

ECTOPARASITE ECOLOGY AND DISEASE EXPOSURE IN BLACK-FOOTED FERRETS
(*MUSTELA NIGRIPES*) AND ASSOCIATED MAMMALS IN CONATA BASIN/BADLANDS
NATIONAL PARK, SOUTH DAKOTA

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Project Abstract:

The black-footed ferret (*Mustela nigripes*; ferret), one of North America's most endangered mammals, persists within prairie dog (*Cynomys* spp.) burrow systems where disease remains a major obstacle to their recovery. While plague (*Yersinia pestis*) is well recognized as a primary threat, tularemia, a bacterial disease caused by *Francisella tularensis* and vectored by ticks and other ectoparasites, also occurs in grassland ecosystems. However, ferrets appear capable of surviving tularemia infection, although the ecological drivers, biological impact, and vector pathways involved in exposure remain unknown. Our objective was to determine variables that explain ferret exposure to tularemia over time, evaluate ectoparasites found on ferrets and their prey, and determine the role of ectoparasites in pathogen transmission to ferrets. We found two mite species on black-footed ferrets that are not parasitic or of pathogenic concern. Serological analyses indicated that 6.3% of ferret serum samples (81/1277) were seropositive for tularemia between 2002-2024. Ferrets recaptured within 5-12 months showed changes in serostatus, indicating that antibodies do not persist long-term. We identified *Ixodes sculptus* and *Ixodes kingi* on ferrets as well as on grasshopper mice (*Onychomys leucogaster*), *Peromyscus* spp., and several other small mammals. A total of 304 (91.4%) ferrets and 173 (27%) small mammals carried at least one tick, and grasshopper mice were more often parasitized (54%) than other small mammal species. Adult *I. sculptus* and *I. kingi* are common on ferrets, whereas small mammals primarily carried larval and nymphal life stages. Unlike many *Ixodes* spp. that quest above ground on vegetation, these ticks appear to be nidicolous, remaining within burrow systems, where ferrets and their prey interact, for one or more of their life stages. Standard surface-based sampling techniques for ticks were ineffective for detecting *Ixodes* spp. in prairie dog colonies. Additionally, no ticks or fleas collected from seropositive ferrets were positive for *F. tularensis* DNA via PCR analysis.

Tularemia periodically emerges and re-emerges in grassland ecosystems, and black-footed ferrets can seroconvert and survive infection. Their antibody responses appear to be short-lived despite repeated exposure and the relationship between these antibodies and pathogen memory remains unknown. Although ticks, mites, and fleas are common on ferrets, none of the ticks collected from seropositive individuals tested PCR-positive for *Francisella tularensis*, suggesting that the specific ticks sampled are unlikely vectors, though transmission could have occurred from ticks that detached prior to surveillance. As a result, experimental vector-competency testing for *Ixodes kingi* and *I. sculptus* remains warranted. These *Ixodes* species occur primarily within prairie dog burrows and on vertebrate hosts rather than on the prairie surface, indicating that effective surveillance should prioritize belowground sampling and host-based collections instead of conventional surface methods. Overall, tularemia is likely not a significant conservation concern for black-footed ferret populations, but continued research into tularemia ecology and vector dynamics is needed.

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Chapter 1: Literature Review and Introduction

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The Grassland Ecosystem and Black-footed Ferrets

Emerging or re-emerging infectious diseases pose significant threats to many wildlife species (Tompkins et al. 2015), particularly endangered species (Smith et al. 2009). Globally, viruses such as *Orthoflavivirus nilense* that causes West Nile Virus (Uelmen et al 2020), bacteria such as *Borrelia burgdorferi* that cause Lyme Disease (Tsao et al. 2021), fungi such as *Pseudogymnoascus destructans*, the causative agent of White Nose Syndrome (Hoyt et al. 2021), and non-infectious agents such as lead intoxication (Pain et al. 2019) are rising in wildlife populations (Tompkins et al. 2011; Cohen et al. 2020). These diseases have complex evolutions that vary with the pathogen involved and geographic, climatic, land use, and ecosystem drivers (Olson et al 2015). Increases in urbanization allow for human-wildlife zoonotic disease interaction (Schell et al. 2020). Climate change may exacerbate potential disease outbreaks (Cohen et al. 2020) and promote vectors (Ostfeld 2009), thereby complicating endangered species conservation.

The grassland ecosystem is considered the most endangered ecosystem in North America (Flores 1996; Martin et al. 2005; Engle et al. 2008), greatly impacting species like prairie dogs (*Cynomys* spp.) and black-footed ferrets (*Mustela nigripes*; referred to hereafter as ferret) that rely on grassland biomes. Once ranging throughout the Great Plains from southern Canada into northern Mexico, ferrets are currently on the IUCN red list (Belant et al. 2015) and listed as endangered under the Endangered Species Act of 1973 (U.S. Fish and Wildlife Service 2019).

Ferrets do not exist outside of prairie dog colonies, rarely leave their colony, and fully subsist within their burrows for their entire lives. Ferrets are prairie dog specialists and therefore rely on robust prairie dog populations throughout their range (Hillman and Clark 1980; Livieri and Anderson 2012). Prairie dogs comprise 75 – 90% of ferret diets throughout their range (United States Fish and Wildlife Service 2019). An estimated decline of nearly 98% of prairie dog populations has occurred since the time of European settlement (Samson and Knopf 1994; Forrest 2005). Due to a dramatic loss in prairie dog populations coupled with sylvatic plague caused by the bacterium *Yersinia pestis* and canine distemper virus caused by *Morbillivirus canis*, black-footed ferrets were feared to be extinct in 1979 (Livieri et al. 2022). In 1981, a small population of black-footed ferrets was discovered by the Hogg family and their dog Shep, near Meeteetse, WY (Forrest et al. 1988; Lockhart et al. 2006). From this population, successful captive breeding and reintroduction practices began (Russell et al 1994). Over 5,100 ferrets have been released since the beginning of the program (United States Fish and Wildlife Service 2019; Livieri et al. 2022). Reintroduction efforts began at Conata Basin/Badlands National Park, South Dakota in 1994, and it is now the largest population of wild ferrets in the world (Livieri et al. 2022). The black-footed ferret recovery program focuses most of the disease mitigation, surveillance, and research efforts on plague because it is the most severe biological threat to in-situ populations (United States Fish and Wildlife Service 2019, Marinari et al. 2024). Plague is vectored by fleas (Gage and Kosoy 2006; Antolin et al. 2002) and causes mass mortality of prairie dogs and ferrets (Abbott and Rocke 2012; Biggins and Eads 2019). Effective vaccines for plague and canine distemper virus have been developed for ferrets (Powell et al. 2005, Wright et al. 2022), plague vaccine baits have been used for prairie dogs (Rocke et al. 2008, Rocke et al.

2010), and burrow systems are “dusted” with the insecticide deltamethrin targeted at reducing flea loads (Eads et al. 2020).

Ferrets have now been reintroduced into Wyoming, South Dakota, Montana, Kansas, Colorado, Utah, New Mexico, Arizona, Canada, and Mexico (United States Fish and Wildlife Service 2019). Ferrets are a flagship species for the prairie ecosystem and are the target of substantial conservation efforts to recover the species (United States Fish and Wildlife Service 2019).

Sustaining wild ferrets remains difficult as few prairie dog colonies are large enough to support a successful ferret population, and disease concerns remain (Santymire et al. 2014).

Ferrets and Their Prey

The endangered black-footed ferret is the only native wild ferret species in North America (Forrest et al. 1988). Ferrets are identified by buff colored fur with a black facemask, black feet, and a black tip on their tail. Similar in size to a mink (*Neovison vison*), they range from 119 to 145 cm in length and weigh between 550-1340 g (Livieri et al. 2022), with slight sexual dimorphism as males can be larger (Anderson et al. 1986). Male home ranges are twice the size of females (Livieri and Anderson 2012; Jachowski et al. 2010). Overall, ferrets are solitary, except during the breeding and kit-rearing periods, starting in mid-March. Females rear litters of one to four kits until early fall when kits are mostly fully independent and on their own (Forrest et al. 1988; Livieri et al. 2022). By the following breeding season, these kits will reach sexual maturity and be able to contribute to the gene pool (U.S. Fish and Wildlife Service 2019). Ferrets are nocturnal, spending 90% of their lifetime underground where they use prairie dog burrows for sleeping, raising young, hunting prey, and escaping from predators and weather (Hillman

1968). Prairie dogs are primary prey but other small rodent species like the northern grasshopper mouse (*Onychomys leucogaster*) and the deer mouse (*Peromyscus* spp.) also are consumed (Brickner et al. 2014; Livieri 2023). Because of a high metabolism, ferrets typically consume one prairie dog every three to four days, approximately 100 prairie dogs per individual ferret per year (Harrington et al. 2003).

Ferrets are a focal species for grassland conservation, with their presence being indicative of a healthy prairie dog system (Miller and Reading 2012). Black-tailed prairie dogs (*C. ludovicianus*; prairie dogs hereafter) are a keystone species within the grassland ecosystem in southwestern South Dakota (Miller et al. 2000; Hoogland 2006). They range in pelage from yellowish, to dark brown, to reddish in color and are characterized by a long tail with a black tip (Hoogland 1995). Similar to other ground squirrels, prairie dogs are diurnal (King 1952) but differ from other burrowing species as they will stay above ground until sunset and will remain underground until sunrise the following morning (Hoogland 1995). Unlike other prairie dog species, they typically do not hibernate (Lehmer et al. 2006) but will remain inactive underground during severe weather (Hoogland 1995). They are mainly herbivorous (Davidson et al. 2012), excluding cannibalism events (Hoogland 1995), and provide beneficial ecosystem services by mixing soil layers and maintaining nutrient dense grasses for grazing ungulate species (Miller et al. 2000; Hoogland 2006).

Prairie dogs live in clusters called colonies (Biggins et al. 2006). Within each colony, prairie dogs occur in harem-polygynous family groups called coteries (Hoogland 2006). Some coteries can be large with a single breeding male, three or four females, and a mixture of juveniles that work together to create and live in burrows (Hoogland 1995). These complex burrow systems can be 3m deep and over 30m long with a variety of tunnel entrances (Sheets et al. 1971).

Temperatures in these systems range from 7 to 24°C with consistent humidity year-round required for prairie species, including parasitic invertebrates (Hoogland 1995; Hixson 1932).

Northern grasshopper mice range across west-central North America and typically occur at low densities (McCarty 1978). These mice have a characteristically short tail and strong odor (Bailey and Sperry 1929, Kurta 2017). They are nocturnal with heightened activity on moonless and cloudy nights (Kurta 2017). They produce two or three litters per year with breeding and birth between September and February. Individuals reach sexual maturity around three months, with a lifespan averaging between a few weeks to a few months in the wild. They are carnivores, with their diet mainly consisting of small insects, but have been documented eating other mice, snakes, and small amounts of plant material (Stapp 1999). They are positively associated with prairie dogs and utilize burrow systems for habitat (Kurta 2017). Previous studies suggest that these mice may be alternate hosts for plague and important for the maintenance and transmission of fleas and plague bacteria within the grassland ecosystem (Stapp et al. 2009; Kraft and Stapp 2013).

Similar in species, the white-footed mouse (*Peromyscus leucopus*) and eastern deer mouse (*Peromyscus maniculatus*) both occur within the southwestern South Dakota grassland ecosystem, and range across the majority of North America (Livieri 2023). Eastern deer mice are nocturnal, spending the daytime in nests in burrows. Both breed throughout the year, but most commonly between March and October, with breeding throughout much of their range thought to be driven by food availability with their lifespan between 12-24 months (Kurta 2017). These mice are omnivorous with seeds, leaves, and arthropods making up their diet (Witmer and Moulton 2012).

Tularemia

Francisella spp. are gram-negative, intracellular, coccobacillary bacteria that can be pathogenic to humans and wildlife worldwide. Antigenically, these bacteria closely resemble *Brucella* spp. (Skyes 2025). Currently, there are two major species, *F. tularensis* and *F. philomiragia* (Yeni et al. 2021). Tularemia, also known as rabbit fever, is caused by *F. tularensis*, which can cause zoonotic cases of the disease. First discovered in Tulare County, California in 1911 by George McCoy, tularemia is now found worldwide (Hirschmann 2018). Four pathogen subspecies persist worldwide, with *F. tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B) most commonly found in North America (Farlow et al. 2005).

Tularemia is a Centers for Disease Control (CDC) reportable bioterrorism agent. Infection can cause a wide range of symptoms that often are relative to the mode of transmission and where the infection is occurring in the body (Centers for Disease Control and Prevention 2024).

Clinical signs in animals range from fever, tachycardia, oral ulceration, lymphadenopathy, and hepatosplenomegaly (Skyes 2025). Mortality may occur as quickly as a few hours after exposure; however, chronic infections with mild symptoms can occur (Skyes 2025). Sepsis can happen if the immune response is not controlled (not localized) which can lead the infected animal/human to have organ failure, low blood pressure, fever, and/or go into septic shock (Kuby 2019). Diagnosis for tularemia is done through bacterial culture, serological testing, and/or PCR assay (Sykes 2025). There were 139 reported cases of tularemia in humans in the United States in 2024 (Centers for Disease Control and Prevention 2025). Human incidence of disease has increased 60% in the United States since 2011 (Bishop et al. 2023). In 2025, ninety-

four positive human cases of tularemia in the United States were reported to the CDC (Centers for Disease Control and Prevention 2025).

Tularemia impacts over 100 wildlife species but is mainly a disease associated with lagomorphs (*Sylvilagus* spp., *Lepus* spp., and *Ochotona* spp.) and rodents (Farlow et al. 2005; Hayes 2005).

Transmission can occur through the bites of ectoparasites, by inhaling or consuming contaminated soil, water, or air particles, via direct contact with an infected animal, or when eating an infected animal. The deer fly (*Chrysops discalis*) was the first vector to be associated with human cases of tularemia (Farlow et al. 2005). Ticks, or tick feces, may be a reservoir of the bacterium (Hayes 2005; Zellner and Huntley 2019), and transmission has been documented through the blacklegged tick (*Ixodes scapularis*), dog tick (*Dermacentor andersoni*), and lonestar tick (*Amblyomma americanum*), and mechanically through deer and horse flies (Maestas 2019). Some suggest species of *Francisella* may be endosymbionts of ticks, creating obligate interactions between the invertebrate and the bacterium (Scoles 2004; Gerhart et al. 2018).

Tularemia abundance in an ecosystem is affected by multiple factors but is mainly associated with the species, both vertebrates and vectors, found within an area. *Francisella tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B) are the most clinically relevant. Type A in North America is highly virulent and pathogenic, with disease caused by a small number of bacteria (Yeni et al. 2021). Lagomorphs are thought to be the reservoirs for Type A in North America, where the cycle is terrestrial and maintained through biting flies/ticks and direct contact with infected animals (Telford and Goethert 2020). Type B occurs in aquatic rodents such as beavers (*Castor* spp.), muskrats (*Ondatra* spp.), and voles (*Microtus* spp.) in North America (Telford and Goethert 2020). It is less virulent and has been isolated in fresh waterbodies, and in waterborne free-living amoebae (Sjostedt 2007; Sharma et al.

2023). *Francisella tularensis* may persist within the environment through infected carcasses, soil, and water for months without a host, surviving through freezing temperatures (Sykes 2025).

Hosts can be exposed to *F. tularensis* through biting vectors, exposure to or eating an infected animal, and contaminated particles in the air, water, and soil (Farlow et al. 2005; Hayes 2005).

Bacteria often are transmitted from ticks to hosts 36–48-hours after attachment (Cook 2015).

Once a tick finds a host, it often searches for an area of thin and warm skin where it uses a chelicerate (saw-like mouthpart) to make an entry incision and hypostome (barbed mouthparts) to saw its way to and lock into a bloodmeal (Cook 2015). At the same time, the tick's mouthparts also excrete saliva containing histamine binders and cytokine inhibitors to temporarily inhibit host immune response in the area and “cement” to help the tick stay in place during host movement (Boulanger et al. 2019; Karim et al. 2021). During this prolonged process, ticks can ingest bacteria from their initial host and transfer them to a new host through salivary pathways during feeding on subsequent hosts (Cook 2015).

