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15 RH: Kinnunen et al. • Toxoplasma Gondii in Squirrels

16 No Evidence of *Toxoplasma Gondii* Infection in Urban and Rural Squirrels in Southern 17 Manitoba, Canada

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ABSTRACT

When wildlife colonizes cities, they can bring parasites that have implications for human health, yet knowledge underlying the ways host-pathogen interactions operate in cities is limited. The Coccidian parasite *Toxoplasma gondii* can infect humans and cause health issues. *T. gondii* also has host species that occur at higher densities in cities than in natural environments, including squirrel species (Sciuridae). Cats and other Felidae are the only known definitive hosts of *T. gondii*. In urban and suburban areas squirrels regularly share their territories with domestic cats where they can encounter infectious oocysts shed in cat feces in contaminated soil or in the food they eat. We hypothesized that urban squirrels might thus be particularly susceptible to *T. gondii* infection compared to squirrels in more natural areas. We investigated this using molecular and serological methods on samples collected from four squirrel species in and around the city of Winnipeg, Manitoba, Canada. We tested a total of 272 tissue samples from 46 squirrels for *T. gondii* DNA using quantitative PCR, and 15 serum samples from grey squirrels (*Sciurus carolinensis*) for *T. gondii* antibodies (IgG) by indirect ELISA. We found no evidence of *T. gondii* infection in squirrels in southern Manitoba. This suggests that squirrels are not important intermediate hosts of *T. gondii* in cities and that the prevalence of *T. gondii* oocysts in the environment in Manitoba is likely low. Consequently, squirrel management to prevent infection to humans or their pet cats is not needed in urban areas with abundant squirrel populations.

KEY WORDS host-parasite interaction, Sciuridae, squirrel, Toxoplasma gondii, urbanization

INTRODUCTION

When wildlife colonizes cities, they can bring parasites that have implications for human health (Mackenstedt et al. 2015). Indeed, previous work has found increased levels of wildlife parasitism in cities compared to rural areas (Deplazes et al. 2004; Reperant et al. 2009; Lehrer et al. 2010; Giraudeau et al. 2014). This increased level of parasitism could reflect both the increased population density of urban host species and higher within and between species contact rates in response to resource provisioning in cities relative to rural areas (Gliwicz et al. 1994; Bradley and Altizer 2007). Contrasting this pattern, in some cases the overall species richness and diversity of parasites can be reduced in cities as parasites with one or a few host species may become extirpated in conjunction with their host species (Bradley and Altizer 2007). Parasites that spread through direct contact or oral-fecal routes are likely to be favored in urban areas (Bradley and Altizer 2007) but knowledge underlying the way host-pathogen interactions operate in cities is still limited (Mackenstedt et al. 2015).

The Coccidian parasite *Toxoplasma gondii* is an interesting parasite within the context of emerging urban ecosystems. This is because it can infect humans and cause health issues, and it has multiple host species that occur in high densities in cities including pets and rodents, with wild and domestic cats (Felidae) being the only known definitive hosts (Elmore et al.

2010). A significant proportion of the human population globally is infected with *T. gondii*, but most healthy people do not experience symptoms of infection (review by Tenter et al. 2000). However, immunocompromised people and pregnant women require medical intervention to avoid serious health issues (Dixon 1992; Tenter et al. 2000; Hill et al. 2005). Consequently, further knowledge of *T. gondii* infection dynamics in cities is needed.

T. gondii has three infectious stages: the tachyzoites, the bradyzoites, and the sporozoites (Hill et al. 2005). The ovoid-shaped tachyzoite can be found inside a host's cells surrounded by a parasitophorous vacuole that protects it from the host defense mechanisms (Hill et al. 2005). This is an active infection stage where the tachyzoites increase within the host body and spread via the bloodstream. After pressure from the host's immune system, intracellular tissue cysts called bradyzoites then form, generally in visceral organs and neural and muscular tissue- particularly the brain, eye, and skeletal and cardiac muscle (Berdoy et al. 2000; Tenter et al. 2000; Hill et al. 2005). This is a chronic infection stage, where the bradyzoites remain within host tissue possibly for the rest of the host's life. When cats feed on infected intermediate hosts, such as rodents, the bradyzoites are released and spread to the intestinal epithelium of the cat for sexual reproduction (Pappas et al. 2009). When a gamete is fertilized, a wall forms around it, making the formed oocyst highly resistant to environmental changes (Dubey et al. 1970). Following this hardening of the oocyst, domestic cats and other Felidae then spread them in the environment in their feces: millions of oocysts can be shed daily for the duration of one to two weeks (Dubey 2001; Fayyad et al. 2016). Each oocyst contains two

