The ecology of anthrax spores: Tough but not invincible

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Abstract

Bacillus anthracis is the causative agent of anthrax, a serious and often fatal disease of wild and domestic animals. Central to the persistence of anthrax in an area is the ability of *B. anthracis* to form long-lasting, highly resistant spores. Understanding the ecology of anthrax spores is essential if one hopes to control epidemics. Studies on the ecology of anthrax have found a correlation between the disease and specific soil factors, such as alkaline pH, high moisture, and high organic content. Researchers initially suggested that these factors influenced vegetative anthrax bacilli. However, subsequent research has shown that vegetative cells of *B. anthracis* have very specific nutrient and physiological requirements and are unlikely to survive outside a host. Review of the properties of spores of *B. anthracis* and other *Bacillus* species suggests that the specific soil factors linked to epidemic areas reflect important environmental conditions that aid the anthrax spores in causing epidemics. Specifically, high levels of calcium in the soil may help to maintain spore vitality for prolonged periods, thereby increasing the chance of spores encountering and infecting a new host. Cycles of runoff and evaporation may collect spores dispersed from previous epidemics into storage areas, thereby concentrating them. Uptake of large doses of viable spores from storage areas by susceptible animals, via altered feeding or breeding behavior, may then allow the bacterium to establish infection and cause a new epidemic. Literature search for this review was done by scanning the Life Sciences Collection 1982–1994 using the keywords "anthrax" and "calcium and spore."

Résumé

L'écologie des spores de l'anthrax : résistants, mais non invincibles

Bacillus anthracis est l'agent causal de l'anthrax, une maladie sérieuse et souvent fatale affectant les animaux sauvages et domestiques. *Bacillus anthrasis* produit des spores de longue durée très résistantes ce qui favorise la persistance de l'anthrax dans une région. Il est essentiel de connaître l'étiologie des spores de l'anthrax afin de pouvoir contrôler les épidémies. Les études écologiques ont démontré une corrélation entre la maladie et certains facteurs du sol tels un ph alcalin, une humidité élevée et un haut contenu organique. Initialement, les chercheurs ont suggéré que ces facteurs influençaient la forme végétative de l'anthrax bacillé. Puis d'autres recherches ont démontré que les cellules végétatives de *B. anthracis* ont des besoins nutritifs et physiologiques spécifiques ce qui rend leur survie peu probable en dehors d'un hôte. Les propriétés des spores de *B. anthracis* et des autres espèces de bacillus suggèrent que les facteurs spécifiques du sol reliés aux régions épidemiques reflètent d'importantes conditions environnementales qui aident les spores d'anthrax à causer une épidemie. Spécifiquement, un haut taux de calcium dans le sol peut favoriser la vitalité des spores pour une longue période augmentant ainsi les possibilités que les spores infectent un nouvel hôte. Les cycles de ruissellement et d'évaporation peuvent ramasser les spores éparses provenant de régions épidemiques et les accumuler dans les sites de réserve. La prise d'une grande quantité de spores viables à partir des sites de réserve par les animaux susceptibles via une alimentation altérée ou lors de leur comportement de reproduction pourrait permettre à la bactérie de provoquer une infection et une nouvelle épidemie. L'étude de la documentation a été effectuée en consultant Life Sciences, de 1982 à 1994 en utilisant les mots clefs « anthrax » et « calcium et spore ».

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Introduction

Bacillus anthracis is the causative agent of anthrax, a serious and often fatal disease of wild and domestic animals, especially herbivores. It can also infect man. The disease occurs primarily as a cutaneous (1), pulmonary (2), or gastrointestinal (1) infection, depending on the route of entry of the *B. anthracis* spores. Upon entry into the host, the spores germinate and the resultant vegetative cells replicate rapidly, enter the bloodstream, and result in septicemia (3). Because the bacterium produces a poly-D-glutamic acid capsule, ingestion by host phagocytes is limited (4). It is thought that death of the host eventually occurs as the result of the action of a tripartite toxin produced by the bacterium (5). Depending on the size of the spore inoculum, the susceptibility of the host species, and the immunological state of the host, the disease can occur as 1 of 3 forms: peracute, acute, or chronic, with the peracute form being the most fulminant infection (6). The peracute form is the most common form observed in ruminants and is almost uniformly fatal.

