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Effects of Experimental Exclusion of Scavengers from Carcasses of Anthrax-Infected Herbivores on *Bacillus anthracis* Sporulation, Survival, and Distribution

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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 left unmanipulated. We found no significant effect of scavengers on soil spore density ($P > 0.05$). Soil stained with terminally hemorrhaged blood and with nonhemorrhagic fluids exhibited high levels of *B. anthracis* spore contamination (ranging from 10^3 to 10^8 spores/g), even in the absence of vertebrate scavengers. At most of the carcass sites, we also found that spore density in samples taken from hemorrhagic-fluid-stained soil continued to increase for >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.

Anthrax , a fatal disease of all mammals and some avian species, is caused by the spore-forming bacterium *Bacillus anthracis*. Herbivorous mammals are most commonly affected, with anthrax causing a burden of major mortality among livestock and wildlife populations 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, and 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, the subsequent sporulation efficiency, the subsequent survival of spores, and putative vegetative extrahost reproduction over time. Lethal-challenge experiments indicate that species less susceptible to *B. anthracis* exhibit greater levels of bacteremia 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*' nonpathogenic 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-CO_2 equilibrium necessary for toxin and capsule production, a process known to be negatively linked to sporulation (5, 12, 13).

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

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

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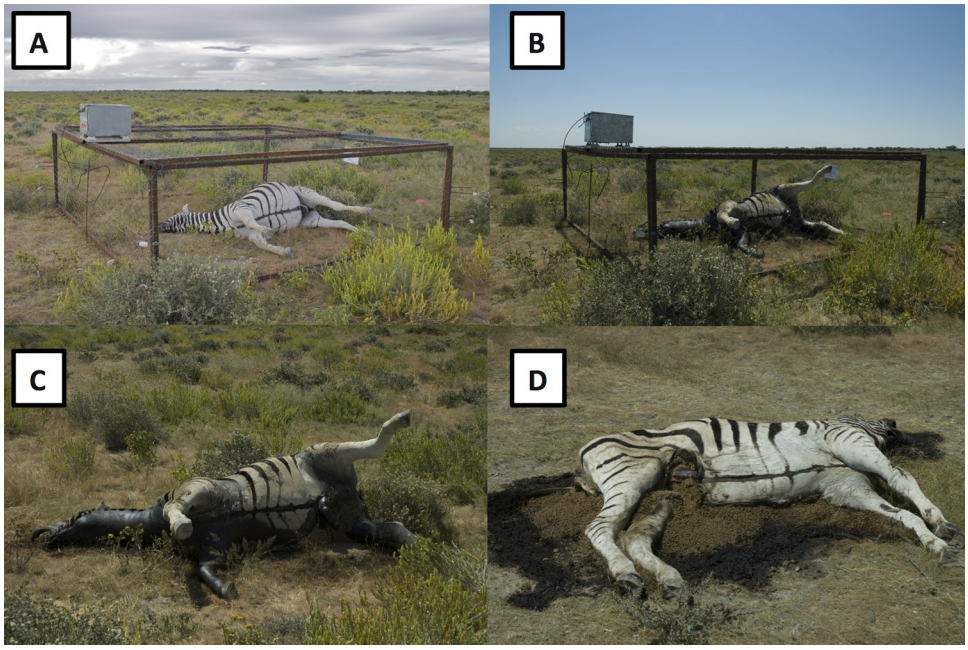

FIG 1 The site of an anthrax-positive zebra carcass that has been experimentally caged from the date of death is shown on the date of death (A) and 4 days later, after substantial bloating and when the cage was removed (B). A closeup of the same carcass (C) better displays the soil saturated with nonhemorrhagic 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 with nonhemorrhagic fluid, as well as substantial maggot activity, 4 days after host death.

at two carcasses, of which one 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 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 to 10% formalin or cover them with locally available obstructive materials (e.g., thorn bushes, tarpaulins, branches) to deter scavengers (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.

MATERIALS AND METHODS

Study area. This study was conducted on the Okaukuejo plains of ENP, where anthrax is seasonally endemic and causes significant mortality in zebras (*Equus quagga*), springboks (*Antidorcas marsupialis*), elephants (*Loxodonta africanus*), wildebeests (*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 jackals (*Canis mesomelas*), spotted hyenas (*Crocuta crocuta*), white-backed vultures (*Gyps africanus*), lappet-faced vultures (*Torgos tracheliotos*), marabou storks (*Leptoptilos crumeniferus*), and occasionally lions (*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 this study if they were found on the date of death (which could be determined accurately because of a separate camera trap study of carcass consumption rates [31]); they were entirely unscavenged or had only minor openings made 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 the absence of any other suspected cause of death (i.e., predation signs). The anthrax diagnosis was later confirmed via selective bacterial culture (29) and confirmation of possession of plasmids pXO1 and pXO2 (33). Eligible carcasses were assigned to different treatments by randomization into blocks of eight that were determined by date of death and were used to ensure the even distribution of carcasses in the case of a small sample size. Initially, only zebra carcasses were considered for inclusion. Because of the rarity of fresh carcasses, the inclusion criteria were modified in May 2009 to allow other herbivore carcasses to be eligible for inclusion if they fit the above criteria.

