**TITLE: Effects of experimental exclusion of scavengers from anthrax-infected herbivore carcasses on *Bacillus anthracis* sporulation, survival and distribution**

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## ABSTRACT

Scavenging of anthrax carcasses has long been hypothesized to play a critical role in the production of the infectious spore-stage of *Bacillus anthracis* after host death, though empirical studies assessing this are lacking. We compared *B. anthracis* spore production, distribution and survival at naturally occurring anthrax herbivore carcasses that were either experimentally caged to exclude vertebrate scavengers or unmanipulated. We found no significant effect of scavengers on soil spore density ($p > .05$). Soil stained by terminally hemorrhaged blood and by non-hemorrhagic fluids exhibited high levels of *B. anthracis* spore contamination (ranging from $10^3$-$10^8$ spores per gram) even in the absence of vertebrate scavengers. At the majority of carcass sites, we also found that spore density in samples taken from hemorrhagic-fluid stained soil continued to increase for longer than 4 days after host death. We conclude that scavenging by vertebrates is not a critical factor in the life cycle of *B. anthracis* and that anthrax control measures relying on deterrence or exclusion of vertebrate scavengers to prevent sporulation are unlikely to be effective.


**Keywords:** anthrax, *Bacillus anthracis*, disease control and management, maggot, putrefaction, scavenger, spore.

## INTRODUCTION

Anthrax is a fatal disease of all mammals and some avian species caused by the spore-forming bacterium *Bacillus anthracis*. Herbivorous mammals are most commonly affected, with anthrax causing a major burden of livestock and wildlife mortality worldwide (1, 2). Upon entry into a susceptible host via ingestion or inhalation, environmentally persistent and metabolically dormant *B. anthracis* spores are transported to the lymph nodes where they germinate. The resulting vegetative cells rapidly reproduce in the blood and produce toxins, killing the host within days (3–5). Along with exposure-related host behavior, species variation in lethal doses, individual-specific variation in immunity, spore production (i.e., sporulation) and survival play a critical role in anthrax transmission (1, 5, 6).

The spore population arising from a carcass site depends on the terminal vegetative cell density at host death, their subsequent sporulation efficiency thereafter, and the subsequent survival of spores and putative vegetative extra-host reproduction over time. Lethal challenge experiments indicate that species less susceptible to *B. anthracis* exhibit greater levels of bacteraemia at death, presumably because their death requires higher toxin concentrations (7, 8). Sporulation success of vegetative cells depends on their local microenvironment. In general, spore-forming bacteria sporulate in response to nutrient-poor conditions (9–11). The sporulation process of *B. anthracis*' non-pathogenic relative, *B. subtilis*, has been used as a model for the former. Yet the particular signals that trigger sporulation in *B. anthracis* remain unknown (9, 10). The exposure of bodily fluids to the atmospheric environment at host death may help trigger sporulation of *B. anthracis* by disrupting the bicarbonate/CO2 equilibrium necessary for

66 toxin and capsule production, a process known to be negatively linked to sporulation (5,
67 12, 13).

68 Upon host death anaerobic putrefying bacteria from the gastrointestinal tract also
69 begin the process of decomposition (14). These bacteria may inhibit sporulation by
70 antagonizing vegetative *B. anthracis* cells. Support for this comes from early
71 experimental work indicating that vegetative reproduction occurred in a variety of media
72 as long as they were sterilized (7, 15), that sporulation in blood taken from
73 experimentally infected anthrax carcasses was greatly reduced when exposed to
74 contaminated air (16), and that the *B. anthracis* vegetative cell population diminished at
75 the onset of putrefaction (17, 18). Processes, such as terminal hemorrhaging and
76 scavenging, that release *B. anthracis*-laden blood into the aerobic environment, provide
77 an escape from microbial competition with anaerobes.

