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Ecology and epidemiology of anthrax in the Etosha National Park, Namibia

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ABSTRACT

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Analysis of mortality records has revealed distinct patterns in the incidence of anthrax in elephant and plains ungulates. The seasonal peak among the former is in November at the end of the dry season, while among the latter it occurs in March towards the end of the rainy season. Among elephants, there has been a notable spread of the disease to the west of the Park. Age and sex analyses indicate that, except for zebra, proportionally greater numbers of adult males die of anthrax among the species predominantly affected; however, zebra carcases are difficult to sex.

In a study to identify possible environmental sources of infection, *B. anthracis* was detected in 3,3% of 92 water and 3,0% of 230 soil samples collected at different times of the year from 23 sites not associated with known cases of anthrax. Slight seasonal differences were noted with 5,7% positives occurring in the cold-dry period (May to August), 3,5% in the hot-dry season (September to December) and 1,4% in the hot-wet season (January to April). Higher rates (26,0% of 73 samples) were found in water from waterholes in the western part of the Park at the time of an outbreak in elephants. The possible importance of scavenger faeces was confirmed with >50% of vulture, jackal and hyaena faeces collected from the vicinity of confirmed anthrax carcases yielding *B. anthracis*, sometimes in substantial numbers, while no spores were found in faeces not associated with known anthrax carcases.

Despite terminal *B. anthracis* levels of usually $> 10^7$ cfu/mℓ in the blood of animals dying of anthrax, spore levels in soil contaminated by such blood at sites of anthrax carcases ranged from undetectable to a few tens of thousands. The rapid loss of viability in soil and water of anthrax bacilli was monitored experimentally and the importance of soil type demonstrated. Survival and extent of sporulation of the bacilli in water were shown to be dependent on the rate at which the blood was diluted out. Other relevant parameters examined were background flora, pH and sunlight.

INTRODUCTION

Anthrax was first diagnosed in the Etosha National Park (hereafter Etosha NP) (Fig. 1) in 1964, although there was evidence to suggest that the disease had been present in the region long before this time (Ebedes 1976). Records of the incidence of anthrax also date to 1964, but no detailed mortality records were kept before 1976. Ebedes (1976) considered that the increased incidence of the disease was the direct consequence of the excavation of gravel pits during construction of tourist roads, thereby (1) retaining water longer, thus causing the animals to delay their normal migration from the area, and (2) providing ideal conditions for the multiplication of *B. anthracis*. He noted that the gravel pits occurred in most of the enzootic areas.

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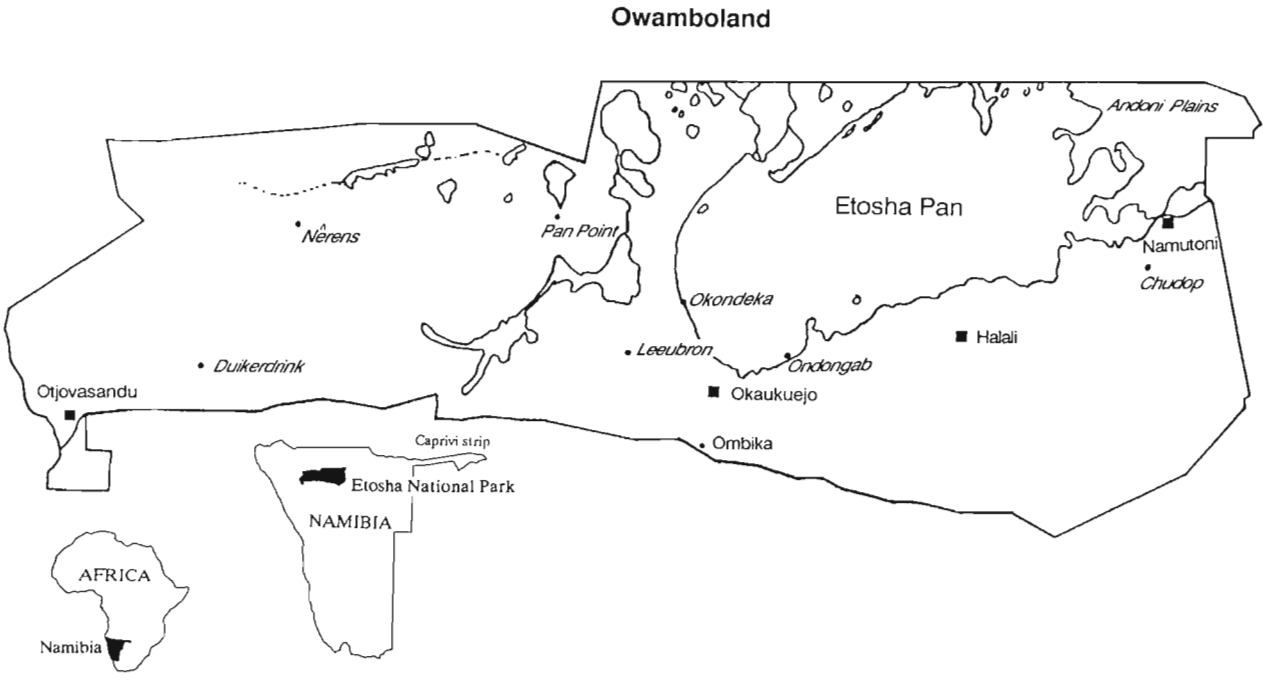

FIG. 1 Map showing camps and the major features in the Etosha National Park, Namibia

Later isolation attempts failed to confirm that gravel pits were or had remained a major source of infection (Turnbull, Hofmeyr, McGetrick & Oppenheim 1986; Turnbull, Carman, Lindeque, Joubert, Hübschle & Snoeyenbos) or that they differed significantly in chemical or microbiological parameters from other water sources (Winter 1985, Turnbull *et al.* 1989); neither could the water be shown to readily support persistence or growth of *B. anthracis* (Turnbull *et al.* 1989).

Enzootic areas can still be differentiated from non-enzootic areas in the Park, and, with the exception of an increased incidence in elephants in the west of the Park, have not greatly changed since first delineated by Ebedes (1976). The sources of infection of the animals in these enzootic areas remain unidentified. The bacteriological studies reported here were aimed at a better understanding of the ecology of *Bacillus anthracis* in the Park and hence of the epidemiology of anthrax.