Electrified cage exclosures. To experimentally exclude all vertebrate scavengers from carcasses, we built an electrified cage exclosure (Fig. 1). The exclosure was constructed from six farm gates (3 by 1.2 m; four were used as side walls, and two were 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 1 cm apart) were offset 10 cm from the side walls at 30 and 70 cm above the ground and powered to 6 to 8 kV by an energizer (125-A, 12-V model; MEPS Electric Fence Systems, Pretoria, South Africa) run off a deep-cycle car battery attached to the exclosure roof.

Sampling procedure. After the completion of the routine protocol for anthrax diagnostics in ENP (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 with terminally hemorrhaged blood and nonhemorrhagic fluid. The samples then collected were (i) soil stained by blood from terminal hemorrhaging, (ii) unstained soil within a 1-m radius of the carcass, (iii) unstained soil within a 3-m radius of the carcass, and (iv) soil stained by nonhemorrhagic fluid. For most of the carcasses, however, the latter region was not evident on the date of death and was mapped and sampled only at later occasions. Soil samples were collected 50 m away from the carcass and used as negative controls for cross-contamination. All soil was collected from the surface (<1 cm deep) with sterile spoons. To reduce the variance due to spatial heterogeneity, each sample consisted of 20 5- to 10-g subsamples distributed randomly throughout the sample area. Fewer subsamples 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 resampled 8 to 11 days after death and again at approximately 1, 6, and 12 months after death. Not all carcasses were sampled at all of the sampling intervals because of logistical constraints. Samples were immediately frozen at −20°C and later thawed, mixed to homogenize subsamples, and aliquoted into 5-g portions.

The spore quantification assay was conducted as follows. After a sterile 2-ml Eppendorf tube to which 1 ml sterile deionized water (SDW) had been added was weighed, approximately 0.5 g of a sample was added to the tube and the tube was weighed again. The tube was then vortexed strongly and put in a heating block at 65°C for 15 min. Following further vortexing, 10^{-1} and 10^{-2} dilutions (0.1 ml transferred to 0.9 ml SDW in a 2-ml Eppendorf tube) 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 the agreement between TSPBA and PLET was good and the use of PLET was discontinued. Colony counts on TSPBA plates were done after overnight incubation at 36 ± 1°C, and those on PLET plates were done after 36 to 48 h of incubation, also at 36 ± 1°C. Those performing the laboratory assays were blinded to each sample's metadata, and all spore densities were quantified in duplicate, with duplicates averaged to yield the final spore densities.

Statistical analysis. We fitted a generalized additive mixed model (GAMM) by 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). On the basis of 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 of the carcasses regardless of species and soil type but also conducted a sensitivity analysis fitting the same model but excluding data from carcasses of outlying species or on 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 were far greater than any variations between sites.

RESULTS

Seven zebra carcasses, one springbok carcass, 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 all were therefore excluded from the analysis. All of the carcasses included in this study were located on the same soil type, except for one outlier (soil C5 is a carbonate-rich loamy regosol/leptosol of aeolian origin, and D2, the outlying soil type, is carbonate-rich eutric-free fluvisol; see reference 35 for more details).

The fitted GAMM is displayed along with sample spore densities (averaged between duplicates) in Fig. 2 on a log(spores-per-gram [spg]) scale; for the tabular data, see the supplemental material. 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 scavengers increased the spore density, though this was not statistically significant ($P > 0.05$). This result did not qualitatively differ when the same analysis was performed while excluding the single wildebeest carcass (0.31 [−0.24, 0.86]), considering the partially scavenged, caged carcass to be in the control group (0.030 [−0.56, 0.62]), or excluding the carcass on an 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, the *B. anthracis* spore density generally increased from about 0 to 100 spg on the date of death to about 10^5 to 10^8 spg 4 days later. Between 4 and 8 days postdeath, the spore density in these samples increased again by factors ranging from 1.9 to 53 at four of six sites. Spore density then generally decayed over the next 1 to 6 months but displayed a slight increase in many samples after a year. In the 1-m radius samples, the spore density began at 0 to 100 spg and displayed an increasing trend over time, though never increasing to $>10^5$ spg, except for one outlier. The 3-m radius samples rarely contained spores and never exceeding 10^4 spg. Spores were not found in soil stained by nonhemorrhagic 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. First, vertebrate scavenging is not critical for spore production at anthrax carcass sites. Second, high *B. anthracis* spore densities (i.e., $>10^5$ spg) were, with rare exception, found only 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 h after host death (16, 19, 20), we noted a relatively consistent trend of spore density increasing between samples taken from blood-stained soil 4 and 8 days after host death. While redistribution of spores between sampling occasions 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 occasions. Given that blood-stained patches

