GRANULOCYTIC ANAPLASMOSIS AND LYME BORRELIOSIS EXPOSURE OF HORSES IN CANADA

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ABSTRACT

A set of studies was designed in order to better understand the exposure of horses in Canada to *Ixodes*-borne diseases, namely equine granulocytic anaplasmosis (EGA, caused by *Anaplasma phagocytophilum*) and Lyme borreliosis (LB, caused by *Borrelia burgdorferi*).

In the first study, equine serum samples submitted to veterinary diagnostic laboratories in SK, MB and ON were tested for antibodies against *A. phagocytophilum* and *B. burgdorferi*, using the point-of-care SNAP® 4Dx® ELISA. Horses seropositive to EGA were found in SK and MB and horses seropositive to LB were found in SK, MB and ON. Overall seroprevalence according to the SNAP® 4Dx® ELISA was 0.53% for EGA and 1.6% for LB. Samples that tested positive for antibodies against *A. phagocytophilum* (n=2) and *B. burgdorferi* (n=6) by SNAP® 4Dx® ELISA and 2 randomly selected subsets of samples that tested negative (n=92 each) were then re-tested using currently recommended serologic methods, and test results were compared. A lack of agreement was found between the SNAP® 4Dx® ELISA and indirect immunofluorescent assay (IFA) for EGA (McNemar test p = 0.000001). Agreement of the SNAP® 4Dx® ELISA and ELISA confirmed with Western Blot (WB) for LB was only fair (Kappa 0.23). Due to the lack of agreement between serologic tests for EGA and LB in the first study, another study to further evaluate the agreement among available serologic tests was conducted.

A set of 50 convenience serum samples submitted to the veterinary diagnostic laboratory in SK was tested by SNAP® 4Dx® Plus ELISA for antibodies against *A. phagocytophilum* and *B. burgdorferi*. Samples were also tested by IFA for antibodies against *A. phagocytophilum* in two referral laboratories, and by IFA, ELISA confirmed with WB and Equine Lyme multiplex assay for antibodies against *B. burgdorferi* in three referral laboratories. Again, test results varied between the different tests. For EGA, all 3 pair-wise test comparisons lacked agreement. For LB, agreement between tests ranged from poor to fair. Differences in test methodology and antigens used, cut-off settings between the laboratories and false positive or false negative results are likely the cause for the different assessment of the same sample as seropositive or seronegative.

In the third study, the goal was to describe potential risk factors for exposure of horses in Canada to EGA and LB. Management factors in horses that tested seropositive or seronegative for EGA or LB, respectively, in the previous studies were evaluated. Horse owners were surveyed with regard to their horses' signalment, timing of pasture housing, and province of residence, travel history, tick infestation history, history of Lyme vaccination and history of previously diagnosed tick-borne disease. Response rate (11.5%) and the number of seropositive horses available for evaluation were low, which precluded statistical analysis. The majority of seropositive horses resided in SK, was pastured in the fall, did not have a recent travel history and had not had visible tick infestation. These observations supported exposure of horses to tick-borne diseases within Canada. Potential risk factors require further investigation.

As information about tick infestation in horses is scarce in general, a passive surveillance study of horse ticks in SK was conducted in 2012 and 2013. A total of 833 ticks from over 86 horses were received. All ticks were *Dermacentor* species, i.e. *D. albipictus, D. andersoni* and *D. variabilis. D. albipictus* ticks were mostly received in February and March, *D. andersoni* mainly in April and June and *D. variabilis* mostly in May and June. Geographic distribution of the species in SK was similar to that previously reported based on active and passive surveillance. No *Ixodes* species were received.

ACKNOWLEDGMENTS

LIST OF TABLES

LIST OF ABBREVIATIONS

AHDL	Animal Health Diagnostic Laboratory
A. phagocytophilum (Ap)	*Anaplasma phagocytophilum*
AP- Ha	*A. phagocytophilum* human pathogenic strain
AP VAR-1	*A. phagocytophilum* variant 1 strain
B. burgdorferi	*Borrelia burgdorferi*
CDC	Centers for disease control and prevention
CVMDL	Connecticut Veterinary Medical Diagnostic Laboratory
DCPAH	Diagnostic Center for Population and Animal Health
D. albipictus	*Dermacentor albipictus*
D. andersoni	*Dermacentor andersoni*
D. variabilis	*Dermacentor variabilis*
EGA	equine granulocytic anaplasmosis
ELISA	enzyme linked immunosorbent assay
***groESL*gene**	the groESL (=groE,groS,groL genes)
HL-60	Human Leukemia-60 (cell line)
IFA	indirect immunofluorescence antibody assay
IL-8	interleukin 8, $\mathbf{IL_1\text{-}\beta}$ – Interleukin$_1$-beta
I. pacificus	*Ixodes pacificus*
I. scapularis	*Ixodes scapularis*
LB	Lyme borreliosis
Msp	major surface protein

msp **gene**	gene encoding Msp
Osp	outer surface protein
P44	protein 44
p44	gene encoding P44
PCR	Polymerase Chain Reaction
RMSF	Rocky Mountain Spotted Fever
rRNA	ribosomal ribonucleic acid
TNF$_\alpha$	tumor necrosis factor alpha
VlsE IR6	variable major protein-like sequence expressed lipoprotein of *B. burgdorferi,* invariable region 6
WB	Western Blot

1. REVIEW OF THE LITERATURE

1.1 Ticks and tick-borne diseases of horses in Canada

1.1.1. Tick infestation of horses in Canada

1.1.1.1. *Dermacentor* species

   D. variabilis and *D. andersoni* are two of the most common tick species in western Canada. *D. variabilis,* also known as the American dog tick, occurs throughout southeastern Saskatchewan (SK), southern Manitoba (MB) and Ontario (ON). A few isolated populations are also found in Nova Scotia (NS) (1). *D. variabilis* is usually found in geographic areas where summers are warm and humid (1). *D. andersoni,* also called the Rocky Mountain wood tick, occurs throughout the southern parts of British Columbia (BC) and Alberta (AB) as well as southwestern SK (1). These ticks are usually found in geographic areas where summers are hot and dry. Habitat areas of *D. variabilis* and *D. andersoni* are mostly separated from each other (1,2); however, occasionally, both species are found together in areas that differ in soil moisture and plant populations, i.e. southern central Saskatchewan. The relative importance of environmental factors for the abundance and distribution of these tick species in the Canadian prairies needs to be further investigated (1).

   D. albipictus, also called the winter tick or the moose tick, is the only *Dermacentor* tick species occurring on only one host. The tick is found all across Canada and its habitat extends further north than that of *D. andersoni* and *D. variabilis* (3). Information regarding infestation of horses with *Dermacentor* species is lacking.

1.1.1.2. *Ixodes* species

   Ixodes species will be described in detail in the following review. *I. scapularis*, the blacklegged tick, has become established in southern ON, Quebec (QC), MB and NS. *I. pacificus*, the Western blacklegged tick, has mainly become established in southern BC. *Ixodes* species are known to infest horses in North America.

1.1.2. Potential tick-borne diseases of horses in Canada

   Equine granulocytic anaplasmosis and Lyme borreliosis are transmitted by *Ixodes* species as will be described in detail in this chapter, and both diseases have been reported in horses in

Canada (4–8). The current distribution and anticipated geographic expansion of the vector *Ixodes* species in Canada pose a substantial risk of encountering the diseases.

Equine piroplasmosis, also called babesiosis, is caused by the blood protozoa *Theileria equi* and *Babesia caballi* in horses. The tropical horse tick, *Dermacentor nitens,* is the natural vector for *Theileria equi* and *Babesia caballi* in the US, but has not been reported in Canada. *Theileria equi* has been experimentally transmitted by *D. variabilis, D. albipictus* and *Rhipicephalus microplus* (formerly known as *Boophilus microplus)* which does not occur in Canada (9). Equine piroplasmosis has not been reported in Canada to date.

1.2 *Anaplasma phagocytophilum* infection

1.2.1. <u>Etiology</u>

The genus *Anaplasma* belongs to the family Anaplasmataceae in the order Rickettsiales (10). *A. phagocytophilum* is an obligatory intracellular bacterium and variants in North America are known to cause clinical disease in humans, dogs and horses but not in cattle (11). Equine granulocytic anaplasmosis (EGA) is caused by a strain of *A. phagocytophilum,* (previously classified as *Ehrlichia equi*) that is closely related to the human pathogenic strain (12,13). EGA is a tick-borne disease transmitted mostly by the ticks of the *Ixodes ricinus* complex which occur worldwide (14,15). The complex includes *Ixodes scapularis* (the blacklegged tick or deer tick) in the eastern regions of North America, *Ixodes pacificus* (the western blacklegged tick) in the western regions of North America, *Ixodes ricinus* in northern Europe and North Africa, and *Ixodes persulcatus* in eastern Europe and temperate regions of Asia (15). *A. phagocytophilum* have serologic cross-reactivity with one another and common serologic tests cannot differentiate among different strains. *A. phagocytophilum* strains differ in their host infectivity and show minor diversity in their nucleotide sequence of the 16S rRNA and the *groESL* genes (10). Differences in the 16S rRNA sequence distinguish between different variants, such as between AP-Ha, which infects humans and whose reservoir is the white footed mouse, and AP Var-1, which is maintained within deer and does not infect humans (16). The *groESL* genes have a structural role and are evolutionarily highly conserved genes among bacteria (10). The pathogenic strains of *A. phagocytophilum* that cause human granulocytic anaplasmosis (HGA) and EGA are mostly identical in their 16S and *groESL* genetic sequences in the midwestern US whereas variation exists in the western US (10,12,13,16–18).

Bacterial cell wall antigens that are expressed inside the mammalian host are commonly encoded by the *p44* gene family (also called *msp2*). The P44 (Msp2) proteins are major surface antigens. The *A. phagocytophilum* genome contains 113 *p44* (*msp2*) genes which encode the P44 (Msp2) proteins (10). The *p44* (*msp2*) genes have a central hypervariable region and terminal conserved sequences. The P44 proteins vary due to a unidirectional gene conversion mechanism and recombination of copies of the gene, which enable antigenic variation and avoidance of the immune system (19).

1.2.2. Hosts and vector ticks

In the eastern and midwestern US, natural reservoirs of *A. phagocytophilum* variants are the vertebrate hosts of the blacklegged tick, *I. scapularis*, which include the white-footed mouse, the white-tailed deer, the grey squirrel and the raccoon (16). Seroprevalence of *A. phagocytophilum* infection in white-footed mice in the eastern US ranges from one to 50% (13). Information about the natural reservoir for *A. phagocytophilum* in the western US is limited; however, the western blacklegged tick, *I. pacificus,* is known to feed on lizards, birds, small mammals and, occasionally, deer and carnivores (13). The prevalence of infection with *A. phagocytophilum* in *Ixodes* species is reported to be 14% and 16% in the midwestern and northeastern US respectively (20), but is lower in the western US where prevalence values of 0.8 - 11% have been reported (13). Only nymphs and adult ticks can transfer *A. phagocytophilum* to mammals (10).

1.2.3. Epidemiology

Equine granulocytic anaplasmosis was first recognized in horses in California in 1969 (21), and was later recognized in other parts of the US and in Europe. In Canada, four cases of EGA have been reported since 1996 (4–7), with three of these diagnosed since 2010. None of the affected horses had a history of travel, suggesting that they acquired the infection within Canada. One of the affected horses resided in Saskatchewan, which is not considered endemic for the vector (i.e. *Ixodes* species). The potential contribution of adventitious ticks to disease occurrence in non-endemic areas is discussed later on in this chapter, in the context of Lyme borreliosis.

Clinical cases of EGA are predominantly reported in the winter months, following the peak activity of the adult stage of the vector ticks in the fall months. This is similar to the disease in dogs but in contrast to human cases, which are reported mainly in the spring and

summer months and correlate with the activity of the nymphal stages of the vector. It is speculated that transmission of the disease by adult ticks to horses and dogs is more efficient (13). It is also possible that humans are more efficient in removing the adult stages of the tick.

1.2.4. Pathogenesis

Members of the Anaplasmataceae family replicate in membrane-bound parasitophorous vacuoles within the cytoplasm of eukaryotic host cells (10). These bacteria are capable of escaping lysosomes by interfering with vesicular movement. As bacteria divide and proliferate, the inclusion vacuoles expand to occupy most of the cytoplasm of the infected cell (10). Subversion of the innate antimicrobial response increases susceptibility of infected individuals to opportunistic infection. *A. phagocytophilum* inhibits spontaneous and induced apoptosis in peripheral neutrophils for up to 96 hours, which enables it to replicate within 24 hours post infection (10). *A phagocytophilum* also induces cell autophagy, thus remodeling the host cell cytoplasmic space and altering nutrient utilization to accommodate its growth (10)

The site of initial replication of the bacteria after inoculation of the dermis during a tick bite is still unknown. In experimentally infected animals, bacteremia was not detected until 72 to 96 hours after intravenous inoculation with infected blood (16). These findings suggest that *A. phagocytophilum* replicates at the tissue level before bacteremia occurs (16). The intracellular bacteria have a tropism toward granulocytes, primarily neutrophils. Endothelial cells are also infected and contribute to inflammation by controlling vascular permeability, movement of leukocytes and production of inflammatory mediators (22).

The pathogenesis of granulocytic anaplasmosis in horses involves the presence of a small number of the organisms in the blood and it is suggested that disease progression is mediated by pro-inflammatory cytokines. In horses that were infected with *A. phagocytophilum* experimentally, up-regulation of expression levels of interleukin (IL)-1β, tumor necrosis factor alpha (TNFα) and IL-8 in peripheral leukocytes was observed (23). Pathologically, the characteristic gross lesions are hemorrhages, petechiae, ecchymoses and edema, which involve the muscles, nerves, brain, heart and kidneys. Histologically, inflammation of the small arteries and veins (9) as well as necrotizing vasculitis with perivascular infiltration of mononuclear cells and, occasionally, neutrophils has been described (21).

1.2.5. Clinical disease in horses

In horses, the period of bacteremia accompanied by high fever lasts for approximately seven days (12,18). Infected horses are often leukopenic, likely due to sequestration of infected granulocytes, and are also thrombocytopenic. Depression, anorexia and distal limb edema are typical clinical signs and ataxia may occur as well (10). Young horses, i.e. those less than 3 years old, tend to have a milder form of the disease (13,24).

Equine granulocytic anaplasmosis is usually self-limiting as long as no concurrent infection with other disease-causing organisms is present. However, a favorable response is seen when horses are treated with oxytetracycline and treatment reduces the duration and severity of the disease (24,25). Currently, there is no commercially available vaccine for EGA. The main factor in preventing EGA is avoiding exposure to the vector (i.e. *Ixodes* species). Reducing exposure in horses may specifically refer to the fall months when the adult stages of *Ixodes* are active. Because horses are more likely to be infested with the adult tick stages (13), it may be necessary to minimize or avoid pasture housing in the fall.

1.2.6. Currently recommended diagnostic tests

A. phagocytophilum is an intracellular bacterium and cannot be detected with Gram staining (10). Romanowsky staining is usually used. The bacteria then stain purple and the characteristic morulae resembling mulberry-like bacterial clumps can be visualized (10). A blood smear may reveal morulae inside the cytoplasm of infected circulating granulocytes in the first week following infection (10). Sensitivity of detection of the organism in a blood smear is relatively low. In human patients with HGA, for example, sensitivity of visual detection of morulae is 60% (26) and diagnosis should be supported with molecular or serologic methods (27). There are no published data regarding the sensitivity of visual detection of *A. phagocytophilum* morulae in horses affected by EGA.

Amplification of *A. phagocytophilum* DNA from whole blood specimens of human patients has an estimated sensitivity of 71.4% (26). Due to a lack of standardization, however, sensitivity and specificity may vary among different PCR assays (27). The use of tetracycline antibiotics prior to testing may further reduce sensitivity (27). There is no published information about the sensitivity of PCR in the diagnosis of EGA in horses.

The indirect immunofluorescence antibody assay (IFA) is a commonly used serologic method for detection of antibodies against *A. phagocytophilum* (27) and is considered the gold

standard of serologic testing for rickettsial diseases (27). Serum antibodies bind to a fixed antigen on a slide and are detected by identification of a fluorescein-labeled anti-antibody. Although IFA remains the principal diagnostic tool for the diagnosis of *A. phagocytophilum* infections, there are no standardized antigens or conjugates, and there is no agreement on what constitutes a positive result among the various laboratories providing these tests (21). In human patients, IFA is estimated to be 94% to 100% sensitive, depending on the timing of sample collection (21). In horses, test sensitivity depends on the horse's stage of infection at the time of sample collection and increases with increased duration of infection (27). Testing paired samples collected 21 days apart further increases diagnostic sensitivity (27). Sensitivity and specificity of the IFA in horses have not been reported in the literature.

A commercially available point-of-care SNAP® 4Dx® ELISA (IDEXX Laboratories, Westbrook, ME) for simultaneous detection of *Dirofilaria immitis* antigen and antibodies to *Borrelia burgdorferi, A. phagocytophilum* and *Ehrlichia canis* was marketed for use in dogs. A peptide derived from the immunodominant P44 protein of *A. phagocytophilum* was used for detection of antibodies against this organism. The test was not labeled for use in horses; however, anti-antibodies that were used in the test were not species-specific (28,29). Chandrashekar et al. (28) reported 100% agreement of the SNAP® 4Dx® ELISA with IFA for detection of antibodies against *A. phagocytophilum*. Samples for this study were obtained from presumed infected horses, i.e. horses that had previously been tested for Lyme borreliosis, and presumed non-infected horses, i.e. horses that resided in a non-endemic area in the US. Lack of gold standard to confirm positive cases and possible cross reactivity were not addressed in this study and may be a limitation of this study.

Recently, a new version of the test that also includes testing for *A. platys* and *E. ewingii* infection and is called SNAP® 4Dx® Plus ELISA has replaced the SNAP® 4Dx® ELISA. According to the manufacturer, comparative trials showed 93.6% agreement between the SNAP® 4Dx® ELISA and the newly developed SNAP® 4Dx® Plus ELISA when canine samples were tested for antibodies against *A. phagoytophilum*.

1.2.6.1. Serologic cross-reactivity

Antibodies against some species of *Ehrlichia* may cross-react with *Anaplasma* antigens (27). Using a competitive ELISA based on recombinant *A. marginale* antigen (Msp5) and an IFA based on *A. phagocytophilum*-infected HL-60 cells, Dreher et al. (30) reported cross-reactivity in cattle experimentally infected with *A. marginale,* and in sheep and horses

6

experimentally infected with *A. phagocytophilum*. Their findings suggest that antibodies against *A. marginale* may produce a false positive result when testing for antibodies against *A. phagocytophilum* and vice versa. Cross-reactivity in horses infected with *A. marginale* in a test for antibodies against *A. phagocytophilum* was not investigated in their study and needs to be evaluated as well. *A. marginale* infection in horses has not been reported and the potential significance of cross-reaction is not clear.

According to the manufacturer, the P44 peptide utilized in the SNAP® 4Dx® and SNAP® 4Dx® Plus ELISA does not have any homology to *A. marginale* Msp2; however, cross-reactivity with antibodies against *A. platys* is possible (Ramaswamy Chandrashekar personal communication). *A. platys* infection in horses has not been reported and the potential significance of this cross-reaction is not clear.

1.2.7. <u>Relationship between seroprevalence and clinical disease</u>

The presence of antibodies against *A. phagocytophilum* in horses does not necessarily indicate a symptomatic infection, but rather may simply indicate previous exposure to the organism. Seroprevalence of EGA, i.e. the overall occurrence of antibodies against *A. phagocytophilum* in horses, may vary between different geographic areas according to the infection rate of the vector ticks.

Although clinical cases of EGA have been reported regularly from the northern coast counties of California, Madigan et al. documented a seroprevalence of only 10% in horses residing in the California Coast Range and the Sierra foothills (31). In clinically normal horses from areas in Minnesota and Wisconsin, where established populations of *I. scapularis* exist, the seroprevalence of EGA was 3.8% and up to 17.6%, respectively (18).

Limitations exist for seroprevalence studies. *A. phagocytophilum* strains vary in their pathogenicity between different hosts and some strains are non-pathogenic. Infection with non-pathogenic strains of *A. phagocytophilum* may therefore induce seroconversion even though clinical disease does not occur (12). As stated above, currently recommended IFA tests cannot differentiate between antibodies produced against specific strains.

1.3 *Borrelia burgdorferi* infection

1.3.1. Etiology

Lyme disease, or Lyme borreliosis (LB), is a multisystemic tick-borne disease caused by the spirochetes of the *B. burgdorferi sensu lato* complex. Lyme borreliosis is the most common vector-borne disease in North America and it is also endemic in parts of Europe and Asia (32–34). *B. burgdorferi* belongs to the family Spirochaetaceae, which also includes species within two genera - *Leptospira* and *Treponema* spp. These spirochetes have a wavelike body and flagella "tail" enclosed between the outer and inner cell membranes (34).

The *B. burgdorferi sensu lato* complex includes a diverse group of bacteria that is distributed worldwide. Three genospecies, namely *B. garnii, B. afzelii,* and *B. burgdorferi sensu stricto* cause the majority of LB in Eurasia (34). *B.burgdorferi sensu stricto* is the only species causing LB in the US (35,36). Lyme disease was first described in 1975 as an epidemic of arthritis among children in Lyme, Connecticut. The causative agent was discovered in 1982 by Burgdorfer et al., when it was isolated from infected *Ixodes* species (37). The disease affects humans and animals, which also serve as infection reservoirs.

1.3.2. Hosts and vector ticks

B. burgdorferi is transmitted to humans and animals by *Ixodes* species which feed on wildlife reservoir hosts, including birds, small mammals (mainly rodents) and large mammals. *I. scapularis* is the main vector in eastern and central North America, and *I. pacificus* is the main vector in western North America (38).

The main reservoir hosts for *B. burgdorferi* in the US are white-footed mice on which larval and nymphal stages feed, some avian species (e.g. passerines) which mainly host nymphal stages, and the white-tailed deer, on which mainly adult but also nymphal stages feed. White-footed mice, some other small mammals, and, rarely, avian species may remain infected and asymptomatic and serve as competent hosts. The white-tailed deer is an incompetent reservoir host, meaning that it does not maintain the infection long-term (34).

1.3.3. Epidemiology

The risk for infection with *B. burgdorferi* is multifactorial and varies with the distribution, density and prevalence of infection in the vector and host populations. In the US, most cases of Lyme borreliosis occur in the north-eastern and north central states (34). In Canada only 2

cases of Lyme borreliosis in horses were reported, in1988 from BC (8), where the vector *I. pacificus* is established (39). According to recent studies, the risk of exposure to Lyme borreliosis in Canada is increasing due to expansion of the geographic range of *I. scapularis*, which is enhanced by ongoing climate changes (38). Over the last decade, more geographically isolated populations of *I. scapularis* have become established in Southern Ontario, Southern Quebec, Southeastern Manitoba, Nova Scotia and New Brunswick (39). The reported prevalence of *B. burgdorferi* infection in *I. scapularis* in endemic areas in the north-eastern US is typically greater than 25% while it is typically less than 25% in south-eastern Canada (38). The prevalence of *B. burgdorferi* infection in *I. pacificus* in endemic areas in BC is usually less than 10% (38). Thus, the risk for Lyme disease in areas where *I. pacificus* is the vector is likely lower than the risk in those areas where *I. scapularis* is the predominant vector (38).

In non-endemic areas, there is still a risk for infection with *B. burgdorferi* as infected ticks can be introduced by migrating birds. These ticks are referred to as adventitious ticks. The prevalence of *B. burgdorferi* infection in ticks carried by migrating birds into Canada is 8 to 15.4% (40). The risk of exposure to Lyme disease in non-endemic areas of Canadian provinces, which is mainly due to tick dispersion by migratory birds, is thus lower than in endemic areas, where the density and infection rate of the ticks is higher.

In 2011, Ogden et al (41) reported that two additional *Borrelia* species, namely *B. myiamotoi* and *B. kurtenbachii*, were identified in *I. scapularis* ticks collected in a national surveillance program extending from Alberta to Newfoundland between 2005 and 2007. *B. kurtenbachii* was identified as an entirely new species whereas *B. myiamotoi* had been previously reported from Connecticut (42). The pathogenicity and clinical relevance of these *Borrelia* species is unknown; however, they may need to be considered when investigating tick-borne diseases in Canada.

Analysis of *B. burgdorferi* DNA obtained from infected ticks collected in surveillance programs suggests that the genetic diversity of *B. burgdorferi* strains in eastern and central Canada is similar to that in the US (34). This indicates that established populations of the vector *I. scapularis* were originally introduced to Canada from the north-eastern US.