Tularemia and Ferrets

In 2015-16, researchers found that 37/79 (47%) of ferrets were tularemia seropositive at Conata Basin/Badlands National Park (T. Livieri and R. Santymire, unpublished data). Matchett et al. (2021) reported 42 antibody positive serum samples collected from adult ferrets from 2004 to 2008. It is unknown whether these animals developed clinical signs of tularemia or how exposure may impact recovery of ferrets long-term. Immunity may not last long without consistent exposure and exposure could have negative reproductive effects. Eliciting an immune response is energetically costly as is reproduction (Wobeser 2006). Fitness costs are more

pronounced in populations when disease is present, therefore coupled with other stochastic events, disease exposure could significantly alter ferret recovery efforts.

The tick species *Ixodes kingi* (Bishopp 1911) occurs on a wide range of rodent and carnivore hosts in South Dakota and has been previously associated with sylvatic maintenance of tularemia (Williams et al. 1991; Durden and Keirans 1996; Hayes 2005). Both *I. kingi* and a similar species, *I. sculptus* (Newmann 1904), parasitize ferrets (Harris et al. 2014; Figure 1.1), with tick prevalence ranging from 68-100% in annual ferret surveys during 2006-2022 (T. Livieri, unpublished data). The role that *I. sculptus* plays in tularemia transmission is unknown (Williams et al. 1991; Durden and Keirans 1996; Hayes 2005). *Ixodes sculptus* may be ground dwelling, laying eggs in soil (Hixon 1932). As ferrets solely reside in prairie dog colonies, these ticks are likely coming from within the burrow system and parasitize ferrets and their prey. Small mammals often are associated with one or more life stage of *Ixodes* spp. ticks (Durden 2006). *Ixodes sculptus* and *I. kingi* have been found on all ferret prey species (Kietzmann 1987; Salkeld et al. 2006) but the ecology, location in the environment, and lifecycles of these two tick species are not well studied and their role in tularemia transmission to ferrets and associated species is unknown.

Tick Behavior

Many *Ixodes* spp. exhibit a three-host lifecycle as explained by Apanaskevich and Oliver (2014) and the Centers for Disease Control and Prevention (2017; Figure 1.2) and may quest on tall grass or exposed surfaces at varying heights to grab onto a host (Troughton and Levin 2007; Tietjen et al. 2020). Ticks spend time off host (Hixson 1932) and have Haller's organs on their

legs used to detect a host while questing on vegetation in the environment (Salomon et al. 2020). Tick collection method success is dependent upon the type of host-seeking behavior shown by an individual tick species (Salomon et al. 2020). For example, *I. scapularis* quests by sitting on vegetation with its arms extended and when a host brushes by, the tick attaches to the host. These ticks also are lured by carbon dioxide to find a host (Tietjen et al. 2020). These ticks tend to quest early in the morning and in the evening to avoid direct sunlight and desiccation. In comparison, *A. americanum* is more aggressive and will “hunt” a possible host by tracking carbon dioxide they emit (Marshall et al. 2025).

Suitable *Ixodes* spp. habitat characteristics often are related to escaping desiccation and starvation (Linske and Williams 2024). *Ixodes scapularis* habitat suitability has been well-studied, and they primarily occupy areas with dense vegetation and high humidity (Maestas et al. 2019; Linske and Williams 2024). Lindsay et al. (1999) determined that density and distribution of leaf litter, as well as host use of the landscape are critical for *I. scapularis* survival.

Additionally, the removal of leaf litter has been shown to reduce nymphal stage *I. scapularis* abundance by increasing desiccation risk (Schulze et al. 1995). *Ixodes ricinus* abundance is typically higher in forested environments that are often cool and humid, with a thick, wet litter layer (Tack et al. 2012). Tick species often exhibit variation in their tolerances of desiccation, which makes vegetation composition and environmental factors such as cloud cover, temperature, and humidity (Apanaskevich and Oliver 2014; Mathisson et al. 2021) important to determine presence/abundance of tick species on the landscape and influence off-host collection methods (Salomon et al. 2020).

South Dakota is comprised of less than 4% total forest statewide (Walters 2016). An assessment of the Conata Basin vegetation structure from MacCracken et al. (1985) shows the prairie

dominated by blue grama (*Bouteloua gracilis*), buffalograss (*Buchloe dactyloides*), western wheatgrass (*Pascoprum smithii*), carex (*Carex spp.*), red threeawn (*Aristida purpurea*), scarlet globemallow (*Sphaeralcea coccinea*), woolly indianwheat (*Plantago patagonica*), and plains prickly pear (*Opuntia polyacuntia*). Activity of prairie dogs often results in little to no vegetation around burrow openings and short vegetation on the prairie dog colony (Bonham and Lerwick 1976, MacCracken et al. 1985). This prairie vegetation structure is conducive for *Dermacentor variabilis* as their highest abundance is found in open-canopy grasslands, however not favorable for well-studied *Ixodes* spp. (Mathisson et al. 2021). Many *Ixodes* ticks exhibit nidicolous behavior at one or more stages within their lifecycle (Gray et al. 2014). Due to the consistency of conditions within the prairie dog burrow system, I think *I. kingi* and *I. sculptus* may be utilizing burrow entrances and/or prairie dog nesting chambers to find hosts and/or reproduce.

Immunology of Francisella spp.

Proteins on a pathogen's surface will impact the general mammalian immune response through two branches of the immune system, innate and adaptive responses (Kuby 2019). Innate immunity is built-in to the mammalian body, protecting the individual through skin, mucosal membranes, and other surfaces (Kuby 2019). Once a foreign protein enters the body, innate white blood cells will combat infections first. After white blood cells are formed, cells can either go through two branches of development through the myeloid or lymphatic lines to become specialized. Myeloid cells include macrophages, mast cells, neutrophils, and myeloid dendritic cells. Macrophages and dendritic cells also are known as professional phagocytes, named for their ability to engulf and destroy microbes. These cells along with B cells can then present antigen peptides back to other adaptive immune cells to induce further specialized responses.

The myeloid lineage cells are mainly involved with innate immune responses and turning on the adaptive side (Kuby 2019).

The clearing of a pathogen in a mammalian host is likely due to the activation of lymphoid adaptive cells. Adaptive immunity is triggered by bacteria, viruses, parasites, and other non-self antigens. Lymphoid lineage cells including lymphoid dendritic cells, B cells (antibodies), and T cells, are involved in adaptive immunity. The adaptive response is initiated through bridging by innate cells requiring specific cytokines, or signals produced by cells to assist with triggering further specific immune responses, including cell activation, differentiation, and cell migration for tissue repair (Kuby 2019). Adaptive responses can be humoral (antibody forward) and/or cell mediated (T cell forward) for specific pathogen recognition and clearing.

Both humoral and cell-mediated responses are induced by *F. tularensis* infection (Koskela et al. 1980). The humoral response involves B cells and antibody production. IgM antibodies are secreted by B cells as the first line of defense against a pathogen. These antibodies recognize lipopolysaccharides found on the cell surface of gram-negative bacteria causing cell division to increase secretion of IgM antibodies to attack *Francisella* spp. antigen in the body (Roberts et al. 2018). Additionally, B-cells can undergo processes to produce IgG antibodies. IgG antibodies are different in structure than IgM antibodies and are involved with immune response memory. A cell-mediated response, or T-cell forward response, is required to clear primary infections of *Francisella* spp. in mammalian hosts (Roberts et al. 2018). There are two main forms of T cells, T helper cells and cytotoxic T cells, both of which produce INF- γ cytokines when activated in response to *F. tularensis* proteins (Sunagar et al. 2016). Cytotoxic T cells can target antigens to trigger a cell death signal of damaged host cells (Kuby 2019). Survival following natural infection with a *Francisella* spp. infection leads to strong cell-mediated immunity against

secondary exposure in human and mouse models (Elkins et al. 2007) however mechanisms for longevity of memory remain unknown.

Replication inside of phagocytic and other immune cells is characteristic of *Franciscella* spp. (Krocava et al. 2017; Abplanalp et al. 2009). This genus has been shown to infect and replicate in macrophages both *in vitro* and *in vivo* (Thorpe and Marcus 1964; Fortier et al. 1994).

Pathogen defenses inside host cells are unknown but appear to involve an ability to escape the encased phagocytic cells and continue replication in the cytoplasm (Clemens et al. 2004), blocking the respiratory burst in neutrophil cells (McCaffrey and Allen 2006), and suppression or delay inflammatory cytokine production (Telepnev et al. 2003; Abplanalp et al. 2009). Type B specifically replicates rapidly within mouse and human dendritic cells while Type A replicates within human myeloid lineage dendritic cells (Bosio and Dow 2005; Nasr et al. 2006).

Oponization, the process of targeting a pathogen for identification and destruction, of bacteria prior to phagocytosis is important to the intracellular opportunities of the bacteria to take over host cells and of the host cells directed response to *F. tularensis* infection (Krocava et al. 2017).

Human monocytes infected with *Francisella* showed reduction in IL-6, TNF-a, and IL-8 chemokines indicating possible cytokine suppression from infected innate immune cells (Bosio and Dow 2005; Gillette et al. 2014). IL-6 is important in the humoral immune response as a proinflammatory cytokine and IL-8 also influences the innate inflammatory response to other innate cells (Kuby 2019). Ferrets naturally have reduced IL-6 when compared to other vertebrates, likely due to inbreeding, possibly increasing their risk of disease when cells become infected with tularemia (Kennedy-Stoskopf et al. 1997). *Francisella tularensis* causes immunosuppression, allowing for bacteria to replicate quickly inside antigen presenting cells while further disrupting antigen presenting adaptive immune cells (Bosio and Dow et al. 2005).

Nothing is known about black-footed ferret immune response to *F. tularensis*, however it is valuable for continuing ferret population recovery.

Previous Accounts of Tularemia in the Prairie Ecosystem

Black-footed ferrets from 6 of 12 recovery sites had antibodies against tularemia when surveyed in 2002-2022 (T. Livieri and R. Santymire, unpublished data). Prairie dogs rarely seroconvert and can suffer high mortality rates (Cherry et al. 2019). Laboratory mice often succumb to disease prior to developing an adaptive response (Roberts et al. 2018). Previous studies of *F. tularensis* dynamics suggest that intervals of multiple years may exacerbate bacterial replication based on specific climatic conditions (Palo et al. 2005; Desvars-Larrive et al. 2016; Ma et. al 2020). Spillover events can cause mortality in other small mammals (e.g., mice, rabbits), which contribute to ferret diets (Brickner et al. 2014). Other prairie carnivores such as the American badger (*Taxidea taxus*) and coyote (*Canis latrans*) have been exposed to tularemia infection, with transmission thought to be during the summer (Williams et al. 1994). Lagomorphs may influence tularemia in this system as well, but due to their widespread populations, sampling remains difficult (Williams et al. 1994). Tularemia outbreaks occur in grasslands with reports of positive tularemia cases in prairie dogs (Cherry et al. 2019; Matchett et al. 2021), voles (Cherry et al. 2019), desert cottontails (*Sylvilagus audubonii*; Matchett et al. 2021) and humans (Harrist et al. 2019) indicating a possible epizootic outbreak in southwestern South Dakota and northeastern Wyoming in summer of 2015. An adult male at that site had a throat mass that contained a live exudate with *F. tularensis* and while the animal otherwise appeared healthy and was administered antibiotics by a veterinarian, it was not observed on subsequent surveys (Figure 1.3).

A cursory look at our 2015-16 data revealed that 52% of seronegative and 43% of seropositive ferrets were observed in subsequent surveys. These pilot data are not conclusive but provide a basis for further investigation into tularemia seroprevalence in ferrets and potential effects on ferrets at Conata Basin/Badlands. Much of ferret research is focused on plague mitigation and recovery, however long-term effects, including reproductive and behavioral changes from tularemia on ferrets, remain unknown. Repeated or chronic stress can impact an animal's overall health. If disease is impacting the animal's health and/or if an animal has long-term elevated glucocorticoid production, this may make them susceptible to diseases (Harper and Austad 2004). Elevated glucocorticoids can suppress reproduction, specifically reduce the production and release of gonadotropin-releasing hormone, which then decreases the synthesis and release of follicle-stimulating hormone and luteinizing hormone, thus, reducing the gonadal hormone production including testosterone in males and progesterone and estrogen in females (Whirlledge and Cidlowski 2017). Further understanding of tularemia dynamics over time may guide insight into other diseases in the prairie ecosystem as well as provide understanding on tularemia risk to ferret recovery. As mechanisms of *F. tularensis* exposure to ferrets remain unknown, we wanted to investigate environmental variables that may influence exposure and ectoparasites found on ferrets within the Southwestern South Dakota prairie ecosystem.

Study Area

Conata Basin, a portion of Buffalo Gap National Grasslands managed by the United States Forest Service, Department of Agriculture and Badlands National Park managed by the National Park Service, Department of the Interior (herby referred to as CB/BADL), is in Pennington, Oglala Lakota, and Jackson counties in Southwestern South Dakota. Prairie dogs occupy around

6,119 ha collectively (Luce 2006). Primary public uses include recreation, hunting and livestock grazing programs within the national grasslands. This collection of short grass prairie is dominated by buffalograss (*Buchloe dactyloides*), blue grama (*Bouteloua gracilis*), and western wheatgrass (*Agropyron smithii*) (Livieri and Anderson 2012). The dominant vertisols and inceptisols feature clay and silt textures with various salt accumulations (Sheets et al. 1971; Web Soil Survey 2024). Because of its lack of cover, the prairie receives direct sunlight and provides little cover for wildlife. Many wildlife species including prairie dogs, black-footed ferrets, deer mice, northern grasshopper mice, prairie rattlesnakes (*Crotalus viridis*), and even small invertebrates like fleas and beetles inhabit prairie dog burrows. On average, winters can be severe, and summers remain hot and dry with unpredictable storm events, with temperatures ranging from 46.7 to -40° C (National Park Service 2021).

Study Population

The Conata Basin/Badlands National Park (CB/BADL) area was identified as a high priority area for black-footed ferret reintroduction in the early 1990s because of the large, contiguous prairie dog colonies and the stable management provided by the two federal agencies (US Forest Service and National Park Service) managing those lands. From 1994-1999, more than 300 captive-born black-footed ferrets were released into CB/BADL and through survival and natural reproduction, the population grew significantly such that reintroductions were no longer needed after 1999. As prairie dog colonies in CB/BADL expanded throughout the early 2000s, the black-footed ferret population increased to a minimum of 355 individuals in 2007, making this population the largest ferret population ever documented and the most successful of the >30 ferret reintroduction sites in North America. It is very likely that this population was functioning

demographically similar to historic populations. One reason for the success of this population was that invasive disease, namely sylvatic plague, which is highly fatal for both black-footed ferrets and prairie dogs, had not entered this ecosystem. However, plague did invade in 2008 and the population suffered a significant decline through 2013 but avoided extirpation due to extensive plague management efforts. Beginning in 2014, the black-footed ferret population began increasing again, reaching ~250 individuals again in 2024. This population is extensively monitored annually by Prairie Wildlife Research, US Forest Service, National Park Service, and their many partners.

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Attachments: Figures and Tables

Figure 1.1: Anesthetized black-footed ferret with feeding *Ixodes sculptus* and *I. kingi*. Photo by Travis Livieri.



Figure 1.2: Three-host lifestyle of Ixodes ticks developed by United States Center for Disease Control (CDC 2014).

