The Infection Prevalence, Seasonality, Supercooling Point, and Overwintering Survival of *Ixodes scapularis* Say (Acari: Ixodidae) in Manitoba, Canada.

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Abstract

The blacklegged tick *Ixodes scapularis* is an ectoparasite capable of transmitting many different human pathogens. In Manitoba, blacklegged ticks have been expanding their range north and west since the discovery of an endemic population in 2006. Although well studied in Eastern Canada, studies in Manitoba are lacking. To address existing knowledge gaps, I determined the seasonality, pathogen prevalence, overwintering success and cold hardiness of blacklegged ticks in Manitoba. My results indicate immature blacklegged ticks feed synchronously, and *Borrelia burgdorferi* and *Anaplasma phagocytophilum* are quite prevalent at two provincial parks in the province. I also found adult blacklegged ticks have a low degree of cold hardiness, but are still capable of successfully overwintering in Manitoba. This work is the first of its kind within the province, and will add to the growing body of literature on blacklegged ticks which can be applied to risk modelling by public health researcher.

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I wish to dedicate this thesis to my family especially my parents who have supported and encouraged my graduate work.

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Chapter 1: General Introduction

In North America one of the most well studied Ixodidae tick species is the blacklegged tick, *Ixodes scapularis* Say. Blacklegged ticks were historically located in the southern United States (Bishopp and Trembley 1945), however, by the 1970's their range expanded to cover parts of the northeastern United States, and southeastern Ontario (Jackson and DeFoliart 1970; Spielman et al. 1985; Watson and Anderson 1976). In Manitoba, blacklegged ticks were first recorded in 1989 (Galloway 1989), and an endemic population was identified in 2006 (R. L. Lindsay, personal communication) (Figure 1). Blacklegged ticks are now well established in southeastern Manitoba and are continuing to spread north and westward.

The life cycle of blacklegged ticks consists of four active life stages: larva, nymph, and adult male and female (Figure 2). Progression from larva to nymph, and nymph to adult requires blacklegged ticks to feed on the blood of a host. Once fed, engorged blacklegged ticks drop from their host, find a suitable microhabitat, (typically within leaf litter), and either molt to their next life stage or in the case of females, deposit eggs. The entire life cycle from egg to adult can take from two to three years in the American Northeast and Midwest (Hamer et al. 2012b; Yuval and Spielman 1990a) to three or four years as observed in southern Ontario (Lindsay et al. 1998). It is likely blacklegged ticks in Manitoba would display a life cycle closer to three or four years, but Lindsay et al. (1998) suggested the life cycle could be extended to four or five years under certain conditions.

Development of blacklegged ticks in northern latitudes is halted by diapause, which results from unfavorable conditions (Belozerov 1982). Before entering diapause, blacklegged ticks will enter the soil-leaf litter interface as it provides a suitable habitat to overwinter. Overwintering tick survival depends on a variety of factors such as winter temperatures (Lindsay

et al. 1998), humidity (Brunner et al. 2012), snow cover (McEnroe 1984) and habitat (Burtis et al. 2014; Lindsay et al. 1998). The most thorough overwintering studies in Canada were conducted in Ontario (Lindsay et al. 1995; 1998), on the first endemic blacklegged tick population in Canada. Currently, the most northerly endemic populations of blacklegged ticks occur in Manitoba. Overwintering studies in Manitoba would be important because they could provide data critical for models on the northward spread of these ticks.

Blacklegged ticks are important from a public health point of view because they are able to carry and transmit a variety of pathogens. The most well-known pathogen blacklegged ticks can transmit is *Borrelia burgdorferi* Johnson et al. emend. Baranton et al. the causative agent of Lyme disease. Passive surveillance in Manitoba has indicated that *B. burgdorferi*, *Borrelia miyamotoi* Fukunaga et al., *Babesia microti* (Franca), and *Anaplasma phagocytophilum* (Foggie) are present in blacklegged ticks (Rochon and Lindsay 2014; 2015). Passive surveillance is the submission of ticks collected from human and domestic animals by the public. Although it is a low cost method for detecting where ticks occur, it cannot be used to determine endemic populations because ticks transported by migratory birds can occur in areas where endemic populations are absent. Thus to determine the prevalence of pathogens in endemic populations active surveillance (the collection of ticks in the field and sampling of host mammals) should be used (Koffi et al. 2012). Extensive active surveillance was performed in Manitoba after blacklegged ticks were first recorded (Galloway et al. 1991) (see Chapter 2.7), and since then no study to determine the infection prevalence of blacklegged ticks through active surveillance has been undertaken. Studying the prevalence of pathogens in endemic blacklegged tick populations from Manitoba is important because it will provide more reliable data that can be used to determine the public's risk of acquiring an infection.

This thesis was conducted to determine the seasonality, pathogen prevalence, overwintering success and cold hardiness blacklegged ticks in Manitoba. Chapter 2 of this thesis is a review of relevant literature on blacklegged ticks, including their range expansion into new areas, and the prevalence of pathogens they carry. In Chapter 3, I outline a study aimed at determining the seasonality and pathogen prevalence of endemic blacklegged ticks at two provincial parks. In Chapter 4, I address knowledge gaps related to cold hardiness by assessing the overwintering survival and cold hardiness of blacklegged ticks. Finally, Chapter 5 serves as a general discussion tying elements of seasonality, pathogen prevalence, and overwintering together.

Chapter 2: Literature Review

2.1 Population Structure

Populations of the blacklegged tick, *Ixodes scapularis* Say (Acari: Ixodidae) exist throughout parts of the United States and Canada. Like all ixodid ticks, blacklegged ticks have four active life stages consisting of larva, nymph, and adult male and female. Larvae and nymphs (collectively referred to as immatures) must consume a blood meal before they can molt to the next stage, while females require blood in order to produce eggs (Lindquist et al. 2016). In eastern and central North America, larvae and nymphs of the blacklegged tick infest a wide range of hosts, including a wide range of passerines (birds of the order Passeriformes) and small mammal species such as the white-footed mouse, (*Peromyscus leucopus* (Rafinesque)) and North American deer mouse (*Peromyscus maniculatus* (Wagner)) (Anderson 1988; Main et al. 1982; Mannelli et al. 1994; Ostfeld et al. 1995; Scott et al. 2001). Adult blacklegged ticks prefer to parasitize white-tailed deer (*Odocoileus virginianus* (Zimmermann)) (Spielman et al. 1979; Watson and Anderson 1976) and other medium sized and large mammal species (Keirans et al. 1996; Zolnik et al. 2015).

In the southern United States, immature blacklegged ticks have distinct host preferences and feed almost exclusively on lizards and snakes (Apperson et al. 1993; Durden et al. 2002; Garvin et al. 2015; Goddard et al. 2015). Questing behavior also differs between southern and northern populations (Arsnoe et al. 2015). For example, nymphs from southern populations rarely emerge from the leaf litter, whereas nymphs from northern localities are more likely to quest at or above the leaf litter surface (Arsnoe et al. 2015). The populations also differ genetically as there are distinct haplotypes present in each of the populations (Norris et al. 1996; Qiu et al. 2002; Rich et al. 1995). Haplotypes are unique sequences in the genome (often the

mitochondrial genome) that are inherited together and can be used to trace lineages. Based on haplotypes, blacklegged ticks from northern populations are said to belong to the American clade, while southern populations belong to the Southern clade (Norris et al. 1996). It has been hypothesized that due to glacial events 18,000 years ago the northern and southern clades of blacklegged ticks became isolated from one another (Qiu et al. 2002). Through time the isolated refugia populations in the north became genetically bottlenecked, and within the last 100 years have started to expand and diversify through events such as deforestation and reforestation (Barbour and Fish 1993; Hewitt 1996).

Throughout the early 1980's and into the mid 1990's some studies suggested a distinct species of tick represented each clade. *Ixodes dammini* represented ticks from the American clade, while *Ixodes scapularis* represented ticks from the Southern clade (Carey et al. 1980; Costero 1989; Spielman et al. 1979; 1985). As more research was published, the validity of *I. dammini* as a species was questioned, and its name was later synonymised (Oliver et al. 1993).

2.2 Seasonality

Once blacklegged ticks are finished engorging on a host they drop off and molt to their next life stage or lay eggs. Although the different stages of blacklegged ticks may feed on the same host species, it would be quite rare for the tick to feed on the same host animal during its development. Larvae, nymphs and females each feed upon a separate host and, as a result this species is considered a three-host tick (Spielman et al. 1985). Depending on the season, the newly molted tick will start host-seeking (also known as questing), or will overwinter at the soil-leaf litter interface in a state of diapause (Lindsay et al. 1998). When ambient air temperatures rise above approximately 4°C (Duffy and Campbell 1994), blacklegged ticks will emerge and resume host-seeking behavior. Depending on the region, the blacklegged tick life cycle can take

from two to three years (ex.: Michigan and eastern Massachusetts) (Hamer et al. 2012b; Yuval and Spielman 1990a) to three or four years (ex.: Long Point, Ontario) (Lindsay et al. 1998) to complete. These differences are mainly linked to larval phenology: at Long Point, fed larvae will overwinter before they molt (Lindsay et al. 1998), whereas in Massachusetts, fed larvae molt into nymphs and then overwinter (Yuval and Spielman 1990a). In the Northeast, peak nymphal activity occurs in May-June and peak larval activity occurs in August-September (Levi et al. 2015). This type of phenology is referred to as asynchronous feeding as larvae and nymphs are feeding at separate times. Synchronous feeding, as seen in the Midwest, occurs when larvae and nymphs feed at the same time, and can co-feed on the same hosts (Gatewood et al. 2009; Hamer et al. 2012b). In the Midwest, peak nymphal and larval activity occurs in June (Hamer et al. 2012b) while in the Northeast (including Canada) the bimodal pattern of larval activity promotes a low level of synchronicity when unfed larvae overwinter and resume host-seeking in the spring (Daniels et al. 1996; Yuval and Spielman 1990a). The asynchronous or synchronous feeding of immatures in a particular region may have large impacts on the efficiency of pathogen transmission. Strains of pathogens that can maintain a long-standing infection in their host should be selected over shorter-lived strains when immatures feed asynchronously (Levi et al. 2015). For instance, hosts infected by persistent strains of *Borrelia burgdorferi* Johnson et al. emend. Baranton et al. by nymphs feeding early in the season would serve to infect larvae feeding later in that same season (Donahue et al. 1987; Hanincová et al. 2008). Synchronous feeding would promote transmission of pathogens that have a shorter duration of infectivity such as *Anaplasma phagocytophilum* (Foggie) (Levi et al. 2015; Levin and Fish 2000). Vertebrate hosts infected with *A. phagocytophilum* remain infected for less than two weeks (Levin and Fish 2000), so larvae that feed at approximately the same time as nymphs have a greater chance of

becoming infected. Warmer autumn temperatures could reduce synchronous feeding by reducing the number of unfed larvae that overwinter. Thus, regions that are expected to warm with global climate change may see less synchronous feeding of immatures, and this would select for strains of pathogens that cause long-lived infections in their hosts (Levi et al. 2015).

Adult blacklegged ticks emerge in the fall after molting from the nymphal stage and quest until they enter diapause when ambient air temperatures reach below approximately 4°C (Duffy and Campbell 1994). Mating can occur both on and off the host however, the female must be mated in order to complete engorgement (Yuval et al. 1990). Females can be mated many times but sperm precedence proceeds from the most recent mating event (Yuval and Spielman 1990b). Sperm precedence influences males to mate guard by inserting their mouthparts into the female. Once completely engorged, mated females will drop off the host and, depending on the season, lay their eggs or overwinter. Adults unable to find a host in the fall will overwinter and emerge in the spring to resume host-seeking (Yuval and Spielman 1990a). Adults that have overwintered and are unable to find a host in the spring perish in late June to early July (Lindsay et al. 1998).

In Manitoba, the seasonality and duration of the life cycle of blacklegged ticks has not been determined. Synchronous feeding of immatures, or lack thereof, has many implications for public health initiatives. The Manitoba Health passive surveillance program has reported a high prevalence of *A. phagocytophilum* in blacklegged ticks (Rochon and Lindsay 2014; 2015), perhaps as a result of the synchronous feeding of larvae and nymphs. It is currently unknown when nymphal blacklegged ticks are active. This is important, as the public should know when they are most at risk of encountering these potentially infected life stages of the blacklegged tick.

2.3 Overwintering Initiation

The distribution of blacklegged ticks in an environment is determined by a variety of landscape and climatic factors (Allan et al. 2003; Brownstein et al. 2003). For climatic factors, humidity and temperature are the most important as they determine the molting and developmental success of these ticks at all life stages (Brownstein et al. 2003; Harris 1959). For instance, if it is too dry, ticks could desiccate. Temperature, when combined with humidity, is important because winters that are too cold and dry have the potential to decrease overwintering survival (Brownstein et al. 2003; Platt et al. 1992). Alternatively, higher mean temperatures between April and October may decrease the time between successive life stages, as observed in the Upper Midwest of the United States (Platt et al. 1992). To survive the daily and seasonal temperature fluctuations ticks use both diapause and quiescence. Diapause is a pre-adaptive behavior that halts development before unfavorable conditions occur, whereas quiescence is a halting of development directly related to unfavorable environmental conditions (Belozerov 1982). Diapause has its advantages as it not only synchronizes the tick life cycle with favorable seasons, but it also allows ticks to resist unfavorable environmental conditions (Belozerov 1982). There are many different kinds of diapause (inactivity of unfed ticks, delay in engorgement, delay of oogenesis in females) but they can all be grouped into two basic categories: behavioral diapause, exhibited by unfed ticks, and morphogenetic diapause, exhibited by fed ticks (Belozerov 1982). Unfed ticks engage primarily in host-seeking activities and behavioral diapause suppresses this activity. In contrast, engorged ticks seek microhabitats for moulting or egg deposition. Morphogenetic diapause blocks these developments, such as delaying the egg-laying of engorged adult females (Belozerov 1982). The interplay of behavioral and

morphogenetic diapause is important for ticks whose life cycles are extended for two or more years such blacklegged ticks (Belozerov 1982).

2.4 Photoperiod

The processes controlling the inception, regulation, and termination of diapause in *I. scapularis* and other Ixodidae is not entirely understood. The response of living organisms to the seasonal changes of day length is known as photoperiodism. The induction and regulation of diapause in ticks has been linked to photoperiodism in numerous studies (Belozerov and Naumov 2002; Belozerov et al. 2002; Smith and Cole 1941). There are two types of day length reactions, long-day and short-day. In long-day reactions, diapause is initiated by shorter day lengths, whereas in short-day reactions diapause is initiated by longer day lengths. A combination of reactions is also possible, and is termed a two-step reaction. For instance Belozerov et al. (2002) found that blacklegged tick nymphs exhibit a behavioral diapause controlled by the combination of short-day and long-day reactions. For ticks exhibiting behavioral diapause in unfed stages, short-day reactions are found more commonly in southern species while long-day reactions are found in ticks with life cycles longer than two years (Belozerov 1982). The “critical photoperiod” or “critical day length” is defined as the length of photoperiod eliciting a 50% induction of diapause (Tauber and Kyriacou 2001). In general many species of insects can detect slight changes in day length, and once day length is shorter than the critical photoperiod these insects will enter diapause (Tauber and Kyriacou 2001). Temperature can affect the length of the critical photoperiod (Belozerov 1982). For example, ticks will remain active during shorter days if the temperatures are warm, while they would enter diapause at the same photoperiod if temperatures were cooler.

The interaction between temperature and photoperiodism on blacklegged tick development is not well understood. While the link between photoperiodism and nymphal blacklegged tick diapause is clear (Belozerov and Naumov 2002; Ogden et al. 2004) there is confounding evidence for larvae, where diapause may be controlled by photoperiodism (Belozerov and Naumov 2002) or development may depend on temperature alone, without diapause (Ogden et al. 2008a).

2.5 Overwintering Survival

After the initiation of diapause, ticks still must survive cool temperatures. In general, arthropods have been placed into two simple categories of low temperature survival: freeze tolerant and freeze intolerant. Freeze tolerant arthropods are defined as being able to withstand ice formation inside their bodies, whereas freeze intolerant arthropods prevent the formation of ice inside their bodies (Salt 1961). There are other methods to classify arthropod responses to cold based on the limits of freeze tolerance (Bale 1993; 1996), but for the purpose of this work, the broad categories will be followed.

To prevent the formation of ice inside their bodies, ticks use many different methods. For instance, certain behaviors and physiological adaptations before the onset of winter can help to prevent ice formation. Behaviorally, blacklegged ticks will seek a favorable spot under leaf litter where they are better protected from the cold, and physiologically ticks will enter diapause and increase the level of cryoprotectants such as sorbitol, glycerol and antifreeze proteins within their bodies (Neelakanta et al. 2010; Yu et al. 2014). The antifreeze glycoprotein IAFGP has been detected in *I. scapularis* (Neelakanta et al. 2010). Interestingly, work by Neelakanta et al. (2010) suggests that when blacklegged ticks infected with *A. phagocytophilum* overwinter, the

expression of IAFGP is increased. These results imply that blacklegged ticks are more freeze tolerant when they are infected with *A. phagocytophilum*.

Many studies that have looked at different aspects of tick survival at or below freezing temperatures. Cold hardiness studies can be grouped into three different types: direct chilling injury, which induces death in response to chilling episodes less than two hours; indirect chilling which is similar to direct chilling, but the chill episodes occur over days or weeks; and inoculative freezing whereby contact with external ice can increase the temperature of ice formation in the body (Burks et al. 1996b; Lee and Baust 1987). Usually these studies are compared to the supercooling point of the arthropods. The supercooling point is the point at which tissues spontaneously freeze (Lee and Denlinger 1985). In experiments it is defined as the lowest temperature reached before the release of the latent heat of fusion (Lee and Baust 1987). The supercooling point therefore represents the lowest survivable temperature for freeze intolerant species. Factors that can affect the supercooling point include contact with moisture, water content, level of engorgement, ice nucleating agents, and concentration of cryoprotectants (Somme 1982). A study by Dautel and Knülle (1996) looked at the supercooling point of nine species of hard and soft ticks, and found that all but one species had a mean supercooling point within the range of -17°C to -23°C. Of the nine species, *Ixodes ricinus* (Linnaeus) had the highest mean supercooling point value for unfed adults (-11.1°C). Another tick from the genus *Ixodes*, *Ixodes uriae* White was found to have mean supercooling point values ranging from -7.1°C to -8.4°C for adults (Lee and Baust 1987). The only published literature on the supercooling point of blacklegged ticks where a life stage is specified is by Burks et al. (1996a) who determined nymphs have a supercooling point of -21.7°C. Determining the supercooling point of blacklegged ticks is important work as the supercooling point indicates the lower lethal

temperature. If the adult stage of these ticks has a relatively high supercooling point, their ability to overwinter in more northerly locations may be limited.

The ability of ticks to successfully survive overwinter is an important consideration when modeling their range expansion. Overwintering studies involve placing a known number of ticks in outdoor enclosures, and determining the number that survive throughout winter. Survival overwinter has been assessed for every life stage of blacklegged tick. A high proportion of larvae (Lindsay et al. 1998), and nymphs have been found to successfully overwinter (Brunner et al. 2012; Lindsay et al. 1995). Between 38.6 to 73.6% unfed nymphs survived overwinter (Lindsay et al. 1995), and up to 56.6% of fed nymphs successfully molted after overwintering (Lindsay et al. (1998). In New York State, Brunner et al. (2012) likewise reported high survival of overwintering nymphs (>80%). Unfed adult survival rates have ranged from 19.6 to 78.3%, while the survival of fed females has ranged from 15 to 100% (Lindsay et al. 1995; 1998) Studies on the overwintering survival of blacklegged ticks west of Ontario are currently lacking. In Canada, Manitoba represents both a new ecozone and the western edge of blacklegged tick expansion. Work by Lindsay et al. (1995); (1998) has proven useful in predicting the survival of these ticks in various habitats in Ontario, but Manitoba represents an unstudied area. The repeated collection of blacklegged ticks year after year from endemic areas is evidence these ticks can survive overwinter in Manitoba. However, data on the overwintering survival of blacklegged ticks under prairie conditions are needed for accurate models predicting expansion north and westward. Furthermore, these studies are warranted because the prairies are climatically much different than parts of eastern North America where blacklegged ticks are present.

2.6 Long Range Dispersal

In Canada, blacklegged ticks are an invasive species, and were not observed until 1904 when females were taken from a man in Bracebridge, Ontario (Nuttall and Warburton 1911). This early report likely represented adventitious ticks transported by passerines as there are no other published reports of established blacklegged tick populations in Canada until Watson and Anderson (1976) found the first endemic population in 1972 at Long Point, Ontario. Adventitious ticks are ticks that attach to migratory animals, most typically birds, and detach from their hosts in areas where they are not yet endemic (Ogden et al. 2008a).

The ability of birds to transport ixodid ticks long distances is well documented (Anderson and Magnarelli 1984; Hoogstraal et al. 1963; Ogden et al. 2008b; Weisbrod and Johnson 1989). In Connecticut, over a quarter of the tick-infested birds caught were transient birds that migrate south during the winter (Anderson and Magnarelli 1984). As well, 0.6% of migratory birds sampled in eastern Minnesota were infested with blacklegged tick larvae and nymphs (Weisbrod and Johnson 1989). Based on an estimate of 3 billion northward-migrating birds in eastern and central Canada, Ogden et al. (2008b) put the number of blacklegged tick immatures transported by migratory birds each spring at approximately 50 to 175 million. The potential role of migrating birds to disperse ticks is a function of six different factors: 1) distance travelled, 2) speed of travel, 3) time spent travelling, 4) routes travelled, 5) number of migratory stopovers, and 6) time spent at each stopover (Weisbrod and Johnson 1989). Although these factors account for the ability of birds to disperse ticks, the ticks themselves are not taken into account. Bird dispersal would also depend on the duration of the feeding period of attached ticks, and the grooming behavior of the host bird (ability of ticks to go undetected). When these factors are favorable for ticks, rare cases of extremely long distance tick dispersal can occur. For example, a

blacklegged tick nymph was removed from a Swainson's thrush in Slave Lake, Alberta, 1,760 km northeast from the closest population, and two larvae of *Amblyomma longirostre* Koch were found on a Traill's flycatcher in Manitoba, approximately 5,000 km north from the closest endemic population (Scott et al. 2001).

Reports of blacklegged ticks in provinces other than Ontario increased in the early 1990's (Barker et al. 1992; Bell et al. 1992; Cawthorn et al. 1990; Galloway 1989; Lankester et al. 1991). In 1995, birds captured at Thunder Cape, Ontario were found infested with blacklegged ticks (all larvae), while small mammals trapped at the same location were not infested (Klich et al. 1996). This provided direct evidence that migratory birds were dispersing blacklegged ticks (larvae) across Lake Superior into Canada. It has been hypothesized that migrating birds disperse larvae southward and nymphs northward (Madhav et al. 2004); however, the pattern may vary between tick populations in the Midwest compared to the Northeast which display different patterns of seasonal abundance (Madhav et al. 2004; Weisbrod and Johnson 1989).