Anthrax is global in its geographical distribution and is endemic to many parts of southern Europe, Asia, Africa, North and South America, and Australia (7). The disease has been described since biblical times, when, it is believed, it was the 5th and 6th plagues suffered by the

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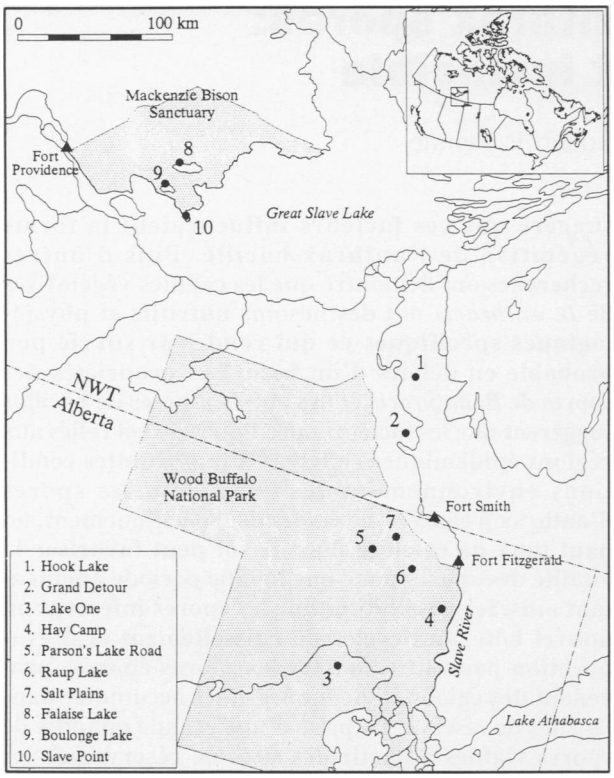

Figure 1. Areas of anthrax epidemics in bison in northern Canada from 1962 to 1993.

livestock of the Egyptians (8). Throughout the 19th century, the disease caused widespread losses among European livestock and was probably spread at this time to the New World by colonists (7). Even today, despite large-scale vaccination programs, removal of infected carcasses and contaminated materials, and extensive monitoring programs, outbreaks of the disease continue to cause significant losses in domestic and wild animal populations in many parts of the world.

In northern Canada, anthrax was first diagnosed during an outbreak among bison (*Bison bison*) in the Hook Lake region of the Northwest Territories in 1962 (Figure 1) (9). During the next 2 summers, deaths due to anthrax among bison continued in the region and began to disseminate, first to the Grand Detour area, then to the Hay Camp and Lake One regions of Wood Buffalo National Park. Between 1962 and 1978, 1098 deaths were attributed to sporadic summer outbreaks of anthrax within these 4 areas; this is a minimum estimate of actual losses, reflecting only the number of carcasses found by cleanup crews (10). After the summer of 1978, no further deaths due to anthrax were observed in the park and surrounding environs until the summer of 1991, when at least 34 bison died in the Salt Plains, Raup Lake, and Parson's Lake regions of the park (11,12). Recently, a large epidemic occurred during August 1993 in the bison herds within the Mackenzie Bison Sanctuary around Falaise Lake, Boulogne Lake, and Slave Point (Figure 1); this resulted in at least 172 bison deaths (Elkin and Gates, personal communication).

Like other *Bacillus* spp., *B. anthracis* can form metabolically inactive endospores in response to nutrient-poor conditions (13). Each vegetative *Bacillus* cell forms 1 spore and undergoes lysis after the process is complete. Thus, sporulation is not a form of replication but, rather, a resting stage that protects the *Bacillus* until conditions are again favorable for growth. While vegetative *Bacillus* cells do not survive well in adverse environments, the endospores can resist prolonged exposure to chemical disinfectants, desiccation, and extremes in pH, temperature, pressure, and ultraviolet and ionizing radiation (13,14). When released into the environment, *B. anthracis* spores can persist for decades before infecting a new host. *Bacillus anthracis* spores released intentionally during biological warfare trials on Gruinard Island in northwest Scotland during World War II remained viable in the soil for over 40 y before the island was successfully decontaminated (15). In the Kruger National Park, *B. anthracis* has been recovered from bones estimated to be approximately 200 y old (16).