MATERIALS AND METHODS

Incidence data

Mortalities are recorded routinely by research and management staff who, on finding a carcase, collect blood and/or tissue specimens; alternatively, where few remains have been left by scavengers, or when the carcase is in an advanced stage of decomposition, soil samples are collected from the site of the carcase. Where possible, confirmation of diagnosis was made on the basis of blood or tissue smears and/or culture of *B. anthracis* from the blood/tissue specimens or from the soil samples as described below. Diagnosis of anthrax from a blood smear, once extensive putrefaction has taken place, is often difficult and, if only limited sporulation has occurred, available culture techniques may be too insensitive for isolation of *B. anthracis*. Some of the cases have to be classed as suspected anthrax on the basis of visual evidence such as haemorrhages from natural openings exuding thick, dark, poorly clotting blood and the absence of signs of predation.

Environmental samples not associated with known anthrax cases

Soil and water from 23 sites comprising five springs, four natural rainwater pans, five boreholes and nine gravel pits in a part of the Park which included one of the major anthrax-enzootic areas, were sampled ten times during the study period (four times during the hot-wet season, and three times in each of the cold-dry and hot-dry seasons). At each site soil samples (2–3 g) were taken from several points around and across dried-out or partially dried-out water holes by means of a sterilized spoon, and pooled in a single, sterile specimen container. When water was present, small samples (approximately 10 mℓ) were collected from the perimeter of the water hole and pooled into a single, sterile 500-mℓ Schott bottle. The Schott bottles were placed in a cool box containing

ice packs, for transport back to the laboratory, where they were held in a refrigerator until they could be tested. Twelve additional soil and 75 further water samples were collected on an incidental basis from sites not associated with a specific anthrax carcase.

Faecal specimens

Faecal specimens were collected from the vicinity of sites of carcases or around watering points and were examined in the same way as soil samples.

Persistence studies

Thirteen sites of carcases confirmed as having been victims of anthrax, were permanently marked with metal stakes to permit the level of *B. anthracis* in the soil to be monitored with time. Over ensuing months, soil samples were collected by pooling numerous small subsamples (about 2–3 g) into a single sterile container.

Experimental contamination of soil

Several experiments were performed to study sporulation by *B. anthracis* in soil contaminated with blood from animals that had died of anthrax. The approaches and conditions are described with each experiment under Results. At predetermined sampling times, a small quantity (about 1,5 g) of soil was collected and suspended in phosphate-buffered saline (PBS) in a 1:2 (w/v) ratio. Ten-fold dilutions were made and 0,25 mℓ of each dilution spread on polymyxin-lysozyme-EDTA-thallous acetate agar (PLET) plates to obtain a total *B. anthracis* count. The dilutions were then heated at 62,5 °C for 15 min and again plated onto PLET to obtain a spore count. After incubation for 48 h at 37 °C, the plates were examined and the numbers of *B. anthracis* per g of soil calculated. A proportion of the colonies counted were checked for correct identification by phage and penicillin-sensitivity testing.

The fate of *B. anthracis* in water

At daybreak water samples were collected from the Okondeka spring and the Okaukuejo gravel pit, both of which are sited in an anthrax enzootic area and have, in the past, yielded *B. anthracis* not associated with a known case of anthrax.

Approximately 100 mℓ of each sample was filtered through 0,45-μm low-protein-binding membrane filters (Schleicher & Schuell, Dassel, Germany) and 10 mℓ of the filtrates were pipetted into each of seven 100-mℓ bottles; 20 mℓ were pipetted into another bottle (bottle 1). In two trials, 5-mℓ volumes of blood from EB(1)91.3.26PML and LA(1)91.4.25RD were added to bottle 1 giving a 1:5 dilution of the blood in that bottle. (Codes indicate species, number of this species examined by a particular operator that day, date and initial of operator.) Two-fold dilutions to 1:640 were made in the remaining bottles by serial transfers of 10-mℓ volumes.

The bottles were incubated at room temperature (27–29,5 °C) and total and spore counts assessed at the intervals shown in Fig. 5. Counting was done by transferring 20-μℓ aliquots from each bottle to 180 μℓ (1:10) PBS (containing 40 U/mℓ of polymyxin B in the second trial, to reduce secondary contamination) in the first lane of a 96-well, sterile tissue-culture plate and serially diluting further transfers of 20 μℓ in 180 μℓ through five or six dilutions. Three 20-μℓ drops from each dilution were then placed on nutrient-agar plates with incubation and counting according to the normal drop-count method (ICMSF 1978). This constituted the total *B. anthracis* count. The dilution plates were then held at 62,5 °C for 15 min in a shallow water bath and the drop count repeated to obtain the number of spores. A proportion of the colonies counted were again checked for correct identification by phage and penicillin-sensitivity tests.

Susceptibility of spores to ultraviolet irradiation and sunlight

Spore suspensions of strain LA(1)89.8.18ML were prepared by washing the growth of 44-h cultures on sporulation-agar plates with sterile, distilled water. The spores were centrifuged, washed once and resuspended in distilled water. The final suspension was held at 62,5 °C for 15 min in a water bath and diluted to approximately 5×10^9 spores/mℓ.

Spore "traps" were prepared by means of fine, sterile sewing thread [Zwichy 120 (SH 90021)] wound round two-pronged forks. About five turns of thread were wound around each fork, each turn being held in place with a drop of glue on each prong. The traps were exposed to the ultraviolet (UV) light of a class-2 safety cabinet for 30 min. Sterility was confirmed by culture on nutrient agar of two transverse strands of the thread from a proportion of the traps.

The traps were dipped into the spore suspension and dried by standing them vertically in the safety cabinet for 1 h. They were then exposed to UV irradiation by being placed in stands on the floor of the cabinet approximately 350 mm from the UV source [Philips 30W G10T8 M9 (UV-C)], or to sunlight, by being placed at the requisite angle in the open in a stand within a high-sided dish. Exposure to the sun occurred between 11:45 and 15:15.

At the end of the chosen exposure time, duplicate transverse lengths of the threads were cut from the traps with flamed scissors and forceps and transferred to the surfaces of nutrient-agar plates. Growth of *B. anthracis* after overnight incubation at 37 °C around the threads was scored as 0–4+. Unexposed

refrigerated controls were examined at the beginning and end of the experiments and shaded controls were included in the sunlight experiment.

Bacteriology

From 1964 to 1986, diagnosis of anthrax in Etosha was performed by the examination of blood/tissue or carcase-exudate smears, or purely on visual signs. Some smears and tissue samples were sent to the Central Veterinary Laboratory in Windhoek, Namibia, for confirmation. Since 1987, attempts to diagnose anthrax in Etosha NP has also included culture of blood and tissue samples from carcases and soil from carcase sites. Confirmation by culture was essential when blood smears could not be collected because of the decomposition or desiccation of a carcase.