As migratory birds use defined flyways for their north and south migration, adventitious ticks in Eastern Canada are likely transported from areas along the Atlantic flyway (Morshed et al. 2005). Likewise, birds travelling along the Mississippi flyway are transporting ticks from the Midwest into Central Canada (Morshed et al. 2005). There are indeed genetic similarities between blacklegged tick populations in the Canadian Prairie Provinces and American Midwest, and between Atlantic populations in Canada and Northeast populations in the United States (Krakowetz et al. 2014a).

2.7 History in Manitoba

In Manitoba, blacklegged ticks were first reported in 1989 when an adult female was removed from a woman near Gunton, Manitoba (Figure 1), and subsequently sent to the

University of Manitoba for identification (Galloway 1989). In that same year, an engorged adult female pulled from a dog was also submitted. In part due to these observations, active surveillance for blacklegged ticks was initiated at 148 localities across southern Manitoba (Galloway et al. 1991). A total distance of 350 km was covered by drag sampling, and 1,566 hosts (42 mammalian, 6 avian species) were examined for ticks. In total, two larvae and one nymph were removed from small mammals collected at one locality near Marchand (Figure 1). Subsequent sampling at this locality failed to detect any other blacklegged ticks and this study demonstrated that blacklegged tick populations were not widespread within Manitoba during the early 1990's (Galloway et al. 1991; and personal communication). Based on the criteria for defining the status of blacklegged ticks in an area, Marchand could not be classified as an endemic site (Laboratory Centre for Disease Control 1991). In 2006, the first endemic blacklegged tick population within the province was discovered at Buffalo Point (R. L. Lindsay, personal communication). Since its initial documentation in Manitoba and the subsequent discovery of an endemic population years later, blacklegged ticks have been identified as far north as Flin Flon (Galloway unpublished data). While the more northerly blacklegged tick submissions likely represent adventitious ticks, endemic populations are now in the southeastern part of the province from the Ontario border to Brandon (Manitoba Health Healthy Living and Seniors 2017). Manitoba likely represents the impending fate of the other Prairie Provinces, Saskatchewan and Alberta that as yet only have reports of adventitious ticks (Lindsay et al. 1999b).

2.8 Pathogens

Like other ticks in the genus *Ixodes*, blacklegged ticks are carriers of many pathogens. In Ontario, the pathogens *Anaplasma phagocytophilum*, *Babesia microti* (Franca), *Borrelia*

burgdorferi, Borrelia miyamotoi Fukunaga et al., Deer tick virus (genotype of Powassan virus), and an *Ehrlichia muris*-like agent are of great concern (Nelder et al. 2016). This concern is also reflected in Manitoba, where three of these pathogens are reportable: *A. phagocytophilum*, *B. microti*, and *B. burgdorferi*. However, *B. miyamotoi*, and Powassan virus are also monitored in *I. scapularis* within the province (Manitoba Health Healthy Living and Seniors 2017)

Anaplasma phagocytophilum

Anaplasma phagocytophilum is classified in the family *Anaplasmataceae* (order *Rickettsiales*) which also contains *Ehrlichia muris*-like species. Both species are small gram-negative bacteria that infect vacuoles in the cytoplasm of blood cells (Rikihisa 1991). *A. phagocytophilum* infects granulocytes while other *Anaplasma* spp. target erythrocytes, monocytes, or platelets (Rar and Golovljova 2011). The life cycle of *A. phagocytophilum* involves *Ixodes* spp. vectors and vertebrate hosts that become reservoirs when they are persistently infected (Rar and Golovljova 2011; Rikihisa 1991). A number of tick species found in Canada can transmit *A. phagocytophilum*, including *I. scapularis*, *Ixodes pacificus* Cooley and Kohls and *Ixodes spinipalpis* Hadwen and Nuttall (Barlough et al. 1997; Burkot et al. 2001; Drebot et al. 2001; Lindquist et al. 2016; Pancholi et al. 1995), while in Europe *I. ricinus* is the main vector (Woldehiwet and Scott 1993). Currently this pathogen has only been shown to be transstadially transmitted as there is no evidence of transovarial transmission (Gray et al. 2002; Oliveira and Kreier 1979). Blacklegged ticks can carry a non-pathogenic variant strain of *A. phagocytophilum* known as Ap-Variant 1 (Courtney et al. 2003; Massung et al. 2002). This strain differs from the pathogenic form of *A. phagocytophilum* (Ap-ha) by only two base pairs in a highly conserved area of the 16S rRNA gene sequences used for genotyping (Courtney et al. 2003; Rar and Golovljova 2011). The two strains show differences in the vertebrate hosts they

are able to infect. Ap-Variant 1 has been detected in white-tailed deer but does not infect mice (Massung et al. 2003; Reichard et al. 2009), while Ap-ha is pathogenic to mice, but is unable to infect white-tailed deer (Reichard et al. 2009). Where Ap-ha is present, white-footed mice are the main reservoir (Michalski et al. 2006). Blacklegged ticks have also been shown to carry other *A. phagocytophilum* strains, but very little work has been done on them to date (Massung et al. 2002; Michalski et al. 2006).

Human granulocytic anaplasmosis (HGA), the disease caused by *A. phagocytophilum*, was first described in 1992 when a patient from Wisconsin was admitted to the hospital after suffering from a flu like illness (Chen et al. 1994). In Canada, *A. phagocytophilum* was first reported following its detection by PCR from blacklegged ticks collected at Long Point, Ontario (Drebot et al. 2001). Few studies have differentiated between the two strains of *A. phagocytophilum*, and unless otherwise stated, *A. phagocytophilum* infection rates do not discern between Ap-Variant 1 and Ap-ha (Krakowetz et al. 2014b). The prevalence of *A. phagocytophilum* in blacklegged ticks varies between regions. In the American Northeast, infection rates can range from 2.3% to 15% for females and 0% to 1.5% for males (Courtney et al. 2003; Telford et al. 1996). In areas of Connecticut and Rhode Island infection rates are slightly higher with 20% of adults and 16.27% of nymphs testing positive (Massung et al. 2002). In Wisconsin, which lies more westward, 10% of adult blacklegged ticks are positive for *A. phagocytophilum*, and two of the 12 sequenced ticks were positive for Ap-Variant 1 (Michalski et al. 2006). In Canada, 1.3% of the 12,606 blacklegged ticks submitted across Canada between 2007 and 2010 were positive for *A. phagocytophilum* (Krakowetz et al. 2014b). Interestingly, Manitoba had the second highest prevalence of *A. phagocytophilum* (5.6%, $n = 570$), after Saskatchewan (40%, $n = 10$). Furthermore, of 17 sequenced *A. phagocytophilum* positive ticks

from Manitoba, two were positive for Ap-Variant 1 (Krakowetz et al. 2014b). Similarly of 4,938 ticks submitted in 2012 to the National Microbiology Laboratory (NML) from across Canada, 0.8% were positive for *A. phagocytophilum* (Dibernardo et al. 2014). Based on passive surveillance data, approximately 7.4-10.1% of the blacklegged ticks within the province are infected with *A. phagocytophilum* (Rochon and Lindsay 2014; 2015)

Babesia microti

Babesia parasites belong to the phylum Apicomplexa, and are known as haemoparasites as they inhabit the bloodstream of their host, infecting erythrocytes (Yabsley and Shock 2013). *Babesia* parasites are transmitted by the bite of infected ixodid ticks and have a broad host range, infecting hundreds of different mammal species and some bird species (Yabsley and Shock 2013). Humans are not natural hosts for *Babesia* species, and are thus termed accidental hosts. The disease caused by *Babesia* species in humans is called human babesiosis, which was first reported in North America in 1966 (Scholtens et al. 1968). In Canada, the first locally acquired case of babesiosis from a tick bite occurred in Manitoba in 2013 (Bullard et al. 2014). In Europe, human babesiosis is associated with *Babesia divergens* M'Fadyean and Stockman, while in North America the major zoonotic species is *B. microti*. Both species cause a wide range of symptoms in humans, and if left untreated can lead to death, especially in patients with weakened immune systems (Gray et al. 2010). Symptoms of babesiosis include fever, fatigue, chills, sweats, and headaches (Krause 2002). An estimated one third of infected individuals, including adults and children, remain asymptomatic (Hunfeld et al. 2008; Krause et al. 1992; 2003). Since *B. microti* infections can occur in people without symptoms, inadvertent transmission via blood transfusions has been reported, especially in parts of the United States where *B. microti* is the most reported transfusion-transmitted pathogen (Young et al. 2012).

In North America, blacklegged ticks are the vectors responsible for the vast majority of *B. microti* transmission to humans (Spielman 1976; Yabsley and Shock 2013). However other ixodid ticks such as *Ixodes angustus* Neumann, *Ixodes muris* Bishopp and Smith, and *I. spinipalpis* are also able to transmit the pathogen (Burkot et al. 2000; Goethert et al. 2003). As transovarial transmission is absent in *B. microti*, only nymphal and female adult ticks are able to transmit the pathogen (Gray et al. 2002; 2010; Walter and Weber 1981). Primary reservoir hosts for *B. microti* include *P. leucopus* and *Microtus pennsylvanicus* (Ord) (meadow voles) (Healy et al. 1976; Telford and Spielman 1993). In areas such as Manitoba, where white-footed mice are absent, *P. maniculatus* likely serves as a reservoir host as it does for *B. burgdorferi* (Rand et al. 1993). Although *B. microti* is able to infect a large number of mammal hosts, many carry different strains of *B. microti*, some of which are non-pathogenic to humans (Goethert et al. 2006; Yabsley and Shock 2013). For instance (Goethert et al. 2003) found a non-pathogenic genotype of *B. microti* in a red-backed vole (*Myodes gapperi* (Vigors)) from Maine. More recent research has revealed that *B. microti* is likely a species complex consisting of many different strains (Goethert et al. 2006; Hunfeld et al. 2008; Nakajima et al. 2009).

In Canada, screening for the pathogen via passive tick surveillance was only initiated in 2012 (Bullard et al. 2014). As well, of the provinces where blacklegged ticks are emerging, babesiosis is only provincially reportable in Manitoba (Nelder et al. 2016). Blacklegged ticks infected with *B. microti* were first discovered in Manitoba in 2010 when 6 of 326 ticks tested positive (Bullard et al. 2014). Samples from rodents in Manitoba have also been positive for *B. microti* with both tick and rodent samples showing 98% homology with GenBank samples from the United States (Bullard et al. 2014). In addition, approximately 1%-2.7% of the blacklegged ticks collected by passive tick surveillance in Manitoba were positive for *B. microti* (Rochon and

Lindsay 2014; 2015). In Ontario, Werden et al. (2015) did not detect *B. microti* in any of the blacklegged ticks collected and tested from a recently established population from along the St. Lawrence River.

In the United States, rates of *B. microti* infection in blacklegged ticks are frequently low. A review by Nelder et al. (2016) reported Connecticut had the highest prevalence of *B. microti* at 6.7%, followed by New Jersey at 6.5% and Massachusetts at 5.3%, all life stages combined. However, *B. microti* infection rates as high as 9% and 20% in adults have been reported in some localities in New Hampshire (Walk et al. 2009) and New York (Tokarz et al. 2010), respectively.

Borrelia

Borrelia is a bacterial genus in the Spirochaetes phylum, which contains many species of considerable zoonotic importance. The genus can be broken down into two major clades, each containing species transmitted by ticks (Barbour 2014). The first clade contains *Borrelia* species causing Lyme disease, which are exclusively transmitted by ixodid ticks, while the second contains species that cause relapsing fever (Hue et al. 2013). *Borrelia burgdorferi* and *B. miyamotoi* belong to the first and second clades respectively, and are becoming more common in blacklegged ticks from Manitoba (Dibernardo et al. 2014; Ogden et al. 2006b; 2013).

B. miyamotoi was first detected in *Ixodes persulcatus* Schulze in 1995 from a northern area of Japan (Fukunaga et al. 1995). It was not until 2011 that the first case of human infection with *B. miyamotoi* was published (Platonov et al. 2011). Symptoms of *B. miyamotoi* often include fever, fatigue, headache, myalgia, chills, and nausea (Molloy et al. 2015; Platonov et al. 2011).

Current vectors of *B. miyamotoi* include six *Ixodes* species from Europe, Eurasia, and North America (Krause et al. 2015). In North America, the two main tick vectors are *I. pacificus*

and *I. scapularis* (Mun et al. 2006; Scoles et al. 2001). Ticks acquire the pathogen when they feed on an infected host or in rare cases when the pathogen is passed from mother to offspring via transovarial transmission. Transovarial transmission of *B. miyamotoi* has been documented through the observation of the pathogen in larvae from wild caught females (Scoles et al. 2001). More recently Rollend et al. (2013) found transovarial transmission rates of 1.4%, 2% and 6% in larva reared from wild caught females from three sites in Connecticut. The main reservoir host for *B. miyamotoi* in the northeastern United States is the white-footed mouse (Barbour et al. 2009; Bunikis and Barbour 2005; Scoles et al. 2001). Where white-footed mice are absent, the closely related North American deer mouse is the most likely reservoir (Rand et al. 1993). The bacteria has also been detected in northern cardinals (*Cardinalis cardinalis* (Linnaeus)), American robins (*Turdus migratorius* Linnaeus), and hermit thrushes (*Catharus guttatus* (Pallas)) (Hamer et al. 2012a). More recently white-tailed deer have been implicated as a potential reservoir host (Han et al. 2016).

In Canada, *B. miyamotoi* has been detected in blacklegged ticks from many provinces including Manitoba. Dibernardo et al. (2014) found 23 of 4,938 ticks (0.5%) from across Canada positive for *B. miyamotoi*. More specifically Manitoba had two (1.2%) of 170 submitted ticks test positive (Dibernardo et al. 2014). In the United States, where the pathogen has been circulating longer and tick populations are well established, prevalence rates can be as high as 10% in some areas (Wormser and Pritt 2015). Most studies however have found rates ranging from 0% to 4.7% (Barbour et al. 2009; Hamer et al. 2012a; Nelder et al. 2014; Scoles et al. 2001; Tokarz et al. 2010). Indeed these prevalence rates are close to the 2.8%-6.4% prevalence in Manitoba (Rochon and Lindsay 2014; 2015), and co-infection with *B. burgdorferi* occur (Barbour et al. 2009; Dibernardo et al. 2014; Tokarz et al. 2010). The low prevalence of *B.*

miyamotoi in blacklegged ticks may be attributed to less efficient horizontal transmission rates compared to *B. burgdorferi* (Barbour et al. 2009). As suggested by Rollend et al. (2013) the mean infection prevalence of *B. miyamotoi* (1.9%) is similar to the rate of transovarial transmission of the pathogen in blacklegged ticks.

Borrelia burgdorferi, the agent of Lyme disease in North America, was first isolated from blacklegged ticks in 1981 from Shelter Island, New York (Burgdorfer et al. 1982). Although Lyme disease was described in Europe as early as 1883 by Alfred Buchwald (Buchwald 1883), it was not formally named and recognized until 1975 in the town of Old Lyme, Connecticut (Steere et al. 1977). In North America, Lyme disease is the most commonly reported vector-borne disease (Caulfield and Pritt 2015) with an increasing number of cases occurring in Canada (Bouchard et al. 2015). The initial onset of early Lyme disease includes flu-like symptoms such as fever, chills, fatigue, and headache (Knapp and Rice 2015), and 80% of patients experience an *erythema migrans* skin lesion commonly referred to as the "bulls-eye rash" at the site of the tick bite (Aguero-Rosenfeld et al. 2005). Early disseminated Lyme disease can occur in patients who are not treated for early Lyme disease. Early disseminated symptoms include muscle pain, as well as neurological and cardiac symptoms (Knapp and Rice 2015). The final stage of Lyme disease is called late disseminated Lyme disease and occurs several months after infection (Knapp and Rice 2015). The late disseminated stage consists of Lyme arthritis and neurological symptoms (Wormser et al. 2006).

Although many *Ixodes* species can harbour the pathogen, the main vectors in North America are *I. pacificus* west of the Rocky Mountains and *I. scapularis* east of the Rocky Mountains (Ogden et al. 2014). Like *B. miyamotoi*, ticks acquire *B. burgdorferi* when they feed on an infected host. There was some controversy over whether *B. burgdorferi* can be transmitted

transovarially, but this has been resolved (Rollend et al. (2013). It is likely earlier studies that reported low rates of *B. burgdorferi* in unfed larvae (Magnarelli et al. 1987; Piesman et al. 1986) were actually detecting *B. miyamotoi* infections (Rollend et al. 2013; Scoles et al. 2001). The observation that *I. scapularis* larvae can partially feed and resume host-seeking behaviour would also erroneously support the hypothesis that *B. burgdorferi* can be transmitted transovarially (Hamer et al. 2010; Rollend et al. 2013).

Reservoir hosts for *B. burgdorferi* include a diversity of small mammals such as deer mice, white-footed mice, chipmunks (*Tamias striatus* (Linnaeus)), masked shrews (*Sorex cinereus* Kerr), and meadow voles (Brisson et al. 2008; LoGiudice et al. 2003; Mather et al. 1989; Rand et al. 1993). Many species of birds such as the American robin, song sparrow (*Melospiza melodia* (Wilson)), and northern cardinal also serve as reservoir hosts for *B. burgdorferi* (Ginsberg et al. 2005; Richter et al. 2000; Stafford et al. 1995). White-tailed deer, the primary hosts for adult blacklegged ticks, (Piesman et al. 1979) are not competent reservoirs for the spirochete and therefore cannot infect ticks (Telford et al. 1988). Over 16 different *B. burgdorferi* lineages have been identified based on their *ospC* genotype, with four strains causing most of the infections in humans (Khatchikian et al. 2015). Mounting evidence supports that *B. burgdorferi* strains are suited towards particular hosts, which would also affect the infectivity of strains in their non-typical host (Brisson and Dykhuizen 2004; Hanincova et al. 2013; Vuong et al. 2014). In Canada, Ogden et al. (2011) found a wide diversity of *B. burgdorferi* strains with most being identical to those from the United States, and suggests that the strain diversity of *B. burgdorferi* in Canada is due to both refugial populations and migratory birds (Ogden et al. 2008b; 2011).

The impact of migratory birds on the spread of Lyme disease is still not entirely understood. Anderson and Magnarelli (1984) were the first to report *B. burgdorferi* infected immatures retrieved from migratory birds in North America. Infected larvae are likely the most important pathway for *B. burgdorferi* establishment as they can transmit the bacteria to competent reservoir hosts after molting (Ogden et al. 2008b). Nymphs however, would be unlikely to spread the infection because they molt into adults, which almost exclusively feed on deer. The role migratory birds play in the introduction of *B. burgdorferi* into newly established tick populations has been called into question "(1) birds carry few larvae, (2) birds do not seem to greatly amplify infection in the ticks they carry, and (3) birds may acquire ticks mostly from regions where *B. burgdorferi* infection prevalence is low, or (4) birds are generally zooprophylactic (divert vector bites from the reservoir host) reducing infection prevalence in the ticks they introduce" (Ogden et al. 2008b).

The phenology of larvae and nymphs can play a large role in the spread of pathogens into Canada. For instance in the midwestern United States, the period between nymphal and larval parasitism is shorter compared to the Northeast (Hamer et al. 2012b). Birds in the Midwest are thus more likely than birds in the Northeast to be simultaneously parasitized by nymphs and larvae, allowng larvae to acquire *B. burgdorferi* from co-feeding nymphs. This can lead to a higher prevalence of *B. burgdorferi* in blacklegged tick immatures (Brinkerhoff et al. 2011) that drop from birds and molt to their next life stage (nymph or adult). For example, Ogden et al. (2008b) studied parasitized birds in the eastern half of Canada, where very few localities would experience birds flying from the Midwest, and they thus report different findings compared to other studies (Brinkerhoff et al. 2011; Scott et al. 2012).

In the United States, Lyme disease has been nationally notifiable since 1991 while in Canada the disease has only more recently (2009) been nationally notifiable (Kulkarni et al. 2015). The 18-year gap of disease reporting between the two countries is likely why there are far more studies on Lyme disease incidence and *B. burgdorferi* prevalence published for the United States. Nevertheless, *B. burgdorferi* has been well studied and documented in southeastern Canada (Bouchard et al. 2011; Lindsay et al. 1997; Nelder et al. 2014). In Manitoba passive surveillance studies by Dibernardo et al. (2014) and Ogden et al. (2006b) have reported *B. burgdorferi* prevalence in blacklegged ticks of 8.8% and 9.7% respectively (all life stages combined). In both studies Manitoba's prevalence was the lowest among studied provinces. The low prevalence of *B. burgdorferi* in ticks from Manitoba was likely due to their recent establishment in the province (Dibernardo et al. 2014). Active surveillance of emerging populations in Canada has yielded prevalence rates of approximately 7.7% in adults (Ogden et al. 2010) whereas Lindsay et al. (1997) found prevalence rates of 17%-35% in questing nymphs at more established sites at Long Point, Ontario. Through passive surveillance Manitoba has recently had prevalence rates of between 23.7% and 26.9% (nymphs and adults combined). In the northeastern and midwestern United States the prevalence of *B. burgdorfer*i is often greater than 30% (all life stages combined) (Nelder et al. 2016). For instance in southern Connecticut Tsao et al. (2004) reported 32.5% (in 1999) and 38.6% (in 2002) of nymphal blacklegged ticks positive. The high prevalence of *B. burgdorferi* in nymphs from southern Connecticut is similar to that reported in ticks from New York (Daniels et al. 1998; LoGiudice et al. 2003).

As mentioned above, no studies have reported the prevalence of *B. burgdorferi*, *B. miyamotoi*, *A. phagocytophilum*, or *B. microti* from active surveillance within Manitoba. Passive surveillance is a good early indicator, but it can be difficult to determine the travel history of

submitters. As well, passive surveillance rarely includes the submission of immature blacklegged ticks which are important in determining the prevalence of pathogens in a region. In Manitoba, true pathogen prevalence rates derived from ticks and their mammal hosts is currently lacking. An area where the public is considered at risk of acquiring pathogens is currently assessed by the presence or absence of ticks, and not pathogens. Without pathogen prevalence data, it will be impossible for public health officials to accurately assess risk areas.

2.9 Collection Methods

The field collection of ticks is a key component of many studies on tick biology, ecology and the epidemiology and epizootiology of tick-borne pathogens. Most often researchers employ drag sampling, flagging, carbon dioxide baiting and/or animal trapping to collect ixodid ticks (Falco and Fish 1992; Sonenshine 1993). To drag sample, the researcher pulls a white flannel cloth over an area of vegetation, and then checks the cloth for ticks (Milne 1943; Philip 1937). If the area of cloth in contact with vegetation and the distance the drag has travelled are multiplied, the area of sampling can be deduced (Rochon et al. 2012). A similar method to dragging is flagging whereby the researcher swings a piece of cloth attached to a pole back and forth as they walk (Rulison et al. 2013). With regards to efficiency, Dantas-Torres et al. (2013) found that flagging and dragging out performed one another under different circumstances. For example Dantas-Torres et al. (2013) collected more adult *Rhipicephalus turanicus* (Pomerantsev) by dragging while most *Ixodes ricinus* (Linnaeus) were collected by flagging. Climate, habitat characteristics, and tick behavior are all factors that can affect whether dragging or flagging should be employed (Dantas-Torres et al. 2013). In some instances either method is acceptable as is the case when sampling for blacklegged tick nymphs (Rulison et al. 2013).