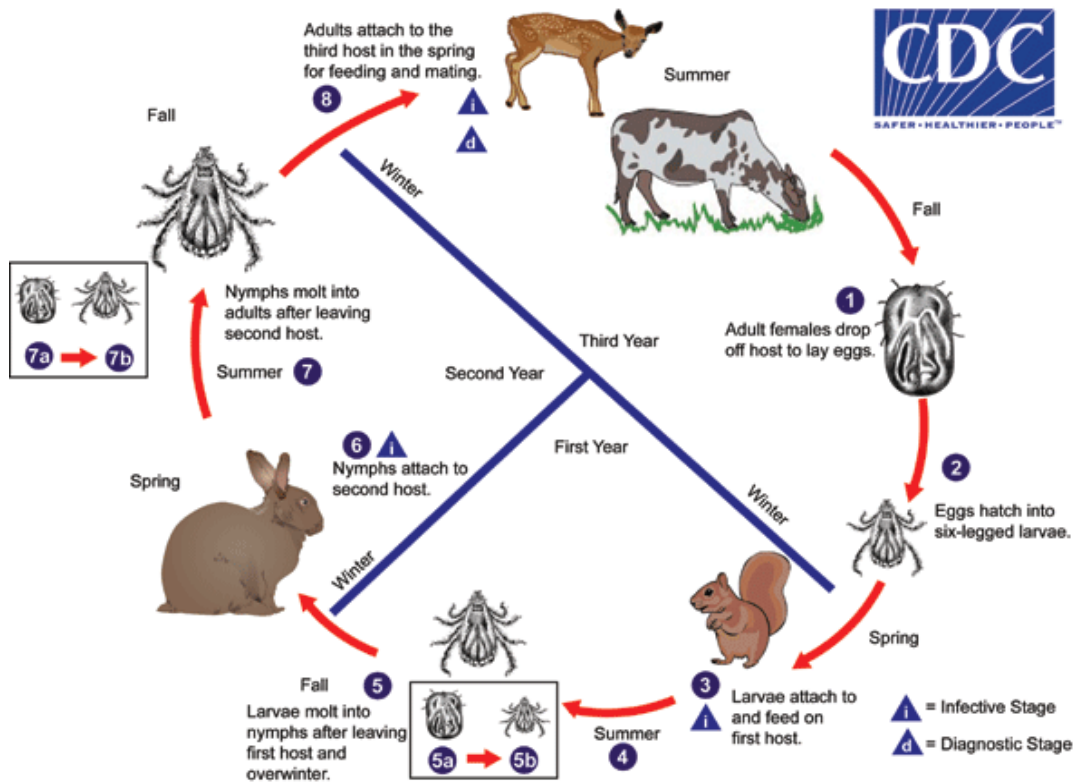


Figure 1.3: An adult male black-footed ferret with a throat mass that contained exudate with live *Francisella tularensis* at Conata Basin/Badlands National Park, South Dakota in spring 2016. Note attached ticks and fleas near the infection site. Photo by Travis Livieri.



Chapter 2: Mites on Endangered Black-footed Ferrets (*Mustela nigripes*) and their Prey in South Dakota

Written in style for Journal of Parasitology with coauthors Drs. Travis Livieri, Sarah Orlofske, and David Eads

ABSTRACT: Mite occurrence can affect mammalian hosts by reducing fitness, causing dermatitis, and contributing to secondary infections which may have consequences for small, endangered populations. Black-footed ferrets (*Mustela nigripes*) were historically reported to host several mite taxa, but the captive-breeding period likely eliminated these ectoparasites from the founding reintroduced population. Since reintroduction, ferrets have been exposed to mites, fleas, and ticks endemic to black-tailed prairie dog (*Cynomys ludovicianus*) colonies, yet mite occurrence in current wild ferret populations has not been documented. Our objective was to identify and estimate the prevalence of ear and body mites parasitizing black-footed ferrets and prairie dogs in Conata Basin/Badlands, South Dakota, home to the largest population of reintroduced black-footed ferrets. We sampled ear and body mites from ferrets during 2010–2014 and 2022–2024 and collected body mites from prairie dogs in 2023. We did not detect *Otodectes* spp. ear mites on any ferrets. However, two genera of mites, *Androlaelaps* spp. and *Pygmephorus* spp., were identified on the body of ferrets across multiple years, and *Androlaelaps* spp. also were documented on prairie dogs. Logistic regression indicated that mite presence on ferrets was positively associated with flea presence, while mite abundance was negatively associated with tick presence. Our findings suggest these mites are widespread, likely phoretic, and pose minimal direct health concerns for black-footed ferrets or prairie dogs.

INTRODUCTION:

Mite (Arachnida: Acari) infestations can harm mammals by decreasing host fitness through dermatitis and secondary infections, reducing body condition, and transmitting pathogens (Mullen and OConnor, 2019). The impacts of mites on individuals can be severe, which could have consequences for small, endangered host populations (Foley et al., 2013). Ear mites, *Otodectes* spp., are prevalent among free-ranging carnivores worldwide and commonly affect the skin surface of domestic dogs, cats, and ferrets (*Mustela putorius furo*; Patterson and Kirchain, 1999; Miller et al., 2006; Mullen and OConnor, 2019). Although they often are asymptomatic, ear mites in domestic ferrets can cause inner ear inflammation and head shaking, potentially leading to severe secondary bacterial and yeast infections that may be life-threatening (Miller et al., 2006; Marini et al., 2007).

Black-footed ferrets (*M. nigripes*) are endangered carnivores with a global free-ranging population of ~400 individuals (Livieri et al., 2022). Historically, two mite genera, *Pygmephorus* spp. and *Macrocheles dimidiatus*, were documented from a black-footed ferret in South Dakota (Boddicker, 1968) and Erickson (1973) mentioned ear mites among the ectoparasites infesting wild black-footed ferrets. From 1985-87 all black-footed ferrets were removed from the wild for captive breeding and subsequent reintroduction back into the wild (Livieri et al., 2022). The relatively sterile captive breeding environment likely eliminated any ectoparasite infestations (Gompper and Williams, 1998) that may have carried over into the wild reintroduced populations of black-footed ferrets. Currently, wild black-footed ferrets have likely been infested with mites and other ectoparasites endemic to the area and habitat, including fleas and ticks that can transmit pathogens (Harris et al., 2014).

Black-footed ferrets prey mostly on prairie dogs (*Cynomys* spp.) and inhabit prairie dog burrows where invertebrates, including ectoparasites, can be relatively abundant (Wilcomb, 1954; Sheets et al., 1971; Witmer et al., 2006). Ectoparasites often parasitize both ferrets and prairie dogs. For instance, black-tailed prairie dogs (*C. ludovicianus*; prairie dogs) and black-footed ferrets in South Dakota are both commonly parasitized by *Oropsylla hirsuta* fleas (Harris et al., 2014, Eads et al., 2023). Additionally, Eads et al. (2020) found that prairie dogs with high flea intensities were co-parasitized by mites, presumably from the genus *Androlaelaps* based upon Kietzmann's (1987) finding that 12% of prairie dogs were infested with *Androlaelaps fahrenheitzi*, a common fur mite of mammals. Thus, it is likely that black-footed ferrets are commonly parasitized by mites; however, to our knowledge, no studies have reported on ectoparasitic mites found in populations of reintroduced black-footed ferrets.

Our objective was to identify and estimate the prevalence of ear and body mites infesting black-footed ferrets and their prey in Conata Basin/Badlands National Park (CB/BADL), South Dakota. This site (43°48'07"N 102°13'39"W) contains >7,000 ha of black-tailed prairie dog colonies across 1,000 km² and supports the largest reintroduced population of black-footed ferrets (Livieri et al., 2022). We analyzed factors that may be associated with mite prevalence on black-footed ferrets including sex, age, month, tick prevalence, flea prevalence, and colony treatment with insecticides. We additionally hypothesized that presence of mites and other ectoparasites (ticks and fleas) would be positively correlated. Selected prairie dog colonies in the study area are treated with insecticides, including DeltaDust® (Envu Environmental Science U.S. LLC, Cary, North Carolina) and fipronil-treated grain (Scimetrics Ltd. Corp., Wellington, Colorado) to reduce potential plague transmission from flea vectors (Eads et al., 2020; Eads et al., 2023). We also sampled prairie dogs for mites to estimate mite prevalence and overlap with

black-footed ferrets. Implications of this research include identifying potential overlap in ectoparasite occurrence across predator and prey species, and insight into the potential influences of mites and other ectoparasites on black-footed ferret and prairie dog health.

METHODS AND MATERIALS:

The CB/BADL area in southwestern South Dakota, managed by the U.S. Forest Service and National Park Service, consists of shortgrass prairie with diverse wildlife, including carnivores, small mammals, reptiles, and invertebrates. This area is home to the largest population of wild black-footed ferrets in the world. Since reintroduction efforts in the 1990's, it has been monitored annually.

Black-footed ferret samples

We sampled mites from black-footed ferrets during annual population monitoring in late summer/fall of 2010-2014 and 2022-2024. Nocturnal black-footed ferrets were located using spotlights and captured in custom box traps before being anesthetized with isoflurane (Biggins et al., 2006; U.S. Fish and Wildlife Service, 2019). Mites were sampled from ferrets trapped on 11 prairie dog colonies, and detection of tick and flea presence was recorded (Harris et al., 2014). We inserted a cotton-tipped swab in the right ear to remove cerumen and debris. Swabs were stored in mineral oil (Miller et al., 2006). We opportunistically collected body mites from black-footed ferrets in 2011, 2013, 2014, and 2022, and systematically sampled ferrets occupying 26 prairie dog colonies for body mites in 2023 and 2024. During anesthesia, we used fine-tipped tweezers to collect mites dislodged during flea combing, observed on the anesthesia surface, and observed on the body of the animals during processing. Mites were then stored in 80% ethanol.

All procedures followed the guidelines of Sikes et al. (2016) and the terms of U.S. Fish and Wildlife Service Endangered Species Recovery Permit #TE064682-1 and authorization from South Dakota Game, Fish and Parks.

Prairie dog samples

We sampled body mites from prairie dogs on three colonies, of which two were occupied by black-footed ferrets in summer 2023. Prairie dog handling methods are discussed in Eads et al. (2020) and Matchett et al. (2021). Briefly, we combed mites and other ectoparasites from live trapped, anesthetized prairie dogs, and counted the number of mites detected per sampling occasion (Eads et al., 2020). The primary objective of the Eads et al. (2020) study was to evaluate the efficacy of systemic insecticides for ectoparasite control and plague mitigation with prairie dogs (detection of any effect of insecticide treatments on mite abundance or prevalence). In most cases, we placed mites back on the prairie dogs to reduce any removal effect (Eads et al., 2020), although we collected mites from three prairie dogs for identification. Following recovery from anesthesia, prairie dogs were released at point of capture as per approved IACUC protocol guidelines (2015–07 U.S. Geological Survey).

Ear swab examination and mite identification

Five swabs were analyzed at Colorado State University Veterinary Diagnostic Lab (CSU) and the remaining 53 swabs at the University of Wisconsin – Stevens Point. Ear swabs were shaken and searched under a stereomicroscope (Olympus SZ60 40-60X magnification). Additionally, mites collected from black-footed ferrets and prairie dogs were observed under a microscope. Permanent slides were created by mounting mites in CMC-10 medium (Masters Chemical Co., Wood Dale, Illinois). Voucher specimens of body mites from ferrets were deposited in the

University of Wisconsin – Stevens Point Parasitology Museum Collection (UWSP-PARA 14751 – 14763). Mites were identified to genus using keys from Furman and Catts (1980) and Smiley and Whitaker (1984) based on differences in morphological features (setae pair numbers, chelae, development of corniculi). Specimens were photographed using an Olympus BX60 microscope with a Leica MC170 HD digital camera.

Data analysis

In addition to species observations, we used logistic regression to create generalized linear models and Poisson generalized linear models using the “lme4” package (Bates et al. 2015) in R™ version 4.4.1 (R Core Team 2024) to identify variables that explain mite presence and abundance on ferrets (Table 2.1) in 2023 and 2024 respectively. Treatment with insecticides including DeltaDust® and fipronil grain presence were included as covariates to account for their potential influence on mite presence. Due to the observational nature of this study, we considered all model combinations as hypotheses that could explain presence/absence or abundance of mites. We ranked these models using AIC and evaluated significant variables using an alpha value of 0.05 (Burnham and Anderson 2004). To better understand which variables explain mite presence and abundance, any variable where confidence intervals on parameter estimates overlapped zero were considered insignificant in all models within four Δ AIC units from the best approximating model.

RESULTS:

In 2010 and 2012, we collected ear swab samples from 58 black-footed ferrets (16 adults (2F.14M), 37 juvenile (17F.20M)). We did not detect *Otodectes* spp. ear mites on black-footed

ferrets in CB/BADL, South Dakota. Body mites were opportunistically collected from five black-footed ferrets in 2011, 2013, 2014 and 2022. In 2023-24, we searched for mites on 165 black-footed ferrets [26 adults (17F.9M), 139 juveniles (56F.83M)] occupying 25 prairie dog colonies across CB/BADL and found mites on ferrets occupying 16 of those colonies. The searched colonies were spatially distributed across 912km² (24km N-S x 38km E-W) of our study area, suggesting these mites are well-distributed and common within our study area.

We found two genera, *Androlaelaps* spp. (Suborder: Mesostigmata) and *Pygmephorus* spp. (Suborder: Prostigmata), on black-footed ferrets in 2011, 2013, 2014, 2002, 2023, and 2024. On average, 0.242 individual mites infested 50 black-footed ferrets across both sampling methods. The prevalence of mites on black-footed ferrets in 2023 was 33% (22.8-43.5 95% CI). The mean intensity of mites on ferrets in 2023 was 4.8 (*SD* = 7.9) with a range of 1-29 and median intensity of one. In 2023, 84% of all ferrets had at least one tick on them, while 54% of ferrets had at least one flea. Prevalence of mites on ferrets in 2024 was 21.3% (12.9-31.8 95% CI) with range 1-6 mites. Mean intensity on ferrets in 2024 was 2 (*SD* = 1.33) with a median intensity of two. In 2024, 93% of the ferrets also had one or more ticks and 35% had fleas.

We also opportunistically collected 77 *Androlaelaps* spp. mites from eight black-footed ferrets in 2011, 2013, 2014, and 2022, with the highest intensity observed in 2022 with 36 mites (Figs. 2.1-2.3). Body mites were documented on ferrets of all age/sex ratios over multiple years. Two *Pygmephorus* spp. were detected as well, one on an ear swab found at CSU and one combed in 2014 (Fig. 2.4). Interestingly, we observed probable uropodid mites attached to five *Oropsylla hirsuta* (Siphonaptera: Ceratophyllidae) fleas combed from three black-footed ferrets in 2023 and one in 2024 (Fig. 5). These four individuals were all coinfecting with *Androlaelaps* spp.

We detected 717 mites on black-tailed prairie dogs during 144 of 724 sampling occasions (20% prevalence, 17.0-23.0% CI) on three separate prairie dog colonies. In 2023, we opportunistically collected and identified 10 *Androlaelaps* spp. mites on three prairie dogs. The mean intensity of mites on black-tailed prairie dogs was 4.9 ($SD = 7.0$) with a range of 1-50 and median intensity of two.

The top-ranked logistic model that we used to examine mite presence on ferrets included flea detection (FLEA) and fipronil treatment (FIP) as explanatory variables (Table 2.2). FLEA presence was a significant predictor of mite detection ($p = 0.002$; Table 2.3), but the confidence intervals on FIP estimate overlapped 0 indicating that it may not explain mite detection (Table 3). For all models within four ΔAIC units, the only variable where parameter estimates did not overlap zero was FLEA. Ferrets infested with fleas were more likely to harbor mites. The highest-ranked Poisson model that we used to examine factors affecting mite abundance included detection of other ectoparasites, specifically ticks (TICK) and fleas (FLEA; Table 2.4), with TICK presence as a significant predictor and confidence interval for FLEA estimate overlapping zero ($p = 0.003$; Table 2.5). For all models within four ΔAIC units, the only variable where parameter estimates did not overlap zero was TICK. Ferrets exhibited higher mite abundance when ticks were not detected.

DISCUSSION:

The absence of *Otodectes* spp. ear mites in sampled black-footed ferrets suggests that this ectoparasite may currently be rare or absent in the CB/BADL population. This finding contrasts with earlier reports of ear mites in historical wild black-footed ferrets (Erickson, 1973),

supporting the hypothesis that the captive breeding phase likely reduced or eliminated some historical ectoparasites from the founding population used for reintroductions, reflecting concerns that conservation practices may have led to unintentional parasite loss (Gompper and Williams, 1998; Lymbery and Smith, 2023).