For the control of epidemics, it is important to understand not only the pathogenesis and interactions of *B. anthracis* with host animals but also the ecology of the spores. In this review, we discuss the ecology of anthrax spores, with particular reference to anthrax outbreaks in bison in northern Canada, and the physiological requirements for calcium of *Bacillus* spp. in the laboratory. We present a new "storage area" hypothesis to explain the conditions associated with areas of repeated anthrax outbreak. The literature search was done by scanning the Life Sciences Collection 1982–1994 using the keywords "anthrax" and "calcium and spore."

Ecology of anthrax spores

One of the most frequently cited studies of the ecology of anthrax is that of Van Ness (17), who made extensive observations of areas of repeated anthrax epidemics in livestock in the southern United States. From these observations, Van Ness concluded there were 2 microenvironments where anthrax generally occurred. These incubator areas were, characteristically, low-lying depressions, where standing water collected and devitalized plant life, or rocklands, which are dried-up water courses or hillside seeps where organic matter accumulates during runoff.

Van Ness also suggested that local geological features have an important effect on the occurrence of anthrax within a given area. For example, in a 1957 outbreak centered in Craig County, Oklahoma, anthrax infections were observed only in association with soils that were developed from, or influenced by, limestone. The disease was, however, absent from an irregular chain of shale and sandstone hills that dissected the area, even though the hills also contained suitable incubator microenvironments. Anthrax is believed to have been introduced to Craig County from previously infected areas of Louisiana and Texas, as the county straddles what was once the Sedalia Trail, a cattle drive route out of these 2 states. Before reaching Craig County the trail forks, with a 2nd route leading into the Ozark uplift. Cattle from Texas and Louisiana were driven over both of these trails, yet anthrax never developed along the Ozark route, a fact that Van Ness attributed to the Ozark's more acidic soil (17). Other geological studies of epidemic areas in the lower Mississippi Valley and the coastal regions of Louisiana and Texas indicated that outbreaks were most frequently associated with calcareous soils, rich in nutrients (17). Other researchers have found

similar correlations between anthrax outbreaks and similar soil conditions in England and Wales (18).

Based on his observations, Van Ness (17) postulated his controversial "incubator area" hypothesis, which states that under conditions of alkaline pH, high soil moisture, and the presence of organic matter, *B. anthracis* may undergo cycles of spore germination, vegetative cell outgrowth, and resporulation that can cause an overall increase in spore numbers and lead to new outbreaks of anthrax. The debate over this hypothesis involves his conclusion that these microenvironments promote spore cycling. Van Ness postulated that such cycling occurs, in order to explain the incubator area-associated conditions of alkaline soil, high organic content, and high soil moisture, and a previously observed correlation of outbreaks with ambient temperatures above 15.5°C (19). These factors, he believed, would have little influence on static spores; therefore, they must be influencing the more sensitive vegetative cells. Experimental evidence, however, indicates that the vegetative cells of *B. anthracis* have very specific nutrient and physiological requirements, and survive poorly outside a host or complex artificial medium (20–22). Experimental germination of *B. anthracis* in the environment has only been successful in soil or water that has been artificially enriched with animal blood or viscera (21–23). Even when adequate nutrients are provided to trigger germination and outgrowth of *B. anthracis* spores, the vegetative cells are very susceptible to antagonism from other bacterial species present (20,22–25). This antagonism, though poorly characterized, leads to an overall decline in the number of anthrax spores, even when other conditions favor growth. It has also been noted that growth of *B. anthracis* outside a host leads to a rapid loss of virulence. This is the basis for the successful development of many anthrax vaccines (26). The Sterne strain, for example, was made avirulent after incubation under 30% CO_2 on horse serum nutrient agar for 24 h (27). Outside the laboratory setting, it is difficult to imagine a microenvironment better suited for successful germination and multiplication of *B. anthracis* than that within a host (26).