While introduction of the *Ixodes* vector from the US into Canada is progressing, it appears that establishment of Lyme borreliosis in Canada is lagging behind the establishment of tick vector populations. This is likely due to dilution of the infection in these populations. Dilution

refers to a decrease in prevalence of the pathogen within a vector population, which is due to either an increase in naïve vector populations, an increase in the non-competent host population (e.g. deer), a decrease in the competent and infected host population, an increase in the non-infected competent host population or a combination of several of these factors (39,41).

1.3.4. <u>Pathogenesis</u>

Genes encoding lipoproteins account for a significant portion of the *B. burgdorferi* genome and these genes show different expression levels in culture, in the vector tick and in the mammalian host (34). The outer surface protein A (Osp A) is expressed in culture and in the unfed tick gut to mediate attachment of the spirochete, whereas Osp B is expressed in host tissue and in culture and Osp C is expressed only when the spirochete is transferred to a mammalian host (34,43). Osp C is expressed early in the infection and is linked to invasiveness of *B. burgdorferi*, whereas Osp F is expressed later, during chronic infection.

While the host tick is feeding, typically for 24-72 hours, the spirochetes replicate in the mid-gut of the tick and move to its salivary gland (36). During this period, the spirochetes down-regulate expression of Osp A and up-regulate expression of Osp C. The spirochetes are transmitted after moving from the mid-gut of the feeding tick to its salivary gland due to influx of blood and an increase in temperature. This is the time when the spirochetes are metabolically active and express Osp C (43).

A lipoprotein named variable major protein-like sequence expressed (Vls E) is required for persistence of *B. burgdorferi* infection in the competent mammalian host and is important for immune response evasion by modulation of the bacterial gene expression (34,43). Although the *B. burgdorferi* genome encodes for a large variety of lipoproteins, a limited number is expressed at any given time of infection of a mammalian host, reducing the number of potential targets for the immune response. The early antibody response against Osp C provokes down-regulation of Osp C expression, enabling evasion of the antibody response. Migration within connective tissue may further protect the organism from the host's humoral response (43,44). Cytolytic activity attributed to *Borrelia* may explain how *B. burgdorferi* escapes macrophages lysosomal compartmentalization (43).

Pathogenicity of *B. burgdorferi* inside the mammalian host involves utilization of host proteases in order to traverse extracellular tissues (36). *B. burgdorferi* infection in mammalian

hosts involves migration, adhesion and immune evasion of the pathogen. The typical red circular skin lesion ("erythema migrans") at the site of the tick bite is the result of an interaction between the spirochete and the host's immune response. Lymphocytes, dendritic cells, macrophages and plasma cells, all producing pro-inflammatory mediators, are seen on histology of these lesions (36). The organism may be found in blood during the very short bacteremia, cerebrospinal fluid, heart, retina, brain and meninges, muscle, bone, spleen and liver, accounting for the multiple symptoms associated with the infection (34,36,43). However, the fact that many infected hosts remain asymptomatic indicates that the clinical picture is determined by the interaction between the spirochete and host and not by infection with *B. burgdorferi* alone (36).

1.3.5. Clinical disease in horses

The clinical picture of Lyme borreliosis in horses is variable and includes arthritis, lameness, muscle tenderness, anterior uveitis, encephalitis, abortion, low grade fever and lethargy (43). Persistence of clinical signs associated with synovitis is attributed to an auto-immune cross-reactivity response to *B. burgdorferi* DNA. This means that antibodies against *B. burgdorferi* may attack synovial membrane components, causing progressive arthritic lesion (43). The variation in clinical signs may be associated with variation in the individual immune response or co-infection with other pathogens, e.g. *A. phagocytophilum,* which is transmitted by the same vector (44,45).

Intravenous oxytetracycline and per oral doxycycline are commonly used for treatment of Lyme disease in horses (44). Oxytetracycline, possibly due to its higher blood and tissue concentration, showed better therapeutic results than doxycycline in experimentally infected ponies (46). The same observations were recorded in naturally infected horses (44). Early diagnosis and treatment initiation are preferable (44).

Similar to EGA, the key factor in the prevention of Lyme borreliosis is avoidance of exposure to the vector. Although a variety of canine vaccines using the Osp A and Osp C antigen are commercially available (47,48) and are anecdotally used in an off-label fashion by some horse owners in the US, the efficacy of the vaccine in horses has not been reported. Currently, there are no commercially available vaccines for prevention of Lyme disease in horses.

1.3.6. <u>Currently recommended diagnostic tests</u>

Diagnosis of Lyme disease is challenging. The diagnosis is typically made by taking into account a history of tick infestation in endemic areas, manifestation of typical clinical signs and serologic evidence of infection. Erythema migrans, which is pathognomonic for Lyme disease in humans, is not a characteristic finding in horses (43,44). With lameness being the most common clinical sign in infected horses, a variety of differential diagnoses need to be ruled out systematically (43,49,50).

Interpretation of serologic tests for *B. burgdorferi* infection is challenging. The ELISA and IFA are considered sensitive tests for detecting anti- *B. burgdorferi* immunoglobulins in horses, with the ELISA reported to detect relatively more seropositive horses (43). Due to a relative low specificity of these tests, a two-tier approach has been developed, and is similar to the recommended serodiagnostic approach to Lyme disease in humans. Initial screening with a sensitive technique (i.e. ELISA or IFA) is followed, if positive, with a more specific Western Blot (WB) to detect antibodies to specific *B. burgdorferi* antigens (43,51). Due to the slow multiplication of the spirochete, a detectable antibody response may take 3-8 weeks to develop, and the sensitivity of combined tests may be low in the first few weeks of infection (43,51). As many seropositive horses are clinically normal, a single positive serologic test result is not sufficient to differentiate active infection from exposure and repeating the test after 3 weeks is recommended (43). If the horse was acutely infected, the second titer is expected to be higher, whereas the titer of a previously exposed horse may wane or stay similar in a repeated test, but will not increase.

The SNAP® 4Dx® ELISA and SNAP® 4Dx® Plus ELISA (IDEXX Laboratories) use a synthetic C6 peptide resembling the invariable region of the membrane protein VlsE to detect antibodies against *B. burgdorferi*. Anti-C6 antibodies are expressed during natural *B. burgdorferi* infection and can therefore distinguish natural infection from an antibody response to vaccination, which results in production of anti-OspA antibodies but not anti-C6 antibodies (52). According to a study evaluating the test's performance in serum samples from 164 horses (28), sensitivity and specificity of the SNAP® 4Dx® ELISA relative to a commercially available Western Blot kit were 100% and 95%, respectively. Samples for this study were obtained from presumed infected horses, i.e. horses that had previously been tested for Lyme borreliosis, and presumed non-infected horses, i.e. horses that resided in a non-endemic area in the US. Lack of gold standard to confirm positive cases and possible

cross reactivity were not addressed in this study and may be a limitation in this study. The reported sensitivity and specificity of the SNAP® 4Dx® ELISA in horses experimentally infected with *B. burgdorferi* was 63% and 100% respectively (29).

The Equine Lyme multiplex assay is based on antigen-labeled fluorescent beads. The multiplex assay detects antibodies against three *B. burgdorferi* antigens, namely Osp A, Osp C and Osp F, in horse serum (48). The use of these antigens enables earlier diagnosis - as early as 2-3 weeks post infection (53,54) - compared to the IFA or ELISA with WB confirmation, where the earliest time of antibody detection averages 5 to 6 weeks post infection. The Lyme multiplex assay is quantitative and, according to the manufacturer's website information (55), the antibody profile allows an assessment of the stage of infection, vaccination status and treatment success in horses. In addition, the multiplex assay agreed with C6 ELISA testing of serum samples from infected and non-infected horses (48,54).

In experimentally infected ponies, culture of *B. burgdorferi* has been reported from skin biopsy samples of tick bite sites, and post mortem from joint capsules, muscles and lymph nodes (56). Manion et al. (57) reported isolation of viable spirochetes from the urine of two clinically normal horses. Molecular detection of *B. burgdorferi* DNA by PCR from synovial tissue or skin biopsy samples may improve sensitivity and specificity of detecting active *B. burgdorferi* infection (43,44). Information about possible cross-reactivity affecting serologic tests for LB in horses is lacking, however, the potential for cross-reactivity should be considered.

1.3.7. <u>Relationship between seroprevalence and clinical disease</u>

Although the seroprevalence of Lyme borreliosis in horses in some endemic areas in the northeastern United States may reach 60% (45,58–60), clinical disease associated with *B. burgdorferi* infection in horses is uncommon. Approximately 10% of seropositive horses develop clinical disease (45,58).

1.4   *Ixodes*

The family Ixodidae is the largest tick family contains approximately 650 tick species (14). Ixodid ticks are characterized by a dorsal plate called scutum. With 245 species, the genus *Ixodes* is the largest in the family Ixodidae (14). The *Ixodes ricinus* complex comprises 14 species and includes *I. scapularis* and *I. pacificus* in North America, *I. ricinus* in Europe and *I. persulactus* in eastern Europe and Asia (14,61). The species in the *I. ricinus* complex are

responsible for many *Ixodes* borne diseases in humans and animals. The ticks serve as a primary vector for *B. burgdorferi* and *A. phagocytophilum* infection in humans and animals and *Babesia microti* infection in humans (14).

1.4.1. *Ixodes scapularis* and *Ixodes pacificus*

I. scapularis is morphologically similar to *I. pacificus* (62,63); however, the species differ in their geographic distribution and feeding preferences. *I. scapularis* is found in the northeastern and midwestern US while *I. pacificus* is found on the west coast. In Canada, established populations of *I. scapularis* are known in southern Ontario and Quebec, Nova Scotia New Brunswick and southeastern Manitoba (38,39,64). Established populations of *I. pacificus* are distributed in southern British Columbia (38,39).

1.4.2. Life cycle

I. scapularis and *I. pacificus* are three-host ticks and each tick stage feeds on a different host. The life cycle of *Ixodes* involves four life stages, namely the egg, larva, nymph and adult, and usually spans two years (3). Tick activity differs dramatically with season and life stage (14) and in colder areas, the life cycle may take up to 3 years to complete (3).

The life cycle of *I. scapularis* has been described (Division of Vector Borne Infectious Disease, Atlanta, GA) and is shown in Figure 1. The larva hatches from the egg in the summer and feeds on small vertebrates, primarily the white-footed mouse, for 3-5 days. Larvae may also feed on birds or reptiles. The engorged larva then drops off the host to the ground, overwinters and molts to the nymph stage in the early spring. The nymph feeds on the second host (small mammals or birds) for 3 to 4 days, drops off and stays dormant until late summer or early fall, at which time it molts to an adult tick. The adult tick is active in the fall and winter months as long as ambient temperature exceeds 0° C. Adult female and male ticks seek out a larger vertebrate host to feed and mate on, the female feeding for 5 to 7 days before dropping off, overwintering and laying eggs in the spring before it dies. The male scarcely feeds and can stay on the host for a longer time period before dying.

Unlike *I. scapularis* larvae, the larvae and nymphs of *I. pacificus* prefer to feed on lizards (65). In general, both *I. scapularis* and *I. pacificus* are considered to have a "non-specific" feeding habit, meaning that they not only feed on their natural reservoir hosts but may also feed on humans (14) and horses (43,66).
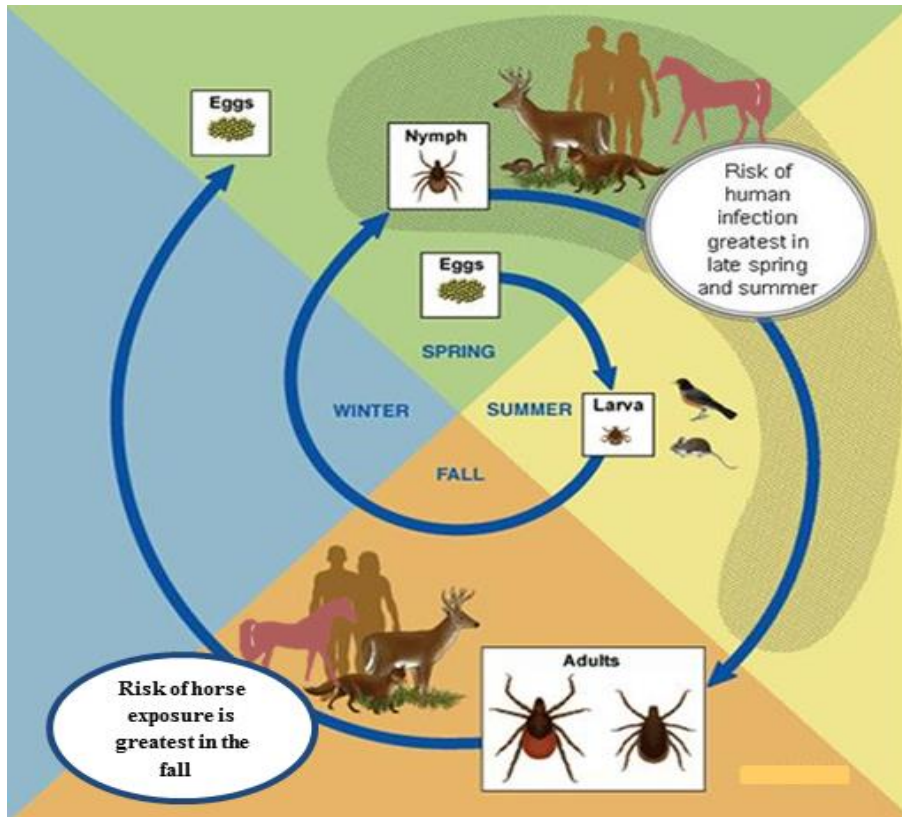
Figure 1.1 *Ixodes scapularis* life cycle adapted with permission from the Center for Disease Control (Division of Vector Borne Infectious Disease, Atlanta, GA)

1.4.3. Pathogen transmission

A tick is considered a vector for a pathogen if the tick feeds on a vertebrate host, is able to acquire the pathogen during a blood meal, maintains the pathogen through one or more life stages, and transmits the pathogen to another host during the next blood meal (61). Transovarial transmission of *A. phagocytophilum* and *B. burgdorferi* infection is considered insignificant in *Ixodes*. Larvae are therefore typically not infected upon hatching but may get infected when feeding on a reservoir host, and may transmit infection in the subsequent blood meal, as a nymph. Infected nymphs will molt to infected adults but adult females will not transmit the infection to the eggs (14).

The asynchronous seasonal activity of nymph and larval stages of *I. scapularis* appears to play a major role in maintaining efficient transmission cycles of *B. burgdorferi* (67). Infected nymphs are active in the spring and early summer and transmit the infection to rodents. Infected rodents transmit the infection to non-infected larvae, which are active in mid- to late summer. Larvae molt to infected nymphs in the following spring and account for a high

15

prevalence of *B. burgdorferi* infection in questing nymphs (67). Lindsay's study of duration of *B. burgdorferi* infection in white-footed mice suggested that efficient transmission of *B. burgdorferi* between different stages of the ticks is possible due to occurrence of co-feeding, which denotes the feeding of two tick stages on the same host (68). During co-feeding, infected nymphs feed between May and July and transmit infection to white-footed mice which remain infective for 3 weeks. The mice then transmit the infection to un-infected overwintered tick larvae that feed during June and July (68,69).

Compared to a low transmission rate of *A. phagocytophilum,* the transmission rate of most strains of *B. burgdorferi* from acutely infected rodents to ticks is considered high (>50%)(67). While the majority of *B. burgdorferi* species are transmitted with high efficiency from infected white-footed mice throughout their entire life span, some *A. phagocytophilum* strains may have a short duration of infection in rodents (67). For a pathogen to survive, the time in which a rodent is infective must be consistent with the seasonal activity of larval and nymph stages and with the gap between their activity periods (67).

In laboratory settings, *I. pacificus* nymphs were more competent vectors than *I. scapularis* nymphs for *A. phagocytophilum,* meaning they were better able to maintain the infection. However, the reported transmission efficiency for strains of *A. phagocytophilum* in these tick species does not necessarily correlate with the known geographic differences in disease prevalence. The fact that *A. phagocytophilum* prevalence in *I. pacificus* is considerably lower than that in *I. scapularis* in nature, alongside the finding that fewer human granulocytic anaplasmosis cases are reported in the western US, suggests that location-specific prevalence of *A. phagocytophilum* infection may involve other factors besides strain transmissibility and vector competence. Suggested factors include differences in host tropism and pathogenicity (65).

1.4.4. Prevalence of tick infection and potential for co-infection

1.4.4.1. United States

In the northeastern and midwestern US, the frequency of *B. burgdorferi* infection in *I. scapularis* ranges from 35 to 72%, and that of *A. phagocytophilum* infection ranges from 1 to 16% (20). The reported prevalence of infection with *B. burgdorferi* and *A. phagocytophilum* in *I. pacificus* ticks in the western US is 1.6 - 6.5% and 4.7%, respectively (66,70,71).

The reported prevalence of *B. burgdorferi* infection in *I. scapularis* collected by passive and active surveillance in ON and QC ranges from 4.9 to 13.2% (39,64). Interestingly, the prevalence of *B. burgdorferi* infection in *I. scapularis* collected from birds migrating northward from the US into Canada was 8 to 15.4% (40). The prevalence of *A. phagocytophilum* infection in *I. scapularis* ticks in eastern Canada was 1.4%, similar to the infection prevalence in ticks that were collected from migrating birds in the same study (40). These data suggest that invasion of tick-borne pathogens into tick populations in Canada is likely due to infected ticks arriving on migrating birds from the US.

1.4.4.3. Potential for co-infection

Generally, co-infection with *A. phagocytophilum* and *B. burgdorferi* is common and geographically widespread, both in the tick vector and in the vertebrate hosts (66). However, the types of co-infecting organisms within the tick vary. The most prevalent (61%) dual infection reported by Steiner et al. (20) was that of *B. burgdorferi* and the *Ixodes*- specific Rickettsial endosymbiotic organisms. Other dual infections were less common. Co-infection with *A. phagocytophilum* and *B. burgdorferi* in nymphal or adult *I. scapularis* may be acquired simultaneously from a co-infected host or in two consecutive feedings (14,20). The reported prevalence of co-infection with *A. phagocytophilum* and *B. burgdorferi* in *I. scapularis* ticks from areas endemic for Lyme disease reached 28% in the north-eastern US and 2% in the midwestern US (14). In northern California, the reported prevalence of co-infection with *A. phagocytophilum* and *B. burgdorferi* in *I. pacificus* ticks was approximately 1% (14). Triple co-infection, e.g. with *A. phagocytophilum, B. burgdorferi* and *B. microti,* was even less common (14).

Levin and Fish (72) demonstrated that the presence of *A. phagocytophilum* and *B. burgdorferi*, respectively, in *I. scapularis* does not prevent the tick from acquiring or transmitting the other organism to a white-footed mouse. On the other hand, primary infection with either *A. phagocytophilum* or *B. burgdorferi* in immune-competent mice appears to inhibit acquisition and transmission of the second agent, suggesting interference between the organisms in vertebrates. The different interaction between the agents in the vertebrate host is likely mediated by the host's immune response (73).

The prevalence of co-infection with *A. phagocytophilum* and *B. burgdorferi* in non-human mammalian hosts varies with geographic location, season and type of host (14). Results of

antibody detection and molecular methods (PCR) indicated that prevalence of co-infection in white-footed mice in Connecticut was approximately 50% but varied between different areas in the state (14). In the Western US, dual infection with *A. phagocytophilum* and *B. burgdorferi* was also detected in other mammals such as deer mice and Mexican wood rats (14). In experimental dual infection studies in animals, an increase in severity of Lyme borreliosis was observed (14). This suggests impaired immune function in animals infected with *A. phagocytophilum*. In human patients, co-infection with *A. phagocytophilum* and *B. burgdorferi* appears to be associated with more severe clinical signs of longer duration, or with persistence of both infections (74).

1.4.5. Current geographic range of the vectors in Canada

Ixodes are distributed throughout woodland and grass lands. The risk of *I. scapularis*-borne diseases is emerging in Canada and is following the northward pattern of geographic expansion of the tick vectors that was seen in the northeastern and midwestern US (75). The influences determining the geographic distribution and abundance of *Ixodes* vary and include the presence of woodland and bushy habitats, presence and density of suitable hosts for the different life stages, and presence of suitable environmental temperatures and moisture conditions for tick development and activity (14,76). These factors affect the survival rate of ticks and the establishment of new tick populations (77). An established tick population is defined as a population of reproducing ticks, meaning that all life stages can be found in at least two subsequent years (78).

The geographic distribution of *I. scapularis* in Canada appears to originate from two foci in the northeastern and Midwest US (79). The tick was introduced into Canada due to local host migration and distant migration on migratory birds (39,40,79). The geographic expansion of the vector is likely the reason for observing an increased incidence of Lyme disease in Canada in recent years (79). Up to 1997, a single known established population of *I. scapularis* ticks in Canada was present at Long Point, ON. By 2008, the number of established *I. scapularis* populations had risen to 13, and included those at the Great Lakes shores in ON, Wood Lake shore in MB and the coast of NS (40,79). Most of the known Canadian *I. scapularis* populations are geographically isolated from one another and are therefore less likely to result from local migration of local hosts (40). However there is also evidence suggesting establishment of populations in a wider area of QC, similar to the pattern of expansion observed in the US (39).

1.4.6. Potential causes of range expansion

The three major factors contributing to the current and anticipated further range expansion of tick habitats within Canada are local spread, distant migration and climate changes (75,79). It is suggested that for a new population of ticks to be established, a threshold number of ticks is required (76,77).

1.4.6.1. Local spread

The white-tailed deer constitutes the major source of a blood meal for the gravid female *I. scapularis* in the eastern and north central regions of the US. As the primary host for the reproducing stage, which will produce thousands of eggs, the deer's presence is essential for the establishment and spread of an *I. scapularis* population (80). Although deer are a non-competent reservoir for Lyme disease and cannot transmit the infection to feeding ticks, increased incidence of Lyme borreliosis in humans in the US was associated with the presence of deer (80). Resurgence of the white-tailed deer population over the past decades also contributed to the expansion of the geographic range of *I. scapularis* populations in much of the eastern United States (14). Although not quantified, the rapid increase in the number of reported human cases of Lyme borreliosis in the US since the early 1980s is associated with the dramatic growth of the deer population in the 20th century (80). Deer exclusion studies, which involve deliberate elimination of individuals in the deer population in certain areas, have shown a reduction in tick abundance in those areas where deer populations were reduced (80). An increase in residential deer sightings was associated with an increase in clinical cases of Lyme disease in humans and an increase in the number of dogs testing seropositive for Lyme disease (80). In addition to its importance for Lyme Disease, the white-tailed deer is also one of the main reservoirs for *A. phagocytophilum* variants in the US (16). Deer are usually infected with the *A. phagocytophilum* Var - 1 strains, which are non-human strains (10). The general increase in tick abundance is suggested as the cause for increased incidence of Lyme in areas with high deer density, due to increased abundance of the ticks.

As discussed earlier, geographically isolated populations of *Ixodes* occur on the southeast shore areas of MB, ON and the coast of NS. These populations are therefore less likely to have resulted from range expansion of local hosts (40). However, tick migration from established Canadian populations via local host migration can contribute to the establishment of new tick populations in Canada. Deer population density is suitable and is of great importance in that deer can carry high numbers of ticks, including gravid females, to distant

areas (75). Considering that climate changes are likely to favor reproduction of the migrating ticks, this may be an important route for establishment of new populations of the vector in Canada. Success of tick invasion with regard to host abundance, host diversity and geographic variation in Canada needs to be investigated further (75)

1.4.6.2. Migrating birds

Migratory passerines are hosts for *I. scapularis* in North America and may carry *I. scapularis* northward into Canada during their spring migration (40). These migrating birds mostly carry nymphs and may disperse *I. scapularis* over a considerable distance within Canada. The ticks that are carried by migrating birds and dropped in distant areas are called adventitious ticks. Adventitious nymphs may survive through molting, and infected adult ticks may then transmit tick-borne infections to the next host they quest (40,79). However, the risk for encountering these infected ticks is likely low due to their relatively lower density compared to the density of reproducing populations. Adult ticks are also in general less likely to transmit disease to humans due to their larger size and ease of identification and removal by humans (40). It is possible that infected adventitious ticks pose a greater risk for other large mammals such as horses, which are less likely to remove them.

1.4.6.3. Climate changes

In laboratory studies, inter-stadial developmental periods of *I. scapularis* (i.e. the length of time between molts) decreased significantly with increasing ambient temperature (81). Inter-stadial development time of ixodid ticks usually decreases with increasing ambient temperatures; however, onset and termination of the dormant stage is complex and relates also to an increase in day length, exposure to extreme ambient temperatures and humidity levels. Variation of ambient temperature within narrow ranges (e.g. within $5\text{-}10^0$ C for the interval to first egg production and within $10\text{-}20^0$C for the interval from egg deposition to hatching of larvae) appears to produce significant variation in developmental rates. Above 20^0C, a further increase in ambient temperature appears to produce smaller changes in development (81). Incubation temperatures $> 30^0$C had a detrimental effect on developing ticks (81). Climate change is anticipated to increase the number of days with an ambient temperature $> 0^0$C and thus may enhance the ticks' survival and reproduction efficiency (40). Anticipated climate changes may therefore lead both to geographic expansion of existing populations and to an increased survival of new populations.