Body mites were found on black-footed ferrets across our study area and in different years, suggesting these may be common. *Androlaelaps* spp. belong to Laelapidae, a family composed of many common fur mite species thought to feed on microarthropods living in nesting environments of various North American bird and rodent species (Whitaker et al., 2007).

Androlaelaps fahrenheitsi has been identified on black-tailed prairie dogs in South Dakota and *Mustela* spp. (Whitaker et al., 1974; Kietzmann, 1987, Whitaker et al., 2007). *Androlaelaps* spp. are not known to have clinical importance or vector diseases, and likely are not parasitizing black-footed ferrets or their prey but may be phoretically using these mammals for transportation (Whitaker et al., 1974).

Our detection of *Pygmephorus* was likely incidental and not parasitizing the ear (Dastych et al., 1991; Dastych et al., 1992). They are common on many North American burrowing rodents, and are easily recognized by their large claws on their first pair of legs used for grasping onto hair (Whitaker et al. 2007; Boddicker 1968). While specific feeding behavior of this genus remains under-studied, it is unlikely that they feed on ferrets as a host. Whitaker et al. (2007) documented *P. spickai* on black-footed ferrets and Boddicker (1968) documented *Pygmephorus* spp. as well on other mammal species including deer mice and voles in South Dakota.

Patterns of mite occurrence on black-footed ferrets appear to be influenced by interactions with other ectoparasites. The association between mite and flea presence suggests that concurrent infestations may be common, likely due to shared environmental conditions (Ventura et al.,

2022). The negative association between mite abundance and tick presence further supports potential competitive taxa occupying similar host niches. Collectively, our findings highlight the complexity of ectoparasite interactions within the prairie dog burrow ecosystem as mites, fleas, and ticks all utilize the burrow system differently, albeit perhaps slightly (Krasnov et al., 2010). For example, mites may be using ferrets for movement while ticks and fleas acquire blood meals for survival and reproduction from these mammal species (Dhooria, 2016). Ferrets are mainly solitary and can have home ranges between 42-65 ha in females and 86-132 ha in males (Livieri and Anderson, 2012). These mites may have adapted to utilize ferrets on the landscape, as ferrets may travel across their entire home range in one night, or perhaps this relationship is occurring by chance. These mites are likely phoretic, relying on a host for travel through a commensal relationship (Seeman and Walter, 2023).

We documented five fleas with attached deutonymphs of uropodid mites. Phoretic mites are thought to rarely use fleas as a means of dispersal, usually choosing beetles or flies within a burrow system (Schwan and Corwin, 1987; Bajerlein et al., 2024). The individuals we detected may have been exploiting *O. hirsuta* to be carried across the grassland on larger mammals (e.g., carnivores hunting prairie dogs; Walter and Proctor, 1999). This relationship is not well studied and has not been documented previously on fleas collected from black-footed ferrets in South Dakota.

Our observations suggest that prairie dogs and black-footed ferrets are sharing mites. The prairie dog burrow system maintains a relatively consistent temperature and humidity (Wilcomb, 1954) making it an ideal location for invertebrates to develop, breed, find hosts, and persist. These conditions may provide the opportunity for spillover of mites between prairie dogs and black-footed ferrets, as indicated by *Androlaelaps* spp. found on both species overlapping three prairie

dog colonies and deutonymphs found on fleas shared by both host species. Additionally, mites have been documented moving between predators and prey during predation events (Gakuya et al., 2011). Body mites are not host specific to black-footed ferrets and may be accidentally traveling on ferrets and prairie dogs when searching for invertebrates in the burrows (Whitaker et al., 2007; Seeman and Walter, 2023). Typically, phoresy occurs in the deutonymph or adult life stage in mites (Seeman and Walter, 2023). These mites are currently an unlikely concern for disease transmission to black-footed ferrets or their prey.

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ATTACHMENTS

Table 2.1: Variables used in models to explain mite presence and abundance on black-footed ferrets.

Variable Name	Variable Type	Variable Information
Mite Detection	Binomial	Mite presence or absence
Mite Count	Count	Number of mites
SEX	Binomial	1=Female; 0=Male
AGE	Binomial	1=Adult; 0=Juvenile
FLEA	Binomial	Flea detection (y/n)
TICK	Binomial	Tick detection (y/n)
DUST	Binomial	Sample site treated with DeltaDust® (y/n)
FIP	Binomial	Same site treated with fipronil grain (y/n)
YEAR	Binomial	1=2023 samples; 0= 2024 samples

Table 2.2: Model selection based on AIC for mite presence on ferrets in 2023 and 2024.

	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
MitePresenceFipFlea	4	194.38	0	0.28	0.28	-93.07
MitePresenceSexAgeFipFlea	6	195.52	1.13	0.16	0.43	-91.49
MitePresenceFlea	3	196.62	1.24	0.15	0.58	-94.74
MitePresenceTreatmentFlea	5	195.88	1.5	0.13	0.71	-92.75
MitePresenceTickFlea	4	196.36	1.98	0.1	0.81	-94.05
MitePresenceSexAgeFlea	5	196.94	2.56	0.08	0.89	-93.28
MitePresenceAll	9	197.61	3.23	0.05	0.95	-89.23
MitePresenceFipYear	4	200.25	5.87	0.01	0.96	-96
MitePresenceTreatmentYear	5	201.7	7.32	0.01	0.97	-95.66
MitePresenceFip	3	202.07	7.69	0.01	0.97	-97.96
MitePresenceTreatment	4	202.24	7.86	0.01	0.98	-97
MitePresenceTreatmentTick	5	202.46	8.08	0	0.98	-96.04
MitePresenceSexAgeFip	5	202.81	8.43	0	0.99	-96.22
MitePresenceTreatmentSexAge	6	203.04	8.66	0	0.99	-95.26
MitePresenceTickYear	4	204.96	10.58	0	0.99	-98.36
MitePresenceTick	3	205.02	10.64	0	0.99	-99.43
MitePresenceSexAgeTick	5	205.21	10.82	0	1	-97.42
MitePresenceYear	3	205.36	10.98	0	1	-99.61
MitePresenceSexAgeYear	5	206.01	11.63	0	1	-97.82

Table 2.3: Parameter estimates and confidence intervals for variables in the most approximating model explaining mite presence on ferrets in 2023 and 2024. Variables for fipronil application (FIP) and flea presence (FLEA), with FLEA being significant and confidence intervals not overlapping zero.

Coefficients	Estimate	Std. Error	t value	Pr(> t)	2.50% CI	97.5% CI
Intercept	0.21313	0.0469	4.545	1.07 e-05	0.121	0.305
FIP	-0.16995	0.09334	-1.821	0.07046	-0.353	0.012
FLEA	0.21937	0.0697	3.148	0.00196	0.083	0.356

Table 2.4: Model selection based on AIC for mite abundance on ferrets in 2023 and 2024.

	K	AICc	Δ AICc	AICcWt	Cum.Wt	LL
MiteCountTickFlea	4	894.47	0	0.31	0.1	-443.11
MiteCountTickYear	4	894.15	0.23	0.27	0.58	-443.23
MiteCountTick	3	895.15	0.69	0.22	0.8	-444.5
MiteCountSexAgeTick	5	896.52	2.05	0.11	0.91	-443.27
MiteCountTreatmentTick	5	898.92	4.45	0.03	0.94	-444.27
MiteCountAll	9	898.94	4.47	0.03	0.97	-439.89
MiteCountFlea	3	901.23	6.76	0.1	0.98	-447.54
MiteCountYear	3	902.46	7.99	0.1	0.99	-448.16
MiteCountSexAgeFlea	5	903.87	9.4	0	0.99	-446.75
MiteCountAge	3	904.6	10.13	0	0.99	-449.22
MiteCountTreatmentFlea	5	904.79	10.32	0	0.99	-447.21
MiteCountSexAgeYear	5	905.29	10.82	0	1	-447.45
MiteCountFip	3	906.02	11.55	0	1	-449.93
MiteCountSex	3	906.05	11.58	0	1	-449.95
MiteCountDust	3	906.05	11.58	0	1	-449.95
MiteCountTreatmentYear	5	906.45	11.98	0	1	-448.04
MiteCountSexAge	4	906.7	12.23	0	1	-449.22
MiteCountTreatment	4	908.1	13.63	0	1	-449.93
MiteCountTreatmentSexAge	6	910.93	16.46	0	1	-449.2

Table 2.5: Poisson regression for the most approximating model explaining mite abundance on ferrets in 2023 and 2024. Variables for flea presence (FLEA) and tick presence (TICK), with TICK being significant and confidence intervals not overlapping zero.

Coefficients	Estimate	Std. Error	t value	Pr(> t)	2.50% CI	97.5% CI
Intercept	2.8755	0.8611	3.339	0.00104	1.187743	4.563269
FLEA	0.9703	0.5841	1.661	0.09861	-0.17453	2.1152
TICK	-2.5458	0.8515	-2.99	0.00323	-4.21467	-0.87685

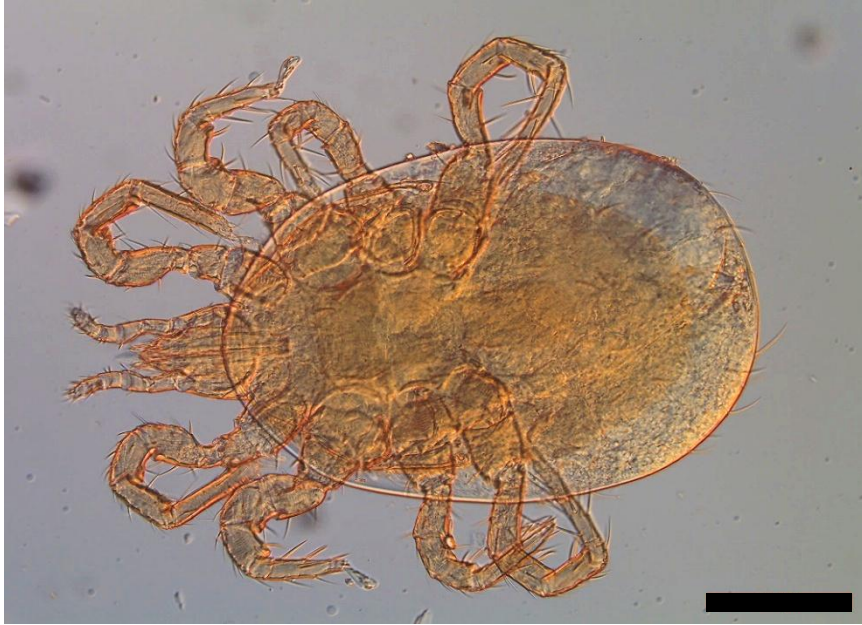


Figure 2.1: *Androlelaps* spp. mite showing morphological characteristics on dorsal side including the teeth of chelae. Scale bar equals 200 μm . Photo by Madisen Hartlaub.



Figure 2.2: *Androlelaps* spp. mite showing transparent tectum flap and cornuculi development. Scale bar equals 100 μm . Photo by Madisen Hartlaub.



Figure 2.3: *Androlelaps* spp. mite showing single pair of setae on genito-ventral plate. Scale bar equals 100 μm . Photo by Madisen Hartlaub.

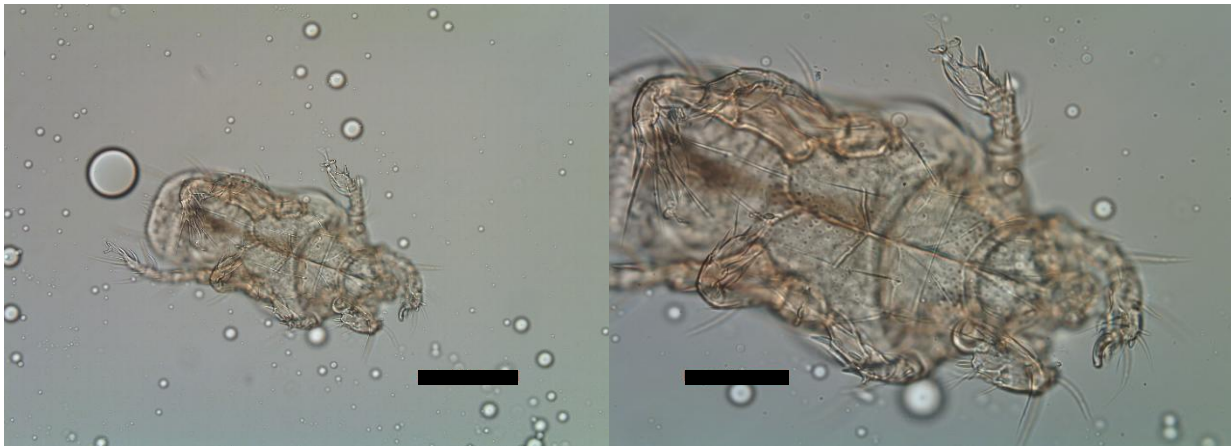


Figure 2.4: *Pygmephorus* spp. dorsal view showing large tibiotarsus I claw, tergite C not covering prodorsum, and tarsus IV with claws. Scale bar equals 100 μm and 50 μm respectively. Photos by Madisen Hartlaub.

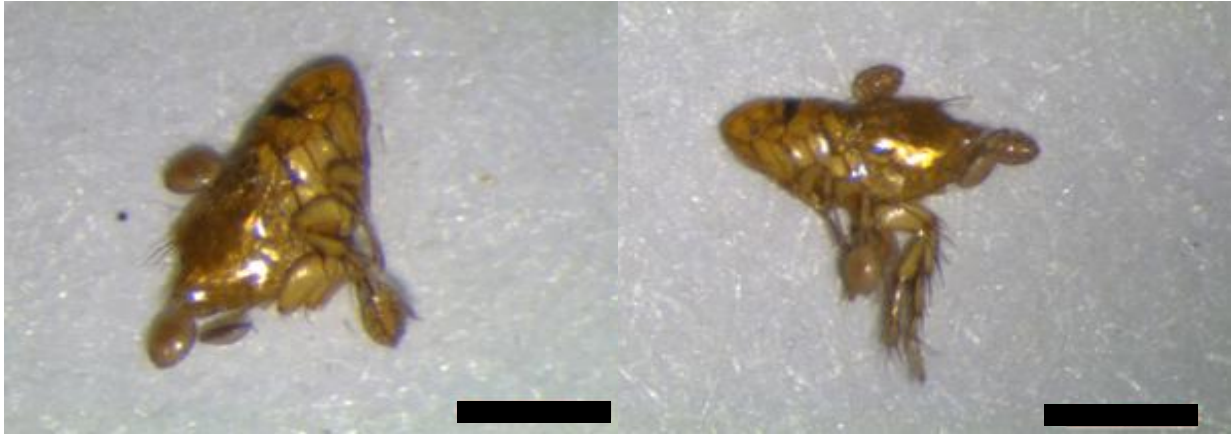


Figure 2.5: *Oropsylla hirsuta* parasitized by 4 uropodid mites. Scale bar equals 1 mm. Photos by Madisen Hartlaub.

CHAPTER 3: SEROLOGICAL EVIDENCE OF BLACK-FOOTED FERRET EXPOSURE TO TULAREMIA FROM 2002-2024

Written in the style of Journal of Wildlife Diseases with co-authors Drs. Travis M. Livieri, Rachel M. Santymire, and Shelli A. Dubay.

ABSTRACT: Black-footed ferrets (*Mustela nigripes*) face multiple infectious disease threats that cause population decline, including bacterial diseases such as plague. Tularemia, caused by *Francisella tularensis*, affects humans and wildlife and is transmitted through ectoparasites, contaminated prey, or environmental reservoirs. Although ferrets are exposed to tularemia, its effects on ferret health and pathways of exposure remain unknown. We aimed to assess long-term tularemia exposure in wild black-footed ferrets and examine how demographic and temporal factors affect seropositivity. Between 2002 and 2024, we collected 1,277 serum samples from 879 individual ferrets. All sera were tested using microagglutination inhibition at the Wyoming State Veterinary Laboratory with titer $\geq 1:128$ considered seropositive. Overall, 6.3% of samples were seropositive, with juvenile males exhibiting the highest likelihood of exposure, particularly in 2015–2016. Recapture data showed that some ferrets seroconverted within five months. Although repeated exposure may impose sublethal effects, ferret populations persisted and even increased during periods of elevated tularemia activity. These results provide the first long-term assessment of tularemia in black-footed ferrets and support the need for additional research on environmental factors and ferret immune response dynamics.

