Ecology of *Francisella tularensis* in Sympatric Rodent and Lagomorph Species in Urban and Non-urban Settings in Prairie Canada

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By

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LIST OF ABREVIATIONS

APZ ……...…… Assiniboine Park Zoo

ARS ……... American red squirrel

CWHC ……..... Canadian Wildlife Health Cooperative

MB …… Manitoba

PDS …….…….. Prairie Diagnostic Services

PHAC …….……. Public Health Agency of Canada

RGS ……... Richardson's ground squirrel

SK …….. Saskatchewan

U of S …….. University of Saskatchewan

VIDO ……... Vaccine and Infectious Disease Organization

WCVM ……... Western College of Veterinary Medicine

WTJR ……..……..... White-tailed jackrabbit

DEFINITIONS

Habitat Definitions

Aquatic habitat: a location was considered aquatic if it was within approximately 250 m of a natural body of water.

Non-urban: a location was considered non-urban if it was comprised predominantly of agricultural land, native habitat, or sparsely populated residential area (e.g., farmhouse and buildings)

Terrestrial habitat: a location was considered terrestrial if it was further than 250 m from a natural body of water.

Urban habitat: a location was considered urban if it was within or surrounded by a densely populated residential or commercial area, including city parks or experimental agricultural lands

Ecological Roles

Sentinels: animal species that may be used during active or passive surveillance to detect diseases or hazards that may impact wildlife or human populations.

The following definitions were used as outlined by Friend (2006)

Recipients: animal "species required for circulation of *F. tularensis* as amplification hosts for maintaining the epizootic; they are often the same species as the ***donors***." They are "highly susceptible to infection, and develop high levels of bacteria in blood, and other tissues." They "maintain sufficient levels of *F. tularensis* for infection of vectors that feed on them, for contamination of environments with infective levels in feces, or urine, and for direct contact transmission."

Donors: animal "species that are the primary source for infection of reservoir hosts that maintain *F. tularensis* in nature." In some cases, these species "may develop chronic infections and serve as ***reservoirs***." They "provide for maintenance of *F. tularensis* in nature during interepizootic periods, provide circulation of pathogen among species within enzootic foci and are persistently infected throughout adult life, and may pass infection to progeny in some instances."

Reservoirs: In the US, has been regarded as “primarily ticks;” but can be any animal “species that continually maintain *F. tularensis* in nature.”

CHAPTER 1. Introduction and Literature Review

1.1 Brief Rationale

How the pathogen, *Francisella tularensis*, is maintained in endemic areas in the Canadian Prairies, and whether there are low levels of mortality in mammal populations between epidemics is not known. Urban wildlife may increase the risk of transmission to pets and people, and transmission dynamics are likely to be different between urban and non-urban settings due to different species composition, differing predator pressure and altered environments. As climate changes continue to progress, shifts may occur in the environment, including shifts in arthropod ranges, population densities of certain species, and state of aquatic habitats. All of these could affect how pathogens are maintained and spread, potentially leading to an increase in water-borne, food-borne and vector borne diseases in many areas of the world, including North America (Harvell, 2002; Nakazawa et al., 2007; Relman et al., 2008; Redshaw et al., 2013). Understanding the current status of *F. tularensis* in endemic or sporadic areas could be beneficial in deciding the potential need for pointed surveillance, as has been recommended in several publications (Sagurova et al., 2019) especially since natural tularemia foci can persist in specific areas but are not always stationary and can spread to other areas with suitable conditions (Pikula, 2003).

In this dissertation, the main goal was to investigate the tularemia status (endemic vs non-endemic) in various habitats and settings across Saskatchewan (SK) and Manitoba (MB) during interepizootic cycles. To achieve this goal various ecological aspects were considered, such as potential reservoir, donor, or recipient species (rodents and lagomorphs), and potential sentinels (mesocarnivores and mosquitoes). Confirming the presence of *F. tularensis*, and any other information about the ecology at these locations, would increase our understanding of this bacterium in Prairie Canada as well as the possible impact on various species, including humans. Our data could potentially inform future tularemia research as well as focused surveillance for endemic areas where risks of transmission to humans or human-owned animals may be higher.

1.2 Literature Review

1.2.1 Background Information on Tularemia

Tularemia, a zoonotic disease caused by the bacterium *Francisella tularensis,* is endemic in North America and in many other parts of the northern hemisphere. It was first reported by McCoy in 1911 as a plague-like disease in California ground squirrels (*Otospermophilus beecheyi*) that was transmissible to other mammals. Further characterization of the novel disease was done by McCoy and Chapin through experiments, which confirmed the agent as a non-motile rod bacterium capable of causing fatal disease in both ground squirrels and rats and appeared to be transmitted via fleas (Mccoy & Chapin, 1912). Shortly thereafter, Parker et al. (1924) extended the list of susceptible animals, adding jackrabbits (*Lepus* species), snowshoe hares (*Lepus americanus*), cottontail rabbits (*Sylvilagus* species) and woodchucks (*Marmota monax*). The first report of human tularemia occurred just two years after McCoy's initial publication, during a large wild rabbit die-off in Kentucky, where the bacterium was likely contracted from dressing contaminated rabbit meat (Wherry and Lamb, 1914).

Since then, much has been discovered about the disease process and its agent. The ecology of the disease is complex, with at least four strains of *F. tularensis* reported in North America with varying pathogenicity, vectors, host range, and geographic distribution. The two main subtypes: *F. tularensis tularensis* (Type A), which historically has been present solely in North America, is also the most virulent, and *F. tularensis holarctica* (Type B), which is found predominantly in Europe and Asia, but also North America (Cross and Penn, 2000). Co-existence of both subtypes occur in North American environments (Rausch et al., 1969; Miller, 1974; Hansen et al., 2011). Although subtype A is often attributed to most human clinical cases (Hansen et al., 2011), a post-outbreak Utah study recovered *F. tularensis* Type AI, AII and B, occasionally in the same rabbit carcasses, leading to the conclusion that localized outbreaks can involve multiple subspecies or clades (Petersen et al., 2008). Historically, Type A has been associated with a terrestrial cycle, with main reservoirs postulated to be lagomorphs (Glass, 1948; Guerrant et al., 1976; Mörner, 1992; Cross and Penn, 2000). Type B has been mainly associated with a water-borne cycle, its main reservoirs likely aquatic rodents like muskrats (*Ondatra zibethicus*), beavers (*Castor canadensis*), or voles (European water vole (*Arvicola terrestris*), *Microtus* and *Clethrionomys* species, Glass, 1948; Mörner, 1992), and well-vegetated

aquatic habitats have been reported to be better at maintaining higher levels of the pathogen (McKeever et al., 1958; Roth, Foley and Wright, 2017). Although the above mentioned are historically accepted geographical extent and reservoir species for both subtypes, new locations continue to be added to the literature; for example, *F. tularensis holarctica* was recently diagnosed in Tasmania and Sydney, Australia (Eden et al., 2017; Jackson et al., 2012).

Infected wild lagomorphs have historically played an important role in transmission of the bacterium to humans and domestic animals, as evidenced by numerous case studies and reports citing direct or indirect interactions with rabbits as source of the disease (McNabb, 1930; Black and Thomson, 1958; Rohrbach et al., 1991; Ohara et al., 1996; Pérez-Castrillón et al., 2001; Hauri et al., 2010; Lang and Kleines, 2012; Mailles and Vaillant, 2014; Otto et al., 2015). However, some publications, like Telford and Goethert (2011), began to encourage updated surveillance and studies to investigate possibly skewed views on tularemia ecology, particularly the accepted sweeping role of lagomorphs as both main reservoir and transmitting species. Others have suggested that tularemia has no real or defined reservoir and instead there are various biological niches (e.g., ticks, rodents, etc.) that allow the pathogen to persist in the environment (Genchi et al., 2015). This is supported by an investigation into the 2007 outbreak in Spain, where disease was associated with a diverse group of animals as reservoir, such as lagomorphs, sheep (*Ovis aries*), rodents, and canids (Martín et al., 2007). It was proposed that the unusual diversity of *F. tularensis* sources, in this case, could be attributed to atypical climatic and environmental circumstances (Martín et al., 2007), and serves as a reminder that reservoir roles may vary not only from region to region but also temporally.

A 1992 ecological review claimed tularemia had been reported in more than 250 species, including humans, various mammals, birds, fishes, amphibians, arthropods, and protozoa (Mörner, 1992). The wide range of animals that can be infected has certainly contributed to the difficulty in pinning down which ecological role each species may play in natural endemic foci.

1.2.2 General Tularemia in Rodents and Lagomorphs

Outbreaks of tularemia have been reported in lagomorphs (Wherry and Lamb, 1914; Klock et al., 1973; Mörner and Krogh, 1984; Decors et al., 2011), voles (Guryčová et al., 2001; Cherry et al., 2019), prairie dogs (Avashia et al., 2004; Petersen et al., 2004), and various mice

species (Guryčová et al., 2001; Wobeser et al., 2007; Dobay et al., 2015; Origgi et al., 2015) in various North American, European, and Asian countries. Different studies looking at susceptibility found that parental and inhalation exposures often lead to high and rapid mortality rate in small mammals, while ingestion yielded lower mortalities (Stagg et al., 1956). Mortality can be high in certain populations, but outbreaks generally self-exhaust (Dobay et al., 2015; Origgi et al., 2015; Wobeser et al., 2007).

Common gross lesions in lagomorphs include splenomegaly (62-80%, Hestvik et al., 2017) and multifocal grayish foci in one or more organs (88%, Gyuranecz et al., 2010), while histological lesions are primarily granulomatous and seen in lymph nodes (Elezi et al., 2018; Korro and Cara, 2021), kidneys (Elezi et al., 2018), lungs (Elezi et al., 2018), spleen (Korro and Cara, 2021) and liver (Korro and Cara, 2021), although the latter appears variable (Elezi et al., 2018). Similarly, gross lesions, when present, in small rodents include splenomegaly (Hestvik et al., 2018), and multifocal necrotic foci in spleen, liver or both (Nelson et al., 2014), while histology reveals primarily multifocal necrosis in the spleen, liver, or lymph nodes (Nelson et al., 2014).

Understandably, if there is an increase in epizootic activity in a region, this will translate into a higher risk for humans to be exposed to the pathogen (Guryčová et al., 2001; Ecke et al., 2020). When looking at transmission from animal to humans, various case studies or other research modalities have shown that direct contact, such as a bite or handling of animals without gloves (MacKinnon, 1947; Mörner, 1992; Avashia et al., 2004; Ulu-Kilic et al., 2013; Pedati et al., 2015); indirect contact, such as consuming water or food contaminated by urine or feces (Mörner, 1992; Pérez-Castrillón et al., 2001; Cerny, 2001; Reintjes, 2002; Avashia et al., 2004; Kantardjiev et al., 2006 (likely but unconfirmed); Larssen et al., 2011; Grunow et al., 2012; Yesilyurt et al., 2012; Ulu-Kilic et al., 2013; Esmaeili et al., 2021); or inhalation from aerosolization, such as seen during mowing, hay cutting, etc. (Dahlstrand et al., 1971; Feldman et al., 2001, 2003; Berrada and Telford III, 2011; Mailles and Vaillant, 2014; Pedati et al., 2015), can play large roles in human cases, whether individual or outbreak situation. Wild rabbits remain, in many locations, the primary mammal species cited as exposure risk factor (Gürcan et al., 2006).

As expected, surveillance studies are often carried out in areas of concern or known endemic foci. Many of these studies are conducted by serology, polymerase chain reaction (PCR), or a combination of these and other methods. Which rodent or lagomorph species are considered most valuable as sentinels for surveillance depends largely on geographical location and which *F. tularensis* subspecies is present, as this will infer which cycle (terrestrial vs aquatic) the pathogen is likely to follow. Many studies have looked at lagomorphs, especially European brown hares (*Lepus europaeus*), and found high seroprevalence (e.g., 5.8% Treml et al., 2001; 6% Winkelmayer et al., 2005; 6.5% Treml et al., 2007; 5.1% Gyuranecz et al., 2011; 9.6-11% Elezi et al., 2016 & 2018; 23.3% Korro and Cara, 2021) or infection prevalence (Moinet et al., 2016; 11.8% Tomaso et al., 2018) in local populations. However, there are a few studies that have found no or low prevalence of *F. tularensis* in their lagomorph populations (0% Hotta et al., 2012; 3.9% Mostafavi et al., 2018), reinforcing the need for each region to accurately identify appropriate sentinels. Other select regions saw high seroprevalence in vole species (9.2% Rossow et al., 2014; 14% Bártová et al., 2020; 14% Ecke et al., 2020; 32% Elashvili et al., 2015), muskrats (14.4% Macieira, 2019) or beavers (21% Mörner and Sandstedt, 1983).

Many published surveillance studies exist (Burroughs et al., 1945; Mörner and Sandstedt, 1983; Tärnvik et al., 1996; Gyuranecz et al., 2011; Gabriele-Rivet et al., 2016; Hestvik et al., 2017; Korro and Cara, 2021), and one area of great interest is to attempt to find animals that could carry the disease with minimal or no gross lesions, as these could fill a reservoir niche in various geographical locations. Over the years, several species, including meadow voles (*Microtus pennsylvanicus*, Burroughs et al., 1945), deer mice (*Peromyscus maniculatus*, Burroughs et al., 1945), brown rats (*Rattus norvegicus*, Burroughs et al., 1945), yellow-necked mouse (*Apodemus flavicollis,* Hestvik et al., 2018), European brown hares (Winkelmayer et al., 2005; Gyuranecz et al., 2010, 2011; Hestvik et al., 2017; Korro and Cara, 2021), Mountain hares (*Lepus timidus,* Hestvik et al., 2017), prairie dogs (Cherry et al., 2019; Petersen et al., 2004), snowshoe hares (Akerman & Embil, 1982; Gabriele-Rivet et al., 2016), muskrats (Macieira, 2019), and gray squirrels (*Sciurus griseus*, Nelson et al., 2014) were reported to test positive on serology, PCR, or other tests while they had minimal to no gross lesions on necropsy, were apparently healthy hunter-harvested or were still alive with no apparent disease. Some of these findings were in contrast with other publications where deer mice (Wobeser et al., 2007) and

yellow-necked mice (Bandouchova et al., 2009) were established as more susceptible to infection. More research is needed on the variability in pathological presentation, taking into consideration differences among species, geographical locations, and subspecies, or strains.

Experimental studies have also investigated survivability and shedding of bacteria, including Bell and Stewart (1975) who established that meadow voles infected by ingestion could develop chronic tularemic nephritis. This chronic carrier state could result in shedding of the pathogen and contamination of the aquatic habitat. The authors later further postulated that although voles, muskrats and beavers often thrive even in *F. tularensis* contaminated waters, heavy burden from several chronic shedders could lead to the epizootic outbreaks occasionally seen in these species (Bell and Stewart, 1983). Another experimental study by Olsufjev et al. (1984) found comparable results, whereby voles that were orally infected and survived, became immune, seroconverted, and exhibited long-term persistence of infectious agent, and occasional bacteriuria.

On the other hand, many studies have investigated rodents that do not appear to play a reservoir role during inter-epizootic periods but appear to show sharp increase in *F. tularensis* prevalence during population eruptions. Such events have been noted in common voles (*Microtus arvalis*) in Spain and appear to support this species as a recipient; causing spillover leading to zoonotic outbreaks (Rodríguez-Pastor et al., 2017). Rodents as recipients causing spillover is supported by Roth et al. (2017), who postulated in their Californian study that high rodent densities (as well as their ectoparasites) could be an amplification mechanism. Further support for the role of rodent density in tularemia outbreaks, including in humans, was seen in several other publications (Hörnfeldt, 1994; Tärnvik, 1996; Cerny, 2001; Efimov et al., 2003; Gürcan et al., 2006; Kantardjiev et al., 2006; Komitova et al., 2010; Larssen et al., 2011; Gyuranecz et al. 2012; Rossow et al., 2014; Pedati et al., 2015; Luque-Larena et al., 2017; Rodríguez-Pastor et al., 2018). In contrast, surveillance in other regions, where vole species held the highest infection prevalence across rodents, e.g., Georgia study, did not demonstrate similar prevalence correlation with population density (Elashvili et al., 2015). Other publications commented on rodent population density increases that were not "natural" or cyclical, such as natural disasters (earthquakes), human conflicts (war zones) and changes in agricultural landscapes. All of these events can contribute to changing the established ecology and tip the

scale towards increased rodent numbers, sometimes eruptively, which contributes to increases pathogens in those populations, thereby increasing risk of disease in humans or their domesticated animals (Reintjes, 2002; Kantardjiev et al., 2006; Grunow et al., 2012; Jareño et al., 2015).

Recent *F. tularensis* cases were discovered fortuitously while other disease conditions were the focus, for example two fox squirrels (*Sciurus niger*) were found to be positive for tularemia during a *Baylisascaris* outbreak investigation (Vincent et al., 2020), as well as one black-tailed and one Gunnison's prairie dog (*Cynomys ludovicianus* and *C. gunnisoni*) were discovered to have active infections during a thymic lymphoma study (Butler et al., 2020).

1.2.3 The History of Tularemia in Canada

In the early 1930's, almost simultaneous cases of human tularemia in Ontario and recovery of the bacterium from a snowshoe hare in BC led to the recognition that tularemia had endemic pockets already established across Canada (McNabb, 1930; Parker, Hearle and Bruce, 1931). A cluster of cases were seen between 1940 and 1942 in a county in Alberta, where two people and three animals (one sheep, one jackrabbit and one ground squirrel) were culture positive (Bow and Brown, 1943). The authors published another report in which ground squirrels from three widely separated areas in Alberta were diagnosed with tularemia (Bow & Brown, 1946). A survey in the western provinces yielded one positive house mouse (*Mus musculus*) in BC and 11 animals in Alberta, including seven ground squirrels, two meadow vole, one deer mouse and one rabbit, further confirming the bacterium's presence (Humphreys and Campbell, 1947). By 1958, six human cases had been documented in BC, with transmissions attributed to a scratch from a cat (*Felis catus*), skinning of (two) lagomorphs and (one) coyote (*Canis latrans*), one tick bite and one lab acquired, leading the authors to conclude that a reservoir for the pathogen was likely already established in the province (Black and Thomson, 1958).

The first demonstration of *F. tularensis* in Canadian waters came on the heels of an epizootic event in aquatic mammals in Alberta (winter 1952-1953); the agent was isolated from beaver and muskrat tissues (Banfield, 1954). Since then, tularemia has been confirmed in eight rodent and two lagomorph species; beavers, muskrats and snowshoe hares were the most common species infected, and both muskrats and hares were associated most commonly with

zoonotic transmission (Wobeser et al., 2009). Rabbit-associated human tularemia was more prevalent before 1950, with muskrat-associated cases increasing in importance between 1950 and 1980 (Martin et al., 1982). Further support for muskrat-associated risk was given by the serological study in Québec trappers in 1995, where 27% of trappers catching 100+ muskrats had titers, while those catching less than 100 were all negative (Lévesque et al., 1995).

Sporadic tularemia cases are most common (Gattereau et al., 1970; Walker and Moore, 1971; Plourde et al., 1992; Isaac-Renton et al., 2010), but outbreaks are also reported, including those in humans (Bow and Brown, 1946; Ford-Jones et al., 1982), muskrats and beavers (Labzoffsky and Sprent, 1952; Fyvie et al., 1959), deer mice (Wobeser et al., 2007), and even non-human primates at the Assiniboine Park Zoo (APZ) in the 1970's (Preiksaitis et al., 1979; Nayar et al., 1979). Another non-human primate case occurred at the same zoo in 2015 (C. Berkvens, personal communication, 08-31-2021) and will be further discussed in a later section.

A survey published by Bruce (1978) looked at the seroprevalence in Richardson's ground squirrels (*Urocitellus richardsonii,* RGS) in Alberta and SK and reported 1.5% of sera had agglutinins and postulated that these squirrels could play a role in the ecology of the disease in these provinces. It was hypothesized that if animals were infected before hibernation and developed active disease in spring, this could lead to a bacteremic population while vectors were present to spread the pathogen (Bruce, 1978). These findings were in contrast with a 1976 serological survey of small rodents in Alberta which had found no positives (Zarnke & Yuill, 1981), although it was acknowledged that false negatives could have occurred. Ground squirrels were found to be a possible source of transmission of *F. tularensis* in the non-human primate outbreak in 1979, at the APZ in Winnipeg, MB, as bacteria was recovered from multiple organs and fleas of rodents found near the affected exhibit (Nayar et al., 1979).

The role of snowshoe hare in tularemia ecology in Alberta was questioned when very low serological prevalence (two of 1543, 0.13%) was found, but at the time it was unknown whether this was due to the species not playing a role in the ecology of the disease or high susceptibility leading to rapid death without production of measurable titers (Hoff et al., 1970). A couple decades later, a study from Québec showed antibody prevalence in snowshoe hares (0.6% (0.1–2.1%)) and *F. tularensis* DNA was detected in two of the seropositive hares (Gabriele-Rivet et al., 2016). These findings coupled with a negligible prevalence in muskrats (0% (0.0–0.9%))

supported a terrestrial cycle hypothesis for this region (Gabriele-Rivet et al., 2016). The study by Gabriele-Rivet et al. diverged from, not only the Alberta publication by Hoff et al., but also an earlier study that found no seropositive snowshoe hares in Québec (Cayouette, 1993). This highlights the complexity of the pathogen's ecology among various locations and years. Indeed, a good example of such differences can be seen in a 1980's serological survey from PEI and NS, where a prevalence of 1.6% in snowshoe hares was seen in NS, but no positives were found in nearby PEI (Akerman & Embil, 1982).

Since 2006, the CWHC has recorded 27 cases across Canada, with most occurring in beavers, muskrats, or snowshoe hares. In SK, including a white-tailed jackrabbit (*Lepus townsendii*) and an American red squirrel (*Tamiasciurus hudsonicus*) in 2017, three beavers were positive in 2007, one red squirrel in 2008 and another beaver in 2011 (CWHC database, 2021). This is not to say that no other animals were infected in the past 15 years, but that perhaps it is more likely for the disease to be picked up in more "typical" species since the person doing the necropsy is more likely to test for it.