A dynamic population model of *I. scapularis* was developed by Ogden et al. (77) in order to simulate the effects of temperature change on tick survival and seasonality. The model considered the effects of changes in rodent and deer density, meteorological data from stations near endemic populations of *I. scapularis*, humidity and protection by litter layer. Increased tick die-out due to a steady increase in mortality of all life stages was associated with decreasing ambient temperatures. Conversely, maximum numbers of ticks in self-sustaining balanced populations were associated with the mean annual number of degree days > 0 (77). The threshold for establishment of new *Ixodes* populations was mapped and indicated that there are regions in Canada which currently experience temperature conditions suitable for *I. scapularis* establishment, and that these are more extensive than the currently recognized distribution of *I. scapularis* populations (77). These areas also overlap with the areas of highest human population density in southeastern Canada. Environmental conditions were found to be suitable for tick establishment in Southern AB, SK and MB where *I. scapularis* are mostly introduced by migrating birds to date (77). The tick population trends shown in the model suggest that increasing ambient temperatures with anticipated climate change will expand the northern and western ranges of *I. scapularis*. The model also demonstrated that the tick population in NS has become established where unfavorable temperatures exist, suggesting that tick mortality and host finding rates are optimal in the Maritimes (77).

In a subsequent study, a tick population model based on two global climate models predicted that tick abundance will almost double by 2020 and that threshold numbers needed to establish new tick populations will decrease during the next decades (76). The degree of range expansion and survival of new populations are anticipated to be evident in the next two decades (76).

Risk maps for predicting the expansion of *I. scapularis* habitats in Canada were created using a simple risk algorithm for new *I. scapularis* populations to occur. The maps include slow and fast scenarios and both indicate an increased risk of *I. scapularis* establishment in currently low-risk areas, as well as northward and westward expansion of existing endemic *I. scapularis* populations (82).

Surveillance data from the Public Health Agency of Canada support an increase in the geographic range and number of established *I. scapularis* populations in much of Southern Canada (Public Health Agency of Canada, http://www.phac-aspc.gc.ca/id-mi/tickinfo-

eng.php, last accessed on September 21, 2013). Established populations have been reported in NB, QC, NS, Ontario and MB. Established populations of *I. pacificus* were reported in southern BC.

1.4.7. <u>Relationship between endemic tick populations and endemic tick-borne disease</u>

As defined by Health Canada, endemic areas for Lyme disease, in which the risk for Lyme disease is greatest, are those areas where populations of the tick vector have been established and where there is evidence that the established tick populations are transmitting Lyme disease (78). Based on active surveillance data, the Public Health Agency of Canada reported that established populations of *I. scapularis* which are endemic for Lyme disease occur in limited areas in Canada (Public Health Agency of Canada http://www.phac-aspc.gc.ca/id-mi/tickinfo-eng.php, last accessed on September 21, 2013). The Public Health Agency of Canada currently reports six Lyme endemic areas in NS, two in NB, five in QC, seven in ON and three in MB. Three more areas in MB are reported as suspected Lyme endemic areas. Established populations of *I. pacificus* are known to occur in some areas of southern BC (mainly Vancouver Island) and others are suspected over a wider region. To date, Lyme borreliosis is reportedly not endemic in BC.

Migrating birds could be significant in the establishment of endemic transmission cycles of *I. scapularis*-borne pathogens by introducing infected ticks to non-endemic areas or by increasing the pathogen load in endemic areas. Ogden et al. (40) estimated that the prevalence of tick infestation in migrating birds was 2.2% and infestation density on average was 1.6 ticks per bird. They concluded that migrating birds account for the dispersion of 50 to 175 million *I. scapularis* ticks across Canada each spring. In the same study, 15.4% and 1.4% of *I. scapularis* nymphs collected from migrating birds were PCR positive for *B. burgdorferi* and *A. phagocytophilum,* respectively. This prevalence of *A. phagocytophilum* infection was consistent with that reported in *I. scapularis* nymphs in the northeastern US (40). Birds are mostly zooprophylactic for *B. burgdorferi*, meaning they reduce the presence of infection in the ticks they carry. This was established based on spring bird observations in and east of Long Point, ON (40). The prevalence of *B. burgdorferi* infection in subset of nymphs collected from birds that migrated via the east coast (8.3%) was significantly lower than that of *B. burgdorferi* infection in questing nymphs in the north eastern US (>30%) (40).

A subsequent study predicting the rate of *B. burgdorferi* invasion into established *I. scapularis* tick populations in Canada used a simulation model of *I. scapularis* populations

and *B. burgdorferi* transmission (64). The simulation model identified that the number of introduced ticks, both infected and non-infected, was the most important factor in determining the speed of *B. burgdorferi* invasion after *I. scapularis* has been established. The model predicted greater numbers of introduced infected nymphs in central Canada versus eastern Canada (64).

Seasonal activity of the immature stages of *I. scapularis* differs between the north-eastern and midwestern US, which likely affects the risk of introducing tick-borne diseases through adventitious ticks in Canada. Larval and nymphal activity is synchronous in the Midwest whereas in the northeast, nymphs are active in the spring and larvae are active in the summer (64). Northward migrating birds therefore disperse both active larvae and nymphs from the midwestern US to central Canada in the spring, whereas tick dispersion from the northeastern US into eastern Canada is composed mainly of nymphs with only a non-significant number of larvae introduced. The higher number of infected larvae introduced into central Canada results in a higher number of infected nymphs after molting, which then feed on competent small mammal hosts that can maintain and are able to transmit infection. Introduced infected nymphs, on the other hand, molt into adult ticks after leaving the migratory bird host, and these adults typically feed on a non-competent large mammal host that is unable to further transmit the infection. Ogden et al. therefore hypothesized that a five-year gap will occur between tick invasion and *B. burgdorferi* invasion into eastern Canada compared to a much shorter gap in central Canada (64).

The term "dilution effect" refers to the inhibition of pathogen transmission cycles which occurs when non-competent host species are added to host populations, making the pathogen less abundant and less likely to persist (83). Thus the number of new infections and the overall reproduction ability of the pathogen will be reduced (83). Increased biodiversity, increased host species richness and evenness between competent hosts, which are typically more abundant, and non-competent hosts should result in reduced pathogen abundance (83,84).

The prevalence of *B. burgdorferi* infection in adult ticks collected through passive surveillance in QC before 2004 was 13.2% (85), similar to the prevalence reported in engorged *I. scapularis* nymphs collected from northward migrating birds (15.4%) (40). The authors indicated that, after 2004, the number of submitted ticks increased each year, which was mostly due to increased submissions from areas close to the US border. During the same

time period, the prevalence of *B. burgdorferi* infection in the submitted ticks declined to 4.9% (39). This finding was accompanied by active surveillance studies in southern QC (June-October, 2007-2008), in which prevalence of *B. burgdorferi* infection in many emerging *I. scapularis* populations was either undetectable or was low at most sites (7.7%) (39). These findings suggest that the establishment of *I. scapularis* populations free of *B. burgdorferi* may dilute the infection prevalence in the adventitious ticks dispersed from the US, and will increase the overall proportion of uninfected ticks in the environment (39).

As hereby described, expansion of the geographic range of *Ixodes* is multifactorial and influenced by environmental conditions, ongoing processes, as well as limitation factors. However, the emergence of the tick is continuously evident in recent decades and the concern over *Ixodes* borne diseases is therefore increased.

1.5. Hypotheses and Objectives

The first goal in this work was to characterize the current level of horse exposure to EGA and LB in Canada. This work is described in chapter 2. The terms seropositive, seronegative and seroprevalence in this and all other studies refer to the presence (or absence) of antibodies in a serum sample. The second goal was to understand whether a point-of-care ELISA is comparable to currently recommended serologic tests for EGA and LB in horses. The hypotheses and objectives for this chapter are as follows:

Hypothesis 1: That based on a point-of-care ELISA, seroprevalence of EGA and equine LB in SK, MB and ON is low.

Objective 1: To estimate the seroprevalence of EGA and LB in horses in SK, MB and ON by testing convenience serum samples submitted to provincial diagnostic laboratories using a point-of-care ELISA.

Hypothesis 2: That the assessment of a sample as seropositive or seronegative for EGA and LB does not differ between a point-of-care ELISA and laboratory-based serologic tests.

Objectives 2.1: To evaluate agreement between a point-of-care ELISA and laboratory-based IFA for EGA based on an inter-rater agreement statistic and a comparison of the proportion of positive tests, using convenience serum samples.

Objective 2.2: To evaluate agreement between a point-of-care ELISA and laboratory-based ELISA/WB combination for LB based on an inter-rater agreement statistic and a comparison of the proportion of positive tests, using convenience serum samples.

As agreement between serologic tests varied in the first study, agreement was further investigated between all available serologic tests for EGA and LB in chapter 3. The goal of this work was to establish whether different serologic tests will result in the same assessment of a sample as seropositive or seronegative. The hypotheses and objectives for this chapter are as follows:

Hypothesis 3: That the assessment of a sample as seropositive or seronegative for EGA or LB, respectively, is independent of the specific testing method used.

Objective 3.1: To evaluate agreement among a point-of-care ELISA and two laboratory-based IFAs for EGA based on an inter-rater agreement statistic and a comparison of the proportion of positive tests, using convenience serum samples.

Objective 3.2: To evaluate agreement among a point-of-care ELISA, laboratory-based IFA, laboratory-based ELISA/WB combination and laboratory-based Lyme multiplex assay for LB based on an inter-rater agreement statistic and a comparison of the proportion of positive tests, using convenience serum samples.

The goal of the third study was to better understand the risk for exposure of horses in Canada to EGA and LB. Management factors were compared between horses that tested seropositive or seronegative to EGA and LB in chapters 2 and 3. The hypothesis and objectives for this chapter are as follows:

Hypothesis 4: That the signalment, management and clinical history do not differ between horses that tested seropositive or seronegative for EGA or LB, respectively.

Objective 4.1: To obtain information regarding signalment, management and clinical history for horses whose serum samples were tested for EGA and LB in previous studies, using mail-out surveys.

Objective 4.2: To compare the signalment, pasture access, tick infestation and tick-borne disease history, travel history and Lyme disease vaccination status between horses testing seropositive or seronegative for EGA or LB, respectively.

Finally, as data concerning the ticks infesting horses in SK are not available, describing the ticks that may be found on horses in SK was the goal of the fourth study. This study is described in chapter 5. The hypothesis and objective for this chapter are as follows:

Hypothesis 5: That tick species known to be established in SK, as well as adventitious tick species can be found on horses in SK.

Objective 5: To describe the species, sex, life stage, state of engorgement and geographic location of acquisition of ticks submitted from horses in SK over a 2-year period.

Transition to chapter 2

Horses may be exposed to the causative agents of equine granulocytic anaplasmosis and Lyme borreliosis when infested by the vector ticks, *I. scapularis* and *I. pacificus*, which are the most common competent vectors transmitting the pathogens to horses in North America (16,18,43,50,86).

As discussed earlier in the thesis, diagnosed cases of EGA and LB have been reported in Canada from provinces with and without established populations of the vector. However, the prevalence of tick-borne infections in horses in Canada has not been reported to date. Thus, there is a need to better understand the prevalence of tick-borne infections and the risk of exposure to the vectors and the causative organisms in horses in Canada.

The aim of the first study was to estimate the seroprevalence of EGA and LB in horses in Canada. A point-of-care ELISA was used to screen equine serum samples from 3 provinces, namely ON, MB and SK. The hypotheses were that based on a point-of-care ELISA, the seroprevalence of EGA and LB SK, MB and ON is currently low, and that the assessment of a sample as seropositive or seronegative for EGA and LB does not differ between a point-of-care ELISA and laboratory-based serologic tests.

The author of this thesis collaborated with Drs. K. Lohmann, H. Burgess, T. Epp and N. Chilton in planning the study, handling samples and reviewing the results and statistical analysis.

The author of this thesis performed the testing for antibodies against *A. phagocytophilum* and *B. burgdorferi* using a point-of-care ELISA kit according to the manufacturer's instructions. Selected samples were further re-tested at the Connecticut Veterinary Medical Diagnostic Laboratory. Recording of test results, data analysis and reporting of the results were performed by the author of the thesis. Results were presented as a research poster during the graduate student poster day at the Western College of Veterinary Medicine, University of Saskatchewan, SK (March 13, 2013) and at the ACVIM Forum, American College of Veterinary Internal Medicine, in Seattle, WA (June 12-15, 2013). This chapter will be submitted for publication in the Canadian Veterinary Journal which will hold copyright.

2. SEROPREVALENCE OF EQUINE GRANULOCYTIC ANAPLASMOSIS AND LYME BORRELIOSIS IN HORSES IN CANADA AS DETERMINED BY A POINT-OF-CARE ELISA

2.1 Abstract

Equine granulocytic anaplasmosis (EGA) and Lyme borreliosis (LB) are tick-borne diseases transmitted by the *Ixodes* species. While *Ixodes*-borne diseases are an emerging concern in Canada, the risk of exposure to horses is poorly understood. A point-of-care ELISA for detection of antibodies against *A. phagocytophilum* and *B. burgdorferi* is licensed in dogs and reportedly accurate in horses. The objectives of the study reported here were to estimate the seroprevalence of EGA and LB in Saskatchewan (SK), Manitoba (MB) and Ontario (ON) and to investigate agreement between a point-of-care ELISA and laboratory-based serologic tests.

Convenience serum samples obtained from veterinary diagnostic laboratories in SK (n=202), MB (n=140) and ON (n=34) were tested using the SNAP® 4Dx® ELISA (IDEXX Laboratories, Inc., Westbrook, ME) and a seroprevalence estimate with 95% confidence interval was reported overall and for each province. Seropositive samples for EGA (n=2) or LB (n=6) and two randomized subsets of seronegative samples (n=92 each) were re-tested by indirect immunofluorescence antibody assay (IFA) for EGA, or whole cell ELISA confirmed with Western Blot (WB) for LB, in a commercial veterinary diagnostic laboratory. Antibody titers $\geq 1:80$ for IFA and $\geq 1:160$ for whole cell ELISA (when WB confirmed) were considered positive. Agreement between the SNAP® 4Dx® ELISA and each laboratory-based serologic test was assessed by comparing the proportion of positive test results (McNemar's test) and by inter-rater agreement testing (Kappa statistic).

Based on the SNAP® 4Dx® ELISA results, the overall seroprevalence of EGA was 0.53% (95% CI: 0.09 -2.12%) while the provincial seroprevalence was 0.49% (95% CI: 0.02-3.1%) in SK, 0.71% (95% CI: 0.03-4.5%) in MB and 0% (95% CI: 0-12.6%) in ON. Overall seroprevalence of LB was 1.6% (95% CI: 0.65-3.6%) while the provincial seroprevalence was 0.49% (95% CI: 0.02-3.15%) in SK, 2.9% (95% CI: 0.96-7.15%) in MB and 2.94% (95% CI: 0.15-17%) in ON. The proportion of positive results differed significantly between serologic tests for EGA but not between serologic tests for LB. For LB, test agreement was considered fair according to a Kappa value of 0.23 (95% CI: -0.03 - 0.50).

Conclusions: While the SNAP® 4Dx® ELISA yielded expected seroprevalence estimates, test results failed to agree (for EGA) or showed only fair agreement (for LB) when the test was compared with laboratory-based serologic tests. This may be attributable to false positive (possibly due to cross-reactivity) or false negative test results. Agreement between the SNAP® 4Dx® ELISA and laboratory-based serologic tests in horses requires further investigation.

2.2 Introduction

Granulocytic anaplasmosis is caused by *Anaplasma phagocytophilum* and has been reported in horses, dogs and humans in Canada (6–10). Equine granulocytic anaplasmosis (EGA) is characterized by fever, anorexia, depression and distal limb edema (10,16). The main hematological abnormalities are neutropenia and thrombocytopenia (24,87). Three case reports from British Columbia (BC) (4), Nova Scotia (NS) (5) and Saskatchewan (SK) (6), and one report from New Brunswick (NB) (7) describe the occurrence of the disease in horses in Canada.

Lyme borreliosis (LB) is caused by *Borrelia burgdorferi* and has been reported in horses, dogs and humans in Canada (8,38,88–91). Lyme borreliosis in horses is characterized by lameness, joint effusion, muscle tenderness, depression and generally decreased performance (43,44,50). Low-grade fever, laminitis or uveitis may also be present (44). While the disease has been reported in only two horses from BC to date (8), it is possible that cases are overlooked due to the rather non-specific clinical signs observed in horses.

The blacklegged tick, *Ixodes scapularis,* and the Western blacklegged tick, *Ixodes pacificus,* are the principal vectors for EGA and LB in eastern and western Canada, respectively (88). Southern Quebec, southern Ontario (ON) and southeast MB are endemic for *I. scapularis* while a few areas in BC are reported to be endemic for *I. pacificus* (39). In Canada, presence of *A. phagocytophilum* and *B. burgdorferi* in *Ixodes* and infections in humans and veterinary species have been reported from areas with and without established populations of the vector ticks (4–6,38,64,89–94). Disease exposure in non-endemic areas is suggested to occur due to adventitious ticks, which are ticks that are carried into an area by migrating animals or humans.

Risk maps for the emergence of the tick vector *I. scapularis* suggest that climate changes will accelerate range expansion of the vector in eastern and central Canada (82). Other contributing factors are local host migration and distant migration by birds migrating to Canada in the spring (40,76,77,82). Geographical range expansion predictions anticipate the emergence of new cases of LB in humans and animals in areas with and without established *Ixodes* populations (64,76,77,81). Studies focused on *Borrelia* phylogeographics, currently known endemic areas of *Ixodes* in the US, and bird migration into Canada (including analysis of their parasitic infection status) indicate an association between the population pattern of *I. scapularis* in the US and its expansion within Canada (40,41,64,76,77,95). These studies

30

suggest that the risk for exposure to *Ixodes*-borne diseases is emerging in Canada. Hence, the risk for horse exposure in Canada, both in currently endemic and non-endemic areas, is expected to increase.

The currently recommended diagnostic test to detect antibodies against *A. phagocytophilum* is the indirect immunofluorescence antibody assay (IFA) (27). Antibodies against *B. burgdorferi* in horses may be detected by IFA, ELISA confirmed with Western Blot (WB) or a Lyme multiplex assay using immunofluorescence beads (43,51,54). The presence of antibodies in blood may indicate active infection associated with active production of antibodies against the existing organism. However, the presence of antibodies may also indicate exposure and persistence of detectable antibody levels in the absence of active infection. Antibodies to *A. phagocytophilum* may persist for up to 2 years in horses (25).

A point-of-care SNAP® 4Dx® ELISA (IDEXX Laboratories, Westbrook, ME) is labeled for the detection of antibodies against the *A. phagocytophilum* P44 antigen and the *B. burgdorferi* C6 antigen, respectively, in dogs. According to the manufacturer, the test methodology is not species-specific and the test performs well in equine samples (28).

Interpretation of serologic test results in non-endemic areas is challenging. False positive result may occur due to cross-reactivity of antibodies to similar organisms (30,96). Sensitivity for detection of antibodies varies between the different serologic tests and may be time-dependent. That means that the expression of different immunogens of the pathogen and, hence, antibody production, varies according to the different stages of infection, as in the case of *B. burgdorferi* (27,48,53,97–99). Another challenge when interpreting serologic tests is the differentiation between active infection and exposure as discussed earlier.

Although the vector *I. scapularis* is endemic in some areas of Canada, the prevalence of exposure to EGA and LB among horses (as detected by the presence of measurable antibody titers) is unknown. In endemic areas in the midwestern and northeastern US, seropositive horses are common and the majority of seropositive horses are asymptomatic (18,25,45,58–60,100). Increased evidence of tick-borne morbidity in horses appears to be related to the emergence of the tick vector in Canada (75). The frequency of exposure of horses in Canada to tick-borne organisms is poorly characterized and requires further investigation. One benefit of estimating seroprevalence in horses is that it provides a baseline for monitoring the behaviour of these tick-borne diseases in horses. As discussed, the anticipated increase in the

rate of exposure requires tight monitoring and application of control measures when indicated.

For this study, it was hypothesized that based on a point-of-care ELISA, the seroprevalence of EGA and equine LB in SK, MB and ON is currently low. We estimated seroprevalences by testing convenience serum samples submitted to provincial diagnostic laboratories. Seroprevalence in this study was defined as the proportion of samples that tested positive for the presence of antibodies against *A. phagocytophilum* (seroprevalence of EGA) or *B. burgdorferi* (seroprevalence of LB).

It was also hypothesized that the assessment of a sample as seropositive or seronegative does not differ between a point-of-care ELISA and laboratory-based serologic tests. Agreement between serologic was assessed by comparison of the proportion of positive tests and by use of an inter-rater agreement (Kappa) statistic.

2.3 Materials and Methods

2.3.1 <u>Serum samples</u>

Equine serum samples were obtained from submissions to diagnostic laboratories in MB (Manitoba Agriculture, Food and Rural Initiatives Veterinary Diagnostic Services, Winnipeg), SK (Prairie Diagnostic Services, Saskatoon) and ON (Animal Health Laboratory, Guelph). To estimate seroprevalence in each province with 95% confidence, a required sample size of 100 samples per province was calculated based on an estimate of at least 100,000 horses in each of the 3 provinces and an estimated seroprevalence of 2% for each disease in horses in Canada.

The laboratories were asked to collect serum samples that were submitted between October and December 2011 and that contained at least 1ml serum. The collection period was chosen to maximize chances for positive serologic results. Horses are more likely to be infested with the adult stage of *I. scapularis,* which is active in the fall months, starting in September (13). Samples were categorized according to the month of submission and the province from which they originated. Taking time for seroconversion into account, we tested all the samples submitted in November first. If the number of samples submitted in November from an individual province did not reach 100, we then tested samples submitted in December. Samples submitted in October were only tested if those submitted in November and December did not add up to 100.

Samples were kept frozen at -20°C between collection and analysis in the investigator's laboratory. The serum samples were supplied in accordance with the laboratory's confidentiality rules and the study was approved by the Animal Research Ethics Board, University Committee on Animal Care and Supply (UCACS), at the University of Saskatchewan (protocol # 20120015).

2.3.2 SNAP® 4Dx® ELISA testing

Before testing, serum samples were thawed at room temperature and then centrifuged (Eppendorf 5702 centrifuge, Eppendorf, Mississauga, ON) for 10 minutes at 4400 rpm. The samples were tested for the presence of antibodies against *B. burgdorferi* and *A. phagocytophilum* using the SNAP® 4Dx® ELISA according to manufacturer's instructions.

Briefly, 3 drops of serum and 4 drops of conjugate were mixed thoroughly in a sample tube. The entire content of the sample tube was added to the sample well. The sample was allowed to flow across the results window for 30-60 seconds. When the first color change appeared in the activation circle, the activator was pushed firmly until it was flush with the device body. The test result was read at 8 minutes. Any change in colour in the sample spots in the activation window was interpreted as a positive result, i.e. the presence of specific antibodies. Samples were re-frozen immediately following testing.

2.3.3 Laboratory-based serologic tests

Samples were re-tested at the Connecticut Veterinary Medical Diagnostic Laboratory (CVMDL). The frozen samples were shipped to the laboratory on ice overnight. All samples testing seropositive for EGA by SNAP® 4Dx® ELISA and 92 randomly selected seronegative samples were re-tested using an *A. phagocytophilum* IFA. All samples testing seropositive for LB by SNAP® 4Dx® ELISA and 92 randomly selected seronegative samples were re-tested using a whole cell B. *burgdorferi* ELISA confirmed by WB. The laboratory's guidelines for interpretation of test results are summarized in Table 2.1. Samples were considered seropositive for EGA when the IFA titer was ≥80. Samples were considered seropositive for LB when a positive *B. burgdorferi* ELISA titer (≥1:160) was confirmed by a positive WB. Samples with equivocal or negative WB results were considered seronegative regardless of the ELISA titer. Samples with an ELISA titer <1:160 were considered seronegative and were not re-tested by WB.

2.3.4   Statistical analysis

To estimate seroprevalence, the number of seropositive samples for EGA and LB by SNAP® 4Dx® ELISA was recorded and the overall and individual provincial proportions of positive samples were calculated with their 95% CI. Each sample was considered as representing a different horse.

The number of seropositive and seronegative samples was recorded for each serologic test used. The two-tailed McNemar's test (VassarStats software, Website for Statistical Computation; http://www.vassarstats.net/index.html) was used to compare the proportion of positive test results between paired tests and $p < 0.05$ was considered significant. When the McNemar's test indicated no significant difference between the proportion of positive results, an inter-rater agreement was calculated, using the Kappa statistic (©2013 GraphPad Software, Inc., San Diego, CA). The magnitude of Kappa was interpreted as follows: ≤ 0 poor, 0.01-0.2 slight, 0.21-0.4 fair, 0.41-0.6 moderate, 0.61-0.8 substantial, 0.81-1 almost perfect agreement (101).