INTRODUCTION:

The black-footed ferret (*Mustela nigripes*) is the only native wild ferret species in North America and one of the most endangered mammals in North America (Forrest et al. 1988; U.S. Fish and Wildlife Service 2019). Ferrets are nocturnal carnivores that rely on prairie dogs (*Cynomys* spp.) for prey, shelter, and survival within the Great Plains grassland ecosystem (Hillman 1968; Brickner et al. 2014). As ferrets spend 90% of their lives underground in prairie dog burrow systems, they share close ecological and pathogen connections with their prey and associated ectoparasites (Livieri 2023). Disease threats within this system, primarily sylvatic plague (*Yersinia pestis*) and canine distemper virus, have critical implications for ferret recovery and ecosystem health (Miller and Reading 2012).

Tularemia, caused by the bacterium *Francisella tularensis*, is a zoonotic disease of global concern that affects over 100 species of mammals, particularly lagomorphs and rodents (Farlow et al. 2005; Hayes 2005). Two subspecies, *F. tularensis tularensis* (Type A) and *F. tularensis holarctica* (Type B), occur in North America. Type A is highly virulent and typically associated with terrestrial cycles maintained by lagomorphs and tick vectors, while Type B is less virulent and mainly associated with aquatic rodents (Telford and Goethert 2020). Transmission occurs through arthropod bites, ingestion of infected prey, or environmental exposure through contaminated soil or water (Hayes 2005). *Francisella tularensis* can persist in the environment not only through associations with free-living amoebae, such as *Acanthamoeba castellanii*, but also as free-living bacteria in soil, thus surviving outside a host (Abd et al. 2003). These environmental systems may serve as important reservoirs for tularemia. Contaminated soil and water have been implicated in causing tularemia in both wildlife and humans (Al Dahouk et al. 2005, Steffan et al. 2020). Tularemia is a Centers for Disease Control and Prevention (CDC)

reportable disease, and human cases have increased across the United States over the past decade (Bishop et al. 2023; CDC 2025).

Black-footed ferrets are hosts for several tick species, including *Ixodes kingi* and *I. sculptus*, both of which commonly parasitize ferrets and their prey within prairie dog colonies (Durden and Keirans 1996; Harris et al. 2014). These ticks may contribute to sylvatic maintenance of *F. tularensis* within burrow systems, yet their ecology and role in transmission remain understudied. Palo et al. (2005) showed that climate variability may influence the timing of tularemia outbreaks in both humans and wildlife. Pathogen re-emergence has been tied to such environmental changes (El-Sayed and Kamal 2020). Similar climate-driven intervals may help explain the periodic tularemia epizootics observed in prairie species, including black-footed ferrets. Previous surveys have detected antibodies to *F. tularensis* in black-footed ferrets at multiple recovery sites, including up to 47% seropositivity at Conata Basin/Badlands National Park (CB/BADL), South Dakota in 2015-2016 (Matchett et al. 2021; T. Livieri and R. Santymire, unpublished data), suggesting that ferrets are repeatedly exposed within this system. An increase in human cases during 2015 (Harrist et al. 2019; Pedati et al. 2015) further suggests that a broader climate driver may be influencing tularemia presence in the environment and elevating exposure rates in both wildlife and humans (Cherry et al. 2019). However, the sources and persistence of infection, as well as its potential physiological and reproductive impacts on ferrets, are unknown.

Black-footed ferrets appear to seroconvert and survive tularemia infection, whereas prairie dogs experience high mortality rates and localized colony declines (Cherry et al. 2019). Numerous other susceptible wildlife species, including cottontail rabbits (*Sylvilagus floridanus*) and small mammals such as mice and voles, often succumb to infection prior to the development of

detectable antibodies, thereby complicating surveillance efforts (Roberts et al. 2018). Tularemia exposure and subsequent antibody production can impose energetic and reproductive costs (Wobeser 2006), and immunological limitations, such as reduced cytokine production, that may increase susceptibility in ferrets (Kennedy-Stoskopf et al. 1997). Spillover events can cause mortality in other small mammals, such as mice, voles, and rabbits, which contribute to ferret diets (Brickner et al. 2014). Other prairie carnivores such as the American badger (*Taxidea taxus*) and coyote (*Canis latrans*), have been exposed to tularemia infection, potentially during the summer (Williams et al. 1994). Surveillance conducted in CB/BADL has documented multiple host species for tularemia, including coyotes, badgers, striped skunks (*Mephitis mephitis*), and red foxes (*Vulpes vulpes*, Williams et al. 1991, 1994; Table 3.1). Lagomorphs may influence tularemia in this system as well, but due to their widespread populations, sampling remains difficult (Williams et al. 1994). Given the ecological overlap between ferrets, their prey, and ticks, assessing *F. tularensis* seroprevalence and environmental correlates provides insight into pathogen maintenance in prairie ecosystems.

The objective of this study was to determine the seroprevalence of tularemia in black-footed ferrets within CB/BADL, the site of the largest extant population of ferrets, and to explore intrinsic and ecological factors that may influence exposure. This work contributes to understanding multi-host disease dynamics in prairie ecosystems and informs management strategies for continued ferret recovery. We hypothesized that seroprevalence would vary by year given yearly climate variability.

METHODS:

The CB/BADL area in southwestern South Dakota, managed by the U.S. Forest Service and National Park Service, consists of shortgrass prairie with diverse wildlife, including carnivores, small mammals, reptiles, and invertebrates. This area is home to the largest population of wild black-footed ferrets in the world following successful reintroduction efforts.

We sampled free-ranging black-footed ferrets inhabiting prairie dog colonies in CB/BADL on six study sites called subcomplexes (Philips et al. 2020; briefly: a “subcomplex” is defined as a cluster of prairie dog colonies spaced ≤ 1.5 km from each other), during August through November with sporadic sampling in spring during March. We located ferrets using spotlights at night and then specially designed live-traps were inserted into prairie dog burrows occupied by the ferrets (Biggins et al. 2006; Black-Footed Ferret Recovery Implementation Team 2016).

Upon capture, we anesthetized the ferrets with isoflurane, recorded morphometric data, collected fleas and ticks (Harris et al. 2014), inserted a PIT microchip, administered vaccines (F1-V plague vaccine and Purevax canine distemper), and drew 1–2 mL of blood from the cranial vena cava (Quesenberry et al. 2019). We separated serum from blood clots and then froze the serum for storage at -80F at Georgia State University. We sent all sera to the Wyoming State Veterinary Laboratory for microagglutination inhibition testing, which uses live *Francisella tularensis* antigen and diluted ferret sera to quantify antibodies to detect an individual’s exposure to tularemia. Serum samples with titers $\geq 1:128$ were considered positive (Brown et al. 1980), and high titers indicated recent exposure (Brown et al. 1980; Botzler and Brown 2014).

We assessed the relationships between ferret serostatus and a suite of explanatory variables using a generalized logistic mixed model using the “lme4” package (Bates et al. 2015) in R™ version 4.4.1 (R Core Team 2024) which accounts for binomial infection status and non-independent

year-to-year repeated sampling (Bolker et al. 2009). Models included serostatus, sex, and age (juvenile or adult) as indicator variables and year and season (fall or spring) as continuous variables. We constructed a model using the full dataset, including season as a covariate to account for potential seasonal variation in exposure. However, as sampling effort in the spring was limited, we also developed a separate model using only fall samples to evaluate both overall patterns and season-specific effects on ferret tularemia serostatus. We included a random effect of site in both models to account for pseudoreplication, spatial variation, and differences in sampling capabilities for this endangered species. Additionally, we examined recaptured ferrets by giving each new sample an individual identification code to assess changes in serostatus over time, including transitions from seropositive to seronegative and vice versa. We identified ferrets for which we had serological data for at least two sampling periods. We summarized these data to determine the length of time that passed between sampling events to determine how quickly serostatus changed for individual ferrets.

RESULTS:

We collected 1,277 serum samples from 879 individual ferrets between 2002 and 2024. Overall, 6.3% of samples (n=81) were seropositive throughout sample collection (Table 3.2). We recaptured 398 ferrets during at least one subsequent sampling event, with 74% of recaptures occurring within one year after initial capture. Thirty-nine recaptured individuals exhibited changes in serostatus between 5 and 12 months after initial sampling (Table 3.3). Seroconversion occurred within 5 months and antibodies were no longer detected within six months of seropositive results. Seropositive samples were distributed across six subcomplexes, indicating no spatial variation in ferret exposure.

Our generalized logistic mixed model incorporating all serum samples (fall and spring) indicated that β_{sex} (0.043; 95% CI: 0.967—0.016), β_{age} (0.004; -1.273—0.238), β_{season} (0.002; -2.332—0.374), and β_{year} ($<2e-16$; 0.017-0.027) all affected ferret exposure to tularemia (Table 3.4). Juvenile male ferrets had a higher likelihood of having tularemia antibodies during spring and seropositive ferrets were most common in 2015-2016 (Table 3.4). When we applied the same model to fall samples only, β_{sex} (0.011; 95% CI: -1.133—0.076), β_{age} ($1.53e-05$; -1.465—0.394), and β_{year} ($<2e-16$; -0.019—0.014) again significantly explained variation in tularemia exposure (Table 3.5).

DISCUSSION:

Our study provides the first long-term assessment of tularemia exposure in black-footed ferrets. Across our 23-year data set, seroprevalence was low, consistent with prior reports of repeated but low-level exposure within ferret populations (Matchett et al. 2021). Seropositive individuals were detected at numerous study sites, suggesting that tularemia exposure is widespread among prairie dog colonies rather than localized to specific areas. These burrow systems function as shared microhabitats for multiple species, facilitating pathogen circulation through both vertebrate hosts and ectoparasites. Recapture data revealed dynamic serostatus transitions, with some ferrets converting from seronegative to seropositive and others losing detectable antibodies over six months. These patterns indicate that exposure does not always result in long-term immunity. Little is known about tularemia antibody duration in black-footed ferrets or other mammalian hosts. Serological testing can be helpful for wildlife disease surveillance by determining prevalence of disease over time, however further experimental infection studies are

needed to understand antibody interactions with memory in infected individuals (Gilbert et al. 2013).

Juvenile male ferrets exhibited higher likelihood of seropositivity, particularly in spring of 2015-2016. Male home ranges are twice the size of females, increasing their probability of encountering infectious agents through contact with ectoparasites, contaminated soil or water, and potentially infected conspecifics or prey items (Livieri and Anderson 2012). Mammalian immune systems are less developed in juvenile individuals when compared to adults, making it difficult for juveniles to effectively respond to pathogens (Kuby 2019). Gabriele-Rivet et al. (2016) found that subadult coyotes were more likely to be seropositive than juvenile or adult age groups, and they posited that older coyotes may be more capable of clearing infection and seroconverting. Black-footed ferret breeding behavior may contribute to seasonal variation in pathogen exposure. Although ferrets are generally solitary, the breeding and subsequent kit-rearing periods, beginning in mid-March, brings individuals into closer contact (Forrest et al. 1988). Increased interactions during this period could facilitate transmission or exposure through shared burrow systems, deceased prey, or ectoparasites.

Further, our fall-only model confirmed that sex, age, and year explained exposure and most positive samples occurred between 2015 and 2016, suggesting that an epizootic event likely occurred during that time. Year-to-year variation may reflect underlying changes in environmental conditions that influence pathogen persistence and host–vector dynamics.

Climatic variability strongly influences the timing and intensity of tularemia epizootics across wildlife and human systems (Palo et al. 2005; Cherry et al. 2019). Environmental factors such as increased precipitation can enhance the survival of *F. tularensis* in moist soils, potentially prolonging environmental reservoirs. Palo et al. (2005) showed that fluctuations in the North

Atlantic Oscillation (NAO) index were associated with increased tularemia cases in humans and wildlife, likely through changes in watershed conditions and runoff patterns that elevate bacterial density in water and soil. Wetter conditions also can promote vegetation growth, which in turn supports higher abundance or activity of small mammal hosts and tick vectors (MaCabe and Bunnell 2004; Previtali et al. 2009).

Although ferrets survive tularemia infection more successfully than their prey, repeated exposure may impose sublethal effects, including energetic or immunological costs that could affect reproduction and population growth. *Francisella tularensis* and *Yersinia pestis* may share patterns on the landscape by both emerging under favorable ecological conditions. Unlike plague epizootics, tularemia does not currently appear to pose a threat to ferret recovery. Notably, despite nearly half of sampled individuals showing evidence of exposure during 2015–2016, ferret populations continued to increase during this time (Livieri 2023).

Management strategies should continue to monitor environmental conditions, prey abundance, and ectoparasite populations to better anticipate disease risks. It is interesting to note that ferrets may effectively combat some bacterial pathogens while plague remains extremely pathogenic for them. The need for further investigation into *Francisella tularensis* infection in ferrets is warranted to understand these cellular pathogen dynamics within the host and their specific immune responses to these bacteria.

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ATTACHMENTS:

Table 3.1: Dr. E. S. Williams reports from University of Wyoming tularemia surveillance in CB/BADL in free-ranging carnivore species (Williams et al. 1991; Williams et al 1994).

Species	Year	Total Samples	Tularemia Seropositive	Percentage Positive
Coyote	1993	20	5	25%
Coyote	1994	21	7	33%
Coyote	1991	36	7	19%
Coyote	1995	10	4	40%
Coyote	1996	11	0	0
Coyote	1997	35	7	20%
Coyote	1998	20	8	40%
Coyote	1999	18	5	28%
Badger	1993	39	12	31%
Badger	1994	12	3	23%
Badger	1991	15	5	34%
Badger	1995	12	3	25%
Badger	1996	4	0	0
Badger	1997	9	2	22%
Badger	1998	3	3	100%
Badger	1999	3	0	0
Raccoon	1993	12	1	8%
Raccoon	1991	9	4	44%
Striped Skunk	1993	8	2	25%
Striped Skunk	1994	7	2	29%
Striped Skunk	1991	18	7	39%
Striped Skunk	1995	8	0	0
Red Fox	1993	3	0	0
Red Fox	1991	2	0	0
Least Weasel	1996	1	0	0
Bobcat	1997	3	0	0
Bobcat	1999	1	0	0

Table 3.2: Sex and age composition, total proportions, and annual tularemia seroprevalence with 95% confidence intervals of sampled black-footed ferrets in 2002–2024.

Year	Sex (F/M)	Age (J/A)	Total Prevalence	Seroprevalence (95% CI)
F2002	53/41	58/36	4/94	0.04 (0.01-0.11)
S2003	2/21	23/0	0/23	0.00 (0.00-0.15)
F2003	10/15	15/0	0/15	0.00 (0.00-0.22)
F2005	5/9	13/1	0/14	0.00 (0.00-0.23)
F2006	58/34	53/39	2/92	0.02 (0.00-0.08)
F2007	55/47	58/44	0/102	0.00 (0.00-0.04)
F2009	47/38	68/17	5/85	0.06 (0.02-0.13)
F2010	14/11	21/4	1/25	0.04 (0.00-0.20)
F2011	30/24	39/15	5/54	0.09 (0.03-0.20)
F2012	56/45	59/42	6/101	0.06 (0.02-0.12)
F2013	29/11	22/18	0/40	0.00 (0.00-0.08)
S2014	7/7	1/13	0/14	0.00 (0.00-0.23)
F2014	44/61	61/26	2/87	0.02 (0.00-0.08)
F2015	56/45	75/26	34/101	0.34 (0.25-0.44)
S2016	14/26	0/30	15/30	0.50 (0.31-0.69)
F2016	58/47	71/34	4/105	0.04 (0.01-0.09)
F2017	58/26	46/38	1/84	0.01 (0.00-0.06)
F2018	11/4	8/7	0/15	0.00 (0.00-0.22)
S2022	9/12	21/0	2/21	0.95 (0.01-0.30)
F2023	40/33	59/14	0/73	0.00 (0.00-0.05)
F2024	55/48	85/18	0/103	0.00 (0.00-0.04)

Table 3.3: Recaptured ferrets that changed serostatus (seropositive = P/seronegative = N) across multiple trapping events. F indicates fall sampling and S indicates spring sampling.