1.2.4 Tularemia Surveys Around the World

Human tularemia cases, either sporadic or as outbreaks, occur worldwide. Examples of countries with well-documented cases include: Spain (Allue et al., 2008; Bellido-Casado et al., 2000), Bulgaria (Kantardjiev et al., 2006; Komitova et al., 2010), Sweden (Christenson, 1984; Dryselius et al., 2019), Germany (Kaysser et al., 2008), Kosovo (Grunow et al. 2012), Ukraine (Zlenko, 2020) and Japan (Ohara, 1998). Surveys are often initiated in response to these occurrences to elucidate the epidemiology and ecology of *F. tularensis* leading to these human cases. Seroprevalence in humans are often tied to occupational activities, such as spending time in the forest for work or leisure (Philip et al., 1962; Jurke et al., 2015). Studies looking at seropositivity in humans in endemic areas or in people engaging in tasks that increase risks of exposure have a wide range of prevalence, from 4% in Germany (Jurke et al., 2015) to 4-17.5% in Alaska (Philip et al., 1962; Hansen et al., 2011). In most studies, human seropositivity prevalence were well correlated with wildlife tested in the same region (Hansen et al., 2011; Gyuranecz et al., 2012; Tomaso et al., in 2018).

Re-emergence of *F. tularensis* around the globe has been well documented in various areas. For example, in Germany, human tularemia had only occurred sporadically since the 1950's and the pathogen was not detected in a survey of European brown hares from 1998-2000 (Frandölich et al., 2003). Human cases spiked between 2004 and 2007, and follow-up studies detected 2.1 to 10.9% prevalence in small rodents (Kaysser et al., 2008), and confirmed *F. tularensis* Type B in lagomorphs (Runge et al., 2011; Müller et al., 2013). These studies lead to the confirmation of re-emergence and the identification of hot spots in the country (Müller et al., 2013). Recent molecular studies have reaffirmed relatively high prevalence (11.5%) in European brown hares (Tomaso et al., 2018), as well as high and novel molecular diversity of *F. tularensis* Type B along with broad host species diversity (Schulze et al., 2016) which supports an established and flourishing *F. tularensis* presence. A study in Spain, found genetic evidence that, at least in some areas, re-emergence of *F. tularensis* could be due to persistent local foci (Ariza-Miguel et al., 2014). Besides re-emergences, increases in the number of cases have been documented in already endemic regions. Examples include Arkansas, USA, where many human tularemia cases have occurred over the years (Boyce, 1975; Taylor et al., 1991; Nelson et al., 2013; Lee-Lewandrowski et al., 2020) recorded 93 cases (33.9% of total cases across the US) in 2019; its highest number of cases in 10 years (Centers for Disease Control and Prevention, 2021). Another example would be the Swedish outbreak in 2019, which was the largest the country had seen in 50 years (Dryselius et al., 2019). Seemingly rising numbers of human tularemia make studies such as a Swedish retrospective study which showed infected bank voles (*Myodes glareolus*) retrieved from owl nest boxes preceded a human outbreak (Ecke et al., 2020), especially valuable, as they grant not only further information on possible species roles, but also potential surveillance methods.

Surveys have been conducted for *F. tularensis* seroprevalence, infection prevalence or both in small rodents in many countries where tularemia is of historical or current concern, with variable results (see Table 1.1.).

Table 1.1. *Francisella tularensis* surveys in small rodents around the world

Publication	Location	Species of small rodent	Type of test	Prevalence
Zlenko et al., 2020	Ukraine (three regions)	Various, positive samples found in house mice, Striped field mice (*Apodemus agrarius*), bank voles, and wood mice (*Sylvaemus sylvaticus*)	PCR	Infection prevalence per regions: 0.4%, 0.9% and 2.6%
Pourhossein et al., 2015	Iran	Indian gerbil (*Tatera indica*)	Serology	Seroprevalence: 11.1%
Mostafavi et al., 2015	Iran	Various, most common genus *Apodemus*, *Mus* and *Meriones*	Serology and PCR	Infection and seroprevalence: 0%
Mostafavi et al., 2018	Iran	Various, positive sample found in *A. witherbyi*, house mouse, and *Chionomys nivalis*	PCR	Infection prevalence: 0.7%
Hemati et al., 2020	Iran	Various, most common species Persian jird (*Meriones persicus*), and Libyan jird (*Meriones libycus*), also those with positive samples (including *M. vinogradovi*)	Serology and PCR	Seroprevalence: 0.7 % Infection prevalence: 1.2%
Jeske et al., 2019	Central Europe	Various, most common species field voles (*Microtus agrestis*), common voles, and bank voles	PCR	Infection prevalence Switzerland: 3% common voles Germany: 2.9% field voles, 1.3% bank voles Czech Republic: 0%
Zhang et al., 2006	China (five regions)	Various, positive sampled found in Korean field mice (*Apodemus peninsulae*), Striped field mice, Eurasian hamster (*Cricetus migratorius*), greater long-tailed hamster (*C. triton*), Siberian chipmunk (*Eutamias sibiricus*), Libyan jirds, and grey-sided voles (*Clethrionomys rufocanus*)	PCR	Infection prevalence per regions: 0%, 1.8%, 6.6%, 10.0% and 11.7%

Publication	Location	Species of small rodent	Type of test	Prevalence
Bártová et al., 2020	Czech Republic	Yellow-necked mice, bank voles, common shrew (*Sorex araneus*), wood mice, striped field mice, common voles and one European mole (*Talpa europaea*)	Serology	Seroprevalence: 7% (Bank voles: 14%)
Christova and Gladnishka, 2005	Bulgaria	Black rats (*Rattus rattus*), house mice, striped field mice	PCR	Infection prevalence: 21.9% (rats: 23.5%, house mice: 20.8%, striped mice: 0%)

Some of these surveys may be difficult to extrapolate to other regions, as the composition and species diversity included in each survey is highly dependent on the regions. For example, some studies reported finding positive jirds (*Meriones* species, Zhang et al., 2006; Hemati et al., 2020), species that are native to the Middle East and Asia, but not Europe or North America. While it is possible for other small native rodents to fill similar niches in other geographical locations, it is likely that variable biological factors make direct extrapolations to local wildlife ill-advised. Some ecological studies have found significant differences in *F. tularensis* prevalence between small rodent species, and sexes (Bártová et al., 2020), or correlations between animal density and natural foci (Pikula et al., 2004). These correlations may be pertinent in understanding which species may play important donor or reservoir roles, and how population densities may influence the persistence of *F. tularensis* in the environment, and alternatively, how *F. tularensis* may affect population densities of certain rodent species.

1.2.5 Tularemia in Mesocarnivores

Cats and Dogs

Although cases of tularemia are reported in both cats and dogs (*Canis familiaris*) (Baldwin et al., 1991; Liles and Burger, 1993; Woods et al., 1998; Kwit et al., 2020; Kittl et al., 2020), it is likely that the disease is still underreported (Meinkoth et al., 2004). The lack of reports could be due to empirical treatment with antibiotics, past diagnostic tests giving

ambiguous results (Baldwin et al., 1991) or that further diagnostics are often only attempted for severe unresolved disease. Infected animals also often receive more thorough diagnostics if there is a potential or confirmed transmission to a human, which has been reported more often from cat-associated contacts than dog (Liles and Burger, 1993; Capellan and Fong, 1993; Larson et al., 2014). Surveys investigating prevalence of tularemia in cats and dogs have found relatively high seroprevalence compared to the number of symptomatic cases seen in veterinary clinics (Leighton et al., 2001; Magnarelli et al., 2007; Roth et al., 2017). Contact with wild rodents or rabbits remain the most common route of exposure for cats and dogs (Woods et al., 1998; Meinkoth et al., 2004; Kwit et al., 2020).

Wildlife

Serological surveys have been performed in several locations to determine possible role of mesocarnivores in various environments that could have different tularemia cycles or ecology.

For example, in North America, opossums (*Didelphis* species, McKeever et al., 1958; Roth, Foley and Wright, 2017), foxes (*Vulpes vulpes*, McKeever et al., 1958), raccoons (*Procyon lotor*, McKeever et al., 1958; Bischof and Rogers, 2005; Berrada, et al., 2006), skunks (*Mephitis mephitis*, McKeever et al., 1958; Berrada et al., 2006), coyotes (Bischof & Rogers, 2005; Gabriele-Rivet et al., 2016) and bears (*Ursus americanus* and *U. arctos*, Binninger et al., 1980; Chomel et al., 1998; Stephenson et al., 2015; Ramey et al., 2019) appear to have high seroprevalence in many populations across the continent. Many of these species have also appeared in case reports as source of transmission to humans (Francis, 1937; Taylor et al., 1991; Chomel et al., 2016). A seroprevalence study by Martin et al. in 1977 at Pike Lake, SK, had one American mink (*Neovison vison)* with measurable agglutinins. Although only four mink were tested, therefore one cannot comment on the statistical significance, it may indicate a high seroprevalence in this species at that location (Martin et al., 1982).

Studies in Sweden and Germany found similar trends in mesocarnivores, whereas antibodies were found in bears (Hestvik et al., 2019), lynxes (*Lynx lynx*, Hestvik et al., 2019), raccoon dogs (*Nyctereutes procyonoides*, Hestvik et al., 2019; Kuehn et al., 2013), foxes (Hestvik et al., 2019; Kuehn et al., 2013; Otto et al., 2014), wild boar (*Sus scrofa*, Hestvik et al., 2019; Kuehn et al., 2013; Otto et al., 2014), wolves (*Canis lupus*, Hestvik et al., 2019) and

wolverines (*Gulo gulo*, Hestvik et al., 2019). The Swedish study concluded that all predator or scavenger species could serve as sentinels in their region (Hestvik et al., 2019), while German studies favored foxes as sentinels (Kuehn et al., 2013; Otto et al., 2014). A large serological survey in Western Iran found a higher seroprevalence in people exposed to foxes (hunting or eating meat) (25%) which was significantly higher than other groups of people (8.65%) (Esmaeili et al., 2014). This finding also appeared to support foxes as playing a key role in various locations. While over-generalization may be tempting to make, when speaking about prevalence of *F. tularensis* antibodies within a population, some caution is warranted. Indeed, seropositivity can be highly location-specific, as a Japanese serological study by Hotta et al. (2012) demonstrates whereas 1.9% of Japanese black bears (*Ursus thibetanus japonicus*) population between 1998-2009 were positive, all of which were collected in the Iwate prefecture.

Tularemia in Other Noteworthy Animal Species

A special note will be made for captive wildlife species (e.g., zoos or research centers), where non-native animals may encounter infected native wildlife or vectors. Several reports exist of seroconversion, disease or both, e.g., hippopotamus (*Hippopotamus amphibius*) at Berlin Zoo (Kuehn et al., 2013), and numerous non-human primates in Europe (Hoelzle et al., 2004; Mätz-Rensing et al., 2007), the United States (Beckwith, 2006; Guthrie et al., 2012) and Canada (Preiksaitis et al., 1979, Nayar et al., 1979). The latter occurred in 1978 at the APZ in MB, where a group of seven individuals were affected, culminating in the death of four monkeys: three tamarins (*Saguinus* species) and one talapoin (*Miopithecus talapoin*) (Preiksaitis et al., 1979, Nayar et al., 1979). A veterinarian suffered a bite from an infected talapoin and developed symptoms consistent with tularemia, which was subsequently confirmed via agglutinins (Preiksaitis et al., 1979). Recovery of the bacterium from rodents and their fleas near the affected exhibit appeared to indicate a potential source for the outbreak (Nayar et al., 1979). Following these events, non-human primates were placed on prophylactic antibiotics and focused rodent control protocols were established in the primate exhibit areas, both endeavours put in place to prevent the recurrence of the disease (C. Berkvens, personal communication, 10-04-2021). Despite these efforts, tularemia was confirmed in a cotton-top tamarin (*Saguinus oedipus*) at the same zoo in 2015, sparking discussion and musing over the ecology of the pathogen in the native

wildlife, with renewed interest in how the organism was maintained in the wild rodent populations found in the zoo and surrounding park.

Another special note will be made concerning livestock species. Although not mentioned frequently in conversations about tularemia, various reports exist about livestock exposure to *F. tularensis,* as well as case reports of tularemia disease in these species, with occasional association with human seropositivity. Examples include a long-term serological survey around Great Salt Lake Desert where 31% of cattle (*Bos taurus*) and 24% of sheep had titers (Thorpe et al., 1965), the first report of the bacterium in Iran in 1973 when eight sheep and three oxen were seropositive (Arata et al., 1973), a 2020 seroprevalence study in Jordan that found a significant correlation between ruminant ownership and human seropositivity (Obaidat et al., 2020), and episodes of abortions and neonatal deaths reported in 1997 and 2007 in sheep flocks in Wyoming and South Dakota (O'Toole et al., 2008).

1.2.6 Mosquitoes

Sweden commonly experiences tularemia outbreaks (Dahlstrand et al., 1971; Payne et al., 2005; Wik, 2006; Dryselius et al., 2019), and unsurprisingly, there are many studies and surveys that have taken place in this country to elucidate reservoir, vectors and any biotic or abiotic environmental risk factors (Eliasson et al., 2002; Eliasson & Bäck, 2007; Hestvik et al., 2019).

Mosquitoes have long been discussed as potential vectors, and in some regions, such as Sweden; their role in transmission has been confirmed through many case reports and studies (Christenson, 1984; Eliasson et al., 2002; Payne et al., 2005; Wik, 2006; Eliasson and Bäck, 2007; Svensson et al., 2009; Rydén et al., 2012). In contrast, studies in other countries with different climates and potentially different tularemia subtypes have either failed to show detectable bacterial DNA in mosquitoes or have shown ambiguous correlation to confirm possible transmission (Hubálek et al., 1996; Hubálek & Halouzka, 1997; Roth et al., 2017). The possibility of detecting the pathogen's DNA within the sample, as demonstrated in the Alaskan mosquito study by Triebenbach et al. (2010), does still give hope that, even if the insect does not hold a vector role in North America, it may be used as a sentinel. The mosquito's non-existent vector role in North America, as per the literature, does have exemptions as there was a

confirmed case of mosquito-transmitted tularemia in Canada (Silverman et al., 1991). Other studies have investigated retention of the bacterium from larvae to adult and found that mosquito larvae exposed to *F. tularensis* bacteria in their natal waters could harbor the pathogen into adulthood (Bäckman et al., 2015; Lundström et al., 2011; Thelaus et al., 2014).

As climatic changes continue, prevalence and distribution of certain arthropods are likely to expand (Hartemink & Takken, 2016; Rydén et al., 2009). Bloodsucking arthropods, such as mosquitoes, have been implicated in emerging or re-emerging diseases, and climatic changes could cause an increase of these diseases, including tularemia, in already endemic areas or introduce them to new ones (Dahmana & Mediannikov, 2020; Nakazawa et al., 2007; Redshaw et al., 2013; Rydén et al., 2009).

1.3 Objectives and Hypotheses

The over-arching objective of this research was to conduct active surveillance over several years and confirm the presence of tularemia and its status (endemic vs non-endemic) in various habitats and settings across SK and MB. To achieve this goal, various ecological aspects were considered in two separate chapters, such as potential reservoir, donor, or recipient species (rodents and lagomorphs) in Chapter 2, and potential sentinels (mesocarnivores and mosquitoes) in Chapter 3. Specific objectives and hypotheses for each section were:

CHAPTER 2 – Presence of Tularemia in Key Rodent and Lagomorph Species

The main goal in this section was to investigate areas where tularemia had been previously diagnosed and in areas where high rodent populations would likely support disease. The study aimed to discover serological or molecular evidence of *F. tularensis* in trapped small rodents, squirrels, and lagomorphs, from both non-urban and urban settings, and in both aquatic and terrestrial habitats in MB and SK. Given the repeated, recent detection of tularemia in some of our study areas we hypothesized:

1) We would detect evidence of tularemia infections in rodent and lagomorph species during active surveillance over an extended period

2) There would be differences in prevalence of seropositivity among rodent and lagomorph species given their differing susceptibility to infection

3) There would be differences in prevalence of infection among urban and non-urban environments (to be assessed if sample sizes are adequate)

CHAPTER 3 – Validity of Mosquitoes and Mesocarnivores as Sentinels for Tularemia

The main goal in this section was to investigate areas where tularemia had been previously diagnosed, focusing on the same locations where rodents and lagomorphs were trapped. The study aimed to discover serological evidence of *F. tularensis* in mesocarnivores or molecular evidence in mosquitoes, from both non-urban and urban settings, and in both aquatic and terrestrial habitat of MB and SK. Given the repeated, recent detection of tularemia in some of our study areas we hypothesized:

1) We would detect evidence of tularemia infections in either or both mesocarnivores and mosquito species during active surveillance over an extended period

2) Mesocarnivores would be good sentinels for current and past tularemia activity in an area, as seen in other geographical locations (Berrada et al., 2006; Otto et al., 2014; Gabriele-Rivet et al., 2016; Hestvik et al., 2019)

3) Mosquitoes would be good sentinels for current or rising tularemia activity in an area, as supported by Triebenbach et al. (2010)

CHAPTER 2. Presence of Tularemia in Key Rodent and Lagomorph Species

2.1 Abstract

Francisella tularensis, the bacterium causing tularemia, is endemic in rodents and lagomorphs in the Canadian Prairies. Recent cases include two wild animals in Saskatoon Saskatchewan (SK) in 2017, and a tamarin at the Assiniboine Park Zoo (APZ), in Winnipeg Manitoba (MB) in 2015. The latter was the second incidence of tularemia being diagnosed in non-human primates at the zoo. Little is known about the disease's ecology in Prairie Canada, hence this study attempted to determine the prevalence, and assess potential candidates for reservoir species in SK and MB.

Rodents and lagomorphs (possible reservoir species) were live trapped across SK (2018-2020) and MB (2018-2019). Locations included non-urban and urban settings, with a mix of terrestrial and aquatic habitats. Animals caught were sampled for blood and ectoparasites, then euthanized for tissue collection or released. A microagglutination test (MAT) was used to determine antibody status, while an in-house polymerase chain reaction (PCR) test was used on tissues to detect *F. tularensis* DNA.

A total of 730 blood samples from rodents and lagomorphs were tested: 12 animals were positive (ten from MB and two from SK, of which nine were Richardson's ground squirrels (RGS), two American red squirrels (ARS) and one prairie vole). A total of 1,036 tissue pools underwent PCR testing. None of the tissues assessed had detectable *F. tularensis* DNA.

Presence of antibodies, along with negative PCR seems to indicate squirrels can be infected and survive. Although all animals with titers had seemingly cleared the infection, there may be individuals that may fail to do so and become chronic carriers (reservoir species). On the other hand, should morbidity within these squirrel species increase they may, in certain conditions, play a recipient (spillover) or donor role in their habitat.

2.2 Introduction

2.2.1 Background on *Francisella tularensis*

Tularemia, a zoonotic disease caused by the bacterium *Francisella tularensis,* is endemic in North America and in many other regions of the northern hemisphere (Farlow et al., 2005). The ecology of the disease is complex, with at least four strains of *F. tularensis* reported in North America with varying pathogenicity, vectors, host range, and geographic distribution (Farlow et al., 2005). The disease garners attention when it causes clinical disease in people or domestic pets (Farlow et al., 2005), although this may be under-reported as most common forms are responsive to antibiotics (Caspar et al., 2018), causes noticeable mortality in wildlife species (Wobeser et al., 2007), or is detected in a new location (Eden et al., 2017; Jackson et al., 2012). Despite the sporadic attention given to tularemia, little is known about how the pathogen is maintained in the wild, what species acts as reservoir hosts in various settings, and its effect on population dynamics.

In North America there are two main subspecies of *F. tularensis*, Type A (subspecies *tularensis*) and Type B (subspecies *holarctica*) (Jellison, 1974). Type A appears to be associated with wild lagomorphs (Farlow et al., 2005). Much of the research on tularemia typing, and vector-host relationships has been done in the US. In Canada, seroprevalence surveys suggests Type A tularemia is endemic to the southern Prairie Provinces (Leighton et al., 2001) and a recent study detected Type A in two hares in Québec (Gabriele-Rivet et al., 2016). Type B tularemia has a Holarctic distribution and appears to be more widely distributed in North America than Type A (Jellison, 1974). It is reportedly associated with aquatic habitats and animals such as muskrats, beavers, and voles as primary hosts (Mörner, 1992). A study in Northern California found that well-vegetated areas with standing water appeared to be ideal habitats for tularemia enzootic persistence, and their findings of seropositivity in feral cats, opossums and rodents support their hypotheses that mesocarnivores may facilitate the spread of *F. tularensis*, and high densities of rodents and their fleas may be a mechanism for amplification and spillover (Roth, Foley and Wright, 2017).

2.2.2 *Francisella tularensis* in Rodents and Lagomorphs

In Europe, tularemia seems to persist in the brown hare and spills over into rodent species during epizootics (Gyuranecz et al., 2011), making the brown hare particularly important as surveillance for environmental risk (Moinet et al., 2016). A recent German study spanning from 2009 to 2014 found 100 positive brown hares (out of 848), bacterial isolates were cultivated from 25 hares, and all belonged to Type B, (Tomaso et al., 2018) also reinforcing the usefulness of hares as a species for surveillance. A comprehensive study on population dynamics during an outbreak suggested that tularemia can cause severe population decimation in small rodents, although the study also concluded that the outbreak self-exhausted in a few months even without antibiotic intervention (Dobay et al., 2015). A study investigating the association of human tularemia and vole population increases in Spain found that the prevalence of *F. tularensis* in common voles increased to 33% during population fluctuation, while the bacterium was not detected when the population density was lower (Rodríguez-Pastor et al., 2017). This could indicate that these small rodents may not play a reservoir role, but an amplification role during population fluctuation, and cause a spillover into the environment. Harvestable species (such as hare), once infected, could come into contact with humans. Their findings confirmed that tularemia prevalence increased as population numbers increased, and that voles act as agents for zoonotic outbreaks (Rodríguez-Pastor et al., 2017). A study in Georgia looked at 57 years of data and found that isolation of *F. tularensis holarctica* was highest in voles and *Dermacentor marginatus* ticks, which could indicate that these species contribute to the persistence of tularemia in the environment (Elashvili et al., 2015). It is unknown whether the roles of small rodents vary depending on geographical location.

A case study from Washington state on 15 tularemia positive gray tree squirrels highlighted the need to consider tularemia in any dead rodent, even when no lesions are seen. Only two of the squirrels had gross lesions, seven simply had microscopic lesions, while the remaining six had no lesions and were only diagnosed by PCR (Nelson et al., 2014). A similar case study, this time in two infected yellow-necked mice, described splenomegaly as the only gross lesion seen in one of the mice, and hepatic necrotic foci appreciated only on histology (Hestvik et al., 2018). Both case studies demonstrate the difference in pathogenicity and presentation of the disease within rodent populations. This highlights the need to learn more

about variability to further our understanding about rodents' potential role as reservoir or transmission to humans or domestic species.