2.4 Results

2.4.1 Samples

A total of 626 serum samples were received. Three hundred and seventy-seven serum samples that originated from SK were submitted to Prairie Diagnostic Services. Two hundred and fifteen serum samples that originated from MB were submitted to Prairie Diagnostic Services and Manitoba Agriculture, Food and Rural Initiatives Veterinary Diagnostic Services. A total of thirty-four samples that originated from ON, all submitted in October, were submitted to the Animal Health Laboratory (Table 2.2).

Three hundred and seventy-six serum samples were tested with the SNAP® 4Dx® ELISA. Due to the fact that a low number of samples originated from ON, more than 100 samples originating from SK and MB were tested (Table 2.2). All samples submitted from one province within one month, starting in November were tested. As only 34 samples were received from the MB lab, all of them were tested, including 6 from October (Table 2.2). In total, 202 samples originating from SK, 140 samples originating from MB and all 34 samples originating from ON were tested (Table 2.2).

2.4.2 <u>Seroprevalence according to SNAP® 4Dx® ELISA testing</u>

Overall, 2 samples were seropositive for EGA and 6 samples were seropositive for LB (Table 2.3). No sample was seropositive for both EGA and LB. Based on the SNAP® 4Dx® ELISA results, the overall seroprevalence of EGA was 0.53% (95% CI: 0.09-2.12%) and the overall seroprevalence of LB was 1.6% (95% CI: 0.65-3.6%). Table 2.3 shows the individual provincial seroprevalences.

2.4.3 <u>Agreement between the SNAP® 4Dx® ELISA and laboratory-based serologic tests</u>

Of the samples testing seropositive for EGA by SNAP® 4Dx® ELISA, one sample tested weakly positive (1:160) and the second sample tested strongly positive (1:10,240) by IFA. Of the samples testing seronegative for EGA by SNAP® 4Dx® ELISA, 67 samples tested negative (<1:80), 24 samples tested weakly positive (1:80-1:320) and one sample tested moderately positive (1:1280) by IFA (Table 2.4; also see Table 2.1 for test interpretation guidelines).

Of the 6 samples that tested seropositive for LB by SNAP® 4Dx® ELISA, 3 were weak positive by WB and 3 were equivocal by WB and therefore categorized as seronegative for the purposes of agreement testing (Table 2.5; also see Table 2.1 for test interpretation guidelines). All of the SNAP® 4Dx® ELISA negative samples that tested positive by WB (n=11) were weakly positive by WB. One sample tested moderately positive by ELISA (1:1280) but was negative by WB. Repeated testing was done in the laboratory with identical result. This sample was interpreted to be seronegative for LB and the moderately high titer by whole cell ELISA was assumed to be due to cross-reactivity.

According to the two-tailed McNemar's test, the proportion of seropositive samples was significantly different between the SNAP® 4Dx® ELISA and laboratory-based IFA for EGA (p <0.000001), indicating a bias effect. The difference in the proportion of positive results was not significant (p = 0.057) between the SNAP® 4Dx® ELISA and ELISA/WB for LB. Agreement between the SNAP® 4Dx® ELISA and whole cell ELISA/WB for LB was fair, as indicated by a Kappa of 0.23 (95% CI: -0.03 - 0.50) (Table 2.6).

2.5 Discussion

The objectives for this study were two-fold. The first goal was to estimate the seroprevalence of EGA and LB in horses in SK, MB and ON using a point-of-care (SNAP® 4Dx®) ELISA. This study reports the first seroprevalence estimate for EGA and LB of horses in Canada. Seroprevalence estimates for both EGA and LB fit well with the expected seroprevalence, suggesting that horses in Canada are currently at low risk for exposure to EGA and LB.

Overall and provincial seroprevalences for EGA and LB were low which supports our first hypothesis (Table 2.3). In comparison to these results, the reported seroprevalence of EGA in the midwestern US is 3.8% in *Ixodes* non-endemic areas and up to 17.6% in *Ixodes* endemic areas (13). The reported seroprevalence of EGA in an *Ixodes* endemic area in California is 10% (13). The reported seroprevalence of LB in horses in *Ixodes* endemic areas in the US is up to 60% (45,58–60).

As established populations of *Ixodes* are known to occur in southeastern MB and in southern ON (39), one may expect an increased risk for exposure of horses in these provinces, with a higher seroprevalence of EGA and LB relative to an area where established populations of *I. scapularis* have not been detected such as SK. However, established populations of *Ixodes* in ON and MB are mostly isolated (39,75) and the level of exposure may differ between regions in each province. While we have no data about the distribution of the tested horses within the provinces, exposure of horses may also be explained by exposure to adventitious ticks introduced by migrating birds or local hosts. Indeed, the provincial and overall seroprevalence of EGA and LB found in our study was similar to the reported prevalence of *A. phagocytophilum* infection in *I. scapularis* ticks (1.4%) collected from migrating birds arriving in Canada (37). Since no established populations of *I. scapularis* have been detected in SK, it may be assumed that the most likely explanation for finding seropositive horses in this province would be exposure to adventitious ticks. However, the occurrence of unrecognized established populations of *Ixodes* in SK cannot be ruled out. Other possible explanations for seropositivity in horses from presumed non-endemic areas include travel of horses to endemic areas prior to sample collection, or collection of samples from horses that were imported from an endemic area. The same is also true for horses from endemic areas which may have been exposed outside their current area of residency. As no historical data, including travel history were available for the horses from which samples

originated, it is not certain whether the seropositive horses were all exposed to the diseases within Canada. Most of the serum samples used in this study were originally submitted for equine infectious anemia testing, which suggests that they originated from a specific population of horses that is likely to compete and, possibly, travel. It is therefore possible that some seropositive horses were exposed to EGA or LB outside of Canada.

Due to the limited sample size, the difference in seroprevalence for EGA and LB among provinces was not assessed. It is worth mentioning that the seroprevalence estimate for ON needs to be interpreted with caution as it was based on a very small sample size which decreased the accuracy of the estimate. Another potential limitation of the seroprevalence estimates in the study is the source of the samples used. As there was no control over the origin of the samples, it is not certain that each sample represented an individual horse. However, as the majority of samples was originally submitted for equine infectious anemia testing, horses are typically tested once per season, and received samples were submitted in a time period of three months, it is most probable that the majority of samples represented different horses.

The second aim was to assess the agreement between test results obtained by a point-of-care ELISA and those obtained from standard laboratory-based serologic tests. When testing for antibodies against *A. phagocytophilum*, the lack of agreement between the tests was caused by the high proportion (27%) of seronegative samples by SNAP® 4Dx® ELISA that were seropositive by the *A. phagocytophilum* IFA (Table 2.4) as was indicated by a significant difference in the proportion of positive results between tests (McNemar's test, Table 2.6). The differences between the test results may be attributable to differences in test methodology, such as the use of different immunogens. The SNAP® 4Dx® ELISA uses a peptide derived from the P44 protein, also known as the major surface protein 2 (MSP2), which is expressed on the cell membrane of *A. phagocytophilum*. In comparison, the *A. phagocytophilum* IFA uses whole *A. phagocytophilum* organisms in HL-60 cells fixed on slides (28). The whole *A. phagocytophilum* organisms may cross-react with antibodies against other, similar rickettsia (45), which may result in false positive results. The fact that titers for the majority of IFA seropositive samples were weak positive may support non-specific reactivity, possibly due to cross-reactivity, implying false positive results in the IFA. Conversely, while being highly specific, the SNAP® 4Dx® ELISA may yield false negative results as it only detects antibodies against one major surface protein of *A. phagocytophilum*. It is also possible that exposed or previously diseased horses maintain positive antibody titers

37

that are detectable by IFA but not by SNAP® 4Dx® ELISA. Reportedly, antibodies against *A. phagocytophilum* in horses can be detected by IFA for up to 2 years (25). The mildly positive titers in the majority of IFA positive samples in our study may therefore suggest exposure without active infection, or resolving infection.

When testing for antibodies against *B. burgdorferi*, the McNemar's test indicated that the difference in the proportion of seropositive results between the SNAP® 4Dx® ELISA and ELISA/WB combination was not statistically significant. While the McNemar's test assesses the difference in the proportion of positive results, Kappa assesses the agreement between both positive and negative results between the tests. Agreement testing indicated fair agreement according to a Kappa value of 0.23 (95% CI: -0.03 - 0.50). Variation in test results may be explained by differences in test methodology and the different immunogens used.

The SNAP® 4Dx® ELISA uses a synthetic C6 peptide resembling the invariable region (IR6) of the membrane protein VlsE of *B. burgdorferi*. Anti-VlsE antibodies are expressed during natural *B. burgdorferi* infection (28). *B. burgdorferi* VlsE is only expressed in the mammalian host, at 7 to 21 days post invasion by *B. burgdorferi,* and anti-VlsE antibodies may be detected as early as 3-4 weeks following infection (40,41). It is therefore possible that some samples testing negative by SNAP® 4Dx® ELISA but positive by ELISA/WB were from horses in the early stages of infection, at which time the SNAP® 4Dx® ELISA might yield a false negative result. The whole cell ELISA/WB combination may detect antibodies produced during early infection, such as those produced against the outer surface protein C (Osp C). The Osp C is only expressed when the spirochete is transferred to a mammalian host (34,43) and an antibody response may be detected as early as 2-3 weeks after infection (54). An early antibody response may be supported by the fact that all the samples that were seronegative by SNAP® 4Dx® ELISA but seropositive by WB were weakly positive (Table 2.1 and Table 2.5). However, it was previously suggested that spirochetes may not always express Osp C while in horses (45). Another study reported that antibodies to Osp C were detected in less than 25% of clinically diseased horses suggesting a short term duration of these antibodies in the circulation (54). Differences in antibody response between horses should be considered.

A second explanation for the occurrence of SNAP® 4Dx® ELISA–negative but ELISA/WB-positive samples is the detection of exposed rather than actively infected horses with the latter test (41). This is supported by the laboratory guidelines for interpretation of

weak positive WB results that may indicate resolving infection (Table 2.1). While recommendations for WB use and interpretation in horses are not standardized, it is likely that the type of antigens expressed by *B. burgdorferi* is similar in different mammals (34,43). Previously reported *B. burgdorferi* antigens that are used for WB testing of equine samples include Osp A, Osp B, Osp C, Osp F, VlsE, flagellin and others (45,54), which are similar to antigens that are used for testing of human samples according to Centers for Disease Control and Prevention (CDC) recommendations (51). Unfortunately, we are not certain about the specific antigens that were used to define WB results as positive or negative in the diagnostic laboratory used for this study. While WB may not differentiate between active infection, previous exposure or resolving infection according to the commonly used antigens, the point-of-care ELISA only detects anti-C6 antibodies compatible with active infection (28,46,52).

Another potential explanation for SNAP® 4Dx® ELISA-negative but ELISA/WB positive samples is vaccination. The Outer surface protein A (Osp A) of *B. burgdorferi* is expressed in culture and in the unfed tick gut and mediates attachment of the spirochete to the tick gut (43). During movement of the spirochete to the salivary gland of the tick during a blood meal, the spirochete down-regulates expression of Osp A and up-regulates expression of Osp C. Currently available canine vaccines against Lyme borreliosis contain Osp A and some contain Osp C of *B. burgdorferi* (48). Although there are no commercially available Lyme vaccines labeled for horses (44), extra-label vaccination of horses with the canine Lyme vaccine is reported anecdotally. In vaccinated animals that develop adequate levels of anti-Osp A or anti-Osp C antibodies, the organism is typically controlled effectively upon exposure and expression of VlsE may not occur. Hence, vaccinated animals, and vaccinated animals exposed to *B. burgdorferi* are less likely to react on the SNAP® 4Dx® ELISA (28) as it tests for antibodies against C6 which resembles VlsE. Conversely, reactivity to Osp A and Osp C may be detected by WB in these animals. While reactivity to Osp A in the WB is commonly regarded as indicating vaccination (48,86), OspC reactivity in the WB is mostly interpreted as a marker for early infection (54). Thus, it is possible that some of the WB-positive samples originated from Lyme vaccinated horses.

Finally, the occurrence of samples testing negative by SNAP® 4Dx® ELISA but positive by ELISA/WB may be explained by false positive results obtained with the latter test. While the whole cell *B. burgdorferi* ELISA/WB combination is considered to be highly sensitive, its specificity may be lower in non-endemic areas or when it is used as a screening test alone (45,51). This is due to possible non-specific reactivity with antibodies against spirochete

flagellin or other heat shock proteins (45). Cross-reactivity with antibodies produced against similar spirochetes may therefore occur and could result in false positive test results (45,51). According to CDC recommendations, the two-tier approach is not recommended for screening but rather as a diagnostic tool for clinical patients, due to the potential false positive results especially in non-endemic areas (51). Although the CDC recommendations concern testing in human patients, the two-tier testing approach used in horses in this study is similar with regard to methodology and antigens used.

Three of the six samples testing seropositive for LB by SNAP® 4Dx® ELISA were negative when re-tested with the ELISA/WB combination. All three samples showed equivocal reactivity on the *B. burgdorferi* WB, which is considered insufficient in specificity and quantity and requires a follow-up sample for confirming or ruling out active infection (Table 2.1). It is possible that these 3 samples were taken from horses in the early stages of infection when antibody levels were low but sufficient to lead to a positive result on the qualitative SNAP® 4Dx® ELISA. Re-testing of the horses at a later time would have provided valuable information; however, this was not possible in the context of this study. On the other hand, these results may represent false positive results by SNAP® 4Dx® ELISA, which have previously been reported (54) when the test was compared to both an ELISA/WB combination and a Lyme multiplex assay defined as the gold standard by the authors.

When using serologic testing in populations with a low seroprevalence, as we suspect is the case with *Ixodes*-borne diseases of horses in Canada, the positive predictive value of a test is expected to be low and the negative predictive value of a test is expected to be high (104). That means that while we can be more certain that negative results are true, we are less sure that positive results are true and this suggest that some false positive results may have been obtained in our study. Yet, the finding of seropositive horses for EGA and LB in this study and previously reported cases of EGA in Canada support the need to consider EGA and LB in veterinary practice in Canada.

Although seroprevalence results according to the SNAP® 4Dx® ELISA were consistent with the expectation, supporting our first hypothesis, a lack of agreement between the SNAP® 4Dx® ELISA and laboratory based serologic tests for EGA and LB was found. Therefore, the second hypothesis was rejected. While the SNAP® 4Dx® ELISA reportedly differentiates active infection from exposure, the IFA and the ELISA/WB combination may not differentiate active infection from exposure (44,45,51) and may seem to be more adequate

40

for use in a seroprevalence studies. However, as discussed previously, these highly sensitive laboratory-based serologic tests may be more prone to false positive results in non-endemic areas (45,51).

An important limitation in this study is the absence of validated gold standard serologic tests for EGA and LB in horses. Although these serologic tests are commonly used in horses no validation studies have been reported. The way samples were categorized as seropositive or seronegative for the purposes of agreement testing may represent another limitation of the study. For the WB, the equivocal and negative results were grouped together as seronegative and all positive levels of WB results were grouped together as seropositive. All positive IFA titers were grouped together as seropositive. A different cut-off for categorization as seropositive or seronegative could potentially have changed the assessment of agreement between the test results. It is further important to consider that assessment of samples as seropositive or seronegative in the absence of any clinical information does not allow drawing any conclusions with regard to the diagnostic usefulness of the evaluated tests. In a clinical setting, the interpretation of equivocal or weak positive test results benefits from additional clinical data and equivocal test results may be readily confirmed or ruled out through follow-up testing or testing of convalescent samples. These were unavailable in the setting of this study. While test results may differ between available serologic tests, one should consider relevant clinical data as well as specific guidelines for the interpretation of titer magnitude when making a clinical diagnosis of EGA or LB.

In summary, provincial and overall seroprevalence of EGA and LB according to the SNAP® 4Dx® ELISA was low. It is likely that a larger sample size from ON would have increased the accuracy of the seroprevalence estimate for that province. It was found that the assessment of a sample as a seropositive or seronegative for EGA and LB differed between the point-of-care ELISA and laboratory-based serologic tests. Further evaluation of the agreement between the SNAP® 4Dx® ELISA and available serological tests is thus warranted. Future studies should include an evaluation of the agreement between the SNAP® 4Dx® ELISA and all available serologic tests.

2.6 Tables

Anaplasma phagocytophilum IFA

<1:80	Negative
1:80-1:320	Weak positive
1:640-1:2,560	Moderately positive
1:5,120-1:20,480	Strongly positive

Borrelia burgdorferi ELISA

≤1:80	Negative
1:160-1:640	Weak positive
1:1,280-1:5,120	Moderate positive
1:10,240-1:40,960	Strong positive

Borrelia burgdorferi WB

Negative	No specific antibody reactivity
Equivocal	Some antibody reactivity, insufficient quantity and specificity (follow up sample in 3-4 weeks should show specific antibodies if infection is active)
Weak	Positive reactivity to a small number of specific antigens, usually early infection but also resolving infection (recommend testing of a convalescent sample)
Moderate	Shows multiple *Bb* specific antibody bands and some non-specific bands, quantity and intensity less than "strong", consider re-testing if treating
Strong	Shows multiple *Bb* specific (as well as non-specific) antibody bands, usually accompanied by high ELISA titer, indicates chronic Bb infection (many months duration), consider re-testing if treating

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Table 2.1. Reference guidelines for interpretation of test results provided by the Connecticut Veterinary Medical Diagnostic Laboratory (CVMDL). IFA = Indirect immunofluorescence antibody assay, ELISA = Enzyme linked immunosorbent assay, WB = Western blot.

Laboratory	Originated from SK	Originated from MB	Originated from ON
PDS	175 (October)	75 (October)	
	131 (November)	*57* (November)	
	71 (December)	*49* (December)	
MB Lab		*6* (October)	
		19 (November)	
		9 (December)	
AHL			*34* (October)
Total submitted	377	215	34
Total tested	**202**	**140**	**34**

Table 2.2. Available serum samples and those actually tested, by province of origin, month of submission and diagnostic laboratory to which they were submitted. PDS= Prairie Diagnostic Services, Saskatoon SK. MB Lab= Agriculture, Food and Rural Initiatives Veterinary Diagnostic Services, Winnipeg, MB. AHL= Animal Health Laboratory, Guelph, ON. Bolded italic numbers represent numbers of samples that were tested.

SNAP® 4Dx® ELISA	SK (n=202)	MB (n=140)	ON (n=34)	Overall (n=376)
EGA (positive)	1	1	0	2
Seroprevalence (95% CI)	0.49% (0.02-3.1)	0.7% (0.03-4.5)	0% (0-12.6)	0.53% (0.09-2.1)
LB (positive)	1	4	1	6
Seroprevalence (95% CI)	0.49% (0.02-3.1)	2.9% (0.92-7.6)	2.94% (0.15-17)	1.6% (0.65-3.6)

Table 2.3. Total number and proportion of samples seropositive for EGA and LB by SNAP® 4Dx® ELISA. EGA= Equine granulocytic anaplasmosis, LB= Lyme borreliosis, SK = Saskatchewan, MB = Manitoba, ON = Ontario.

Sample	SNAP	IFA
1	+	1:160
2	+	1:10,240
3-13	-	1:80
14-24	-	1:160
25-26	-	1:320
27	-	1:1280

Table 2.4 Samples seropositive for EGA by one or two serologic tests. Titers are indicated as negative (-) or positive (+), positive titers are specified where applicable. SNAP= SNAP® 4Dx® ELISA. IFA= Indirect immunofluorescence assay.

Sample	SNAP	ELISA / WB
1	+	1280 / +
2-3	+	2560 / +
4-6	-	160 / +
7-12	-	320 / +
13-14	-	640 / +

Table 2.5 Samples seropositive for LB by one or two serologic tests. Titers are indicated as negative (-) or positive (+), positive titers are specified where applicable. SNAP= SNAP® 4Dx® ELISA. ELISA= Enzyme linked immunosorbent assay, WB= Western Blot.

		IFA for EGA				ELISA & WB for LB		
		+	-	Total		+	-	Total
SNAP® 4Dx® ELISA	+	**2**	0	2	+	**3**	3	6
	-	25	**67**	92	-	11	**81**	92
	Total	27	67	94	Total	14	84	98

p<0.000001   p=0.057

Kappa = 0.23 (-0.03 - 0.50)

Table 2.6. Comparison between the SNAP® 4Dx® ELISA and IFA for EGA, and between the SNAP® 4Dx® ELISA and ELISA/WB combination for LB. For the comparison of the proportion of positive tests by McNemar's test, p<0.05 was considered significant. The Kappa statistic (shown with 95% CI of the estimate) indicated fair agreement for LB. IFA = Indirect immunofluorescence assay, ELISA = Enzyme linked immunosorbent assay, WB = Western blot.

2.7 Acknowledgements

Transition to chapter 3

In the previous study, equine serum samples were tested for antibodies against *A. phagocytophilum* and *B. burgdorferi* using a point-of-care ELISA and the results were compared to those of a laboratory-based *A. phagocytophilum* IFA and *B. burgdorferi* ELISA confirmed with WB, respectively. Lack of agreement was found when a point-of-care ELISA was compared with IFA for EGA. Agreement was fair (Kappa 0.23) when the point-of-care ELISA and ELISA confirmed with WB were compared for LB. It was found that testing with laboratory-based serologic tests yielded a significantly higher number of positive results than the SNAP® 4Dx® ELISA when testing for EGA but not for LB. In the next study, the goal was to compare results of testing equine serum samples by a point-of-care ELISA and additional serologic tests in more than one diagnostic laboratory to further understand the agreement between available serologic tests. The hypothesis was that the assessment of a sample as seropositive or seronegative for EGA or LB, respectively, is independent of the specific testing method used.

The author of the thesis took part in obtaining funds for the second study and study planning in collaboration with Drs. K. Lohmann, T. Epp. H. Burgess, and managed sample collection, handling and testing the new set of equine serum samples. Subsequent to in-house testing, sample management and shipment of samples to the referral laboratories was done by the author. Recording and analysis of results was done by the author. This chapter will be submitted for publication in the Journal of Veterinary Diagnostic Investigation, which will hold copyright.

3. COMPARISON BETWEEN AVAILABLE SEROLOGIC TESTS FOR DETECTING SEROPOSITIVITY FOR EQUINE GRANULOCYTIC ANAPLASMOSIS AND LYME BORRELIOSIS IN HORSES IN CANADA

3.1 Abstract

To investigate agreement between available serologic tests for detection of seropositive samples for equine granulocytic anaplasmosis (EGA) and Lyme borreliosis (LB), 50 equine serum samples were tested using a point-of-care ELISA (SNAP® 4Dx® Plus ELISA, IDEXX Laboratories, Inc., Westbrook, ME) and laboratory-based serologic tests. Laboratory-based tests included two indirect immunofluorescence antibody assays (IFA) for antibodies against *A. phagocytophilum* and an IFA, an ELISA confirmed with Western Blot (WB), and an equine Lyme multiplex assay for antibodies against *B. burgdorferi*. Samples were assessed as seropositive or seronegative according to the manufacturer's instructions for the SNAP® 4Dx® Plus kit and each diagnostic laboratory's guidelines. Results were compared between individual pairs of tests by comparing the proportion of positive results using the McNemar's test and by using Kappa statistic for assessment of agreement. For detection of antibodies against *A. phagocytophilum,* the proportion of seropositive samples differed significantly between all 3 pairs of tests indicating lack of agreement between test pairs of serologic test for EGA. For detection of antibodies against *B. burgdorferi,* the proportion of seropositive samples was significantly different for 2 of the 6 pairs of tests indicating lack of agreement between these pairs. For the other test comparisons, agreement ranged from poor to fair as was indicated by Kappa values. It was concluded that assessment of samples as seropositive or seronegative for EGA and LB, respectively, differs between available serologic tests, likely due to differences in test methodology. Further investigation of the adequacy of available serologic tests for assessing disease exposure is warranted.

3.2 Introduction

Equine granulocytic anaplasmosis (EGA) caused by *Anaplasma phagocytophilum*, and Lyme borreliosis (LB), caused by *Borrelia burgdorferi,* are important tick-borne diseases in horses. In North America, both organisms are transmitted by the same vector ticks *Ixodes scapularis* and *Ixodes pacificus* (13,16). Co-exposure to both organisms in horses was previously reported in the US (45).