Another frequently used method in the collection of ticks is trapping a live host and checking it for attached ticks (Hamer et al. 2012b; Hazler and Ostfeld 1995; Mather et al. 1989; Rudenko et al. 2011). Small mammal trapping utilizes smaller traps which can fit small mammals from 1-500 grams (Falco and Fish 1992). A bait ball made of rolled oats and peanut butter is placed inside the trap to entice animals. Small mammals frequently come into contact with vegetation at a level immature ixodid ticks quest at; small mammal trapping therefore serves to collect only immature ticks (Ginsberg and Ewing 1989). In one of the only comparison studies of small mammal trapping, Falco and Fish (1992) found that significantly fewer nymphs were recovered via mammal trapping compared to CO_2 and dragging methods however, the method of tick collection used did not significantly affect the number of larvae retrieved. Small mammal trapping, although time consuming, is favorable for immature collection as it is a more sensitive method to detect blacklegged tick populations especially when the abundance of ticks is low (Ginsberg and Ewing 1989), as would occur when populations first establish in a new area (Ogden et al. 2014). For larger animals, trapping techniques vary, but can involve the use of medium sized traps and foot snares (Levin et al. 2002; Zolnik et al. 2015). Birds will often be infested with immature ticks, and can be collected using a variety of collection devices, including mist nets. However, as most birds readily disperse over short, medium and long distances, the origin of attached ticks can be hard to precisely define (Ginsberg et al. 2005; Scott et al. 2012).

Chapter 3: The Seasonality of *Ixodes scapularis* Say and Prevalence of Pathogens at Two Provincial Parks in Manitoba

3.1 Abstract

The blacklegged tick, *Ixodes scapularis*, is a relatively recent invasive species in Manitoba. Although many studies have addressed the seasonality of blacklegged ticks in endemic regions, none have been conducted in Manitoba, where the range of this tick is expanding. Passive surveillance has identified four pathogens present in blacklegged ticks in Manitoba: *Borrelia burgdorferi*, *B. miyamotoi*, *Babesia microti*, and *Anaplasma phagocytophilum*. While these data can provide information on the emergence of pathogens into new areas, they are not suited to determine the prevalence of pathogens within populations. We identified two provincial parks in eastern Manitoba with known populations of blacklegged ticks to conduct a seasonality and pathogen prevalence study. Mammal trapping was conducted in 2015 and 2016, and tick dragging was conducted from 2014 to 2016. Ticks, rodent ear biopsies, and rodent blood samples were collected and screened for the four aforementioned pathogens. During the entire sampling period we recovered three nymphs, and their activity overlapped with larvae. Overall 32% (19/60) and 12% (7/60) of adult ticks were infected with *B. burgdorferi* and *A. phagocytophilum*, respectively. In addition, one southern red-backed vole and the two larvae infesting it tested positive for an unknown *Borrelia spp*. Our findings indicate nymphs and larvae feed synchronously which may favor the proliferation of short-lived pathogens. At both provincial parks, we suggest nymphs are using larger mammals and/or diurnal mammals. Finally, we suggest continued active surveillance at both provincial parks to assess the prevalence of pathogens over time, and the emergence of new pathogens.

3.2 Introduction

The blacklegged tick, *Ixodes scapularis* Say, is a vector of many pathogens in North America. The bacteria *Borrelia burgdorferi* Johnson et al. emend. Baranton et al., *B. miyamotoi* Fukunaga et al., and *Anaplasma phagocytophilum* (Foggie), and the haemoparasite *Babesia microti* (Franca) are of greatest concern. The causative agent of Lyme disease (*B. burgdorferi*) is by far the most well-known and studied of the four and is the most reported vector borne disease in the United States (Bacon et al. 2008). *Borrelia miyamotoi* is the causative agent of tick borne relapsing fever, and has only been documented in humans since 201l (Platonov et al. 2011). The haemoparasite *B. microti* causes human babesiosis, while the bacterium *A. phagocytophilum* infects granulocytes causing human granulocytic anaplasmosis (HGA) (Rar and Golovljova 2011). In Canada reservoir hosts for these pathogens include many small mammals such as white-footed mice (*Peromyscus leucopus* (Rafinesque)), North American deer mice (*P. maniculatus* (Wagner)), eastern chipmunks (*Tamias striatus* (Linnaeus)), masked shrews (*Sorex cinereus* Kerr), and meadow voles (*Microtus pennsylvanicus* (Ord)) (Brisson et al. 2008; Goethert et al. 2003; LoGiudice et al. 2003; Mather et al. 1989; Rand et al. 1993).

In Manitoba the first endemic population of blacklegged ticks was found in 2006 at Buffalo Point (R. L. Lindsay, personal communication).Within the last decade, blacklegged ticks have expanded their range to cover most of the southeastern and south central Manitoba (Manitoba Health Healthy Living and Seniors 2017). Tests on ticks submitted to the blacklegged tick passive surveillance program and locally acquired human infections have revealed that all four pathogens are present in the province (Bullard et al. 2014; Dibernardo et al. 2014). The prevalence of infection in nymphal and adult blacklegged ticks submitted to the passive surveillance program in 2015 (n = 387) was: *A. phagocytophilum* (8.8%), *B. burgdorferi*

(25.3%), *B. miyamotoi* (4.6%), and *B. microti* (1.9%) (Rochon and Lindsay 2015). As the number of Lyme disease and HGA cases continue to rise, it is important for public health officers to educate the public on tick bite prevention. Educational campaigns by agencies require specific knowledge of when and where the public is most at risk of coming into contact and acquiring an infection from blacklegged ticks to best orient their message.

The seasonality of blacklegged ticks has been studied in Ontario (Lindsay et al. 1995; 1998), where climate and vegetation differ from Manitoba. In addition, the origin of the ticks appears to be different, with endemic blacklegged tick populations in Manitoba likely arising from adventitious ticks from the midwestern United States transported by migratory birds (Krakowetz et al. 2014a; Morshed et al. 2005). Through passive surveillance data, the seasonality of adult blacklegged ticks in Manitoba is generally known, but data on nymphs are lacking. Nymphal seasonality has been well studied in the American Northeast and Midwest, but as the climate of Manitoba is cooler than that of the midwestern United States, it is currently unknown how this may influence nymphal seasonal activity.

The seasonal activity of immature blacklegged ticks plays a large role in pathogen transmission (Levi et al. 2015). If larvae and nymphs of blacklegged ticks are active at the same time synchronous feeding can occur, as observed in the midwestern United States (Gatewood et al. 2009; Hamer et al. 2012b). Synchronous feeding has the potential to favor transmission of pathogens that cause short-lived infections in their hosts such as *A. phagocytophilum* (Levi et al. 2015; Levin and Fish 2000). If larvae and nymphs do not share the same activity period, asynchronous feeding occurs, which favors the transmission of pathogens causing longer-lived infections in their hosts (Levi et al. 2015). This type of activity period occurs in northeastern

North America and is favorable for *B. burgdorferi* transmission (Donahue et al. 1987; Gatewood et al. 2009; Hanincová et al. 2008).

Blacklegged ticks can carry a variety of pathogens, which may have influence the emergence of pathogens into new areas. In the American Northeast, blacklegged tick co-infections of *B. burgdorferi* and *B. microti* occur more often than expected by chance alone (Hersh et al. 2014). Recent research suggests that co-infected mice are better able to transmit *B. microti* to blacklegged ticks than mice infected with *B. microti* alone (Dunn et al. 2014). *B. burgdorferi* increases the suitability of reservoir hosts for *B. microti* thus facilitating its emergence to new localities (Diuk-Wasser et al. 2016). Similarly, the presence of *A. phagocytophilum* may increase the cold hardiness of blacklegged ticks, thereby increasing survivability in colder environments (Neelakanta et al. 2010).

In the northeastern United States the prevalence of *B. burgdorferi* can often reach as high as 52% (all life stages combined) (Hutchinson et al. 2015; Nelder et al. 2016). The prevalence of *B. burgdorferi* in the Midwest is more variable with reports of localities with up to a 72% prevalence in adults (Steiner et al. 2008) and others reporting as low as 33.9% (all life stages combined) (Nelder et al. 2016). Both regions also show variability in the prevalence of *A. phagocytophilum* ranging from 6% to 22%, and *B. miyamotoi* which is most prevalent in Connecticut (4.7%) (all life stages combined) (Nelder et al. 2016). For *B. microti*, states along the northeastern Atlantic coast often show the highest prevalence (<5%) (all life stages combined) (Nelder et al. 2016). Most of these blacklegged tick populations have been established for many more years than the endemic populations of Manitoba, and while comparisons with other regions can be helpful, local data is essential for public health initiatives. Our first objective is to determine the seasonality of blacklegged ticks in Manitoba by collecting

adult and immature ticks from two provincial parks. Our second objective also is to determine the infection prevalence of *A. phagocytophilum*, *B. burgdorferi*, *B. miyamotoi*, and *B. microti* in ticks and small mammals sampled within these provincial parks. We hypothesize that 1) the seasonality of blacklegged ticks in Manitoba will be similar to that of the midwestern United States, and 2) the prevalence of the four aforementioned pathogens will be lower than in other regions in eastern Canada and the Northeastern or midwestern United States where blacklegged ticks have been established longer.

3.3 Material and Methods

Locations

Beaudry Provincial Park (BPP, 50.0244°N 96.8836°W) and Birds Hill Provincial Park (BHPP, 49.8539°N 97.4733°W) are both located in southeastern Manitoba near the city of Winnipeg (Figure 1). Both parks were chosen as study sites due to their proximity to Winnipeg, suitable blacklegged tick habitat, and established populations of blacklegged ticks (the detection of host-seeking ticks at these sites over multiple years). BPP is located 10 km west of Winnipeg and is approximately 8.1 km², with much of its area lying along the Assiniboine River. BHPP is located outside Winnipeg at approximately 24 km to the northeast and encompasses 35.1 km² of land, predominately a mixed forest of poplars and oak. Since the late 2000's, blacklegged ticks from both parks have been have routinely been submitted to the Manitoba Blacklegged Tick Passive Surveillance Program (unpublished data).

Small Mammal Trapping

Small mammals were trapped on an alternating biweekly basis between the two parks, starting at BPP, from June 16, 2015 until October 14, 2015. At each site 78 Sherman live traps

(H. B. Sherman Traps, Tallahassee, FL) baited with hamster mix (and rolled oats and peanut butter later in the season) were placed in an area of forest where minimal public intrusion would occur. At BPP traps were spaced 10 m apart in five transects to form a grid. At BHPP, five line transects were established, but due to space restrictions, transects were not equally spaced from one another. Trapping in 2016 began May 17, 2016 at BPP and finished on August 30, 2016 at BHPP. At BPP we moved our trapping grid to a more suitable location within the park. At BHPP we moved two transect lines to more suitable habitat. Bacon grease was added to the peanut butter and rolled oats baits in 2016.

Small mammal trapping and handling protocols were approved by the University of Manitoba Fort Garry Campus Animal Care Committee (Protocol F15-012/AC 11052). Traps were set in the evening just before dark and were checked for mammals at sunrise the next day. Trapped mammals were anesthetized with isoflurane (Fresenius Kabi, Richmond Hill, Ontario) in an induction chamber, and the sex, weight and age were determined, where possible. Deer mice weighing 16g or greater and southern red-backed voles (*Myodes gapperi* (Vigors)) 18g or larger were considered adults (Dracup et al. (2015).

The age (juvenile or adult) of mammals other than deer mice and southern red-backed voles captured in this study were not recorded. Each mammal was checked for ticks, which were removed and stored in separate vials kept in a cooler. A 2 mm ear biopsy (Michi-Crown, Bay City, Michigan) and up to 300 µL of blood were also taken from the saphenous vein of each mammal. Ear biopsies were stored in 70% ethanol in separate vials and blood was collected and stored in Microvette® CB 300 vials with EDTA (Sarstedt AG & Co., Nümbrecht, Germany). Once at the University of Manitoba, ticks, ear biopsies and blood samples were transferred to a -20°C freezer until pathogen testing. Mammals were marked for recapture by tattooing their foot

pads using the Aramis Laboratory Animal Microtattoo System with green tattoo paste (Ketchum Manufacturing Inc, Brockville, Ontario), following a marking system traditionally used for toe clipping (Boggess et al. 2004). All mammals were released at their site of capture after processing. Due to the associated risks of Sin Nombre virus (Hantavirus) transmission in Manitoba (Lindsay et al. 2001), traps with a captured southern red backed-vole or deer mouse were washed with 10% bleach, and new cotton was used. Nitrile gloves along with North® 7700 series half mask respirators with North® 7580 P100 particulate filters were used when handling animals and traps.

The intensity, prevalence, and mean density of immature ticks on mammals were calculated for each site and tick species. Intensity (I) is the number of ticks per infested animal, and prevalence (P) is the proportion of captured animals that are infested. When I and P are multiplied, their product is the mean density (M) or number of ticks per animal (Jones and Kitron 2000).

Population size of deer mice and red-backed voles was estimated using the Chapman modification of the Lincoln-Petersen method as it is more accurate when trappability (the percentage of all individuals known alive that were actually captured during a particular trapping event) (Tuyttens et al. 1999) is a concern (Jones and Kitron 2000; Mares et al. 1981): $N = [(M_i + 1)(n_i + 1) / (m_i + 1)] - 1$ where N is the number of animals in a population, M_i is the number of marked animals in the population, n_i is the number of animals captured in sample i, and m_i is the number of marked animals in sample i. If fewer than five mammals were recovered the first night of trapping, traps were set for a second night of trapping. Trapping success for each night was calculated by dividing on the number of traps containing an animal by the number of traps set.

Tick Dragging

Drag sampling was conducted on a weekly basis at both provincial parks starting at BPP on May 09 2014, and July 30 2014 at BHPP. Sampling was terminated when daily temperatures fell below -5°C for more than one week, or if snow fell. For the years 2015 and 2016, drag sampling at both parks began as soon as snow receded from walking trails at both parks, which was May 05 2015, and April 20 2016. Drags consisted of a 1x1 m piece of white flannel cloth attached to plastic dowels at both ends, with one end fitted with a rope. Researchers pulled drags behind themselves for 10 m and then visually inspected the drag for ticks. Each 10 m sample was considered a quadrat, and 200 quadrats were sampled during each period for a total sampled area of 2,000 square-meter (Rochon et al. 2012). Sampling location within each park varied each week (Appendix B). Collected ticks were put into vials and then stored individually at -20°C until pathogen testing. Density (*D*) of ticks per 10 m at each park during sampling periods was determined by the formula $D = T/Q$. Where T is equal to the number of ticks retrieved during the sampling period, and Q is the number of quadrats sampled.

Pathogen Detection

Ticks were identified to species, life stage and sex using dichotomous keys (Clifford et al. 1961; Coley 2015; Durden and Keirans 1996; Keirans and Litwak 1989) and only blacklegged ticks were further processed for pathogen detection. DNA was extracted from ticks, ear biopsies, and blood from small mammals using DNeasy® Blood and Tissue Kits (Qiagen, Valencia, California) following the appropriate manufacturer's protocol.

Extracted DNA from ticks, ear biopsies, and blood samples was tested for *A. phagocytophilum*, *B. burgdorferi*, and *B. miyamotoi* using a quantitative real-time PCR protocol developed at the National Microbiology Laboratory (Public Health Agency of Canada,

Winnipeg, Manitoba) (Dibernardo et al. 2014). Extracts were first screened using a duplex PCR reaction for *Borrelia spp*. and *A. phagocytophilum* (Courtney et al. 2004). The *Borrelia spp.* primers target the 23S rRNA region of the *Borrelia* genus while the *A. phagocytophilum* primers target the associated *msp2* gene (Table 1). *Borrelia* positive samples were then tested for *B. burgdorferi* using ospA primers, and for *B. miyamotoi* using glpQ primers (Ullmann et al. 2005). Samples positive for *Borrelia spp.* but negative for both *B. burgdorferi* and *B. miyamotoi* were sent to the National Microbiology Laboratory for further testing and species confirmation. Positive msp2 samples were confirmed for *A. phagocytophilum* by repeating the assay. Tick and blood extracts were also screened for *B. microti* using cctŋ primers that target the *Babesia*-group parasites (Nakajima et al. 2009). Positive extracts are then confirmed as *B. microti* using Bm18S primers.

Data Analysis

All statistical procedures were done using SAS/STAT® University Edition (SAS Institute Inc. 2016) with the significance level set to 0.05. Two sample Z-tests were used to analyze tick abundance, tick sex bias, and mammal sex bias. Two-sample *t*-tests were used to analyze pathogen prevalence data between sites.

3.4 Results

Mammal Trapping

We trapped small mammals for 46 nights over the two-year period (n = 29 in 2015, n =17 in 2016) between the two provincial parks. We did not find any animals from 2015 in the 2016 captures but we are uncertain if the tattoo would have lasted over the winter. Trapping success varied between years and sites with BHPP having a higher average trapping success. The

average trapping success at BPP increased from 2% in 2015 to 6% in 2016, while at BHPP trapping success increased from 7% to 22%. Between the two parks, 62 (19 BPP, 43 BHP) individual mammals were trapped in 2015 and 139 (32 BPP, 107 BHPP) individual mammals were trapped in 2016. At both sites the rodents most frequently collected were southern red-backed voles followed by the masked shrew (*Sorex cinereus*) at BPP and the deer mouse at BHPP (Table 2). Other species that were trapped included the meadow vole (*M. pennsylvanicus*), the American red squirrel (*Tamiasciurus hudsonicus* (Erxleben)), the Eastern chipmunk (*T. striatus*), and the brown rat (*Rattus norvegicus* (Berkenhout)). Recaptures were more frequent at BHPP compared to BPP in both years (Table 2). Bi-weekly population estimates during both years could only be done for the southern red back vole at both sites, and deer mouse at BHPP (2015) due to the small number of captures for all other species. Our largest estimated population of southern red back voles occurred at BHPP (n = 80 in 2015, n = 307 in 2016), and populations at both parks were highest in August. The total number of southern red-backed voles trapped (sites combined) increased by 270% between 2015 and 2016 (n = 56 in 2015, n = 151 in 2016), and more females than males ($P < 0.001$) were caught in 2016 (n = 115 females, n = 34 males). The population of deer mice at BHPP fluctuated throughout the 2015 season and was estimated to be as high as 15 individuals.

The blacklegged tick (n = 50), American dog tick (*Dermacentor variabilis* (Say) n = 112), and mouse tick (*Ixodes muris* Bishopp and Smith n = 1) were the only tick species found infesting mammals. We also removed flea and louse species (Appendix A). Of the 50 blacklegged ticks collected on mammals, three were nymphs and 47 were larvae. The lone *I. muris* was a nymph, and one damaged *Ixodes* larva was unidentifiable. In total 37 nymphs and 75 larvae of *D. variabilis* were also collected from the captured rodents.

To calculate the intensity, prevalence, and mean density of ticks infesting mammals we pooled all mammal species together due to the low number of trapped mammals. The intensity, prevalence, and mean density for both tick species increased from 2015 to 2016 at BPP but decreased for BHPP (Table 3). Only the American red squirrel, deer mouse, and southern red-backed vole were infested with blacklegged ticks. We removed three blacklegged tick nymphs from two mammals (one American red squirrel and two deer mice) in 2015, and found none in 2016. More blacklegged tick larvae were removed from southern red-backed voles than any other species at BPP for 2015 (two larvae) and 2016 (14 larvae), and at BHPP for 2016 (six larvae). In 2015, more larvae were removed from deer mice (16 larvae) at BHPP.

During the 2015 season we collected 22 larvae and three nymphs of the blacklegged tick. The seasonal activity of larvae combined from both parks can be seen in Figure 3. Larvae were found from June 22 to August 18, and nymphs were observed over a short period between June 17 and 23 in 2015. During the 2016 season, the activity period of larvae was similar and we collected 25 larvae from June 22 to August 30, but we did not observe any blacklegged tick nymphs. Peak activity occurred from July 6-12 in 2015 and August 15-21 in 2016.

Tick Dragging

Due to the small number of blacklegged ticks collected using both collection methods, we pooled ticks from both sites. During the 2014, 2015, and 2016 season we drag sampled for a total of 28,500 square-meters between the two provincial parks. Tick species present included blacklegged ticks, American dog ticks, and the rabbit tick (*Haemaphysalis leporispalustris* Packard). From 2014 to 2016 we collected a total of 93 blacklegged ticks. There was no sex bias in collected blacklegged ticks across all years and both sites (Table 4). Only the adult stage of blacklegged ticks and American dog ticks were collected on our drags at both sites, however one

H. leporispalustris larva was collected at BHPP. Blacklegged tick density was greatest at BPP in 2014 with 0.008 ticks/10 m^2 (Table 4).

Adult blacklegged ticks displayed two activity periods during each of our yearly sampling periods (Figure 4). In 2014 the spring cohort peaked the first week of June, and the fall cohort peaked in the third week of October. In 2015 the spring cohort peaked during the second week of June and the fall cohort peaked during the third week of October. In 2016 the spring cohort peaked during the last week of May, and the fall cohort peaked during the last week in September. Tick abundance during spring and fall activity periods was not statistically different between years ($P = 0.9588$).

Pathogens

We tested a total of 60 blacklegged tick adults, 46 larvae, and two nymphs for the pathogens *A. phagocytophilum*, *B. burgdorferi*, *B. miyamotoi*, and *B. microti* (Table 5). Six adults were collected after testing, and 27 adults were used in a different experiment. Due to the low number of recovered ticks we combined years when analyzing our data. Adult blacklegged ticks had a high prevalence of *B. burgdorferi* infection at BPP (36%) compared to BHPP (19%). Infection with *A. phagocytophilum* in adults was similar between both parks (Table 5). Of the 26 adult ticks positive for a pathogen, three were co-infected with *B. burgdorferi* and *A. phagocytophilum*. Of all the adult ticks positive for *A. phagocytophilum*, 43% were co-infected with *B. burgdorferi*. We found no *B. miyamotoi* or *B. microti* infection in any ticks across all life stages. We were able to test two of the three nymphs collected from BHPP; one tested positive for *A. phagocytophilum*. Larvae recovered at BHPP had a prevalence of 10% infection with *B. burgdorferi*, and 20% with *A. phagocytophilum*. Larvae recovered at BPP showed no infectivity for either pathogen. One larva removed from a deer mouse at BHPP was co-infected with *B.*

burgdorferi, and *A. phagocytophilum*. Three other larvae infected with *A. phagocytophilum* were removed from the same animal. Two larvae taken from a red-backed vole at BPP were positive for either *Borrelia bissettii* Postic et al., or *Borrelia carolinensis* Rudenko et al.; however, because of limited sequence data on DNA extracted from these ticks, the precise species infecting them could not be determined.

Due to the small number of animals processed the first season we combined trapping years. At BPP we extracted DNA from 47 ear biopsies and 45 blood samples, while at BHPP we extracted DNA from 158 ear biopsies and 162 blood samples. Biopsies and blood samples from the same animal were not always concordant. *Anaplasma*-positive blood samples were associated with positive biopsies, with one exception, but *Anaplasma*-positive biopsies were not always associated with a positive blood sample. No blood samples were positive for *B. burgdorferi*. *Babesia microti* and *Borrelia miyamotoi* were never amplified from mammal blood or ear biopsies.