78 Given the above body of evidence, it is commonly held that "if the carcass is not
79 opened the anaerobic decomposition and acidification will kill the contained vegetative
80 cells within 4 days resulting in minimal environmental contamination" (5), with this time
81 window extrapolated from experiments suggesting that *B. anthracis* vegetative cells
82 sporulate or die within 72 hours of host death (16, 19, 20). To our knowledge, however,
83 only anecdotal evidence exists supporting the inability of vegetative cells to survive or
84 sporulate in unopened carcasses (1, 2, 5, 21–26). Nevertheless, scavenging is frequently
85 considered to play a critical role in the production of spores at carcass sites. The single
86 study to empirically assess the relationship between scavengers and spore production
87 found no significant difference between spore contamination at two carcasses, one of
88 which was minimally scavenged while the other had been fully scavenged (27).

Additionally, because vegetative cells do not survive scavengers' digestive systems (1, 20, 28, 29), scavengers may not only facilitate contamination but may also cleanse carcass sites. Consumption of carcass material early after host death may reduce the quantity of vegetative cells available to sporulate. Nevertheless, unscavenged carcasses are generally thought to exhibit low levels of contamination so the existing consensus is that preventing scavenging minimizes spore production (1, 2, 5). When burning of carcasses (considered the optimal carcass decontamination measure) is logistically infeasible, the current recommended approach is therefore to either spray carcasses with considerable quantities of 5-10% formalin or cover them with locally-available obstructive materials (e.g., thorn bushes, tarpaulins, branches) (1, 2, 30).

Given that microbiological dynamics at naturally occurring carcasses may differ substantially from laboratory models, we decided to further investigate the role of scavengers in spore production. We assessed how these factors differed between carcasses experimentally excluded from vertebrate scavengers and unmanipulated control carcasses, taking advantage of endemically-occurring anthrax in the herbivores of Etosha National Park (ENP), Namibia, to enable access to naturally occurring anthrax-generated carcasses.

## METHODS

### Study area

This study was conducted on the Okaukuejo plains of ENP where anthrax is seasonally endemic and causes significant mortality in zebra (*Equus quagga)*, springbok (*Antidorcas marsupialis*), elephant (*Loxodonta africanus*), wildebeest (*Connochaetes taurinus*), and

occasionally other herbivores (20). The carrion produced by anthrax deaths feeds a diverse assemblage of vertebrate scavengers including most frequently black-backed jackal (*Canis mesomelas*), spotted hyena (*Crocuta crocuta*), white-backed vulture (*Gyps africanus*), lappet-faced-vulture (*Torgos tracheliotos*), marabou storks (*Leptoptilos crumeniferus*) and occasionally lion (*Panthera leo*), black crows (*Corvus capensis*), pied crows (*Corvus albus*), and various other raptor species (20, 31, 32).

**Carcass inclusion criteria and randomization**

Zebra carcasses found between March 2009 and March 2010 were eligible for inclusion in the study if they were found on the date of death (which could be determined accurately due to a separate camera trap study of carcass consumption rates (31)), they were entirely unscavenged or had only minor openings by scavengers at the anus, eye, or abdomen and the probable cause of death was anthrax as determined by blood smear microscopy, terminal hemorrhaging, lack of clotting, and absence of any other suspected cause of death (*i.e.* predation signs). Anthrax diagnosis was later confirmed via selective bacterial culture (29) and confirmation of possession of pXO1 and pXO2 plasmids (33). Eligible carcasses were assigned to treatments using block randomization in blocks of eight where blocks were determined by date of death and were used to ensure even distribution of carcasses in case of small sample size. Initially, only zebra carcasses were considered for inclusion. Due to the rarity of fresh carcasses, inclusion criteria were modified in May 2009 to allow other herbivore carcasses to be eligible for inclusion if they fitted the above criteria.

**Electrified cage exclosures**

To experimentally exclude all vertebrate scavengers from carcasses we built an electrified cage exclosure (Figure 1). The exclosure was constructed from six 3 x 1.2 m farm gates (four as sidewalls and two used as roofs) covered in diamond mesh fencing. A skirt of chicken wire was then wrapped around the sides to prevent intrusion by digging. Four electric fencing wires (two pairs of positive and grounded wires 1cm apart) were offset 10cm from the sidewalls at 30 cm and 70cm above the ground and powered to 6-8 kV by an energizer (125 A 12 V model, MEPS Electric Fence Systems, South Africa) run off of a deep cycle car battery attached to the exclosure roof.