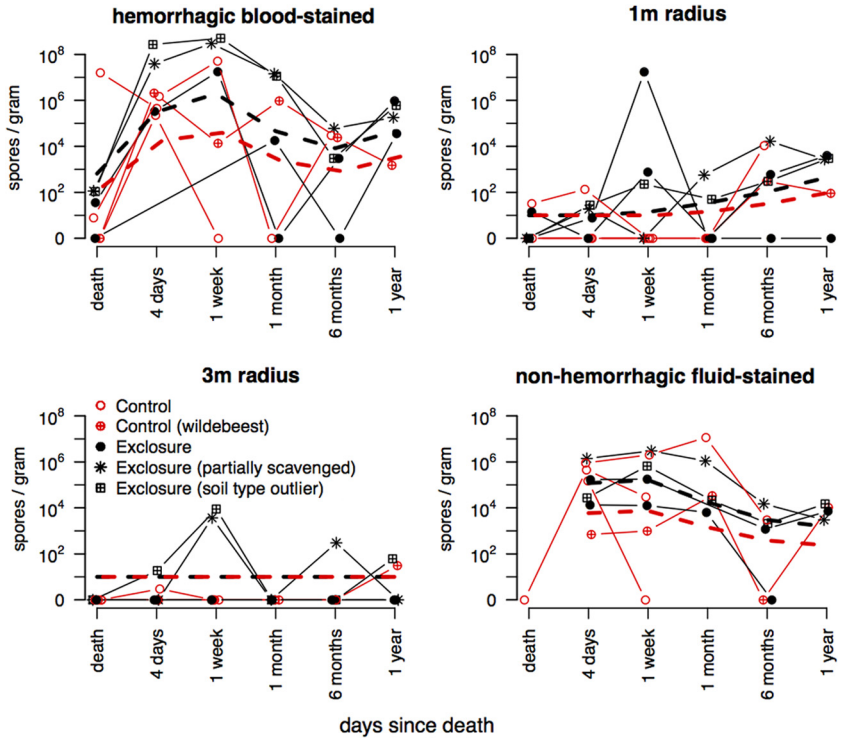

FIG 2 Numbers of spg plotted on a log scale by days since host death, experimental exclosure (black) or control (red) treatment, and sample area (with panels showing results for soil collected from hemorrhagic-fluid-stained soil, samples of soil unstained by carcass fluid and taken from within 1- and 3-m radii of the carcass, and soil stained by nonhemorrhagic fluid). Each solid line is from a single carcass, with points representing samples. The dashed lines show the GAMM fitted to the data. Carcasses in the exclosure treatment were excluded from vertebrate scavenging up until the second sampling point (4 days after death), while control carcasses were unmanipulated. The asterisks represent a carcass in the exclosure treatment group that had been scavenged for approximately an hour prior to being caged. All carcasses were those of plains zebras, except for one blue wildebeest. All carcasses were on the same soil type, except for one carcass (outlier).

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 4 days after host death would not have captured the entire sporulation time frame. Nevertheless, continuation of sporulation 4 to 8 days after host death cannot explain the similarity in spore concentrations between experimental treatment groups since comparable or greater spore densities were already found in samples at the 4-day sampling occasion (i.e., before scavenging could have occurred in the exclosure group).

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

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

The consistently high spore densities found in soil saturated with nonhemorrhagic carcass fluid was an unexpected result, particularly at caged carcasses, given the common assumption that vegetative *B. anthracis* cells would not be able to exit unscavenged carcasses except via terminal hemorrhaging. Extravasation of liquid from the carcass can occur only through natural orifices, except to the extent to which the skin is ruptured (14). That unscavenged, caged carcasses exhibited substantial visible areas of soil clearly saturated with carcass fluids suggests that vertebrate scavenging is not necessary for skin to rupture (Fig. 1). Carter and Tibbett (14) noted 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 time scale on 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 (Fig. 1), which may have compensated for the former's 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 the temperature and skin thickness. Blowflies have been suspected to play an important role in anthrax transmission in Kruger National Park, South Africa, because of 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 the direct ingestion of contaminated soil (6, 20).

Soil spore density persisted in all sample types, though with varying consistency, as found in previous studies (20). While contamination levels generally decreased in the months following host death, samples from fluid-saturated soil still occasionally exhibited densities as high as 10^5 to 10^6 spg a year after host death. Slight increases in contamination levels found in soil within a 1-m radius around the carcass are likely due to the mixing of fluid-saturated soil and nearby soil over the course of the year or are simply an artifact of sampling noise, though we cannot exclude vegetative reproduction in the soil.

Given the logistical limitations of a field experiment, we were unable to assess several other relevant factors affecting spore production and distribution. Temperature affects both vegetative cell survival and sporulation efficiency (16). The ambient temperature during the first 8 days after host death was in the range allowing sporulation (15 to 38°C) but varied more with the time of day than between carcasses, and thus we were unable to include this in our analysis. Further, carcass and ambient temperatures may differ substantially, in large part because of heat generated by maggot activity (38). In addition to soil spore density in each of the four sample areas, the exposure risk to susceptible hosts will additionally depend on the area of contamination and a host's behavioral propensity to approach that area (6). The area of fluid-saturated soil changes dynamically while the carcass is consumed and may be distributed at satellite sites by scavengers. Soil that has been incidentally contaminated via movement of carcass materials will cover an even wider area and is even more difficult to measure but will have a much lower spore density, which may render it irrelevant to the transmission process.

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

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