sporocysts that each contain four sporozoites—the last infectious stage of *T. gondii* (Hill et al. 2005). Cats and intermediate hosts can become infected by consuming carcasses infected by *T. gondii* cysts, or by coming into contact with oocyst contaminated soil, water, or food.

Urbanization can increase the risk of an animal being exposed to *T. gondii* (Conrad et al. 2005; Lehrer et al. 2010; Ballash et al. 2015). Many squirrel species (Sciuridae) are ubiquitous in cities, where they are commonly found at much higher densities than in natural environments (Parker and Nilon 2008). In urban and suburban areas squirrels regularly share their territories with domestic cats (Baker et al. 2008; Sims et al. 2008) and collect and cache their food in backyards and gardens where they can easily encounter infectious oocysts shed in cat feces in contaminated soil or in the food they eat. This may make urban squirrels particularly susceptible to parasite infection compared to their rural counterparts. After being infected squirrels act as intermediate hosts for the parasite, and the parasite can remain within the host body in tissue cysts for the rest of the host's life. The infection can be asymptomatic, or squirrels can acquire toxoplasmosis (Dubey et al. 2006; Jokelainen and Nylund 2012). Many *T. gondii* strains isolated from nature are of low virulence, leading to subclinical, mild toxoplasmosis that may not kill the animal, but can make prey—such as a squirrel—susceptible to predation by cats (Dubey and Frenkel 1973), thus enabling the parasite to complete its life cycle (Dubey and Frenkel 1973; Dubey et al. 2006). Squirrels may thus act as a source of infection to cats, in a similar way to other prey species (Afonso et al. 2007). As such squirrels may play a role in *T. gondii* population and infection dynamics in cities. Our

aim in this paper was to survey the prevalence of *T. gondii* infection in squirrel (Sciuridae) populations in and around the city of Winnipeg, Manitoba, Canada. We specifically asked whether squirrels are important intermediate hosts of *T. gondii* and whether *T. gondii* infection is more common in a city than in more natural habitats.

T. gondii has been found in many wild animals particularly in farms and natural areas (e.g., Tizard et al. 1978; Smith and Frenkel 1995; Gray fox (*Urocyon cinereoargentus*), Lindsay et al. 2001; Red fox (*Vulpes vulpes*), Wanha et al. 2005), yet we know relatively little about the prevalence of *T. gondii* in wild animals in cities (but see e.g., Frenkel et al. 1995; Conrad et al. 2005; Murphy et al. 2008; Lehrer et al. 2010; Mercier et al. 2013; Dubey et al. 2014; Ballash et al. 2015). Squirrels are among the many species capable of getting a *T. gondii* infection, but our knowledge is limited, and studies have mostly focused on acute, fatal cases with little information existing on host-pathogen dynamics during chronic, latent infection (Jokelainen and Nylund 2012). Toxoplasmosis has been found in the Eastern grey squirrel (*Sciurus carolinensis*; Jacobs et al. 1962 (2/24 positive individuals); Walton and Walls 1964 (1/8); Smith and Frenkel 1995 (2/5); Dubey et al. 2006 (3/3)), Western grey squirrel (*Sciurus griseus*; Soave and Lennette 1959 (1/1)), and Eurasian red squirrel (*Sciurus vulgaris*; Jokelainen and Nylund 2012 (3/19); Fayyad et al. 2016 (1/1)). The sample size in these studies has typically been low and none examined the prevalence of *T. gondii* in urban squirrels.