**Sampling procedure**

After following routine protocol for anthrax diagnostics in Etosha National Park (sterile throat swabs in the nasal turbinates), two 0.5 m metal fence stakes were inserted into the ground on an axis running from the mouth to the anus, 2 m from the animal on either side. The carcass location was mapped out on a coordinate system based on these stakes, noting locations saturated by terminally hemorrhaged blood and non-hemorrhagic fluid. Soil was then collected from: (1) soil stained by blood from terminal hemorrhaging; (2) unstained soil within a 1 m radius of the carcass; (3) unstained soil within a 3 m radius of the carcass; and (4) soil stained by non-hemorrhagic fluid. For most carcasses, however, the latter region was not evident on the date of death and only mapped and sampled at later occasions. Soil samples at 50 m away from the carcass were collected as negative controls for cross-contamination. All soil was collected from the surface (< 1 cm deep) using sterile spoons. To reduce the variance due to spatial heterogeneity, each sample was comprised of twenty 5-10 g sub-samples distributed randomly throughout the sample area. Fewer sub-samples were taken when the area to be sampled was

prohibitively small. Following sampling, exclosures were deployed on carcasses assigned to the exclosure group.

Carcasses were resampled again 4 days after death. For carcasses in the exclosure group, the exclosure was removed during this visit to permit resampling and to allow scavenging to commence. Carcasses were then re-sampled 8-11 days after death and again at approximately 1, 6, and 12 months after death. Not all carcasses were sampled at all sample intervals due to logistical constraints. Samples were immediately frozen at -20 °C and then later thawed, mixed to homogenize subsamples, and aliquoted into 5 g samples.

The spore quantification assay was conducted as follows. After weighing sterile 2 ml eppendorf tubes to which 1 ml sterile deionized water (SDW) had been added, approximately 0.5 g of a sample was added to each tube and the tubes weighed again. The tubes were then vortexed strongly and put in heating block at 65 $^{o}$C for 15 min. Following further vortexing, $10^{-1}$ and $10^{-2}$ dilutions (0.1 ml transferred to 0.9 ml SDW in 2 ml eppendorfs) were made and 0.1 ml volumes of these and of the undiluted sample were spread on duplicate trimethoprim-sulfamethoxazole polymyxin blood agar (TSPBA) and, initially, polymyxin-lysozyme-EDTA-thallous acetate agar (PLET) plates (2). Later it became apparent that agreement between TSPBA and PLET was good and use of PLET was discontinued. Colony counts on TSPBA plates were determined after overnight incubation at 36 ± 1 $^{o}$C and those on PLET after 36-48 h incubation, also at 36 ± 1 $^{o}$C. Those performing the laboratory assays blinded to each samples' metadata and all spore densities were quantified in duplicate, with duplicates averaged to yield the final spore densities analyzed averaged.

**Statistical Analysis**

We fitted a generalized additive mixed model (GAMM) using the R package 'gamm4' to spore density data to assess the effect of experimental exclusion of scavengers as well as to determine how spore density varied spatially and temporally (34). Based on a preliminary exploration of residual plots, we chose to fit log(spore density) with a Poisson link function and each carcass modeled as a random effect. We modeled experimental group as a fixed effect with no interactions. We used a sampling occasion by sample area interaction smoothing term to flexibly model distinct temporal trends in spore density for each sample area. We chose a smoother of basis 4 using Akaike's Information Criterion. Given our small carcass sample size, our primary analysis included all carcasses regardless of species and soil type but conducted a sensitivity analysis fitting the same model but excluding data from carcasses of outlying species or at outlying soil types. While several other environmental factors are known to be relevant to sporulation and survival (i.e., temperature, UV radiation, humidity, and rainfall), we were unable to include them in our analysis because diurnal and seasonal variations within sites varied far more than any variations between sites.