Dissemination of spores

A common misconception about *B. anthracis* is that its vegetative cells produce spores upon exposure to atmospheric oxygen. The reason for this misconception is the observation that *B. anthracis* in an intact carcass is destroyed within 48 to 72 h by putrefactive processes (28,29). As stated previously, sporulation is a starvation response and occurs independently of the presence of oxygen (13,30). Metabolism of the aerobic *Bacillus*, on the other hand, is influenced by oxygen (30). In the circulatory system of a living host, *B. anthracis* is exposed to aerobic, nutrient-rich, conditions that are optimal for growth. When the host dies, the internal environment becomes anaerobic and the bacillus is held in a static condition. Meanwhile, anaerobic bacteria from the gastrointestinal tract start to rapidly decompose the carcass. Better suited to the conditions now present in the host, the anaerobic bacteria eliminate the vegetative cells of *B. anthracis* through the antagonistic interactions mentioned previously (20,22–25,31). Only if the carcass is opened can the vegetative *B. anthracis* escape to an aerobic environment. More importantly, opening of the carcass also promotes drying of tissues and aerosolization of body fluids, both of which introduce the vegetative cells of *B. anthracis* to nutrient-depleted microenvironments, where they can then undergo sporulation.

In nature, carcasses are rarely left undisturbed long enough for putrefaction to eliminate all of the vegetative cells of *B. anthracis*. Instead, scavengers open the carcass and introduce the vegetative cells to microenvironments where nutrients are limited and sporulation can proceed (7). Because carnivores are less susceptible to anthrax than are herbivores and can ingest spores without developing the disease, they can act as carriers by dispersing ingested spores in their feces over large distances (22,32,33). Avian scavengers, such as vultures (various spp.), herring gulls (*Larus argentatus*), and ravens (*Corvus corax*), have been especially implicated in this route of dissemination, because they may carry spores over considerable distances (32,33).

During an anthrax epidemic, many other vectors act to disperse spores. Infected animals shed spores in their feces and urine (8). Various insects, such as mosquitoes and tabanids (deer, horse, and stable flies), have been implicated in the transmission of spores from animal to animal (7,33–36) and from animal to vegetation (7,16,33,37). Spores can be spread from infected areas through soil adhering to the coat of wallowing animals (bison) (33,38), or in dried blood and viscera adhering to the fur and feathers of scavengers that have fed on infected carcasses (33). Water and wind action can also contribute to the dispersion of spores from carcasses and other infected material (8,33,39). Clearly, there are many opportunities during the course of an anthrax outbreak for spores to be dispersed over large areas.

Concentration of spores by water

Despite the debate over Van Ness's incubator hypothesis (17), the facts still support his observations that epidemics of anthrax are associated with low-lying depressions and rockland seep areas with a high moisture and organic content in the soil and with an alkaline pH, hereafter referred to as storage areas. It has also been observed that anthrax epidemics generally occur during dry summer months following prolonged periods of heavy rain (6,7). This weather pattern and the high moisture content of storage areas suggests that water plays an important role in the ecology of anthrax.

One possible role for water may be in the collection and concentration of spores in storage areas. The prolonged period of rain preceding an epidemic promotes runoff and pooling of standing water. *Bacillus anthracis* spores have a high surface hydrophobicity (40) and, as a result, could be carried along in clumps of organic matter by runoff to collect in the standing pools. Coconcentration of organic matter by runoff may explain the high organic content of soils associated with storage areas. Spores also have a high buoyant density (41) and clumps of organic matter carrying large numbers of spores may float, remaining suspended in the standing water. Dry weather would lead to evaporation of the standing water and concentration of the floating spores as the pools shrank. Storage areas may collect spores from the surrounding areas, because they would be the