Due to the zoonotic nature of *T. gondii* and its negative effects on pregnant women, children and people with compromised immune systems monitoring of the various sources of infection risk is important. Public health organizations, such as the World Health Organisation recommend epidemiological data collection of *T. gondii*, yet regular monitoring of the infection in humans or animals is rare. We hypothesized that, due to higher population densities of both squirrels and cats in cities, urban squirrels may act as intermediate hosts of *T. gondii* and that urban squirrels will have a higher prevalence of *T. gondii* infection than rural squirrels. We tested these hypotheses by using molecular and serological methods on samples collected from squirrels in and around the city of Winnipeg, Manitoba, Canada.

STUDY AREA

We conducted live trapping of red (*Tamiasciurus hudsonicus*) and grey squirrels (*Sciurus carolinensis*) in one urban and one rural site between 5th June and 1st August 2019 (Fig. 1). The urban site is located in the city of Winnipeg, Manitoba, Canada, and consists of an ~10 ha park located on the University of Manitoba campus and a suburban neighborhood next to the park. The study site is bordered by the Red River and two major highways with high amounts of car traffic. Winnipeg is the largest city in the province of Manitoba with a population of 778,489 and a total land area of 464,33 km^2 (Statistics Canada 2016). Winnipeg lies 239 meters above sea level and has high seasonal climatic variation, with temperature varying from the extremes of around −24 °C to −33 °C between January to March to around +30 °C to +35 °C between June to September (Environment Canada 2020b). The rural site is a ~34 ha

forest patch next to an active honey-farm, near the twin cities Morden and Winkler in southern Manitoba (49°24'01.1"N, 98°00'29.2"W), bordered by agricultural land.

METHODS

We used live traps (Tomahawk Live Trap Co., Tomahawk, WI, USA) to capture squirrels at the study sites. After capture, squirrels were handled in a canvas capture bag and we recorded the weight (g), body and tail length (cm), skull width (cm), age (adult or juvenile), reproductive status, and sex for each individual. We collected a minimum of 500 µL of blood from the femoral vein of each squirrel and stored the sample on ice until processing. Each squirrel was pit tagged between the shoulder blades with passive integrated transponder (PIT) tags. Squirrels were then released at the place of capture. Our protocol was approved by the University of Manitoba animal care and use committee.

We also collected squirrel carcasses from trappers, wildlife rehabilitation centers, and pest control companies during the years of 2017 to 2019. Twenty of the carcasses were from urban locations within Winnipeg, 20 from rural locations approximately 30 to 250 km from Winnipeg (Fig 2). Six carcasses were from unknown locations from Manitoba. We had 25 American red squirrels (*Tamiasciurus hudsonicus*); 16 Eastern grey squirrels (*Sciurus carolinensis*); four Northern flying squirrels (*Glaucomys sabrinus*); and one Least chipmunk (*Neotamias minimus*).

To best detect *T. gondii* whether in an acute or chronic stage and to follow recommendations from previous studies (see review by Galeh et al. 2020), we combined molecular and serological methods to investigate *T. gondii* prevalence in squirrels.

Molecular Methods

Upon necropsy, we collected the entire liver, spleen, brain, heart, kidneys, and lungs, and stored tissues separately at −20 °C until further analysis. As *T. gondii* is a cyst-forming parasite, the detection probability of *T. gondii* can differ between organs (Elmore et al. 2016). Consequently, we tested multiple samples per individual from two to six different organs to maximize the probability of detecting the parasite.

Cell lysis.—Cell lysis was done by first adding 3 ball bearings to each 2 mL screw-cap tube containing 0.6 mL of ATL buffer, and adding 100 mg of frozen tissue or pipetting 0.1 mL of sample (if liquid such as thawed brain) to the tube. We placed the samples in a BeadBeater for 3 minutes after which they were quickly centrifuged. We added 70 µL of Proteinase K to the ATL lysate. We incubated the lysate at +56 °C for 1-3 hours during which the tubes were intermittently inverted several times. We centrifuged the lysate quickly and added 0.6 mL of AL buffer. We inverted the tubes several times and incubated at +70 °C for 10-30 minutes in a dry block. We then again inverted the tubes intermittently several times. The samples were then centrifuged for 3 minutes at 10,000 x *g*. After the completion of the cell lysis, we used 100 µL of the ATL/ProtK/AL lysate to continue the extraction.