Blood smears

Smears were stained with polychrome methylene blue stain (M'Fadyean 1903) (BDH Chemicals, Poole, England) or CAM's Quick Stain [C.A. Milsch (Pty) Ltd, Krugersdorp, RSA], which gives results similar to a Wright-Giemsa stain. Blood smears were examined under oil immersion for characteristic capsulated anthrax bacilli. With M'Fadyean's stain, the dark-blue-stained bacterial cells were surrounded by pink capsular material. Quick Stain, while not always showing up the capsule, did highlight the characteristic square-ended shape of the anthrax bacilli better than M'Fadyean's stain. This was advantageous in smears from decomposed carcases in which, as previously recognized (Sterne 1959), the capsule loses its affinity for methylene blue during putrefaction and may no longer be visible.

Cultures

The principal isolation medium for *B. anthracis*, PLET (Knisely 1966), was used according to the methodology of Carman, Hambleton & Melling (1985) and Turnbull *et al.* (1989). Culturing procedures were used when carcases were too old for diagnosis by means of blood smears, or for isolation of *B. anthracis* from environmental and faecal samples. Specimens and samples were heated for the dual purpose of killing non-sporing background flora and heat-shocking spores to induce germination.

Water samples were examined by heating aliquots at 62,5 °C for 15 min in a waterbath and spreading 0,25-mℓ volumes on the surfaces of four PLET plates.

In the case of other samples (soil, faeces, etc.), a few g of samples were suspended in two volumes (w/v) of sterile, distilled water and, after vigourous shaking and heating at 62,5 °C for 15 min, 0,25-mℓ volumes of the undiluted samples and of 10^{-1} and 10^{-2} dilutions were spread on PLET plates. After incubation at 37 °C for 30–48 h, colonies on the PLET plates suspected of being *B. anthracis* were subcultured on nutrient-agar plates and tested for penicillin and gamma-phage sensitivity. Phage- and/or penicillin-sensitive isolates were tested for capsule production by inoculating a small amount of the growth into 2 mℓ of heparinized horse blood. After incubation at 37 °C for about 5 h, a M'Fadyean-stained smear was prepared, and capsulated *B. anthracis* looked for.

RESULTS

Incidence

Confirmed and suspected cases of anthrax recorded in Etosha NP since 1964 are presented in Table 1. Anthrax deaths have now been recorded in ten of the 13 large herbivorous mammals in the Park and one herbivorous bird (ostrich—*Struthio camelus*). The only cases in carnivores were two cheetahs recorded as suspected anthrax in 1970 (Ebedes 1976) and two confirmed cheetah mortalities in 1993 (to November). Cheetahs occasionally get the disease elsewhere in Namibia (Jäger, Booker & Hübschle 1990; Jäger & Lindeque 1990).

Anthrax in the Etosha NP typically occurs as sporadic cases interspersed with small-scale outbreaks. Distinct epizootics also occur, but only on rare occasions. Three intense and localized outbreaks of anthrax have occurred in Etosha since 1976—November 1981 to January 1982, when about 200 elephants (*Loxodonta africana*) died of anthrax in western Etosha NP; March to April 1984, when an estimated 276 Burchell's zebra (*Equus burchelli*), 115 blue wildebeest (*Connochaetes taurinus*), 31 gemsbok (*Oryx gazella*) and 21 springbok (*Antidorcas marsupialis*) died on the Andoni plains and north-eastern section of the Etosha pan; and October to December 1989, when an estimated 101 elephants died in north-western Etosha. Anthrax was not recorded in western Etosha prior to the outbreak of November 1981 to January 1982.

Yearly incidence of anthrax

The proportion of anthrax deaths relative to population estimates (Table 2) shows a significant decrease over time in the incidence of anthrax in wildebeest since 1968 (linear-regression analysis: $R^2 = 0,56$; $P < 0,01$), no trend for the other plains ungulates, and an increase among elephant since 1972 ($R^2 = 0,38$; $P < 0,05$). No correlation between population and the proportion of the population dying from anthrax could be demonstrated for any of the species, implying that anthrax is not density dependent in the Etosha NP.

TABLE 1 Confirmed and suspected cases of anthrax in the Etosha National Park, Namibia, during the period 1964–1992[a]

Year[c]	Species affected[b]											Total conf. cases	Total susp. cases	Total
	EB	CT	AM	LA	OG	TO	TS	GC	SC	DB	EZ			
< 1988	1 201	741	180	258	30	4	21	8	6	10	2			2 461
1988	12	13	11	47	2	0	1	0	0	0	3	57	32	89
1989	25	18	16	83	2	1	2	1	0	1	1	113	37	150
1990	24	11	2	46	1	0	0	1	0	2	0	58	29	87
1991	28	10	10	8	0	1	0	0	0	0	0	45	12	57
1992	21	18	14	32	1	0	0	5	0	0	0	64	27	91
TOTAL	1 311	811	233	474	36	6	24	15	6	13	6			2 935

[a] 1964–1976 from Ebedes (1970; 1974; 1976), 1976–1992 from mortality records held at Etosha
[b] EB, Burchell's zebra (*Equus burchelli*); CT, blue wildebeest (*Connochaetes taurinus*); AM, springbok (*Antidorcas marsupialis*); LA, elephant (*Loxodonta africana*); OG, gemsbok (*Oryx gazella*); TO, eland (*Taurotragus oryx*); TS, kudu (*Tragelaphus strepsiceros*); GC, giraffe (*Giraffa camelopardalis*); SC, ostrich (*Struthio camelus*); DB, black rhinoceros (*Diceros bicornis*) and EZ, Hartmann's zebra (*Equus zebra hartmannae*)
[c] Data for years 1964–1987 combined

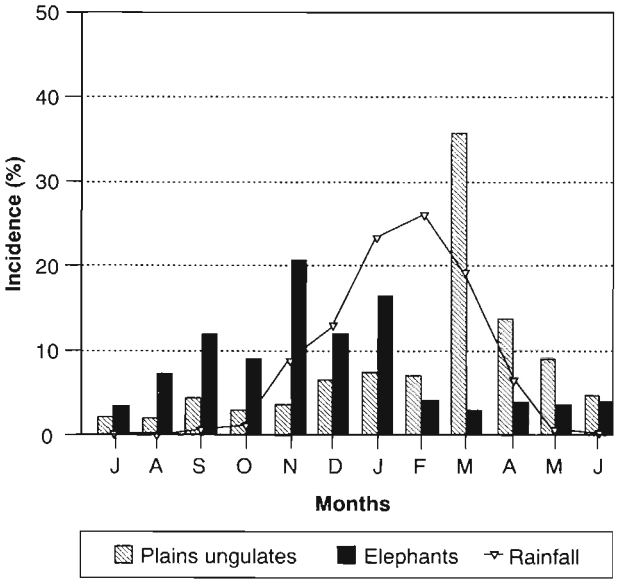

FIG. 2 Monthly recorded incidence of anthrax in plains ungulates and elephants (1976–1992) together with mean monthly rainfall given as a % distribution in the Etosha National Park, Namibia