## RESULTS

Seven zebra, one springbok, and one wildebeest carcass were included in this study. Four zebra carcasses were in the exclosure treatment, while the rest of the carcasses were in the control group. All caged carcasses were entirely unscavenged except one that had minor openings at the abdomen and anus. All nine carcasses were anthrax positive as confirmed by culture and genetic diagnostics. However, none of the

soil samples taken from the springbok carcass contained *B. anthracis* spores and were therefore excluded from the analysis. All carcasses included in the study were located in the same soil type except for one outlier (soil C5 is a carbonate-rich loamy regosol/leptosol of aeolian origin, and D2 is carbonate-rich eutric-free fluvisol; see (35) for more details).

The fitted GAMM is displayed along with sample spore densities (averaged between duplicates) in Figure 2 on a log(spg) scale with the tabular data provided as an online supplementary file. The estimated coefficient (95% Wald confidence interval) associated with the exclosure treatment was 0.30 (-0.16, 0.75), indicating that the average effect of excluding scavenger increased spore density, though this was not statistically significant ($p > 0.05$). This result did not qualitatively differ when performing the same analysis but excluding the single wildebeest carcass (0.31 (-0.24, .86)), considering the partially scavenged, caged carcass to be in the control group (0.030 (-0.56, .62)), or excluding the carcass in outlying soil type (0.23, (-0.41, 0.86)).

The fitted smoother functions of spore density over time differed among sample areas. In soil stained by terminally hemorrhaged blood, *B. anthracis* spore density generally increased from about 0-100 spores per gram (spg) on the date of death to about $10^5$-$10^8$ spg 4 days later. Between 4 days to 8 days post-death spore density in these samples increased again by factors ranging from 1.9-53 at four of six sites. Spore density then generally decayed over the next 1-6 months but displayed a slight increase in many samples after a year. In the 1m radius samples, spore density began at 0-100 spg and displayed an increasing trend over time, though never increasing greater than $10^5$ spg except for one outlier. The 3m radius samples rarely contained spores and never

exceeding $10^4$ spg. Spores were not found in soil stained by non-hemorrhagic fluid on the date of death for the single carcass at which such soil was visible at that time. At subsequent samples, spore density in this sample type varied between $10^3$ and $10^7$ spg, except for three samples that tested negative.

## DISCUSSION

Our results yielded two major conclusions. Firstly, vertebrate scavenging is not critical for spore production at anthrax carcass sites. Secondly, high *B. anthracis* spore densities (i.e. > $10^5$ spg) were, with rare exception, only found in soil stained by either blood or other carcass fluids.

As a further speculation we note that while it has previously been noted that *B. anthracis* sporulation occurs within the first 72 hours after host death (16, 19, 20), we noted a relatively consistent trend of spore density increasing between samples taken four days and 8 days after host death in blood-stained soil. While redistribution of spores between sampling occasion and sampling error could explain these patterns, we note that the soil spore density in several samples increased by tens of times between the 4 and 8 day sampling occasion. Given that blood-stained patches mapped out on the date of death were relatively small and surrounded by soil with lower spore concentrations, we would expect spores in such patches to be diluted and not concentrated over time. We believe further work is warranted to examine the duration of sporulation.

If sporulation continued after 4 days, our experimental exclusion of scavengers for only four days after host death would not have captured the entire sporulation time frame. Nevertheless, continuation of sporulation 4-8 days after host death cannot explain

249 the similarity in spore concentrations between experimental treatment groups since
250 comparable or greater spore densities were already found in samples at the 4-day sample
251 (i.e., before scavenging could have occurred in the exclosure group).

252 Thus, the absence of any significant quantitative differences and apparent
253 qualitative similarity between experimentally caged and control carcasses for the first 4
254 days after host death is at odds with the long-held view that scavenging plays a
255 significant role in *B. anthracis* spore contamination at carcass sites. While our sample
256 size was small due to the logistical difficulties associated with locating fresh anthrax
257 carcasses before vertebrate scavengers arrive, the similar spore concentrations at even a
258 few caged carcasses suggests that carcasses do not need to be opened by vertebrate
259 scavengers for large-scale spore production to occur.