2.2.3 *Francisella tularensis* in SK and MB

Recent reports of tularemia in Western Canada include a white-tailed jackrabbit (WTJR) and ARS in residential parks in Saskatoon, SK, in 2017. In 2015, a cotton-topped tamarin was diagnosed with tularemia at the APZ in Winnipeg, MB. This is the second time tularemia has been diagnosed in non-human primates at the zoo; the earlier occurrence resulted in transmission to a zoo veterinarian (Preiksaitis et al., 1979). In 2005, tularemia was responsible for a widespread die-off of deer mice during a population eruption in southwestern SK. The pathogen's subspecies was determined to be *holarctica*, but the identity of the maintenance host in the area is unknown (Wobeser et al., 2007).

2.2.4 Rationale, Objectives, and Hypotheses

Tularemia is endemic in wildlife species in the Canadian Prairies, diagnosed occasionally as individual cases and rarely as larger die-offs. How the pathogen is maintained in endemic areas, and whether there are low levels of mortality in mammal populations between epidemics, is not known. Recent detection of cases in urban wildlife may be the result of increased detection and submission for necropsy or could be potentially the result of different transmission dynamics between urban and non-urban settings due to differing species composition, differing predator pressure, and altered environments. As climate change continues to progress, shifts may occur in the environment, including shifts in arthropod ranges, population densities of certain species, and state of aquatic habitats; all of which could affect how pathogens are maintained and spread, potentially leading to an increase in water-borne, food-borne and vector borne diseases in many areas of the world, including North America (Harvell, 2002; Nakazawa et al., 2007; Redshaw et al., 2013). Understanding the current status of tularemia within the areas it has been detected could be beneficial in deciding potential need for more pointed surveillance, as has been recommended by several publications (Sagurova et al., 2019). This may be especially important since it has been observed, in other locations, that natural tularemia foci can persist in specific

areas, but are not always stationary, and can occur in other areas with suitable conditions (Pikula, 2003).

Our objectives were to conduct active surveillance over several years in areas where tularemia had been previously diagnosed and in areas where high rodent populations would likely support disease occurrence looking for evidence of its presence through trapping of small rodents, squirrels, and lagomorphs, from both non-urban and urban settings, and in both aquatic and terrestrial habitats in MB and SK. Given the repeated, recent detection of tularemia in some of our study areas we hypothesize:

1) We will detect evidence of tularemia infections in rodent and lagomorph species during active surveillance over an extended period

2) There will be differences in prevalence of seropositivity among rodent and lagomorph species given their differing susceptibility to infection

3) There will be differences in prevalence of infection among urban and non-urban environments (to be assessed if sample sizes are adequate)

2.3 Material and Methods

2.3.1 Research and Animal Care Permits

Provincial trapping permit for SK (Ministry of Environment: 18FW079) and MB (Wildlife and Fisheries: WB21987).

Animal use protocol 20180018 as approved by the U of S Animal Research Ethics Board and biosafety permit VPH-03 for all laboratory procedures.

2.3.2 Study Sites

Samples from rodents, lagomorphs or both were obtained from a total of eight unique locations in SK between 2018 and 2020, of which half were in an urban setting (Saskatoon) and the other half in non-urban settings (Dundurn RM and Grasslands National Park), while samples were obtained from nine unique locations in MB between 2015 and 2020, of which two were

urban park locations, four were non-urban settings (Melita and Hartney area) and three were rural communities. Note that all MB samples available for 2015-2017 and 2020 were tissues only (no sera).

See tables 2.1. and 2.2. for details and figures A.1. and A.2. in the appendix for maps.

Table 2.1. All trapping locations in SK with description of habitat and year available

Location	Specific location	GPS	Description	Years available
U of S campus, Saskatoon	East road, VIDO building	52.1368, -106.6243	Urban terrestrial Fragmented lawns between parking lots and buildings with occasional bushes and trees. Over one km away from SK river	2018, 2019 and 2020
	Seminary area	52.1395, -106.6382	Urban aquatic Grounds near Meewasin trail, fields, and wooden area ~100 to 300 m from SK river	2018, 2019 and 2020
	Sculpture Park	52.1350, -106.6391	Urban aquatic Park near Meewasin trail with lawn and small fragmented wooden areas ~75 to 250 m from SK river	2018, 2019 and 2020
	Agricultural field	52.1475, -106.6300	Urban aquatic Cultured field and surrounding grounds near Meewasin trail ~75 to 300 m from SK river	2019 and 2020
Dundurn RM	Indi Lake	51.7015, -106.5172	Non-urban aquatic (Severe drought receded shoreline substantially) Field with grazing cattle 0 to 200 m from marsh	2018, 2019, and 2020
	Rural farm	51.7395, -106.5026	Non-urban terrestrial Fields with grazing cattle, farm equipment and debris. Natural body of water 500+ m.	2019 and 2020
	Seasonal pond	51.7651, -106.5269	Non-urban aquatic Amidst tall grass fields, thick vegetation and trees surround the pond	2019 and 2020
Grasslands National Park	Frenchman Valley Campground	49.1503, -107.5107	Non-urban terrestrial Low brush vegetation, grassland near campground. Natural body of water 500 m away.	2018*

*Carcasses submitted by Frenchman Valley Campground pest control in 2018

Table 2.2. All trapping locations in MB with description of habitat and year available

Location	Specific location	GPS	Description	Years available
Assiniboine Park, Winnipeg	APZ	49.8696, -97.2429	Urban terrestrial Zoo with exhibits, buildings, and manicured lawn. One artificial pond on premise, several smaller artificial bodies of water scattered throughout, and Assiniboine river ~250 to 700 m from all locations within the zoo	2018, 2019 and 2020*
	Lyric theatre	49.8740, -97.2304	Urban aquatic Open manicured lawns of surrounding park grounds, within ~250 m from Assiniboine river	2016*, 2017*, 2018, 2019 and 2020*
Stonewall	Not available	50.8102, -97.1473	Not available	2015*
Brandon	Not available	49.8423, -99.9620	Not available	2015*
East Selkirk	Not available	50.1394, -96.8394	Not available	2020*
Melita and Hartney area	Farm near Elva	49.2205, -101.1988	Non-urban terrestrial Large open field near farm equipment and buildings, small wooden area on edge of field and two artificial bodies of water within 500 m	2018 and 2019
	Abandoned field near Broomhill	49.4222, -101.1692	Non-urban terrestrial Large grazing field with natural body of water over 500 m away	2018 and 2019
	Field near Broomhill	49.3611, -101.1230	Non-urban aquatic Large open grazing field with abandoned farm buildings with natural bodies of water running through location (all trapping within 200 m)	2018 and 2019
	Feedlot farm near Hartney	49.5610, -100.4639	Non-urban terrestrial Large open cattle grazing field with many buildings on property and natural body of water ~600 m away.	2019

*Carcasses submitted by Assiniboine Park staff in 2015, 2016, 2017 and 2020

2.3.3 Animal Capture and Handling

Trapping at each location was repeated daily, nightly or both until either a) 60 rodents were caught, b) 10 trapping days, nights, or both (approximately two work weeks) were completed or c) population density was extremely low and unlikely to yield satisfactory numbers even with additional trapping time.

Overnight trapping spanned from ~20:00 (up to two hours before sunset) to ~6:30 (up to three hours after sunrise). Day trapping would begin within three hours of sunrise and end either at 14:00 or when ambient temperatures could potentially cause heat stress to trapped animals.

Sherman traps (H.B. Sherman Traps, Inc., Tallahassee, Florida, USA) were used for small rodents (e.g., mice, voles, etc.). Small live traps (Tomahawk Live Trap, Hazelhurst, Wisconsin, USA) or burrow traps (Wobeser and Leighton, 1979) were used for squirrels, while large Tomahawk live traps were used for lagomorphs.

Figure 2.1. Sherman traps for small rodents

Figure 2.2. Burrow trap with RGS, *courtesy of Morgan Kelley*

Once a location was chosen, overnight traps were placed in areas where target species were either spotted, or where habitat appeared favorable. The number and location of each trap types were recorded. When possible, rabbit traps were placed, wired open and baited for a few days prior to active trapping. This process was an attempt to lure any wildlife in the area with bait and habituate them to the traps prior to them being set. Once active trapping began, traps were baited and opened, up to two hours before sunset (weather-dependent) including both small rodents, and rabbit traps. All overnight traps were checked within three hours of sunrise (range of 4:30 to 7:00); any empty traps were closed for the day, any non-target species were released, and all target species caught were processed. Traps were either replaced with fresh clean traps, or if the trap was not soiled; it was placed back on location, if more target species were expected in the area. All traps that were soiled or needing repair were placed in the vehicle for appropriate attention.

While overnight traps were being checked and processed, active day trapping of ground squirrels began. A mix of appropriate traps were set up in or near burrows. Regular checks were made, ranging from every thirty to sixty minutes, depending on density of population, wariness of individuals and ambient temperatures. Traps containing animals were covered with a sheet to provide shade and lower stress caused by nearby workers while processing.

Anesthesia and Field Sample Collection

Lagomorphs were given an intramuscular sedation prior to their removal from the traps, while small rodents and squirrels were manually restrained for masked induction. All animals were anesthetized with isoflurane for physical examination, blood sampling and ectoparasite collection. As much blood as possible was collected from each animal as they were to be subsequently euthanized. Euthanasia was performed using chemical overdose whilst still under anesthesia (see appendix for specific anesthetic, euthanasia, and sampling protocols).

Figure 2.3 RGS masked induction, *courtesy of Morgan Kelley*

Figure 2.4 Blood sampling from anesthetized deer mouse, *courtesy of Morgan Kelley*

2.3.4 Laboratory Protocols

Necropsies, sampling, and tissue storage were done within the Prairie Diagnostic Services (PDS) Saskatoon, SK. The facility is certified containment level 2. Carcasses and subsequent tissues were handled as potentially containing a Risk Group 3 (RG3) pathogen.

Surfaces were disinfected with a 1% bleach solution followed by DNAase between each individual animal. Disposable scalpels and forceps were used and discarded after each carcass.

Individual carcasses were weighed, sexed, and examined for any external abnormalities, then opened for removal of the kidneys, liver, spleen, and lungs. A small piece of each organ was excised, macerated, and mixed to make a pooled sample of roughly 25 mg. Pooled samples were sent for DNA extraction and PCR testing. Remaining tissues were divided in two: one half was frozen and the other fixed in formalin for follow-up histopathology, if needed. Remaining non-target tissues and carcasses were disposed of and clean up completed following protocols for RG3.

Blood collected from the field or provided by a 3rd party was processed, and the sera aliquoted for testing, while any remaining were stored in a -80^{o}C freezer. A microagglutination test (MAT) was performed at PHAC's National Microbiology Laboratory in Winnipeg following a modified protocol as outlined in Sato et al. (1990). A sample was considered positive when titers were ≥ 1:128 (PHAC, 2009).

When appropriate, formalin-fixed tissues including liver, lung, spleen, and kidney were processed through a general trimming protocol and the slide(s) stained with hematoxylin and eosin for histological examination.

An in-house assay was developed and validated to test all available pooled mammal tissues (liver, kidney, spleen, lung) as well as pooled mosquito samples. Gene target *fopA* and *lpnA* were chosen for the assay, based on literature and validation using previously diagnosed individuals. The confirmed positive animals (ARS and WTJR) had frozen tissues available for use as positive control, however due to large amount of testing required, a synthetic gene target for real-time PCR was designed (Sigma), utilizing a *fopA* amplicon generated from gene coding sequences cloned into a vector. This plasmid DNA was subsequently used as positive control. Genomic DNA extractions, DNA level confirmation and real-time PCR were done in-house. All

extracted genomic material from mammal samples were subsequently also evaluated by the National Microbiology Lab (PHAC). A Ct value ≤ 37.0 °C, and valid melt peak and melt temperature was considered positive and confirmed by Sanger sequencing and Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cg).
(See Appendix A for full laboratory protocols).

Statistical calculations

When appropriate, Fisher's exact tests of independence were performed to look for relations between factors (such as urban versus non-urban habitat and sex) that may affect an animal having a positive test result. A result was considered significant if the p value was equal to or less than .05. Incidences and odd ratios were calculated and reported with 95% confidence intervals. All calculations were completed using Epitools (https://epitools.ausvet.com.au).

2.4 Results

2.4.1 Number and Species of Individuals With Samples Available

A total of 1,037 rodents and 16 lagomorphs, sampled between 2015 and 2020, had tissues, sera or both available for testing.

Table 2.3 Demographics, (province, habitats, sex, and age) of sampled rodents and lagomorphs between 2015 and 2020 (N=1,053)

Species	**Province**		**Habitat**							**Sex**			**Age**		
	MB	SK	Urban terrestrial	Urban aquatic	Non-urban terrestrial	Non-urban aquatic	Urban Unknown	Non-urban Unknown	Unknown	Female	Male	Unknown	Adult	Juvenile	Unknown
RGS (N=864)	424	440	336	246	199	83	-	-	-	558	304	2	520	339	5
Deer mouse (N=98)	17	81	20	52	8	17	-	-	1	48	48	2	69	29	-
Voles (N=28)	19	9	1	5	14	6	-	-	2	5	8	15	10	4	14
House mouse (N=25)	24	1	23	1	-	-	-	-	-	5	12	8	6	17	2
Lagomorphs (N=16)	2	14	2	3	-	-	11	-	-	7	3	6	5	4	7
Red squirrel (N=10)	9	1	8	2	-	-	-	-	-	6	4	-	9	1	-
Muskrat (N=5)	3	2	-	3	-	2	-	-	-	1	1	3	1	1	3
Other* (N=7)	5	2	1	-	1	-	-	3	2	3	3	1	5	1	1

*Two northern flying squirrels (*Glaucomys sabrinus)*, two 13-lined ground squirrels (*Ictidomys tridecemlineatus)*, one beaver, one white-footed mouse (*Peromyscus leucopus)* and one northern short-tailed shrew (*Blarina brevicauda)*

2.4.2 Results of Testing

MAT results

Of the 730 rodent and lagomorph sera tested across both provinces between 2018 and 2020*, a total of 12 rodents (1.6% overall prevalence) were found to have measurable antibodies to *F. tularensis*. Of these, the majority (nine out of 12) were RGS, and the most common location was the APZ. The prevalence and confidence interval of species tested were as follows: ARS 22.2% (N = 9, 95% CI 6.3% - 54.7%), voles 16.7% (N = 6, 95% CI 3.0% - 56.4%), RGS 1.4% (N = 633, 95% CI 0.8% - 2.7%), deer mice 0% (N = 69, 95% CI 0% - 5.3%), house mice 0% (N = 2, 95% CI 0% - 65.8%), lagomorphs 0% (N = 9, 95% CI 0% - 29.9%). The sole beaver and 13-lined squirrel available were both negative. The full list of positive rodents, along with their relevant information is found in Table 2.4.

There was no significant difference between seropositivity rates between RGS captured in non-urban or urban environments when samples were pooled across years (N = 633, Fisher's exact test p = 0.496). The urban RGS were 2.1 times more likely to be seropositive than non-urban RGS (OR = 2.1 (95% CI 0.4 - 10.1)), however, as mentioned above, this was not significant. There was no significant difference between adult male and female RGS captured at the APZ (N = 120, Fisher's exact test p = 0.631). Male RGS were 1.6 times more likely to be seropositive than female RGS at the APZ (OR = 1.6 (95% CI 0.3 - 9.3)), however, as mentioned above, this was not significant.

A more detailed breakdown of the serological findings will be discussed in the next section.

*Due to SARS-COV2 travelling restrictions, blood samples were not available for MB in 2020.

Table 2.4. All rodents with measurable antibodies

Year	Location	Date sampled	Species	Titer	Signalment	Physical and gross exam
2018	APZ, MB	May 8, 2018	RGS	1:128	Female adult	small wound under eye, pregnant
	APZ, MB	May 9, 2018	RGS	1:128	Female adult	marked fat deposits, pregnant
	APZ, MB	May 16, 2018	RGS	1:128	Female adult	marked fat deposits, lactating
	U of S, SK	June 25, 2018	RGS	1:128	Female adult	mild-moderate fat deposits
2019	APZ, MB	May 8, 2018	Red squirrel	1:128	Female adult	apparently healthy
	APZ, MB	May 8, 2018	Red squirrel	1:128	Female adult	apparently healthy
	APZ, MB	May 11, 2018	RGS	1:128	Female adult	marked fat deposits
	APZ, MB	May 13, 2018	RGS	1:128	Male adult	scabbed wound over shoulder, moderate fat deposits
	APZ, MB	May 13, 2018	RGS	1:128	Male adult	mild-moderate fat deposits
	Melita, MB	July 4, 2018	RGS	1:128	Male juvenile	moderate-marked fat deposits, respiratory issues during anesthesia
	Melita, MB	July 10, 2018	RGS	1:256	Male juvenile	moderate fat deposits
2020	U of S, SK	July 31, 2020	Vole	1:128	Female adult	late pregnancy, a cuterebra was found subcutaneously on the flank

Table 2.5. All serological results of rodents and lagomorphs (N=730); numerator is # of positives, denominator is total # tested

Locations	Specific location	Species	Year	Month/Day of trapping	Female Adult	Male Adult	Female Juvenile	Male Juvenile	Female unknown age	Male unknown age	Unknown sex juvenile
APZ, MB	In-zoo	RGS (N=55)	2018	May	3/43	0/12	-	-	-	-	-
	Lyric field	RGS (N=5)	2018	May	0/3	0/2	-	-	-	-	-
	In-zoo	RGS (N=47)	2019	May	1/34	2/13	-	-	-	-	-
	Lyric field	RGS (N=13)	2019	May	0/11	0/2	-	-	-	-	-
	In-zoo	ARS (N=9)	2019	May	2/5	0/4	-	-	-	-	-
	In-zoo	Deer mice (N=2)	2019	May	-	-	0/2	-	-	-	-
	In-zoo	House mice (N=1)	2019	May	0/1	-	-	-	-	-	-
	In-zoo	Meadow vole (N=1)	2019	May	-	0/1	-	-	-	-	-
Melita, MB	Farm, field, old barn	RGS (N=62)	2018	July	0/13	0/5	0/23	0/16	0/4	0/1	-
	Farm	RGS (N=12)	2019	July	0/5	0/2	0/2	1/3	-	-	-
	Field	RGS (N=6)	2019	July	0/1	-	0/4	1/1	-	-	-
	Old barn	RGS (N=24)	2019	July	0/1	0/5	0/12	0/6	-	-	-
	Feedlot	RGS (N=19)	2019	July	0/3	0/5	0/4	0/7	-	-	-
	Feedlot	Deer mice (N=2)	2019	July	-	0/2	-	-	-	-	-
	Old barn	Deer mice (N=2)	2019	July	0/1	-	-	0/1	-	-	-
U of S, SK	Park, field near river	RGS (N=13)	2018	June and July	1/7	-	0/3	0/3	-	-	-
	Near VIDO building	RGS (N=31)	2018	June and July	0/18	0/4	0/5	0/4	-	-	-
	Park, field near river	RGS (N=53)	2019	June and August	0/18	0/15	0/12	0/8	-	-	-
	Near VIDO building	RGS (N=65)	2019	June	0/11	0/3	0/34	0/17	-	-	-
	Park, field near river	RGS (N=30)	2020	July	0/15	0/11	0/3	0/1	-	-	-
	Near VIDO building	RGS (N=17)	2020	July	0/11	0/5	0/1	-	-	-	-
	Near VIDO building	Deer mice (N=3)	2019	June	0/2	-	0/1	-	-	-	-
	Park, field near river	Deer mice (N=13)	2019	July and August	0/4	0/5	0/1	0/3	-	-	-
	Park, field near river	Meadow voles (N=2)	2019	July and August	-	0/2	-	-	-	-	-
	Park, field near river	Deer mice (N=20)	2020	July and September 1st	0/12	0/4	0/2	0/2	-	-	-

	Park, field near river	House mice (N=1)	2020	September 1st	-	-	0/1	-	-	-	-
	Park, field near river	Prairie vole (N=1)	2020	July 31st	1/1	-	-	-	-	-	-
	Near VIDO building	Deer mice (N=3)	2020	July	0/1	0/2	-	-	-	-	-
Locations	Specific location	Species	Year	Month/Day of trapping	Female Adult	Male Adult	Female Juvenile	Male Juvenile	Female unknown age	Male unknown age	Unknown sex juvenile
Urban field, SK		RGS (N=64)	2019	June	0/10	0/1	0/20	0/33	-	-	-
		RGS (N=4)	2020	July	0/2	0/2	-	-	-	-	-
		Prairie vole (N=1)	2019	June	0/1	-	-	-	-	-	-
		Deer mice (N=3)	2019	June	0/1	-	-	0/2	-	-	-
		Deer mice (N=7)	2020	July	0/2	0/3	0/1	0/1	-	-	-
Indi area, SK	Indi Lake	RGS (N=24)	2018	May-July*	0/17	0/6	0/1	-	-	-	-
	Indi Lake	RGS (N=6)	2019	May and June	0/1	0/1	0/2	0/2	-	-	-
	Indi Lake	RGS (N=8)	2020	July	0/2	0/3	0/2	0/1	-	-	-
	Indi Lake	Deer mice (N=3)	2020	July	0/1	0/2	-	-	-	-	-
	Farm	RGS (N=66)*	2019	May and June	0/15	0/1	0/27	0/22	-	-	0/1
	Farm	RGS (N=8)	2020	July	0/1	0/6	-	0/1	-	-	-
	Farm	Deer mice (N=1)	2020	July	0/1	-	-	-	-	-	-
	Seasonal pond	Deer mice (N=9)	2019	September	0/2	0/5	-	0/2	-	-	-
	Seasonal pond	Meadow vole (N=1)	2019	September	-	-	-	0/1	-	-	-
	Seasonal pond	Deer mice (N=1)	2020	July	-	0/1	-	-	-	-	-
Grandora, SK	Sandyridge gas bar	13-Lined squirrel (N=1)	2018	July	-	-	-	0/1	-	-	-
Saskatoon, SK	Urban park	RGS (N=1)	2019	June	-	0/1	-	-	-	-	-
	Urban areas	W-T JR (N=6)	2019	May-October	0/1	0/2	0/3	-	-	-	-
Unknown SK (likely Saskatoon)		W-T JR (N=2)	2019	June	-	-	0/1	-	-	-	0/1
		W-T JR (N=1)	2020	Summer	1 animal of unknown sex and unknown age						
Unknown SK		Beaver (N=1)	2020	Summer	1 animal of unknown sex and unknown age						

*most animals were caught in late May or early June

PCR Results and Positive Controls Information

None of the 1,036 tissue pools tested had detectable *F. tularensis* DNA. All in-house PCR results were confirmed by the National Microbiology Lab (PHAC). The information on the two positive cases used as controls is included below.