Borrelia burgdorferi was the only pathogen detected in mammals at BPP, and only from red-backed voles (Table 5). At BHPP, *A. phagocytophilum* and *B. burgdorferi* were detected, and both pathogens were more prevalent in deer mice compared to red-backed voles. Three other mammals were infected with pathogens at BHPP. One ear biopsy from an Eastern chipmunk was co-infected with *A. phagocytophilum* and *B. burgdorferi*, and two samples from American red squirrels were positive for *B. burgdorferi* and *A. phagocytophilum*, respectively. At BPP, one red-backed vole and its attached blacklegged tick larvae were positive for either *Borrelia bissettii* or *B. carolinensis*. In total, three out of 201 individual mammals (1.5%) were co-infected with *B. burgdorferi* and *A. phagocytophilum*.

Four mammals infected with a pathogen at their initial capture were recaptured in subsequent trapping sessions. Over a period of 14 days, two mammals infected with *A. phagocytophilum* had lost their infection one mammal maintained its *B. burgdorferi* over the same period. The last recaptured mammal was co-infected and maintained its *B. burgdorferi* infection 28 days after it was first capture, but was no longer positive for *A. phagocytophilum*.

Only one of four larvae collected from a deer mouse infected with *A. phagocytophilum* and *B. burgdorferi* was also co-infected; two larvae were only infected with *A. phagocytophilum*, and targeted pathogens were absent from one tick. Similarly, of eleven larvae removed from a co-infected mouse two were positive for *B. burgdorferi*, and one was positive for *A. phagocytophilum*. One larva removed from a *B. burgdorferi* positive mammal, and one larva removed from an *A. phagocytophilum* positive mammal had no evidence of infection with either pathogen.

3.5 Discussion

Mammal Trapping and Seasonality

Mark recapture studies are most informative when individual animals within the study population can be unambiguously identified. Ear tagging may artificially increase the number of larval ixodid ticks on mice by reducing their grooming efficiency (Ostfeld et al. 1993), and as such has the potential to overestimate host use by ticks. Although ear tattooing is quick and uncomplicated (Linder and Fuelling 2002), we needed access to ears for ear biopsies, and so could not use this method. We opted for footpad tattooing as this method is quick, relatively painless, requires minimal equipment, and the markings last long enough to be reliable. Although tattoos typically remain visible for 15 months under laboratory conditions, there are limited data on the longevity of the tattoos on marked wild animals over multiple seasons. As there are no

data on the longevity of the tattoos on marked wild animals over multiple seasons, we assumed the markings did not remain visible the following year. Furthermore, due to predation and other environmental factors, most small rodents (i.e., voles and mice) generally live less than one year in the wild (Blair 1948; Ostfeld 1988), and red-backed voles and *Peromyscus* species are no exception (Blair 1948; Boonstra and Krebs 2012; Burt 1940; Schug et al. 1991).

Trapping success was much greater in 2016 compared to 2015. At BHPP, the number of trapped red-backed voles increased our trapping success substantially. In 2015, we estimated a population of 80 red-backed voles within our trapping area at BHPP, compared to 307 in 2016. In a study involving mammal trapping at BHPP Burachynsky and Galloway (1985) also observed a large variation of southern red-backed voles between years. The reason for large population fluctuations for this species remains unknown. In Manitoba red-backed vole populations are not cyclical (Boonstra and Krebs 2012; Mihok et al. 1985) and a mast year seems unlikely to affect population size (Dracup et al. 2016; McCracken et al. 1999).

Surprisingly, we captured few deer mice at either site, and this was especially evident at BPP where we captured only four mice in two years. Previous trapping at BHPP indicated deer mice captures varied from location to location (Burachynsky and Galloway 1985), so it is possible our traps were placed in an area with lower deer mouse abundance. Deer mice populations are known to fluctuate based on the seed crop availability in their habitat (Falls et al. 2007), and it is possible populations were in the lower part of a cycle. Our bias towards adults in 2015 could indicate a population that had previously undergone large increases perhaps from a mast year the previous season.

Current Public Health Agency of Canada guidelines define endemic sites as "Locations where both ticks and Lyme disease have been confirmed over multiple years of active field

surveillance. Confirmation is based on both: (1) active field surveillance revealing the presence of all life stages of the ticks at multiple visits over more than one year, (2) detection of the Lyme disease pathogen in ticks or animals collected from the site (Public Health Agency of Canada 2017)". Based on these guidelines, the collection of a single tick does not define an area as endemic. For instance the collection of a single nymph or adult could be the result of a bird-transported tick that subsequently molted from larva to nymph, or nymph to adult (Klich et al. 1996; Ogden et al. 2008b). Similarly, the detection of *B. burgdorferi* in a small number of animals may be from parasitism by bird transported ticks, and not local residents. Researchers could collect adventitious ticks or *B. burgdorferi* positive animal samples and mistakenly declare the area as endemic. Due to the number of adults, and larvae collected in both years, and presence of *B. burgdorferi*, we feel confident in declaring both parks endemic for blacklegged ticks.

Although we only recovered three nymphs during mammal trapping, their presence during larval activity indicates that Manitoba does experience some degree of synchronous feeding of immature blacklegged ticks, based on the nymphs collected. This was somewhat expected as studies in the midwestern United States have observed synchronicity of the immatures stages (Gatewood et al. 2009; Hamer et al. 2012b). Our mammal trapping indicates that larvae are active from the third week in June to the end of August and possibly later, and while the general pattern was similar between 2015 and 2016, there was a noted difference. In 2015, the activity was unimodal with a peak in early July (Figure 3), while in 2016 a second peak of activity was clearly observed at the end of August. It is possible the small number of larvae we recovered (n = 47) does not allow us to accurately depict immature blacklegged tick seasonality. Other similar studies often report the collection of 600-1000 larvae from captured

hosts (Falco and Fish 1992; Hamer et al. 2012b; Jones and Kitron 2000; Kitron et al. 1991). In the Northeastern United States, larval activity is bimodal with a small peak in early summer and a larger peak in the fall (Daniels et al. 1996; Ostfeld et al. 1996; Yuval and Spielman 1990a). However, Simmons et al. (2015) found that larvae in midwestern Pennsylvania displayed only a single peak in late July. In the American Midwest, larvae also show bimodal activity but the greatest activity occurs mid spring and the second, smaller peak occurs in early fall (Hamer et al. 2012b; Hoen et al. 2009). Tick phenology can vary based on environmental conditions (Gray 2008; Hamer et al. 2012b), and it is possible that larval activity is influenced by a shorter season in Manitoba: larvae are active from May-October in the Northeast and Midwest (Fish 1993; Hamer et al. 2012b; Hoen et al. 2009; Simmons et al. 2015) but we did not find any evidence of activity before the later part of June. Snow cover also retreated earlier in 2016 than it did in 2015, which may have allowed larvae to begin questing. Nevertheless, the activity period of larvae in Manitoba has implications for pathogen transmission. If larval and nymphal seasonal activity overlaps for long periods of time, transmission of pathogens causing shorter-lived infections such as *A. phagocytophilum* would be favoured (Hamer et al. 2012b; Hoen et al. 2009; Levi et al. 2015; Lindsay et al. 1998).

We recovered three blacklegged tick nymphs in 2015: one from an American red squirrel at BPP, and two from a deer mouse at BHPP. It is possible we started trapping too late in 2015 to obtain nymphs, but in 2016 we started trapping one month earlier and found no nymphs infesting mammals. Another reason for the low number of recovered nymphs may be due to the level of establishment of blacklegged ticks at both provincial parks. Even though the number of recovered larvae, nymphs and adults was small, we recovered all three life stages at BHPP, which is enough to indicate an established population. The lack of larvae or nymphs on drags,

and consistently low number of recovered blacklegged ticks from all life stages, is indicative of recently established populations at both parks.

We trapped large numbers of red-backed vole throughout the year with no blacklegged tick nymphs present. We therefore speculate that southern red-backed voles are a rarely used host for *I. scapularis* nymphs in Manitoba, and other mammals may support the nymphal population. Many different mammal species have been found harboring nymphal blacklegged ticks (Table 6). Our traps were small and ill-suited to catch larger rodents, and the traps were set at night, favoring nocturnal animals. Across our entire sampling period we captured seven larger rodents (American red squirrel, Eastern chipmunk, brown rat). It is unlikely this was due to a scarcity in larger mammals but more so a result of our methodology. Grey squirrels (*Sciurus carolinensis* Gmelin), red squirrels, eastern chipmunks, and raccoons (*Procyon lotor* (Linnaeus)) can all support large numbers of blacklegged tick nymphs, sometimes more than white-footed mice (Godsey et al. 1987; Main et al. 1982; Mannelli et al. 1993b; Schmidt et al. 1999; Slajchert et al. 1997). In a study performed in the midwestern United States, chipmunks and mice contributed equally to feeding nymphs, despite chipmunk density being an order of magnitude lower compared to mice (Hamer et al. (2012b). Ground squirrels are diurnal (Choromanski-Norris et al. 1989; Quantstrom 1971; Rongstad 1965), are hosts for a number of *Ixodes* ticks (Gregson 1956; Holland 1944; Lindquist et al. 2016; Ostroff and Finck 2003; Salkeld et al. 2006), and many species are present at BHPP, including Franklin's ground squirrels (*Poliocitellus franklinii* (Sabine)), Richardson's ground squirrels (*Urocitellus richardsonii* (Sabine)), and thirteen-lined ground squirrels (*Ictidomys tridecemlineatus* (Mitchill)). Many Franklin's and thirteen-lined ground squirrels were captured in the vicinity of BHPP by using larger traps, and setting traps for 24 hours (Burachynsky and Galloway 1985). No blacklegged

ticks were recovered, but this study predates the first record of blacklegged ticks in the province by at least five years, and no studies have since recorded blacklegged ticks on these hosts. To determine if ground squirrels or other diurnal small-medium sized mammals harbour blacklegged tick nymphs, traps of varying sizes, set for 24 hours should be used whenever possible.

Adult *I. scapularis* exhibited two activity periods, one in the fall, and another in the spring, as documented in other endemic regions (Hamer et al. 2012b; Lindsay et al. 1998; Simmons et al. 2015; Yuval and Spielman 1990a). Newly moulted adults were active from mid-September to late October, and in the spring from early May to the beginning of July, which is similar to the adult activity period described in northeastern North America, and southern Ontario (Lindsay et al. 1998; Yuval and Spielman 1990a). The number of adults active in the fall was not significantly different from the number of ticks active in the spring. This is important because blacklegged ticks active in the fall and the following spring, are part of the same cohort (Lindsay et al. 1998). This finding also adds to a growing body of literature that suggests unfavorable winters have less of an impact on spring tick abundance than previously thought (Brunner et al. 2012; Simmons et al. 2015).

All adult ticks sampled were removed from the environment, and it is hard to determine what effect this removal had on the local tick populations. Adult blacklegged ticks disperse short distances (>3 m) from their point of release (Falco and Fish 1991; Goddard 1993). Thus, repeatedly dragging the same area could have some effect on tick abundance. Instead of removing ticks from the environment, we could have employed mark recapture techniques (Goddard 1993; Kramer et al. 1993), but this would have prevented us from testing ticks for

pathogens. The potential effects of removing ticks may have been mitigated because we changed dragging sites within the parks each week, meaning we did not repeatedly sample the same area. Conversely, removing adults from the population may not have had a large impact on the abundance of subsequent adult generations. Mark-recapture studies in New York state reported small populations with little year to year variation in population size, indicating that blacklegged tick populations can be maintained with very few individuals, in part because females lay many eggs (Daniels et al. 2000). We collected few adults at Beaudry and Birds Hill, even though year after year the public submits adults from both parks via passive surveillance (Rochon and Lindsay 2014; 2015). Adventitious ticks are undoubtedly contributing to the population, but it is also likely a small number of resident individuals are maintaining tick populations from year to year.

Pathogens

We used specific primers targeting the species of interest with no known reactivity to other species in our confirmatory tests (Courtney et al. 2003; Nakajima et al. 2009; Persing et al. 1990; Ullmann et al. 2005). The selected primers were also sensitive (Courtney et al. 2004; Ullmann et al. 2005). Because the *msp2* assay cannot differentiate between strains, we were unable to determine Ap-Variant 1 and Ap-ha infectivity (Courtney et al. 2004). Future studies could use a TaqMan SNP genotyping assay (Krakowetz et al. 2014b) as an added step to differentiate strains, when a positive *A. phagocytophilum* sample is detected (Werden et al. 2015). At this time Ap-Variant 1 is not considered likely to infect humans (Massung et al. 2003). Differentiating between the two strains would more accurately elucidate the public's risk of acquiring Human granulocytic anaplasmosis in a given area.

Pathogens detected in ticks, blood, and ear biopsies were successfully amplified using a protocol developed by the National Microbiology Laboratory (Public Health Agency of Canada). We collected both ear biopsies and blood samples from trapped mammals because *Borrelia* species are known to infect the ears of rodents (ear biopsies) (Sinsky and Piesman 1989) and *A. phagocytophilum* and *B. microti* are parasites of blood cells (Chen et al. 1994; Rikihisa 1991; Western et al. 1970; Yabsley and Shock 2013). Interestingly, four ear biopsies were positive for *A. phagocytophilum* while the associated blood samples were negative. Ixodid ticks are most often found on or around the ears of smaller hosts, and initial pathogen infections resulting from tick bites tend to be localized (Radzijevskaja et al. 2011). It is possible the amplified *A. phagocytophilum* in ear biopsies were due to recent localized infections, but we cannot confirm this because we could not collect subsequent samples from these individuals. There is also the possibility ear biopsies were positive for *A. phagocytophilum* due to contamination. This is unlikely though, as the ear punch was sterilized between use, and controls monitoring reagent contamination remained negative. All but one ear biopsy sample contained *A. phagocytophilum* when blood samples were positive, and as expected, *B. burgdorferi* was only amplified from ear biopsies. In future studies, if blood samples cannot be drawn we recommend the use of ear punches as a reliable indicator of both *A. phagocytophilum* and *B. burgdorferi* infection status.

We found *B. burgdorferi* to be twice as prevalent in adult ticks collected at BPP compared to BHPP. Perhaps more passerine birds stop at BPP than at BHPP during the spring migration, bringing in more infected nymphs into the park. At both parks it is likely we were sampling ticks from two different origins: those that are endemic and bird-borne ticks introduced on an annual basis. By determining the prevalence of different *B. burgdorferi* strains present in our adult and immatures, we may have been able to accurately compare the rate of bird-borne

tick introductions between parks (Ogden et al. 2008b; 2011). If more infected nymphs are transported into BPP as compared to BHPP, this would help explain why only 4% of mammals and none of the larvae recovered at BPP were positive for *B. burgdorferi*, while 36% of adults were positive. Both Madhav et al. (2004) and Weisbrod and Johnson (1989) speculate that migrating birds will disperse more nymphs northward than larvae. BPP is located next to a large oxbow lake, and hence may attract more migrating birds than BHPP. Ticks dispersed by these birds along the Mississippi flyway could originate from sites where ticks and pathogens have been established for many more years as compared to Manitoba. Minnesota, Michigan, Illinois, and Wisconsin are all along the Mississippi flyway and have established tick populations (Hamer et al. 2012b; Lee et al. 2014; Mannelli et al. 1993a). At BHPP 10% of larvae and 7% of mammal ear biopsies tested positive for *B. burgdorferi*, indicating this pathogen may not be well established.

Compared to recent studies from the midwestern United States, we report a lower infection prevalence of *B. burgdorferi* (32%) in adult ticks, but overall higher prevalence of *A. phagocytophilum* (12%) (Courtney et al. 2003; Hamer et al. 2012b; Hutchinson et al. 2015; Steiner et al. 2008). For instance, prevalence of *A. phagocytophilum* in Pennsylvania, and Indiana was reported but be 1-5%, but as high as 14% in Wisconsin (Steiner et al. (2008). Our prevalence data for *B. burgdorferi* is higher than what was reported in Québec, but the prevalence of *A. phagocytophilum* was similar (Bouchard et al. 2013; Ogden et al. 2010).

Through passive surveillance in Manitoba for the years 2014 and 2015, we calculated the prevalence of *A. phagocytophilum*, *B. burgdorferi*, *B. microti*, and *B. miyamotoi* in adults and nymphs (combined) to range from 5.8-10.1%, 23.7-26.9%, 1.0-2.7% and 0.2-6.4% respectively (Rochon and Lindsay 2014; 2015). The average prevalence of *B. burgdorferi* in adult ticks from

BPP (36%) and BHPP (19%) is quite similar to that found via passive surveillance in Manitoba; however the prevalence of *A. phagocytophilum* is slightly higher at our parks (11% BPP, 13% BHPP). As well, we found no *B. microti* or *B. miyamotoi* in either of our parks whereas passive surveillance data indicates both pathogens are present in the province. The differences observed between active and passive surveillance were expected as passive surveillance covers a much larger area and adventitious tick submitted to the program can reduce the certainty of pathogen endemic areas (Ogden et al. 2006b). The passive surveillance program received 552 and 478 blacklegged ticks in 2014 and 2015, respectively (Rochon and Lindsay 2014; 2015). In our active surveillance at the two parks, our sample size was small and may not have been sufficient to capture rare pathogens like *B. miyamotoi* and *B. microti*.

Even though we recovered few deer mice, we found over one-third were positive for *B. burgdorferi*. Few studies have tested the prevalence of pathogens in deer mice, as most studies occur in the northeast and midwestern United States where the deer mouse is absent. Our reported 38% infection prevalence from blood and ear punch samples for deer mice is higher than reported studies in British Columbia (7.8%) (Morshed et al. 2015) and Québec (1.8%) (Bouchard et al. 2011), even though the authors used serological testing methods that look at antibody prevalence. *Borrelia burgdorferi* prevalence in white-footed mice varies by site and year (Hamer et al. 2010). Studies on *B. burgdorferi* infection from white-footed mouse skin biopsies have reported lows of 0-26% in New York state (Oliver et al. 2006), and highs of 76% in Connecticut (Barbour et al. 2009). Our recorded infection prevalence is similar to a coastal site in Lower Michigan (42.6%) (Hamer et al. 2010). Deer mice also had a high prevalence of *A. phagocytophilum* with 23% of our ear biopsies testing positive. This is similar to the 9.2-20.6%

prevalence reported for deer mice in Colorado where *I. spinipalpis* is the only known vector (DeNatale et al. 2002; Zeidner et al. 2000).

The low prevalence of *B. burgdorferi* in red-backed voles is interesting, and may indicate they do not play a large role in the enzootic maintenance of this pathogen. Red-backed voles can become infected with *B. burgdorferi* when syringe inoculated (Bey et al. (1995), but their capacity to infect feeding ticks has not yet been determined (Russart et al. 2014). In our study, none of the six *B. burgdorferi*-positive red-backed voles were infested with tick larvae, so we were unable to document transmission from voles to attached ticks. However, we did collect *A. phagocytophilum* positive larvae from positive red-backed voles, which is a likely indication of successful transmission to ticks. The prevalence of *A. phagocytophilum* in red-backed voles in Minnesota can reach up to 17% (Walls et al. (1997), which is much higher than our 2%. As far as we know this is the first study to provide evidence of blacklegged ticks acquiring *A. phagocytophilum* from red-backed voles.

We found evidence of *B. bissettii* or *B. carolinensis* in blacklegged ticks and a red-backed vole at BPP in 2016. *B. bissettii* is widespread throughout the United States, infecting multiple vertebrate hosts and *Ixodes* tick species (Diuk-Wasser et al. 2006; Eisen et al. 2009; Golovchenko et al. 2016; Oliver 1996; Postic et al. 1998). Blacklegged ticks are known vectors of *B. bissettii* (Diuk-Wasser et al. 2006) but the pathogenicity of this bacteria in humans remains undetermined. Unlike *B. bissettii*, *B. carolinensis* is not as wide spread in America. Until now, this bacterial species had only been identified in *Ixodes minor* (Neumann) and a limited number of vertebrate hosts (Foley et al. 2014; Rudenko et al. 2011). The pathogenicity of *B. carolinensis* in humans is also unknown. Because both host and larvae were positive the two *Borrelia* species, we were not able to determine the direction of transmission. To the author's knowledge,

transovarial transmission has not been studied in either *B. bissetti*i or *B. carolinesis*. In the absence of transovarial transmission, the red-backed vole would have infected the larvae. Determining the direction of transmission in this tick-host dynamic could have been better documented had we recorded the engorgement status of the collected ticks. For example, pathogen-positive unengorged larvae would likely have acquired the infection transovarially. Further sampling of ticks and mammals at BPP could help in determining the prevalence of these seemingly rare bacteria and provide further information on its host diversity.

In summary, we provide evidence that in Manitoba, the seasonal activity of nymphs and larvae overlap and this has implications for the establishment of some pathogens. Similar to midwestern (Hamer et al. 2012b) and eastern North America (Yuval and Spielman 1990a), adult blacklegged ticks displayed two activity periods, one in the spring and another the fall. We showed red-backed voles are a rarely used host for *I. scapularis* nymphs and that other hosts likely feed blacklegged tick nymphs in Manitoba. As well, we provide evidence of red-backed voles as reservoir hosts for *A. phagocytophilum*. Finally, we report the presence of *B. bissettii* or *B. carolinensis* in larvae and a red-backed vole at BPP.

3.6 Acknowledgements

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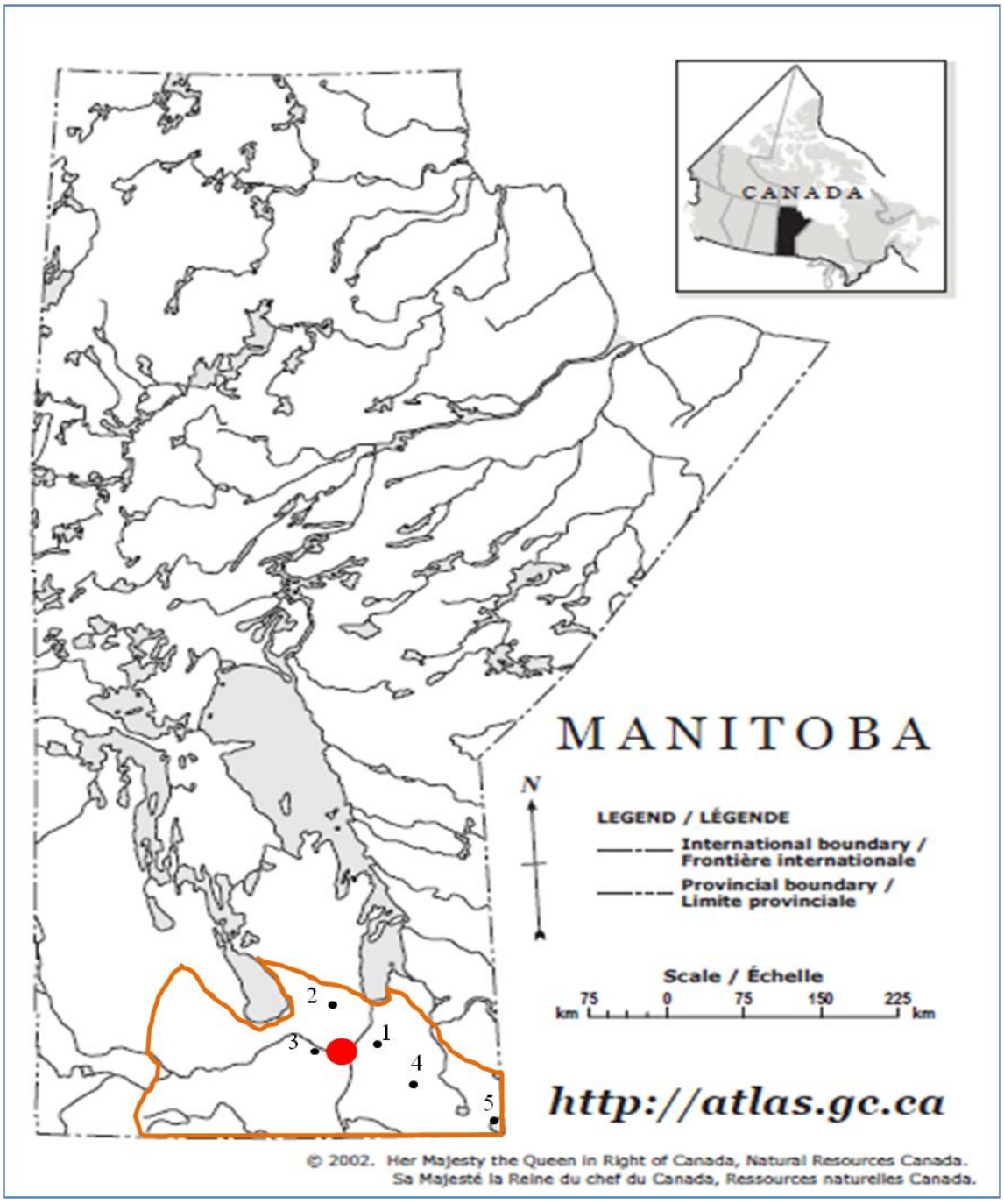

Figure 1. Map of Manitoba, Canada. Numbers indicate the following localities: 1, Birds Hill Provincial Park; 2, Gunton; 3, Beaudry Provincial Park; 4, Marchand; 5, Buffalo Point. Large red circle indicates city of Winnipeg. Orange line encompasses the current range of blacklegged ticks.