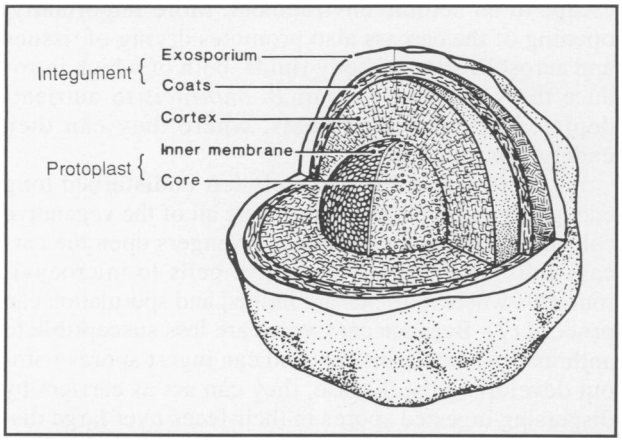

Figure 2. Generalized structure of bacterial endospores. Note not all *Bacillus* spp. have exosporia (42; reproduced with permission of Dr. K. Johnstone and Blackwell Scientific Publications).

last to experience evaporation. At the same time, receding water levels may allow the floating spores an opportunity to come into contact with, and adhere to, resurfacing vegetation through hydrophobic interactions. Thus, we suggest that successive cycles of runoff and evaporation may slowly concentrate anthrax spores into storage areas. Once in the storage area, evaporation may aid in the redistribution of spores from the soil environment onto resurfacing vegetation, thereby increasing the chances of spores infecting susceptible herbivores.

Physiology of spores

Spores, despite their resistance and dormancy, are not static and do interact with their surroundings. The most obvious site of interaction with the environment is the spore's surface layers, which are the exosporium and spore coat (Figure 2). These layers, also called the spore integument, are responsible for the spore's surface hydrophobicity (40,43) and protect the inner spore from the effects of harmful chemicals, disinfectants, and degradative enzymes (42).

Inside spores, the cortex, in response to changes in pH or ionic strength, may undergo significant changes in volume that exert a pressure difference on the spore core and "squeeze out" water (13,44). One of the results of this low core water content is the high buoyant density of endospores (13). The reduced water content of the core also confers resistance to heat and ultraviolet light to the core components and is believed to contribute to spore dormancy (45). Despite the high osmotic pressure exerted by the cortex, the core is not completely dry and contains water that is in equilibrium (13,46).

It has been shown that small molecules dissolved in water can passively diffuse across the outer spore layers and be exchanged between the core and the environment (47). Passive diffusion of molecules in spores is primarily a function of the molecular size and charge of the particle (48). The apparent uptake-exclusion threshold of *B. cereus* T spore protoplasts, that is, spores with their exosporium, coat, and cortex layers removed (Figure 2), corresponds to a molecule with an average molecular weight of 280 and an equivalent hydrodynamic radius of 0.6 nm (49). Thus, even the spore's innermost core can interact with the environment. When initially formed, spores may be highly resistant and dormant, but, over prolonged periods of exposure to the environment, small molecules involved in resistance or dormancy may be damaged or lost. Since they are metabolically inactive, spores are unable to repair damage or renew stores of lost molecules and, as a result, may lose their ability to germinate or maintain resistance and dormancy.

Role of calcium

Calcium may be one component of spores that is affected by prolonged interactions with the environment. Calcium cations have been shown to be important participants in both germination and the maintenance of dormancy. The 3rd condition that Van Ness (17) correlated with the incubator area microenvironments was alkaline pH. In his studies, however, alkaline pH was a direct result of calcareous soils, and it may be the high calcium levels in these soils that is the actual predisposing condition. High levels of calcium in the soil may buffer the spore's supply of calcium and help to maintain viable spores in the environment for longer periods of time, thereby increasing their chance of coming into contact with, and successfully infecting, a new host.

Many studies have examined the importance of calcium in spore germination. Spores produced in calcium-deficient media (50–52) or converted via ion exchange to an H+ form, where exchangeable cations in the spore are removed and replaced with protons (53–56), respond poorly to specific organic germinants. When these calcium-deficient spores are exposed to calcium-supplemented germination solutions or reloaded with calcium (a process whereby spores are incubated with high concentrations of calcium for extended periods of time), normal germination is observed (50–56).