Tularemia Cases in 2017

I. A young-of-the-year male ARS weighing 107.3g was found with an injured leg and tail at a golf course in Saskatoon, SK, in mid-June 2017. The animal was dead on arrival to the wildlife clinic and submitted to the CWHC. On necropsy, the only abnormal finding was a large, dark, and firm spleen. On histology, there were necrotic foci in the liver, spleen, and lymph node. The slide was positive on immunohistochemistry for *F. tularensis*. The National Microbiology Lab (PHAC) confirmed the diagnosis and subtype as *holarctica.* Tissues from this animal were used as positive control for the present study, and continuously yielded adequate Ct values.

II. An adult lactating female WTJR weighing 4.1kg was found unable to walk in a residential park in Saskatoon, SK, in early-May 2017. The animal was brought to the wildlife clinic where the lagomorph was found to be disoriented and unable to stand, although no fractures could be palpated or visualized on radiographs. The animal was euthanized due to poor response to treatment and submitted to the CWHC. On necropsy, notable findings included multifocal hepatic and splenic necrosis. *F. tularensis holarctica* was isolated from the tissues by the National Microbiology Lab (PHAC). Tissues from this animal were used as positive control for the present study, and continuously yielded adequate Ct values.

2.5 Discussion

Although no active tularemia was detected during this study, multiple rodents across various habitats and both provinces were found to have measurable antibodies to *F. tularensis*, confirming our first hypothesis that evidence of tularemia activity would be detected. Five locations across the two provinces were trapped intensively and of these three had evidence of seropositive rodents supporting the endemic nature of this disease on the prairies. Given the low

prevalence of seropositivity, small samples sizes may have prevented detection of tularemia at the two negative sites.

Small sample sizes also hindered statistical comparisons among certain species. Although a total of 1,053 individuals from 14 different rodent and lagomorph species were sampled, RGS accounted for the majority (864 individuals), leaving the remaining 13 species with insufficient samples for calculations. Due to this, comparison between species to address the second hypothesis was not possible. However, findings qualitatively appeared to be consistent with our hypothesis that seroprevalence would vary among species. Of the populations tested by MAT, 1.4% (N = 633, 95% CI 0.8% - 2.7%) of RGS, 22.2% (N = 9, 95% CI 6.3% - 54.7%) of ARS, and 16.7% (N = 6, 95% CI 3.0% - 56.4%) of voles were serologically positive for *F. tularensis* while none of the deer mice (N = 69, 95% CI 0% - 5.3%), house mice (N = 2, 95% CI 0% - 65.8%), lagomorphs (N = 9, 95% CI 0% - 29.9%) or sole beaver and 13-lined squirrel had detectable *F. tularensis* antibodies or DNA. A detailed discussion will be presented about each group.

2.5.1 Squirrels

The presence of antibodies in 1.4% (N = 633, 95% CI 0.8% - 2.7%) of RGS tested, with the majority in MB, along with a negative PCR indicates these populations were exposed, at some point, to *F. tularensis*, and at least a few individuals did not succumb to the bacterium. This finding mirrors those seen in previous case studies published on prairie dogs in the United States (Petersen et al., 2004; Cherry et al., 2019) and an older survey on ground squirrels in Alberta, Canada (Bruce, 1978).

While most serologically positive animals were adult female RGSs (five), it was also true that they made up the majority of caught animals in most locations. Statistical analysis of females and males serostatus at the APZ for 2018 and 2019 had no statistical significance. The observed skewed sex ratio is likely in part due to the difference in lifespan between males (one to three years) and females (three to four years) of this species (Michener, 1989; Reimer, 2018). At the APZ in 2018, three (of 43) females trapped within the zoo were serologically positive, while none (of the 12) males had titers.

Considering the time of year, it is highly likely that the positive RGS were exposed the previous summer, prior to hibernation, or any previous seasons since it was not possible to accurately age these rodents. A previous publication discussed the possibility of active disease resurging post-hibernation in exposed RGS (Bruce, 1978). The resulting bacteremia, which would last until the animal cleared the pathogen or died, could serve as reservoir for arthropod vectors or infection to other mammals (Bruce, 1978). Although titers were indeed found in some RGS in spring, the lack of detectable DNA in tissue pools would seem to indicate there were no pathogens present for re-activation, which Bruce (1978) had alluded to. This could either be due to the exposure having been long cleared, an inadequate level of detectable DNA from dilution of pooled samples, or the wrong organ having been sampled. Although Gabriele et al. (2016) found certain animals in their study, namely the snowshoe hare, had detectable DNA in individual organs but not pooled samples, it is highly likely in the cases of the individuals tested in this study that the pathogen had been cleared.

On another note, all seropositive squirrels, including RGS and ARS, had titres of 1:128 (eight RGS and two ARS) or 1:256 (one RGS). A serological survey done in black-tailed prairie dogs showed a range of titres, from 1:128 to 1:4,096 (Petersen et al., 2004). If we consider black-tailed prairie dogs and RGS are closely enough related phylogenetically (Harrison et al., 2003), their immune response and ability to develop robust titres should be similar. With this principle in mind, the lack of variability found in our study, which is in contrast with Petersen et al. (2004), may be due to timing of sampling post-infection. Unlike Petersen at al. (2004) who were able to do serial sampling within a colony that was experiencing an active tularemia outbreak, our study was only able to sample individuals once and the local population over approximately 10 days, thus making it impossible to follow-up and observe titre changes in individuals or populations. Cherry et al. (2019) who conducted a serosurvey in black-tailed prairie dogs' post-outbreak, only found one individual (2% prevalence) with a titre of 1:256. This post-epizootic setting and its finding of a low prevalence with relatively lower titre appears consistent with our own findings.

Although we hypothesized there would be differences in seroprevalence among different age-sex classes we were unable to detect differences, likely due to small sample sizes. In late June, of 2018, at the U of S campus, one adult female (out of 7) had titers while the other six

RGS tested (three males and three females, all juveniles) had no detectable antibodies. No adult males were caught at that location in 2018. In contrast, there were proportionally more males in the APZ in May 2019 that had titers (two out of 13) compared to females (one out of 34). Similarly, two young males in the Melita area in July 2019 had titers, and since only a few young males were caught at each specific location; these positives were a substantial proportion of tested individual (one out of three, and one out of one). As stated previously seropositives detected in May likely indicated these animals were exposed prior to hibernation; however, finding titers in juveniles suggests the disease had circulated relatively recently in these locations.

With a larger sample size, differences in seroprevalence among sex-age classes would be expected given the social behavior of RGS. Female RGS frequently co-exist with other female kin, such as mother-daughters or sisters (Michener, 1979), and this closeness may increase the likelihood of infected blood-sucking arthropods, such as fleas, to circulate within the social group. Males are solitary and aggressive towards other squirrels (Reimer, 2018), which reduces contact time with conspecifics, thus decreasing transmission risks that require sustained interaction. Blood-sucking arthropods, such as ticks, also likely play a role in transmission. As well RGS are one of the few rodent species that will eat carrion, including conspecifics (Reimer, 2018), and although not investigated previously, consuming carcasses dead of tularemia may also be a route of transmission. Although home ranges often only span 20-40m, the size fluctuates with season, age, and sex (Michener, 1977), and dispersal of the juveniles, especially males, has been known to span up to 10 km (Reimer, 2018). This dispersion behavior could contribute to pathogen movement within the environment, influencing the metapopulation dynamics in both non-urban and urban settings.

ARS, often seen sharing the same urban locations as the RGS, could potentially also play a similar role in the ecology of tularemia in these milieus, as some of the individuals sampled at the APZ also had titers, which accounted for 22.2% (N = 9, 95% CI 6.3% - 54.7%) of ARS tested. Since these positive individuals at the zoo were released, tissues were not collected and tested by PCR, making it impossible to determine if the squirrels were carrying an active infection at time of sampling. While tree squirrels have been known to eat meat (Burt, 1972; Rubin 2012), they prefer seeds and nuts when plentiful, and it is likely that an urban park and

zoo habitat would have bountiful food where consumption of a diseased or dead animal would be less likely. It is more likely that exposure would occur via vectors. The home range of a red squirrel can be 2-20 times larger than that of a ground squirrel (Rubin 2012). An infected tree squirrel could potentially move the pathogen much further in comparison to a ground squirrel. Both squirrel species have been noted to fall prey to skunks, raccoons, mustelids, foxes, and the occasional domestic cat (Burt, 1972; Saunders, 1988; Reimer 2018). Since both squirrel species at the APZ had titers and had either appeared to have cleared the infection previously or had unconfirmed disease status, also considering the previous finding of an infected ground squirrel and its fleas (Nayar et al., 1979); it would appear that they play at the very least, a ***donor role*** at this location. Depending on the status of the population, individual variations and other factors, these species may play a ***recipient role*** where the circulating pathogen is amplified, and the risk of transmission to other species (e.g., zoo animals or humans) is increased. On the other hand, if individuals are infected and unable to clear the bacterium, for any number of reasons, they may become chronic carriers and become ***reservoirs*** in an endemic focus, either during interepidemic or epizootic periods, the latter which was perhaps the case in 1978 (Preiksatis et al., 1979).

Another consideration arising from these findings is the potential use of squirrels for surveillance purposes, maybe even more so in locations where tularemia is believed to be endemic. As indicated in the methods section, ground squirrels were provided in part by pest control endeavours, as these animals often reside on lands that are used by humans for recreational purposes (e.g., parks, campgrounds, zoo, etc.) and their presence can cause conflict due to direct human-animal contact and unsightly or costly damages to grounds. Consistent access to carcasses from pest control agencies, could potentially be a reliable source for either tissue (if looking for traces of *F. tularensis* DNA), blood or extracted sera (if looking for antibodies). Furthermore, there should be thorough histological assessment, PCR testing or both in rodents that are found moribund or dead in SK or MB for unexplained reasons, since there may not be typical gross lesions at necropsy, as seen in the 2017 ARS case in Saskatoon.

2.5.2 Small Rodents

Voles

Although the sample size of voles was low (six sera available and 22 tissue pools), one pregnant adult female prairie vole (*Microtus ochrogaster*), caught on U of S campus in 2020 had measurable antibodies but did not have detectable *F. tularensis* DNA, making the prevalence 16.7% (N =6, 95% CI 3.0% - 56.4%) overall among the population across both provinces and species (prairie and meadow voles). Voles have been reported to play a role in tularemia ecology in other publications, either as ***reservoirs*** (Glass, 1948; Mörner, 1992) or ***recipient*** (Rodríguez-Pastor et al., 2017). Taking into consideration voles' roles in other geographical locations, it is possible that voles found in Prairie Canada could fulfill similar niches. The seropositive animal was in apparent good health and body condition, which may suggest the rodent was either not taxed by the bacterium while infected or the infection had been cleared for a while and the animal had fully recuperated. Even so, considering the average prairie vole will live less than one year in the wild (VanderLinden, 2002) and that the home range is assumed less than 1,000sq.m (Kurta, 1995); the positive rodent was likely exposed earlier in 2020 where it was caught, or nearby. The area in question was an approximately 225sq.m pile of rubble from which various small rodents (prairie voles, meadow voles, deer mice and house mouse) were captured in 2019 and 2020, as well as sightings of short-tailed weasels (*Mustela erminea)*. This pile of rock was near grass fields, in which RGS were trapped yearly, approximately 50m from the Meewasin trail and a wooded area where skunk, raccoon and WTJR were either observed or trapped, as well as being less than 200m from the SK river. Considering the previously positive RGS in this area in 2018; it may indicate either an endemic focus with low prevalence or coincidental sporadic cases. Since the preferred habitat of prairies voles appears to overlap with that of the RGS (VanderLinden, 2002; Reimer, 2018), exposure of either species to *F. tularensis* could transfer to the other either via arthropods, water, or carrion ingestion by the RGS. The presence of antibody in a vole, indicating survival following exposure, is not overly surprising, as previous publications have found seroconversions in voles, either experimentally or naturally (Olsufjev, 1984; Bártová et al., 2020). The presence of a seroconverted individual coupled with previous experiments with voles showing they were capable of becoming chronic carriers and shedding bacteria in their urine (Bell and Stewart, 1975; Bell and Stewart, 1983; Olsufjev, 1984) would warrant further investigation to improve sample size in order to narrow the confidence

interval as well as to elucidate whether this rodent species may play a ***donor or reservoir*** role in Prairie Canada. Prairie voles seem to have a more social nature compared to meadow voles, which may increase the likelihood of *F. tularensis* transmission within the population (VanderLinden 2002; Rowe 2017).

Mice

None of the 124 mice or the single shrew had detectable antibodies or *F. tularensis* DNA. The seroprevalence and confidence interval of the two most common species caught were 0% (N = 69, 95% CI 0% - 5.3%) in deer mice, and 0% (N = 2, 95% CI 0% - 65.8%) in house mice. While sample size hindered both the confidence interval and the ability to make significant comparisons, low to no prevalence was expected since there had been no large morbidities and mortalities, which often accompanies a tularemia outbreak in these species. The absence of titers in these species could be due to one or more of the following: an inadequate number of individuals were sampled, sick or recovering animal were unlikely to enter the traps, small rodents' short lifespan made it less likely to capture an individual with titers, or these small rodents can die acutely once infected and therefore do not develop titers. The latter is consistent with a previous publication by Wobeser et al. (2007). The positive vole on U of S grounds in 2020 potentially came in contact with these highly susceptible species (e.g., deer mice), as 11 deer mice were also trapped in the same pile of rubbles that week, and another three deer mice and one house mouse a month later. As none of these mice were positive on serology or PCR, this may indicate previously exposed mice did not survive or the local population was low enough for minimal transmission, leading to a lower morbidity.

2.5.3 Lagomorphs

None of the 16 lagomorphs (two eastern cottontails, *Sylvilagus floridanus,* and 14 WTJR) assessed had detectable antibodies or *F. tularensis* DNA. The seroprevalence for WTJR was 0% (N = 9, 95% CI 0% - 29.9%). Due to the small number of animals tested, it is not possible at this time to rule out a potential contribution in the tularemia ecology in Prairie Canada, especially considering their historical role (McNabb, 1930; Black and Thomson, 1958), role in North America (Hayes et al., 2002; Farlow et al., 2005; Staples et al., 2006), role in other parts of the

world (Gürcan et al., 2006; Lang and Kleines, 2012; Mailles and Vaillant, 2014; Otto et al., 2015) and the diagnosis of tularemia in a Saskatoon WTJR in 2017. Two eastern cottontail carcasses from the APZ in 2018 and 2019 were tested, the same years multiple RGS and ARS had titers for the pathogen. Since there were no sera available for either lagomorphs, failing to recover *F. tularensis* DNA may indicate either an exposure that was cleared, or these individuals were not exposed to the positive rodents sharing their habitat. All other tested lagomorphs were from SK. It is possible that the 14 WTJR were too low a number to detect a low prevalence of circulating *F. tularensis*, that the disease occurs only sporadically in the locations visited, or that this specific lagomorph species does not contribute substantially to the ecology of tularemia at these locations. The latter would be consistent with an aquatic or *F. tularensis* subspecies *holarctica* cycle, although further investigation with a larger sample size would be required to confirm this hypothesis.

2.5.4 Urban Versus Non-urban Comparisons

We found no statistical difference between the prevalence of seropositivity in RGS between urban and non-urban settings, but this result has limitations and biases. Unfortunately, due to low sample sizes of other rodent species statistical comparisons could not be made. The presence of animals with antibodies appeared to vary spatially and temporally. Statistical analyses for RGS showed a significant difference between provinces in 2019, more specifically between both cities sampled that year (Saskatoon versus Winnipeg). No other analyzes, including other years or locations, were significant for RGS, although these findings may be limited due to biases and location sample sizes. Qualitative observations were made about locations' prevalence over the course of the study, such as several positive rodents every year (e.g., APZ), sporadic and sparse number of positives (e.g., U of S campus and Melita) or none detected at all (e.g., urban agricultural field and Dundurn RM). These findings partially support the first hypothesis: evidence of *F. tularensis* infections in rodent species was detected in many of the locations, although a few of these locations had no positive rodents and positive rabbits were not found in any of the locations.

2.5.5 Conclusions

This study found evidence of *F. tularensis* exposure in rodents from multiple locations, confirming the presence of *F. tularensis*. Furthermore, spatiotemporal pattern of these serological positives appears to indicate that there may be a natural endemic focus at the APZ, while a low prevalence or sporadic activity may account for the seropositive animals seen at the U of S campus and southern rural MB locations.

Taking into consideration that all but one serologically positive individual were squirrels, there is some evidence to support RGS and ARS as having at the very least a ***recipient*** role, but they may have the potential for a ***donor or reservoir*** role in the right conditions, as has been previously reported in prairie dogs (Petersen, 2004). Further investigation into ectoparasites (vectors) for assessing risk of transmission, may be warranted.

While voles have been hypothesized or shown to play various ecological roles in other geographical locations, their role could not be determined in this study due to low sample numbers. However, considering it was nonetheless possible to find one vole with measurable antibodies, they may play a role similar to squirrels.

The habitat preference of all positive rodents (see table A.2 for species information) did appear to overlap at the locations they were sampled; urban fields or rural pastures with nearby mixed wooded areas (except one location in rural MB). This may be purely coincidental or may indicate a habitat where the pathogen is more likely to circulate. While the location on the U of S campus where a seropositive animal was found in 2018 and 2020 was approximately 200 m from a large body of water, all positives in MB were 350 m to over one km away from any natural body of water. Though there was not enough data to analyze these findings, it appears there may be aquatic versus terrestrial difference between SK and MB, or perhaps the proximity to water may not be a factor for endemicity or presence of *F. tularensis* at the locations visited. Further investigation of rodents utilizing aquatic habitats (voles, muskrats, and beavers) would likely help create a better understanding of *F. tularensis*' ecology in both provinces. non-urbanAlthough statistical significance was not found, a qualitative comment can be made that 10 of the 12 positive rodents were in urban settings. This further breaks down into two positive urban rodents in SK versus none in rural SK (2018-2020), and eight positive urban rodents in MB versus two in rural MB (2018-2019). These findings appear to suggest a higher prevalence

in urban habitats, although they may be purely coincidental, or situational. However, should this setting difference be real, it may be attributed to potentially higher population density and diversity in these fragmented habitats scattered between buildings and other human-made barriers.

CHAPTER 3. Validity of Mosquitoes and Mesocarnivores as Sentinels for Tularemia

3.1 Abstract

Francisella tularensis, the bacterium causing tularemia, is endemic in rodents and lagomorphs in the Canadian Prairies. Recent cases include two animals in Saskatoon Saskatchewan (SK) in 2017, and a tamarin at the Assiniboine Park Zoo (APZ), in Winnipeg Manitoba (MB) in 2015. The latter was the second incidence of tularemia being diagnosed in non-human primates at the zoo. Little is known of the ecology of this disease in Prairie Canada. This study attempted to determine tularemia's temporal and spatial prevalence in mesocarnivores and to assess which species would be best for detecting tularemia activity in an area (sentinel species) and to compare it to mosquitoes which have been reported elsewhere as effective sentinels.

Mesocarnivores were trapped at the same locations as rodents and lagomorphs (see chapter 2), and additionally at other locations of interest, from 2018 to 2020. Any animals caught were sampled for blood and ectoparasites. Meanwhile, mosquitoes (possible sentinels or vectors) were also collected from these same locations, but also supplemented by local mosquito programs. A microagglutination (MAT) test was used to determine antibody status of mesocarnivores, while in-house PCR was used on mesocarnivore tissues and mosquitoes to detect *F. tularensis* DNA.

A total of 29 blood samples were tested, and four animals were positive (two from MB and two from SK); including two skunks, one mink and a raccoon. A total of 105 mosquito pools were tested, and all were negative.

Although the number of mesocarnivore sera available was low, each year at least one animal had *F. tularensis* antibodies. This supports endemic presence of the pathogen in wildlife populations and the hypothesis that mesocarnivores could be good sentinels for tularemia activity. In contrast, none of the mosquito samples tested yielded positive results. While it was not possible to rule out mosquitoes as potential vectors, it seems they are not useful sentinels in locations where *F. tularensis* prevalence is low.

3.2 Introduction

3.2.1 Background on *Francisella tularensis*

Tularemia, a zoonotic disease caused by the bacterium *Francisella tularensis,* is endemic in North America and in many other regions of the northern hemisphere (Farlow et al., 2005). The ecology of the disease is complex, with at least four strains of *F. tularensis* reported in North America with varying pathogenicity, vectors, host range, and geographic distribution (Farlow et al., 2005). The disease garners attention when it causes clinical disease in people or domestic pets (Farlow et al., 2005), although this may be under-reported as most common forms are responsive to antibiotics (Caspar et al., 2018), causes noticeable mortality in wildlife species (Wobeser et al., 2007), or is detected in a new location (Eden et al., 2017; Jackson et al., 2012). Despite the sporadic attention given to tularemia, little is known about how the pathogen is maintained in the wild, what species acts as reservoir hosts in various settings, and its effect on population dynamics.

In North America there are two main subspecies of *F. tularensis*, Type A (subspecies *tularensis*) and Type B (subspecies *holarctica*) (Jellison, 1974). Type A appears to be associated with wild lagomorphs (Farlow et al., 2005). Much of the research on tularemia typing, and vector-host relationships has been done in the US (Farlow et al., 2005). In Canada, seroprevalence surveys suggests Type A tularemia is endemic to the southern Prairie Provinces (Leighton et al., 2001) and a recent study detected Type A in two hares in Québec (Gabriele-Rivet et al., 2016). Type B tularemia has a Holarctic distribution and appears to be more widely distributed in North America than Type A (Jellison, 1974). It is reportedly associated with aquatic habitats and animals such as muskrats, beavers, and voles as primary hosts (Mörner, 1992). A study in Northern California found that well-vegetated areas with standing water appeared to be ideal habitats for tularemia enzootic persistence, and their findings of seropositivity in feral cats, opossums and rodents support their hypotheses that mesocarnivores may facilitate the spread of *F. tularensis*, and high densities of rodents and their fleas may be a mechanism for amplification and spillover (Roth, Foley and Wright, 2017).