TABLE 2 Incidence of anthrax in springbok, Burchell's zebra, blue wildebeest and elephant as a percentage of the population in the Etosha National Park, Namibia (only calculated for years when there is a census population estimate)

Year	Spring-bok	Blue wildebeest	Burchell's zebra	Elephant
1968		2,77		
1969		1,51		
1970		1,36		
1972		3,25		0,23
1973	0,10	1,68	0,58	0,31
1974	0,10	0,95	0,34	0,00
1976	0,01	0,45	0,25	0,00
1977		0,56	0,16	
1978		0,53	0,35	0,00
1982		0,23	0,28	3,33
1983				0,42
1984	0,09	2,49	2,20	0,37
		0,46[a]	0,50[a]	
1985				1,52
1986			0,72	
1987	0,06	0,16	0,33	0,79
1990				3,00

[a] Excluding epidemic at Andoni during March 1984

Seasonal incidence of anthrax

The peak in mortalities among the plains ungulates occurred in March, towards the end of the rainy season, in contrast to a peak incidence in elephants in November, at the end of the dry season (Fig. 2). Estimating the decomposition rate of an elephant carcase—hence the approximate time of death—is frequently difficult. The incidence shown in Fig. 2 represents the dates on which the carcases were found; the actual seasonality of elephant-anthrax mortalities would probably be much more pronounced than it appears in the figure.

Spatial pattern of anthrax mortalities within the Etosha National Park

Maps showing the distribution of anthrax mortalities in the plains ungulates have been presented in earlier reports (Ebedes 1976; Turnbull *et al.* 1986; 1989). This distribution has not changed greatly since the 1960s and it would appear valid to refer to distinct enzootic areas within the Park; for the plains ungulates, these areas coincide closely with their wet-season distribution. Elephant mortalities, however, have shown a recent westward spread following a major outbreak in 1981–1982. This extension of the

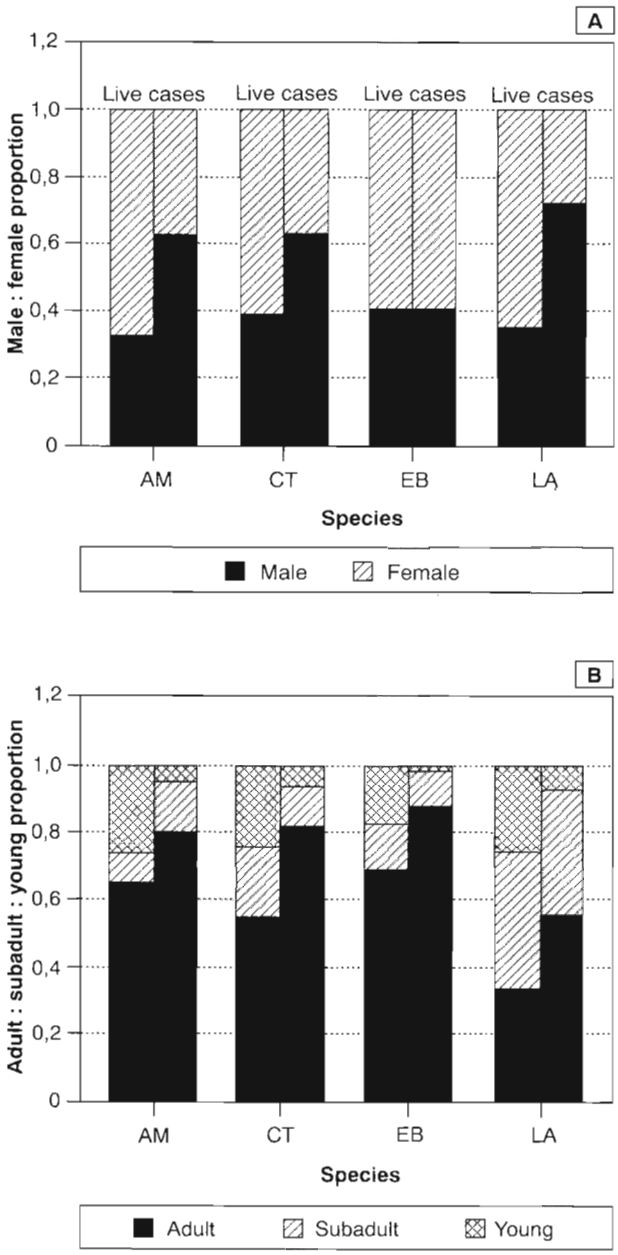

FIG. 3 Sex (A) and age-class (B) ratios of anthrax cases compared to respective live populations of Springbok (AM), blue wildebeest (CT), Burchell's zebra (EB) and elephant (LA) in the Etosha National Park, Namibia

distribution of the disease would seem to be the result of an increase in the incidence of anthrax in elephants, a highly mobile species (Lindeque & Lindeque 1991).

Age and sex ratios

The adult male:female proportions of the observed anthrax cases and the respective live populations in the Park from 1976–1990 are illustrated in Fig. 3A. Fig. 3B shows the adult:subadult/juvenile:young proportions among the same set of anthrax cases, again with the comparative ratios in the respective live populations.

The age distribution of carcases is likely to be heavily biased towards adults, as larger carcases have a greater chance of being discovered, and the carcases of younger animals disappear more rapidly. Valid statistical analyses are therefore not feasible. The raw data suggest that proportionally greater numbers of adult males die from anthrax in all species except zebra; however, zebra carcases are particularly difficult to sex without opening up the carcase and the associated risks that procedure produces of human infection and environmental contamination.

Environmental survey

As part of an environmental survey, a total of 92 water samples and 230 soil samples from a pre-selected set of 23 sites not associated with known anthrax deaths were examined at different times and seasons over a three-year period. The sites chosen were gravel pits, natural pans, springs and boreholes. *B. anthracis* was detected in three of the water samples (3,3 %) and seven of the soil samples (3,0 %). Five of the positive samples—four soil (8–80 spores/g) and one water (1 spore/mℓ)—were from the Adamax gravel pit. The remaining positives consisted of one sample each of Grunewald gravel-pit water (1 spore/mℓ), Leeubron borehole soil (80 spores/g), Gemsbokvlakte borehole water (1 spore/mℓ), Okondeka spring soil (10 spores/g) and Leeubos natural-pan soil (4 spores/g). Isolation of anthrax spores from Adamax gravel pit and Okondeka fountain has been reported before (Turnbull *et al.* 1989; the positive 1986 sample in Table 11 of that paper was from Adamax). In terms of season, isolation rates were 5/88 samples (5,7 %) in the cold-dry period (May to August), 3/87 (3,4 %) in the hot-dry season (September to December) and 2/147 (1,3 %) in the hot-wet season (January to April).