Ixodes species are endemic in the Northeast, Midwest and on the west coast of the US. Evidence for the presence of established populations of *Ixodes* in Canada is increasing in recent years (39,75,85) and *Ixodes* populations have been reported from Ontario (ON), Quebec (QC), Nova Scotia (NS), New Brunswick (NB), Manitoba (MB) and British Columbia (BC). It is anticipated that the geographic range of *I. scapularis* in Canada will expand in the upcoming decades and will extend further west and north (41,64,75,77,82). Cases of EGA in Canada have been reported in horses without history of recent travel, residing in areas with and without established populations of the vector (4–7). Lyme borreliosis has been reported in horses in Canada (8) but travel history for these horses was not reported. Concern over *Ixodes-* borne diseases in Canada is increasing and information about the seroprevalence of the diseases in horses in Canada was reported earlier in chapter 2.

Clinical diagnosis of EGA or LB depends on the manifestation of typical clinical signs, geographic location and likelihood of exposure to the tick vector, and diagnostic testing. Detection of antibodies is important when studying the seroprevalence of these diseases and when defining the rate of exposure in a specific population or location.

The indirect immunofluorescence antibody assay (IFA) is a commonly used serologic test for detection of antibodies against *A. phagocytophilum* (27) and is considered the gold standard of serologic testing for rickettsial diseases (24,27). Serum antibodies bind to *A. phagocytophilum* present in infected cells, e.g. HL-60 cells (27,105), on a slide and are then detected by addition of a fluorescein-labeled species-specific anti-antibody that binds the serum antibodies. Although IFA remains the principal diagnostic tool for the diagnosis of antibodies against *A. phagocytophilum,* there are no standardized antigens or conjugates, and there is no agreement on what constitutes a positive test result among the different laboratories that offer serologic testing (27). IFA tests may detect different antibodies at different stages of infection or time from exposure. Levels of antibodies are likely to increase with increased duration of infection (27).

The enzyme-linked immunosorbent assay (ELISA) and IFA are considered sensitive tests for detecting antibodies against *B. burgdorferi* in horses, with the ELISA reported to detect relatively more seropositive horses (43). To increase specificity, a two-tier approach has been adapted in horses. This method is similar to the recommended serodiagnostic approach to Lyme disease in humans (45,51). Initial screening with a sensitive technique, i.e. a whole cell ELISA or IFA, is followed, if positive, with a more specific Western Blot (WB) to detect antibodies against separated *B. burgdorferi* antigens (43,51). The CDC recommends the use of specific antigens to detect *B. burgdorferi* antibodies, which includes the use of at least 2 out of 3 antigens for detection of IgM and the use of at least 5 out of 10 antigens for detection of IgG (27,32,51). Due to the slow multiplication of the spirochete in the host, a detectable antibody response may take 3-8 weeks to develop, and the sensitivity of the ELISA/WB combined test may be low in the first few weeks of infection (48).

The Equine Lyme multiplex assay (Animal Health Diagnostic Center, Cornell University, Ithaca, NY) uses antigen-labeled beads and detects antibodies against three *B. burgdorferi* antigens, namely the Outer surface proteins (Osp) A, C and F, in horse serum (48). *B. burgdorferi* Osp A is expressed while the spirochete is in the tick, Osp C is expressed during transmission to mammals and Osp F is expressed later during infection in the mammalian host (53). The use of these antigens allows detection of antibodies as early as 3 weeks post infection (53), which compares favorably to the IFA or ELISA with WB confirmation, where the earliest time of antibody detection averages 5 to 6 weeks after experimental infection in ponies (56). The Lyme multiplex assay also allows an assessment of the stage of infection, vaccination status and treatment success in horses if testing is repeated over time (48,53,55,106). In addition, the multiplex assay showed good agreement with C6 ELISA tests, including the SNAP® 4Dx® ELISA (IDEXX Laboratories, Inc., Westbrook, ME) in dogs and horses, using samples from both infected and non-infected animals (48,53).

The point-of-care SNAP® 4Dx® ELISA uses a peptide derived from the immunodominant P44 protein of *A. phagocytophilum* for detection of antibodies against *A. phagocytophilum*. For detection of antibodies against *B. burgdorferi*, synthetic C6 peptide resembling the invariable region (IR6) of the *B. burgdorferi* membrane protein VlsE is used. Antibodies against VlsE are only produced during natural *B. burgdorferi* infection and testing can therefore distinguish natural infection from the antibody response to vaccination. The latter results in production of anti-Osp A but not anti-C6 antibodies (52). According to the manufacturer (28), the SNAP® 4Dx® ELISA is not species-specific although it is currently

52

only licensed for use in dogs. In a study evaluating the test's performance using 164 equine serum samples from endemic and non-endemic areas of the US (28), both sensitivity and specificity of the SNAP® 4Dx® ELISA relative to a commercially available *A. phagocytophilum* IFA were 100%. The relative sensitivity and specificity of the SNAP® 4Dx® ELISA compared to a *B. burgdorferi* Western Blot were 100% and 95%, respectively (26). In horses experimentally infected with *B. burgdorferi,* a sensitivity of 63% and specificity of 100% for the SNAP® 4Dx® ELISA were further reported (29). Recently, the SNAP® 4Dx® ELISA has been replaced by an extended version of the same test, namely the SNAP® 4Dx® Plus ELISA, which adds testing for antibodies against *Ehrlichia ewingii* and *Anaplasma platys* (107). According to the manufacturer (Ramaswamy Chandrashekar, personal communication), the test methodology for detection of antibodies against *A. phagocytophilum* and *B. burgdorferi* has not changed.

The positive and the negative predictive value of serologic tests depend on the prevalence of the diseases which is important to consider when using serologic tests in areas with a low seroprevalence of the disease, as is the case for EGA and LB in horses in Canada (104). That means that while we can be more certain that negative results are true, we may be less sure that positive results are true.

In 2011, a small seroprevalence study using equine serum samples from SK, MB and ON was conducted by the authors. Samples were tested for the presence of antibodies against *A. phagocytophilum* and *B. burgdorferi.* As part of that study, results obtained by testing with the SNAP® 4Dx® ELISA were compared to results obtained when testing the same samples at one commercial veterinary diagnostic laboratory. We found lack of agreement between the SNAP® 4Dx® ELISA, the *A. phagocytophilum* IFA and the *B. burgdorferi* ELISA confirmed with WB, respectively. It was therefore suspected that results of serologic tests for EGA and LB may differ depending on test methodology, especially when applying these tests in non-endemic populations with a low expected seroprevalence.

As concerns over an increased incidence of tick-borne diseases in Canada may result in more frequent testing, and clinicians wishing to identify seropositive (or seronegative) horses may conceivably submit samples for testing with any of the available serologic tests, it would be helpful for the equine veterinarian to understand whether the available tests are comparable. The aim of the study reported here was therefore to further investigate the agreement between available serologic tests when classifying serum samples as seropositive

or seronegative for EGA and LB. The hypothesis for this study was that the assessment of a sample as seropositive or seronegative for EGA or LB, respectively, is independent of the specific testing method used. The agreement was evaluated between a point-of-care ELISA and two laboratory-based IFAs for EGA, and between a point-of-care ELISA, IFA, Lyme multiplex assay and ELISA/WB combination for LB. Agreement between tests was evaluated by use of an inter-rater agreement statistic and a comparison of the proportion of positive tests, using convenience serum samples.

3.3 Materials and methods

3.3.1. Study design

Fifty anonymous equine serum samples that were originally submitted for equine infectious anemia testing in April 2013, were obtained from Prairie Diagnostic Services Inc., Saskatoon, Saskatchewan. Only samples that contained at least 3.5 ml of serum following separation from cells were collected. The samples were divided into 3 aliquots of 1ml each and 1 aliquot of 0.5 ml and frozen at -20 ℃ until analysis. One aliquot (0.5 ml) of each serum sample was tested in the principal investigator's laboratory for presence of antibodies against *A. phagocytophilum* and *B. burgdorferi* using the point-of-care SNAP® 4Dx® Plus ELISA (IDEXX Laboratories, Inc., Westbrook, ME). The remaining 3 aliquots of each sample (1 ml each) were tested at 3 commercial veterinary diagnostic laboratories for antibodies against *A. phagocytophilum* and *B. burgdorferi* as described below. Samples were sent to the diagnostic laboratories frozen and on ice packs, using overnight courier shipment.

3.3.2. SNAP® 4Dx® Plus ELISA testing

Serum samples were thawed at room temperature and centrifuged (Eppendorf 5702 centrifuge, Eppendorf, Mississauga, ON) at 4400 rpm for 10 minutes. Samples were tested according to manufacturer's instructions. Briefly, 3 drops of serum and 4 drops of conjugate were mixed thoroughly in a sample tube. The entire content of the sample tube was added to the sample well. The sample was allowed to flow across the results window for 30-60 seconds. When the first colour change appeared in the activation circle, the activator was pushed firmly until it was flush with the device body. The test result was read at 8 minutes. Any change in colour in the sample spots in the activation window was interpreted as a positive result, i.e. the presence of antibodies. Samples were re-frozen immediately following testing.

3.3.3. Laboratory-based serologic testing

Samples were tested by *A. phagocytophilum* IFA at two veterinary diagnostic laboratories: the Michigan State University Diagnostic Center for Population & Animal Health, Lansing, MI (DCPAH, from here on referred to as IFALAB1) and the Connecticut Veterinary Medical Diagnostic Laboratory, Storrs, CT (CVMDL, from here on referred to as IFALAB2). The laboratories used commercial IFA kits manufactured by Fuller (IFALAB1) and VMRD (IFALAB2), respectively. Test results were interpreted as seropositive or seronegative according to each laboratory's guidelines (Table 3.1). Titers ≥1:160 were considered positive for IFALAB1 and titers ≥ 1: 80 were considered positive for IFALAB2.

Samples were tested with the equine Lyme multiplex assay at the Animal Health Diagnostic Center, Ithaca, NY (AHDL), *B. burgdorferi* IFA at the DCPAH and *B. burgdorferi* ELISA confirmed with WB at the CVMDL. Test results were interpreted as seropositive or seronegative according to each laboratory's guidelines (Table 3.2). For the equine Lyme multiplex assay, samples with a positive anti- Osp C titer (>1000) or positive anti-Osp F titer (>1250), were considered seropositive. For the *B. burgdorferi* IFA, samples with a titer ≥ 1: 160 were considered seropositive. For the *B. burgdorferi* ELISA confirmed with WB, only samples that tested positive by WB were considered seropositive. Negative and equivocal WB results were considered negative, regardless of the ELISA titer. Samples that had an ELISA titer <1:160 were considered seronegative and were not re-tested by WB.

3.3.4. Statistical analysis

The number of seropositive and seronegative samples was recorded for each test used. The proportion of positive test results was compared between pairs of tests using a two-tailed McNemar's test (VassarStats software, Website for Statistical Computation; http://www.vassarstats.net/index.html) and p<0.05 was considered significant. When the McNemar's test indicated no significant difference between the proportions of positive result, an inter-rater agreement was calculated for each pair-wise comparison of test results, using the Kappa statistic (©2013 GraphPad Software, Inc., San Diego, CA). The magnitude of Kappa was interpreted as follows: Kappa ≤0 poor, 0.01-0.2 slight, 0.21-0.4 fair, 0.41-0.6 moderate, 0.61-0.8 substantial, 0.81-1 almost perfect agreement (101).

3.4 Results

3.4.1 Results of serologic testing for EGA

All 50 samples were seronegative for EGA by SNAP® 4Dx® Plus ELISA. When tested at IFALAB1, 6 samples were seropositive (1:320) and 44 samples were seronegative. When tested at IFALAB2, there were 15 seropositive results (1:80-1:320) and 35 seronegative results. Only 4 samples were seropositive in both laboratories. Interestingly, serum titers for these 4 samples differed between the two laboratories. At IFALAB1, all positive titers were 1:320, while at IFALAB2, 3 titers were 1:80 and one was 1:320 (Table 3.3).

3.4.2. Agreement between serologic tests for EGA

A significant difference in the proportion of positive tests was found in each pair-wise comparison of tests (Table 3.4) indicating lack of agreement between the tests.

3.4.3. Results of serologic testing for LB

Thirty-one samples were seronegative on all tests. Individual test results for those samples testing positive on one or more tests (n=19) are presented in Table 3.5. Fourteen samples were seropositive based on only one test, 4 were seropositive based on 2 tests and only one sample was seropositive based on 3 tests. None of the samples was seropositive based on all 4 tests.

3.4.4. Agreement between serologic tests for LB

The two-tailed McNemar's test revealed that the proportion of positive tests differed significantly when comparing the SNAP® 4Dx® Plus ELISA to the IFA or to the Lyme multiplex assay, indicating lack of agreement. For the other pair-wise test comparisons, Kappa values indicated agreement that ranged from poor to fair (Table 3.6).

When considering a positive result in any of the tests as evidence of seropositivity, 7 samples were seropositive for both EGA and LB.

3.5 Discussion

The investigation of the agreement between serologic tests for antibodies against *A. phagocytophilum* and *B. burgdorferi* in horse serum included comparisons between the point-of-care SNAP® 4Dx® Plus ELISA and serologic tests available at 3 veterinary diagnostic laboratories. Tests were compared using two methods: Kappa to investigate inter-rater agreement and the McNemar's test to compare the proportion of positive test results.

For the serologic tests for EGA, the McNemar's test indicated a significant difference between the proportions of positive results for each pair-wise comparison, suggesting lack of agreement between test results. This likely resulted from differences in test methodology. While the SNAP® 4Dx® Plus ELISA detects antibodies against a single antigen, namely P44 (Msp2), both IFAs use whole cell *A. phagocytophilum* in infected cells. The whole *A. phagocytophilum* organisms may cross-react with antibodies against other, similar rickettsia (45), which may result in false positive results. All positive titers at IFALAB1 were 1:320. Interestingly, the instructions for the Fuller *A. phagocytophilum* IFA kit (used in Lab 1) specify that titers of 1:160-1:320 suggest either early infection, past exposure or may reflect cross-reactivity. Cross-reactivity may occur with antibodies that were produced against similar organism such as other *Anaplasma* species (45). As it is expected that each laboratory will validate a commercial test, standardize it and develop its own guidelines for test interpretation, guidelines provided by the diagnostic laboratory rather than those provided by the manufacturer of the test kit were used for test interpretation in this study.

Another potential explanation for the difference in the proportion of positive results is that the SNAP® 4Dx® ELISA, while being highly specific, may yield false negative results as it only detects antibodies against one major surface protein of *A. phagocytophilum*. It is also possible that exposed or previously diseased horses maintain positive antibody titers that are detectable by IFA but not by SNAP® 4Dx® ELISA. Reportedly, antibodies against *A. phagocytophilum* in horses can be detected by IFA for up to 2 years (23).

The lack of agreement between the 2 commercial IFA tests was somewhat surprising as it was expected that the tests would perform similarly. While the principal methodology is similar, IFALAB1 and IFALAB2 represented different commercially available IFA kits. Possible explanations are the use of different strains and conjugates and use of different calibration and reading systems in the individual labs, although, presumably, each lab sets up and validates its test based on known positive and negative samples. It is worth noting that the

IFALAB1 only defined samples as negative, inconclusive or positive whereas IFALAB2 used different categories for positive titers. Both labs further based their interpretation as seropositive or seronegative on different cut-off titers, which may also differentiate between the tests.

As test methodology may impact on the occurrence of false positive and false negative results both have to be considered given the results obtained. The positive predictive value of diagnostic tests in low-prevalence populations, such as the one tested here is expected to be low and, thus, it is more probable that the IFA resulted in a considerable number of false positive results.

The cut-off IFA titer values that were used to categorize samples as seropositive or seronegative for the purposes of agreement testing may represent a limitation of the study. A different cut-off may have changed the categorization of samples and could potentially have resulted in better agreement between the test results. However, when a cut-off titer of $\geq 1:160$ was used for both IFA tests, and also when a higher cut-off for defining seropositive samples (such as >1:320) was used, the agreement between tests was still poor at best, which supports the observed lack of agreement (data not shown).

For the serologic tests for LB, the McNemar's test indicated that the proportion of positive results was significantly different for only two of six pairs of tests, namely the SNAP® 4Dx® Plus ELISA and IFA, and the SNAP® 4Dx® Plus ELISA and Lyme multiplex assay. For the other pairs, the difference in the proportion of positive results was not statistically significant, while Kappa indicated lack of agreement. While the McNemar's test assesses the difference between the proportion of positive results between the tests, Kappa assesses agreement between both positive and negative results. Although a low prevalence of seropositive results was expected in the population tested, adjustment of Kappa values was not deemed necessary or appropriate (101,108).

When comparing the SNAP® 4Dx® Plus ELISA to a whole cell *B. burgdorferi* IFA, the significant difference in the proportion of positive results was likely due to differences in test methodology. The SNAP® 4Dx® Plus ELISA uses synthetic C6 peptide resembling the invariable region (IR6) of the membrane protein VlsE of *B. burgdorferi,* whereas the whole cell IFA detects a variety of antibodies that are directed against different immunogens of *B. burgdorferi* and thus may yield a higher number of positive results. While the VlsE antigen is considered to be expressed only during active infection, the whole cell *B. burgdorferi* IFA

may also detect antibodies due to exposure alone (44). While the IFA is highly sensitive (43,45,51), false positive results may occur due to non-specific reactivity with antibodies against flagellin or heat shock proteins of similar organisms (45), such as other *Borrelia* species or other similar spirochetes. While 2 other *Borrelia* species, namely *B. miyamotoi* and *B. kurtenbachii* have been previously reported to infect *I. scapularis* ticks in Canada (41), their clinical significance is not clear. Further investigation of *Borrelia* variants is therefore warranted and may enable better interpretation of serologic tests.

Anti-VlsE antibodies may not be detected until 3-4 weeks following infection (40,41) and it is therefore possible that some samples testing negative by SNAP® 4Dx® ELISA were from horses in the early stages of infection, at which time the SNAP® 4Dx® ELISA would yield a false negative result. These samples may have been detected by IFA as the latter is expected to detect early antibodies such as those produced against the outer surface protein C (Osp C)(43,45). Osp C is only expressed when the spirochete is transferred to a mammalian host (34,43) and an antibody response may be detected as early as 2-3 weeks after infection (54). However, it was previously suggested the spirochete may not always express Osp C while in horses (45). Another study reported that antibodies to Osp C were detected in less than 25% of clinically diseased horses suggesting a short term duration of these antibodies in the circulation (54). Differences in the antibody response between horses should therefore be considered.

When comparing the SNAP® 4Dx® Plus ELISA and the Lyme multiplex assay, the McNemar's test indicated a significant difference in the proportion of positive results. Again, differences in test methodology were most likely the cause for the variation in test results. While the SNAP® 4Dx® Plus ELISA detects anti-C6 antibodies indicating active infection, the Lyme multiplex assay detects antibodies against 3 surface antigens of *B. burgdorferi,* namely the Osps A, C and F, which are expressed in different stages of infection (48). Detection of these different outer surface proteins reportedly allows the differentiation of early infection, chronic infection and an immune response to vaccination (48). Interestingly, the only sample that was positive by SNAP® 4Dx® Plus ELISA yielded an equivocal result in the Lyme multiplex assay (Osp F). According to the laboratory guidelines, a single equivocal Osp F value suggests a non-specific reaction. This result was surprising given that a previous study in horses described a strong correlation between the detection of antibodies against C6 in the SNAP® 4Dx® ELISA and detection of antibodies against Osp F in the Lyme multiplex assay (54). One possible explanation is a false positive result in the SNAP®

4Dx® Plus ELISA; however, as this sample was the only sample that was positive in 3 of 4 tests, the possibility of a false negative result in the Lyme multiplex assay may seem more likely.

The four samples that had a positive Osp C titer in the Lyme multiplex assay and were negative in all other tests (Table 3.4) may indicate a very early antibody response (54) which was not detected by the other tests. It has also been suggested that the antibody response to Osp F starts earlier than the response to C6 (54) in horses, which may explain why all samples testing positive for anti-Osp F antibodies were negative by SNAP® 4Dx® Plus ELISA.

The best agreement, which was still only fair (Kappa 0.31), was obtained when the SNAP® 4Dx® Plus ELISA was compared with the ELISA/WB combination. In contrast to this finding, a relative sensitivity of 100% and relative specificity of 95% for the SNAP® 4Dx® ELISA compared to a *B. burgdorferi* WB in horses were previously reported (28). The previous study used samples that were initially submitted for Lyme testing and originated from potentially infected horses in endemic areas. Thus, the positive predictive value may have been higher than in the study reported here, where samples were collected anonymously and information about the horses was not available. The positive predictive value of serologic tests for Lyme borreliosis in non-endemic areas such as Canada is likely low.

The variation in test results observed here may be explained by differences in test methodology and the different immunogens used. The SNAP® 4Dx® ELISA uses a synthetic C6 peptide to detect anti-VlsE antibodies as early as 3-4 weeks following infection (40,41). It is therefore possible that some samples testing negative by SNAP® 4Dx® ELISA but positive by ELISA/WB were from horses in the early stages of infection, at which time the SNAP® 4Dx® ELISA might yield a false negative result. The whole cell ELISA/WB combination may detect antibodies produced during early infection, such as those produced against the outer surface protein C (Osp C) which may be detected as early as 2-3 weeks after infection (54). An early antibody response may be supported by the fact that all the samples that were seronegative by SNAP® 4Dx® ELISA but seropositive by WB were weakly positive (Table 3.1).

A second explanation for the occurrence of SNAP® 4Dx® ELISA–negative but ELISA/WB-positive samples is the detection of exposed rather than actively infected horses with the latter test (41). This is supported by the laboratory guidelines for interpretation of weak positive WB results that may indicate resolving infection. While recommendations for

WB use and interpretation in horses are not standardized, it is likely that the type of antigens expressed by *B. burgdorferi* is similar in different mammals (34,43). Previously reported *B. burgdorferi* antigens that are used for WB testing of equine samples include Osp A, Osp B, Osp C, Osp F, VlsE, flagellin and others (45,54), which are similar to antigens that are used for testing of human samples according to Centers for Disease Control and Prevention (CDC) recommendations (51). Unfortunately, we are not certain about the specific antigens that were used to define WB results as positive or negative in the diagnostic laboratory used for this study. While WB may not differentiate between active infection, previous exposure or resolving infection according to the commonly used antigens, the point-of-care ELISA only detects anti-C6 antibodies compatible with active infection (28,46,52).

Another potential explanation for SNAP® 4Dx® ELISA-negative but ELISA/WB positive samples is vaccination. The Outer surface protein A (Osp A) of *B. burgdorferi* is expressed in culture and in the unfed tick gut and mediates attachment of the spirochete to the tick gut (43). Upon movement of the spirochete to the salivary gland of the tick during a blood meal, the spirochete down-regulates expression of Osp A and up-regulates expression of Osp C. Currently available canine vaccines against Lyme borreliosis contain Osp A and some contain Osp C of *B. burgdorferi* (48). Although there are no commercially available Lyme vaccines labeled for horses (44), extra-label vaccination of horses with the canine Lyme vaccine is reported anecdotally. In vaccinated animals that develop adequate levels of anti-Osp A or anti-Osp C antibodies, the organism is typically controlled effectively upon exposure and expression of VlsE may not occur. Hence, vaccinated animals are less likely to react on the SNAP® 4Dx® ELISA (28) as it tests for antibodies against C6 which resembles VlsE. Conversely, reactivity to Osp A and Osp C may be detected by WB in these animals. While reactivity to Osp A in the WB is commonly regarded as indicating vaccination (48,86), OspC reactivity in the WB is mostly interpreted as a marker for early infection (54). Thus, it is possible that some of the WB-positive samples originated from Lyme vaccinated horses.

Finally, the occurrence of samples testing negative by SNAP® 4Dx® ELISA but positive by ELISA/WB may be explained by false positive results obtained with the latter test. While the whole cell *B. burgdorferi* ELISA/WB combination is considered to be highly sensitive, its specificity may be lower in non-endemic areas or when it is used as a screening test alone (45,51). This is due to possible non-specific reactivity with antibodies against spirochete flagellin or heat shock proteins (45). Cross-reactivity with antibodies produced against similar spirochetes may therefore occur and could result in false positive test results (45,51).

According to CDC recommendations, the two-tier approach is not recommended for screening but rather as a diagnostic tool for clinical patients, due to the potential for false positive results, especially in non-endemic areas (51). Although the CDC recommendations concern testing in human patients, the two-tier testing approach used in horses in this study is similar with regard to methodology and antigens used.

It is worth mentioning that the majority of samples testing seropositive in the ELISA/WB combination, including the one that was also positive by the SNAP® 4Dx® Plus ELISA, were weakly positive by WB. Weakly positive WB results suggest early or resolving infection with recommendation for follow-up sampling in 3-4 weeks. Accepting only moderately positive WB as positive would have actually resulted in better agreement with the SNAP® 4Dx® Plus ELISA (data not shown).