3.2.2 *Francisella tularensis* in Mesocarnivores

A study in Québec found that coyotes had a higher seroprevalence (2.9%) than muskrats (0%) which would seem to suggest a terrestrial pattern consistent with Type A, (Gabriele-Rivet et al., 2016) but also worth noting is the potential for a longer-lived carnivore to act as a sentinel for the area. Indeed, a study in Massachusetts found that skunks and raccoons were frequently seroreactive, whereas white-footed mice, cottontail rabbits, deer, and rats were not (Berrada et al., 2006), making mesocarnivores good candidates for serological surveillance.

3.2.3 *Francisella tularensis* in Mosquitoes

Another possible valuable tool for tularemia surveillance may be mosquitoes, as an Alaskan study found that *F. tularensis* DNA could be detected in pools of these insects, although their role as vectors was uncertain. (Triebenbach et al., 2010). Mosquitoes have also been found to be important vectors of tularemia infection in other parts of the world, such as in Sweden where numerous surveys and case studies have established their role (Christenson, 1984; Eliasson et al., 2002; Payne et al., 2005; Wik, 2006; Eliasson and Bäck, 2007; Svensson et al., 2009; Rydén et al., 2012). Although the insect's role in *F. tularensis* ecology in North America is not established, there have been sporadic cases of mosquito-transmitted tularemia in Canada (Silverman et al., 1991). As climate changes continue, prevalence and distribution of certain arthropods are likely to expand (Rydén et al., 2009; Hartemink and Takken, 2016). Bloodsucking arthropods, such as mosquitoes, have been implicated in emerging or re-emerging diseases, such as tularemia, and climatic changes could cause an increase of these diseases in already endemic areas or introduce them to new regions (Nakazawa et al., 2007; Rydén et al., 2009; Redshaw et al., 2013; Dahmana and Mediannikov, 2020).

3.2.4 *Francisella tularensis* in SK and MB

Recent reports of tularemia in Western Canada include a white-tailed jackrabbit (WTJR) and American red squirrel (ARS) in residential parks in Saskatoon in 2017. In 2015, a cotton-topped tamarin was diagnosed with tularemia at the APZ in Winnipeg. This is the second time tularemia has been diagnosed in non-human primates at the zoo; the earlier occurrence resulted in transmission to a zoo veterinarian (Preiksaitis et al., 1979). In 2005, tularemia was responsible

for a large widespread die-off of deer mice during a population eruption of this species in southwestern SK. The subspecies was determined to be holarctica, but the origin of the outbreak was not determined (Wobeser et al., 2007).

3.2.5 Rationale, Objectives, and Hypotheses

How the pathogen is maintained in endemic areas in the Canadian Prairies, and whether there are low levels of mortality in mammal populations between epidemics is not known. Urban wildlife may increase the risk of transmission to pets and people, and transmission dynamics are likely to be different between urban and non-urban settings due to different species composition, differing predator pressure and altered environments. As climate changes continue to progress, shifts may occur in the environment, including shifts in arthropod ranges, population densities of certain species, and state of aquatic habitats, all of which could affect how pathogens are maintained and spread, potentially leading to an increase in water-borne, food-borne and vector borne diseases in many areas of the world, including North America (Harvell et al., 2002; Relman et al., 2008; Nakazawa et al., 2007; Redshaw et al., 2013). Understanding the current status of tularemia within the areas it has been detected could be beneficial in deciding potential need for more pointed surveillance, as has been recommended by several publications (Sagurova et al., 2019). This may be especially important since it has been observed, in other locations, that natural tularemia foci can persist in specific areas, but are not always stationary, and can occur in other areas with suitable conditions (Pikula, 2003).

Our objectives were to conduct active surveillance over several years in areas where tularemia had been previously diagnosed and in areas where high rodent populations would likely support disease occurrence. The study aimed to look for evidence of tularemia presence through trapping of mesocarnivores and mosquitoes, from both non-urban and urban settings, and in both aquatic and terrestrial habitat of MB and SK. Given the repeated, recent detection of tularemia in some of our study areas we hypothesized:

1) We will detect evidence of tularemia infections in either or both mesocarnivores and mosquito species during active surveillance over an extended period

2) Mesocarnivores will be good sentinels for current and past tularemia activity in an area, as seen in other geographical locations (Berrada et al., 2006; Otto et al., 2014; Gabriele-Rivet et al., 2016; Hestvik et al., 2019)

3) Mosquitoes will be good sentinels for current or rising tularemia activity in an area, as supported by Triebenbach et al. (2010)

3.3 Materials and Methods

3.3.1 Research and Animal Care Permits

Provincial trapping permit for SK (Ministry of Environment: 18FW079) and MB (Wildlife and Fisheries: WB21987).

Animal use protocol 20180018 as approved by the U of S Animal Research Ethics Board.

Biosafety permit for all laboratory procedures: VPH-03.

3.3.2 Study Sites

Trapping for mesocarnivores, mosquitoes or both occurred at a total of 19 unique locations in SK between 2018 and 2020, of which 14 were in an urban setting (Saskatoon) and the other five were in non-urban settings (Dundurn RM, Corman Park RM and Yorkton). Trapping for mesocarnivores, mosquitoes or both occurred at a total of seven unique locations in MB between 2016 and 2020, of which three were urban locations (Winnipeg), and the other four were in non-urban settings (Melita and Hartney area). Note that MB samples available for 2016 were tissues only (no sera).

See tables 3.1. and 3.2. for details and figures A.1. and A.2. in the appendix for maps.

Table 3.1. All SK locations of mesocarnivore trapping and known locations of blood, tissues, or both from third party submission

Location	Specific location	GPS	Description	Years trapped
U of S campus, Saskatoon	East road, VIDO building	52.1368, -106.6243	Urban terrestrial Fragmented lawns between parking lots and buildings with occasional bushes and trees	2018, 2019 and 2020
	Seminary area	52.1395, -106.6382	Urban aquatic Grounds near Meewasin trail, fields, and wooden area ~100 to 300 m from SK river	2018, 2019 and 2020
	Sculpture Park	52.1350, -106.6391	Urban aquatic Lawn and small fragmented wood areas near Meewasin trail ~75 to 250 m from SK river	2018, 2019 and 2020
	Agricultural field	52.1475, -106.6300	Urban aquatic Cultured field and surrounding grounds near Meewasin trail ~75 to 300 m from SK river	2019 and 2020
	Beef research station	52.1534, -106.6203	Urban terrestrial Grounds near farm buildings, and ~525 m from the SK River	2018
Meewasin Valley properties, Saskatoon	Northeast Swale	52.1714, -106.5855	Urban terrestrial Mostly sparse wood areas, low brush vegetation, over 200 m from frozen body of waters	2019
	Natural Grasslands	52.1638, -106.5914	Urban terrestrial Sparse wood areas and low brush vegetations, only nearby natural body of water ~450 m	2019
Diefenbaker Park, Saskatoon	Along SK river	52.0979, -106.6894	Urban aquatic Large park along the SK River, mix of wooden area, open field and manicured lawn, trapping occurred exclusively in tree canopy covers, distance from river ~10-100 m	2019 and 2020

Location	Specific location	GPS	Description	Years trapped
Dundurn RM, SK	Indi Lake	51.7015, -106.5172	Non-urban aquatic* Field with grazing cattle 0 to 200 m from marsh (Severe drought receded shoreline substantially)	2018, 2019, and 2020
	Rural farm	51.7395, -106.5026	Non-urban terrestrial Fields with grazing cattle, farm equipment and debris	2019 and 2020
	Seasonal pond	51.7651, -106.5269	Non-urban aquatic Amidst tall grass fields, thick vegetation and trees surround the pond	2019 and 2020
Corman Park RM, SK	Private rural property	52.0454, -106.8157	Urban terrestrial Farm property with paddocks, pasture, and buildings, no natural body of water	2019
Yorkton, SK	Unknown	Unknown	Likely non-urban terrestrial. Animal found on side of highway and brough to wildlife clinic	2018

Table 3.2. All locations in SK from which mosquitoes were trapped or made available by third parties

Location	Specific location	GPS	Description	Years available
U of S campus, Saskatoon	East road, VIDO building	52.1368, -106.6243	Urban terrestrial Fragmented lawns between parking lots and buildings with occasional bushes and trees	2018, 2019 and 2020
	Seminary area	52.1395, -106.6382	Urban aquatic Grounds near Meewasin trail, fields, and wooden area ~100 to 300 m from SK river	2019 and 2020
	Sculpture Park	52.1350, -106.6391	Urban aquatic Park near Meewasin trail with lawn and small fragmented wooden areas ~75 to 250 m from SK river	2019 and 2020
	Agricultural field	52.1475, -106.6300	Urban aquatic Cultured field and surrounding grounds near Meewasin trail ~75 to 300 m from SK river	2019 and 2020
Diefenbaker Park, Saskatoon	Along SK river	52.0979, -106.6894	Urban aquatic Large park along SK River, with a mix of wooden area, open manicured lawn.	2019 and 2020
Saskatoon Parks Branch		52.1415, -106.6953	Urban terrestrial Area with nearby fields, vegetation, and buildings (no natural body of water within 2 km)	2019 and 2020
Woodlawn Cemetery, Saskatoon		52.1504, -106.6552	Urban terrestrial Both open lawn and tree canopy cover areas, ~750-1000 m from the SK River	2019 and 2020

Location	Specific location	GPS	Description	Years available
Umea Park, Saskatoon		52.1670, -106.6310	Urban terrestrial Area with mostly open fields, ~650 m from the SK River	2019 and 2020
Nutana Kiwanis Park, Saskatoon		52.1024, -106.6138	Urban terrestrial Area with mostly open fields, over one km from a large natural body of water	2019 and 2020
Gordie Howe, Saskatoon	Includes golf course and campground	52.1065, -106.6924	Urban terrestrial Area with mostly fields with sparse trees, within 500 m of the SK River	2019 and 2020
Forestry Farm, Saskatoon		52.1556, -106.5837	Urban terrestrial Area with a mix of wooden area, open field, and manicured lawn	2019 and 2020
Dundurn RM	Indi Lake	51.7015, -106.5172	Non-urban aquatic* (*Severe drought receded shoreline substantially) Field with grazing cattle 0 to 200 m from marsh	2018, 2019, and 2020
	Rural farm	51.7395, -106.5026	Non-urban terrestrial Fields with grazing cattle, farm equipment and debris	2019 and 2020
	Seasonal pond	51.7651, -106.5269	Non-urban aquatic Amidst tall grass fields, thick vegetation and trees surround the pond	2019 and 2020

Table 3.3. All MB locations of mesocarnivore trapping and known locations of blood, tissues, or both from third party submission

Location	Specific location	GPS	Description	Years available
Assiniboine Park, Winnipeg	APZ	49.8696, -97.2429	Urban terrestrial Zoo with exhibits, buildings, and manicured lawn. One artificial pond on premise, several smaller artificial bodies of water scattered throughout, and Assiniboine river ~250 to 700 m from all locations within the zoo	2018, and 2019
Melita and Hartney area	Farm near Elva	49.2205, -101.1988	Non-urban terrestrial Large open field near farm equipment and buildings, small wooden area on edge of field and two artificial bodies of water within 500 m	2018, and 2019
	Abandoned field near Broomhill	49.4222, -101.1692	Non-urban terrestrial Large grazing field with natural body of water over 400 m away	2018, and 2019
	Field near Broomhill	49.3611, -101.1230	Non-urban aquatic Large open grazing field with abandoned farm buildings with natural bodies of water running through location (all trapping within 200 m)	2018, and 2019
	Feedlot farm near Hartney	49.5610, -100.4639	Non-urban terrestrial Large open cattle grazing field with many buildings on property	2019

Table 3.4. All locations in MB from which mosquitoes were trapped or made available by third parties

Location	Specific location	GPS	Description	Years available
Winnipeg	APZ	49.8696, -97.2429	Urban terrestrial Zoo with exhibits, buildings, and manicured lawn. One artificial pond on premise, several smaller artificial bodies of water scattered throughout, and Assiniboine river ~250 to 700 m from all locations within the zoo	2019 and 2020
	Kings Park	49.7978, -97.1246	Urban aquatic Mix of wooden area and large manicured lawns, abutting the Red River	2020
	Fort Whyte	49.8205, -97.2294	Urban aquatic Mix of open fields and wooden areas near multiple bodies of water (Lake Devonian, Muir Lake, and wetlands)	2020
Melita and Hartney area	Farm near Elva	49.2205, -101.1988	Non-urban terrestrial Large open field near farm equipment and buildings, small wooden area on edge of field and two artificial bodies of water within 500 m	2019
	Abandoned field near Broomhill	49.4222, -101.1692	Non-urban terrestrial Large grazing field with natural body of water over 400 m away	2019
	Field near Broomhill	49.3611, -101.1230	Non-urban aquatic Large open grazing field with abandoned farm buildings with natural bodies of water running through location (all trapping within 200 m)	2018, and 2019
	Feedlot farm near Hartney	49.5610, -100.4639	Non-urban terrestrial Large open cattle grazing field with many buildings on property	2019

3.3.3 Animal Capture and Handling

Trapping at each location was repeated nightly until either a) 10 trapping nights (approximately two work weeks) were completed, b) a target species was successfully caught, and further trapping would be unlikely to lead to the capture of another individual, or c) bait outside of the trap was left untouched for several nights in a row.

Graduate student

During the warm field season (May to September), overnight trapping of mesocarnivores spanned from approximately 20:00 (up to two hours before sunset) to approximately 6:30 (up to three hours after sunrise). In contrast, mesocarnivore traps were left open 24hrs during the milder field season (September to November), were checked every morning and closed for the weekend. Mosquito traps were left opened and running 24hrs.

APZ staff

Overnight trapping of mesocarnivores spanned from 16:00 to 8:00 (approximately, depending on staff availability).

Tomahawk traps were used for mesocarnivores. Megacatch mosquito trap (2018), was subsequently replaced by BG-Sentinel trap with flow-by CO^2 (2019-2020). Mosquito surveillance programs in Saskatoon and Winnipeg used CDC light traps.

Figure 3.1. An urban raccoon recuperating after anesthesia

Figure 3.2. Mosquito trap with battery and CO^2 cannister

Once a location was chosen, traps were placed in areas where target species were either spotted, or habitat was favorable. Mesocarnivore traps were placed in areas with vegetation cover, and the mosquito trap was placed on even ground, preferably near a water source and a tree or post against which the CO^2 cannister could be cinched to. When possible, mesocarnivore traps were placed, wired open and baited, on location for a few days prior to active trapping. This process was an attempt to lure any wildlife and habituate them to the traps. Once active trapping began, traps were baited and opened, up to two hours before sunset (weather-dependent). All overnight traps were checked within 3hours of sunrise (range of 4:30 to 7:00am); any empty traps were closed for the day, any non-target species were released, and all target species caught were processed. Traps were either replaced with fresh clean traps, or if the trap was not soiled; it was placed back on location, if more target species were expected in the area. As previously stated, hours of setting and checking traps by the APZ staff dif fered from the above-mentioned schedule: setting commonly occurred at 16:00 and checks shortly after arrival to the zoo, typically 8:00 (C. Berkvens, personal communication, 10-04-2021).
(Full trapping protocols available in Appendix A).

Anesthesia and Fieldwork Sample Collection

Mesocarnivores

Mesocarnivores were given an intramuscular sedation of ketamine and dexmedetomidine (medetomidine at the APZ) prior to their removal from the traps, then given flow-by oxygen +/- isoflurane. Once an adequate plane of sedation was reached, a physical exam was done then blood and ectoparasites were collected. Blood draws attempted to maximize amount extracted while remaining below 1% of body weight. Once all procedures were performed, the animals were given atipamezole to reverse sedation before release (see appendix for specific anesthetic and sampling protocols).

Figure 3.3. Graduate student with pole syringe approaching a tomahawk trap containing a wild skunk, *courtesy of Morgan Kelley*

Figure 3.4. Jugular venipuncture for blood sampling in an anesthetized wild raccoon, *courtesy of Morgan Kelley*

Mosquitoes

The catch bag from the mosquito trap was retrieved every weekday morning. A replacement bag was affixed to the intake funnel for continued collection, the battery replaced as needed, and the CO^2 cannister was checked to ensure adequate pressure and contents for continued trapping.

3.3.4 Laboratory Protocols

Necropsies, sampling, and tissue storage were done within the Prairie Diagnostic Services (PDS) Saskatoon, SK. The facility is certified containment level 2. Carcasses and subsequent tissues were handled as potentially containing a Risk Group 3 (RG3) pathogen. Surfaces were disinfected with a 1% bleach solution followed by DNAase between each individual animal. Disposable scalpels and forceps were used and discarded after each carcass. Individual carcasses were weighed, sexed, and examined for any external abnormalities, then opened for removal of the kidneys, liver, spleen, and lungs. A small piece of each organ was excised, macerated, and mixed to make a pooled sample of roughly 25 mg. Pooled samples were sent for DNA extraction

and PCR testing. Remaining tissues were divided in two: one half was frozen and the other fixed in formalin for follow-up histopathology, if needed. Remaining non-target tissues and carcasses were disposed of and clean up completed following protocols for RG3.

The mosquito collection bag was placed in the freezer for rapid euthanasia of the insects. Subsequently, the bag was emptied in a petri dish for sorting. Any non-mosquito insects were discarded. Males were identified, counted, and excluded from testing. Female mosquitoes were counted and identified to the genus level utilizing keys (Thielman and Hunter, 2007; APHC, 2016) then randomly chosen for testing. Pooled samples were crushed using a vial and pestle, then sent for PCR test.

Blood collected from the field or provided by a 3rd party was processed, and the serum aliquoted for testing. A microagglutination test (MAT) was performed at PHAC's National Microbiology Lab in Winnipeg following a modified protocol as outlined in Sato et al., 1990. A sample was considered positive when titers were ≥ 1:128 (PHAC, 2009).

An in-house assay was developed and validated to test all available pooled mammal tissues (liver, kidney, spleen, lung) as well as pooled mosquito samples. Gene target *fopA* and *lpnA* were chosen for the assay, based on literature and validation using previously diagnosed individuals. The confirmed positive animals (ARS and WTJR) had frozen tissues available for use as positive control, however due to large amount of testing required, a synthetic gene target for real-time PCR was designed (Sigma), utilizing a *fopA* amplicon generated from gene coding sequences cloned into a vector. This plasmid DNA was subsequently used as positive control. Genomic DNA extractions, DNA level confirmation and real-time PCR were done in-house. All extracted genomic material from mammal samples were subsequently also evaluated by the National Microbiology Lab (PHAC). A Ct value ≤ 37.0 °C, and valid melt peak and melt temperature was considered positive and confirmed by Sanger sequencing and Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cg).
(See Appendix A for full laboratory protocols).

3.4 Results

3.4.1 Number and Species of Individuals With Samples Available

A total of 33 mesocarnivores were sampled between 2018 and 2020. Four had only tissues available (two skunks, one raccoon and one short-tailed weasel), while 29 individuals had sera available (nine skunks, nine raccoons, eight foxes, two feral cats and one mink).

A total of 14,904 mosquitoes, caught between 2018 and 2020 by the graduate student or made available by mosquito surveillance programs in Saskatoon and Winnipeg, were used for pooled testing.

Table 3.5. Number of individual mesocarnivores sampled. Includes both animals caught by graduate student and carcasses, or blood made available by third parties.

Year	Urban MB	Non-urban MB	Urban SK					Non-urban SK		
			Campus Agriculture location	Northeast Swale	Diefenbaker Park	Other campus location	Another city location	Indi Lake	Brightwater	Other non-urban location
2018	6 raccoons 6 foxes 2 skunks *2 skunks*	NC	NT	NT	NT	1 raccoon	1 fox	1 skunk	NT	1 mink
2019	4 skunks	1 raccoon	NC	1 skunk	2 feral cats 1 fox	NC	1 raccoon	NC	NC	NC
2020	NT	NT	*1 weasel*	NT	NT	NC	NC	NC	1 skunk	*1 raccoon*

NC: nothing caught **NT**: no trapping *tissues made available*

Table 3.6. Demographics, (province, habitats, sex, and age) of sampled mesocarnivores between 2018 and 2020 (N=33).

Species	**Province**		**Habitat**						**Signalment**				
	MB	SK	Urban terrestrial	Urban aquatic	Non-urban terrestrial	Non-urban aquatic	Urban Unknown	Non-urban Unknown	Female adult	Male adult	Female juvenile	Male juvenile	Unknown
Skunks (N=11)	8	3	9	-	-	2	-	-	6	3	1	1	-
Raccoon (N=10)	7	3	8	-	1	1	-	-	5	1	-	1	3
Foxes (N=8)	6	2	6	1	-	-	1	-	1	-	1	-	6
Feral cats (N=2)	-	2	-	2	-	-	-	-	0	1	-	1	-
Mink (N=1)	-	1	-	-	-	-	-	1	1	-	-	-	-
Short-tailed weasel (N=1)	-	1	1	-	-	-	-	-	1	-	-	-	-

Table 3.7. Total number of mosquitoes (prior to sexing) available. Includes insects caught by graduate student and those made available by third parties.

Year	Urban MB		Non-urban MB	Urban SK			Non-urban SK		Total
	APZ	Other locations		Campus	City parks	Agricultural field	Indi Lake	Other non-urban locations	
2018	NC	NT	2	6	NT	NT	63	NT	71
2019	1	NT	482	108	156	11	NC	32	790
2020	6,897	5,350	NT	377	1,342	1	36	40	14,043
Total	6,898	5,350	484	491	1,498	12	99	72	14,904

NC: none caught NT: not trapped

Aedes genus was the most common, followed by *Culiseta* and *Culex,* with *Anopheles* being the least common.

3.4.2 MAT Results for Mesocarnivores

Four animals had measurable antibodies to *F. tularensis*. The remaining 25 sera had no or below threshold titers (all serological results in table 3.6).

In 2018

One young male skunk from APZ (49.8734, -97.2442) with titers 1:512. The animal was caught by pest control and was in apparent good health. Blood was drawn prior to euthanasia.

One adult female mink, weighing 700g, was found near Yorkton (51.3084, -102.6490). The serum (titers 1:1024) was provided by the WCVM wildlife clinic; the animal was found injured on the side of the highway, and presumed hit by a car. A broken pelvis was observed on radiographs, and the animal was euthanized due to poor prognosis. The animal was necropsied by a wildlife veterinary pathologist, and no abnormalities, other than trauma-related, were noted grossly or on histopathology. Tissue was not kept for PCR.