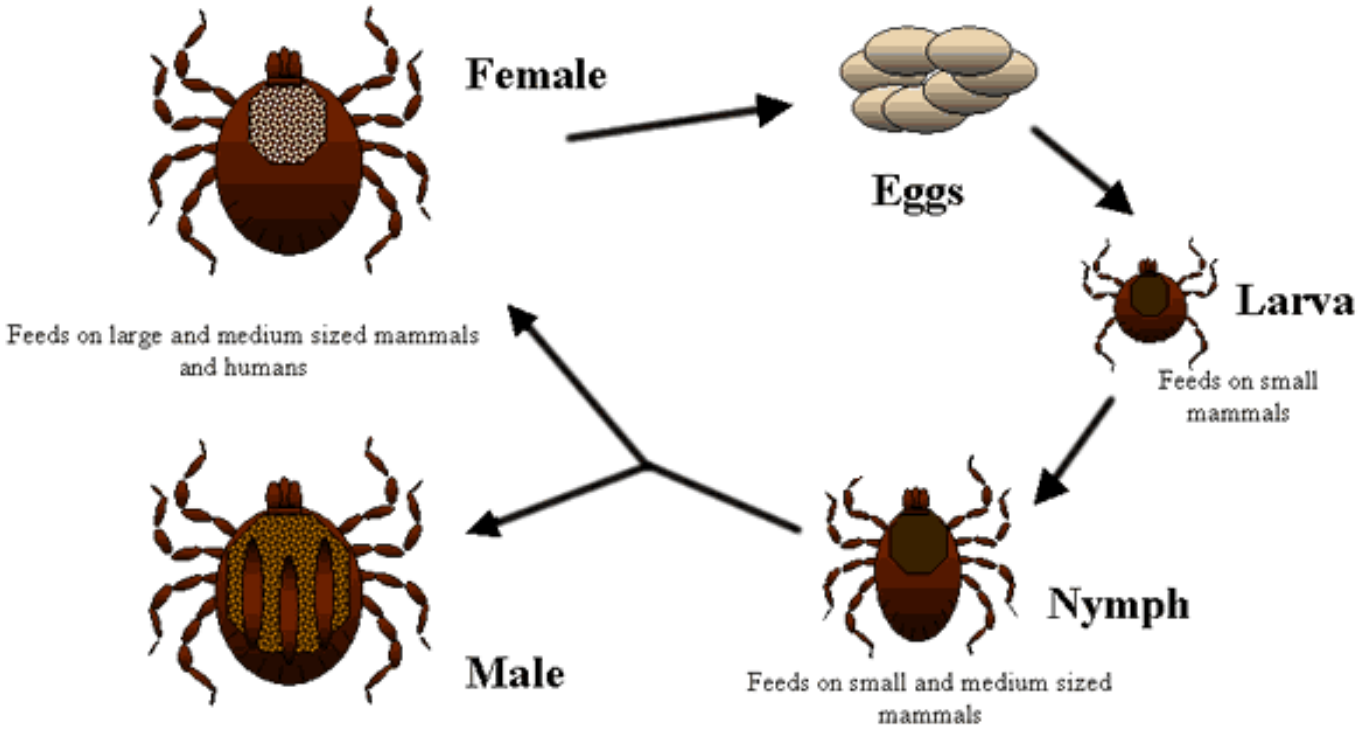

Figure 2. Life stages of ticks in the family Ixodidae, such as *Ixodes scapularis*, the blacklegged tick. Public domain image from the United States Center for Disease Control)

Table 1. List of sequence, 5' to 3', of primers and probes, with dye and quencher, used in the real time PCR assays for detection and sequencing of pathogens from extracted blood, and ear biopsies of mammals, and ticks.

Primer (target)	Source/ Reference	Forward Primer	Reverse Primer	Probe
			Borrelia burgdorferi detection	
Bb 23S (*23S*)	Courtney et al. 2004	CGCGTCTTAAAAGGGCGATTTAGT	GCTTCAGCCTGGCCATAAATAG	FAM-AGATGTGGTAGACCCGAAGCCGAGTG-TAMRA
ospA (*ospA*)	TIB Molbiol	CTGGGGAAGTTTCAGTTGAAC	TTGGTGCCATTTGAGTCGTA	FAM-CTGCAGCTTGGAATTCAGGCACTT-BBQ
			Anaplasma phagocytophilum detection	
Ap msp2 (*msp2*)	Courtney et al. 2004	ATGGAAGGTAGTGTTGGTTATGGTATT	TTGGTCTTGAAGCGCTCGA	VIC-TGGTGCCAGGGTTGAGCTTGAGATTG-TAMRA
			Borrelia miyamotoi detection	
glpQ (*glpQ*)	Ullmann et al. 2005	GATAATATTCCTGTTATAATGC	CACTGAGATTTAGTGATTTAAGTTC	FAM-CCCAGAAATTGACAACCAC-BBQ
			Babesia microti detection	
Bm cctη (*cctη*)	Nakajima et al. 2009	CAAGTTGGAGGCAATTCATAGC	CACAGCTTCCCAAACAAGAGTC	FAM-ACGAGTCCTCCTGTTGCTTTGGCCA-BBQ
Bm 18S (*18S*)	Applied Biosystems	AGCCATGCATGTCTTAGTATAAGCTTT	CACGGTTATCCATGTAAAACGAACA	FAM-AATGGCTCATTAAAACAGTTATAG-TAMRA

Table 2. Summary of small mammal trapping conducted in Beaudry and Birds Hill Provincial Parks during 2015 and 2016.

Species	2015				2016				Total	
	Beaudry		Birds Hill		Beaudry		Birds Hill			
	n	recap.	*n*	recap.	*n*	recap.	*n*	recap.	*n*	recap.
Southern red-backed vole (*Myodes gapperi*)	9	3	47	15	29	5	120	22	205	45
Meadow vole (*Microtus pennslyvanicus*)	0	0	1	0	4	0	2	0	7	0
Deer mouse (*Peromyscus maniculatus*)	3	0	14	4	2	1	4	0	23	5
Brown rat (*Rattus norvegicus*)	2	1	0	0	0	0	0	0	2	1
Cinereus shrew (*Sorex cinereus*)	7	0	0	0	2	0	1	0	10	0
American red squirrel (*Tamiasciurus hudsonicus*)	1	0	1	0	0	0	2	0	4	0
Eastern chipmunk (*Tamias striatus*)	0	0	0	0	0	0	1	0	1	0
Total	22	4 (18%)	63	19 (30%)	37	6 (16%)	130	22 (17%)	252	51 (20%)

n = number of times animal of this species was trapped; recap. = number of animals recaptured.

Table 3. Intensity, prevalence, and mean density of *Ixodes scapularis* and *Dermacentor variabilis* from mammals trapped in Beaudry and Birds Hill Provincial Parks in 2015 and 2016.

Tick Species	Year	Mammals[a]	Ticks	Intensity	Prevalence (%)	Mean Density
			Beaudry Provincial Park			
I. scapularis	2015	3	3	1.0	14	0.14
	2016	9	18	2.3 ± 0.62	24	0.54
D. variabilis	2015	4	8	2.0 ± 1.0	18	0.36
	2016	10	53	5.3 ± 3.4	27	1.43
			Birds Hill Provincial Park			
I. scapularis	2015	9	22	2.8 ± 1.2	14	0.39
	2016	5	**7**	1.4 ± 0.24	4	0.06
D. variabilis	2015	9	41	5.1 ± 2.9	14	0.72
	2016	8	10	1.1 ± 0.16	6	0.07

[a] All host species pooled.

Mammals = the number of trapped mammals with ticks (recaptured not included); Ticks = number of ticks removed from infested mammals; Intensity = number of ticks per infested mammal; Prevalence = proportion of trapped mammals that were infested; Mean density = number of ticks per infested mammal

Table 4. Number and density of *Ixodes scapularis* ticks collected by drag sampling at Beaudry and Birds Hill Provincial Parks during 2014, 2015, and 2016.

Year	Site	Ticks					Density (ticks/10 m^2)
		Males	Females	Total	z	p	
2014							
	Beaudry	15	25	40	1.265	0.206	0.008
	Birds Hill	4	2	6	0.817	0.414	0.0024
2015							
	Beaudry	3	1	4	1	0.317	0.0008
	Birds Hill	10	15	25	-1	0.317	0.005
2016							
	Beaudry	1	4	5	-1.342	0.180	0.0009
	Birds Hill	5	8	13	-0.832	0.405	0.0024
Total		38	55	93	--	--	0.0033

z-statistic and p-values indicate there is no sex-bias in the sample

Table 5. The prevalence of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in ticks, ear biopsies, and blood samples from deer mice and red-backed voles during 2015 and 2016.

Pathogen	Ticks			Ear Biopsy*		Blood Sample	
	Adults	Nymphs	Larvae	*P. maniculatus*	*M. gapperi*	*P. maniculatus*	*M. gapperi*
	Beaudry						
A. phagocytophilum	11% (44)	0% (0)	0% (16)	0% (2)	0% (30)	0% (2)	0% (27)
B. burgdorferi	36% (44)	0% (0)	0% (16)	0% (2)	7% (30)	0% (2)	0% (27)
	Birds Hill						
A. phagocytophilum	13% (16)	50% (2)	17% (30)	27% (11)	2% (138)	7% (14)	1% (130)
B. burgdorferi	19% (16)	0% (2)	10% (30)	45% (11)	3% (138)	0% (14)	0% (130)

Number of samples tested (*n*) indicated in parentheses.

* Ear biopsies not included: Eastern chipmunk (BHPP) co-infected with *A. phagocytophilum*, and *B. burgdorferi*; American red squirrel (BHPP) positive for *B. burgdorferi*; American red squirrel (BHPP) positive for *A. phagocytophilum*; Red-backed vole (BPP) positive for a *Borrelia* spp.

Table 6. Mammalian hosts of nymphal *Ixodes scapularis* with a known range in Manitoba.

Common Name	Scientific Name	Reference
American black bear	*Ursus americanus*	(Zolnik et al. 2015)
American red squirrel	*Tamiasciurus hudsonicus*	(Bouchard et al. 2011; Carey et al. 1980; Main et al. 1982)
Deer mouse	*Peromyscus maniculatus*	(Bouchard et al. 2011)
Eastern chipmunk	*Tamias striatus*	(Bouchard et al. 2011; Main et al. 1982; Schmidt et al. 1999)
Eastern cottontail rabbit	*Sylvilagus floridanus*	(Kollars et al. 1999; Main et al. 1982; Mannelli et al. 1993a)
Eastern grey squirrel	*Sciurus carolinensis*	(Godsey et al. 1987; Main et al. 1982; Mannelli et al. 1993a)
Groundhog	*Marmota monax*	(Carey et al. 1980; Godsey et al. 1987; Main et al. 1982)
Masked shrew	*Sorex cinereus*	(Main et al. 1982)
Meadow jumping mouse	*Zapus hudsonicus*	(Bouchard et al. 2011; Carey et al. 1980)
Meadow vole	*Microtus pennsylvanicus*	(Carey et al. 1980; Main et al. 1982)
Brown rat	*Rattus norvegicus*	(Main et al. 1982)
Raccoon	*Procyon lotor*	(Main et al. 1982; Mannelli et al. 1993a)
Red fox	*Vulpes vulpes*	(Anderson 1988)
Short-tailed shrew	*Blarina brevicauda*	(Main et al. 1982)
Southern red-backed vole	*Myodes gapperi*	(Carey et al. 1980; Godsey et al. 1987; Main et al. 1982)
Striped skunk	*Mephitis mephitis*	(Carey et al. 1980; Godsey et al. 1987)
White-footed mouse	*Peromyscus leucopus*	(Bouchard et al. 2011; Main et al. 1982; Schmidt et al. 1999)
White-tailed deer	*Odocoileus virginianus*	(Telford et al. 1988)
Woodland jumping mouse	*Napaeozapus insignis*	(Main et al. 1982)

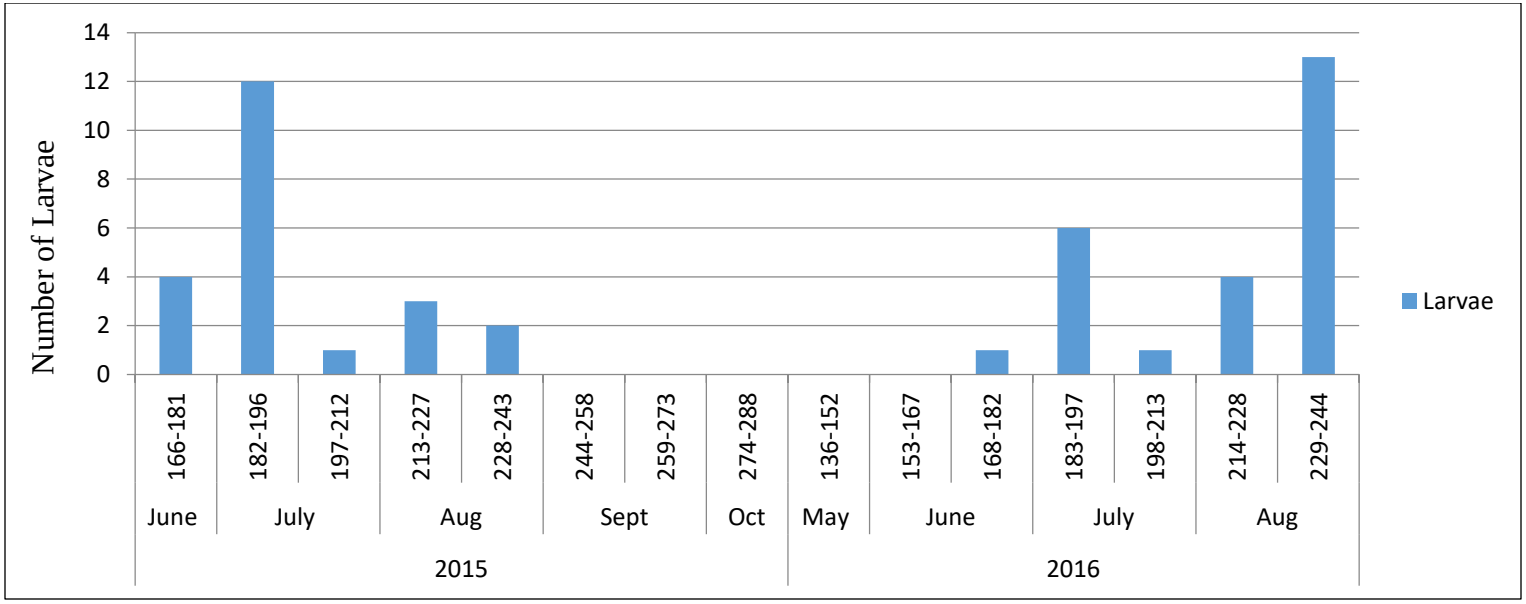

Figure 3. Number of larvae retrieved from mammals trapped at Birds Hill and Beaudry Provincial Parks in 2015 and 2016. Numbers on the x-axis represent Julian dates for 2015 and 2016, consecutively, and data are presented by two-week intervals to include trapping results from both parks.

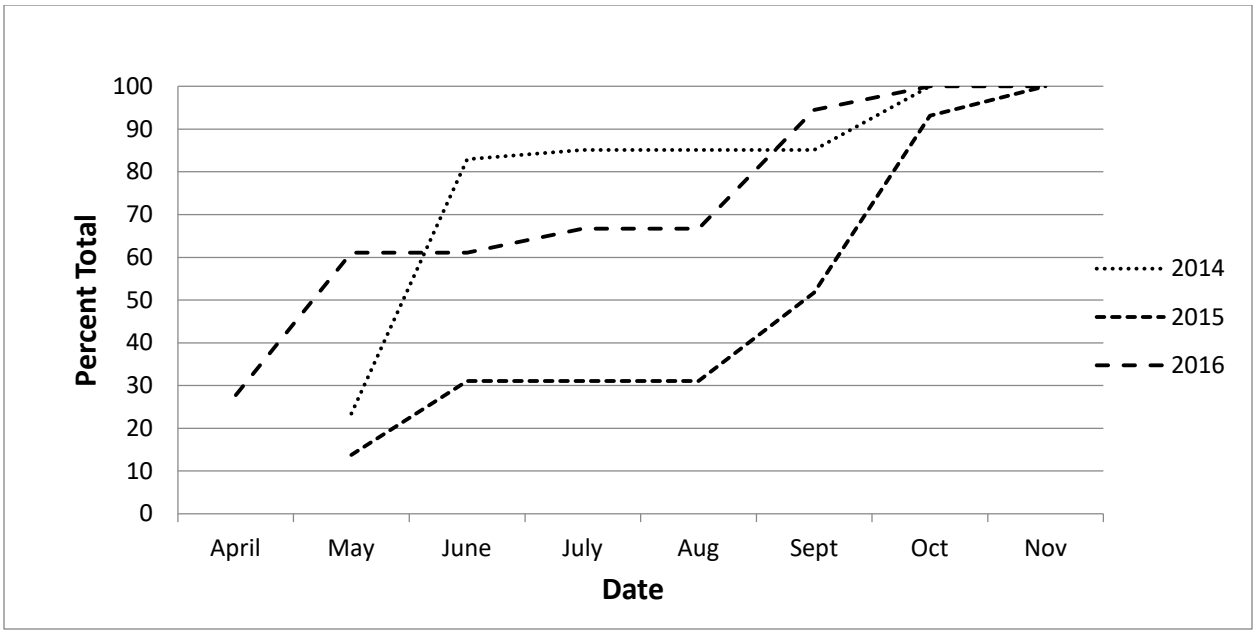

Figure 4. The seasonal host-seeking activity of adult blacklegged ticks sampled by dragging at Birds Hill and Beaudry Provincial Parks. Data are cumulative proportions of ticks caught for each month of sampling in three consecutive years.

Chapter 4: The Overwintering Survival, Supercooling Point, and Freeze Tolerance of Unfed Adult *Ixodes scapularis*

4.1 Abstract

In Manitoba, the range of blacklegged ticks is expanding north and west putting more people at risk of acquiring tick borne infections. For blacklegged ticks to expand their range, they must successfully overwinter. This survival is often dependent on factors such as microhabitat humidity, temperature, and the tick's cold hardiness. One measure of cold hardiness is the supercooling point, or the lower lethal temperature for freeze intolerant arthropods. To better predict the northward spread of blacklegged ticks, we determined their overwintering survival in two different field conditions, and cold hardiness under two different pre-treatments. At the end of October, 2015, 180 adult male and female ticks were placed in enclosures at a forest edge and forest site to overwinter in Winnipeg, Manitoba. Ticks from each site were brought indoors and assessed for survival at the end of January, February, March, April, and May. We also tested the supercooling point of adult blacklegged ticks held at 15°C for 24 hours or at 15°C followed by 5°C for 24 hours. Our forest edge site provided significantly better overwintering survival (69%) than our forest site (53%). No blacklegged ticks survived freezing past their supercooling point (-11.7°C ± 5.0), and ticks held at 15°C had a lower supercooling point (-15.7°C ± 2.5) than those held at 5°C. These findings suggest habitat plays a larger role in the overwintering survival of blacklegged ticks than does the tick's cold hardiness. Thus, the spread of these ticks northward will in part depend on the availability of suitable microhabitats.

4.2 Introduction

The blacklegged tick, *Ixodes scapularis* Say, is a species of hard tick in the family Ixodidae. These ticks are considered of public health importance as vectors of the zoonotic

pathogens *Borrelia burgdorferi* Johnson et al. emend. Baranton et al., *B. miyamotoi* Fukunaga et al., *Anaplasma phagocytophilum* (Foggie), and *Babesia microti* (Franca). Blacklegged ticks have the ability to acquire and/or transmit these pathogens when larvae, nymphs or females feed. Like other ixodid ticks, blacklegged ticks spend >90% of their time off the host, at the soil leaf litter interface (Burtis et al. 2016; Needham and Teel 1991). In the northern United States and in southern Canada, fed and unfed larval, nymphal and adult blacklegged ticks will overwinter in these specialized microhabitats in one of their three active life stages (Lindsay et al. 1998; Yuval and Spielman 1990a).

The first documented blacklegged tick in Manitoba was recovered north of Winnipeg in 1989 (Galloway 1989), while the first endemic population in the province was found at Buffalo Point in 2006 (R. L. Lindsay, personal communication). In the subsequent decade, blacklegged tick populations have become established in the southcentral and southeastern part of the province, and are expanding their range north and west (Manitoba Health Healthy Living and Seniors 2017). Limits to northern range expansion are in part climate-based, with temperature and humidity playing large roles (Dergousoff et al. 2013; Leighton et al. 2012; Lindgren et al. 2000; Ogden et al. 2005). Temperature and humidity are important factors because cold and dry winters (winters with low precipitation) have the potential to decrease overwintering survival of all life stages of *I. scapularis* (Brownstein et al. 2003; Platt et al. 1992). Currently the most northerly documented blacklegged tick population occurs slightly above 50° latitude within Manitoba (Manitoba Health 2015). However, active and passive surveillance data indicate that adventitious ticks are present as far north as Bowsman, Manitoba (52.2371°N, 101.205°W). As Manitoba is part of a new ecozone for these ticks in Canada, the potential limits of their northern range are of the utmost importance. Studies assessing the cold hardiness, and overwintering

survival of blacklegged ticks will therefore play a large role in modelling their northward expansion.