Total aerobic bacterial counts, spore counts and pH in the water samples were also determined. Large variations in values obtained from one type of site made it difficult to compare values between the different types of sites. Nonparametric Kruskal-Wallis test criteria based on ranks, performed to detect any differences between the four water types for the three parameters in the hot-wet season (sample sizes for the other seasons were too small), showed no significant differences in aerobic bacterial and spore counts. The pH values were found to be significantly different ($P < 0,05$) with mean values of 9,17 for springs, 8,36 for natural pans, 8,20 for gravel pits and 8,01 for boreholes.

In addition to the samples collected as part of the planned survey, 12 incidental soils from dried-up

TABLE 3 Results of a survey on the presence of anthrax spores in the faeces of black-backed jackals, vultures and hyaenas in the Etosha National Park, Namibia

Animal species and visibility of spores	Number tested	Number positive	Count/g (cfu/g)	
			Mean	Range
Jackal				
In vicinity of carcase	25	18	626	2–4 480
Away from a carcase	9	0		
Vulture				
In vicinity of carcase	18	9	34	2–174
Away from a carcase	4	0		
Hyaena				
In vicinity of carcase	5	3	6 740	2–20 000
Away from a carcase	1	0		

waterholes, ten from boreholes, two from gravel pits, as well as 73 water samples were examined. No anthrax spores were detected in any of the soil samples, but 19 water samples (26%) were positive (1–22 spores/mℓ). The majority of these, however, were taken in the western part of the Park during anthrax outbreaks in elephants. Three distinct bodies of water at boreholes were tested; the reservoir water that comes directly from the ground and is not available to animals for drinking (0/4 positive); water which flows out from the reservoir into a saucer-shaped concrete trough (14/38 positive); and the overflow that forms the mudhole (5/17 positive). The results indicated that the troughs have a greater tendency to become contaminated with *B. anthracis*. Results of resampling over 1–5-month periods suggested, however, that the contamination was transient.

Faecal specimens

Faecal samples from 34 black-backed jackals (*Canis mesomelas*), 22 vultures (*Gyps africanus*, *Torgos tracheliotus*, *Trigonoceps occipitalis*) and six hyaenas (*Crocuta crocuta*) were tested for the presence of anthrax spores (Table 3). Half the samples tested did contain spores and all these were collected from the vicinity of known anthrax carcases. Due to big variances, no significant difference between counts from the three species was found (Kruskal-Wallis ANOVA $P > 0,05$).

Terminal *B. anthracis* blood counts in victims of anthrax

Levels of *B. anthracis* in the blood of nine Burchell's zebra, four elephants and one springbok that had succumbed to anthrax, were determined. In some cases the animals were seen before death and the time between death and collection was established. Other carcases were estimated as < 12–18 h old from their condition at the time the blood was collected. Counts ranged from 3×10^6 to 7×10^8 cfu/mℓ in the zebra, $2,5 \times 10^6$ to 2×10^9 cfu/mℓ in the elephant with the exception of one in which a level of only 330 cfu/mℓ was found about 3 h after death, and that in the springbok was $2,5 \times 10^8$ cfu/mℓ. One of the elephants had been lying in water for about 3 d before blood collection; despite an ambient temperature of approximately 30 °C, putrefaction was not very advanced and few putrefactive bacteria were apparent in blood smears.

Levels and persistence of *B. anthracis* at sites of anthrax carcases

The levels of anthrax spores found in soil samples collected from the sites of 106 carcases are summarized in Table 4. To study the persistence of freshly shed *B. anthracis*, 13 of the sites were marked with metal stakes placed at points where the soil had been contaminated by the animal's terminal haemorrhages. Contamination levels of the soil were then monitored at the time and periodically thereafter. The results obtained at nine of these sites are presented in Table 5. On five occasions it was possible to collect blood from the dead animal and to determine terminal blood levels of *B. anthracis*. All other cases were confirmed with M'Fadyean's stain smears which revealed large numbers of *B. anthracis*. According to Lincoln, Walker, Klein, Rosenwald & Jones (1967), large numbers of *B. anthracis* on a smear represent blood counts of 10^7–10^9 cfu/mℓ. The emergent picture was one of a remarkably low survival rate among the very large numbers of *B. anthracis* reaching the soil during the terminal haemorrhage. On only a small proportion of occasions did the soil become substantially contaminated. Despite fluctuations apparent in counts at several of the sites, the overall impression is one of numbers declining with time.

The soil types in the Etosha NP are classified into sandy, deep, karstveld, miscellaneous rock types and saline pan (Le Roux, Grunow, Morris, Bredenkamp & Scheepers 1988). Carcases are rarely found on pans or areas of miscellaneous rock types. The 106 samples from carcase sites were from the other three soil types. A nonparametric Kruskal-Wallis analysis of variance based on ranks, showed that spore counts on the karstveld soils were significantly higher than those on the deep or sandy soils ($P < 0,01$) even when the analysis was repeated and results from sites of elephant carcases only were used to exclude species-related bias.

Experiments on sporulation of *B. anthracis* in soil

The events occurring in soil contaminated with blood from victims of anthrax shortly after their deaths, were studied on four occasions. In the first study at the site of AM(1)91.2.28LS (with terminal blood *B.*

TABLE 4 *Bacillus anthracis* spore counts in 106 soil samples associated with anthrax carcases in the Etosha National Park, Namibia

B. anthracis Spore counts/g	No. of samples
1–10	27
11–100	31
101–1 000	26
1 001–10 000	11
10 001–100 000	7
>1 000 000	4

anthracis level of 2,5 x 10^8 cfu/mℓ), soil samples (karstveld type) were taken at 0, 2, 4, 6, 8, 24, 32, 48 and 72 h and at 1, 4 and 7 months. Spores (6400/g) were first detected at 8 h (0,33% of the total count) and accounted for 75% of the total count of 9,6 x 10^6/g at 24 h; complete sporulation (8,3 x 10^6/g) was achieved between 32 and 48 h. This level of residual soil contamination, as indicated above, was unusually high.

In four other studies, blood from two zebras, EB(1)-91.3.26PML and EB(1)91.3.28PML, and two elephants, LA(1)91.4.25RD and LA(1)91.9.12PM, with terminal blood *B. anthracis* levels of 2,8 x 10^8, 1 x 10^8, 5 x 10^8 and 2,6 x 10^6 cfu/mℓ, respectively, were transported back to the laboratory where 15-mℓ volumes were used to contaminate 100-g samples of karstveld-type soil in 12 x 12 x 3-cm weighing dishes left in the open or, alternatively, the soil in three cordoned-off, 12 x 12-cm sites on the ground.