Ferret ID	F2011	F2012	F2013	S2014	F2014	S2015	F2015	S2016	F2016	F2017	F2018
12-010	-	-	N	-	N	-	P	-	-	-	-
12-016	-	N	N	-	N	-	-	-	P	-	-
12-017	-	-	-	N	-	-	-	P	-	-	-
12-032	-	P	N	-	-	-	-	-	-	-	-
13-008	-	-	N	-	-	-	P	N	-	-	-
13-020	-	-	-	-	N	-	P	-	N	-	-
14-002	-	-	-	-	N	-	P	-	-	-	-
14-004	-	-	-	-	N	-	P	-	-	-	-
14-006	-	-	-	-	N	-	P	P	-	-	-
14-010	-	-	-	-	N	-	P	-	-	-	-
14-016	-	-	-	-	N	P	-	-	-	-	-
14-017	-	-	-	-	-	-	P	N	-	-	-
14-021	-	-	-	-	N	-	-	P	N	-	-
14-026	-	-	-	-	N	-	P	-	-	-	-
15-006	-	-	-	-	-	-	P	P	-	-	-
15-008	-	-	-	-	-	-	P	P	-	-	-
15-010	-	-	-	-	-	-	N	P	N	-	-
15-015	-	-	-	-	-	-	P	-	N	-	-
15-021	-	-	-	-	-	-	P	-	N	-	-
15-022	-	-	-	-	-	-	P	-	N	N	-
15-030	-	-	-	-	-	-	N	P	-	N	-
15-033	-	-	-	-	-	-	P	-	-	N	-
15-034	-	-	-	-	-	-	P	N	N	N	-
15-036	-	-	-	-	-	-	P	-	N	-	-
16-003	-	-	-	-	-	-	-	-	N	P	-
16-040	-	-	-	-	-	-	-	-	P	N	N
B11-02	N	P	-	-	-	-	-	-	-	-	-
B12-16	-	-	-	-	N	-	-	-	P	-	-
B13-08	-	-	-	-	N	-	P	-	-	-	-
B14-02	-	-	-	-	N	-	P	-	-	N	-
B14-05	-	-	-	-	N	-	N	P	N	-	-
B14-07	-	-	-	-	N	-	P	-	-	-	-
B14-12	-	-	-	-	N	-	P	-	-	-	-
B14-20	-	-	-	-	N	-	P	-	N	-	-
B14-22	-	-	-	-	N	-	N	P	-	-	-
B15-02	-	-	-	-	-	-	N	P	-	-	-
B15-03	-	-	-	-	-	-	N	P	-	-	-
B15-04	-	-	-	-	-	-	N	P	N	N	-
B15-10	-	-	-	-	-	-	P	N	-	-	-

Table 3.4: Generalized logistic mixed model parameter estimates and 95% confidence intervals for predictors of tularemia exposure in black-footed ferrets in Conata Basin/Badlands National Park from 2002-2024 using fall and spring samples. Significant predictors included Sex, Age, Year and Season.

Coefficients	Estimate	Std. Error	z-value	P-value	2.5% CI	97.5% CI
(Intercept)	-40.920	3.46	-11.836	<2e-16	-47.696	-34.144
Sex	-0.491	0.243	-2.026	0.043	-0.967	-0.016
Age	-0.755	0.264	-2.859	0.004	-1.273	-0.238
Year	0.020	0.002	12.040	<2e-16	0.017	0.024
Season	-1.052	0.349	-3.042	0.002	-2.332	-0.374

Table 3.5: Generalized logistic mixed model parameter estimates and 95% confidence intervals for predictors of tularemia exposure in black-footed ferrets Conata Basin/Badlands National Park from 2002-2024 using only fall samples. Significant predictors included Sex, Age, and Year.

Coefficients	Estimate	Std. Error	z-value	P-value	2.5% CI	97.5% CI
(Intercept)	-53.687	3.228	-16.632	< 2e-16	24.655	36.431
Sex	-0.607	0.239	-2.544	0.011	-1.133	-0.076
Age	-1.035	0.239	-4.324	1.53e-05	-1.465	-0.394
Year	-0.026	0.002	16.088	<2e-16	-0.019	-0.014

CHAPTER 4: IXODES SCULPTUS AND IXODES KINGI FOUND ON BLACK-FOOTED FERRETS AND THEIR PREY

Written in style for Journal of Parasitology with coauthors Drs. T. Livieri and S. Dubay, and Z. Wilson.

ABSTRACT: Black-footed ferrets (*Mustela nigripes*), a highly specialized predator of prairie dogs (*Cynomys* spp.), inhabit burrow systems that support a diverse community of small mammals and ectoparasites, including *Ixodes* spp. ticks. We investigated the occurrence, host associations, and potential role of *Ixodes kingi* and *I. sculptus* in tularemia transmission within the Conata Basin/Badlands National Park ecosystem in South Dakota, USA. From 2022–2025, we sampled 304 ferrets and 640 small mammals, recording ticks on 92% of ferrets and 27% of small mammals. Larvae and nymphs were primarily found on small mammals, while adult ticks were concentrated on ferrets, consistent with a three-host life cycle. Generalized logistic models indicated host species and co-occurrence with fleas as significant predictors of tick abundance. Real-Time PCR assays on ticks and fleas from seropositive ferrets did not detect *Francisella tularensis*, suggesting these *Ixodes* species may not directly drive tularemia epizootics. These results highlight that burrow systems are likely critical microhabitats for tick populations and potential pathogen circulation.

INTRODUCTION:

Ticks are ecologically and medically significant vectors of bacterial, viral, and protozoan pathogens in wildlife systems (Sonenshine 1991; Eisen and Paddock 2021). Black-footed ferrets

(*Mustela nigripes*) occupy a specialized ecological niche within North American grasslands, relying almost exclusively on prairie dogs (*Cynomys* spp.) as their primary prey and therefore specializing in prairie dog burrow habitat (Hillman 1968; Livieri 2023). These burrows create a stable microhabitat that favors ectoparasites, including ticks in the genus *Ixodes*, that may parasitize ferrets and other co-occurring small mammal prey species (Sheets et al. 1971; Hoogland 1995).

Two tick species, *Ixodes kingi* (Bishopp, 1911) and *I. sculptus* (Neumann, 1904), have been documented on black-footed ferrets in South Dakota (Harris et al. 2014) and their prey, including black-tailed prairie dogs (*C. ludovicianus*) and other small rodents (Durden and Keirans 1996). *Ixodes kingi* has been associated with transmission of *Francisella tularensis*, the causative agent of tularemia (Hayes 2005; Williams et al. 1991). Black-footed ferrets are exposed to tularemia, but exposure routes are unknown (Chapter 3, Matchett et al. 2021). While less is known about *I. sculptus*, it also parasitizes small mammals in prairie ecosystems and may contribute to pathogen circulation through shared burrow use. In South Dakota, Maestas et al. (2019) reported *I. sculptus* from thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*), plains pocket gophers (*Geomys bursarius*), deer mice (*Peromyscus maniculatus*), and northern grasshopper mice (*Onychomys leucogaster*) in Lyman County, swift foxes (*Vulpes velox*) in Pennington County, and other ground-dwelling rodents and carnivores in Custer, Harding, Hyde, Jackson, and Brookings Counties (Easton 1983).

Most *Ixodes* spp. ticks exhibit a three-host life cycle, with larvae, nymphs, and adults all feeding on different hosts (Apanaskevich and Oliver 2014). However, probable nidicolous, nest or burrow-using, species such as *I. kingi* and *I. sculptus* may spend the majority of their off-host time within burrows, increasing opportunities for finding hosts among cohabitating vertebrates

(Hixson 1932). Prairie dogs and other small mammal species, including deer mice (*Peromyscus* spp.) and northern grasshopper mice (*Onychomys leucogaster*) which may serve as hosts for immature ticks and as reservoirs for *F. tularensis*, inhabit prairie dog burrows (Kietzmann 1987; Stapp et al. 2009; Kraft and Stapp 2013). Other prairie species, such as American badgers (*Taxidea taxus*), coyotes (*Canis latrans*), and raccoons (*Procyon lotor*), also carry *Ixodes* spp. ticks (Williams et al. 1991, Maestas et al. 2019). Shared use of burrows by multiple host species creates a potential pathway for pathogen maintenance and spillover to black-footed ferrets. Prairie dog colonies support increased small mammal abundance and diversity relative to adjacent grasslands without prairie dogs (Agnew et al. 1986). Therefore, host density is a fundamental ecological factor influencing pathogen transmission dynamics (McCallum et al. 2001).

Despite repeated detection of these ticks on ferrets and other prairie mammals in South Dakota, little is known about their natural histories, host associations, or capacity to vector pathogens in prairie dog colonies. Understanding the ecology, host associations, and vector potential of *I. sculptus* and *I. kingi* is therefore critical for elucidating the dynamics of tularemia in prairie ecosystems. The objective of this study was to investigate the occurrence of *Ixodes sculptus* and *Ixodes kingi* on black-footed ferrets, prairie dogs, deer mice, northern grasshopper mice, meadow voles (*Microtus pennsylvanicus*), hispid pocket mice (*Chaetodipus hispidus*), meadow jumping mice (*Zapus hudsonius*), and thirteen lined-ground squirrels to better understand their natural history and ecology in the prairie ecosystem. We hypothesize that these ticks have a three-host lifecycle, and we expect to find larval and nymphal stages on small mammals and adults on ferrets. We also hypothesize that these ticks are contributing to transmission of tularemia to ferrets, therefore ticks collected from seropositive ferrets will contain tularemia DNA.

METHODS:

The Conata Basin/Badlands National Park (CB/BADL) in southwestern South Dakota, managed by the U.S. Forest Service and National Park Service, consists of shortgrass prairie with diverse wildlife, including carnivores, small mammals, reptiles, and invertebrates. This area is home to the largest population of wild black-footed ferrets in the world following successful reintroduction efforts and ferrets are monitored annually.

We sampled free-ranging ferrets inhabiting prairie dog colonies in CB/BADL on six subcomplexes (Phillips et al. 2020) during late summer and fall (Biggins et al. 2006). We located ferrets using spotlights at night and then specially designed live-traps were inserted into prairie dog burrows occupied by the ferrets (Biggins et al. 2006b; Black-Footed Ferret Recovery Implementation Team 2016). Upon capture, we anesthetized ferrets with isoflurane, recorded morphometric data, sampled fleas and ticks (Harris et al. 2014), inserted a PIT microchip, administered vaccines (F1-V plague vaccine and Purevax canine distemper), and drew 1–2 mL of blood from the cranial vena cava to extract sera for tularemia antibody testing (Quesenberry et al. 2019; Chapter 3). We combed each ferret over a white pail for 20 seconds and conducted a systematic 30-second search from anterior to posterior and dorsal to ventral to collect ticks and fleas (Harris et al. 2014). We removed attached ticks at the base of the mouthparts using forceps to ensure all identifiable characteristics were preserved. All collected ticks and fleas were placed in 80% ethanol for subsequent identification in the laboratory.

We sampled small mammals for ticks across four prairie dog colony subcomplexes, each containing six 150×150 m² plots. Ferret numbers in an area were positively correlated with prairie dog burrow density (Livieri 2007) therefore we randomly selected six plots per subcomplex from those that met criteria for historical ferret density, serostatus, and active

burrow density (Biggins et al. 1993). We recorded active prairie dog burrows on each plot using a Garmin GPS unit. Burrow density was then calculated as the number of active burrows per 150 m². We deployed 100 Sherman traps (7.62 × 7.62 × 22.86 cm) baited with sweetfeed along 15 × 15 m within each plot to live-trap small mammals during four trapping events (Eads et al. 2016; Goldberg et al. 2022). We marked animals with a unique ear tag (National Band and Tag Co., Newport, Kentucky). We sampled ticks from each individual by combing for 30 seconds and conducting a systematic one-minute search from anterior to posterior and dorsal to ventral (Ostfeld et al. 1996; Harrist et al. 2014; Eads et al. 2020). We removed attached ticks with forceps at the attachment site to ensure the mouthparts remained intact for identification. All ticks were preserved in 80% ethanol, and we recorded the location, date, and time of each collection. After sampling, individuals were released back at their capture site as per approved National Park Service permitting and IACUC protocol guidelines (BADL-2024-SCI-0018; 2024-11 University of Wisconsin-Stevens Point). Prairie dogs were not prioritized for sampling due to their extensive grooming behavior; however, systematic sampling conducted by the USGS and other researchers allowed us to identify ticks to species. Ticks collected from all methods were identified to sex, life stage, and species using keys based on differences in morphological features (anal groove location, distinctness of cornua and lateral carinea, hypostome teeth and dentation and shape of basis capitulum and porous area; Cooley and Kohls 1945; Kerians and Clifford 1978; Furman and Catts 1982). We identified tick larvae to genus, nymphs to species, and adults to sex and species. Some nymphal and adult specimens lacked one or more diagnostic characteristic and these individuals were identified to genus or sex and genus.

To investigate the role of ticks in the epizootiology of tularemia in the prairie ecosystem, ticks and fleas were collected from black-footed ferrets in 2015 and 2016, and DNA was extracted

from these ectoparasites for Polymerase Chain Reaction (PCR) analysis. PCR assays were conducted at the National Wildlife Health Center to estimate the prevalence of *Francisella tularensis* and to identify probable vectors among ectoparasite species collected from both seropositive and seronegative ferrets. Primers used to target the 23kDa gene of *F. tularensis* were as follows: forward primer: 5'- tga gat gat aac aag aca aca ggt aac a -3' and a reverse primer: 5'- gga tga gat cct ata cat gca gta gg -3' with a 5' - /5HEX/ccca ttc atg/ZEN/tga gaa ctg/3IABkFQ -3' probe. During PCR, target DNA sequences were amplified through repeated cycles of denaturation, primer annealing, and extension, in which a thermal cycler separated DNA strands by heating, cooled samples to allow primers to bind, and reheated them to enable Taq polymerase to synthesize new complementary strands (Botzler and Brown 2014). Each cycle doubled the amount of target DNA and was repeated 30 or more times to generate sufficient product for detection. This approach allowed determination of which ectoparasite samples contained *F. tularensis* DNA, thereby contributing to a clearer understanding of the role ticks may play in tularemia transmission within ferret burrow systems.

We used Poisson regression models using the “lme4” package (Bates et al. 2015) in R™ version 4.4.1 (R Core Team 2024) to assess factors influencing tick occurrence on small mammals. Specifically, we included burrow density of sample plot, flea presence or absence, host sex, age, and species to explain tick abundance. We evaluated how variation in active burrow density on prairie dog colonies affects tick host selection, co-parasitism of ticks and fleas, and how host sex and age may influence patterns of tick abundance on small mammal hosts. We ranked models using Akaike’s Information Criterion and assessed whether confidence intervals on parameter estimates overlapped zero to determine explanatory variables. Additionally, we used a Pearson’s chi-square test for heterogeneity using the “chisq.posthoc.test” (Beasley and Schumacker 1995)

in R™ to compare total larvae, nymph, and adult tick life stages on ferrets compared to small mammals to investigate differences in host usage by these ticks throughout their lifecycle.

RESULTS:

Between 2022 and 2025, we sampled 304 ferrets and found ticks on 279 of them (91.8%). Tick abundance per individual ranged from 0 to 41. We recaptured 20 ferrets across multiple years that harbored ticks during each capture, totaling 91.4% of all sampled ferrets having ticks. In summer 2024, we captured 640 small mammals: 103 northern grasshopper mice, 530 *Peromyscus* spp., one meadow vole, and six hispid pocket mice. We detected ticks on 173 individuals (27%), and tick abundance ranged from 0 to 47.