In Ontario, adult blacklegged ticks molt from nymphs to adults in August (Lindsay et al. 1998) and are usually active throughout the fall until seasonal temperatures drop to 4°C (Duffy and Campbell 1994). Adults that do not feed in the fall will overwinter, emerge in the spring, and continue to host-seek until mid-June (Lindsay et al. 1998). Females that feed in the fall will overwinter engorged and lay their eggs in the spring. Thus, like other hard ticks in North America, blacklegged ticks must overwinter to complete their life cycle (Barnard et al. 1985; Garvie et al. 1978). The overwintering process for blacklegged ticks involves finding a suitable habitat in the leaf litter and entering a state of diapause. It is reasonable to assume that the successful overwintering of blacklegged ticks is in some part related to their cold hardiness.

Cold hardiness is the ability to survive cool temperatures. Living organisms can be placed into one of two general categories: freeze tolerant or freezing intolerant. Freeze tolerant organisms are able to withstand ice formation inside their bodies through various means (Salt 1961; Somme 1982). Freeze intolerant organisms are unable to withstand ice formation inside their bodies and must block the formation of ice to survive (Salt 1961). Arthropods can use both behavioural and physiological adaptations before the onset of winter to help prevent ice formation. Behaviourally, ticks will seek a favourable spot under leaf litter where they are better protected from the cold. Physiologically, ticks will enter diapause, and also increase the level of cryoprotectants such as sorbitol, glycerol and antifreeze proteins within their bodies (Neelakanta et al. 2010; Yu et al. 2014). This freeze avoidance is achieved by supercooling body fluids (Salt 1961). Supercooling occurs when a fluid is lowered below its freezing point without solidifying. Although arthropods can supercool their body fluids by increasing the concentration of

cryoprotectants, there is still a temperature at which their body fluids will freeze (Somme 1982). This temperature is referred to as the supercooling point and results in the spontaneous freezing of tissues causing a release of latent heat. The supercooling point is measured by lowering the temperature of an organism (an insect, for example) at a constant rate, usually 1°C/minute (Salt 1966; Sinclair et al. 2015). The lowest temperature recorded before latent heat is released is measured as the supercooling point (Lee and Denlinger 1985; Lee and Baust 1987).

The supercooling point can provide a baseline in determining the freeze tolerance strategy used by an arthropod (Renault et al. 2002; Sinclair et al. 2015). For example freeze intolerant species cannot survive temperatures lower than their supercooling point (Salt 1961). Factors like sex, size, age, or the level of engorgement of an insect can affect its supercooling point (Renault et al. 2002; Somme 1982). Published studies on the supercooling point of blacklegged ticks are sparse. While it has been previously studied in immature ticks, it is uncertain how the values will compare to the supercooling point of adults due to their size difference (Burks et al. 1996b; Schmid 1986).

Studies on the survival of blacklegged ticks at low temperatures indicate they are quite susceptible to cool temperatures. For instance, nymphs acclimated to 4°C do not survive being held for two hours at temperatures lower than -11°C (Burks et al. (1996b), and 50% of unengorged adults die when held for two hours at approximately -12°C (Vandyk et al. 1996). Localities in Canada where blacklegged ticks are endemic routinely experience winter air temperatures below these thresholds for survival. Ticks are able to survive in these cold climates because the microhabitat they live in can protect them from cold and dry conditions (Burks et al. 1996b; Lindgren and Gustafson 2001). Evidence supports this as microhabitats under leaf litter generally do not reach temperatures below -5°C (Burks et al. 1996b; Burtis et al. 2016; Daniels

et al. 1996; Yuval and Spielman 1990a). When suitable microhabitats are sparse or lacking, the overwintering survival of blacklegged ticks is adversely affected (Burtis et al. 2014; Lindsay et al. 1998). It has been postulated for other tick species such as *Dermacentor variabilis* (Say), that the amount of snow accumulation and cover plays a role in overwintering survival (Berry 1981; McEnroe 1984). However, Burtis et al. (2016) found no difference in the overwintering survival of engorged blacklegged tick larvae in study plots with manipulated snow cover. The effect of snow cover on blacklegged tick survival may be more pronounced in regions that experience early intermittent snow cover (Burtis et al. 2016). Humidity could also affect the overwintering survival of blacklegged ticks. Soils saturated with moisture increase the likelihood of blacklegged ticks coming into contact with external ice crystals which promote inoculative freezing (Brunner et al. 2012; Burks et al. 1996b). On the other hand, winters with low precipitation (dry winters) have the potential to kill blacklegged ticks as they are unable to replenish water lost from transpiration (Burks et al. 1996b; Ostfeld and Brunner 2015).

Recent studies have modeled the projected range expansion of blacklegged ticks northward by using a combination of variables such as degree days, global climate change data, and climatically suitable habitat (Brownstein et al. 2005; Leighton et al. 2012; Ogden et al. 2006a). In some models the range of blacklegged ticks is predicted to expand to cover up to approximately half of Manitoba by the 2050's, and approximately 75% by the 2080's (Ogden et al. 2006a). However, models are only as good as the data used to build them, and currently there are no studies addressing the overwintering survival of adult blacklegged ticks on the Canadian prairies. This is important since Manitoba represents the first Prairie Province with established blacklegged tick populations. Models predicting the expansion of blacklegged ticks northward will also require information on the ability of all life stages to successfully tolerate freezing

temperatures. In Manitoba freezing temperatures can arrive suddenly, and daily winter temperatures can dip to -40°C. It is unknown how the supercooling point of these ticks would be affected by a sudden cool period compared to gradual cooling. If adult blacklegged ticks are freeze intolerant and their supercooling point is high, their ability to survive overwinter in the northern regions of Manitoba may be compromised.

In the present study, we investigated aspects of cold-hardiness in unfed adult blacklegged ticks from Manitoba. We first determined the supercooling point of both sexes under two different temperature pre-treatments, and secondly determined if females are freeze tolerant. We hypothesized that ticks exposed to a cooler temperature would have a lower supercooling point due to a rapid cold hardening response. As well, we hypothesised females would be freeze intolerant like other tick species studied thus far. We also build on the work of Lindsay et al. (1995) who determined the overwintering survival of blacklegged ticks in Ontario, as we sought to determine the overwintering survival of unfed adults in Manitoba. Our objective was to assess the monthly rate of survival of adult blacklegged ticks in two different habitat types (forest and forest edge). In southern Manitoba, forest habitat often consists of small patches situated close to agricultural fields, and consequently, the edge represents a relatively large portion of the treed area. As forests have more canopy cover, they produce more leaf litter than their surrounding edge habitat, and could provide a more suitable microhabitat for blacklegged ticks. We hypothesized that blacklegged ticks would have greater survival throughout the winter in our forest habitat as the forest floor would provide better insulative properties.

4.3 Methods

Location

The tick collection site was located approximately 85 km southeast from the city of Winnipeg, on private land adjacent to the Roseau River. The riparian habitat is a deciduous forest dominated by a variety of poplars and cottonwoods (*Populus balsamifera* Linnaeus, *P. tremuloides* Michx., *P. deltoids* Bartr. ex Marsh), bur oaks (*Quercus macrocarpa* Michx.), and Manitoba maples (*Acer negundo* Linnaeus.), with some saskatoon berry bushes (*Amelanchier alnifolia* (Nuttall)) and chokecherry bushes (*Prunus virginica* Linnaeus.).

Tick Collection

Host-seeking adult blacklegged ticks were collected by dragging a flannel sheet on the ground (Rochon et al. 2012). Adult ticks collected from Beaudry and Birds Hill provincial parks (n = 27) were also used in this experiment. Collected ticks were transported to the University of Manitoba, identified to species (Keirans and Litwak 1989), and sorted by sex into Corning® CentriStar™ centrifuge tubes with pierced lids fitted with muslin squares. The tubes were stored at 95% RH a desiccator containing an oversaturated KNO_3 solution (Winston and Bates 1960) and kept in an incubator held at 15°C in total darkness.

Overwintering Survival

Our study sites were located at an area called "The Point" at the University of Manitoba where blacklegged tick populations are not known to be present. The Point serves as a research area for field crop studies, and is enclosed by woodland composed of oaks and Manitoba maples. Two areas roughly 200 meters apart were chosen as study sites due to their different canopy cover and hence potentially different snow accumulation. Site one known henceforth as the

"forest edge site" was located in an open grassy area approximately ten meters from the forest. Site two henceforth known as the "forest site" was located within the forested area. The two sites were intended to mimic different overwintering scenarios encountered on the Canadian prairies where the forest edge site would potentially receive more snow and have less leaf litter, and the forest site, sheltered by the forest canopy, would receive less snow cover but have more leaf litter available.

Tickaria were constructed similarly to those of Lindsay et al. (1995) and Yunik et al. (2015) to allow ticks to be in contact with leaf litter and soil. This is important as studies indicate ticks have higher survival when they are able to move in and out of the soil layer as opposed to being confined in one place (Bertrand and Wilson 1996; Brunner et al. 2012; Padgett and Lane 2001). Tickaria were constructed from 4 litre plastic pails (Prowestern Plastics LTD.) with snap tight lids. Four 3.5 mm diameter holes drilled into the bottom of each pail and lid, and covered with a plastic mesh glued into position allowed moisture to flow through the pails (Figure 3 A). Soil cores the same size and depth as the pails were taken from each site and placed into respective pails destined for that site. Thus, each pail fit into the hole left from the corresponding soil core (Figure 1 C). Six centrifuge tubes with their bottoms removed (Figure 1 B) were placed into each pail. The open bottom of the tubes were pushed into the soil core while cloth mesh was added to the lids to allow moisture to flow through but not allow ticks to escape (Figure 1 D) Within each pail, three tubes each contained three female ticks and three tubes each contained three male ticks.

On 9, 19, and 24 October ticks were collected from the aforementioned site near Winnipeg, MB. In total, 360 ticks collected on these days were used in this experiment. Ticks were sorted into groups of 10 and stored at 15°C and 95% RH. The total number of ticks used

was 360 (180 per site). At each site, 10 pails were placed into the ground spaced approximately 50 cm apart in a 2 x 5 grid where their corresponding soil core was taken. Pails from the forest site were placed outside on 28 October, 2015, and pails from the forest edge site were placed outside 30 October, 2015.

On 14 January, 16 February, 13 March, 14 April, and 11 May 2016, one pair of pails from each site were brought into the lab and placed in a fridge at 5°C, 95% RH. Pails retrieved in May were placed in a fridge at 15°C, 95% RH to mimic outdoor temperatures. All pails (except those retrieved in May) were held at 5°C to maintain the ticks' cold hardiness. The pails were opened one at a time and tick survival was assessed in a 5°C cold room on the day of retrieval from the field. Surviving ticks were separated based on sex and stored in an incubator at 5°C, 95% RH for future supercooling point studies. Ticks that showed no signs of movement were separated based on sex and stored at 5°C, 95% RH for 24 hours, and then reassessed for survival using the breath test. The breath test involves blowing on ticks releasing CO_2 in their vicinity, which ticks should respond to with movement. If no movement or signs of life were seen, ticks were assumed dead. Any tick missing from the tickaria were counted as "missing" and removed from the study.

Supercooling Point with Pre-treatment

We tested the freeze tolerance and the supercooling point of unfed adult blacklegged ticks held under two different conditions. Ticks used in the 15°C treatment were held under conditions described above for 3-3.5 months before the supercooling point was determined. Ticks used in the 5°C treatment were moved from 15°C to 5°C (95% RH) for 24 hours prior to the supercooling point and freeze tolerance test. Holding ticks at 5°C for 24 hours before testing

their supercooling point created conditions that should illicit a rapid cold hardening response in pretreated ticks.

Five males and five females were individually placed into pipette tips with a thermocouple positioned in direct contact with the tick. To prevent an artificial freezing event due to condensation, the end of each pipette tip was removed allowing air to circulate. Each pipette tip containing tick and thermocouple was placed into a wooden block containing ten holes, one for each pipette tip. The block was then placed into a Styrofoam box containing inflatable packing plastic to reduce the amount of air within the container. The entire container was then placed into a -40°C freezer and cooled at a calculated rate of approximately 1°C/minute. Temperature of each tick were measured every second (HOBO® data loggers, Onset Computer Corp, Bourne MA). Ticks were chilled to approximately -25°C before ending the experiment. The lowest temperature recorded prior to the release of latent heat was recorded as the supercooling point.

Freeze Tolerance

Twenty females that had been held in our laboratory in a desiccator at 15°C, 95% RH, for 5-5.5 months were placed into desiccators at 95% RH within an incubator set to 5°C for 24 hours before freezing tolerance was tested. The same setup as described in the supercooling point determination above was used, however ticks were tested individually and not in groups of ten. A computer connected to a HOBO® data logger was used to monitor for the latent release of heat in real time. As soon as a temperature increase was detected, the tick was quickly removed from the freezer and exposed to room temperature so that temperatures below its supercooling point were not reached. After warming to room temperature, tick survival was assessed by breathing on them to evoke a response. Those that showed no response were kept in centrifuge

tubes and held at 15°C, 95% RH for 24 hours. Any ticks still showing no signs of life after 24 hours were classified as dead.

Supercooling Point of Overwintering Ticks

Live ticks retrieved from the overwintering tickaria were stored at 5°C, 95% RH. Ticks were held for no longer than 24 hours after being assessed for survival. Using the same setup described above, the supercooling point of overwintering ticks was tested.

Data Analysis

All statistical analyses were performed using the GLM procedure in SAS/STAT® University Edition (SAS Institute Inc. 2016). Tukey's method was used in conjunction with the general linear model to assess overwintering survival. The GLM procedure uses the least squares method to fit general linear models, while Tukey's method is a post-hoc test used to compare all possible pairs of means to determine which groups means are different. The GLM procedure was appropriate because the overwintering samples were independent, and the data was normally distributed with fixed effects. The Tukey's method was used because it reduces the probability of making a type 1 error, tests all pairwise differences, and is simple to compute. All significance thresholds were set to 0.05.

4.4 Results

Overwintering

We found ticks overwintering in our forest edge habitat had a statistically greater survival rate (69% ± 6.2) (mean ± Standard Error Mean) compared to those in our forest site (53% ± 4.7) (F = 8.72, P = 0.0062). However, when comparing sites from the same month, neither the forest nor forest edge sites were significantly better for tick survival in any given month (Figure 4). The

survival of ticks through winter (i.e., to April and May) did not differ by sites or months. At our forest edge site, ticks assessed in January ($P < 0.001$) and February ($P = 0.0025$) had greater survival than those assessed in March. We obtained soil temperature recorded at a depth of 2.5 cm, and air temperature data from a weather station located 200 meters from the forest edge site in an open field (Figure 5). Air temperatures at The Point ranged from -31°C to 18°C between the end of October and mid-May, when the experiment ended. Freezing temperatures were first recorded on November 11, and were last recorded April 14. Soil temperatures ranged from -4.1°C to 14.3°C, with the first freezing temperatures recorded on November 29, and the last on April 12. A number of pails from both sites were found with fewer ticks than they initially had. Ticks that could not be found ("Missing Ticks") were removed from the study (Figure 4).

Supercooling Point

Throughout the entire experiment no tick survived freezing of their body tissues past their supercooling point. The mean (± Standard Error Mean) supercooling point of females from the freeze tolerance study was -11.7°C (± 1.1). There was high variability between individual ticks supercooling with the highest supercooling point at -5.5°C, and the lowest at -18.9°C.

Out of the 75 ticks tested under different pre-treatment regimes, 66 provided reliable supercooling point readings. Nine readings were inconclusive, likely due to the thermocouple losing contact with the surface of the ticks. There was no statistical difference in the supercooling point between sexes ($F = 0.61$, $P = 0.4387$) so we combined them for further analysis. Ticks held at 15°C had a mean supercooling point of -15.7°C (± 0.46) while ticks under the 5°C pre-treatment had a mean supercooling of -12.0°C (± 0.89) ($F = 11.62$, $P = 0.0011$). Ticks held at 15°C show a unimodal distribution of their supercooling point clustered around

-15°C, while those held at 5°C show a bimodal distribution clustered around -7°C and -17°C (Figure 4).

We obtained supercooling point results for 101 ticks originating from the forest edge site, and 65 from the forest site (Figure 6). There was no statistical difference in supercooling points between sexes ($F = 0.64$, $P = 0.4262$), date of retrieval ($F = 1.95$, $P = 0.1050$) or sites ($F = 3.58$, $P = 0.0603$).

4.5 Discussion

We found blacklegged ticks are able to successfully overwinter in Manitoba in two different habitat types. In the forest and forest edge habitats, survival was never less than 28%. Although we found overall survival was greater in the forest edge habitat, on any given month there was no difference between sites. Due to the design of our study, the percentage of ticks that successfully overwintered can only be determined from the months after the last freezing temperatures were recorded. From a nearby weather station located at The Point, we know freezing air and soil temperatures were last recorded April 14, which is the same day we removed our April ticks. We cannot tell if ticks we collected before April would have perished had they been left in their tickaria, so we cannot say they successfully overwintered. For this reason, we consider ticks surviving in April and May as surviving over the entire winter. The survival rate of successfully overwintered blacklegged ticks (April and May) was 55% at the forest site, and 69% at the forest edge site. These survival rates are similar to Lindsay et al. (1998) (47%) who overwintered unfed adults at Long Point, Ontario. However, our rates are much higher than the 26%-32% survival found by Lindsay et al. (1995) in their northern Ontario sites. Winnipeg is located only slightly further north than these northerly sites, so the survival rate should have been similar if air temperature alone was a limiting factor. Differences in snow

cover and available refugia likely cause year to year variation in survival between sites and years (Lindsay et al. 1998). Although air temperatures reached a low of -31°C, soil temperatures were never colder than -4°C. Other studies have found similar results, with soil temperatures never dipping below -5°C even in the midst of cooler air temperatures (Burks et al. 1996b; Rosendale et al. 2016).

We observed no difference in the overwintering survival of unfed males and unfed females. In certain years and locations, female blacklegged ticks have better survival than males (Lindsay et al. 1998), but others found no difference (Bertrand and Wilson 1996; Lindsay et al. 1995). For example ticks held at more northerly locations showed no difference in overwintering survival between sexes, whereas female ticks held at Long Point, Ontario were more successful than males (Lindsay et al. 1995). Most of the "missing" ticks that were removed from the analysis were males. To make sure these "missing" males did not affect our results, we also ran an analysis where "missing" ticks were counted as dead ticks. The resulting analysis also determined there was no difference in the overwintering success between sexes at our study locations. In a recent study of overwintering of American dog ticks (*D. variabilis*) performed a few hundred meters from our study sites, more unfed females (n = 170) survived overwinter compared to males (n = 99) (Yunik et al. 2015). Although *D. variabilis* and *I. scapularis* can occupy the same habitat, sexual dimorphism is less pronounced in *D. variabilis*, making it harder to draw comparisons between species. We expected females would have greater overwintering success compared to males, because of their larger size and hence greater energy reserves to survive until spring (Daniels et al. 1989).

At the forest edge site, ticks assessed in January and February had greater survival than those assessed in March (Figure 4). During our retrieval of forest edge ticks in March we noticed

ice formation inside our buckets, with some ticks encased in ice. Inoculative freezing, in which ticks come into contact with ice, is known to cause high mortality (Burks et al. 1996b). In the later part of February and early part of March (Figure 5), we experienced large amplitude in daily temperatures, with warm day temperatures melting snow followed by freezing nightly temperatures. It is possible some pooling snow melt water got into our forest edge buckets and froze some of our tick specimens. Tick survival assessed in April and May was no different than tick survival assessed in March. Water could have got into these tickaria as well and formed ice, but due to the warm temperatures during collection, we would not have seen ice present. Choosing areas that aren't prone to pooling melt water could possibly alleviate this problem in future overwintering studies.

Allowing ticks access to soil and leaf litter was a critical factor in the design of our tickaria. Ticks that are allowed accesses to soil and leaf litter have greater survival than those confined to unnatural conditions (Bertrand and Wilson 1996; Padgett and Lane 2001). Brunner et al. (2012) attributed their higher overwintering success in part to the design of their overwintering enclosures, which allowed ticks an opportunity to seek microhabitats similar to natural conditions. Similarly our design allowed ticks access to a natural microhabitat whilst providing adequate airflow. To disturb ticks as little as possible we used destructive sampling to assess monthly winter survival. The energy loss of ticks disturbed during diapause is unknown, but we felt repeated disturbances could affect true survival rates. This difference in methodology may be why our overwintering success is higher than other studies. A draw back to our destructive sampling was that we were left with a smaller sample size to assess overwintering survival unless we assumed that monthly rates of overwintering survival would continue until winter had passed.

Our finding of a high overwintering success rate in two different habitat types in Manitoba could shed some light on the ability of these ticks to overwinter on the rest on the Prairie Provinces. Our sites were reminiscent of habitat common to the prairie ecozone that encompasses most of southern Manitoba, Saskatchewan, and Alberta. Blacklegged ticks have sporadically been found in Saskatchewan and Alberta but to date no endemic populations have been identified (Anstead and Chilton 2011; Lindsay et al. 1999b; Ogden et al. 2006b; Scott et al. 2012). In 2007, blacklegged tick larvae were recovered from northern pocket gophers (*Tomomys talpoides* Richardson) in Calvet, Saskatchewan (Anstead and Chilton 2011). It was determined these ticks were probably offspring of adults that had successfully overwintered (Anstead and Chilton 2011). Like our overwintering sites, Calvet Saskatchewan is located in the prairie ecozone (Gauthier and Wiken 2003; Wiken and Areas. 1996). Due to a warming climate, blacklegged ticks were predicted to become established in southern Manitoba and Saskatchewan by the 2020's (Ogden et al. 2006a). Our overwintering results and the likely overwintering survival of adult blacklegged ticks in southern Saskatchewan indicate that adult ticks have the ability to overwinter on the prairies in both forest and forest edge habitats.

Our data demonstrate that unfed blacklegged tick females are freezing intolerant, which is consistent with our hypothesis since all tick species studied so far are also freezing intolerant (Burks et al. 1996b; Dautel and Knülle 1996; Lee and Baust 1987; Schmid 1986). Although males were not specifically tested for freeze tolerance it is likely they too are freezing intolerant. To date no published literature shows that male ticks can survive freezing when females cannot or vice versa. Since blacklegged tick adult females are freezing intolerant, the supercooling point theoretically represents the lowest temperature that these ticks could survive. It is unlikely blacklegged ticks would survive to these temperatures as many tick species perish above their

supercooling point (Burks et al. 1996a; 1996b; Yu et al. 2014). For instance Burks et al. (1996b) exposed nymphal *Amblyomma americanum* (Linnaeus) and *D. variabilis* to -4°C for 2 hours and observed 80% and 87% mortality, respectively. For ticks that overwinter, mortality is likely caused by inoculative freezing at temperatures well above their supercooling point (Burks et al. 1996b).

The mean supercooling values under all treatments are higher than those recorded for unfed blacklegged tick nymphs, which is expected as smaller arthropods should have a lower supercooling point (Burks et al. 1996a; 1996b; Johnston and Lee 1990; Somme 1982). As males are smaller than females, we should expect them to have a lower supercooling point; however, within each treatment we did not observe supercooling point differences between sexes. Our observations are corroborated by Dautel and Knülle (1996) who found no difference in the supercooling point between males and females of the closely related tick *Ixodes ricinus* (Linnaeus). Although males are smaller than females, the difference may not be large enough to cause a significant difference in the observed supercooling point (Dautel and Knülle 1996).