260 If extravasation of carcass fluids indeed plays an important role in environmental
261 spore contamination, then scavengers' disarticulation and movement of carcasses may
262 reduce contamination around the original carcass site with compensatory contamination
263 of satellite sites, which were not sampled in our study. However, satellite site
264 contamination levels are likely to reflect those found in our 1 m or 3 m radius sampling
265 zones (in which carcass materials were also dragged and eaten) which exhibited much
266 lower levels of contamination ($< 10^5$ spg) compared to soil saturated in blood or other
267 carcass fluids (up to $10^8$ spg, respectively).

268 The consistently high spore densities found in soil saturated by non-hemorrhagic
269 carcass fluid was an unexpected result, particularly at caged carcasses given the common
270 assumption that *B. anthracis* vegetative cells would not be able to exit unscavenged
271 carcasses except via terminal hemorrhaging. Extravasation of liquid from the carcass can

only occur from natural orifices except to the extent by which the skin is ruptured (14). That unscavenged, caged carcasses exhibited substantial visible areas of soil clearly saturated by carcass fluids suggests that vertebrate scavenging is not necessary for skin to rupture (Figure 1). Carter and Tibbett (14) note that both the bloating caused by gases produced via anaerobic metabolism during putrefaction and maggot feeding activity are capable of independently rupturing carcass skin. In addition to allowing carcass fluid to purge into the soil, skin ruptures also allow air into the carcass and may thereby facilitate sporulation both inside and outside the carcass. While we do not know the timescale at which ruptures occurred in our study, it is clear that a substantial population of vegetative *B. anthracis* cells survived the putrefactive phase up until skin rupture or sporulated beforehand.

Our exclusion of vertebrate scavengers permitted a substantial increase in blowfly activity at caged carcasses (Figure 1), which may have compensated for the formers' role in opening the carcass and facilitating *B. anthracis* spore production. However, bloating alone (i.e. without maggot activity) may be sufficient to rupture skin, depending on temperature and skin thickness. Blowflies have been suspected to play an important role in anthrax transmission in Kruger National Park, South Africa, due to their propensity to ingest material at carcass sites and then regurgitate it on vegetation at heights preferred by the browsing species most frequently infected in that system (36, 37). In ENP, however, while we observed similar blowfly feeding preferences (but far fewer flies), the outbreaks occur primarily in grazers and appear more likely to be due to direct ingestion of contaminated soil (6, 20).

294 Soil spore density persisted in all sample types, though with varying consistency
295 as found in previous studies (20). While contamination levels generally decreased in the
296 months following host death, samples from fluid-saturated soil still occasionally
297 exhibited densities as high as $10^5$-$10^6$ spg a year after host death. Slight increases in
298 contamination levels found in soil within a 1 m radius around the carcass is likely due to
299 mixture of fluid-saturated soil and nearby soil over the course of the year, or simply an
300 artifact of sampling noise, though we cannot exclude vegetative reproduction in the soil.
301 Given the logistical limitations of a field experiment, we were unable to assess
302 several other relevant factors affecting spore production and distribution. Temperature
303 affects both vegetative cell survival and sporulation efficiency (16). Ambient
304 temperatures during the first 8 days after carcass death were in the range allowing
305 sporulation (15-38°C), but varied more with time of day than between carcasses and thus
306 we were unable to include this in our analysis. Further, carcass and ambient temperatures
307 may differ substantially, in large part due to heat generated by maggot activity (38). In
308 addition to soil spore density in each of the four sample areas, the exposure risk to
309 susceptible hosts will additionally depend on the area of contamination and a host's
310 behavioral propensity to approach that area (6). The area of fluid-saturated soil changes
311 dynamically while the carcass is consumed, and may be distributed at satellite sites by
312 scavengers. Soil that has been incidentally contaminated via movement of carcass
313 materials will cover an even wider area and is even more difficult to measure but will
314 have much lower soil spore density, which may render it irrelevant to the transmission
315 process.