At the same time, the spore coat layer has been implicated in spore germination in response to certain organic germinants. The coat layer of a spore can be removed through either harsh chemical extraction, such as treatment with sodium dodecyl sulfate (SDS) and dithiothreitol (DTT), or mutagenesis, as in the case of *B. cereus* 10LD mutants. Studies of SDS-DTT treated and 10LD mutant *B. cereus* spores have shown that both are unable to germinate in response to inosine (a common germinant for wild type spores). Further, 10LD mutants could not germinate and SDS-DTT-treated spores could only germinate slowly in response to L-alanine, another common germinant (57). The germinability of both types of coat-modified spores was restored, however, when the germination medium was supplemented with exogenous calcium. Although other divalent cations were also shown to help restore germinability, the restoration was much more pronounced with calcium. From their findings with coat-modified *B. cereus* spores, Shibata *et al* (57) hypothesized that the diminished or lost germinability of the coat-modified spores was due to the loss of calcium associated with the spore integuments.

Despite its involvement with germination, calcium associated with the integument comprises only a small fraction of the total spore calcium content of *Bacillus* species (58,59). The vast majority of spore calcium, greater than 98%, is located in the spore core region

(58–60). Along with dipicolinic acid (DPA), calcium forms an extensive salt lattice that immobilizes the enzymes, DNA, and other components of the core. This immobilization is thought to play an important role in metabolic dormancy and heat resistance of core components (45). *Bacillus cereus* mutants that produce spores lacking DPA have 10- to 20-fold less calcium than do wild-type spores (61) and, although the mutant spores have normal heat resistance when freshly formed, the resistance is lost upon storage for 2 wk at 4°C (62). Furthermore, the rates of spore thermal death and loss of DPA on heating appear to be related: heat-sensitive *Bacillus* strains lose DPA more quickly than do heat-resistant strains (63). Thus, while not necessarily being the cause of spore heat resistance, the calcium-DPA lattice appears essential for the maintenance of thermoresistance (63).

In the case of *B. anthracis* spores, it is possible that exchange of core water along with diffusion of protons or other cations into the core from the environment could result, over time, in the leaching of calcium cations and a disruption of the salt lattice. This could, in turn, disrupt spore dormancy and heat resistance. Below a certain threshold of calcium loss, the spore may be left "weakened" and unable to germinate, or it may germinate prematurely in the environment and die off as a result of inadequate nutrients or antagonism from other bacterial species. In contrast, in calcium-rich environments, exogenous levels of calcium may buffer the calcium supply of *B. anthracis* spores until the opportunity to infect a new host presents itself.

Whether required in the spore integument to aid in the germination response or in the core to maintain spore dormancy and resistance, calcium appears to be very important in the continued spore–vegetative cell–spore cycling of all *Bacillus* spp. In both these locations, calcium interacts with the environment and may be lost over prolonged periods. The spores of most *Bacillus* spp. probably encounter germination conditions after relatively short periods of time and, therefore, calcium loss to the environment would not usually be a problem. *Bacillus anthracis* spores, however, may exist for years in the environment before the opportunity arises to infect a living host, the only apparent viable vegetative cell habitat. We hypothesize that high levels of calcium in the soil may act to buffer the internal supplies of anthrax spores and greatly extend their viability.

Epidemiology of anthrax with respect to bison

In every anthrax outbreak in bison in northern Canada, mortality has been much higher among sexually mature males than females and immature bison (11). Of the 48 confirmed anthrax deaths in bison in Wood Buffalo National Park in 1978, 87.5% were adult male and 4% adult female (4 scavenged carcasses were of unknown gender and no immature bison fatalities were observed) (12). In the 1991 outbreak in the park, in which 34 carcasses were recovered, 58.8% were adult bulls, 26.4% adult females, and 14.7% immature bison (11). In the Mackenzie Bison Sanctuary epidemic of 1993, approximately 80% of the carcasses found were adult bulls (Elkin and Gates, personal communication). Any hypothesis to explain anthrax epidemics in northern Canada must take into account the increased susceptibility of sexually mature bulls.