Blacklegged ticks have higher supercooling point values compared to most other unfed adult tick species studied (Burks et al. 1996a; Dautel and Knülle 1996; 1997). For instance, the supercooling point of *D. variabilis*, which can be found in the same localities as *I. scapularis*, is approximately -22.1°C (Dautel and Knülle 1996). One species of tick with a higher recorded supercooling point than *I. scapularis* is *I. ricinus*, a close relative and also a vector of the pathogens associated with Lyme disease in Europe. Interestingly, the mean supercooling point (±SD) of field collected *I. ricinus* (-11.6 ± 3.0°C) was higher than lab reared specimens (-19.5 ± 2.8°C) (Dautel and Knülle 1997). This suggests that growing or rearing conditions may have an impact on the supercooling point, making it hard to draw comparisons to other studies which

have used laboratory raised ticks (Burks et al. 1996b; Dautel and Knülle 1996; Needham et al. 1996; Yu et al. 2014).

By holding ticks at 5°C for 24 hours before testing their supercooling point, we created conditions that could elicit a rapid cold hardening (RCH) response. The results indicate that pretreated ticks were less cold hardy than those that were not pretreated. This finding is contrary to what we expected, as we thought a cold hardening response would lead to lower supercooling point s compared to ticks held at 15°C (i.e., not pre-treated). It could be argued our 5°C pre-treatment was too long, as RCH takes place within minutes or hours (Teets and Denlinger 2013). However, we believe that holding our ticks for 24 hours is closer to RCH than it is to seasonal cold hardening which would require days to weeks for induction (Teets and Denlinger 2013). Tick studies employing longer pre-treatments (7-15 days) have not shown a change in the supercooling point (Lee and Baust 1987; Needham et al. 1996), where as our shorter induction time did illicit a response. The difference in supercooling points between our two treatments is likely from physiological or biochemical changes taking place in the pre-treated ticks (Coleman et al. 2014).

It is also interesting that ticks held at 15°C have a unimodal distribution of their supercooling point whereas pre-treated ticks have a bimodal distribution, the reason for which remains unclear. Ice nucleators and differences in body composition are some causes of bimodal supercooling point distributions (Sinclair et al. 2015). A similar high and low supercooling point distribution has been observed in the larvae of *Cisseps fulvicollis* (Hübner) (Lepidoptera: Arctiidae) (Fields and McNeil 1986). Larvae of this species exhibit a dual cold-hardiness with high supercooling points indicative of freezing tolerance and low supercooling point s indicative of freezing intolerance (Fields and McNeil 1986). Higher supercooling points are indeed

generally representative of insects that are freeze tolerant (Zachariassen 1982). However, the results of our freezing tolerance experiment show that both high and low supercooling point s associated with blacklegged ticks are not from freeze tolerance in ticks. It could also be argued that our high supercooling point values in the range of -3°C to -8°C for pre-treated ticks, are artificial freezing events due to condensation (Sinclair et al. 2015). We do not believe this is the case though, as great care was taken to ensure condensation was not present in our tubes, and ticks were not in contact with moisture. Condensation was never observed on any of the tubes containing ticks prior to supercooling point testing or immediately following supercooling point readings.

The supercooling point of insects can change seasonally, resulting from the concentration of cryoprotectants present in the insect (Somme 1982). We found that ticks from both overwintering habitats showed no difference in their supercooling point throughout the winter and into spring. An intermittent thawing period in mid-March also seemed to have no effect on the seasonal supercooling point (Figure 5). This lack of seasonal change has also been observed in *Ixodes uriae* (White) (Lee and Baust 1987), and *Dermacentor marginatus* (Sulzer) (Dautel and Knülle 1996). To be sure that blacklegged ticks show no seasonal change in the supercooling point, we would need to determine supercooling points during the summer and into fall. If the supercooling point remains unchanged, *I. scapularis* could be said to exhibit a permanent state of cold hardiness, much like *I. uriae* (Lee and Baust 1987).

The use of the supercooling point to determine low temperature survival in field conditions is often unreliable and in most cases has little ecological significance (Baust and Rojas 1985; Nedved et al. 1998; Renault et al. 2002). Ticks have been found to die at temperatures well above their supercooling point from chilling injury rather than freezing (Burks

et al. 1996b). Despite its drawbacks, the supercooling point is relevant because it can elude a species freeze tolerance strategy, and indicate if biochemical or physiological changes are taking place between treatments (Sinclair et al. 2015). We believe our supercooling point studies were designed with this in mind, and are important for future cold hardiness studies that could address ecological questions. For example it would be interesting to expand the work of Neelakanta et al. (2010), and determine the ability of *A. phagocytophilum* positive adults to survive various cold exposures. In this example knowing the supercooling point of unfed adults is important, because it provides a baseline temperature expand on.

In summary, we determined that unfed blacklegged ticks can successfully overwinter in both forest and forest edge habitats, with forest edge habitats providing greater overwintering success. Soil temperatures were always higher than seasonal supercooling points, meaning the supercooling point likely has no effect on the overwintering ability of these ticks in Manitoba. We also determined that pre-treating ticks by holding them for 24 hours at 5°C increased their supercooling point. During the fall in Manitoba, warm periods are often interrupted with cooler temperatures lasting one or two days. Our findings suggest these cold snaps do not make blacklegged ticks more cold tolerant than ticks with no prior exposure to the cold. Following the suggested workflow put forth by Sinclair et al. (2015), future cold hardiness studies on blacklegged ticks should look at other measures such as the critical thermal minimum, and chill coma recovery. Chill coma is the loss of movement in an insect due to cool temperatures, and chill coma recovery is the amount of time it takes for the insect to resume movement after warming (Sinclair et al. 2015). Because an immobile insect will eventually die, these measures are critical aspects of cold hardiness and would add to the results obtained here. Future overwintering studies in Manitoba should look at the other aspects of this tick not explored here.

The life cycle of blacklegged ticks can range from 2-3, to 3-4 years depending on their location (Hamer et al. 2012b; Lindsay et al. 1998; Yuval and Spielman 1990a). Determining the length of this tick's life cycle in Manitoba would require overwintering fed and unfed ticks of all life stages. This is important work as the life cycle of these ticks has many implications for disease transmission. Pathogens present in a 3-4 year tick life cycle have the potential to be maintained longer in the environment (in vectors and hosts) compared to those in a 2-3 year life cycle. If blacklegged ticks in Manitoba display a 3-4 year life cycle, this could put the public at greater risk of encountering pathogen positive ticks compared to a 2-3 year life cycle.

4.6 Acknowledgments

There are many people who assisted us in this study. Dr. Fields provided insight, assistance, materials, and lab space for supercooling point determinations. Kaitlyn Watts collected ticks. Lisa Babey collected ticks and helped with the placement of overwintering tickaria. We also thank the department of Plant Science for allowing us access to The Point, and Georges Beaudry for permission to collect ticks on his property.

Figure 5. A) 4 Litre plastic pails with snap tight lids. Four 3.5 mm diameter holes were drilled into the bottom of each pail and lid, and covered with a plastic mesh glued into position to allow moisture to flow through the pails. B) Centrifuge tubes with their bottoms removed, and mesh cap to allow air flow. C) 4 Litre plastic pail positioned in a hole left from a soil core. Soil core can be seen next to pail. D) 4 Litre plastic pail with inserted centrifuge tubes.

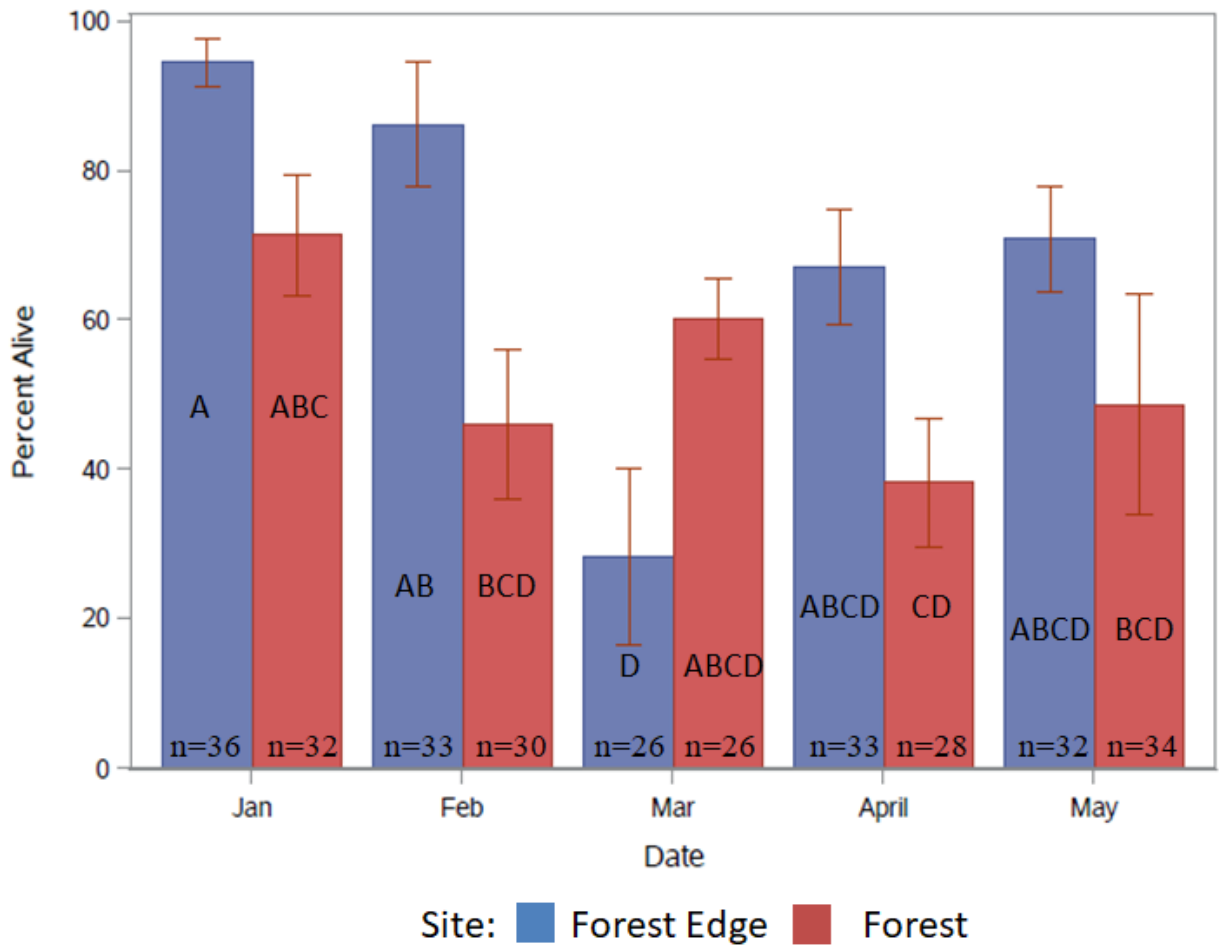

Figure 6. Survival of overwintering adult male and female ticks at forest and forest edge sites at The Point (University of Manitoba, Winnipeg). Data analysed using Proc GLM and Tukey's method. Values are mean ± SEM. Means with the same letter are not significantly different. Subsets of ticks were retrieved from the field on January 14, February 16, March 13, April 14, and May 11, 2017. Starting number of ticks each month for each site is equal to 36.

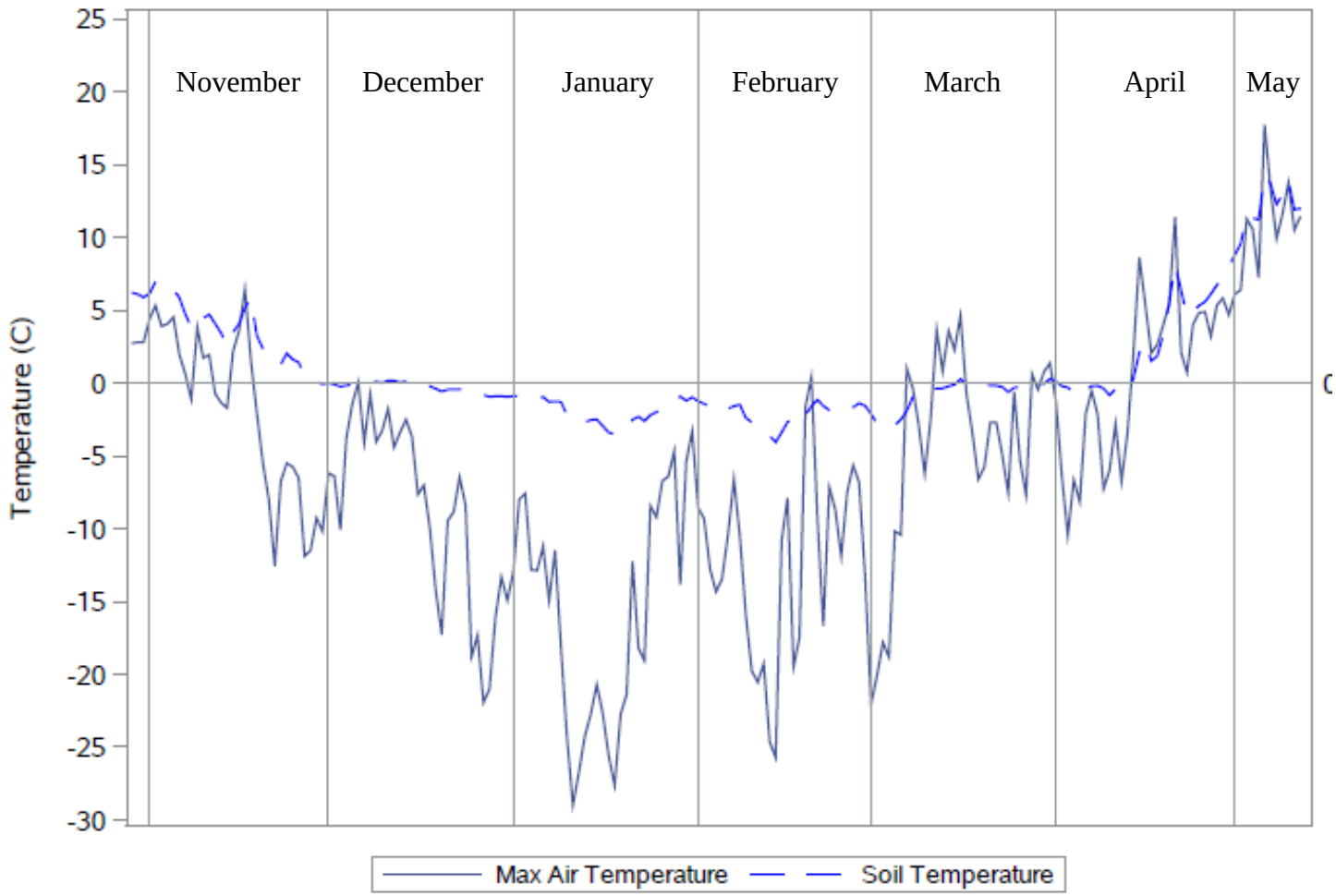

Figure 7. Maximum air and mean soil temperatures recorded at 'The Point' between October 28/2015 and May 11/2015 (*n* = 197).

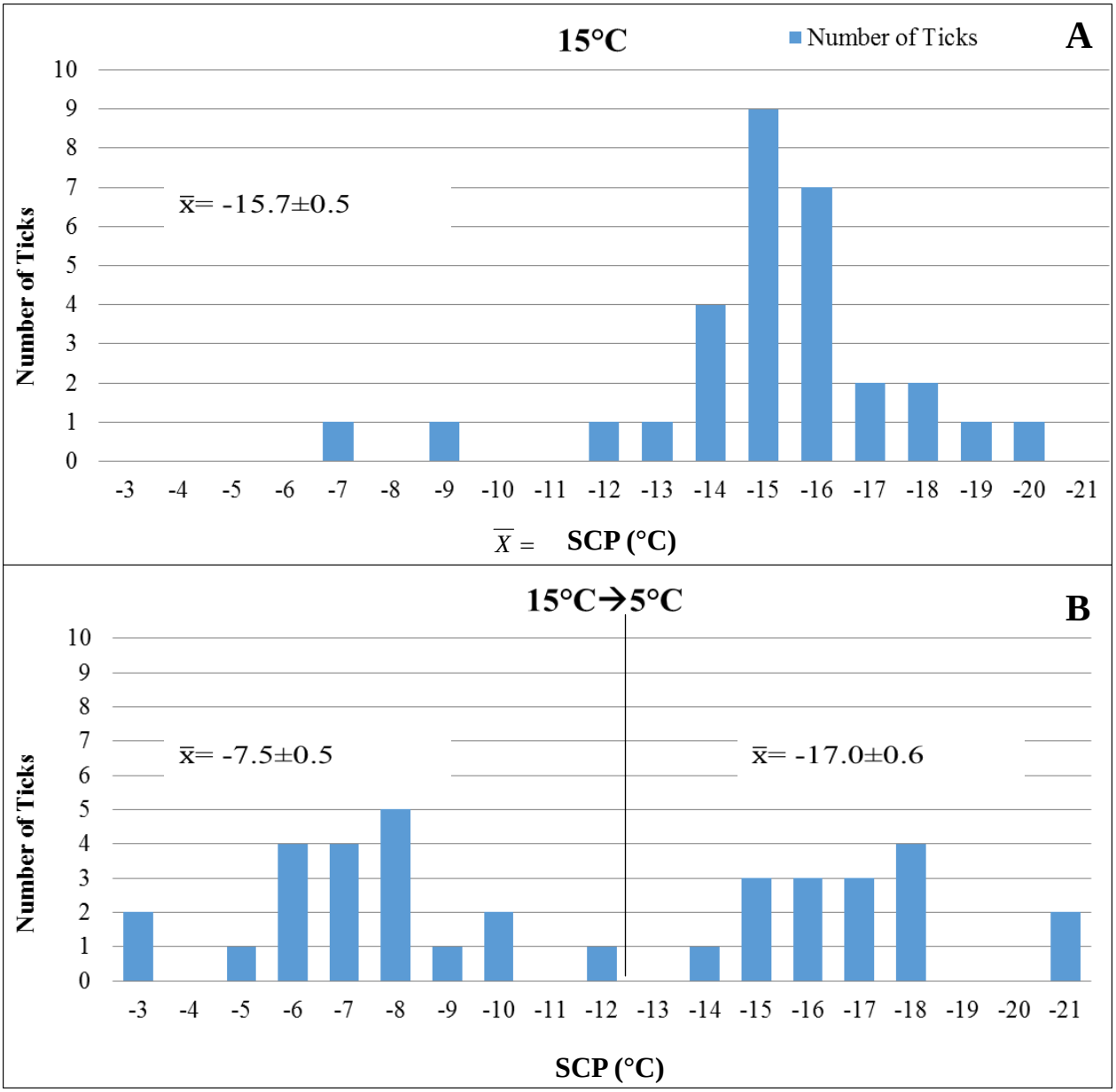

Figure 8. A) Supercooling point (SCP) distribution of ticks held at 15°C (*n* = 33). B) Supercooling point distribution of ticks held at 15°C for 3-3.5 months then held at 5°C for 24 hours (*n* = 30). Vertical line separates data into two distributions with mean +/- SEM.

Chapter 5: General Discussion

The blacklegged tick *Ixodes scapularis* Say has been well studied in eastern Canada (Lindsay et al. 1995; 1999a; Watson and Anderson 1976), and in the midwestern (Hamer et al. 2012b; 2014; Platt et al. 1992), and eastern United States (Piesman and Spielman 1979; Qiu et al. 2002; Sakamoto et al. 2014). Blacklegged ticks have been reported in Manitoba since 1989 (Galloway 1989), and established since 2006 (R. L. Lindsay, personal communication), but have received sparse scientific study within the province. By tick dragging and mammal trapping I was able to determine the seasonality of adults and larvae at two provincial parks. I also determined the pathogen prevalence of *Borrelia burgdorferi*, *B. miyamotoi*, *Anaplasma phagocytophilum*, and *Babesia microti* in ticks and mammals using quantitative real-time PCR. Finally, I contributed to our understanding of blacklegged tick cold-hardiness by determining the overwintering success and supercooling point of adult blacklegged ticks.

5.1 Insights

Mammal Trapping and Seasonality

Through mammal trapping I determined larval and nymphal host-seeking activity periods overlap in Manitoba, indicating a degree of synchronicity. To what extent this occurs remains unknown due to the small number of nymphs we recovered. As mentioned previously, the synchronicity of larvae and nymphs has implications on the prevalence of pathogens in the environment. If future studies in Manitoba determine larvae and nymphs have a high degree of synchronous feeding, then shorter-lived pathogens such as *A. phagocytophilum* will be favored in the environment (Levi et al. 2015). This would be concerning for public health officials as Powassan virus, an emerging pathogen that has a mortality rate of 10% (Ebel 2010), is expected to become more prevalent when immatures feed synchronously (Levi et al. 2015).

Although synchronous feeding of larvae and nymphs may be the current situation in Manitoba, it may not be so in the future. In the American Northeast, warmer autumns decrease the synchronicity of larvae and nymphs, because fewer larvae will feed in the spring (Levi et al. 2015). This has implications on pathogen transmission rates as longer lived pathogens such as *Borrelia burgdorferi* are expected to increase (Levi et al. 2015). The number of days above 5°C are expected to increase in Manitoba as a result of global climate change (Agriculutre and Agri-Food Canada 2014). Warmer temperatures will mean shorter winters (shorter period with snow cover), and therefore a longer period for tick activity and development. If larvae are able to host-seek for an extended amount of time in autumn, then like in the Northeast fewer larvae will feed in the spring. For Manitoba, the consequences of a warming climate may be a greater risk of acquiring Lyme disease.

Pathogens

The prevalence of *A. phagocytophilum* (10% of adults in both parks, and 12 % of larvae in Birds Hill provincial park) we report is similar to the prevalence calculated from ticks submitted to the Manitoba blacklegged tick passive surveillance program in 2014, where the minimum and maximum infection prevalence of *A. phagocytophilum* in blacklegged ticks was 7.4%-10.1%. (n = 387 tick pools) (Rochon and Lindsay 2015). Manitoba had 21 confirmed or probable cases of human granulocytic anaplasmosis in 2015, but since anaplasmosis is not reportable in Ontario and Quebec, it is difficult to compare case numbers in Canada. The border states of North Dakota, Minnesota, and Wisconsin reported 3, 613, and 547 confirmed or probable cases in 2015 (Minnesota Department of Health 2017; North Dakota Department of Health 2016; Wisconsin Department of Health Services 2017). The large number of confirmed or probable cases in Minnesota and Wisconsin is indicative of a high prevalence of Ap-ha.

Although anaplasmosis cases are of concern, the number of reported Lyme disease cases has been rising in Manitoba since 2009 (Manitoba Health Healthy Living and Seniors 2017). During my seasonality study from 2014-2016, 56 cases of Lyme disease were diagnosed in Manitoba (Manitoba Health Healthy Living and Seniors 2017). Based on ticks submitted to the blacklegged tick passive surveillance program, the minimum and maximum prevalence of *B. burgdorferi* in blacklegged ticks was 23.7-26.9% (Rochon and Lindsay 2015). We found 31.7% of our adult ticks infected with *B. burgdorferi*, which is higher than the mean prevalence associated with passive surveillance.