Nucleic acid extraction and real-time PCR.—We did nucleic acid extraction for *T. gondii* using 5X MagMAX viral Isolation kit (Applied Biosystems AMB1836-5). Primers and probes for real-time PCR were designed according to the protocol of De Craeye et al. (2011) (see SI Table S1 for primers and probes). PCR product size was 106 bp. We used cellular r18S (ribosomal RNA gene) as an internal control of all PCRs. Real-time PCR was conducted using TaqMan Fast Advanced Master Mix (Applied Biosystem) in Applied Biosystems™ 7500 Real-Time PCR System. Each PCR reaction had concentrations of 10 µM of T2 /F primer, 10 µM of T3/R primer, and 5 µM of probe in the master mix. Thermo cycling Program: Initial denaturation and activation of the Taq polymerase at +95 °C for 2 minutes, followed by 45 cycles at +95 °C for 5 seconds and +60 °C for 33 seconds. We analyzed the results using 7500 System SDS Software.

Serological Methods

We collected a minimum of 500 µL of blood from 15 individual grey squirrels and stored samples on ice. All samples were processed within 12 hours. We centrifuged the collected blood samples at 3500 rpm for 15 minutes and froze the serum at −20 °C until used for testing.

Enzyme-linked immunosorbent assay.—We used enzyme-linked immunosorbent assays (ELISA) to detect serum antibodies (IgG) against *T. gondii.* As species-specific conjugates are not available for squirrels, a commercially available ELISA kit was used for testing the samples (Multi-species ID Screen Toxoplasmosis Indirect kit, IDVet, Grabels,

France) following manufacturer's instructions. Briefly, we diluted samples and negative and positive kit controls in a 1:10 ratio on sample dilution buffer. Then, we transferred 100 µL of the diluted sample to each well by duplicates, incubated the plate for 45 minutes at room temperature, and washed 3 times. We diluted conjugate in a 1:20 ratio and added 100 µL of the conjugate to each well. The plate was then incubated at room temperature for 30 minutes and washed 3 times again. Afterward, we added 100 µL of substrate solution to each well and incubated the plate for 15 minutes in the dark at room temperature. The reaction was stopped by adding 100 µL of stop solution to each well and the plate was read at 450 nm in a spectrophotometer. Results were calculated using the optical density (OD) values of the samples and kit controls and expressed as S/P (Sample to Positive Ratio**)** percentage (S/P%) using the following formula

$$S/P\% = \frac{(OD_{sample} - OD_{NC})}{(OD_{PC} - OD_{NC})}$$

where NC is negative control and PC positive control.

We considered samples with S/P% less or equal to 40% negative; samples with S/P% between 40 and 50% doubtful or inconclusive; and samples with an S/P% higher than 50% positive, following the kit's protocol. The multi-species ELISA kit we used has been successfully used to detect *T. gondii* antibodies in wildlife (Roqueplo et al. 2011; Sharma et al. 2019b), has high sensitivity and specificity, and does not cross-react with other coccidian parasites—a factor known to limit the specificity of serological assays (Hirota et al. 2010).

RESULTS

We tested a total of 272 tissue samples from 46 squirrels from four squirrel species for *T. gondii* DNA using quantitative PCR. *T. gondii* DNA was not detected on any of the 272 tissue samples on any of the organs (liver; heart; brain; lung; spleen; kidney) sampled from any of the 46 squirrels.

We also tested a total of 15 samples of blood sera from grey squirrels for *T. gondii* antibodies (IgG) by indirect ELISA. Two of the samples did not have enough volume for testing. The results were negative—no *T. gondii* antibodies were detected in any sample (see SI Table S2).

DISCUSSION

We hypothesized that, due to higher population densities of both squirrels and cats in cities, urban squirrels may act as intermediate hosts of *T. gondii* and have a higher prevalence of *T. gondii* infection than rural squirrels. However, we found no evidence of *T. gondii* infection in squirrels in southern Manitoba.