Anthrax epidemics in bison occur during hot, dry weather between late June and early September (10). This period roughly corresponds with the bison rut, which occurs from mid-August to early September (10). The wet spring usually preceding an anthrax epidemic leads to increased insect harassment for the bison herds later in the summer. Drought brought on by the hot, dry weather results in bison concentrating around decreasing supplies of feed and water. Gainer and Saunders (26) suggested that these 4 factors, high temperatures, breeding activity, high levels of insect harassment, and concentration of bison, may act to alter or modify the bison's immunity to disease. According to their modified resistance hypothesis, low oral doses of spores or parenteral infection of *B. anthracis*, which would not cause anthrax in a healthy animal, result in lethal infections, because seasonal conditions modifying host resistance leave the animals in a weakened immunological state (26). The hypothesis was developed to explain the apparent lack of large numbers of *B. anthracis* spores recovered from the soil of affected areas in northern Canada (26). To explain the difference in mortality among sexes, Gainer and Saunders suggested that the immune response of adult bulls is compromised by their breeding activity (26). These researchers suggested that an increased susceptibility to disease may be reflected in the weight loss commonly observed in rutting bulls. While the above conditions probably do have an effect on a bison's susceptibility to disease, the extent of their effect remains unknown. The search for anthrax spores in the soil of affected areas was very limited and the apparent lack of spores upon which this hypothesis was based may be a result of the sampling. It should be noted that, while sexually mature bulls appear weakened during the rut, anthrax outbreaks often begin before the rut, when the bulls are at their fittest (Elkin and Gates, personal communication).

As mentioned previously, insects have been implicated in the transmission of anthrax from animal to animal (7,33,35,36). Many of the anthrax epidemics in northern Canada ceased with the arrival of cooler weather (11; Elkin and Gates, personal communication). The halting of epidemics by colder weather has been attributed to the reduced temperatures decreasing the number of biting insects in the area, thus removing them as vectors (11). Despite this hypothesis and the large populations of biting insects present during epidemics, biting insects as vectors of infection are thought to be of minor importance in epidemics in northern Canada. If the spread of anthrax by insects was the key to a continuing epidemic, then one would expect equal representation in bison mortalities between sex and age classes, as biting insects do not discriminate.

Besides breeding activity modifying resistance, a simpler possibility that may account for the higher anthrax mortality among adult bulls is their breeding behavior. Rutting aggression of bulls, such as increased stamping and wallowing, may greatly increase their chances of exposure to anthrax spores (12). In storage areas, this behavior may create large dust clouds of aerosolized spores. The aerosolized spores may enter the

bulls' respiratory tract through either normal respiration or snorting, another common rutting behavior, and establish disease. Arrival of colder weather during an epidemic would bring about an increase in humidity that could decrease the amount of anthrax spore-containing dust kicked up by the bulls, therefore halting the epidemic.

The prolonged drought generally accompanying an epidemic causes a wilting of vegetation, forcing bison to graze closer to soil that is potentially infected with spores. This altered feeding behavior has also been implicated in anthrax infection (64). It should be noted that the devitalized plant life occurring in storage areas is also wilted. If such plants are already coated with anthrax spores as a result of evaporation, wilting would force grazing herbivores into even more intimate contact with spores concentrated in storage areas. Thus, while conditions that modify host resistance and accompany anthrax outbreaks probably account to some extent for increased susceptibility to the disease, breeding and altered grazing behavior may play more important roles in the establishment of infection.

To date, all investigations into the presence of anthrax spores in the environment have had to rely on direct plating techniques. As these techniques are time-consuming and labor-intensive, the resulting sample size of the investigations has been limited. These techniques also require *B. anthracis* to germinate and successfully multiply on laboratory media. Inability of *B. anthracis* to grow in vitro, however, does not necessarily imply that it is unable to do so in vivo and cause disease. It is actually difficult to recover *B. anthracis* from environmental samples (65); often, it is necessary to passage the suspected sample through a laboratory animal first to recover the organism (26). We hope that the development of *B. anthracis*-specific assays not requiring direct germination of the bacterium, such as enzyme-linked immunosorbent assays, direct fluorescent assays, and polymerase chain reaction assays, which are currently being assessed in our laboratory, will allow large-scale investigations to delineate anthrax spore concentrations and distribution in the environment. These investigations will greatly aid in a better understanding of anthrax spore ecology, and the roles played by altered host resistance and behavior in the epidemiology of the disease.

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