As *Peromyscus* spp. and northern grasshopper mice were the most abundant species, we restricted our models to these two hosts to simplify interpretation. Our best approximating model explaining tick abundance included β_{species} ($<2e-16$; 95% CI: 3.861-5.620), β_{sex} (0.053; -1.287-0.007, β_{flea} presence (0.005; 0.088-0.506), and β_{burrow} density (0.084; -0.038-0.613) however, only species and flea presence were significant (Table 4.1) and these two variables appeared in all models within four ΔAIC units of our best model (Table 4.2). Grasshopper mice had more ticks than *Peromyscus* spp., and ticks were more abundant on small mammals with fleas.

All life stages belonged to the genus *Ixodes*, yielding a total of 2,495 ticks across all host species (Table 4.3). Of these, 1,780 ticks were collected from black-footed ferrets, including 36 larvae, 227 nymphs, 1,052 adult females, 211 adult males, and 225 unidentifiable adults (Figures 4.1 & 4.2). Small mammals carried 719 ticks, consisting of 517 larvae and 177 nymphs and no adult ticks. A chi-square analysis comparing tick life stages found on ferrets versus small mammals

was highly significant ($\chi^2 = 1448.4$, $df = 2$, $p < 2.2e-16$). Ferrets harbored substantially higher numbers of adult ticks, whereas small mammals were parasitized primarily by larvae (Figure 4.3).

We analyzed 52 ticks and 37 fleas collected in 2015–2016 from 16 seropositive and nine seronegative black-footed ferrets using Real-Time PCR. This sampling occurred during a documented period of elevated tularemia exposure indicative of an epizootic event (Chapter 3). Six samples were lost due to failed extractions, and the PCR assay did not detect *F. tularensis* in any of the remaining tick or flea samples.

DISCUSSION:

Tick occurrence is likely widespread and common within the prairie dog burrow ecosystem, which highlights the importance of the burrow system for microhabitats that support tick survival and reproduction. Adult ticks were predominantly found on black-footed ferrets, while larvae and nymphs were concentrated on small mammals, consistent with a three-host life cycle (Apanaskevich and Oliver 2014). Northern grasshopper mice were significantly more likely to carry ticks compared to *Peromyscus* spp. Unlike other small mammals, grasshopper mice are insectivores/carnivores and spend more time actively hunting in burrows, moving through multiple chambers, and encountering a large range of microhabitats where ticks may quest (Stapp 1999). These behaviors likely increase their exposure to ticks compared to *Peromyscus* spp., which may have more limited movement within burrows or forage primarily above ground (Kurta 2017).

A positive association between flea presence and tick abundance shows potential co-parasitism effects through shared use of prairie dog burrows. Northern grasshopper mice, which carry more

ticks, also carry more ectoparasites including fleas (Caron-Levesque et al. 2023). Their role as hosts for multiple parasites may have important implications for pathogen maintenance and persistence within small mammal communities (Stapp et al. 2009; Kraft and Stapp 2013).

While ticks were highly prevalent on ferrets and small mammals, our PCR results did not detect *Francisella tularensis* DNA in the sampled ectoparasites, suggesting that ticks may not have been a primary driver of the observed tularemia epizootic. Ticks were collected in the fall, which may not correspond to the period when ferrets were exposed to the pathogen; if ticks were not attached during peak exposure, they may not have had the opportunity to acquire or transmit the bacteria. Additionally, ticks were preserved in ethanol prior to analysis, and degradation of DNA over time could have contributed to the negative PCR results. Therefore, while our results indicate that ticks were likely not responsible for this specific epizootic, we cannot rule out their potential role in tularemia transmission in this system. The high prevalence of ticks on ferrets and the concentration of larval and nymphal ticks on small mammals demonstrate that these ectoparasites occupy positions in the ecosystem where host interactions could facilitate pathogen transfer. To fully assess the role of *Ixodes* ticks in disease dynamics within prairie dog colonies, targeted laboratory vector competency studies and experimental exposure trials are needed to determine whether these ticks can acquire, maintain, and transmit tularemia.

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Attachments:

Table 4.1: AIC model selection for Poisson regression models explaining tick abundance on small mammal species in Conata Basin/Badlands National Park, South Dakota in 2024 and 2025. Significant variables included Species and Flea presence within all top models.

Model:	K	ΔAICc	AICc Wt	Cum. Wt
Species + Sex + Flea + Burrow Density	6	0	0.25	0.25
Species + Sex + Age + Flea + Burrow Density	7	0.61	0.19	0.44
Species + Sex + Flea	5	0.97	0.16	0.60
Species + Sex + Age + Flea	6	1.56	0.12	0.72
Species + Flea + Burrow Density	5	1.73	0.10	0.82
Species + Age + Flea + Burrow Density	6	2.23	0.09	0.91
Species + Flea	4	2.23	0.08	0.99
Species + Sex + Age	6	6.54	0.01	1.00

Table 4.2: Poisson regression model parameter estimates and 95% confidence intervals from the top model for predictors of tick abundance on small mammal species. Significant predictors included Species (Northern Grasshopper Mice and *Peromyscus* spp.) and Flea presence.

Coefficients	Estimate	Std. Error	t value	Pr(> z)	2.5% CI	97.5% CI
(Intercept)	0.472	0.259	1.823	0.069	-0.035	0.980
Species	4.741	0.449	10.569	<2e-16	3.861	5.620
Sex	-0.640	0.330	-1.938	0.053	-1.287	0.007
Flea	0.297	0.107	2.790	0.005	0.088	0.506
Burrow Density	0.287	0.166	1.729	0.084	-0.038	0.613

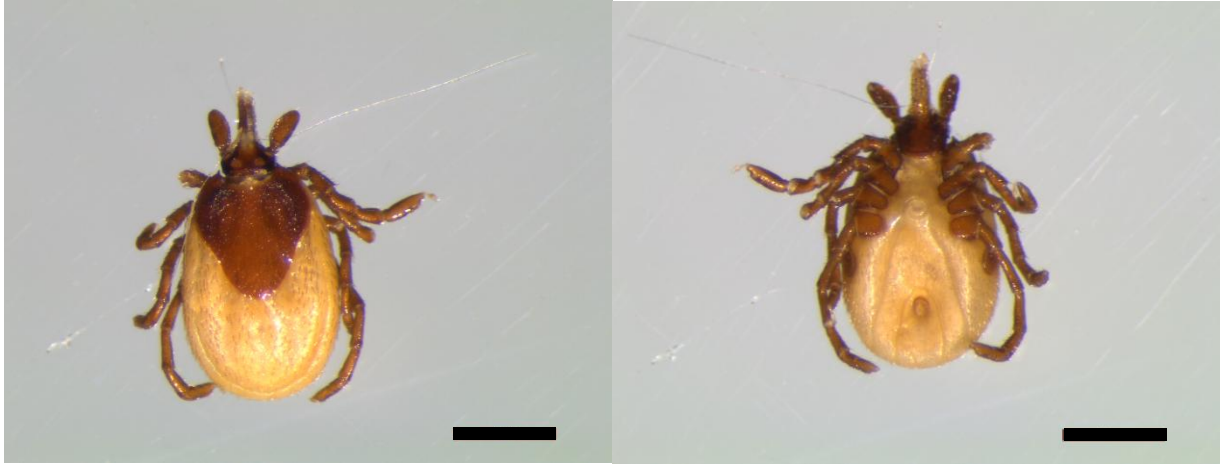


Figure 4.1: Adult female *Ixodes kingi*. Scale bar equals 1mm. Photos by Madisen Hartlaub.



Figure 4.2: Adult female *Ixodes sculptus*. Scale bar equals 1mm. Photos by Madisen Hartlaub

Table 4.2: Identification of Ticks found on black-footed ferrets between 2022-2025 and small mammal species found on prairie dog colonies in 2024 and 2025 in Conata Basin/Badlands National Park, South Dakota.

Host Species	N	<i>Ixodes</i> Larvae	<i>Ixodes sculptus</i> Nymph	<i>Ixodes kingi</i> Nymph	<i>Ixodes Nymph</i> N/A	<i>Ixodes sculptus</i> Adult Female	<i>Ixodes sculptus</i> Adult Male	<i>Ixodes kingi</i> Adult Female	<i>Ixodes kingi</i> Adult Male	<i>Ixodes</i> Adult N/A	Tick Total
Black-footed Ferret	279	36	169	38	20	769	106	283	105	225	1780
Northern Grasshopper Mouse	66	297	108	17	16	0	0	0	0	0	438
<i>Peromyscus</i> spp.	101	171	41	5	5	0	0	0	0	0	222
Meadow Vole	1	0	2	0	0	0	0	0	0	0	2
Hispid Pocket Mouse	6	49	3	1	0	0	0	0	0	0	53
TOTAL:											2495

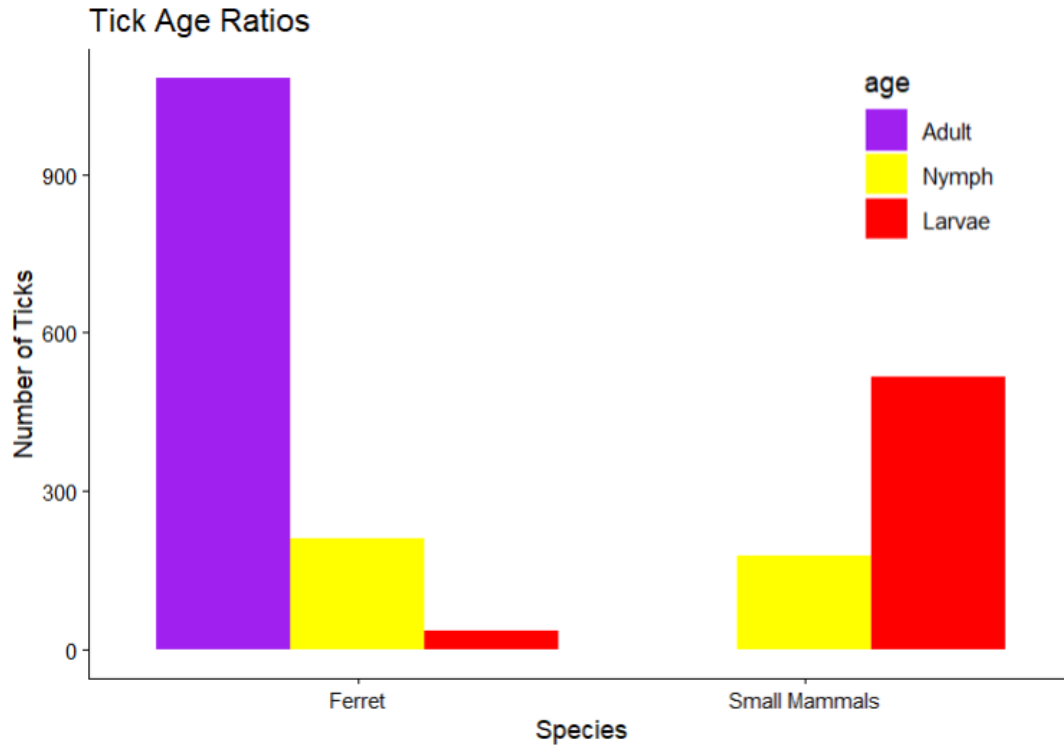


Figure 4.3: Chi-square test for heterogeneity confirmed a significant relationship between tick life stage and prairie dog burrow host (ferrets versus small mammals). Adult ticks were most prevalent on ferrets, whereas immature stages were more prevalent on small mammal hosts.

**CHAPTER 5: TESTING ENVIRONMENTAL TICK SURVEILLANCE METHODS IN
THE PRAIRIE DOG BURROW ECOSYSTEM: THE SEARCH FOR IXODES
SCULPTUS AND IXODES KINGI (ACARI: IXODIDAE)**

Written in style for Journal of Parasitology with coauthors Drs. T. Livieri and S. Dubay

ABSTRACT: Understanding the presence of tick vectors is essential for parasite phenology, wildlife conservation, and public health surveillance, especially as tick-borne diseases continue to rise globally and now account for more than 75% of all vector-borne infections in the United States. Standard environmental sampling methods such as dragging, flagging, and carbon dioxide trapping are widely used for forest-associated *Ixodes* species, but their efficacy for grassland ticks remains largely unknown. Mixed-grass prairie habitat structure is generally unfavorable for well-studied *Ixodes* spp., however understudied species such as *Ixodes kingi* and *I. sculptus* persist and frequently parasitize small mammals and black-footed ferrets (*Mustela nigripes*). We evaluated if conventional off-host sampling methods could reliably detect these species in southwestern South Dakota, USA. Extensive dragging and flagging across prairie dog colonies yielded no *Ixodes* spp., whereas 21 ticks were collected via burrow swabbing. Direct excavation of a prairie dog burrow revealed *I. sculptus* deep within the burrow system, supporting our hypothesis that these ticks exhibit nidicolous behavior and rarely quest on the prairie surface. These results suggest that standard surface-based surveillance is poorly suited for detecting prairie-adapted *Ixodes* spp. and that these ticks likely rely on the stable temperature, humidity, and host availability within burrow systems. As tick-borne diseases and tick ranges continue to expand, developing specialized belowground surveillance methods will be critical to improve monitoring of these understudied grassland ticks within a One Health framework.

INTRODUCTION:

Understanding the distribution of disease vectors such as mosquitoes, ticks, fleas, and horseflies is important for not only parasite phenology but also surveillance targeting wildlife and public health. Of recent concern, tick populations are increasing globally, and tick-borne diseases account for >75% of vector-borne infectious diseases in the United States (Madison-Antenucci et al., 2020; Eisen and Paddock, 2021). Methods of tick surveillance are becoming increasingly important with emerging diseases and tick range expansion (Nuttall, 2022; Gilbert, 2021; Sonenshine, 2018). Often, environmental surveillance for ticks is conducted in locations where heavy tick burdens impact public health, such as forested areas used for recreational purposes. Effective surveillance is critical for tick-borne disease monitoring and prevention in all aspects of the One Health approach (Rowan et al., 2023).

Ticks of clinical importance in the United States include the black-legged tick (*Ixodes scapularis*), western blacklegged tick (*Ixodes pacificus*), *Amblyomma americanum*, *A. maculatum*, *Dermacentor variabilis*, American dog tick (*Dermacentor andersonii*), *Dermacentor occidentalis*, *Rhipicephalus sanguineus*, and soft ticks (*Ornithodoros* spp.; Eisen and Paddock, 2021). For example, *I. scapularis* is a vector for both *Borrelia burgdorferi* (Lyme disease) and *Francisella tularensis* (tularemia) in humans and animals (Hayes, 2005; Kugeler et al., 2024). While many of these ticks are typically associated with forested environments, they occur in states such as South Dakota, despite having less than 4% total forest cover (Walters 2016). Other, lesser-known species also inhabit South Dakota including, *I. kingi* and *I. sculptus* (Maestas, 2019). *Ixodes kingi* occurs on a wide range of rodent and carnivore hosts in South Dakota and has been previously associated with sylvatic maintenance of tularemia (Williams et al., 1991; Durden and Keirans, 1996; Hayes, 2005; Maestas, 2019). *Ixodes sculptus* also has been

reported on many ground dwelling rodents and carnivores with no known clinical associations (Maestas, 2019). Additionally, both species often parasitize black-footed ferrets (*Mustela nigripes*, Harris et al., 2014), an endangered carnivore that survives in prairie dog (*Cynomys* spp.) colonies in western South Dakota. Ticks spend time off-host (Hixson, 1932) and use Haller's organs on their legs to detect hosts while questing on vegetation (Salomon et al., 2020). Successful tick collection methods depend upon predictable questing behavior shown by an individual tick species. For example, *I. scapularis* quests by sitting on vegetation with its legs extended and when a host brushes by, the tick attaches to the host. These ticks also are lured by carbon dioxide to find a host (Tietjen et al., 2020) and tend to quest in early morning and evening to avoid direct sunlight and desiccation. In comparison, *A. americanum* is more aggressive and will "hunt" a possible host by tracking carbon dioxide they emit (Marshall et al., 2025).