In two weighing-dish experiments, vegetative cell counts declined from ± 10^6 and ± 5 x 10^6 cfu/g, respectively, to undetectable within approximately 24 h, with almost no sporulation (a single reading of four spores at 15 h). In the contaminated soil on the ground, vegetative cell counts again declined from initial counts of ± 10^7, 3 x 10^6 and 8 x 10^5 cfu/g, to a few thousand by 48 h. Sporulation was again limited, first being detected at 5 d (260 spores/g) and 1 month (40 spores/g) in two of the trials. At the third site, 48 spores were detected at the zero-time reading; subsequent numbers of spores never exceeded 100/g and the count at 1 month was 10/g.

The last two trials were part of a single experiment to obtain an indication of whether the time of day—and therefore the ambient temperature and exposure to sunlight—played an important role in the extent of sporulation or survival of spores. Contamination of the first site was done at 15:00 (ambient temperature 32 °C, sunlight waning towards an 18:30 sunset) and the other at 09:00 the next day (ambient temperature 21 °C, sunlight waxing from a 07:00 sunrise). No preliminary evidence was apparent of time-of-day differences in numbers of vegetative cells surviving and sporulating.

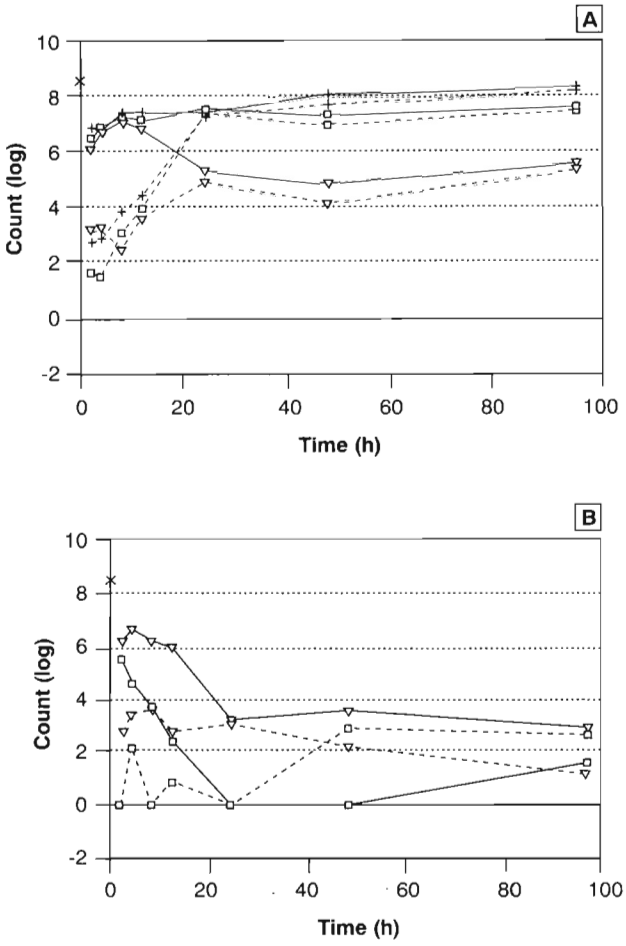

FIG. 4 Fate of *Bacillus anthracis* within blood and mixed with different soils from the Etosha National Park, three sandy soils (A) and two karstveld soils (B). (Solid line = total aerobic count, broken line = spore count, x = contamination level)

All experimentally contaminated soil sites were decontaminated with 10% formalin at the end of the experiments.

To examine the importance of soil type more closely, 15-mℓ volumes of blood from LA(1)91.9.12PM (2 x 10^9 cfu/mℓ) were mixed with 100 g of each of five soil samples, three sandy and two karstveld type, in weighing dishes giving initial contamination levels of 3 x 10^8 cfu/g. Total and spore counts were carried out at intervals over 4 d. The results illustrated in Fig. 4 show that sporulation in sandy soils (Fig. 4A) occurred readily and was complete within 24 h. In the karstveld soils (Fig. 4B), there were substantial decreases in numbers of *B. anthracis*, although some sporulation did occur. The two soil types possessed similar levels of background bacterial flora with total counts all within the range 5 x 10^5 to 10^6 cfu/g and non-anthrax spore counts of 6,5 x 10^4 to 7 x 10^5 cfu/g. The pHs of the karstveld soils were 7,9 and 8,1 and those of the sandy soils were 7,5, 7,6 and 8,2.

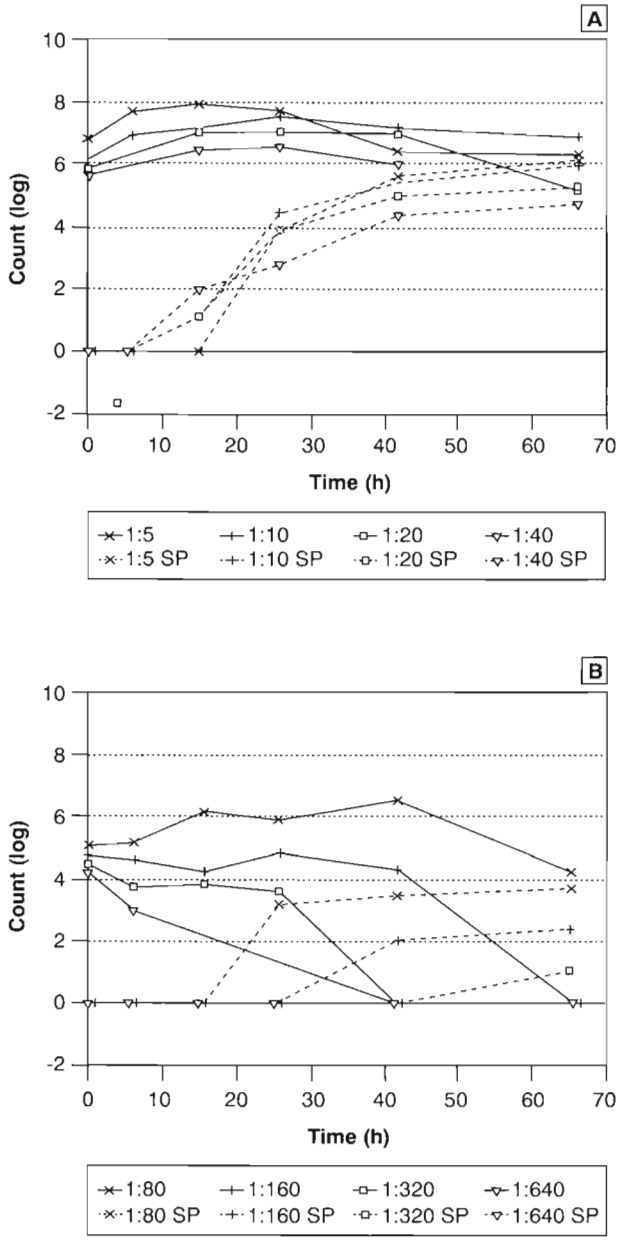

FIG. 5 Fate of *Bacillus anthracis* within the blood of LA(1)91.4.25 RD inoculated at the dilutions shown into Okondeka fountain water (A – dilutions 1:5 to 1:40; B – dilutions 1:80 to 1:640; solid line = total aerobic count, broken line = spore count)