In 2019

One adult female raccoon, weighing approximately 10kg, in non-urban southern MB (49.5602, -100.4642) with titers 1:128. The animal was in apparent good health.

In 2020

One adult female skunk, weighing 2.4kg, in non-urban SK (51.7015, -106.5172) with titers 1:4096. The animal was in great body condition and appeared to be in good health.

Figure 3.5. Adult female skunk whose titers were 1:4096, *courtesy of Morgan Kelley*

Table 3.8. All serological results for mesocarnivores (N=29); nominator is # of positives, denominator is total # tested

Locations	Specific location	Species	Year	Month/Season	Female Adult	Male Adult	Female Juvenile	Male Juvenile	Adult unknown sex	Unknown age and sex
APZ, MB	In-zoo	Fox (N=6)	2018	May-July	-	-	-	-	0/1	0/5
		Skunk (N=2)	2018	May and July	-	-	-	1/1	0/1	-
		Raccoon (N=6)	2018	May-October	0/2	0/1	-	-	-	0/3
		Skunk (N=4)	2019	May	0/3	0/1	-	-	-	-
Melita, MB	Feedlot	Raccoon (N=1)	2019	July	1/1	-	-	-	-	-
Saskatoon, SK	Diefenbaker Park, Saskatoon, SK	Feral cat (N=2)	2019	October	-	0/1	-	0/1	-	-
		Fox (N=1)	2019	October	-	-	0/1	-	-	-
	Beef research area	Raccoon (N=1)	2018	June	0/1	-	-	-	-	-
	Northeast Swale	Skunk (N=1)	2019	November	-	0/1	-	-	-	-
	Other urban area	Raccoon (N=1)	2019	August	0/1	-	-	-	-	-
Dundurn RM Indi area, SK	Indi Lake	Skunk (N=1)	2018	June	-	0/1	-	-	-	-
	Seasonal pond	Skunk (N=1)	2020	July	1/1	-	-	-	-	-
Yorkton area	Outside town, off highway	Mink (N=1)	2018	July	1/1	-	-	-	-	-
Unknown (but likely Saskatoon)		Fox (N=1)	2018	Summer	-	-	-	-	-	0/1

3.4.3 PCR Results

All 105 mosquito pooled samples and the four mesocarnivore tissues did not have detectable *F. tularensis* DNA. In-house PCR results for the mesocarnivores were confirmed by the National Microbiology Lab.

3.5 Discussion

3.5.1 Mesocarnivores

The prevalence of seropositive mesocarnivores was 13.8% (95% CI 5.5% - 30.6%), indicating tularemia was, or had been, present in the area. Three of the four had relatively high titers (1:512, 1:1024 and 1:4096) consistent with relatively recent exposure. High prevalence and ease of sampling makes mesocarnivores a good species for detecting *F. tularensis* activity.

Presence of antibodies in species tested were the highest in skunk (two of nine, 22.2% (95% CI 6.3% - 54.7%)) and mink (only one, 100% (95% CI 20.7% - 100%)) followed by raccoon (one of nine, 11.1% (95% CI 2% - 43.5%)), while no positives were found in either foxes or feral cats (eight (95% CI 0% - 32.4%) and two (95% CI 0% - 65.8%) tested respectively). The presence of measurable titers in both skunks (1:512 and 1:4096) and raccoons (1:128), even though the number of animals sampled was low, is consistent with previous studies (McKeever et al., 1958; Berrada et al., 2006). Skunks appear to be of particular interest considering both the prevalence among those sampled and the high titers. It would likely be beneficial to further investigate serology in skunks, and raccoons as these show the most promise for potential use as sentinel species in SK and MB locations. Unlike studies in Germany (Kuehn et al., 2013; Otto et al., 2014), none of the foxes had titers. However, this may be only true for the locations included in this study, and again, having a low sample size (eight foxes total) and lacking data for most individuals (e.g., age) makes it impossible to appropriately interpret both the species role and serological prevalence in Prairie Canada. On a similar note, while only one mink was sampled and included in this study, this sole representative of the species had a high titer (1:1024). This finding is consistent with a previous finding by Martin et. al (1982) where one mink was positive from four tested at Pike Lake, SK. Mustelids, especially mink, should be further included in investigations or surveillance.

Taking into consideration species information, a few qualitative observations can be made. First, all species with titers prefer habitats that are close to water (Fox, 2001; Schlimme, 2003; Kiiskila, 2014), while foxes and feral cats show no such predilection (Fox, 2007; Anna Toenjes, 2014). Most mesocarnivores lead solitary lives outside of the breeding season, although occasionally, foxes may stay in mated pairs (Saunders, 1988), or raccoon and feral cats may live in small family groups that may overlap the same home range (Saunders, 1988; Anna Toenjes, 2014). Mice and voles are universal prey to all mesocarnivore species sampled (Saunders, 1988). Muskrats will be predated mainly by minks and raccoons (Fox, 2001; Schlimme, 2003), while skunks will eat carrion if chanced upon (Saunders, 1988). Squirrels are associated more so with raccoons, foxes, and domestic cats (Fox, 2001; Fox 2007; Reimer 2018), while rabbits are prey to minks and foxes (Schlimme, 2003; Fox, 2007). The life expectancies of minks (three to four years; Schlimme, 2003), skunks (one to six years; Kiiskila, 2014) and raccoons (five to six years; Fox, 2001) do not appear to differ from those of foxes (three to six years; Fox, 2007), however they are on average vastly higher than any rodents or lagomorphs. This longer lifespan does correlate with a higher chance of being exposed to pathogens in the environment, thus developing antibodies, and potentially gives a longer snapshot into the pathogen activity in the area. It seems appropriate to interpret titers in an adult from these species as having been exposed within four to six years from the sampling date. Although mesocarnivores' longer lifespan increases the time available for potential exposure, these species are larger and more mobile than rodents, making it more difficult to make accurate comments on a specific location. Indeed, home ranges vary widely, from female minks occupying approximately 20.4 hectares territories to raccoons occupying up to 5,000 hectares territories in grassland habitats (Fox, 2001; Schlimme, 2003). Many factors will play into the size of an animal's home range, including sex, age, habitat, and season (Saunders, 1988). This wide range of movement will make it inherently more difficult to decidedly confirm activity in a precise location, but it may also suggest the pathogen can be disseminated to other populations more easily via infected ectoparasites, bacteria shedding in urine or feces, or being predated upon. Even greater distances are often achieved by juveniles dispersing, sometimes staggeringly far as has been recorded in a fox traveling 400 km from its original den (Fox, 2007).

The APZ had sera available for 2018 and 2019. One of two (50%) skunks had titers (1:512), representing one of fourteen (7.14%) mesocarnivores sampled from the APZ grounds in

2018. Considering the habitat is one where the animal likely had plentiful food, the individual's territory was likely smaller and within the confines of the zoo's borders, although some travel outside the zoo's perimeter fence and into surrounding park would be plausible. The APZ provided a unique location for our study where wildlife, zoo animals, and humans all have a potential to interact directly or indirectly with each other in a relatively small area (~33ha). Note that in 2019, four skunks were sampled at the zoo, but none were positive. This could indicate either no tularemia activity since the previous year, or that sampling occurred too early (antibodies not produced yet) or too late (antibody levels fallen below threshold) after exposure.

While traps were set up in July 2018 and 2019 in non-urban southern MB, only one mesocarnivore was caught and sampled. This animal was a raccoon, caught in Hartney RM in 2019, and it had a low but positive titer (1:128). This potentially indicated either an exposure to tularemia in a more distant past or a very recent exposure that did not provide enough time for titers to reach a high level. As the study was not set up for repeat and comparisons of titers; it was not possible to confirm if this was a rising or decreasing titer. The animal was trapped within an abandoned farm building, and many other raccoons were spotted in the area. Considering the habitat (small private feedlot; open pastures with nearby wooded area and many farm buildings) which likely offered bountiful resources, this animal's home range was likely centered around the area it was caught and did not extend too far beyond the farm's property.

The sole mink serum submitted by the WCVM clinic to the project in July 2018, was found off a highway near Yorkton, SK. Since an exact location was not given, inferences about habitat could not be made. Considering that female minks on average have a home range of approximately 20.4 hectares, and that this individual had relatively high titers (1:1024); it would seem to indicate that there might have been tularemia activity during the summer months around the highway where the animal was found.

In July 2020, a skunk with high titers (1:4096) was caught near a seasonal pond in the Dundurn RM. It is likely that this mesocarnivore had been recently exposed. The exact location this animal was caught is prime habitat for the species, however non-urban skunks' territories can be up to 551 hectares (Saunders, 1988; Kiiskila, 2014). Considering this possible home range, tularemia activity in this region likely occurred within two km of the trapping location.

Three of the four animals with antibodies were adult females, of apparent good health. One would expect more adults to have titers specific to a pathogen than juveniles (Pedersen et

al., 2012; Ramey et al., 2019). Whether there are biological factors that would increase a female's risk of exposure to tularemia, is questionable. The majority of the mesocarnivores sampled have solitary social behaviors (Saunders, 1988; Fox, 2001; Schlimme, 2003; Fox, 2007; Anna Toenjes, 2014; Kiiskila, 2014), however females inherently spend a relatively longer amount of time interacting with conspecifics during breeding and rearing season compared to males, who often only interact during breeding season or territorial confrontations. Being in a small family group may increase the risk of one individual encountering *F. tularensis* and potentially transmitting to their kin via infected food or ectoparasites. On the other hand, females have smaller home range, as a rule, therefore may not travel over as much terrain as males, which would put the latter at a higher risk to encounter infected prey or vectors.

Although further studies are required to confirm any of these observations and related considerations, it is reasonable to infer that any serological surveillance to detect tularemia in mesocarnivores should prioritize adult animals and include apparently healthy individuals.

3.5.2 Mosquitoes

Francisella tularensis DNA was not detected in any of the mosquito pools tested across the two provinces. Since various studies have confirmed mosquito can harbor *F. tularensis* and, in the right conditions, transmit the pathogen (Triebenbach et al., 2010; Rydén et al., 2012; Thelaus et al., 2014), failure to detect *Francisella tularensis* DNA through mosquito pools in Prairie Canada could be due to one or more of the following: low prevalence of active cases during inter-epizootic times made it less likely for mosquitoes to feed on infected animals, water burden inter-epizootically from *F. tularensis* is too low for significant larvae infection, the species of mosquitoes in Prairie Canada do not regularly feed on key species, the month or season of active tularemia does not overlap with peak mosquito population or the mosquitoes in Prairie Canada do not harbor the bacterium long enough to be detected by PCR.

3.5.3 Conclusions

Presence of tularemia in many of the locations sampled was confirmed by resident mesocarnivores having antibodies to the bacterium, occasionally with remarkably high titers. This includes the APZ, where previous non-human primate cases occurred, lending credibility to

the hypothesis that this may potentially be an endemic focus. While all mesocarnivores with titers are commonly associated with habitats bordering or near water, analysis was not performed to compare aquatic vs terrestrial prevalence due to low sample size. Despite few sera available, having four positive animals may indicate that most mesocarnivores in Prairie Canada would be useful as sentinel for *F. tularensis*. Therefore, if surveillance is needed in the locations included in this study; non-lethal sampling from mesocarnivores would be adequate. Furthermore, mesocarnivore sera available from veterinary clinics or rehabilitation centers may be screened using MAT to check for antibodies, should there be any concerns about tularemia in the area.

Using mosquitoes as immediate seasonal sentinels was not validated during this study. Continued investigation could be worthwhile, especially to elucidate whether these insects could be useful during rodent or lagomorph population eruption to detect an impending tularemia outbreak. The role of mosquitoes in the ecology of tularemia in SK and MB remains unknown, and further investigation into arthropod vectors (e.g., mosquitoes, ticks, and fleas) is warranted.

CHAPTER 4. General Discussion

4.1 Introduction

Although cases of tularemia have been confirmed in some of the study areas in Saskatchewan (SK) and Manitoba (MB) in the last 10 years, *Francisella tularensis* was not detected by PCR during our study that included samples from 2015 to 2020. However, multiple individuals, representing three mesocarnivore and three rodent species, had measurable antibody titers. Low interepizootic prevalence was expected and is supported by these findings.

4.2 Research Summary

4.2.1 Objectives

The study's main objective was to conduct active surveillance over several years to confirm the presence of tularemia and its status (endemic vs non-endemic) in various habitats and settings across Prairie Canada. To meet this goal, active surveillance was achieved by trapping rodents, lagomorphs, mesocarnivore and mosquitoes. The study was also supplemented by passive surveillance via submissions to the CWHC or directly to the project. As previously established in the methods, surveillance included non-urban and urban settings as well as aquatic and terrestrial habitats. *F. tularensis* past or current activity in a location was confirmed by serum testing for antibodies or tissues for presence of the bacterium's DNA.

Other objectives were to be explored quantitatively if sample sizes allowed statistical data or qualitatively if sample sizes were too low. These secondary goals included:

- Establishing prevalence and differences between habitats or species
- Formulating hypotheses for reservoir, donor, recipient, or vector roles
- Formulating recommendation for surveillance using mesocarnivores or mosquitoes
- Formulating hypothesis about the ecology of tularemia in Prairie Canada

4.2.2 Results

Rodent and Lagomorphs

The consistent presence of antibody titers in tree and ground squirrels, especially at the urban zoo, a location with historical confirmed cases of tularemia, highlighted the potential importance of these rodents in *F. tularensis'* ecology in SK and MB. Although their exact role in the ecology of the bacterium in Prairie Canada could not be confirmed due to sample size and small number of positive individuals, the presence of antibodies indicates that at least some individuals in these populations do not succumb to infections like other highly susceptible rodents. These qualitative observations along with plausible biological factors support both American Red Squirrels (ARS) and Richardson's Ground Squirrels (RGS) as being recipient species with spillover roles and, in the right conditions, the potential to play donor or reservoir roles. These roles may carry important implications for both interepizootic and epizootic periods for humans and other species, such as zoological, or domestic animals. While behavioral and biological differences between ages and sexes may be important in susceptibility to *F. tularensis*, statistical comparisons were not attempted due to sample size and few positive animals. Associations between exposure and signalment could be further explored in future studies.

While voles have been hypothesized or shown to play various ecological roles in other geographical locations (Glass, 1948; Mörner, 1992; Rodríguez-Pastor et al., 2017), their role in this study could not be established due to low sample sizes. However, considering it was possible to find one prairie vole (out of 28 tested) with measurable antibodies, this may indicate a higher prevalence within the vole populations at our sampling locations for the season or year trapped.

None of the 16 lagomorphs (two eastern cottontails and 14 WTJR), 124 mice (98 deer mice, 25 house mice, and one white-footed mouse) and one shrew assessed had detectable antibodies or *F. tularensis* DNA. These results were unsurprising, especially in mice since there had been no large morbidities or mortalities reported during the period of this study, which often accompanies *F. tularensis* circulation in these species. However, due to the small sample size of most of the species assessed, it is not possible at this time to rule out a potential contribution in the tularemia ecology in Prairie Canada. The absence of titers in these species could be due to one or more of the following: an inadequate number of individuals were sampled, sick or recovering animal were unlikely to enter the traps or affected animals may have died acutely

without titers and were inaccessible for testing. While the latter is consistent with a previous publication by Wobeser et al. (2007) for small susceptible rodents, it is likely that lagomorphs of Prairie Canada fell into the first two categories, considering historical evidence.

Mesocarnivores

With an overall prevalence of 13.8% (95% CI 5.5% - 30.6%) across both provinces, these findings confirmed the presence of tularemia in some of the locations trapped, or at least within these species home-range. With three of the four positive mesocarnivores having high measurable titers (1:512, 1:1024 and 1:4096), this appeared to be enough evidence to deem these animals as truly positive and considering life expectancy and level of titers; they were likely exposed in the relatively recent past.

Although the mesocarnivore sample size was low, and even more so when considered at the species level, qualitative observations included:

- Representation of species that prefer habitat with proximity to water (skunks, raccoons, and minks), although not all locations with positive animals were associated with aquatic habitat
- The majority (three of four) of positive animals were in non-urban locations, although the positive raccoon in non-urban MB was intimately associated with a human infrastructure
- All positive animals were sampled in July of their respective years
- The majority (three of four) of positives were adult females in apparent good health and nutritional status

Mosquitoes

Failure to detect *F. tularensis* DNA in pooled mosquito samples from all locations visited confirms it may not be an adequate surveillance method during interepizootic periods. However, it did not rule out the mosquito as a potential vector or having another ecological role in Prairie Canada. Since no detection could have been due to several factors, further research into mosquitoes, and other blood-sucking arthropods, is warranted.

Ecological Aspects

This study found evidence of *F. tularensis* exposure in both rodents and mesocarnivores from multiple locations, confirming the presence of the bacterium. Furthermore, spatiotemporal pattern of these serological positives, although not confirmed statistically, appeared to indicate that there may be a natural endemic focus at the APZ, while a low prevalence or sporadic activity may account for the positives seen at the U of S campus and various non-urban locations.

Many urban areas visited, such as the APZ, provide fragmented habitats that create a mosaic suited to various wildlife species, from the tree dwelling ARS to the grass field habiting RGS and prairie vole, while still providing a large body of water that would fulfill the habitat preferences of many mesocarnivores (e.g., skunks, raccoons, mustelids). These areas potentially create an increase in diversity and density, which would be ideal for the circulation and maintenance of versatile pathogens, like *F. tularensis*. On the other hand, fragmentation of habitat, as seen in agricultural environments, can lead to metapopulation dynamics that also foster disease endemicity and spread (Jousimo et al., 2014). During our study, non-urban areas with serological positives were sporadic and often only had one rodent or one mesocarnivore per location, making it difficult to interpret beyond confirmation of previous activity at these locations. For example, three distinct locations in non-urban MB in 2019 had one individual with titers: two juvenile RGS and one adult raccoon. While the juveniles at the two locations are evidence of activity within the last two months, the adult raccoon may have been exposed in previous seasons, as tempting as it may be to use the titers as support of tularemia activity in the region. Another location, in non-urban SK in 2020, had a healthy adult skunk caught near a seasonal pond, with a high titer (1:4096). While no other positives were found in other species at this location (e.g., small rodents or mosquitoes), significant interpretation was not possible since only one deer mouse was caught over the three nights of trapping (30 Sherman traps used). This appears to be a low number, especially compared to the previous year, when three voles and nine deer mice were caught over three nights (25 Sherman traps used) at the exact same location. While confounding variables may exist, such as time of year (September 2019 vs July 2020), it is possible that small rodents were not as abundant in 2020 at the time of sampling. This possible decrease in small rodent population at this location could be purely coincidental, or if the high antibody titer to *F. tularensis* seen in the skunk is an indication of recent tularemia activity;

resident rodent populations may have been affected. Numerous rodents were caught at other locations in the same rural municipality, ranging from three to six kilometers away from the seasonal pond where this seropositive skunk was found. Although outside of the average non-urban skunk home-range, these locations may be part of the larger metapopulation of the area between Dundurn and Indi Lake. All rodents caught in these additional non-urban locations were also negative on both serology and molecular analysis. Further investigation at this locale could be beneficial to potentially elucidate if this is a focus of tularemia activity. Overall, a qualitative observation can be made about locations with serological positives; most positives, save for a couple of individuals, were in habitats that included or was near (within 300 m) a natural body of water. This may indicate that the cycle of *F. tularensis* in Prairie Canada is mainly an aquatic one.

In summary, ground and tree squirrels (prevalence 1.4% (N = 633, 95% CI 0.8% - 2.7%) and 22.2% (N = 9, 95% CI 6.3% - 54.7%) respectively) appear to hold a **recipient or donor role** in prairie Canada. Whether they have the potential to also, given the right condition, play a **reservoir role** would require further studies. While one vole had titers (prevalence 16.7% (N = 6, 95% CI 3.0% - 56.4%)), this one single point of data is not enough to confidently assign the rodent a role. Considering the titer and vole roles in other geographical locations, a **recipient role** would be the most likely for SK and MB. Mesocarnivores, with their average longer lifespans and larger home ranges, appear to be great candidates for **sentinels**. On the other hand, no positives were found in mice, lagomorphs, and mosquitoes. These findings could potentially mean these species play no role in the Prairie locations visited, or further studies with higher sample size are required. The need for further research with higher sample sizes is likely especially true for the lagomorphs, as a previous tularemia case was confirmed in one individual within Saskatoon city limits in 2017.

4.2.3 Limitations and Future Directions

Biases and Limitations

Several biases are inherent in large-scale ecological studies of wildlife diseases; thus, acknowledgments will be made below for limitations that may have introduced biases.

An attempt was made to try to balance aquatic and terrestrial habitats, however, regional droughts in 2019 and 2020 made some aquatic environments drier than normal or virtually non-existent. For example, in 2018, Indi Lake was adequate and showed sign of various aquatic life (e.g., muskrats, waterfowl, etc.). In contrast, in 2019 the water had receded much earlier in the summer than normal, and by 2020; the body of water was ~30% of its usual size. This trend was consistent across both provinces.

Study areas were chosen based on ease of access, presence of species of interest, and previous good working relations with landowners. Non-urban SK locations were also chosen based on traveling distance between the trapping sites and the U of S, as it was commonly needed to drive to and from these locations at least twice a day. This restricted active trapping to central SK, or up to 50 km away from the U of S (work base).

The live trapping of rodents, especially RGS, possibly selected for healthier individuals, as sick or moribund animals likely stayed hidden. An attempt was made to harvest rodent carcasses when they were chanced upon, or through necropsy submissions.

There was only a single trapping team, thus it was impossible to trap at all locations over the entire season. A decision to move on from one location to another was made either after 60 RGS were caught or once approximately 10 trapping days, nights or both had been completed, whichever came first. This trapping model could potentially create artificial species composition differences. For example, no mosquitoes or juvenile RGS were collected at the APZ in 2018 and 2019 since this location was visited in early May, while abundant mosquitoes and juveniles could be sampled from non-urban MB as trapping occurred in July. An attempt was made to mitigate these discrepancies by collaborating with local mosquito programs and pest control which operated later in the season. Other examples of natural behavior that could have biased trapping included lagomorphs' disinterest in baited traps when other natural food was readily available, and possible different wariness level of mesocarnivores from various habitats and seasons.

Future Research and Recommendations

There are several questions that could be explored in future research, which would provide either new information or improve the current knowledge.