With one-third of adult ticks infected with *B. burgdorferi*, the public may fear they have a substantial risk of acquiring *B. burgdorferi* from adult ticks at Beaudry and Birds Hill provincial parks. Although adults can transmit *B. burgdorferi* to humans, nymphs are considered the principal vector (Falco et al. 1996; 1999; Mather et al. 1996; Mead 2015; Piesman et al. 1987; Spielman et al. 1985). This is due in part to their much smaller size, making them more difficult to find and remove. In theory nymphs should also be more abundant than adults, meaning the public would be more likely to encounter them (Ogden et al. 2015). However, few nymphs are submitted to the Manitoba blacklegged tick passive surveillance program (Rochon and Lindsay 2014; 2015). To prevent tick bites the public should follow some simple prevention strategies: 1) avoid tick infested habitats (Piesman and Eisen 2008), 2) wear clothing that limits tick access to skin (long-sleeve shirts, pants, socks) (Carroll and Kramer 2001), 3) frequently check for ticks before and after outdoor activities (Shadick et al. 1997), and 4) use DEET-based topical repellents (Bissinger and Roe 2010; Katz et al. 2008).

Overwintering Survival and Cold Hardiness

Blacklegged ticks can successfully survive overwinter in two different habitat types in Manitoba. I assumed greater survival would occur at the forest site where presumably more leaf litter would be available for shelter. It was surprising to see a greater overwintering survival at the forest edge site, but this could be a result of greater snow accumulation, providing additional protection from the elements. This finding is important for future range expansion models, as habitats other than the traditional deciduous forest should be considered. Our supercooling point experiments showed adult blacklegged ticks have a relatively high supercooling point, with no difference between sexes.

5.2 Limitations

Mammal Trapping and Seasonality

The lack of recovered nymphs in both trapping years hindered my objective of determining the seasonality of immatures, but provides us with more questions to answer. In other mammal trapping studies a large number of nymphs are routinely removed from the white-footed mice (Hamer et al. 2012b; Oliver et al. 2006), which are almost morphologically indistinguishable from the deer mouse, one of our most trapped species. As discussed previously, we believe that diurnal and/or larger sized mammals are harboring nymphs at both provincial parks. There is precedence for this, as many studies have found larger mammals more heavily infested with nymphal blacklegged ticks than *Peromyscus* species (Hamer et al. 2012b; Main et al. 1982; Schmidt et al. 1999; Slajchert et al. 1997). From observations, Franklin's ground squirrels, Richardson's ground squirrels, and thirteen-lined ground squirrels frequently inhabit Birds Hill provincial park, and could potentially host blacklegged tick immatures. At Beaudry provincial park, raccoons could serve as suitable hosts, and are known to carry nymphs

(Ouellette et al. 1997; Pung et al. 1994). It should also be noted that in studies where alternative hosts to *Peromyscus* species were found, *Peromyscus* species still harboured far more ticks than we recovered from our deer mice. Although other mammals are likely harboring nymphal blacklegged ticks, deer mice should still be seen as a suitable host. In the Southern United States where lizards are the preferred host of blacklegged tick immatures, mice are still parasitized (Apperson et al. 1993).

Pathogens

Although we report a large proportion of ticks infected with *A. phagocytophilum* (10% of adults in both parks, and 12 % of larvae in Birds Hill provincial park), this should be put into context from a public health point of view because we did not differentiate between Ap-Variant 1 and Ap-ha, and Ap-Variant 1 is not known to cause disease in humans (Chen et al. 1994; Massung et al. 2002). It is also important to acknowledge that the number of confirmed or probable cases of anaplasmosis is probably an underrepresentation of the true number of cases. *A. phagocytophilum* is an emerging pathogen which means physicians may not recognize its symptoms. Compared to Lyme disease, anaplasmosis receives little media attention when blacklegged ticks are discussed. As well, anaplasmosis can be hard to track because the symptoms are often mild and flu-like leading to misdiagnosis (Chen et al. 1994).

There is also a limitation in our ability to compare our reported pathogen prevalence with passive surveillance. Differences in pathogen prevalence between our study and passive surveillance are likely due to our small sample size, which came from two localized regions. Passive surveillance data represents the entire province, including places where pathogens are not established. Our prevalence data is still important because it shows passive surveillance may under represent true pathogen prevalence in localized areas.

Overwintering Survival and Cold Hardiness

Determining the supercooling point of adult blacklegged ticks is important for future cold hardiness experiments, but it is likely irrelevant to field settings where ticks rarely encounter such temperatures. This is because soil temperatures during winter are rarely reach below -5°C, (Burks et al. 1996b; Rosendale et al. 2016) which is well above the measured supercooling point of blacklegged ticks. Thus the overwintering mortality we observed was likely a result of desiccation or inoculative freezing (Burks et al. 1996b). A limitation to our overwintering experiment is that it was a very small-scale study with limited replications. As well, our overwintering experiment represents one year of data at only two potentially different habitats. Our habitats are by no means representative of the many different habitats available to blacklegged ticks throughout the province.

5.3 Future Directions

Mammal Trapping and Seasonality

To determine what other hosts are harboring nymphs I would suggest modifying our trapping methodology to enable the collection of a greater diversity of hosts. We used small traps aimed at trapping mice and other small mammals, based on published literature that identified these as common hosts as supporting immature blacklegged ticks; however, this limited the size of mammals we were able to catch. For example, the largest mammal we trapped weighed only 225 g. In addition, we only set traps during the evening just before sundown, and retrieved them shortly after sunrise, in accordance with our animal care protocols. This limited our trapping to nocturnal and crepuscular mammals, although we did occasionally catch American red squirrels which are diurnal (Steele 1998), and likely entered our traps early in the morning. Using a

methodology similar to Burachynsky and Galloway (1985) who set two sizes of traps for 24 hours, would allow us to sample a greater diversity of mammal species.

The low population density of adults and scarcity of nymphs and larvae made it hard to determine the seasonality of all three active life stages. Studies from the northeastern (Daniels et al. 2000; Diuk-Wasser et al. 2006; Falco and Fish 1992) and midwestern (Diuk-Wasser et al. 2006; Hamer et al. 2012b) United States have been able to collect blacklegged tick larvae and nymphs by dragging and/or flagging. At both provincial parks we were unable to collect nymphs and larvae by dragging, and we never used the flagging technique. At other sites in Manitoba where blacklegged ticks are assumed to be more established such as Pembina Valley and Moose Mountain provincial parks, I have sporadically been able to collect nymphs and larvae by dragging and/or flagging. Repeating the mammal trapping study at locations such as these could definitively determine the seasonality of immature blacklegged ticks.

Pathogens

I suggest the monitoring of *A. phagocytophilum* and *B. burgdorferi* at established blacklegged tick localities in Manitoba. More specifically, future studies reporting the prevalence of *A. phagocytophilum* and *B. burgdorferi* in Manitoba should differentiate between strains of each pathogen. Differentiating between *A. phagocytophilum* strains would help determine how big of a risk Ap-ha is in Manitoba, and also aid in determining how best to allocate zoonotic surveillance resources. For example mice seem to be reservoirs for Ap-ha and not Ap-Variant 1 (Massung et al. 2003), whereas deer are competent reservoirs of Ap-Variant 1 but not Ap-ha (Massung et al. 2005; Reichard et al. 2009). If Ap-ha is shown to be quite prevalent in Manitoba, then extensive small mammal monitoring would be poor use of resources. Differentiating between strains of *B. burgdorferi* would allow researchers to better characterize regions

adventitious ticks are being transported from (Ogden et al. 2008b; Ogden et al. 2011). This is important because it could allow public health officials to focus media campaigns on pathogens that will be a concern for Manitoba.

Overwintering Survival and Cold Hardiness

In hindsight, I would like to have overwintered ticks during two winters, and employed a larger number of field sites with more replication. Using more sites and replication would have allowed us to determine the variability in overwintering survival from year to year. For instance Lindsay et al. (1998) overwintered unfed blacklegged ticks on Long Point, Ontario, and found overwintering survival differed between years. Future cold hardiness and overwintering studies should also incorporate fed ticks to determine how engorgement will affect their survival. All life stages of the blacklegged tick can overwinter engorged (Lindsay et al. 1998; Yuval and Spielman 1990a), but how this will affect their cold hardiness and survival in Manitoba remains to be studied.

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Appendix A: Fleas and Lice Removed From Small Mammals

The following flea and louse species were removed from small mammals trapped at Beaudry and Birds Hill provincial parks during 2015 and 2016. Identifications were made my Dr. Galloway at the University of Manitoba. Louse species indicated by "*".

Birds Hill					
Date	**Host**	**Host Sex**	**Species**	**Male**	**Female**
2015-06-22	*P. maniculatus*	N/A	*Orchopeas leucopus* Baker		1
2015-06-22	*M. gapperi*	M	*Peromyscopsylla catatina* Fox		1
			Megabothris quirini Rothschild	2	
2015-08-05	*P. maniculatus*	M	*Orchopeas leucopus*		1
			*Hoplopleura acanthopus** Burmeister		1
2015-08-05	*M. gapperi*	M	*Megabothris quirini*		2
			Peromyscopsylla catatina		1
2015-08-05	*P. maniculatus*	M	*Orchopeas leucopus*	1	
2015-08-18	*M. gapperi*	M	*Peromyscopsylla catatina*		2
			Megabothris quirini		1
2016-08-18	*M. gapperi*	F	*Peromyscopsylla catatina*	1	2
2016-08-31	*M. gapperi*	F	*Peromyscopsylla catatina*		1
2016-08-31	*M. gapperi*	F	*Megabothris quirini*		
2016-09-15	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes* Baker	1	
2016-09-15	*P. maniculatus*	F	*Orchopeas leucopus*		1
2016-09-15	*M. gapperi*	F	*Megabothris quirini*		1
2016-10-13	*M. gapperi*	M	*Peromyscopsylla catatina*	2	
2016-10-13	*M. gapperi*	F	*Peromyscopsylla catatina*		1
2016-10-13	*M. pennsylvanicus*	M	*Peromyscopsylla catatina*	1	
2016-06-22	*M. gapperi*	F	*Ctenophthalmus pseudogyrtes*		1

Birds Hill (continued)

Date	Host	Host Sex	Species	Male	Female
2016-06-22	*P. maniculatus*	M	*Orchopeas leucopus*	2	14
2016-07-06	*M. gapperi*	M	*Peromyscopsylla catatina*	1	
2016-07-20	*M. gapperi*	F	*Peromyscopsylla catatina*	1	
2016-07-20	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
2016-07-20			*Orchopeas leucopus*	1	
2016-07-20	*M. gapperi*	F	*Peromyscopsylla catatina*	5	8
2016-07-20	*M. gapperi*	F	*Megabothris quirini*	1	1
2016-07-20	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
			Megabothris quirini		1
2016-07-20	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*		1
			Peromyscopsylla catatina	1	1
2016-07-20	*P. maniculatus*	M	*Peromyscopsylla catatina*		1
2016-08-06	*M. gapperi*	M	*Peromyscopsylla catatina*	1	
2016-08-06	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
2016-08-06	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
			Peromyscopsylla catatina		1
2016-08-06	*M. gapperi*	M	*Peromyscopsylla catatina*	1	1
			Megabothris quirini	1	
2016-08-06	*M. gapperi*	F	*Megabothris quirini*		1
2016-08-06	*M. gapperi*	F	*Megabothris quirini*		1
2016-08-06	*M. gapperi*	F	*Megabothris quirini*		1
2016-08-06	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
			Megabothris quirini	1	1
2016-08-06	*M. gapperi*	M	*Peromyscopsylla catatina*	2	
			Megabothris quirini		1
2016-08-06	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
2016-08-06	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*		1

Birds Hill (continued)

Date	Host	Host Sex	Species	Male	Female
2016-08-06	*M. gapperi*	F	*Megabothris asio megacolpus* (Jordan)		1
2016-08-06	*M. gapperi*	F	*Megabothris quirini*		1
2016-08-06	*M. gapperi*	F	*Peromyscopsylla catatina*	1	
2016-08-06	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
2016-08-06	*M. gapperi*	M	*Peromyscopsylla catatina*	1	
			Megabothris quirini	3	1
2016-08-06	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
2016-08-06	*M. gapperi*	F	*Peromyscopsylla catatina*	1	
2016-08-06	*M. gapperi*	F	*Megabothris quirini*	1	
2016-08-06	*M. gapperi*	M	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
			Orchopeas leucopus	1	
2016-08-06	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*		1
2016-08-17	*M. gapperi*	F	*Peromyscopsylla catatina*		1
2016-08-17	*M. gapperi*	M	*Peromyscopsylla catatina*	2	2
2016-08-17	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*		2
2016-08-17	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
			Ctenophthalmus pseudagyrtes pseudagyrtes	1	
2016-08-17	*M. gapperi*	F	*Megabothris quirini*	1	1
			Orchopeas leucopus	1	
2016-08-17	*M. gapperi*	F	*Peromyscopsylla catatina*	1	
2016-08-17	*M. gapperi*	F	*Peromyscopsylla catatina*		2
2016-08-17	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
			Peromyscopsylla catatina	1	
2016-08-17	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
			Ctenophthalmus pseudagyrtes pseudagyrtes	1	
2016-08-17	*M. gapperi*	F	*Peromyscopsylla catatina*		1
			Megabothris quirini		1

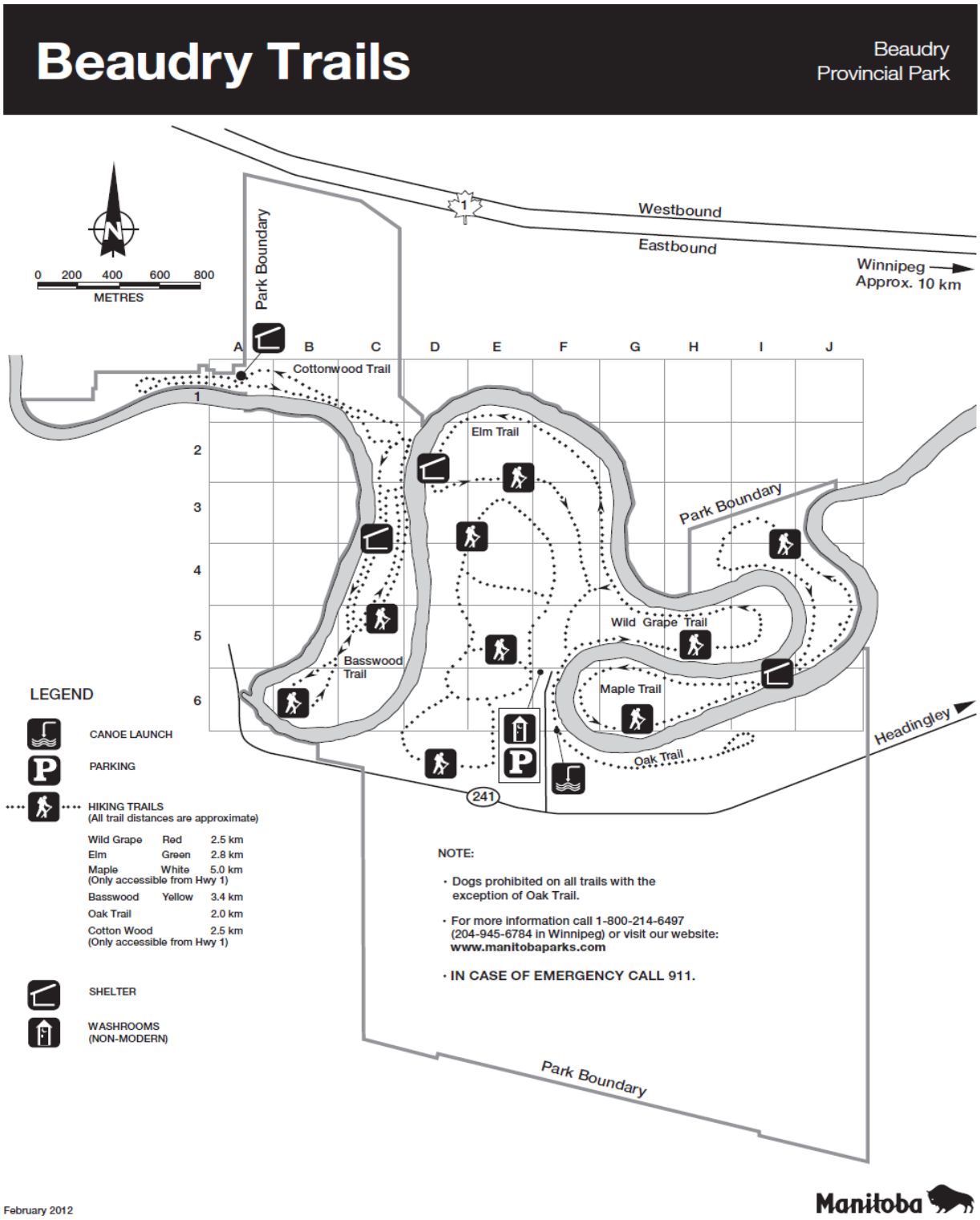
Birds Hill (continued)

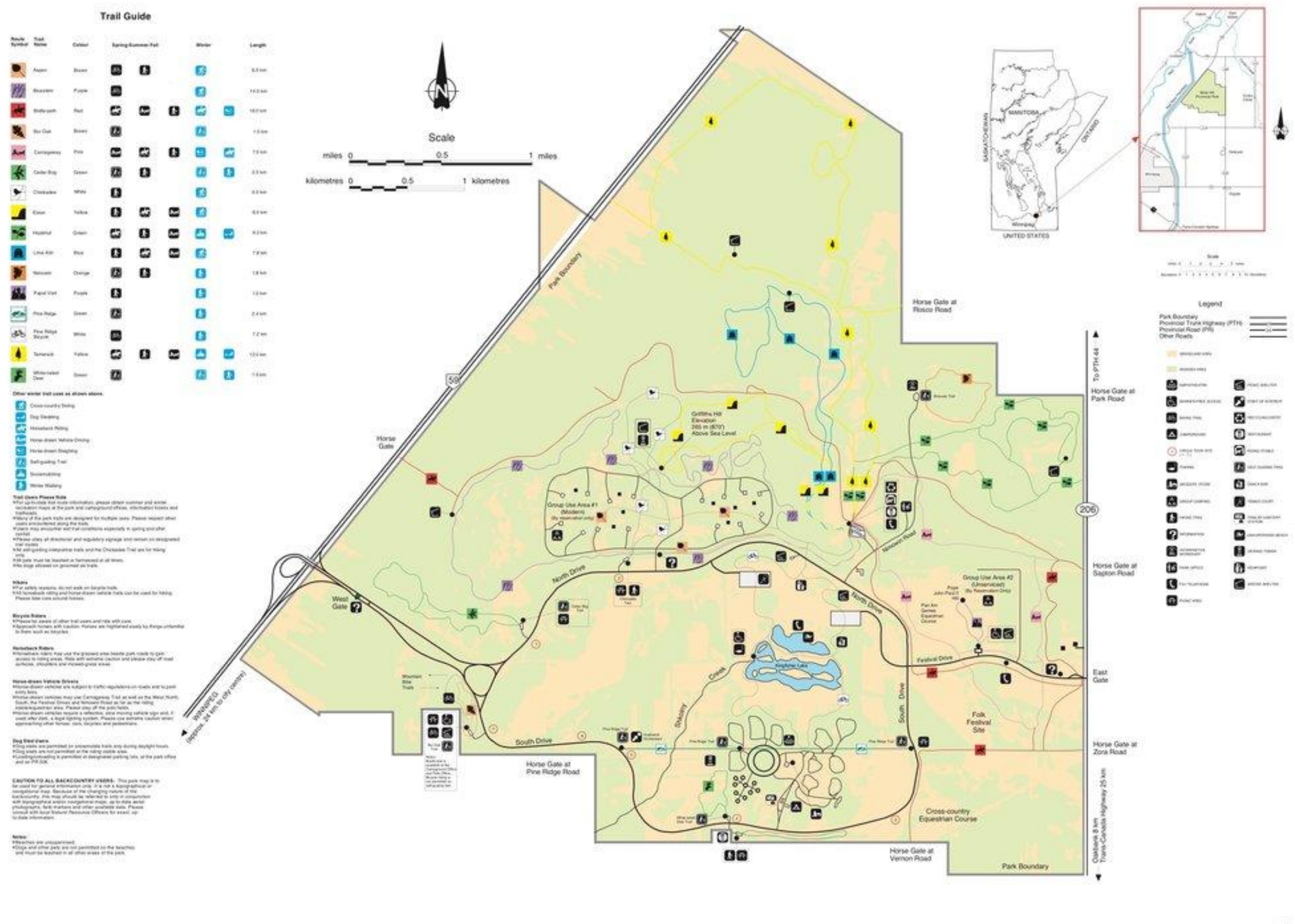
Date	Host	Host Sex	Species	Male	Female
2016-08-30	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
2016-08-30	*M. gapperi*	M	*Ctenophthalmus pseudagyrtes pseudagyrtes*		1
2016-08-30	*M. gapperi*	F	*Megabothris quirini*		1
2016-08-30	*M. gapperi*	M	*Peromyscopsylla catatina*		1
2016-08-30	*M. gapperi*	F	*Orchopeas leucopus*	1	
2016-08-30	*M. gapperi*	M	*Peromyscopsylla catatina*		2

Beaudry

Date	Host	Host Sex	Species	Male	Female
2016-06-30	*M. gapperi*	F	*Megabothris asio megacolpus*		1
			Megabothris quirini	1	
2016-06-30	*P. maniculatus*	F	*Aetheca wagneri* Baker		1
			Megabothris quirini	1	
2016-07-15	*M. gapperi*	F	*Megabothris quirini*		1
2016-07-15	*M. pennsylvanicus*	F	*Megabothris quirini*	1	
2016-07-15	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*		1
2016-08-10	*M. gapperi*	F	*Megabothris quirini*		1
2016-08-10	*M. gapperi*	F	*Corrodopsylla curvata curvata* Rothschild		2
			Megabothris quirini		1
2016-08-10	*M. gapperi*	F	*Ctenophthalmus pseudagyrtes pseudagyrtes*	1	
2016-08-23	*M. gapperi*	F	*Megabothris quirini*		1
2016-08-23	*M. gapperi*	F	*Aetheca wagneri*		1
2016-08-23	*M. gapperi*	F	*Megabothris quirini*		1

Appendix B: Maps of Birds Hill and Beaudry Provincial Parks




Trail Guide
Scale
miles
kilometres
Legend
Horse Gate at
Rosco Road
Horse Gate at
Park Road
Horse Gate at
Sapton Road
East
Gate
Horse Gate at
Zora Road
Horse Gate at
Pine Ridge Road
Horse Gate at
Vernon Road
Park Boundary
Horse
Gate
West
Gate
North Drive
South Drive
Festival Drive
Folk
Festival
Site
Cross-country
Equestrian Course
Kingfisher Lake
Group Use Area #1
Group Use Area #2
MANITOBA
SASKATCHEWAN
ONTARIO
UNITED STATES
Winnipeg