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Confirmation of *Bacillus anthracis* from Flesh-eating Flies Collected during a West Texas Anthrax Season

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ABSTRACT: This case study confirms the interaction between necrophilic flies and white-tailed deer, *Odocoileus virginianus*, during an anthrax outbreak in West Texas (summer 2005). *Bacillus anthracis* was identified by culture and PCR from one of eight pooled fly collections from deer carcasses on a deer ranch with a well-documented history of anthrax. These results provide the first known isolation of *B. anthracis* from flesh-eating flies associated with a wildlife anthrax outbreak in North America and are discussed in the context of wildlife ecology and anthrax epizootics.

Key words: Anthrax, *Bacillus anthracis*, disease transmission, necrophilic flies, Texas, white-tailed deer, wildlife.

Despite its being a zoonotic disease that continues to impact livestock and wildlife, and humans secondarily, worldwide (Smith et al., 2000; Hugh-Jones and De Vos, 2002), the transmission cycle of *Bacillus anthracis*, the causative agent of anthrax, remains poorly understood in wildlife populations. Although studies continue to expand our understanding of the bacterial genome (e.g., Van Ert et al., 2007; Simonson et al., 2009), a more limited literature is available on wildlife epidemiology of the disease, particularly for North America (e.g., Dragon and Elkin, 2001; Blackburn, 2006). Several species of necrophilic flies were implicated as *B. anthracis* spore carriers a century ago (Buchanan, 1907; Graham-Smith, 1914; Morris, 1918). In some of those early studies, researchers experimentally exposed flies (Muscidae and Calliphoridae) to contaminated mouse carcasses and systematically recovered viable spores from fly body parts up to 20 days post-feeding, 14 days from their feces, and vomit for at least 6 days postdeposition (Graham-Smith, 1914). As the flies were exposed to freshly opened carcasses within several minutes of death, it was presumed that individual flies were ingesting vegetative cells (Graham-Smith, 1914). Graham-Smith (1914) also confirmed that viable *B. anthracis* spores fed to larvae could be recovered after pupation to adulthood. Morris (1918) expanded these experiments with guinea pigs to simulate the effects of temperature and putrefaction on *B. anthracis* viability under various environmental conditions; he showed that when carcasses were opened to the environment and flies were allowed to feed, their body parts and feces or vomit nearly always tested positive for *B. anthracis*.

Braack and De Vos (1990) reported similar results to that of Graham-Smith (1914) and Morris (1918) under field conditions. This study reported previously unpublished results by one of the authors confirming the isolation of *B. anthracis* spores from fly feces and vomit deposited on vegetation surrounding disease-positive wildlife carcasses in Kruger National Park, South Africa. Braack and De Vos (1990) also reported that unpublished experiments confirmed spores survive in the digestive tract of flies and were deposited from feces in large quantities on leaves. In this same experiment the authors reported animals fed these recovered spores died. De Vos (1990) attributed anthrax epizootics in greater kudu (*Tragelaphus strepsiceros*) in Kruger National Park to flies and correlated previous large outbreaks in

1960 and 1970 that coincided with large population explosions of blowflies. The Braack and De Vos study (1990) reported that blowflies of the *Chrysomyia* genus contaminate vegetation species important in the diet of browsing ungulates in Kruger National Park by defecating and vomiting spore-positive matter at preferential browsing heights. Their study also demonstrated through experimentation that individual flies were more likely to deposit these materials in close proximity to carcasses. Braack and De Vos (1990) reported *Chrysomyia* flies in general and individuals within the study were more likely to land, rest, and digest material near the carcass (often within 1 m) before any long-distance trips. Although they noted marked flies were trapped upwards of 25–32.5 km away from the carcass, they quantified the greatest feces and vomit droplet counts in close proximity to the carcass where the flies had fed. In at least one US report, Steele and Helvig (1953) confirmed a high abundance of necrophilic flies associated with anthrax-positive cow carcasses, and in at least one case isolated spores from flies collected on a carcass and successfully cultured viable *B. anthracis* from those flies.

In an anthrax outbreak among wildlife, flies will imbibe the body fluids and tissues of dead animals and potentially pick up *B. anthracis* vegetative cells or spores on their body parts as well as ingesting them. Once satiated or disturbed, flies travel to nearby vegetation and defecate or regurgitate, depositing *B. anthracis* on leaves. The deposited spores may be ingested by browsing ungulates, resulting in new infections (Braack and De Vos, 1990). Hugh-Jones and De Vos (2002) extended this hypothesis of necrophilic fly contamination and infection to white-tailed deer (*Odocoileus virginianus*) in North America, based on the white-tailed deer's well known browse feeding habits and the high incidence of anthrax in deer in Texas. White-tailed deer have varied diets in Texas, but during summer months ingest a higher percentage of browse than forbs or grasses (Fullbright and Ortega-S, 2006). Likewise, deer home ranges during the summer months are limited across Texas (Fullbright and Ortega-S, 2006). Blackburn (2006) confirmed limited home ranges of 109.6 or fewer hectares in both genders on a West Texas ranch with repeated anthrax outbreaks, suggesting animals in the vicinity of anthrax-positive carcasses were likely to remain in the area and therefore feed in the area. Despite these speculations, data are unavailable to confirm the presence of *B. anthracis* spores in necrophilic flies in the US during anthrax outbreaks or the possible relationships between flies and deer carcasses. This field report documents the presence of anthrax spores in necrophilic flies collected during the 2005 anthrax season (summer months) in West Texas.