First, while mosquitoes as seasonal sentinels during interepizootic periods could not be validated during this study, continued investigation could be worthwhile, especially to elucidate whether these insects could be useful during rodent or lagomorph population eruption to detect impending tularemia outbreaks. Furthermore, assessing the role of ectoparasites and mosquitoes as potential vectors in the prairie provinces of MB and SK would be important for future studies. This investigation would be crucial, as the literature appears divided on role and types of vectors depending on *F. tularensis* subspecies involved, the habitat studied and geographical locations. Therefore, a study specific to the Canadian Prairies would be more informative than applying other region's data to our environment. Although ectoparasites were collected from trapped animals with the intent to test by PCR, this endeavour was dropped from the current thesis due to time constraints. These collected ectoparasites have been stored and could be further investigated.

Another ecological element that could be worth investigating are abiotic factors, such as water. Environmental testing, prioritizing water samples, is strongly recommended as various studies have shown that this medium may play a role in amplification or reservoir (Hennebique et al., 2019). A related biotic factor would include aquatic protozoans, which have come up as potential reservoirs for *F. tularensis holarctica* (Foley et al., 2010; Hennebique et al., 2019).

Should any tularemia surveillance be attempted in the locations visited in this study, consideration should be given to use mesocarnivores, as serum testing in these species is relatively easier due to their size and can be done as an antemortem test for any individual that is trapped or already in a hospital or rehabilitation center. Another option would be to sample squirrels, maybe even more so in locations where tularemia endemicity is suspected or known. Consistent access to numerous carcasses, such as from pest control agencies, could potentially be a reliable source of samples. Note that should future research include carcasses for antibody testing; a different MAT protocol than the one used in this thesis would need to be established, or the existing one validated for extracts instead of fresh serum.

Finally, since presence of the bacterium has been confirmed in several locations in this study and there may not be typical gross lesions at necropsy (as seen in the 2017 ARS case in Saskatoon), thorough histological assessment, PCR testing or both of any moribund or dead rodent is strongly recommended.

4.3 Concluding Remarks

Our study was the first rodent tularemia survey done in Prairie Canada in almost 50 years and updated our knowledge of *F. tularensis'* ecology in SK and MB. This study further confirmed tularemia is endemic in both provinces. The APZ has indication of relatively constant presence of *F. tularensis* while other areas have indication of either low prevalence (U of S) or sporadic cases (Melita, Dundurn). We were unable to demonstrate a difference between the prevalence of *F. tularensis* in urban and non-urban environments, however this study was the first attempt to assess tularemia activity in urban settings in Prairie Canada and contributed valuable data as well as offering insight on aspects that would be worth investigating further. The seropositive rodents in this study (red squirrels, ground squirrels, and prairie vole) use similar habitat. While mink, skunks, and raccoons have a more aquatic habitat preference, their larger home-range may overlap with the rodents mentioned above. Urban habitats like parks and the zoo may change dynamics such as animal densities and preferred habitats, thus increasing transmission risks between wildlife, people, or human-owned animals, due to an increase in contact.

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APPENDIX A

Supplemental materials and methods

Table A.1. Anesthetic and euthanasia or reversal protocol per species

Animal species	Sedation (based on estimated weight)	Anesthesia	Euthanasia or reversal
Small rodents (e.g., mice and voles)	Isoflurane chamber	Isoflurane and O_2 (as needed)	pentobarbital 0.25 mL intracardiac or intraperitoneal inj. (top up as necessary)
Squirrels	Isoflurane mask		pentobarbital 0.5 mL intracardiac inj. (top up as necessary)
Rabbits	Ketamine 5 mg/kg and dexmedetomidine 0.15 mg/kg intramuscular inj.		pentobarbital 2 mL intracardiac or intravenous inj. (top up as necessary)
Mustelid	Ketamine 8 mg/kg and dexmedetomidine 0.04 mg/mL intramuscular inj.		Atipamezole, intramuscular inj. (mL needed correlated to amount of dexmedetomidine administered and time since sedation began)
Cat	Ketamine 3 mg/kg and dexmedetomidine 0.05 mg/kg intramuscular inj.		Atipamezole, intramuscular inj. (mL needed correlated to amount of dexmedetomidine administered and time since sedation began)
Mesocarnivores (e.g., skunks, raccoons, foxes)	Ketamine 5 mg/kg and dexmedetomidine 0.025 mg/kg intramuscular inj. APZ protocol Ketamine 5 mg/kg and medetomidine 0.05 mg/kg intramuscular inj.		Atipamezole, intramuscular inj. (mL needed correlated to amount of dexmedetomidine administered and time since sedation began)

Complete list of drugs used:

- pentobarbital (Euthanyl, 240 mg/mL)
- ketamine (Narketan, 100 mg/mL)
- dexmedetomidine (Dexdomitor, 0.5 mg/mL)
- medetomidine (Domitor, 1 mg/mL)
- atipamezole (Antisedan, 5 mg/mL or Revertor, 5 mg/mL)

Table A.2. Mammal species included in study

Common name	Scientific name	Average size	Lifespan	Diet	Geographical range and habitat	Behavior and home range	Other info
Deer mouse	*Peromyscus maniculatus* (NB, there is a prairie and woodland subspecies)	11.9-22.2 cm, 10-31 g[2,3,4]	Max: 8 yrs[2,3] Avg: 1 yr[3,4]	Omnivorous[2]	Native to all locations visited[1]; occurs in all terrestrial habitats, but most common in mix-deciduous forest with sparse or open grass-cover[2,3]	Solitary in summer, group in winter, on 242-3,000 sq.m[4]	Nocturnal[2,3,4], most active at twilight[3] Can climb, swim and may forage in shallow water[3,4]. All small mammal predators eat deer mice e.g., foxes, minks, weasels, etc[3]
House mouse	*Mus musculus*	6.5-18 cm, 12-30 g[2,4]	Max: 5 yrs Avg: 1 yr[4]	Omnivorous[2]	Introduced species, present in all locations visited[1,4], lives in close association with humans e.g., houses, barns, etc. Can be found in fields, especially grain (but never too far from buildings)[2,4]	Social[2,4], degree of established hierarchy dependent on sex and resources[4]; rarely travel further than 15 m away from home[4]	Nocturnal, although may be active during day if inside human building. [4] Territorial, not as pronounced in "wild"[4]. Predators include cats, foxes, weasels, etc.[4]
White-footed mouse	*Peromyscus leucopus*	14.1-20.5 cm, 12-31 g[2,3,4]	Max: 3 yrs Avg: 1 yr[4]	Omnivorous[2]	Native to MB locations visited[1,6]; most common in dry forest and brushy areas, but can be present in many other habitats[2,3,4]	Solitary, on 2,000-6,000 sq.m [4]	Nocturnal[4], and territorial[2,4]. Main predators (also investigated) include weasels and foxes[4]

Common name	Scientific name	Average size	Lifespan	Diet	Geographical range and habitat	Behavior and home range	Other info
Meadow vole	*Microtus pennsylvanicus*	12-18.8 cm, 20-68 g[2,3]	Max: 2.5 yrs[3,4] Avg: 3 mo[4]	Mainly herbivore[2,3] occasionally insects	Native to at all locations visited[1]; most common in moist, low areas with grass (high grassland near streams, lakes, or swamps)[2]	Mostly solitary, occasionally communal nests in winter[3], on 160-3,500 sq.m, males typically larger range and female typically only territorial of 38 sq.m [2,3,4]	Active year round, both day and night (peak at dawn and dusk)[2,3] Swims well[3] Prolific animal with tremendous population fluctuation, cyclic species (~4 yrs)[2,3]. Predators include weasels and foxes
Prairie vole	*Microtus ochrogaster*	11.8-18 cm, 22-70 g[2,4]	Max: 3 yrs Avg: 1 yr[4]	Mainly herbivore occasionally insects[2,4]	Native to at all locations visited[1]; common in open grassland or fields[2,4]	Can be solitary, mated pair (usually summer) or small communal group (usually winter); home range not reported[4]	Territorial[2] Active year-round, both day and night[2,4] but mainly crepuscular.[4] Predators include foxes and weasel[4]
Northern short-tailed shrew	*Blarina brevicauda*	9.8-13.2 cm, 12-23 g[2]	Max: 3 yrs Avg: 18 mo[3]	Insectivore[1]	Native to at all locations visited[1]; most common in heavy forests and low swampy areas, but can be present in any habitat[2,3]	Solitary, on 0.1 – 2 ha[3]	Highly active, semifossorial[1]. Predator may sometimes kill but not eat due to skin gland odor[3]
13-lined ground squirrel	*Ictidomys tridecemlineatus*	21.5-30.5 cm, 82-297 g[2,4]	90% of juveniles die before hibernation	Omnivorous[2]	Native to all locations visited[1]; prefers open prairie and grasslands, urban habitats (golf courses, or parks).[2,4]	Not colonial but can concentrate in desirable habitat, on 4.7 ha for males, 1.4 for females[4]	Diurnal, fossorial and territorial.[2,4] Main predators listed in literature are raptors and snakes[4], however likely mesocarnivores as well

Common name	Scientific name	Average size	Lifespan	Diet	Geographical range and habitat	Behavior and home range	Other info
Northern flying squirrel	*Glaucomys sabrinus*	24.5-34.2cm, 74-140 g[2,3,4]	Max: 4yrs[3,4] Avg: <4 yrs[4]	Mainly herbivore[3], lichen, fungi, occasional insect, or bird egg[4]	Native to MB locations visited[1]; wooded areas[2], prefers coniferous or mixed forests but also in deciduous[3]	Solitary, groups of up to 8 in winter.[4] 0.8-31ha; only females are territorial[4]	Strictly nocturnal[3,4]. Predators include weasels, cats,[3,4] raccoons and foxes.[3]
American red squirrel	*Tamiasciurus hudsonicus*	28.3-34.5 cm[2,3] 120-250 g[1,2,3]	Max: 10 yrs[2,3] Avg: 3-5 yrs[3]	Mainly granivore[4], but will eat eggs, insects or mammals[2,4]	Native to all locations visited[1]; common in coniferous or mixed forests, also found in hardwood[2]	Solitary, on 0.24 - 4.8 ha[3,4]	Diurnal, territorial, active year-roung[1,2,3] Predators include foxes, raccoons, weasels and minks [2,3]
Richardson's ground squirrel	*Urocitellus richardsonii*	27.7-30.6 cm, F: 250-400 g M: 350-500 g[4,5]	Max: 7 yrs Avg F: 3-4 yrs M: 1-3 yr[4,5]	Mainly herbivore, occasional insects and carrion[4,]	Native to all locations visited[1]; common in short and mixed-grass prairies, human landscapes like parks, pastures, and crop fields.[5]	Female kinship, on 20-40 sq.m[5] Male solitary and dispersal of 3-10 km from natal site[4]	Diurnal, fossorial and territorial.[4] Predators include weasels, foxes, skunks, and domestic cats[4,]
White-tailed jackrabbit	*Lepus townsendii*	3-4 kg[4] (females a little larger)	8 yrs[4]	Herbivore[3,4]	Native to all locations visited[1]; common in open grasslands, pastures, and fields, but also forested areas[4].	Solitary[1], on 2-3 km diameter[4]	Nocturnal[4]. Predated on by foxes[4].
Eastern cottontail	*Sylvilagus floridanus*	380-485 mm, 825-1800 g[2,3] (females a little larger)	Max: 10 yrs Avg: 15 mo[3]	Herbivore[3]	Native to all MB locations visited[1]; deciduous forest clearings, fields,	Solitary, on 0.9-2.8 ha (up to 40.4 if food or cover scarce)[3]	Most active at twilight and moonlit nights[3]. Predated on foxes, weasels and minks[3]

					farmlands, marshes or residential areas[3]		
Common name	Scientific name	Average size	Lifespan	Diet	Geographical range and habitat	Behavior and home range	Other info
Muskrat	*Ondatra zibethicus*	41.7-62 cm, 680-1,800 g[2,4]	Max: 10 yrs Avg: 3-4 yrs[4]	Mainly herbivore, but supplements with aquatic animals[2,3,4]	Native to at all locations visited[1]; found in wetlands,[1,2] such as marshes, ponds, lakes and streams[2].	Solitary (outside breeding season), communal dens (in winter); territory 30-350 m radius, activity usually within 15 m of den[3]	Primarily nocturnal or crepuscular[3], semi-aquatic[3], and active year-round[3.] Fossorial (underwater burrows)[2]. Main predators are raccoons[3] and minks[2,3]. Other predators; foxes and weasels[3]
American beaver	*Castor canadensis*	87.5-117 cm, 13.6-32 kg[2,4]	10-20 yrs[2,4]	Herbivore[2,3,4]	Native to all locations visited[1]; common around streams and lake shores near deciduous or mixed forests[2,3]	Colony or family unit (1-12, avg 5 animals)[2,3], on 805 m radius [3]	Primarily nocturnal, occasional crepuscular[3,4], fossorial (underwater burrows or dams)[3]
American mink	*Neovison vison*	M: 52-70 cm, 567-1,600 g F: 42-57 cm, 665-1,100 g[2,4]	Max: 10 yrs[4] Avg: 3-4 yrs[11]	Carnivore[2], shrews, mice, voles, rabbits, and muskrats (species of interest).	Native to all locations visited[3,4]; found along streams, rivers and lakes with brush or rocky cover nearby[2,3,4]	Solitary[3,4] except family groups or mated pair[3]. Females live on 7.8-20.4 ha and males 3.2-8.1[3]	Mainly nocturnal[2,3,4], but can forage during the day (especially in winter when caring for young)[3]. Well-adapted semi-aquatic species[3,4]. Foxes occasionally kill minks[3]
Short-tailed weasel	*Mustela erminea*	M: 27.7-33 cm, 67-116 g F: 17-25.5 cm, 25-80 g[2,4]	Max: 7 yrs Avg: 1-2 yrs[3,4]	Carnivore[2], voles and mice mainly, squirrels, shrews, and rabbits less[3,4]	Native to all locations visited[7,8]; primarily riparian woodlands, marshes, and open areas near forests or shrubs[2,4]	Solitary[3,4], on 4-200 ha[3] (avg: 10-40)[3,4], and may travel up to 15 km in one night[4]	Mainly nocturnal[2], active year-round[3]. Good climbers and swimmers[3]. Occasional predators include foxes and other mustelids

Common name	Scientific name	Average size	Lifespan	Diet	Geographical range and habitat	Behavior and home range	Other info
Red fox	*Vulpes vulpes*	92-107 cm, 3.6-7.7 kg[2,3], males are slightly larger[3,4]	Max: 15 yrs Avg: 3-6 yrs[3,4]	Mainly carnivore[2], also fruits, berries, and insects[2,3,4] Mice, voles, squirrels, and rabbits[2,3,4]	Native to all locations visited[1]; prefers broken sparse or edge areas[2,4] such as urban areas, farmlands, mixed shrub, or woodland, and meadows[3,4]	Mated pairs stay together for a year[2,3], otherwise solitary[3,4] on 57.5-1,200 ha[3,4] dispersal of young 10-400 km[4]	Mainly nocturnal, but can be active in dusk or dawn[2,3,4]. Active year-round[3].
Raccoon	*Procyon lotor*	65.5-104 cm, 4.1-16.4 kg[2,3]	Max: 21 yrs Avg: 5-6 yrs[3,4]	Omnivorous, opportunistic [2,3,4]. Mice, voles, rabbits, muskrats and squirrels[3,4]	Native to all locations visited[1]; prefers woodlands, or farmlands, along streams and lakes[2,3] also urban areas[3]	Solitary or family groups (female with young or siblings)[2,3], on 5-5,000 ha (avg: 40-100)[3,4]	Primarily nocturnal[2,3,4], active year-round but partial hibernation in winter[2,3,4]. Occasional predator, foxes[3]
Striped skunk	*Mephitis mephitis*	46.5-81.5 cm, 0.7-6.3 kg[2,3,4]	Max: 10 yrs Avg: 1-6 yrs[3,4]	Omnivorous[2,3] opportunistic[3] Mice, voles, and carrion[3,4]	Native to all locations visited[1]; prefers woods, brushland, open grassland, farmland or urban, close to water[2,3,4]	Solitary[3,4], on 0.02-551 ha[3,4]	Nocturnal[2,4], remain in den during winter[3,4]. Foxes occasionally kill and eat skunk[3,4]
Feral cats	*Felis catus*	76.2 cm, 4.1-5.4 kg[4]	Avg captive: 14 yrs[4] Feral or outdoor 2-5 yrs[9,10]	Carnivore[4], rodents.	Non-native, found in all locations[1,4]; proximity to current or past human habitation[4]	Solitary, or family groups[4]; on 6.1-60.7 ha that often overlap[4]	Territorial, active throughout the day but often mainly nocturnal in the wild[4]. Can fall prey to many different predators[4]

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Figure A.1. Map for all Saskatchewan locations from which rodents, lagomorphs, mesocarnivores, mosquitoes or any combination thereof are available

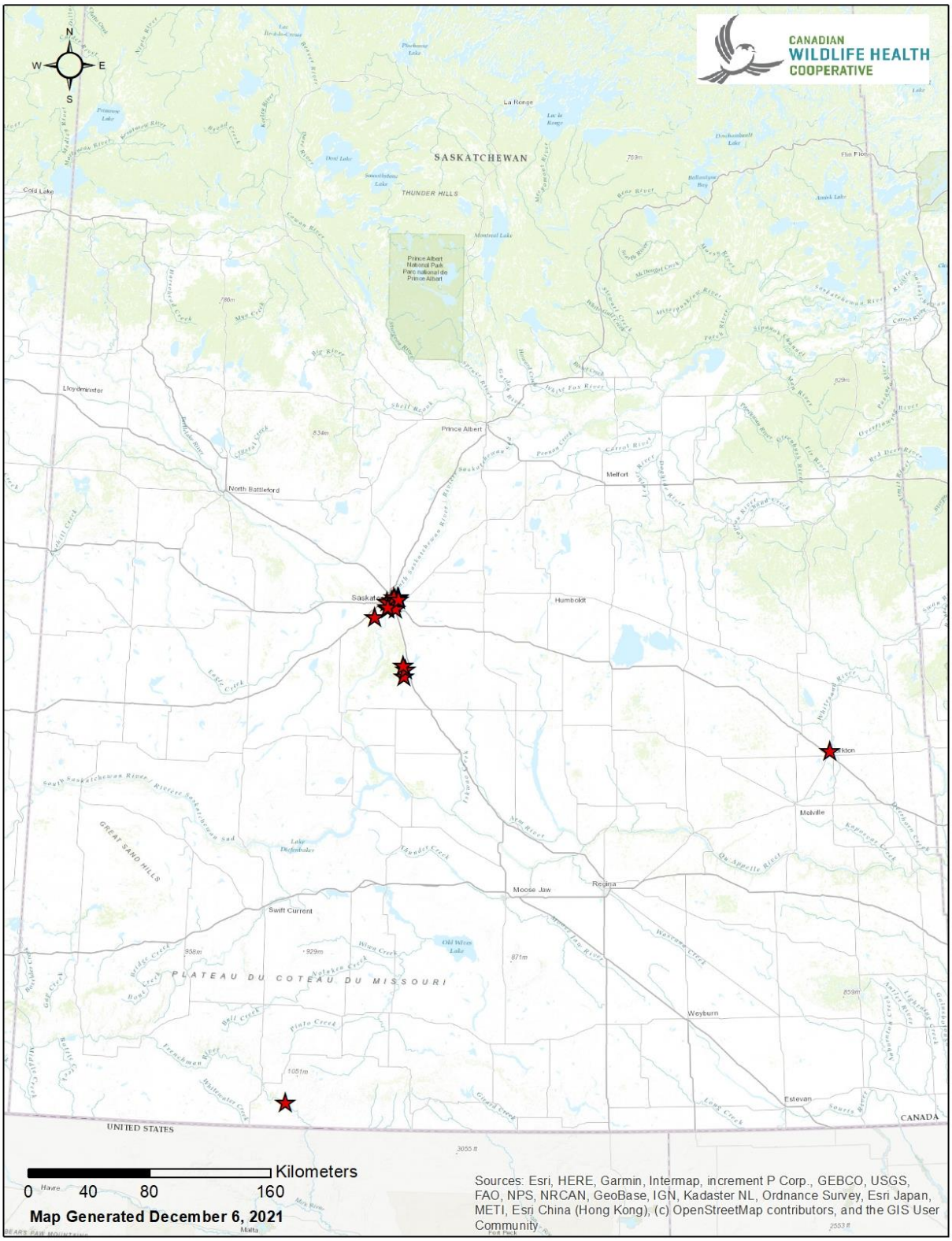
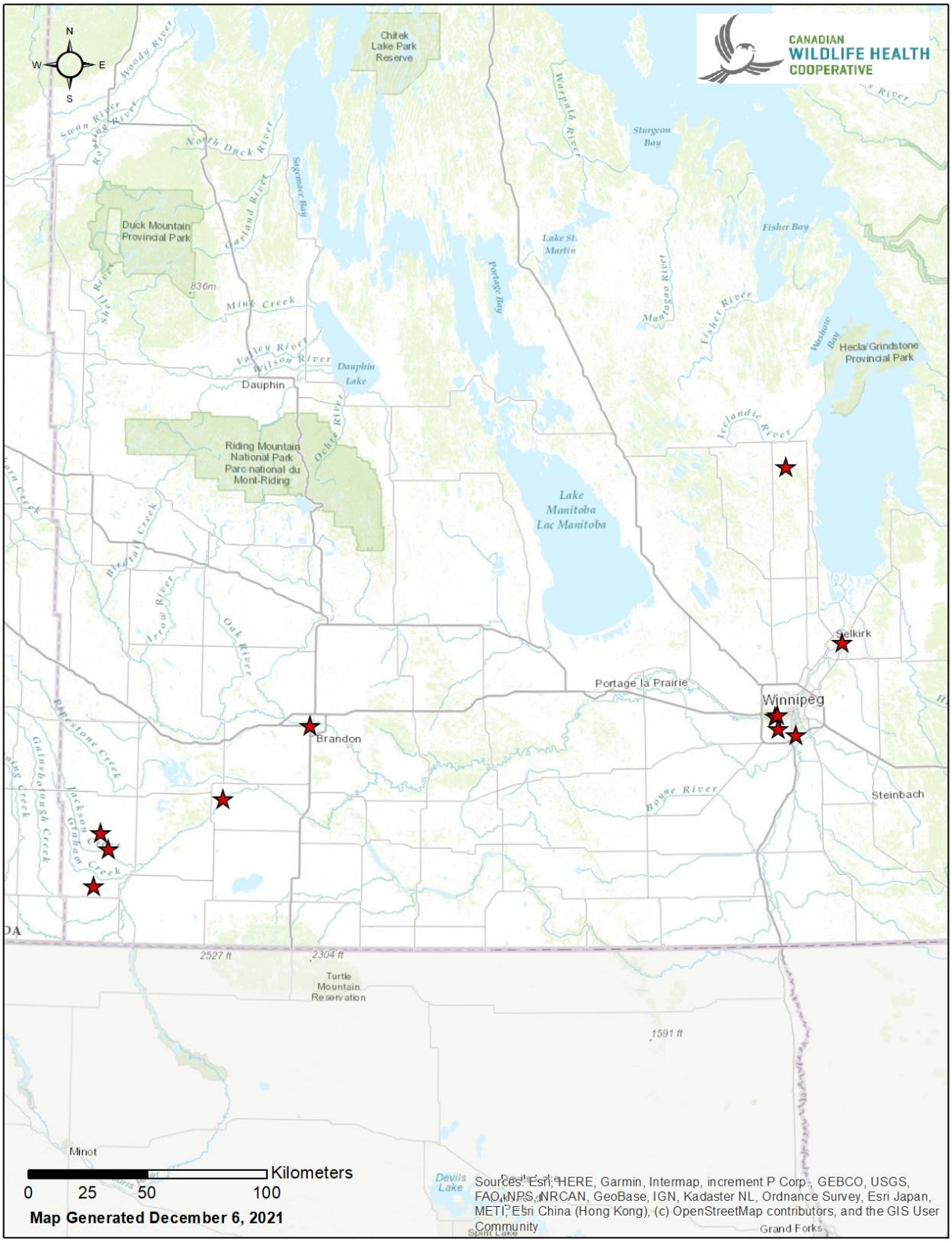