Suitable habitat characteristics for *Ixodes* spp. often are related to escaping desiccation and starvation mortality events (Linske and Williams, 2024). *Ixodes scapularis* habitat suitability has been well-studied, and they primarily occupy areas with dense vegetation and high humidity (Maestas et al., 2019; Linske and Williams, 2024). Methods of collection from the environment have proven effective for sampling various *Ixodes* species, particularly those inhabiting forested or shrubby environments (Maestas et al., 2016; Mathisson et al., 2021). Dragging or flagging methods involve tugging flannel cloth over vegetation and litter to collect questing ticks, whereas carbon dioxide traps, typically baited with dry ice, attract "hunting" ticks from nearby (Marshall et al., 2025). Tick species often exhibit variation in their tolerances of desiccation, which makes vegetation composition and environmental factors such as cloud cover, temperature, and humidity (Apanaskevich and Oliver, 2014; Mathisson et al., 2021) important for estimating the presence and abundance of tick species on the landscape, and influencing off-host collection

methods (Salomon et al., 2020). Environmental factors and host species are the most common variables used to evaluate tick geographic range (Sonenshine, 2018). Additionally, tick questing behaviors are considered when designing methods for off-host sampling and habitat suitability modeling (James et al., 2015). Despite their success in temperate woodland ecosystems, flagging and dragging techniques may be less effective to collect ticks adapted to open or dry habitats, such as those found in grasslands. Grassland tick species remain comparatively understudied, with limited research exploring their ecology, distribution, and the efficacy of traditional sampling methods in these environments.

On black-tailed prairie dog (*C. ludovicianus*) colonies in the grasslands of western South Dakota, vegetation structure is dominated by blue grama (*Bouteloua gracilis*), buffalograss (*Buchloe dactyloides*), western wheatgrass (*Pascopyrum smithii*), carex (*Carex spp.*), red threeawn (*Aristida purpurea*), scarlet globemallow (*Sphaeralcea coccinea*), woolly indianwheat (*Plantago patagonica*), and plains prickly pear (*Opuntia polyacuntia*; MacCracken et al. 1985). Activity of prairie dogs often results in little to no vegetation around burrow openings and short vegetation on the prairie dog colony (Bonham and Lerwick, 1976, MacCracken et al., 1985). This prairie vegetation structure is conducive for the American dog tick (*Dermacentor variabilis*) as their highest abundance is found in open-canopy grasslands, however it is not favorable for well-studied *Ixodes* spp. (Mathisson et al., 2021).

Studies on prairie dog colonies in southwestern South Dakota found that despite a lack of typical tick habitat due to low vegetation structure, both *I. kingi* and *I. sculptus* are commonly found on black-footed ferrets and small mammals but rarely found on prairie dogs (Harris et al. 2014, Chapter 4). We speculate that *I. kingi* and *I. sculptus* exhibit nidicolous behavior and maintain their lifecycles within the prairie dog burrows, rarely using the prairie surface to find a host.

Many *Ixodes* ticks exhibit nidicolous behavior at one or more stages of their lifecycle (Gray et al., 2014). Well studied species such as *Amblyomma limbatum*, *A. albolimbatum*, and *Bothriocoton hydrosauri* parasitize Australian lizards (*Trachydosaurus* spp.) and access hosts by waiting in common lizard shelter, such as burrows, to escape the hot and dry environmental conditions (Gray et al., 2014). Due to the consistency of environmental conditions within the prairie dog burrow system, we suspect *I. kingi* and *I. sculptus* may be using burrow entrances and/or prairie dog nesting chambers to find hosts and/or reproduce. Our objective was to assess standard tick collection methods to sample *Ixodes sculptus* and *I. kingi* in the southwestern South Dakota black-tailed prairie dog ecosystem. We hypothesize that these tick species will be found within the prairie dog burrow system and will not be on the surface because of the threat of desiccation in this arid environment.

METHODS AND MATERIALS:

The Conata Basin/Badlands National Park (CB/BADL) in southwestern South Dakota, managed by the U.S. Forest Service and National Park Service, consists of shortgrass prairie with diverse wildlife, including carnivores, small mammals, reptiles, and invertebrates. This area is home to the largest population of wild black-footed ferrets in the world following successful reintroduction efforts and ferrets are monitored annually.

We sampled ticks from prairie dog colonies in CB/BADL occupied by tick-parasitized ferrets and the surrounding grasslands in summer 2024 and 2025 using dragging/flagging and burrow swabbing techniques. Dragging and flagging followed the standardized protocol of Salomon et al. (2020). We established 90 randomized 100 m transects across 18 plots of prairie dog colonies

in which we sampled 63 transects on the colony and 27 transects on the colony edge/surrounding tall vegetation. We dragged a 1 m² white flannel cloth along each transect and inspected the cloth every 10 m for attached ticks (Figure 5.1). Each transect was dragged a total of six times, three times before 1100 and three times after 1700 to account for potential variation in tick activity. We noted if our drag passed over a burrow entrance, percent cloud cover (Jones 1992), temperature, and humidity and followed Salomon et al. (2020) to avoid trees and bundles of cacti. Additionally, we dragged 2,100 meters in 2024 and 2025 in adjacent areas (off-colony and colony edge) of taller vegetation to capture questing ticks from different questing heights or locations. We used drags to sample three nights from 2300 to 0300 to investigate potential nocturnal tick questing behaviors because temperatures tend to be cooler and humidity higher than during the day. Ticks were collected using fine-tipped forceps and placed in labeled vials for later identification. These flagging efforts followed the same collection and inspection protocols as described above.

We used prairie dog burrow swabbing (Eads 2017) to sample ticks inhabiting host burrows. At each plot, we randomly selected 10 active prairie dog burrows and inserted a 0.3m² flannel square swab attached to a flexible drain snake rod into each burrow (Figure 5.1). Burrows were classified as active if they lacked cobwebs or ingrown vegetation, fresh prairie dog scat was present, had been recently excavated as evidenced by presence of loose, scattered, or wet soil, and/or prairie dogs were present. Swabs were inserted as deep as possible and rotated for 30 seconds to collect ectoparasites sitting in the burrow substrate. Ectoparasites collected were transferred into vials containing 80% ethanol for preservation for further identification. For each burrow sampled, burrow depth, aspect (cardinal direction) of the burrow entrance, and GPS coordinates were recorded to characterize habitat conditions associated with tick presence.

Additionally, we opportunistically excavated one burrow in 2025 to further assess tick presence deep in the prairie dog burrow microclimate. We chose an unoccupied burrow that had been vacant from prairie dogs and ferrets due to a prior plague epizootic. We chose a rim crater burrow shape to increase the chance of finding a burrow tunnel that would descend almost vertically (Hoogland, 1995) rather than the more gradual descent typical of a dome crater burrow. Prior to digging, we measured burrow depth, aspect, and recorded coordinates. A backhoe excavator was used to remove soil from the burrow and place it onto a tarp where we sieved the soil through a 1mm mesh strainer to separate any invertebrates living within the soil. We collected 40 samples per each of the soil levels at 0.3-0.6m, 0.6-1.42m, 1.2-1.8m, 1.8-2.4m, 2.4-3m from the surface. Between each soil level, we also opportunistically collected invertebrates from inside the burrow cavity itself by scraping 0.05-0.07m of soil from the surrounding wall and sieving it through a strainer. Any non-parasitic invertebrates found during this effort were placed in 80% ethanol for later identification. Our goal of this excavation was to find a prairie dog nesting chamber/nesting material to search for ticks (Figure 5.1). Ticks collected from all methods were identified to sex, life-stage, and species using keys based on differences in morphological features (anal groove location, distinctness of cornua and lateral carinea, hypostome teeth and dentation and shape of basis capitulum and porous area; Cooley and Kohls 1945; Kerians and Clifford 1978; Furman and Catts 1982).

We recorded sampling time for each of these methods to approximate the economic cost of each sampling method. We used \$15 per hour for a single technician and then multiplied that by the number of technicians and time each spent sampling using each method. We had two technicians dragging/flagging, small mammal trapping, and burrow swabbing but had three while excavating

the burrow. We compared the cost of burrow swabbing, dragging/flagging, and excavating a burrow to estimate the cost per tick.

RESULTS:

Tick dragging covered a total distance of 56,500 m but yielded no *Ixodes* spp., resulting in a 0% detection rate (Table 5.1). The cost was ~\$6,780 with nearly 222 person hours of invested time (Table 5.2). Dragging took place during an average of 26°C, 48% humidity, 19% cloud cover and when wind speed was ≤ 16 kph. We incidentally collected three adult female *Dermacentor variabilis* ticks in 2025 during 2,100 m of dragging and flagging on off-colony vegetation but these were not considered part of targeted *Ixodes* spp. surveillance. We swabbed 180 burrows on 18 prairie dog colonies between 0800 and 2000 between 5/23/2024 and 8/10/2024. These burrows averaged 2.23m in depth (1m – 4.5m) facing all aspects. We detected *Ixodes* spp. on 10 of 180 swabs with a 5.6% success rate. Twenty-one ticks (6 *Ixodes* spp. larvae and 15 *I. sculptus* nymphs) were recovered from burrows below 2 meters in depth from 3 of our sampling areas (Pinnacles, Agate, and Prairie Wind; Table 5.1). This averaged roughly 36 person hours of work costing ~\$1,080 (Table 5.2). In June 2025, we began to excavate a single southwest facing burrow estimated to be over 3m in length, however, the burrow began to turn eastward at 3m making it difficult to access further. The burrow contained two probable prairie dog nesting chambers, containing grass and bones. This burrow produced two adult female *Ixodes kingi* ticks, recovered below 1.8m in depth from the burrow entrance.

DISCUSSION:

Conventional surface sampling methods are poorly suited for detecting *Ixodes sculptus* and *I. kingi* on prairie dog colonies and surrounding grasslands. Dragging and flagging, despite the labor investment and associated financial cost, did not produce any target ticks and therefore dragging/flagging does not appear to be an effective nor efficient surveillance approach for these species in this environment. While we did collect 10 *Ixodes* ticks from swabbing burrows, this approach alone is not reliable for tick detection (Table 5.2). Direct burrow excavation produced ticks from within an active burrow system, supporting the idea that these species may persist primarily underground (Hixon 1932).

Our results, while not surprising, are striking in their lack of efficiency in comparison to our substantial sampling effort. Schulze et al. (1997) collected 1,467 nymphal and 259 adult *Ixodes sculptus* and *Amblyomma americanum* from only 5,400 meters of dragging in 3 forested environments in New Jersey. We dragged over ten times the distance but collected no *Ixodes* spp. Similarly, Maestas et al. (2016) reported that 23 person-hours (approximately one day) of dragging in eastern South Dakota yielded seven adult *Ixodes scapularis* and more than 430 adult *Dermacentor variabilis*, whereas our surveys required 222 person-hours (over nine full days) and still produced no *Ixodes*. Despite bycatch of three *D. variabilis* on off-colony vegetation, our intensive and costly sampling effort suggests that *I. sculptus* and *I. kingi* are likely persisting within prairie dog burrows and on hosts rather than questing on the colony surface.

Prairie dog burrow systems provide relatively stable temperature and humidity conditions that minimize desiccation risk and offer continuous access to hosts (Hoogland 1995; Hixson 1932). These features may reduce the need for *Ixodes sculptus* and *I. kingi* to quest above-ground,

contributing to the difficulty of sampling these ticks using methods developed for three-host tick species. These ticks likely exhibit nidicolous behavior for at least one or all three life stages, similar to other burrow dwelling species such as *Ixodes rothschildi* found in sea bird nests and soft tick (Argasidae) species such as *Ornithodoros turicata* (Canino et al., 2024; Nuttall., 2022; Gray et al., 2014). Occasionally adult female *I. rothschildi* are found on sea bird hosts but these ticks remain understudied possibly due to nidicolous behavior (Gray et al., 2014).

Ixodes kingi and *I. sculptus* likely exhibit some lifecycle seasonality, typically correlated to local climactic conditions, which should be considered when sampling (Salkeld et al., 2006). In northern Colorado, Salkeld et al. (2006) found *I. kingi* on mice in early May and on ground squirrels in late June, whereas *I. sculptus* first appeared in early July, with nymphal loads of both species peaking in July–August. They concluded that nymphs of *I. kingi* and *I. sculptus* exhibit a unimodal summer activity pattern aligned with the region’s warmest temperatures and peak rainfall. In contrast, Beck et al. (1963) reported a bimodal activity pattern for *I. kingi* nymphs, peaking in spring and winter, in hot, dry desert shrubland and piñon–juniper woodland in Nevada, while *I. sculptus* nymphs showed a unimodal pattern with peak abundance from November to March which should be considered during off host sampling.

Future sampling should include specialized carbon dioxide bait traps placed at burrow entrances or within the burrows themselves (Miles, 1968; Koch et al., 1981). Burrow vacuuming should also be explored, as it has proven useful for soft tick surveillance, although the complexity and length of prairie dog burrow systems pose significant logistical challenges (Canino et al. 2024; Wilcomb 1954). At present, host-based sampling appears to be the most reliable method for detecting these ticks (Chapter 4; Table 5.1), but additional specialized belowground sampling is warranted.

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ATTACHMENTS:

Table 5.1. Success of sampling methods used to collect *Ixodes* spp. ticks from the environment on and off prairie dog colonies in Conata Basin/Badlands National Park, South Dakota.

Sampling Method	Number of Samples	Success Rate for Ticks	<i>Ixodes</i> spp. Ticks Collected
Small Mammal Trapping	633	20%	736
Burrow Swabbing	180	5.6%	21
Dragging/Flagging	57,600 m	0	0
Burrow Excavation	1	-	2

Table 5.2: Average cost in US dollars and person hours per tick sampling method. Average cost per tick was calculated by dividing the average cost of each sampling method by the number of *Ixodes* spp. ticks collected from the environment.

Sampling Method	Person Hours	Average Cost	Average Cost Per Tick
Small Mammal Trapping	792hrs	\$11,880	\$16.14
Burrow Swabbing	72hrs	\$1,080	\$51.43
Dragging/Flagging	452hrs	\$6,780	-
Burrow Excavation	18hrs	\$270	\$90

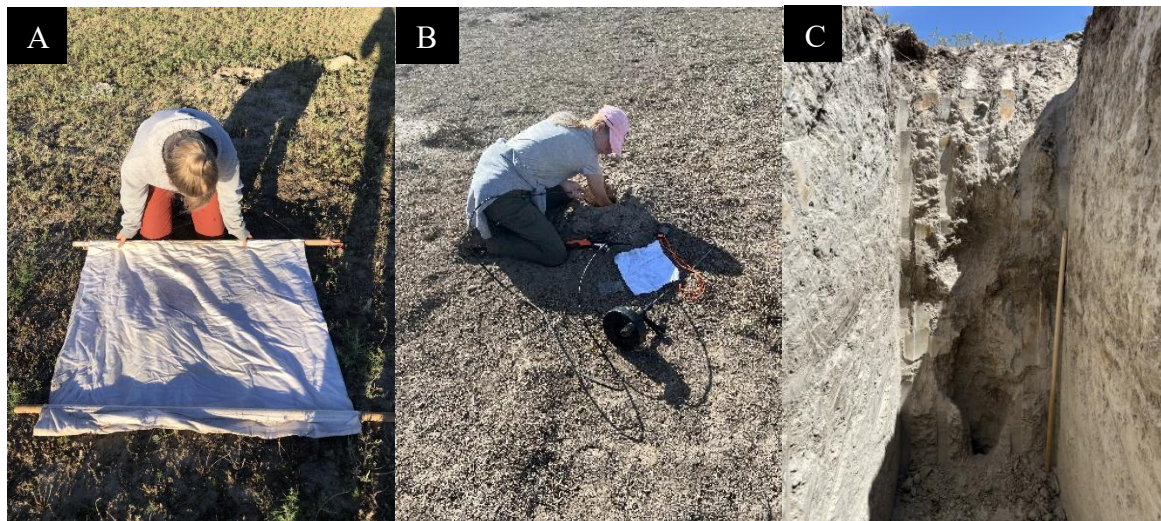


Figure 5.1: Field photos of tick collected techniques: A. Dragging, B. Swabbing, and C. Burrow Excavation