In an attempt to isolate *B. anthracis* from flies during the anthrax season in North America, necrophilic flies were collected from carcasses of eight dead deer between 5 June and 15 October 2005 on a ranch in west Texas with documented cases of anthrax in wildlife (Blackburn, 2006). This ranch, approximately 80.5 km north of Del Rio, Texas, had enzootic anthrax in deer between 2001 and 2005. Large epizootics were well documented in 2001 and 2005, with smaller intermittent incidents of a single or few (one to seven) cases in the intervening years (Blackburn, 2006). During the 2005 outbreak, flies were collected by holding a sterile Whirlpak® bag (Nasco, Fort Atkinson, Wisconsin, USA) over a natural skin tear in the axillary region of the carcass. As flies left the body cavity, they exited the lesion and flew upwards into the bag. The bag was closed after five to 10 flies entered. Flies were frozen at −30 C in the field. Prior to laboratory analysis, flies were identified as members of the Calliphoridae and Sarcophagidae (Diptera) families by the Department of Entomology at Louisiana State University. Flies were then trans-

ported to the Midwest Research Institute for diagnostic analyses.

To test for *B. anthracis*, all flies from a single sampling bag (often between three and 10 flies) were pooled and placed in 1 ml of PBS and ground with a tissue grinder. A portion of the suspension of ground tissues was heat shocked for 30 min at 65 C. This technique kills gram-negative and nonsporulating, gram-positive aerobic bacteria, allowing the best opportunity to recover sporulated *B. anthracis* from the samples. One-hundred–microliter samples of pre– and post–heat-shocked, ground fly suspension were transferred to blood agar plates and incubated at 30 C for 24 hr.

A single colony of *B. anthracis* was isolated from one of the eight pools of flies collected (13%; 95% CI, 0–52%). The colony was nonhemolytic and showed atypical morphology on blood agar, lacking the typical ground glass appearance (colony appeared smooth). Gram stains showed a large, gram-positive bacillus resembling *B. anthracis*. The colony was subcultured and replated on trypticase soy agar. The subculture had morphology typical of *B. anthracis*. The culture was identified using microbial biochemical characterization (Microlog, BiOLOG, Hayward, California, USA) and confirmatory polymerase chain reaction (PCR; PathAlert, Invitrogen Corporation, Carlsbad, California, USA).

This result confirmed necrophilic flies become contaminated with *B. anthracis* after interacting with deer carcasses in West Texas and may subsequently play a role in outbreak promotion. The relatively wide confidence interval suggests the potential for a substantial population of flies feeding on positive carcasses to potentially uptake spores (or vegetative cells) and transport them away from carcasses. Additionally, laboratory efforts were focused on recovering *B. anthracis* spores and may have missed potential vegetative cells present in imbibed blood or on the fly bodies, which very likely reduced the overall recovery rate from these eight samples.

Despite the potential for long-distance travel by necrophilic flies, it is more likely they will defecate or regurgitate within the immediate vicinity (first several meters) of the carcass (Braack and De Vos 1990). Because of this, these flies probably do little to expand the spatial extent of anthrax outbreaks. It is more plausible that necrophilic flies move viable *B. anthracis* (vegetative cells or spores) from carcasses on the ground to nearby vegetation preferred by deer, thereby increasing the number of deer consuming virulent organisms originating from the positive carcass. This phenomenon has previously been described as the Case-Multiplier Hypothesis, whereby multiple deer may be exposed to organisms from a localized index case through ingestion of contaminated browse. This hypothesis would provide a mechanism to support observations of rapid onset of multiple but localized cases in wildlife outbreaks discussed by Hugh-Jones and De Vos (2002). However, this hypothesis still lacks quantitative evidence beyond the isolation of *B. anthracis* in flies. The low recovery rates found in this pilot study suggest greater effort is needed to understand the role that calliphorid and sarcophagid flies may play in transmission and that a more robust field sampling strategy should be coupled with a more exhaustive laboratory effort to recover *B. anthracis* (both spores and vegetative cells).

It is also important to acknowledge the potential of hematophagous flies to mechanically transmit organisms between individual animals. A number of studies have confirmed biting flies can successfully move *B. anthracis* spores between animals. Turrell and Knudson (1987) successfully showed that *Stomoxys calcitrans* (and *Aedes* spp. mosquitoes) could successfully inoculate naive guinea pigs under laboratory conditions. Krishna Rao and Mohiyudeen (1958) confirmed mechanical transmission under field conditions during a large cattle outbreak in India. Although this has not been con-

firmed during North American outbreaks, several studies implicate biting flies in swine (Anonymous, 1951), cattle (Mongoh et al., 2008), and wood bison (Gainer and Saunders, 1989; Dragon and Elkin, 2001). In a case report specific to white-tailed deer, Kellogg et al. (1970) suggested a combination of high deer density and abundant biting flies lead to a large epizootic on Beuhla Island, Arkansas. Ganeva (2004) summarized a number of laboratory studies performed on Tabanidae species and suggested more than 20 species could successfully mechanically transmit spores. Additional work is needed to determine the quantity of *B. anthracis* spores or vegetative cells that various fly species may deposit on leaves (necrophilic flies) or that may be mechanically transmitted during a blood meal (hematophagous flies). These results would also need to be combined with diet studies confirming deer feed on those browse species where necrophilic flies land during summer months. Additionally, there is a need to calculate the median lethal dose for anthrax in North American white-tailed deer.

As many of the wildlife species infected in Africa and North America are browsers during at least part of the year, consumption of spore-contaminated browse is a plausible source for infection. Although it is unrealistic to rule out infection through grazing grasses and low-lying vegetation and the subsequent ingestion of soil, the results of this pilot study, and the well-documented history of necrophilic flies as spore carriers, suggest further research is warranted to define the role of flies in North American wildlife outbreaks of anthrax.

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