Figure A.2. Map for all Saskatchewan locations from which rodents, lagomorphs, mesocarnivores, mosquitoes or any combination thereof are available



Trapping Methodology

1. Small rodents (species weighing less than 50 g)

Humane and safe live trapping of small rodent species using Shermann traps. Care was taken to reduce stress and to prevent injury to the animal.

i. Equipment and materials

- Shermann trap
- Bait (peanut butter and a piece of apple or carrot)
- Nest material (cotton ball)

ii. Procedure

a. Identify location of adequate activity of the target species.

b. Set the Shermann trap

   a. Push the front plate down
   b. Position the plate so that the metal lip is just barely holding it down
   c. Ensure there is no objects under or interfering with the trigger plate in the middle of the trap.
   d. Place cotton ball and a peanut butter smeared apple or carrot at the back of the trap.

c. Place trap in the selected area.

d. Traps will be set in the evening, up to two hours prior to sunset and checked the next morning, within three hours of sunrise. If a target species is present, they will be removed and restrained for the required procedures. If a non-target species is present, they will be released.

e. Animals are induced with isoflurane by utilizing a small mammal mask as a gas induction chamber. Once righting reflex has been loss, they are connected to a small rodent anesthesia mask for the remainder of the procedure.

f. Physical exam is done, noting sex, age, and overall condition of the animal. Proper plane of anesthesia is confirmed by pinching one of the feet and observing for withdrawal reflex. Once unconsciousness confirmed, blood is extracted using a 1mL syringe and 22-gauge needle, inserting into the heart either through the side of the thorax (with the animal on its right side) or going under the sternum (with the animal

on its back). Ectoparasites can be collected before or after euthanasia. Blood and ectoparasites are placed in respective tubes, red top tubes for blood and cryovials for parasites.

g. Euthanasia is performed intracardiac or intraperitoneally with 0.25 mL of pentobarbital (Euthanyl, 240 mg/mL). Top up is done if needed. Death is confirmed by auscultation (with a pediatric stethoscope) followed by cervical dislocation.

h. White medical tape with the animal's study number is placed on the tail or hindlimb; the body and tubes are placed in portable cooler.

2. Squirrels, both ground and tree (weight between 100 g and 1 kg)

Humane and safe live trapping of squirrel species using Tomahawk or burrow traps. Care is taken to reduce stress and to prevent injury to the animal.

i. Equipment and materials

- Tomahawk or burrow trap
- Bait (peanut butter)

ii. Procedure

a. Identify location of adequate activity of the target species.

b. Set the trap

a. Tomahawk trap instructions

i. Release roll hooks that are holding the trap together

ii. Lift the handle to open to the trap's working shape.

iii. Secure backdoor by using roll hooks.

iv. Move U-bar down into position and hold in place with roll hooks.

v. Secure U-bar with roll clips.

vi. Place trap in selected area

vii. Place bait along plate and set trap

b. Burrow trap instructions

i. Ensure trap has no sharp or jutting edges on the inside

ii. Find holes of similar sized as the trap

iii. Place trap so that it rests comfortably in the hole (not buried too far in that it would be difficult to remove, but enough that it does not fall out when animal nudges it)
iv. Ensure the sides of the trap are flush with the hole so no animal can squeeze out
v. Baiting the back of the burrow trap is optional

c. Traps will be checked at a minimum of every hour. If a target species is present, they will be removed and restrained for the required procedures. If a non-target species is present, they will be released. It is recommended to wear gloves while transporting traps containing animals back to processing area.
d. If multiple animals were caught, place sheet over traps to reduce exposure to sun or heat and to lower stress while processing.
e. Animals are moved from the trap to a pillowcase for restraint. Swiftly, the animal is restrained through the cloth using thumb and index on either side of the head, behind the ears. Once restrained, the fabric of the pillow is pulled back to apply an anesthetic mask.
f. Animals are induced with isoflurane. Proper plane of anesthesia is confirmed by pinching one of the feet and observing for withdrawal reflex. A physical exam is done, noting sex, age, and overall condition of the animal. Once full unconsciousness is confirmed, blood is extracted using a 3 mL syringe and 22-gauge needle, inserted intracardiac either through the side of the thorax (with the animal on its right side) or going under the sternum (with the animal on its back). Ectoparasites can be collected before or after euthanasia. Blood and ectoparasites are placed in respective tubes, red top tubes for blood and cryovials for parasites.
g. Euthanasia is performed intracardiac with 0.5 mL of pentobarbital (Euthanyl, 240 mg/mL). Top up is done if needed. Death is confirmed by absence of heartbeat with a stethoscope.
h. White medical tape with the animal's study number is placed on the hindlimb; the body and tubes are placed in a portable cooler.

3. Rabbits

Humane and safe live trapping of lagomorphs using Tomahawk traps. Care is taken to reduce stress and to prevent injury to the animal.

i. Equipment and materials

- Tomahawk trap
- Bait (alfalfa, carrots, apples, or all three)

ii. Procedure

a. Identify location of adequate activity of the target species.

b. Set the Tomahawk trap

   a. Release roll hooks that are holding the trap together
   b. Lift the handle to open to the trap's working shape.
   c. Secure backdoor by using roll hooks.
   d. Move U-bar down into position and hold in place with roll hooks.
   e. Secure U-bar with roll clips.

c. Place trap in the selected area.

d. Provide appropriate bait material and coverage from element.

e. Traps will be checked at a minimum every day. If a target species is present, they will be removed and restrained for the required procedures. If a non-target species is present, they will be released.

f. Animal weight is estimated, and combo of 5 mg/kg ketamine (Narketan, 100 mg/mL) and 0.15 mg/kg dexmedetomidine (Dexdomitor, 0.5 mg/mL) is administered intramuscularly using pole syringe. Sedation is supplemented with oxygen, and isoflurane as needed.

g. Physical exam is done, noting sex, age, weight, and overall condition of the animal. Blood is taken using a 3 mL syringe and 22-gauge needle, and ectoparasites are placed in respective tubes, red top tubes for blood and cryovials for parasites. The tubes are labelled and placed in a cooler.

   - Animal is euthanized with 2 mL of pentobarbital (Euthanyl, 240 mg/mL), either intracardiac or any vein that may be available. The body is posited in a bag that is identified by white medical tape with the animal's study number, before it is placed in a cooler.

h. Traps are rebaited and placed in the same location if it is believed there are other target species or re-located to another area.

4. Mesocarnivores

Humane and safe live trapping of mesocarnivores using Tomahawk traps. Care is taken to reduce stress and to prevent injury to the animal.

i. Equipment and materials

- Tomahawk trap
- Bait (canned fish, peanut butter, eggs, raw meat, or combination)

ii. Procedure

a. Identify location of adequate activity of the target species.
b. Set the Tomahawk trap
    a. Release roll hooks that are holding the trap together
    b. Lift the handle to open to the trap's working shape.
    c. Secure backdoor by using roll hooks.
    d. Move U-bar down into position and hold in place with roll hooks.
    e. Secure U-bar with roll clips.
c. Place trap in the selected area.
d. Provide appropriate bait material and coverage from element.
e. Traps will be checked at a minimum every day. If a target species is present, they will be removed and restrained for the required procedures. If a non-target species is present, they will be released.
f. Animal weight is estimated, and combo of ketamine (Narketan 100 mg/mL) and dexmedetomidine (Dexdomitor 0.5 mg/mL) or medetomidine (Domitor 1 mg/mL) is administered intramuscularly using pole syringe (see table A1).
g. Sedation is supplemented with oxygen, and isoflurane as needed. The animal's plane of anesthesia is monitored throughout the procedure by assessing relevant reflexes, heartrate, and respiratory rate.
h. A physical exam is done, noting sex, age, weight, and overall condition of the animal.

i. Blood is collected via venipuncture of the jugular, cephalic or femoral vein, with a 3mL syringe and 22-gauge needle, then placed in a red top tube. Care is taken to not collect more than 1 % of body weight. Ectoparasites are collected and placed in cryovials. Both tubes are labelled and posited in a cooler.

j. Once blood and ectoparasites are collected, the animal is identified with a visible shaved area or cattle marker then given atipamezole (Antisedan 5 mg/mL or Revertor 5 mg/mL) intramuscularly. The animal is subsequently placed inside the now wired opened trap with a blanket or overlying vegetation for shade in the same location it was caught. The animal is monitored at a distance until it wakes and can leave.

k. Traps are rebaited and placed in the same location if it is believed there are other target species or re-located to another area.

5. Mosquitoes

i. Equipment and materials

- BG-Sentinel mosquito trap (see Figure A.3. for parts)
- BioQuip 12 volt battery
- Bait: continuous CO_2 flow into the trap from cannister

ii. Procedure

a. Identify location of adequate activity of the target species.

b. Set the mosquito trap

a. Release the strap holding the trap collapsed, letting it unfold

b. Place the battery inside the trap body, and connect to fan via battery cables; ensure the blades of the fan are moving

c. Place a catch bag on the intake funnel, opposite the flap entrance

d. Twist in-place the intake funnel to the opening above the fan

e. Secure the white cover of the trap, and ensure the suction of the fan blades is opening the intake funnel flap

f. Secure the CO_2 cannister to a solid object with straps and cinches (e.g., large tree, fence post, etc.)

g. Open the valve of the CO_2 cannister and confirm pressure is adequate

h. Insert the end of the tube into the side of the mosquito trap, ensuring the CO_2 is flowing freely into the trap

i. Rain shield and poles available as needed

c. Place trap in the selected area.

d. Traps will be checked at a minimum every day. If catch bag contains target species, the bag is removed and closed.

e. A new catch bag is placed, battery is switched as needed and the CO_2 cannister is assessed to ensure enough gas content

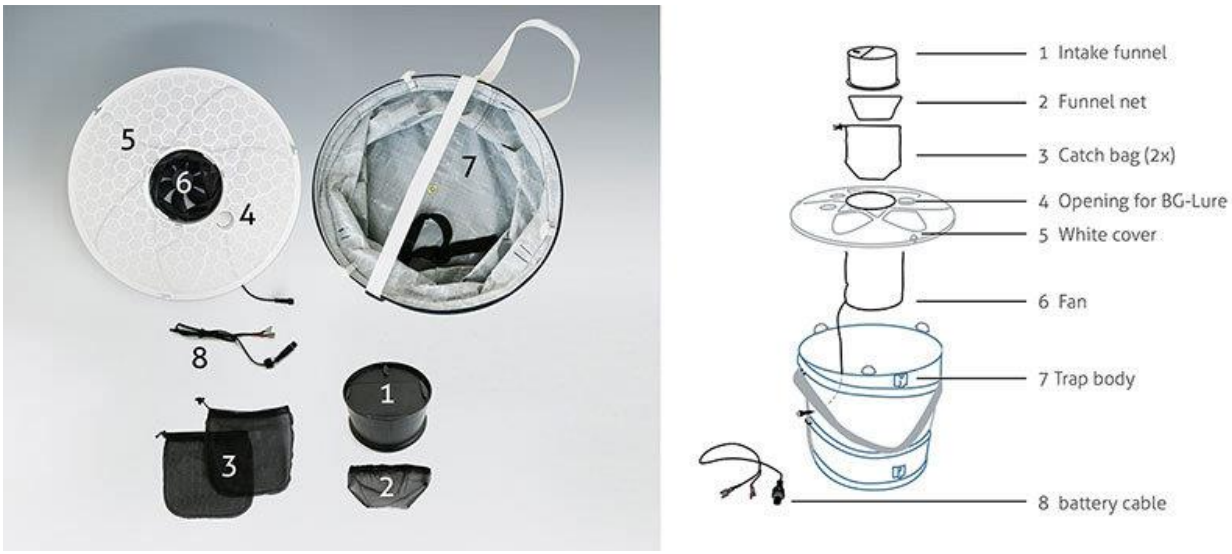


Figure A.3. Schematic of BG-Sentinel 2 mosquito trap https://eu.biogents.com/wp-content/uploads/BG-Sentinel2-basic-version-numbers-with-explosion-72dpi.jpg

6. Safety

Personal protective equipment will be worn as necessary for each species

- Small rodent species: gloves and a N95 or N100 mask are required while handling both animals and traps, or while cleaning equipment
- Squirrels and rabbits: gloves are required while handling animals, and both gloves and a N95 or N100 mask are required while cleaning equipment
- Mesocarnivores: thick leather gloves required for all mesocarnivores, plus eye protection and coveralls for skunks, gloves, and mask while cleaning of equipment

Proper functioning of the traps must be verified prior to setting the traps to reduce the risk of injury to the animals.

Any improper functioning or injuries must be reported to the primary investigator.

It is the responsibility of the principle investigator to ensure that all personnel involved in trapping and handling the equipment are properly trained to do so appropriately and according to the protocol.

All personnel involved are required to have up-to-date immunization, including rabies.

Standard Operating Procedures for Processing and Testing of samples

Serology

Preparation of Blood for Serum Collection and Storage

Blood collected from the field or provided by the WCVM's wildlife clinic is brought back to the laboratory where it is processed.

A. Blood should be allowed to sit in the red top tube for a minimum of 30 min to insure proper clotting. If the blood appears "runny," it should rest for a further 10-15 min. Should the blood remain abnormal in color or viscosity, a note should be made prior to moving on to the next step.
B. The vial is placed in a centrifuge and the blood spun at 2500 rpm for 20 min.
C. Two cryovials per sample are prepared; the first cryovial is 1.5 mL and labelled with the study number only, while the second (any size available) is labeled with the study number, date, and "serum."
D. A minimal amount of 0.1mL of serum is necessary to run the MAT test and is dispensed into appropriately labelled cryovials using an automatic pipette dispenser. In the event the amount of serum is insufficient (e.g., dehydrated, or diminutive animals), combination of animals' sera were combined, and which study numbers were merged was noted.
E. The cryovials are placed in labelled boxes for storage in a -80^{o}C freezer pending testing

Microagglutination Test (MAT)

The MAT was performed at the National Microbiology Laboratory in Winnipeg following the same protocol as outlined in Sato et al. (1990).

Molecular Testing

Genomic DNA extraction

Genomic DNA was extracted from approximately 25 mg of pooled liver, kidney, spleen, and lung of wild animals using Qiagen DNeasy Blood and Tissue Kit (Qiagen) as recommended, except incubation overnight at 56 °C instead of 10 min. DNA quality and concentration were determined using NanoDrop 2000/2000c spectrophotometer (Thermo Scientific, Waltham, MA).

Wild Animals and Screening for Candidate Targets

Tissue obtained from confirmed cases of animal tularemia (Prairie Diagnostic Services, Saskatoon, SK): (1) American red squirrel (ARS), based on culture, histopathology, and immunohistochemical staining using anti-*Francisella tularensis* antiserum; and, (2) white-tailed jackrabbit (WTJR), based on culture and microagglutination test were used in initial evaluation by conventional PCR using *F. tularensis*-specific FopA (43 kDa outer membrane protein A) and lpnA (17 kDA lipoprotein A) genes as described by Wang et al. (2011). Visualization on 1 % agarose gel following fractionation and nucleic acid staining showed product size of 409 bp and 410 bp for FopA and lpnA, respectively. Primer specificity and sequence similarity of isolate from ARS showed a 100 % query coverage and an e-value of 0.0 in BLASTn (hppt://blastncbi.nlm.nih.gov/Blast.cgi), and BLASTn Megablast, and matched 99.72% identity to *F. tularensis* strain 410112 fopA gene partial coding sequences of GenBank accession No. HM371347.1. Similarly, the WTJR showed BLASTn match of 100 % identity to *F. tularensis* subspecies *holarctica* isolate ad FopA gene partial cds KF607098.1. Amplifiable DNA availability in each sample was re-determined by housekeeping genes: YWHAZ (ground squirrel; Williams et al., 2011; Otis et al., 2010), and COX1 (artic hare; Halanych et al., 1999;

Tobe et al., 2009). GenBank accession No. HM371347.1 has been identified by canSNP as *F. tularensis* subspecies *holarctica* B.16 lineage (Wang et al., 2014; Lu et al., 2016).

Real-time PCR

I. Real-time PCR Amplification and Melting Point Analysis

Real-time PCR amplification was performed in 0.2 ml, thin wall, clear tubes (Axygen, Inc. ON, Canada) in CFX Connect PCR system (Bio-Rad Laboratories, Inc., CA, USA) in a 20 ul volume containing 2x IQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., CA, USA), 10 uM each primer, and 2 ul of DNA template. All samples were run in duplicate, and included negative control, no template control and positive control. Thermal cycling was performed with following conditions: 95 °C for 3 min., 40 cycles of 95 °C for 10 sec., 65 °C for 30 sec., 72 °C for 30 sec. (Fujita et al.,2006); followed by melting point analysis with 0.5 C increments (65 C – 95 C). Amplification and melt curve data were analyzed using CFX ManagerTM Software 3.1 (Bio-Rad laboratories, Inc. CA, USA). An amplicon is determined as *Francisella tularensis* positive based on mean Ct value ≤ to 37.0 °C, and valid melt peak and melt temperature (Tm) 82.0 °C. Any amplicons that were determined positive were confirmed by Sanger sequencing and Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cg).

II. Sanger Sequencing and Sequence Assembly and BLAST Analysis

Francisella tularensis-positive amplicons were purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany). Purified amplicons with 5mM primers were Sanger sequenced (Macrogen, South Korea). Generated Sanger sequences were processed and assembled using software PreGap4 and Gap4 (Staden Package version 3.3; http;//staden.sourceforge.net/). Generated consensus sequences were matched for optimized high sequence similarity (megablast) with nucleotide database BLASTn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi, National Center for Biotechnology Information, MD, USA).

III. Construction of Positive Control Plasmid DNA

Synthetic gene target for real-time PCR were designed (Sigma) and used as positive control. FopA amplicon was generated (Bio Basic Inc., ON, Canada) from fopA gene partial coding sequences (GenBank accession No : HM 371347.1) cloned into pUC vector (GenBank

Accession No. Y14837.1) according to standard cloning methods. Two microliters of recombinant plasmid (pUC57-fopA) were transformed into competent Escherichia coil DH5α cells and inoculated into Lurin-Bertani plates containing 100 ul/ml Ampicillin. 0.1 mM IPTG and 0.006% X-Gal, and incubated overnight at 37 °C. Single, white colonies containing the plasmid was inoculated into LB + Ampicillin broth overnight at 37 C, and plasmid was purified using min-prep plasmid extraction kit. 5 ul of purified plasmid was ligated overnight into pGEM-T easy vector system (Promega, WI, USA) using DNA T4 ligase and confirmation of plasmid creation by visualization in 1 % agarose gel. The plasmid concentration was determined by measuring absorbable at 260 and 280 nm in spectrophotometer (NanoDrop 2000/2000c, Thermofisher Scientific)

The sequence similarity showed a 54 % query coverage, e-value of 0.0, and 100 percentage identity in BLASTn using Megablast (hppt://blastncbi.nlm.nih.gov/Blast.cgi), to *F. tularensis* strain 410112 fopA gene partial coding sequence of GenBank accession No. HM371347.1.

Analytical Sensitivity of Real-time PCR

Standard curves were generated to determine the sensitivity of the optimized singleplex real-time PCR using pUC57-fopA gene. Ten-fold serial dilutions of gene construct was prepared in 50mM tris (pH 8.0) and in eight replicates, with concentrations ranging from 2 pg – 2 gg. Each standard curve was analysed on linear regression parameters: coefficient of determination, goodness-of-fit (R2); reaction efficiency (E); slope and y-intercept to reveal linearity, repeatability, and analytical sensitivity of the assay. The number of chromosomal copies for a particular concentration of the gene construct being tested in the real-time PCR that multiplies a single-copy, double stranded DNA (dsDNA) target, the calculation was used: copy number = (mass of dsDNA in grams/(bp length of dsDNA chromosome x 650g/mol) x (Avogadro's number of ~6.022x1023 molecules/mol) as highlighted in Larson et al., 2020.

Table A.3. Gene target and program used for each

Gene Target	Sequence (5'-3')	Program	Reference
fopA	F: CTTGAGTCTTATGTTTCGGCATGTGAATAG R: CCAACTAATTGGTTGTACTGTACAGCGAAG	95 C for 3 min., 40 cycles of 95 C for 10 sec., 65 C for 30 sec., 72 C for 30 sec.	Wang et al., 2011; Fujita et al., 2006
lpnA	F: GCTGTATCATCATTTAATAAACTGCTG R: TTGGGAAGCTTGTATCATGGCACT	95 C for 3 min., 40 cycles of 95 C for 10 sec., 65 C for 30 sec., 72 C for 30 sec.	Wang et al., 2011; Fujita et al., 2006