Slight agreement (Kappa 0.02), was found when the IFA was compared to the Lyme multiplex assay. As discussed earlier, the Lyme multiplex assay detects antibodies against *B. burgdorferi*-specific Osp antigens (Osp A, C, F) that are expressed at different times after infection or after vaccination (48,54). In contrast, the whole cell IFA may also detect antibodies targeting less specific *B. burgdorferi* antigens (45,109) such as flagellin and heat shock proteins. Thus, it is not clear why 8 of the 10 samples that were positive by Lyme multiplex assay were negative by IFA. One possible and partial explanation is that in 4 of these samples, positive results in the Lyme multiplex assay were based on relatively low anti-Osp C titers. Based on previous reports, anti-Osp C antibodies in horses are only detected for a short period of time after infection (45,54), and it is therefore possible that very early or diminishing titers of anti-Osp C antibodies were not detected by the IFA.

When comparing the ELISA/WB combination with the IFA, agreement was considered slight as well (Kappa 0.18). The two-tier approach for serologic testing for LB, as recommended by the CDC (51), uses the more specific WB to confirm positive or equivocal ELISA or IFA results and was designed to improve specificity of diagnostic testing in human patients. The CDC recommends the use of specific bands to detect *B. burgdorferi* antibodies, which includes the use of at least 2 out of 3 specific bands for detection of IgM presence and the use of at least 5 out of 10 specific bands for detection of IgG (27,32,51). As the WB is considered to be more specific than the IFA and the ELISA (43,45,51), the lower number of positive results and higher number of negative results compared with the IFA (Table 3.5) was

expected. As discussed earlier, false positive results in the IFA test may result from cross-reactivity with antibodies against similar spirochetes.

When comparing the Lyme multiplex assay to the ELISA confirmed with WB, the agreement was considered to be poor or the same as agreement by chance alone (Kappa 0). As discussed earlier, WB testing in horses typically includes assessment of reactivity to Osp antigens, some of which are also used in the Lyme multiplex assay. However, the WB is able to detect additional antibodies against other *B. burgdorferi* antigens while the Lyme multiplex assay only detects antibodies against 3 specific *B. burgdorferi* Osp antigens that are expressed at certain times after infection. Thus, it was surprising that a higher number of seropositive samples were obtained by the Lyme multiplex assay. Interestingly, 4 of the 5 samples testing positive by ELISA/WB showed a weak response in the WB, which may indicate early or resolving infection. Only one sample that was weakly positive by WB was Osp F positive in the Lyme multiplex assay. A resolving infection was likely the case in this sample. Another 2 samples that were weakly positive by WB had equivocal titers for Osp F, suggesting non-specific reactivity. According to guidelines from both laboratories, follow-up sampling would have been recommended to conclude whether the infection was active or not. The only sample that was moderately positive by WB was negative for antibodies to any of the Osp antigens. When taking into account all 13 samples that were equivocal by WB (counted as negative), only 2 of them were positive according to Osp C and Osp F reactivity on the Lyme multiplex assay. Thus, even the use of a less conservative cut-off for the categorization of seropositive samples in our agreement analysis would likely not have changed our results (data not shown). In contrast to our study, better agreement between the Lyme multiplex assay and WB when testing horse serum from presumed infected horses has been reported (54). While, presumably, some of the antigens used are overlapping between the tests, one may assume that differences in test methodology, test validation and standardization exist between the tests, which may account for the differences in test results observed in the study reported here.

As was discussed in the context of EGA testing earlier, assessing samples as seropositive or seronegative for LB based on serologic testing at one point in time is limited. While it is reasonable to assume that the categorization of samples as seropositive or seronegative may have affected the agreement analysis, all equivocal test results were categorized according to what was considered most likely to reflect the presence or absence of specific antibodies. Considering low prevalence and laboratory recommendations for interpretation of equivocal results all equivocal results were categorized as negative.

Another limitation of the study is the absence of validated gold standard serologic test for EGA and LB in horses. While these tests are commonly used, validation studies in horses are lacking. It is also important to consider that assessment of samples as seropositive or seronegative was done in the absence of any clinical information and that, therefore, it is not possible to draw any conclusions with regard to the diagnostic usefulness of the evaluated tests. For example, in a clinical setting, interpretation of weak positive results benefits from additional clinical data and infection may be readily confirmed or ruled out with follow-up testing or testing of convalescent samples. These were unavailable in the setting of this study. Previously reported sensitivity and specificity of available serologic tests (25,28,45,46,54) was high when known positive and known negative samples were tested. When using serologic tests in the context of making a clinical diagnosis of EGA or LB, one should consider relevant clinical data as well as specific guidelines for interpretation of titer magnitude.

In summary, agreement between serologic tests varied from poor to fair, thus our hypothesis was rejected. The observed differences may be attributable to the different test methodologies and, specifically, the different antigens that were used. Differences in the categorization of test results as positive or negative may also play a role, such as in the case of IFA tests for EGA. Overall, the laboratory-based serologic tests assessed a higher number of samples as seropositive for EGA and LB compared to the point-of-care ELISA. With regard to LB, one likely explanation for this observation is that the point-of-care ELISA is limited to detecting active infection, while laboratory-based serologic tests may have limited ability to differentiate active infection from exposure. Another potential explanation for the difference between serologic tests for both EGA and LB is a difference in susceptibility to cross-reactivity, which is suggested to happen particularly in whole cell tests. As low seroprevalence in the population of horses from which samples were derived was assumed, it is necessary to consider that these findings are to some extent due to false positive results. Given the observed differences in test results and the difficulties in test interpretation in the absence of clinical signs of disease, general screening of the horse population for tick-borne diseases in non-endemic areas may not be warranted until further information about the adequacy of available serologic tests for assessing exposure versus active infection is available.

3.6. Tables

Testing for antibodies against *A. phagocytophilum*

IFALAB1

< 1:80	Negative
1:80	Inconclusive (recommend re-testing of a convalescent sample in 3-4 weeks)
≥1:160	Positive

IFALAB2

<1:80	Negative
1:80-1:320	Weak positive
1:640-1:2560	Moderate positive
1:5,120-1:20,480	Strong positive

Table 3.1. Reference guidelines for interpretation of test results provided by the referral diagnostic laboratories. IFA = Indirect immunofluorescence assay.

Testing for antibodies against *B. burgdorferi*

***B. burgdorferi* ELISA**

≤1:80	Negative
1:160-1:640	Weak positive
1:1280-1:5120	Moderate positive
1:10,240-1:40,960	Strong positive

***B. burgdorferi* WB**

Negative:	No specific antibody reactivity.
Equivocal:	Some antibody reactivity, insufficient quantity and specificity (follow up sample in 3-4 weeks should show specific antibodies if infection is active).
Weak:	Positive reactivity to a small number of specific antigens, usually early infection but also resolving infection (recommend testing of a convalescent sample).
Moderate:	Shows multiple *Bb* specific antibody bands and some non-specific bands, quantity and intensity less than the "strong", consider re-testing if treating.
Strong:	Shows multiple *B. burgdorferi* specific (as well as non-specific) antibody bands, usually accompanied by high ELISA titer, indicates chronic *B. burgdorferi* infection (many months duration).

***B. burgdorferi* IFA**

<1:160	Negative
1:160-1:640	Weak-moderate /equivocal
>1:1280	Fairly high (recent infection)

Lyme Multiplex Assay

Negative:	Negative values for the antibodies to all 3 Osp antigens are predictive that the horse is not infected. If only one or two values are in the negative range, see interpretation for equivocal or positive values for the corresponding Osp antigen.
Equivocal:	Equivocal values can indicate very early infection or nonspecific serum reaction. If no positive Osp results the horses need to be retested in 2-3 weeks. If 1 or 2 values are in the positive range see interpretation for positive Osp.
OspA (>2000-28,000)	Positive values typically observed in vaccinated animals, may rise during infection. If antibodies to OspC and or OspF are positive along OspA horses should be considered as infected with *B. burgdorferi*.
OspC (>1000-10,000)	Positive values to OspC only indicate early infection. Antibodies to OspA can be also elevated during early infection.
OspF (>1250-26,000)	Positive value to OspF only are predictive for chronic infection stages. Positive values for OspC and OspF in the same sample are indicators for an infection that occurred several weeks ago and is moving towards chronic infection stage.

Table 3.2. Reference guidelines for interpretation of test results provided by the referral diagnostic laboratories. IFA = Indirect immunofluorescence assay, ELISA = Enzyme linked immunosorbent assay, WB = Western blot, Osp = Outer surface protein.

Sample	SNAP	IFALAB1	IFALAB2
1-8	-	-	1:80
9	-	-	1:160
10-11	-	-	1:320
12-13	-	1:320	-
14-16	-	1:320	1:80
17	-	1:320	1:320

Table 3.3. Samples seropositive for EGA by one or two serologic tests. Titers are indicated as negative (-) or positive (+), positive titers are specified where applicable. SNAP= SNAP® 4Dx® Plus ELISA. IFALAB1= Indirect immunofluorescence assay in laboratory 1 (DCPAH). IFALAB2 = Indirect immunofluorescence assay in laboratory 2 (CVMDL).

	IFALAB1		
	+	-	Total
SNAP +	**0**	0	0
-	6	**44**	50
Total	6	44	50

p=0.031

	IFALAB2		
	+	-	Total
SNAP +	**0**	0	0
-	15	**35**	50
Total	15	35	50

p=0.000061

	IFALAB2		
	+	-	Total
IFALAB1 +	**4**	2	6
-	11	**33**	44
Total	15	35	50

p=0.022

Table 3.4. Comparison between pairs of serologic tests for EGA. SNAP= SNAP® 4Dx® Plus ELISA. IFALAB1= *A. phagocytophilum* IFA in laboratory 1. IFALAB2= *A. phagocytophilum* IFA in laboratory 2. Differences between the proportions of positive results were significant for all pairs (p<0.05 on the McNemar's test) indicating lack of agreement between test results.

| Sample | SNAP | IFA | Lyme Multiplex Assay | | | ELISA/WB |
			OspA	OspC	OspF	
1	+	640	-	-	-	1280 / +
2	-	160	-	10,573	-	- / NA
3	-	640	-	1604	-	- /NA
4	-	160	-	-	-	2560 / +
5	-	-	-	-	4134	320/ +
6	-	2560	-	-	-	640 / -
7	-	320	-	-	-	320 / -
8	-	320	-	-	-	320 / -
9	-	640	-	-	-	320 / -
10	-	640	-	-	-	640 / -
11	-	-	2017	1144	-	160 / -
12	-	-	-	1091	-	- / NA
13	-	-	-	1030	-	- / NA
14	-	-	-	1079	-	- / NA
15	-	-	-	-	12,996	160 / -
16	-	-	-	-	4312	-/ NA
17	-	-	-	-	7357	-/ NA
18	-	-	-	-	-	640/ +
19	-	-	-	-	-	320/ +

Table 3.5. Samples seropositive for LB by one or more serologic tests. Titers are indicated as negative (-) or positive (+), positive titers are specified where applicable. NA= Not applicable. SNAP= SNAP® 4Dx® Plus ELISA. IFA= Indirect immunofluorescence assay. OspA,C,F= Outer surface protein A, C, F. ELISA= Enzyme linked immunosorbent assay, WB= Western Blot. ELISA/WB = Only positive ELISA results that were confirmed by WB were considered as positive. Samples with negative ELISA results were not re-tested with WB.

		IFA		
		+	-	Total
SNAP	+	**1**	0	1
	-	8	**41**	49
	Total	9	41	50

p=0.008

		Multiplex Assay		
		+	-	Total
SNAP	+	**0**	1	1
	-	10	**39**	49
	Total	10	40	50

p=0.012

		ELISA/WB		
		+	-	Total
SNAP	+	**1**	0	1
	-	4	**45**	49
	Total	5	45	50

p=0.125
Kappa   0.31 (95% CI: 0.16 - 0.78)

		Multiplex Assay		
		+	-	Total
IFA	+	**2**	7	9
	-	8	**33**	41
	Total	10	40	50

p=1
Kappa   0.02 (95% CI: -0.26 - 0.31)

		ELISA/WB		
		+	-	Total
IFA	+	**2**	7	9
	-	3	**38**	41
	Total	5	45	50

p=0.344
Kappa   0.18 (95% CI: 0.15 - 0.51)

		ELISA/WB		
		+	-	Total
Multiplex Assay	+	**1**	9	10
	-	4	**36**	40
	Total	5	45	50

p=0.267
Kappa   0 (95% CI: 0.26 - 0.26)

Table 3.6. Comparison between pairs of serologic tests LB. For the comparison of the proportion of positive tests by McNemar's test, p<0.05 was considered significant. Kappa values indicated agreement that ranged from poor (0) to slight (0.02 and 0.18) and fair (0.31) between individual pairs of serologic tests. SNAP= SNAP® 4Dx® Plus ELISA. IFA= Indirect immunofluorescence assay. ELISA= Enzyme linked immunosorbent assay, WB= Western Blot.

3.7 Acknowledgements

Transition to chapter 4

The previous studies composing this thesis revealed that samples from horses in Canada may be seropositive for EGA and LB. Based on the first study, seroprevalence of EGA and LB in SK, MB and ON was low. As the samples for the first two studies were provided without identifying information, no information about the tested horses was available. For example, it was not possible to confirm whether seropositive samples originated from horses that were exposed within their province of residence or whether horses may have been exposed during travel within or outside of Canada. Thus, it was of interest to compare management factors such as pasture access, travel history, history of infestation with ticks, and signalment between horses testing seropositive or seronegative for EGA and LB, respectively, in the previous studies. It was hypothesized that management factors would not differ between seropositive and seronegative horses. Mail-out surveys were used to collect this information and anonymity of the horses and horse owners was maintained through collaboration with the diagnostic laboratories that originally supplied the samples.

Survey planning, design and editing of surveys, survey distribution, recording and description of results was done by the author of this thesis in collaboration with the research team (Drs. Lohmann, Epp, Burgess). As no statistical analysis was done in this study it will not be submitted for publication in the format presented here. Instead, a portion of the descriptive data (2011 surveys) will be used as an addition to chapter 2 that will be submitted for publication in the Canadian Veterinary Journal.

4 COMPARISON OF MANAGEMENT FACTORS BETWEEN HORSES TESTING POSITIVE OR NEGATIVE FOR ANTIBODIES AGAINST *ANAPLASMA PHAGOCYTOPHILUM* AND *BORRELIA BURGDORFERI* IN CANADA

4.1 Abstract

Equine granulocytic anaplasmosis (EGA) and Lyme borreliosis (LB) are tick-borne diseases transmitted by the vector ticks *I. scapularis* and *I. pacificus*. Established populations of the ticks have been reported from a few provinces in Canada. Clinical cases of EGA and LB have been reported in horses in Canada. In previous studies (see chapters 2 and 3), serologic evidence of exposure to both EGA and LB in horses in Canada was reported. The aim of this study was to identify potential risk factors for exposure in these same horses. The hypothesis was that management factors would not differ between seropositive and seronegative horses. A total of 392 anonymous surveys were sent to the owners of horses tested in previous studies, via the laboratories that supplied the samples. Surveys included questions concerning signalment of the horse, province of residence, timing of pasture access, visible tick infestation, history of travel and Lyme vaccination, and history of previous diagnosis with a tick-borne disease. A low response rate of 11.5% (45 of 392 surveys returned) and a low number of seropositive compared to seronegative animals precluded statistical analysis. The median age of horses seropositive for EGA was higher than that of the seronegative horses. For both diseases, the majority of seropositive horses was pasture-housed in the fall and had not travelled for at least 12 months prior to sample collection. None of the owners of seropositive horses reported seeing ticks on their horses. Potential risk factors for exposure to tick-borne diseases in Canada were identified in this study and should be explored in future investigations.

4.2 Introduction

Ixodes scapularis and *I. pacificus* are the common vectors for equine granulocytic anaplasmosis (EGA, caused by *Anaplasma phagocytophilum)* and Lyme borreliosis (LB, caused by *Borrelia burgdorferi)* in North America. In Canada, *I. scapularis* populations are currently established in a few locations in southern Ontario (ON) and Quebec (QC), Nova Scotia (NS), New Brunswick (NB), and in south east Manitoba (MB) (39,64). *I. pacificus* is established in a few locations in British Columbia (BC) (40,64,77,81,83,110).

Ixodes-borne diseases are an emerging concern in Canada (38,41,64). Granulocytic anaplasmosis has been reported in horses (4–6), dogs (89,93) and humans (94) in Canada, with EGA reported from areas with (4,5,7) and without (6) established populations of the vector. Reportedly, the affected horses did not travel outside their province of residence for at least a few years before diagnosis, which suggests that these horses were exposed to infected *Ixodes* within Canada. Horses living in non-endemic areas were probably exposed to infected adventitious ticks, for example ticks brought into the province on migrating birds.

Lyme borreliosis has been reported in horses, dogs and humans (8,38,39,89,90,111) in Canada. An increased risk for LB exposure has been reported in humans in eastern Canada, and in dogs in areas bordering the northeastern US (90).

While seroprevalence estimates for EGA and LB were previously reported for SK, MB and ON (see chapter 2), information about risk factors for exposure to these diseases in horses is lacking. The risk of tick infestation and infection with tick-borne organisms is likely increased with increased exposure to the vector such as in pastured horses (50), and it is thus possible that management factors such as travel or outdoor access during periods of vector activity contribute to the risk of encountering tick-borne infections. Confirmed diagnoses of EGA and LB in resident horses are a clear indicator that Canadian horses are at risk for exposure to tick-borne diseases and characterization of risk factors in horses is warranted as the geographic range of the vector tick is predicted to expand further north and west in Canada (39,40,64,77,82,91).

In 2011 a small seroprevalence study of horses residing in SK, MB and ON was conducted (see chapter 2). Horses were tested for the presence of antibodies to *A. phagocytophilum* and *B. burgdorferi* by use of a point-of-care ELISA that is licensed for dogs but is claimed to be non-species-specific (28). Selected samples were also re-tested at a

commercial veterinary diagnostic laboratory using an IFA for EGA and an ELISA/Western Blot combination for LB. Seropositive horses were found in provinces with (ON and MB) and without (SK) known established populations of the *Ixodes* vector. The overall seroprevalence of EGA was 0.53% (95% CI: 0.09-2.12%) and the overall seroprevalence of LB was 1.6% (95% CI: 0.65-3.6%).

In a second study, additional serum samples submitted to Prairie Diagnostic Services Inc. (PDS) in Saskatoon, SK during April 2013 were tested with a point-of-care ELISA as well as different serologic tests offered by commercial veterinary diagnostic laboratories. For EGA, samples were tested with two IFAs and for LB, samples were tested with an IFA, an ELISA/WB combination and a Lyme multiplex assay. Seropositive samples were identified for both organisms.

The aim of the study reported here was to identify potential risk factors for exposure of horses to EGA and LB in Canada. The hypothesis was that management factors do not differ between seropositive and seronegative horses. Mail-out surveys were used to obtain information about signalment, management factors including pasture access, travel history, and clinical history which were then compared between horses that tested seropositive or seronegative for EGA and LB, respectively, in the previous studies.

4.3 Materials and Methods

4.3.1 Serum samples

Samples for the previous 2 studies (n=426) were supplied from laboratory submissions to three provincial veterinary diagnostic laboratories, namely PDS, Saskatoon, Saskatchewan; the Manitoba Agriculture Food and Rural Initiatives Veterinary Diagnostic Services, Winnipeg, Manitoba; and the Animal Health Laboratory (AHL), Guelph, Ontario. A total of 392 submissions were used for the current study; 34 submissions were excluded because one diagnostic laboratory declined participation in the study. To comply with the terms of the original agreements for supplying serum samples to the investigators, specifically to maintain anonymity of the horses and horse owners, all matching of sample identification numbers to individual horses, horse owners and submitting veterinary practices was done by the veterinary diagnostic laboratories rather than the investigators. Details are described under "survey distribution".

For the previous studies, samples were collected in 2011 (n=342) and 2013 (n=50). In 2011, all samples were tested using the SNAP® 4Dx® ELISA (IDEXX Laboratories, Inc., Westbrook, ME); seropositive samples and a random selection of seronegative samples were re-tested for antibodies against A. *phagocytophilum* by laboratory-based IFA and for antibodies against *B. burgdorferi* by laboratory-based ELISA confirmed with WB. In 2013, all samples were tested using the SNAP® 4Dx® Plus ELISA (IDEXX Laboratories), 2 laboratory-based IFAs for antibodies against *A. phagocytophilum* and with laboratory-based IFA, ELISA confirmed with WB and Lyme multiplex assay for antibodies against *B. burgdorferi*. Samples that tested positive for antibodies against *A. phagocytophilum* or *B. burgdorferi* respectively, in any of the serologic tests were considered seropositive for the purpose of this study.

4.3.2   Survey distribution

Information about signalment, management and clinical history was obtained through surveys that were mailed out to the horses' owners. Survey mail-outs were conducted in such a way as to blind the investigators and maintain the anonymity of the horses and horse owners. Briefly, each survey was labeled with one sample identification (ID) number by one of the authors (GS). The diagnostic laboratories matched the sample ID to the horse's name or other identifying information, the owner's name and to the veterinary practice (or veterinary practitioner) who originally submitted the sample. The diagnostic laboratories mailed the surveys to the submitting veterinary practices (or veterinary practitioners), who were asked to pass on the surveys to the horses' owners. Pre-paid envelopes for all mailings were supplied by the investigators.

Each mailed package contained the surveys as well as cover letters from both the investigators and the diagnostic laboratory explaining the study. Pre-paid business reply envelopes for return of the surveys to one of the investigators (GS) were also supplied. Owners were asked to return surveys within 6 weeks of the mail-out date and were allowed an additional 2 weeks to withdraw from the survey analysis. Horse owners responding to the survey were not required to identify themselves or their horse. Their name and contact information as well as the name or identifying information of the horse remained unknown to the investigators unless owners chose to disclose it. Horse owners were also given the option of contacting the investigators if they wanted to know the test results of their own horses. The

study was approved on ethical grounds by the University of Saskatchewan's Behavioural Research Ethics Board (BEH #13-147).

4.3.3   Survey design

Samples for the previous studies were submitted to the diagnostic laboratories in October, November and December 2011 or in April 2013 and the study reported here was performed from June to September 2013. To include information about at least one tick season prior to sample analysis, owners of horses whose serum samples were used in the 2011 study were asked to address the 2011 calendar year, and owners of horses whose serum samples were used in the 2013 study were asked to address the 14 months preceding sampling (January 2012 – March 2013). Survey forms were worded accordingly (Figure 4.1 and 4.2).

The questions in the survey pertained to the signalment (age and sex) of the horses, duration of ownership, timing of pasture access, history of travel, history of diagnosis of EGA or LB by a veterinarian, known history of tick infestation and vaccination status with regard to LB.

4.3.4   Data management and reporting

Horses were classified as seropositive or seronegative for EGA, and as seropositive or seronegative for LB, and survey responses are reported separately for each disease. As the potential risk factors for exposure was of interest, information (e.g. travel history) provided about time frames after sample collection was excluded. Information regarding travel history provided for the month of sample collection was also excluded as the majority of samples were originally submitted for equine infectious anemia testing. Results for these tests are typically required for travel or competition, and any travel in the same month was therefore expected to have taken place after sample collection.

For horses whose serum samples were used in the 2011 study, information concerning age and duration of ownership was adjusted to arrive at the horses' age and duration of ownership at the time of sample collection. For example, if a horse's age was reported as "10 years" on the survey form, the horse was considered to be 8 years of age at the time of sample collection. If age was clearly identified as the age in 2011 on the form, the reported age was recorded. Age and duration of ownership were reported as median and range. The reason for inquiring about duration of ownership was to confirm the age of the horses at the time of sample collection.

Pasture access in the fall was of specific interest due to the presumably higher likelihood of horses to be infested with the adult stage of *Ixodes* which is active from September to December (13). Pasture access was therefore categorized as "pastured in the fall" or "not pastured in the fall". Horses that were reported to be pastured "all year round" or those whose season of pasture access included "fall" were categorized as "pastured in the fall". Horses that were reported to be pastured only in the "spring" or "summer" or that were not pastured were categorized as "not pastured in the fall". Travel history was categorized as "travelled" or "did not travel". "Travelled" referred to travel "outside the province but within Canada", "to the US", or "elsewhere". Travel history for horses that did not travel outside their province of residence was categorized as "did not travel". Time of travel was reported as the month in which travel occurred or, in cases where owners reported travel but did not identify a month, as "unknown time". Diagnosis of EGA or LB by a veterinarian, known tick infestation and vaccination against LB was categorized as "yes" or "no" according to responses. Answers provided as "do not recall" or "do not know" for any questions were considered as "no" answers.

Due to a low response rate, i.e. a low number of surveys returned, the data were not analyzed using statistical tests.