Studies investigating *T. gondii* infection dynamics in cities are still relatively rare considering the parasite can infect most mammalian species, including humans, and wildlife can act as reservoirs for the parasite and as sources of infection. Our study is currently one of the very few studies that have explored the prevalence of *T. gondii* in Sciurids in urban areas. One earlier study from Guelph, Ontario, Canada found no evidence of infection across nine

253 locations within and around the city from grey squirrels (*Sciurus carolinensis*, n= 16, the
254 number of urban captures not specified) and chipmunks (*Tamias striatus*, n= 6) using the
255 Sabin-Feldman dye test (Tizard et al. 1978). Using serological techniques with bioassay and
256 PCR together can give a more reliable estimation of infection rate (Galeh et al. 2020), as the
257 distribution of *T. gondii* tissue cysts can be uneven and vary between organs (Elmore et al.
258 2016) which can lead to detection difficulties using PCR-based methods (Opsteegh et al.
259 2010). The sensitivity and specificity of antibody detection methods can also vary, which can
260 lead to misinterpretation of results and possible false negatives or false positives (Gilbert et al.
261 2013). Work in natural or rural areas also suggests *T. gondii* prevalence in squirrels is low
262 (Jacobs et al. 1962; indirect hemagglutination test: 1 of 265, Burridge et al. 1979; Sabin-
263 Feldman dye test: 2 of 11, Smith and Frenkel 1995; PCR: 3 of 19, Jokelainen and Nylund
264 2012).
265
266 *T. gondii* prevalence in Manitoba, in general, is not well known. To the best of our
267 knowledge, no previous survey of *T. gondii* prevalence in squirrels exists from the province.
268 Serological testing in 1981 reported that of 55,527 pregnant women 129 showed signs of a
269 recent *T. gondii* infection (Sekla et al. 1981). The same study also reported that 19 of 72 cats
270 and one polar bear tested positive for *T. gondii*, but results from 28 other species were all
271 negative. An earlier study done in Manitoba in 1976 found that *T. gondii* prevalence in
272 pregnant women in urban areas was 8.17 %, and 6.29 % in rural areas (Shettigara et al. 1976).
273 The prevalence of *T. gondii* can be high in domestic sheep, pigs, and cattle (e.g., Tizard et al.

1978; Fayer 1981; however see Poljak et al. 2008), yet studies from Manitoba or Saskatchewan, another prairie province with a similar climate to Manitoba, have mostly found low prevalence (Nation and Allen 1976; Smith 1991).

The extreme climatic variations in the region may decrease the viability or infectivity of oocysts and tissue cysts in carcasses, therefore decreasing overall *T. gondii* prevalence in the area (Nation and Allen 1976). The temperature in Winnipeg between June to September can reach extremes of +30 °C to +35 °C (Environment Canada 2020b) with daily average temperatures varying from approximately +20 °C to +13 °C (Environment Canada 2020a). *T. gondii* oocysts are highly resistant to environmental variation but sporulation (i.e. infectivity) is dependent on fixed temperatures and factors such as soil moisture that can influence the time oocysts can survive at high temperatures (Dubey et al. 1970). Additionally, winters in Winnipeg can be cold and windy with extreme temperatures of −24 °C to −33 °C between January to March. Oocysts cannot sporulate and become infective after exposure to −21 °C for 1 day or −6 °C for 7 days (Frenkel et al. 1975). After sporulation, oocysts can withstand lower temperatures better, being able to survive at −21 °C for 28 days (Frenkel et al. 1975), yet oocysts can not sporulate if the conditions are unfavorable (Dubey et al. 1970). It is thus possible that the combination of hot summers and cold winters reduces the viability of oocysts in southern Manitoba. Contact rates between domestic cats and *T. gondii* intermediate hosts may also be lower during the winter in Manitoba as people keep their pets indoors in cold weather. Winnipeg has an average yearly precipitation of 521 mm, but the years 2018 and

2019 were unusually dry in southern Manitoba (Government of Manitoba 2020). This could have influenced oocyst survival in the area during data collection, as oocysts survive better in moist than in dry conditions (Frenkel et al. 1975; Lélu et al. 2012).