316 **Conclusion**

317 By comparing spore concentrations at experimentally caged and unmanipulated
318 naturally occurring anthrax carcasses, we demonstrate that vertebrate scavengers do not
319 play a critical role in the sporulation process of *B. anthracis*. Our results also suggest that
320 contamination of soil by fluid purged from carcasses via putrefactive bloating or maggot
321 activity exhibit soil spore densities close to those in blood-saturated soil. We thus suggest
322 that anthrax control measures aimed at deterring scavengers to prevent sporulation appear
323 unwarranted.
324

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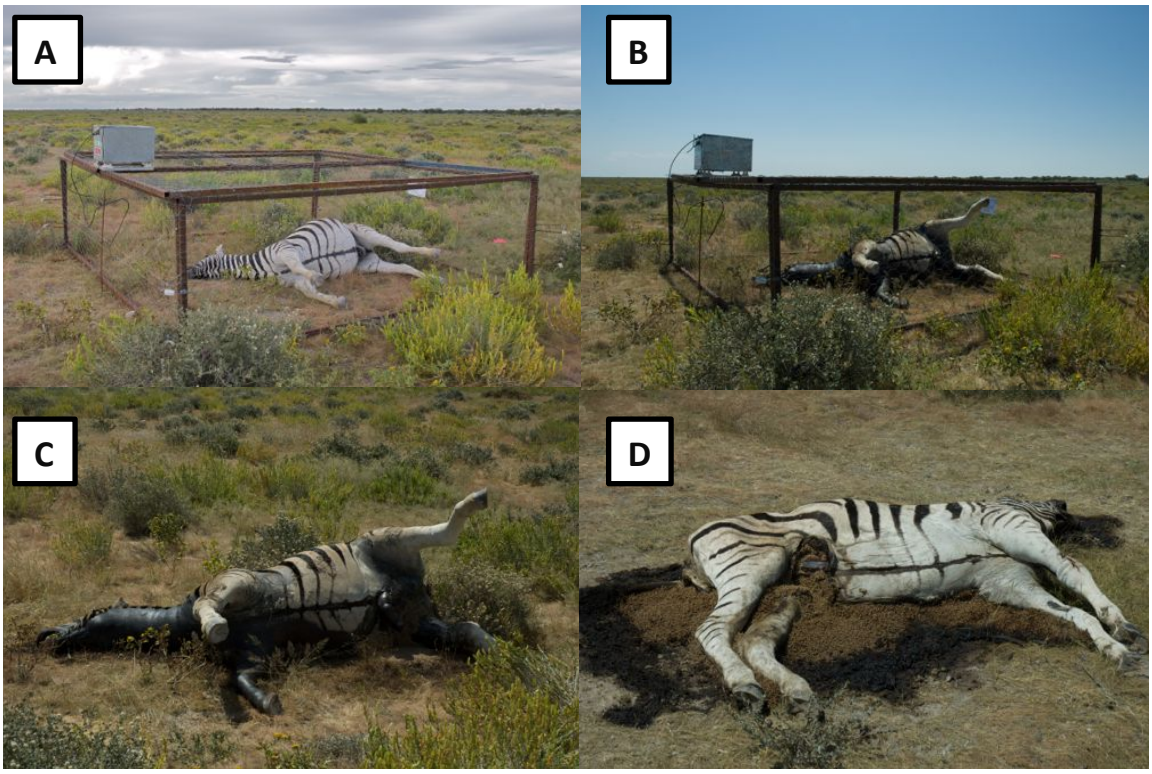
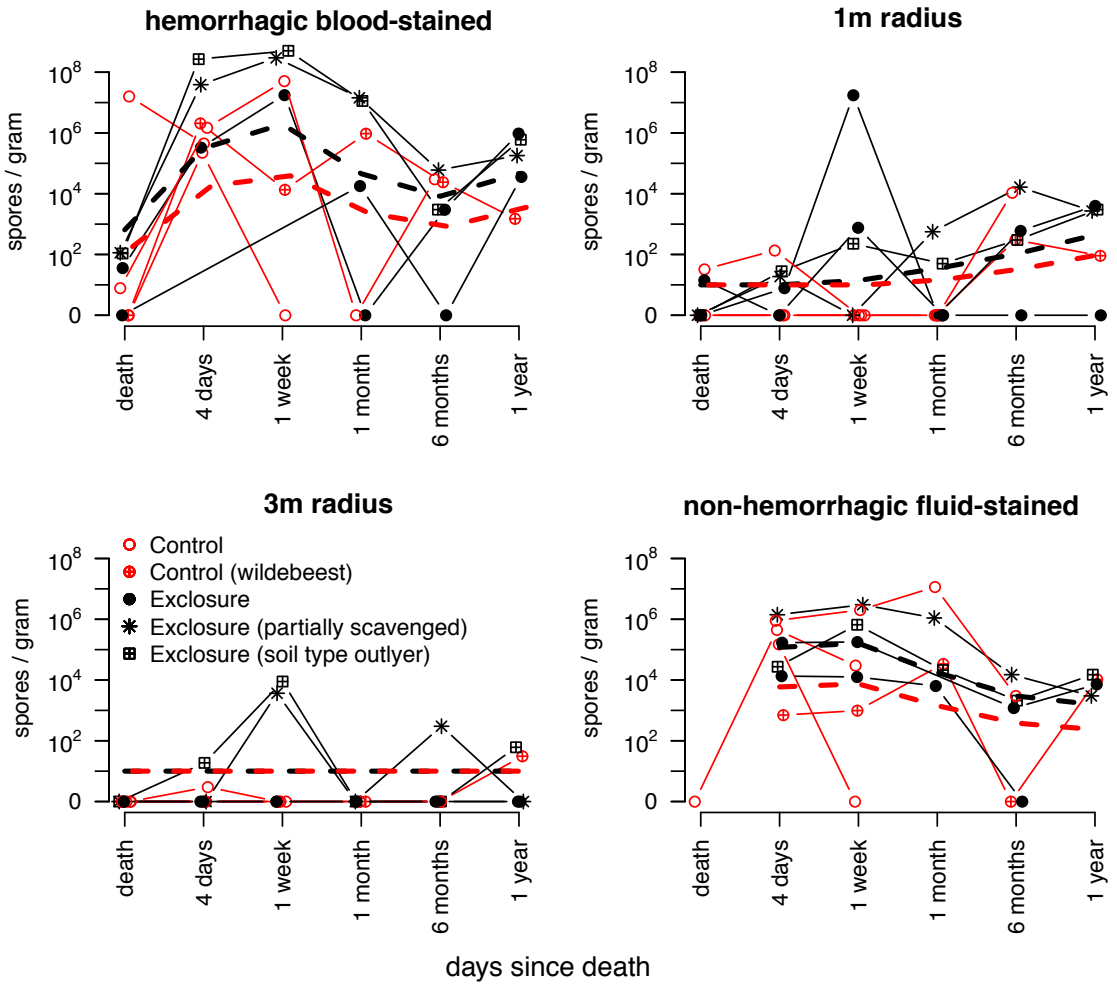