4.4 Results

Response rate was 11.5% with 45 out of 392 surveys returned to us. One returned survey was excluded because the owner did not provide information about a specific horse but rather described the general management of the herd. This left 44 survey responses for descriptive reporting. Thirty-four responses concerned horses that were seronegative for both EGA and LB. Two survey responses concerned horses that were seropositive for EGA but seronegative for LB, and five survey responses concerned horses that were seropositive for LB but seronegative for EGA. Three survey responses concerned horses that were seropositive for both EGA and LB (Table 4.1 and 4.2). Ticks were only reported from horses tested in 2011. None of the horses was diagnosed with EGA or LB by a veterinarian or was vaccinated against LB.

4.4.1 <u>Horses seropositive or seronegative for EGA</u>

The median age of the five horses that were seropositive for EGA (12 years) was higher than the median age of the 39 seronegative horses (3 years) although the age ranges were

similar (Table 4.1). Four geldings and one mare were seropositive for EGA. Mares appeared over-represented in the seropositive group; 20% of horses in the seropositive group were mares while 10% of seronegative horses and 11% of horses overall were mares (Table 4.1). All of the horses seropositive for EGA had access to pasture in the fall while only 77% of the seronegative horses did. When looking at all horses, about 80% had access to pasture in the fall. Interestingly, ticks were reportedly not seen on the horses that were seropositive for EGA. In contrast, owners reportedly observed ticks on 15 of 39 seronegative horses (38%). Ticks were reportedly seen only in the spring and summer months (April to July 2011) (Table 4.1).

All of the horses that were seropositive for EGA and 77% of the horses seronegative for EGA resided in SK. Overall, horses from SK were over-represented in the responses that were received (80%) considering the proportion of horses from SK that were tested in both studies (60%). One of the five horses that were seropositive for EGA traveled to the US; however, the month of travel was not reported (Table 4.1). The proportion of seropositive horses that travelled (20%) appeared lower than that of the seronegative horses (28%).

4.4.2   Horses seropositive or seronegative for LB

For LB, the median age and age range of seropositive and seronegative horses was similar (Table 4.2). Of horses overall, 86% were geldings whereas 75% of the horses that tested seropositive for LB were geldings. This suggests that mares were overrepresented in the seropositive group. Mares represented only 11% of all the horses but 25% of the seropositive horses were mares (Table 4.2). Of horses seropositive for LB, 88% had access to pasture in the fall compared to 78% of the seronegative horses and 80% of all horses. Similar to horses seropositive for EGA, ticks were reportedly not seen on any of the horses seropositive for LB. In contrast, ticks were seen on 15 seronegative horses (42%). Seven of the horses seropositive for LB (88%) resided in SK while 81% of the seronegative horses did. As stated earlier, horses from SK were over represented in the responses. Only one seropositive horse had travelled. The horse had travelled to Pennsylvania and reportedly resided there in the 5 months before sample collection in April 2013 (Table 4.2). This means that the horse spent the fall, when adult stages of *Ixodes* are active, in the US. Eleven of the seronegative horses (31%) had a travel history.

4.5 Discussion

Reported response rates of paper-based surveys are typically over 32% (112) while the response rate in our study was low (11.5%). The response rate for owners of horses sampled in 2011 (10.5%) was lower than that of owners whose horses were sampled in 2013 (18%), which may indicate that a longer time from sample collection affected our ability to reach the owners or their recall. All surveys were sent out by the diagnostic laboratories; however, it is unknown how many surveys were passed on by veterinary practices (or veterinary practitioners) and how many were actually received by the horses' owners. Another limitation of the study, namely the fact that the surveys were an "afterthought" and were not planned from the beginning of the serologic studies, probably reduced compliance.

As identifying differences between horses testing seropositive or seronegative for EGA and LB, respectively, was attempted, it was essential to obtain information from seropositive and seronegative horses. While the proportion of surveys about seropositive horses for EGA (11%) was similar to the proportion of samples that were seropositive for EGA (10%), surveys about horses seropositive for LB were over represented (18%) compared to the proportion of samples that were seropositive for LB (8.5%). Overall the low response rate precluded statistical evaluation, such that it was not possible to truly test the hypothesis. However, the following interesting trends were identified and may warrant further investigation in future studies.

The median age of horses seropositive for EGA was higher than that of seronegative horses although the age ranges overlapped. Although the severity of clinical signs of EGA is considered to be age dependent, with older horses showing more severe clinical signs (4,13,87), the association between age and rate of infection is unknown. As measurable antibody titers may last up to 2 years (25), life time exposure does not necessarily explain a higher rate of seropositivity in the older horses.

Only 2 (20%) seropositive horses (one to EGA and one to LB) travelled, both to the US, while 10 (30%) of the seronegative horses travelled. The fact that more seronegative horses travelled does not support travel as risk factor for exposure. While the timing of travel for the LB-seropositive horse was consistent with the activity time of adult *Ixodes*, the time of travel to the US for the EGA-seropositive horse was not reported. While it is possible that time of travel was relevant to exposure of the EGA seropositive horses it is also possible that the travel to the US occurred after sample collection which would obviously be irrelevant for

82

exposure. Yet, the majority of seropositive horses were not reported to travel out of their province of residence which suggests that they were exposed to the causative organisms within Canada. Thus, these horses may have been exposed to infected *Ixodes* originating from established tick populations such as those known to be present in MB, or they could have been exposed to adventitious ticks arriving in non-endemic areas such as SK. While only one horse that resided in MB was seropositive to LB, the rest of the seropositive horses were from SK and the majority of them did not travel. Thus, one may assume that they were most likely exposed to adventitious ticks in SK. While it appears from our data that the majority of seropositive horses were exposed to adventitious ticks, it is worth mentioning that horses from SK were over-represented in our responses.

For the purposes of this study, seropositive horses were defined as those testing positive for antibodies in any of the serologic tests. It is essential to mention that test results for the same samples differed between the different serologic tests and that the proportion of positive results obtained in laboratory-based tests was higher than would be expected for horses in Canada. The possibility of false positive results should therefore be considered, which may have limited interpretation of our data. Further studies using more stringent criteria are needed in order to more accurately evaluate the risk of exposure in horses in Canada.

The adult stage of *Ixodes*, which is the stage most likely to infest horses, is active the fall (13,61). The majority of seropositive horses had access to pasture in the fall, which may support a higher risk of exposure for pastured horses. However, it was noticed that most of the seronegative horses had access to pasture in the fall as well, and the association of pasture access in the fall and testing seropositive for tick-borne diseases requires further investigation.

It was interesting to note that none of the horses seropositive for EGA or LB were observed to be infested with ticks. On the other hand, 15 of the seronegative horses were reported to have had visible tick infestation. Ticks were seen in the spring or summer that preceded sample collection in 2011. No ticks were reportedly seen by the owners of the horses tested in 2013. Based on the season of observation, it is possible that the ticks that infested these horses were *Dermacentor* species rather than *Ixodes* species that transmit EGA and LB.

D. variabilis and *D. andersoni,* which are among the most common ticks in Canada (1,3,113), are not able to maintain or transmit *A. phagocytophilum* and *B. burgdorferi* (2,114–

117). Spring and summer are the seasons in which the adult stages of *D. variabilis* and *D. andersoni* are active (3) and it is possible that these species were observed on the horses. *D. albipictus* is able to maintain and transmit infection with non-pathogenic strains of *A. phagocytophilum* (115) and non-pathogenic strains may cause seroconversion. While the adult *D. albipictus* ticks are active in the winter, the nymphs are active later in the spring (3). *D. albipictus* is not able to maintain or transmit infection with *B. burgdorferi* (115).

It is possible that *Ixodes*, which are the major vectors for both *A. phagocytophilum* and *B. burgdorferi*, were simply not noticed by horse owners. *Ixodes* are significantly smaller than *Dermacentor* species (adults are 3mm in length versus 6 mm, nymphs are 0.6mm in length versus 1-2mm). The *Ixodes* are also darker and less easily identified.

As none of the horses was reportedly diagnosed with EGA or LB by a veterinarian, it was assumed that none of the horses had clinical signs of these diseases. Infection with *A. phagocytophilum* and *B. burgdorferi* does not necessarily cause disease and asymptomatic seropositive horses have been previously reported (13,50). It is also possible that infection with non-pathogenic strains of *A. phagocytophilum*, while not causing symptomatic infection, resulted in seroconversion. Current serologic tests do not differentiate between strains of *A. phagocytophilum* and it is not possible to differentiate between horses exposed to pathogenic or non-pathogenic strains based on serology.

In summary, we observed that the median age of horses that were seropositive for EGA was higher than that of the seronegative horses. Horses seropositive for EGA and LB resided in provinces with (MB) and without (SK) known established populations of *Ixodes*. The majority of seropositive horses did not travel in the year that preceded sample collection and it is therefore likely that they were exposed to infected vector ticks within Canada. Infestation with infected adventitious ticks carried by humans or animals such as migrating birds may explain exposure in these horses. The majority of seropositive horses had access to pasture in the fall; however, horse owners did not report seeing ticks on the seropositive horses. The small number of returned surveys did not allow for statistical analysis; however our data identify initial trends that can be investigated further in additional studies.

4.6. Figures

SURVEY   Sample ID: _____

Please answer the following questions about your horse (see envelope for horse's name)

1. Age in years: _____

2. Sex (check the one that applies): Mare ☐   Stallion ☐   Gelding ☐

3. When did you acquire the horse (year)? _____

4. In 2011, was your horse on pasture?   No ☐   Yes ☐   Do not recall/know ☐

   If yes, check all that apply: Spring ☐ Summer ☐ Fall ☐ Winter ☐ All year round ☐
   Additional comments:_____

5. In 2011, did a veterinarian diagnose Lyme disease or Equine granulocytic anaplasmosis in
   your horse? No ☐   Yes ☐   Do not recall/know ☐
   Which disease(s) were diagnosed? _____

6. In 2011, did you find one or more ticks on your horse? No ☐   Yes ☐ Do not recall/know ☐
   In which month(s) were ticks found? _____

7. In 2011, which province did your horse reside in? _____

8. In 2011, did your horse travel outside his or her residence province?

   Yes, outside the province but within Canada ☐

   Yes, travelled to the US ☐   No, did not travel outside the province ☐

   Yes, travelled elsewhere ☐   Do not recall/know ☐

   If "Yes", please provide details about the destination and month in which the horse travelled:

Province or US State or other detail (e.g. country)	Month travel occurred

9. In 2011, was your horse vaccinated against Lyme disease?

   No ☐   Yes ☐   Do not recall/know ☐

   If "Yes", please provide details about the dates or frequency of vaccination and the
   vaccine that was used (e.g. brand) _____
   _____

Figure 4.1. Survey for owners of horses whose serum samples were tested for antibodies against *A. phagocytophilum* and *B. burgdorferi* in 2011.

SURVEY   Sample ID: _____

Please answer the following questions about your horse (see envelope for horse's name)

1. Age in years: _____

2. Sex (check the one that applies): Mare ☐   Stallion ☐   Gelding ☐

3. When did you acquire the horse (year)? _____

Please answer the following questions for the time period from January 2012-April 2013:

4. Was your horse on pasture?   No ☐   Yes ☐   Do not recall/know ☐

   If yes, check all that apply: Spring ☐ Summer ☐ Fall ☐ Winter ☐ All year round ☐
   Additional comments:_____

5. Did a veterinarian diagnose Lyme disease or Equine granulocytic anaplasmosis in your horse? No ☐ Yes ☐   Do not recall/know ☐
   Which disease(s) were diagnosed? _____

6. Did you find one or more ticks on your horse? No ☐   Yes ☐ Do not recall/know ☐
   In which month(s) were ticks found? _____

7. Which province did your horse reside in? _____

8. Did your horse travel outside his or her residence province?

   Yes, outside the province but within Canada ☐

   Yes, travelled to the US ☐   No, did not travel outside the province ☐

   Yes, travelled elsewhere ☐   Do not recall/know ☐

   If "Yes", please provide details about the destination and month in which the horse travelled:

Province or US State or other detail (e.g. country)	Month travel occurred

9. Was your horse vaccinated against Lyme disease?

   No ☐   Yes ☐   Do not recall/know ☐
   If "Yes", please provide details about the dates or frequency of vaccination and the
   vaccine that was used (e.g. brand) _____

Figure 4.2. Survey for owners of horses whose serum samples were tested for antibodies against *A. phagocytophilum* and *B. burgdorferi* in 2013.

4.7. Tables

	EGA+ (n=5)	EGA- (n=39)
Age (years): Median (range)	12 (2-18)	3 (0.3-21)
Not reported (n=1)	0	1
Sex		
Gelding (n=38)	4	34
Mare (n=5)	1	4
Stallion (n=1)	0	1
Duration of ownership (years)		
Median (range)	5 (1.5-10)	2 (0-12)
Not reported (n=2)	0	2
Pastured in Fall		
Yes (n=35)	5	30
No (n=9)	0	9
Diagnosed with EGA or LB		
Yes (n=0)	0	0
No (n=44)	5	39
Ticks seen		
Yes (n=15)	0	15
No (n=29)	5	24
Province of Residency		
SK (n=36)	5	31
MB (n=6)	0	6
ON (n=2)	0	2
Travel history		
Travelled (n=12)	1	11
Did not travel (n=32)	4	28
Vaccinated against LB		
Yes (n=0)	0	0
No (n=44)	5	39

Table 4.1. Comparison of signalment, management and clinical history between horses testing seropositive (+) or seronegative (-) for equine granulocytic anaplasmosis (EGA).

	LB+ (n=8)	**LB-** (n=36)
Age (years): Median (range)	3 (2-18)	4 (0.3-21)
Not reported (n=1)	0	1
Sex		
Gelding (n= 38)	6	32
Mare (n=5)	2	3
Stallion (n=1)	0	1
Duration of ownership (years)		
Median (range)	2 (1.5-5)	3 (0-12)
Not reported (n=2)	1	1
Pastured in Fall		
Yes (n=35)	7	28
No (n=9)	1	8
Diagnosed with EGA or LB		
Yes (n=0)	0	0
No (n=44)	8	36
Ticks seen		
Yes (n=15)	0	15
No (n=29)	8	21
Residency		
SK (n=36)	7	29
MB (n=6)	1	5
ON (n=2)	0	2
Travel history		
Travelled (n=12)	1	11
Did not travel (n=32)	7	25
Vaccinated against LB		
Yes (0)	0	0
No (n=44)	8	36

Table 4.2. Comparison of signalment, management and clinical history between horses testing seropositive (+) or seronegative (-) for Lyme borreliosis (LB).

4.8. Acknowledgments

Transition to chapter 5

The previous chapter described management factors in horses that tested seropositive or seronegative for EGA and LB, respectively. The information was obtained by asking horse owners to return anonymous surveys. The study revealed that most of the horses testing seropositive to either organism did not travel in the year that preceded sample collection, and that most of the seropositive horses lived in areas without known established populations of the *Ixodes* vector. Although the majority of the horses were pastured in the fall, which is the time of activity for *Ixodes* adults that typically infest horses, none of the owners of seropositive horses reported that they had seen ticks on their horses at any time of the year.

Published reports or reviews describing the ticks infesting horses are scarce. Thus, findings that horses are indeed exposed to tick-borne diseases in Canada indicated that further investigation of what ticks infest horses in Canada is needed. A passive surveillance study was conducted for the collection and identification of ticks infesting horses in SK, collaborating with Dr. Neil Chilton from the Department of Biology, University of Saskatchewan. The author of the thesis was responsible for receiving tick submissions from horses in SK in 2012 and 2013 and for transferring these to Dr. Chilton's parasitology lab for identification. Dr. Neil Chilton and Mr. James Armstrong identified and categorized ticks. The author recorded tick species, stage and number, and followed up with horse owners, providing identification of the ticks submitted from the horses. We plan to submit this study for publication in the Canadian Veterinary Journal which will hold copyright.

5. PASSIVE SURVEILLANCE FOR HORSE TICKS IN SASKATCHEWAN

5.1 Abstract

Although tick-borne disease has been reported in horses in Canada, systematic descriptions of ticks infesting horses in Canada or elsewhere are rare. The aim of the study presented here was to describe ticks collected by horse owners and veterinarians from horses residing in Saskatchewan (SK) over a two-year period (2012-2013). The hypothesis of this study was that tick species known to be established in SK as well as adventitious ticks can be found on horses in SK. Ticks were categorized according to species, sex, life stage and degree of engorgement. Information about probable geographic locality of tick acquisition, estimated duration of tick attachment and travel history of the horse was requested along with tick submissions. None of the horses reportedly travelled in the 2 weeks prior to tick collection, suggesting that the horses acquired the ticks within SK. The number of submissions and the total number of ticks by species and month was recorded. During the study, a total of 833 ticks were collected from over 86 horses. Ticks were received from February to August 2012 and from February to July 2013. All ticks were *Dermacentor* species (D. *albipictus*, D. *andersoni* and D. *variabilis*). Timing of submission and geographic distribution were consistent with the reported peak activity of *Dermacentor* species and with previous reports of *Dermacentor* species distribution in SK. No *Ixodes* were received and none of the ticks received for this study were expected to put horses at risk for tick-borne diseases. A longer-term surveillance, including active surveillance, of horse ticks in SK may be warranted to further characterize the ticks infesting horses in SK and to monitor predicted changes in tick habitats.

5.2 Introduction

Tick-borne disease occurs after transmission of a pathogenic organism by a vector tick to the tick's host. Ticks are considered a vector for a pathogen if the tick feeds on a vertebrate host, is able to acquire the pathogen during a blood meal, maintains the pathogen through one or more life stages, and transfers the pathogen to another host during the next blood meal (61).

Tick-borne diseases of horses in North America that are of relevance to Canadian horses are equine granulocytic anaplasmosis (EGA), Lyme borreliosis (LB) and equine piroplamosis. Of these, equine granulocytic anaplasmosis caused by *Anaplasma phagocytophilum* and LB caused by *Borrelia burgdorferi* are the only reported tick-borne diseases in horses in Canada to date (4–7). Both pathogens are transmitted by the tick vector *Ixodes scapularis,* which is currently established in a few locations in south east Canada, including areas of Southern Ontario (ON) and Quebec (QC), Nova Scotia (NS), New Brunswick (NB) and south east Manitoba (MB) (38,39,111). *Ixodes pacificus* may also transmit EGA and LB and is currently established in a few locations in southern British Columbia (BC) (38,39). Established populations of *I. scapularis* or *I. pacificus* ticks have not been reported in SK and it is therefore likely that a SK horse diagnosed with EGA in 2010 (6) was exposed to infected adventitious *Ixodes*. Adventitious ticks are those brought into a non-endemic area on migrating humans or animals, such as migrating birds (40,118).

Equine piroplasmosis, also called equine babesiosis, is caused by the blood protozoa *Theileria equi* and *Babesia caballi*. The tropical horse tick *Dermacentor nitens* is the natural vector in the US, but presence of this tick has not been reported in Canada (9). *T. equi* can be experimentally transmitted by *D. variabilis* and *D. albipictus,* both of which are established in Canada, and by *Rhipicephalus microplus* (9) which does not occur in Canada. Equine piroplasmosis is a reportable disease in the US and Canada and has not been reported in Canada to date.

Dermacentor ticks are a genus of the ixodid ticks (hard ticks). The genus contains 33 species and occurs on all continents except Australia (61). In North America, *D. variabilis* and *D. andersoni* are the most important species that infest livestock; *D. albipictus* mostly infests moose (61). *D. variabilis* and *D. andersoni* are two of the most common tick species in Western Canada. *D. variabilis,* also known as the American dog tick, occurs throughout southeastern SK, southern MB and ON (1,119). Isolated populations are also found in NS

(1,3). *D. variabilis* is usually found in geographic areas where summers are warm and humid (1), whereas *D. andersoni,* also called the Rocky Mountain wood tick, occurs throughout the southern parts of BC and AB, and throughout southwestern SK (1,3,119) where summers are hot and dry (1). Usually, *D. variabilis* and *D. andersoni* will not be found in the same habitat (1–3) but occasionally, both species may be found together in areas that differ from their natural habitat, e.g. southern central SK which is dry and cold (1). *D. variabilis* and *D. andersoni* are vectors for Rocky Mountain spotted fever (RMSF), a tick-borne disease caused by *Rickettsia rickettsii* (119,120). RMSF mostly affects humans although dogs may occasionally become ill as well (120). Horses have not been reported to contract RMSF. *D. albipictus*, also called the winter tick or moose tick, is the only *Dermacentor* tick whose life stages all occur on the same host. The tick is found all across Canada and its habitat extends further north than that of *D. andersoni* and *D. variabilis* (3).

Geographic range expansion of ticks is a concern in Canada; potential contributors are local migration of the ticks while on a local host, distant migration on birds migrating northward in the spring, and climate changes that may enable the survival and establishment of ticks in new locations (40,64,77,81,83,110). While passive and active surveillance of ticks collected from humans, companion animals and in the environment has been ongoing at the Department of Biology, University of Saskatchewan, we here report the first tick surveillance study focusing on ticks collected from horses in SK. To the best of our knowledge, published data about ticks infesting horses in SK are lacking. It was hypothesized that tick species known to be established in SK as well as adventitious tick species can be found on horses in SK. The objective of this study was to describe the species, sex, and life stage, stage of engorgement and geographic location of acquisition of ticks submitted from horses in SK over a 2-year period

5.3 Materials and Methods

5.3.1 Study design

Passive surveillance of horse ticks in Saskatchewan took place between January 2012 and September 2013. The study was advertised through the mailing list of the Saskatchewan Veterinary Medical Association, the website of the Saskatchewan Horse Federation and the website of the Western College of Veterinary Medicine (WCVM), University of Saskatchewan. Advertisements were also posted in the reception area of the WCVM Large Animal Clinic and at the WCVM booth during the annual Saskatchewan Horse Expo in

Saskatoon. Veterinarians and horse owners were asked to submit ticks that were found on horses residing in SK.

5.3.2 Tick submissions

Horse owners and veterinarians were asked to submit ticks by mail to two of the authors (GS, KL). The submitters were asked to fill out a submission form for each horse from which ticks were collected, and to submit ticks from each horse separately. The submission form included questions about geographic location of acquisition of the ticks, travel history of the horse within the 2 weeks that preceded collection, date of tick collection and estimated time of attachment of the ticks. The horse's name or other identification, as well as contact information were requested for the purpose of follow-up with the owners. Submitters were asked to submit ticks in sealed containers containing moist tissue to maximize preservation of the ticks.

5.3.3 Tick identification

Ticks were identified and catalogued by two of the authors (NC, JA). Tick species was recorded. Life stage was recorded as larva, nymph or adult and sex was recorded as male or female. Level of engorgement of female ticks was recorded as engorged or non-engorged. Ticks that were not preserved enough to allow their identification (e.g. dried out) were excluded from the study.

5.3.4 Data reporting

A 'submission' was defined as all the ticks from one horse that were received on a specific date. If a package arrived with no identification of the horse, it was considered as one submission. Repeated submissions from the same horse on different dates were counted as individual submissions. For each submission, the total number of ticks as well as the number of ticks by species and stage was recorded. The number of submissions (by species) and the total number of ticks (by species) was recorded and graphed by month (MATLAB R2013a, Mathworks Inc., US). Geographic location of tick acquisition was reported by city, town or by Dominion land survey parameters, according to information given by the submitter. Direction and distance from Saskatoon was described when city, town or a combination of section, township, range and meridian were given. The geographic location of acquisition was mapped manually using a free access royal map of SK and an online free province legal land converter

website (http://www.prairielocator.com/). Relevant travel history concerned travel outside the area of residence within 2 weeks before collection of the tick.

5.4 Results

A total of 833 ticks in 86 submissions were received over the duration of the study. In 2012, all tick submissions occurred between February and August (543 ticks in 60 submissions); no ticks were received in January and from September to December 2012. In 2013, all tick submissions occurred between February and July (290 ticks in 26 submissions); no ticks were received in January, August or September 2013 (Figure 5.3 and 5.4).

All received ticks were identified as *Dermacentor* species and species were identified as *D. albipictus*, *D. andersoni* and *D. variabilis*. Only adult ticks and nymphs were received and no larvae were identified. In both years, adult and nymphal *D. albipictus* were received whereas only adult *D. andersoni* and *D. variabilis* were received. The geographic distribution of the ticks according to species was similar in 2012 and 2013 (Figure 5.5 and 5.6). None of the horses had reportedly travelled outside the area of residence in the 2 weeks prior to collection of the ticks. In 2013, 3 horses were reported to have travelled to neighboring farms, but this was not considered as travel outside the area of residence. Only 4 owners reported the estimated time of attachment of the ticks. These 4 owners suggested that the ticks were attached for approximately 2 hours (*D. variabilis*) one day (*D. andersoni*), 3-5 days (*D. variabilis*) and possibly for 2 weeks (*D. andersoni* and *D. variabilis*).

5.4.1   Ticks received in 2012

The number of submissions (Figure 5.3) and total number of ticks (Figure 5.4) was highest for *D. variabilis*, followed by *D. albipictus* and *D. andersoni*. The number of ticks per submission ranged from one to 94. One submission (in May) contained both *D. albipictus* and *D. variabilis*. The other submissions contained only one species. Two submissions (both in June) contained ticks collected from more than one horse. For another three horses, ticks were received twice, on different dates (April, May and June). On eight occasions, packages containing multiple submissions were received. Three packages were submitted by the same horse owner, containing submissions from one or two horses (all in May).