T. gondii is a cyst forming parasite and the distribution of *T. gondii* tissue cysts can be uneven and vary between organs (Elmore et al. 2016). This may influence the results from molecular methods, and the negative squirrels in our study may have had cysts in other tissues or at a concentration level below the detection limit of the PCR. The sensitivity and specificity of antibody detection methods can also vary—we used ELISA to detect serum antibodies to *T. gondii*, as this method generally has good sensitivity and specificity compared to other serological tests such as the modified agglutination test (Sharma et al. 2019a). As we sampled many different organs and tissues per individual by PCR and used serological methods as an additional test to survey *T. gondii* prevalence in squirrels our results are much less likely to be false negatives.

We note that *T. gondii* antibodies have been found in skunks (*Mephitis mephitis*) and raccoons (*Procyon lotor*) from Saskatchewan (modified agglutination test: average seroprevalence in skunks 15.6%; in raccoons 20.8% in 1999, 12.5% in 2000) and Manitoba (in skunks 28%; in raccoons 27.5%) (Hwang et al. 2007), which implies that although climatic variation may lower the prevalence of *T. gondii* in the prairie provinces of Canada, *T. gondii* is still present in the area. Nonetheless, our results in conjunction with previous studies

suggest that squirrels are often not important intermediate hosts of *T. gondii* in cities and that the prevalence of *T. gondii* oocysts in the environment in southern Manitoba and other prairie provinces is likely low. This knowledge is important as *T. gondii* infection dynamics in cities are relatively unknown, and no previous survey of *T. gondii* prevalence in Sciurids exists from Manitoba.

MANAGEMENT IMPLICATIONS

Cities are now the primary place where people, and our pets, interact with wildlife. Urban wildlife tends to occur in higher densities in cities than in natural habitats and this creates the possibility for increased parasite transmission. When wildlife parasites are a human health concern, such as with *T. gondii*, then management actions are warranted. Our results suggest that squirrels likely do not act as reservoirs for *T. gondii* in cities and therefore do not need to be considered as possible sources of infection to humans or their pet cats. Consequently, no management efforts are needed in cities with abundant squirrel populations.

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549 **FIGURES AND CAPTIONS**

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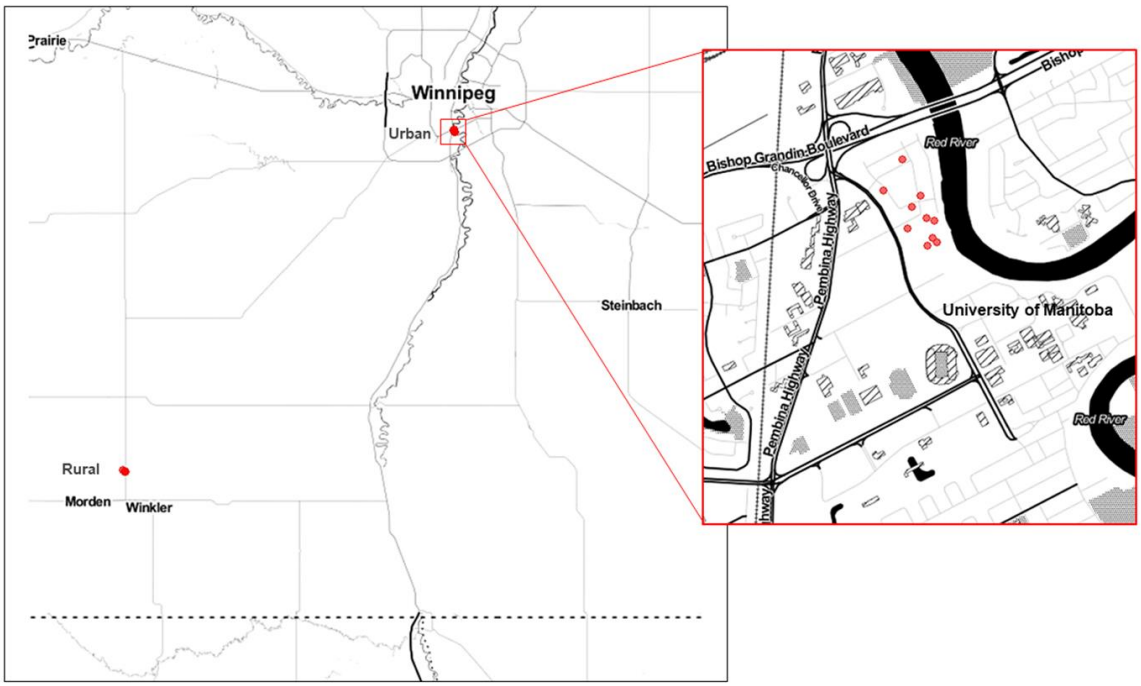