Fate of *B. anthracis* in water

Fig. 5 is representative of events occurring in the four trials following inoculation of Okondeka fountain and Okaukuejo gravel-pit waters with blood from EB-(1)91.3.26PML and LA(1)91.4.25RD. The patterns of events were similar in all four trials. Some multiplication (max. 3,25 log) was seen in the spring water at blood dilutions of 1:5 to 1:320. In them, sporulation had commenced by 15–24 h, and was completed or almost completed by 68 h. At blood dilutions of 1:160 to 1:640 in the spring water, total *B. anthracis* counts declined with an increased rapidity as blood dilution increased, and sporulation was detectable at correspondingly later times until, at 1:640, the vegetative cells had died out before sporulation could occur. In the gravel-pit water it was only at the 1:640 blood dilution that the total counts fell rapidly and late sporulation resulted in a low, final spore count.

The ambient total and spore counts before filtering were assayed and the pH in the 1:5 and 1:640 samples noted at the beginning and end of each trial. Pre-filtration counts in the natural fountain water (total counts 4 and 5,5 x 10^5; spore counts 2 x 10^3 and 1,1 x 10^4) were, in general, one log higher than those in the gravel-pit water (total 8,5 x 10^3 and 1,5 x 10^4; spores 2 and 3 x 10^3). The buffering power appeared to be less in the gravel-pit water, because, although it had similar ambient pH values of ≥ 9, the pH of the gravel-pit water, after addition of blood (pH 6.0), was lower (6,5 in the 1:5 dilution and 8,4 in the 1:640 dilution) than that of the natural fountain water (7,4 at 1:5 and 9,2 at 1:640). To avoid contamination of the pH pro, it was necessary to measure the pH of the blood, and water containing dilutions of the blood, after filtration through a 0,45-μm filter.

Susceptibility to ultraviolet irradiation and sunlight

Although the tests were not carried out to the point of complete sterility, 4+ growth was apparent around the threads collected after 30 min exposure to the UV source. Growth was reduced to 1+ after 60 min. The cabinet temperature during the experiment was 33–35 °C.

Reduced growth around threads exposed to the sun was apparent at 1 h (3+) but not at 30 min (4+). At 4 h, sterility had almost been reached, with one of the duplicate threads yielding no growth and the other producing 10 poorly formed colonies suggestive of damaged cells. The peak angle of the sun to horizontal was 56,5° and the temperature was 34,5 °C around the exposed spores. Shade (27 °C) and refrigerated controls showed 3+ and 4+ growth at 4 h.

DISCUSSION

Incidence and seasonality

The incidence of anthrax has declined in all species except elephant, since the early 1970s, and elephant, Burchell's zebra, blue wildebeest and springbok account for nearly 97 % of all recorded anthrax deaths (Table 1), while representing only some 55 % of all large herbivores in the park. In terms of population size and the number of anthrax deaths

on record, blue wildebeest appears to have been the most susceptible species, followed by elephant, Burchell's zebra and springbok.

Wherever it occurs, anthrax is seasonal in incidence (Kellogg, Prestwood & Noble 1970; De Vos 1971, personal communication cited in Ebedes 1976; Wise & Kennedy 1980; Davies 1983; Prins & Weyerhaeuser 1987), but there is no consistency from location to location with regard to time of year or climatic conditions during peak incidence. The occurrence of the overall peak anthrax activity among Etosha-plains ungulates in the rainy season differs from those in other localities such as the Kruger, Lake Manyara and Luangwa National Parks, where peak mortalities occur during the dry months of the year (Prins & Weyerhaeuser 1987; De Vos 1990; Turnbull, Bell, Saigawa, Munyenyembe, Mulenga & Makala 1991). Traditionally held causes of anthrax outbreaks, such as overgrazing and feeding by cropping close to the ground, are unlikely to be important in the Etosha-plains ungulates, as the overall-peak-mortality period occurs during optimum grazing conditions.

The difference between the pattern of elephant and plains-ungulate mortalities is also of interest. The data suggest that reasons for seasonality in anthrax are more complex than simply climatic conditions, and it is possible that host condition at any particular time plays an important role. This is also supported by the well-recorded observations of anthrax epizootics affecting only one species among several with apparently similar exposure (De Villiers 1943; Pienaar 1961, 1967; Gainer 1987; Prins & Weyerhaeuser 1987); species may undergo different physiological or behavioural cycles which can affect their condition. Of the three distinct outbreaks experienced in the Etosha NP, only the 1984 outbreak involved more than one species. No anthrax deaths were recorded in any other species during the two major elephant epizootics, even though large numbers of Burchell's zebra, springbok, and gemsbok used the waterholes where many of the elephant carcases were found.

Age and sex

With regard to determining age and sex in anthrax carcases, a general indication can usually be obtained from the size and shape the of horns, tusks and body and, in zebras, from the dentition (Smuts 1974). Sub-adult individuals of all the major susceptible species can be difficult to sex, even when the carcases are fresh, unless they are dissected; this is not encouraged when anthrax is the suspected or proven cause of death. A bias towards adult males has also been reported before in elephants in the Etosha NP (Ebedes 1976; Lindeque 1988) and other species in other parks (Pienaar 1961; Moynihan 1963). De Vos (1990), however, found no sex-related difference in susceptibility among animals in the Kruger NP. No adequate explanation has been offered yet to account for apparent sex differences in the incidence of anthrax. No striking differences in behaviour, distribution, condition or diet are apparent during the peak anthrax season. A sex-associated difference in the resistance to anthrax resulting from immunization has been reported in laboratory mice (Ivins, Welkos, Little, Crumrine & Nelson 1992), and these authors suggest that the role of gender in both innate and acquired resistance to anthrax requires further evaluation.

B. anthracis in the environment

Anthrax enzootic areas have been linked to high levels of environmental contamination with anthrax spores (Ebedes 1976; De Vos 1990). Attempts in this and two previous studies (Turnbull *et al.* 1986; 1989) to identify environmental sites in the Etosha NP with substantial concentrations of anthrax spores have persistently failed to reveal particular sites from which the wildlife could be contracting the disease. When *B. anthracis* is found in the Etosha environment it is present only in low numbers and, at least in waterholes, for limited periods. Scavenger activity, especially vultures coming to bathe after feeding on anthrax carcases, may account for the transient contamination of water.