**Figure 1.** The site of an anthrax-positive zebra carcass that has been experimentally caged from the date of death is shown on the date of death (A) and 4 days afterwards after substantial bloating and when the cage was removed (B). A close-up of the same carcass (C) better displays the soil saturated by non-hemorrhagic fluid (the blackened disturbed area surrounding the carcass), which exhibited high levels of *B. anthracis* spore contamination. A carcass that had been slightly opened prior to caging (D) exhibited a larger area of soil saturated by non-hemorrhagic fluid as well as substantial maggot activity 4 days after host death.




460

461 **Figure 2.** Spores per gram plotted on a log scale by days since carcass death,
462 experimental exclosure (black) or control (red) treatments, and sample area (with panels
463 showing results for soil collected from hemorrhagic fluid-stained soil, soil unstained by
464 carcass fluid taken from within a 1m and 3m radius of the carcass, and soil stained by
465 non-hemorrhagic fluid). Each solid line is from a single carcass, with points representing
466 samples. The dashed lines show generalized additive mixed model fitted to the data.
467 Carcasses in the 'exclosure' treatment were excluded from vertebrate scavenging up until
468 second sample (4 days after death) while ‘control’ carcasses were unmanipulated. The
469 asterisks represent a carcass in the exclosure treatment group that had been scavenged for
470 approximately an hour prior to being caged. All carcasses were plains zebra except for

471 one blue wildebeest. All carcasses were in the same soil type except for one carcass as
472 indicated by the legend.