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552 Figure 1. Map of the urban and rural squirrel study sites in southern Manitoba, Canada. Blood
553 was collected from red and grey squirrels from these sites for serological testing of
554 *Toxoplasma gondii* antibodies. The close-up (framed red) shows the 10 urban trapping
555 locations next to the University of Manitoba campus.

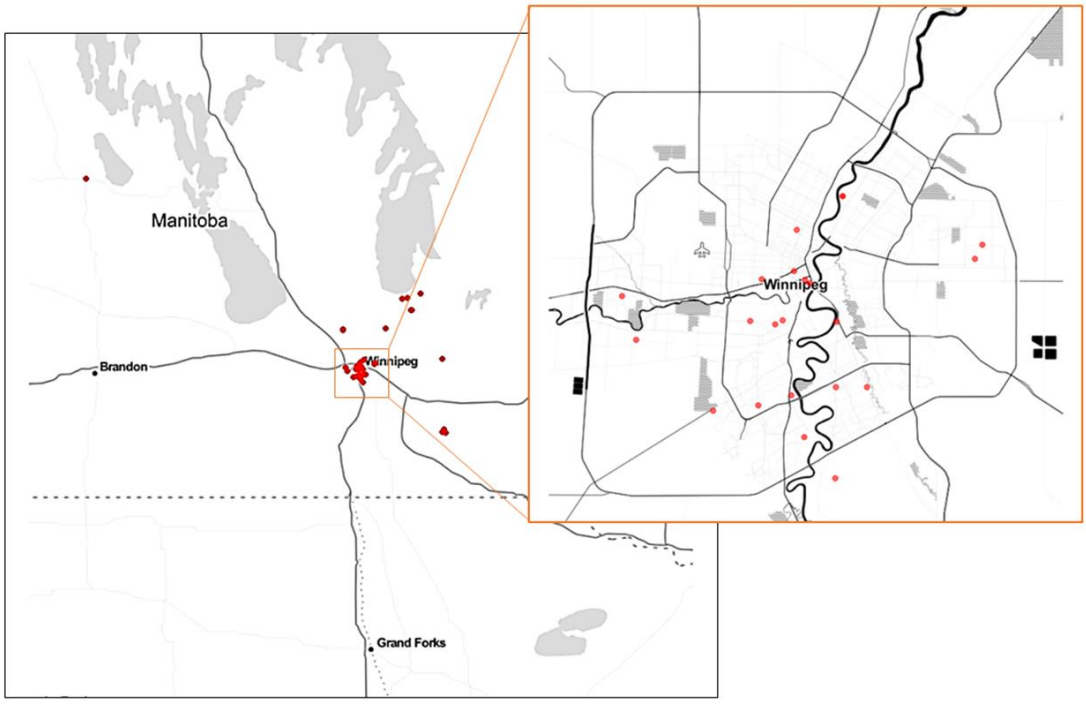

Figure 2. Map showing the sampling locations of squirrel (Sciuridae) carcasses used to collect tissue samples for quantitative PCR detection of *Toxoplasma gondii*. The large map (on the left) shows the urban and rural locations of squirrels from in and around the city of Winnipeg, Manitoba, Canada, and the small map (framed orange) is a close up of the urban locations within the city perimeter.