The importance of gravel pits in the anthrax cycle in Etosha, as hypothesized by Ebedes (1976) is not evident from the results. Although gravel pits accounted for 60% of the positive results, *B. anthracis* was isolated from only two of the nine gravel pits tested. Adamax gravel pit yielded the organism on six occasions, once in the water (1 spore/mℓ) and the rest in soil. This gravel pit, in particular, retains water for as long as two months after the rains have stopped, which makes it a focus for animal concentration. The presence of anthrax spores here is attributable to the large numbers of animals utilizing the waterhole at peak anthrax times. Anthrax spores in the soil probably derive from carcases of animals that died close to the water.

Distribution of *B. anthracis* by scavengers

Positive isolations of anthrax spores were obtained from 72% of the jackal, 60% of the hyaena and 50% of the vulture faeces collected around anthrax carcases (Table 3) with numbers frequently high and potentially able to produce new foci of infection. Levels of anthrax spores in scavenger faeces depend largely on the state of anthrax sporulation in the carcases as vegetative *B. anthracis* probably do not survive transit through the digestive system of vultures and carnivores (Houston & Cooper 1975; Ebedes 1976). Although no statistical differences were found between the spore counts obtained from scavenger

TABLE 5 Monthly rersistence of contamination with *Bacillus anthracis* at the sites of nine anthrax carcases in the Etosha National Park, Namibia (spores/g of soil)—according to animal ID, soil type and pH

Month	SITE 2	SITE3	SITE 4	SITE 5	SITE 7	SITE 9	SITE 10	SITE 12	SITE 13
	EB87-03-21 Karstveld 7,7	EB88-03-05 Karstveld 7,8	EB88-05-03 Karstveld 7,4	AM88-07-26 Karstveld 8,4	EB89-02-21 Sandy 8,2	LA89-08-04 Sandy 7,5	EB90-04-23 Karstveld 7,9	EB90-04-25 Karstveld 7,8	LA90-09-17 Karstveld 7,6
0	700 000 000[a]	50 000 000[a]	[b]	[b]	[b]	24 000	40 000 000[a]	1 440	200 000 000[a]
1	33 800						1 280	4 800	
2				0			1 120		0
3				0		6 600	560		
4			4		200	20 000	480	24	
5	2 400		580						
6		922				35 800			
7		1 934					18 600	0	0
9					50		7 600		0
10			2 480		400			16 000	
11					560				
12					30		3 360		
13					36 800				
14				2			0	32	
15	6				2 000	1 040			
16			0	0	4 400				
17				0					
18	2	3 200	400	0	33 200				0
19	36								
20		52 300	0	0					
21		420			800				
22		38 002	32	0					
23						0			
24		1 200	0						
25				0	560				
26	2	400			800				
27			0						
28				0	2 400				
29		6 400							
30			0	0					
31									
32	0	400	0						
33				80					
34	4	16 800							
35			0						
36	0						0		
37		0							
38	0			0					
40			0						
41	0								
42	0	9 600							
44	0								
49	0								
51	0								
60	0								

[a] Terminal blood *B. anthracis* counts (per mℓ) in the animal that died at that site
[b] Confirmed through blood smear on that date

species (Kruskal-Wallis test, $P > 0,1$), maximum and mean spore counts (Table 3) might reflect specific habits. Vultures usually find carcases first, and would be expected to feed more often on fresh carcases where sporulation has not yet occurred. In contrast, hyaenas are more likely to chew on older scraps of skin and bones, with a higher chance of ingesting large numbers of spores. The importance of vultures as disseminators of anthrax spores may be outweighed by their ability to minimize contamination of the environment through rapid consumption of carcases.

Sporulation and survival of *B. anthracis*

Despite high terminal blood counts in animals that die from anthrax, extensive contamination of soil from haemorrhaged or spilt blood does not always occur and, when it does, spore levels are often relatively low (Tables 4 and 5). It is evident that only a small proportion of the released bacilli sporulate successfully. Temperature and moisture are known to affect the sporulation of *B. anthracis* (Howie 1949; Minett 1950; Davies 1960). Maximum daily temperatures at the time of death of the animals at all

marked sites were nevertheless always above 25 °C, which is conducive to active sporulation. Ambient humidity is probably irrelevant, as over the period in which sporulation will take place, anthrax bacilli are still surrounded by carcase fluids. Other factors therefore account for the low incidence of sporulation as reported here.

It appears that multiplication did not occur at the 13 marked sites (Table 5). Spore numbers at the site of a carcase decline over time. Samples were always taken from the superficial (< 2 cm) soil layers, and declines in numbers may be caused by dispersal due to wind or water. Also, although the soil-contamination experiments which commenced at different times of the day failed to highlight any variations attributable to sunlight, the susceptibility of spores to sunlight was demonstrated on the spore traps; it remains a possibility that even short-term exposure to sunlight may kill spores in the field. Other reports recording similar degrees of susceptibility of anthrax spores to sunlight are summarized by Mitscherlich & Marth (1984). The antagonism of other soil flora may contribute to the decline of *B. anthracis*. Nevertheless, it remains clear that anthrax spores can survive in the Etosha environment for long periods. The rapid decline in viable, shed vegetative bacilli and relatively low numbers of residual spores in the soil-contamination experiments parallels the observations at carcase sites in the field (Tables 4 and 5).

A preliminary clue to the importance of soil type was obtained by the significant difference in spore counts from experiments on sandy and karstveld soils (Fig. 4). The sandy soils showing maximum sporulation had the lowest pH values and the lowest total and spore counts of all five soils. The soil least conducive to survival and sporulation of *B. anthracis* had the highest bacterial level, and the second highest pH value. The results of these experiments differed from those found from the analyses of carcase sites, which showed higher levels of *B. anthracis* contamination on karstveld-type soils. Further studies on factors determining survival and sporulation of *B. anthracis* in soil are needed.

The studies on water experimentally contaminated with infected blood were extensions of previous attempts (Turnbull *et al*. 1989; 1991) at understanding the events occurring after *B. anthracis* is shed by a carcase in a waterhole. The rapid death of the vegetative bacilli, once the protective nature of the blood has been lost by dilution, is in agreement with those previous studies. The results help to account for the low levels of contamination of waterholes associated with anthrax deaths. It would appear that the extent of contamination depends on the rate at which the haemorrhaged or spilt blood is diluted, which, in turn, is dependent on whether the water is flowing—for example, near the spring of a natural fountain—or disturbed—for instance, by other animals utilizing the waterhole.

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