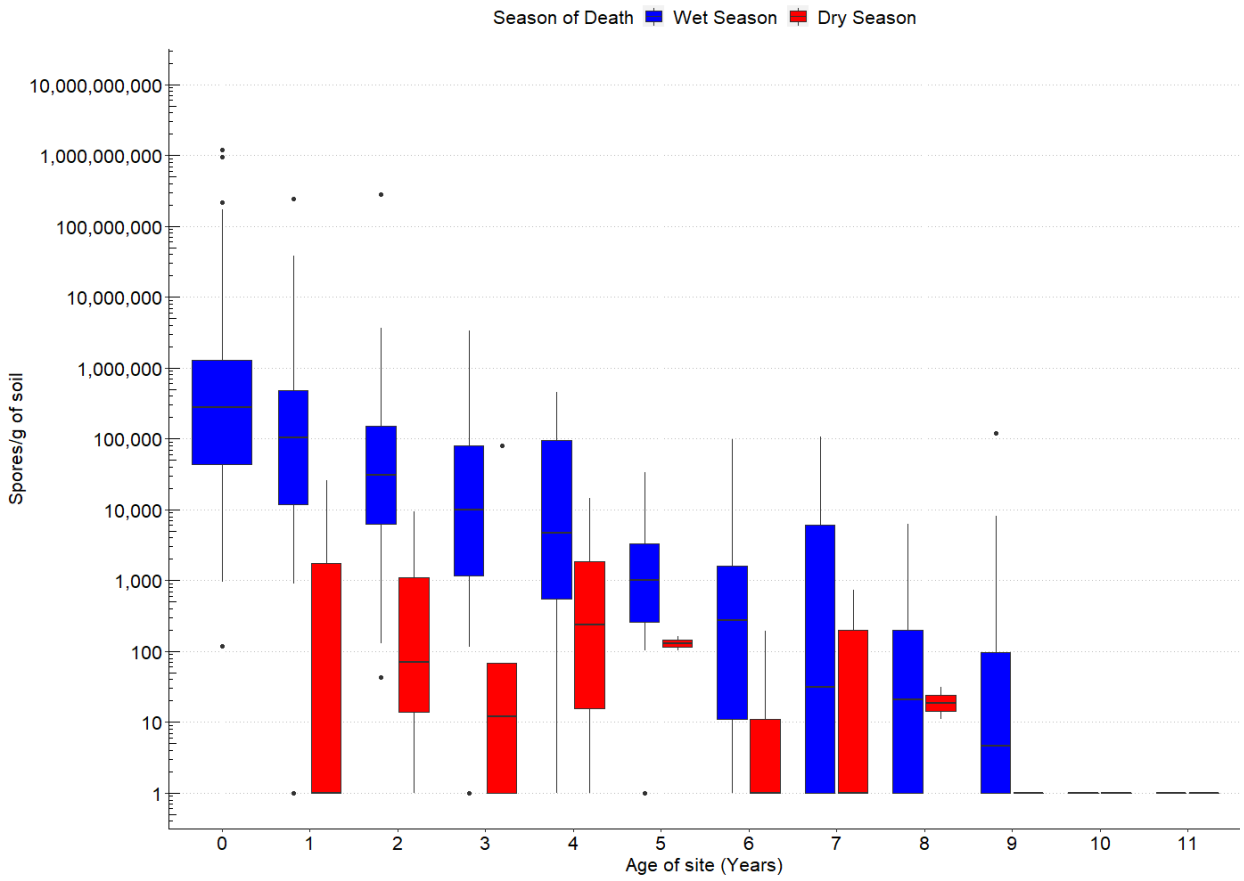

Fig. S1. Decline in *Bacillus anthracis* spore counts over time in surface soils at all monitored anthrax carcass sites in Etosha National Park, Namibia. These box plots include all sample data, including the "false zeros" (counts of 0 spores/g of soil in one year followed by a count account greater than 1 the following year) that were excluded from the statistical analysis and in Fig. 1. The variation in spore concentrations over the 12-year dataset (0 is the year of death). Surface soils from all sites were culture-negative for *B. anthracis* by 10 years after death.