D. albipictus

One hundred and four *D. albipictus* ticks were received in nine submissions; five submissions occurred in February, three in March and one in May (Figure 5.3). The majority

of *D. albipictus* ticks were received in March (Figure 5.4). Both adult (n=62) and nymphal (n=42) stages were identified. Of the adults, there were 36 males, 23 un-engorged females and 3 engorged females. *D. albipictus* ticks were received mainly from the Marsden area (near the AB border) and a few from 100 km northwest and 300 km south of Saskatoon. One submission of *D. albipictus* ticks originated further south in the province, near Assiniboia (Figure 5.5).

D. andersoni

Sixty *D. andersoni* ticks were received in two submissions, one in April and one in June (Figure 5.3). The majority of *D. andersoni* ticks were received in April (Figure 5.4).Only adult ticks were received. There were 26 males, 33 un-engorged females and 1 engorged female. *D. andersoni* ticks were received only from the southwest corner of the province (Figure 5.5), near Swift Current and Maple Creek.

D. variabilis

Three hundred and seventy-nine *D. variabilis* ticks were received in 50 submissions between April and August (Figure 5.3). The majority of *D. variabilis* ticks were received in June (Figure 5.4). Only adult ticks were received; there were 191 males, 81 un-engorged females and 107 engorged females. Most of the *D. variabilis* ticks were received from Saskatoon and areas surrounding Saskatoon (in the radius of 200 km), while some were from the south and south east areas of SK (Figure 5.5), near Regina and Yorkton.

5.4.2 Ticks received in 2013

The number of submissions (Figure 5.3) and total number of ticks (Figure 5.4) was highest for *D. variabilis*, followed by *D. albipictus* and *D. andersoni*. The number of ticks per submission ranged from one to 78. In May, one submission contained both *D. variabilis* and *D. andersoni*. All other submission contained only one species. One horse owner who sent ticks in 2013 had also sent ticks in 2012; however, the horse(s) from which ticks were collected were not identified. Two owners submitted ticks from more than one horse on different dates in April and May, respectively. One submission (in June) contained ticks from unidentified horse(s).

D. albipictus

Eighty-nine *D. albipictus* ticks were received in 5 submissions; 1 submission each occurred in February and March, and 3 occurred in April (Figure 5.3). The majority of *D.*

albipictus ticks were received in March (Figure 5.4). Both adult (n=71) and nymphal (n=18) stages were identified. Of the adults, there were 22 males, 45 un-engorged females and 4 engorged females. *D. albipictu*s ticks were received from Saskatoon and up to 100 km northwest of Saskatoon, as well as from the Lloydminster area, near the border with AB (Figure 5.6).

D. andersoni

Seventy-nine *D. andersoni* ticks were received in 6 submissions, of which 5 occurred in May and one occurred in June (Figure 5.3). The majority of *D. andersoni* ticks were received in May (Figure 5.4). Only adult ticks were received and there were 30 males, 8 un-engorged females and 41 engorged females. *D. andersoni* ticks were received from the southwest and south areas of the province (Figure 5.6), near Maple Creek and Assiniboia.

D. variabilis

One hundred and twenty two *D. variabilis* ticks were received in 16 submissions from May to July (Figure 5.3). The total number of *D. variabilis* ticks (n=60) and the number of submissions (n=7) received in May was similar to those received in June (n = 61 and n=8, respectively) (Figures 5.3 and 5.4). 64 adult males, 20 un-engorged adult females and 38 engorged adult female ticks were received. *D. variabilis* ticks were received from Saskatoon and from areas up to 250 km south, southeast and east of Saskatoon, as well from the south area of the province near Assiniboia (Figure 5.6). One submission in May (Colonsay, SK) originated from a donkey.

5.5 Discussion

The passive surveillance of horse ticks in SK reported here took place between January 2012 and September 2013. The aim of the study was to describe ticks that can be found on horses in SK. All ticks received from horses were *Dermacentor* species.

The total number of tick submissions was lower in 2013 compared to 2012. Due to the fact that a passive surveillance was used to collect ticks, it is challenging to point out accurately why there was a difference in the numbers of tick submissions between 2012 and 2013. However, there are a few possible explanations for this difference. One possible explanation concerns differences in environmental conditions between the first and second year of surveillance, which may have favored development of different tick species and stages in 2012 or inhibited their development in 2013 (3,113,119). When comparing records of air

temperature and relative humidity in SK between 2012 and 2013 (personal communication with Environment Canada, St. Denis station, 40 km east of Saskatoon), it was interesting to note that the average temperature in the winter and spring months varied between the years. January 2012 was less cold than January 2013, with average temperatures of -8.7 ⁰C and -15.0 ⁰C, respectively. The average temperature in February 2012 (-8.9 ⁰C) was similar to that in February 2013 (-11.0 ⁰C). The spring of 2012 was characterized by relatively higher monthly average air temperatures compared to those in 2013. In 2012, the average air temperatures in March and April were -1⁰C and +3.6 ⁰C, respectively, whereas in 2013, these were -11.3 ⁰C and -2.7 ⁰C. In 2013, relative humidity was higher in January (82.8% versus 71.5%), February (86% versus 79%), April (76.2% versus 71.5%) and May (58.8% versus 52%) compared to 2012. Considering the preferred relative humidity conditions in *Dermacentor* species, the differences in the average relative humidity in SK between 2012 and 2013 may *not* explain the differences in the number of ticks received between the years.

Another possible explanation lies in the nature of a passive surveillance study. The success of this study was depended on the interest and willingness to help of veterinarians and horse owners in SK. As owners and veterinarians who submitted ticks were informed with the identity of the ticks they submitted, and as these ticks were probably of little clinical significance with regards to tick-borne diseases to their horses, their interest and motivation to submit new identified ticks may have declined over time. Only one owner submitted ticks in both 2012 and 2013; however, it was unknown whether submissions in both years occurred from one or more horses. Another factor potentially influencing the number of submissions was advertisement effort. While efforts were done to re-advertise the study in 2013, it was not possible to renew posting on the SK Horse Federation website, which was one of the sources used during 2012.

D. albipictus

Receiving *D. albipictus* ticks from horses in SK was not surprising as the ticks are known to occur in this province, and will infest large mammals such as moose, deer, cows and horses (3,121). Although the number of submissions (n = 8) and the total number of ticks (n = 104) received was higher in 2012 compared to 2013 (n = 5 and n = 89, respectively), the general pattern of submissions in both years was consistent with the expected life cycle of the tick. *D. albipictus* is a one-host tick that typically completes its life cycle in one year (3). Larvae hatch in the early fall and immediately start questing for a host. Once on a host, the larvae feed and

immediately upon feeding molt to nymphs. The nymphs remain on the host for the fall and winter months, feed constantly and molt to the reproducing adult stages starting in January. The adult ticks are usually active from January to May, laying eggs in mid-summer (3).

D. albipictus ticks were received only in the months of February, March, April and May, with the highest number of *D. albipictus* ticks received in March 2012 and March 2013. Both nymphal and adult *D. albipictus* ticks were received in February and March of each year. Although it has been reported that nymphs may be found on their host during their molting, from November through February (121), it was somewhat surprising that nymphs were received as late as February and March. It is possible that relative low temperatures in our geographical region delay the ticks' development compared to other regions. Submissions later in the spring (in May 2012 and April 2013) contained only adult stages, mainly engorged females. It is possible that because *D. albipictus* are one host ticks and remain on one host, which ensures a relatively constant environment, variation in winter and fall temperatures between the years of collection did not have a major effect on the ticks' life cycle.

Lower numbers of nymphal compared to adult *D. albipictus* were received in both years. It is possible that the difference between the number of nymphal and adult stages that were received resulted from a failure to identify immature stages, due to their significantly smaller size. The fact that *D. albipictus* are one host ticks which occur on one host is probably the reason why it was the only species of which we received nymphs. While nymphs of the other species may occur on horses, horses are mostly infested with adult ticks. No larvae were received in our study. This was not surprising as this life stage mostly does not occur on horses. However, in the case of *D. albipictus*, in which all stages occur on the host, it is most likely that larvae were not identified due to their smaller size (Figure 5.1).

D. andersoni

Receiving *D. andersoni* from horses in SK was not surprising as *D. andersoni* have been reported to occur in SK and adult stages may infest horses (1,3,61). An increase in the number of submissions of *D. andersoni* was noticed in 2013 (n=6) compared to 2012 (n=2). Only adult *D. andersoni* ticks were received for the study. Adult *D. andersoni* ticks are typically active between April and June, with peak reproductive activity in May (3). In 2012, adult ticks were received in April and again in June; however, according to the accompanying questionnaire, ticks in the second submission may have been collected in late May or early June. In 2013, adult ticks were received in May and June but not in April. As described

earlier, the higher average temperature in April 2012 (+3.6 °C) was likely more conducive to the ticks' activity than the average temperature in April 2013 (-2.7 °C), which was probably not favorable. However, average temperatures in May 2012 and May 2013 were 10.2 °C and 12.9 °C, respectively, and likely favored reproduction activity equally.

D. variabilis

Receiving *D. variabilis* from horses in SK was not surprising as this species has been reported to occur in SK and adult stages may infest horses (1,3,61). *D. variabilis* ticks were received from April to August 2012 and from May to July 2013. Relative to 2012, the overall submission and number of *D. variabilis* was lower in 2013. The pattern of submissions in 2012 suggested that adult ticks were active in April, with tick activity peaking in May and gradually declining throughout June, July and August. This pattern is typical for the activity of adult *D. variabilis* (3). The average ambient temperature in March 2012 (-1.0 °C) and April 2012 (+3.6 °C) indicated favorable conditions for cessation of dormancy and stimulation of adult tick activity (3). In comparison, the average temperatures in March 2013 (-11.0 °C) and April 2013(-2.7 °C) were likely too low to stimulate tick activity. In May 2013, the average ambient temperature was 12.9 °C, which should have favored activity of the reproductive stages of *D. variabilis*. The fact that submissions of *D. variabilis* first occurred in May 2013 (versus April in 2012) and lasted until July suggested that environmental conditions influenced the delayed pattern of submission. The less favorable environmental temperatures may partially explain the lower numbers of adult *D. variabilis* submissions in 2013.

The geographical distribution of tick submissions was consistent between 2012 and 2013 and was compatible with other reports of the distribution of *Dermacentor* ticks in SK, which are based on active surveillance or on submissions of ticks collected from humans and animals other than horses (1,3). *D. variabilis* was primarily distributed in the south and southeast of the province, *D. andersoni* in the southwest of the province and *D. albipictus* was mostly extending further north and west, near the border of AB, between Marsden and North Battleford, but was occasionally submitted from south of Saskatoon.

None of the ticks received for this study were expected to put horses at risk for tick-borne diseases. *D. andersoni* and *D. variabilis* are unable to maintain or transmit *A. phagocytophilum* causing EGA and *B. burgdorferi* causing LB (114,115). Conversely, *D. albipictus* reportedly can be infected with a non-pathogenic strain of *A. phagocytophilum* (115). *D. variabilis* and *D. andersoni* are the main vectors for the Rickettsial agent causing

RMSF (2,119,122); however, RMSF has never been reported in horses. *Theileria equi,* the causative agent of equine piroplasmosis has been experimentally transmitted by *D. variabilis* and *D. albipictus* (9); however, the significance of this form of transmission in the context of naturally occurring disease is not clear. Equine piroplasmosis has not been reported in horses in Canada to date.

In this study, information about any clinical manifestations of tick infestation, nor the horses' physical condition were not requested. It would have been beneficial to collect that information in order to better understand possible clinical manifestations of tick infestation in horses. Two of the owners voluntarily reported that their horses suffered oozing lesions in the areas of tick attachment and that they seemed very itchy. Interestingly, only *D. variabilis* males were received from these horses, and while one male tick was attached (time of attachment unknown), none were engorged. There are no descriptions in the literature that relate the severity of reaction to a tick bite to the sex or life stage of the tick. The fact that only male ticks were identified on these two horses does not rule out the possibility that the observed reaction was due to the bite of another tick. In fact, the observation that only one male tick was attached and none was engorged may suggest that these ticks were not responsible for the observed reactions. It is thus possible that female ticks, which attach for longer periods of time and feed more, caused the reported reactions. Hypersensitivity reactions in response to infestation with *Dermacentor* and *Ixodes* have been reported in horses (123) and the clinical significance of *Dermacentor* infestation in horses warrants further investigation. *D. albipictus* infestation can cause severe anemia and debilitation in moose (121). Similar effects of *Dermacentor* tick infestation in horses are not reported but cannot exclude the possibility that severe infestation might carry health risks.

No ticks were received in the fall and winter months and the end of summer 2013 (January 2012, September-December 2012, January 2013 and August and September 2013). As adult stages of *D. andersoni* and *D. variabilis,* the two species that were most frequently received, have their peak activity in the spring and summer, the lack of submissions in the fall and winter months was expected for these species. It is also possible that many horses are not pastured in these colder months and therefore are less likely to be infested with ticks such as *D. albipictus.* As unfortunately owners were not asked about pasture housing in the submission forms, it is not possible to assess the likelihood of this possibility. Another possible explanation is that fall and especially winter may be associated with decreased outdoor activity of horse owners and, consequently, decreased frequency of grooming and

inspection of their horses. This may explain why *D. albipictus,* whose adults start to be active as early as January (3) were not submitted until February of each year.

No *Ixodes* were received in this study. In general, horses may be infested with adult stages of *Ixodes* in the fall months, or with immature stages (larvae and nymphs) in the spring and summer (3). *Ixodes* species (Figure 5.2) may be difficult to identify due to their smaller size relative to the *Dermacentor* species (Figure 5.1), which may in part explain the fact that no *Ixodes* were received for this study. Established populations of *Ixodes* have not been reported in SK to date; however, adventitious *Ixodes* have likely been responsible for the occurrence of EGA in at least 2 horses that did not travel outside of SK for years before diagnosis (2, and personal communication, Dr. Alain Fafard). Failure to identify *Ixodes* in this study therefore does not rule out the occurrence of these ticks in SK. The rate and prevalence of infection with *A. phagocytophilum* in adventitious *Ixodes* species has been described elsewhere (40,79,90,124,125). It was previously suggested that adventitious *Ixodes* species arriving in areas which are not endemic for *Ixodes scapularis* may gradually establish populations due to anticipated climate change favoring their survival and establishment (64,76,81,82,110). Once established, ticks may further become endemic for tick-borne diseases such as EGA and LB, which would increase the risk for horses in SK to acquire these tick-borne pathogens. The study reported here may serve as a good baseline for future studies that could evaluate changes in tick exposure of horses to monitor the predicted expansion of *Ixodes* habitats in Canada.

A limitation of the study presented here was the fact that complementary active surveillance by collecting ticks from horses in SK was not pursued. Passive surveillance is limited in that collection is not done systematically or done by trained investigators. Passive surveillance may have resulted in over-representation or under-representation of certain locations and horses from which ticks were collected. Observation of ticks on horses may be difficult at times, especially if immature stages are present. It is possible that many ticks went unnoticed. Management factors such as the use of the horses, grooming and inspection habits are likely to vary between owners and trainers. It is possible that information about the study was not equally distributed to horse owners, resulting in a biased submission pattern. This could have potentially caused a bias in the number of ticks submitted, location of submission and number of submissions. It is also possible that other species of ticks infest horses in SK but were not submitted for the study. Yet, this is the first report describing tick species

infesting horses in SK in a time period of 2 years, including the description of geographical distribution from where ticks were collected from horses.

In summary, the findings of this study suggest that a variety of *Dermacentor* ticks infest horses in SK. The absence of relevant travel history of the horses suggests that all of them acquired the ticks within SK. As was expected, and partially supporting the study hypothesis, ticks that are known to be established in SK, i.e. *D. albipictus*, *D. andersoni* and *D. variabilis* were received from horses in SK. *Ixodes* species, which may occur as adventitious ticks in SK, were not received from horses in this study which does not support the second part of study hypothesis. A longer-term surveillance, including active surveillance, of horse ticks in SK may be warranted to further characterize the ticks infesting horses in SK and to monitor predicted changes in tick habitats.

5.6 Figures

Figure 5.1. The American dog tick, *Dermacentor variabilis*. Clockwise (from top left): nymph, larva, male, female. The figure is presented with permission from Laura Harrington (Department of Entomology, Cornell University, Ithaca, NY); pictures were taken by Kent Loeffler. Sizes of *D. albipictus* and *D. andersoni* are similar to those of *D. variabilis.*

Figures 5.2. The blacklegged tick, *Ixodes scapularis*. Clockwise (from bottom left): female, nymph, male. The figure is presented with permission from Laura Harrington (Department of Entomology, Cornell University, Ithaca, NY); pictures were taken by Kent Loeffler.

Size and morphology of *I. pacificus* are similar to those of *I. scapularis.*
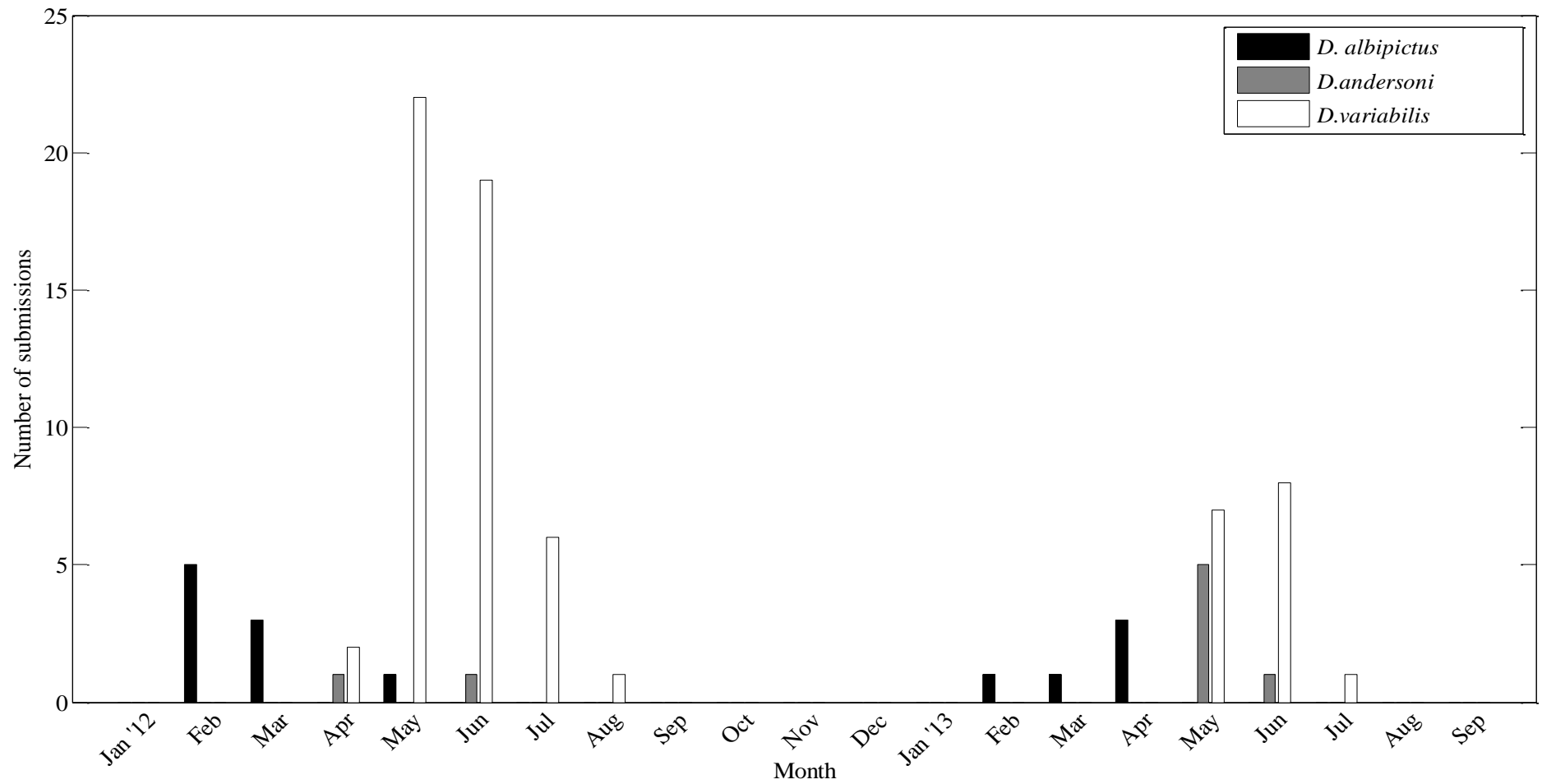
Figure 5.3. Tick submissions (number by species and month) received from horses in Saskatchewan between January 2012 and September 2013. All ticks were *Dermacentor* species.

Figure 5.4. Ticks (number by species and month) received from horses in Saskatchewan between January 2012 and September 2013. All ticks were *Dermacentor* species.

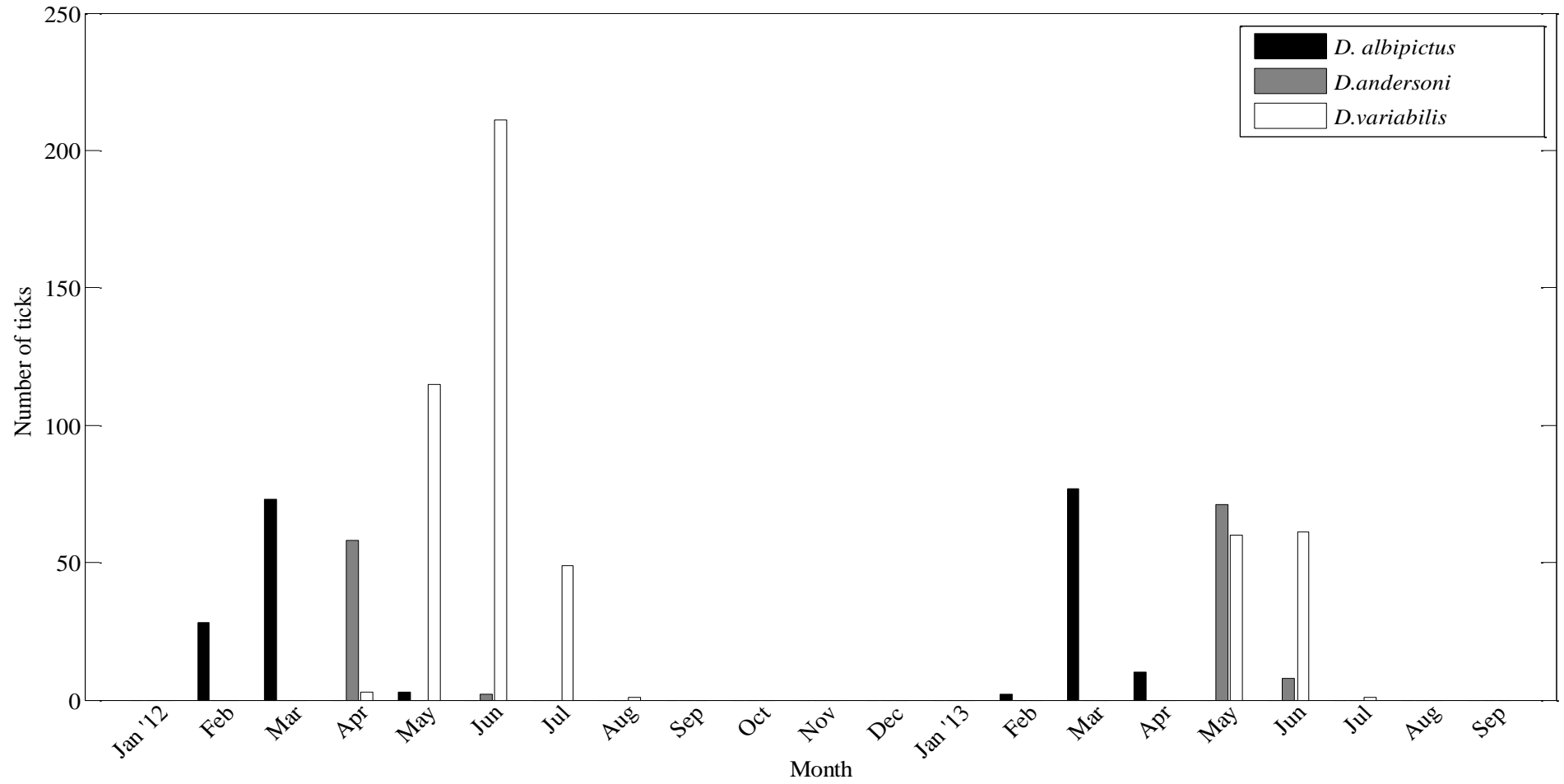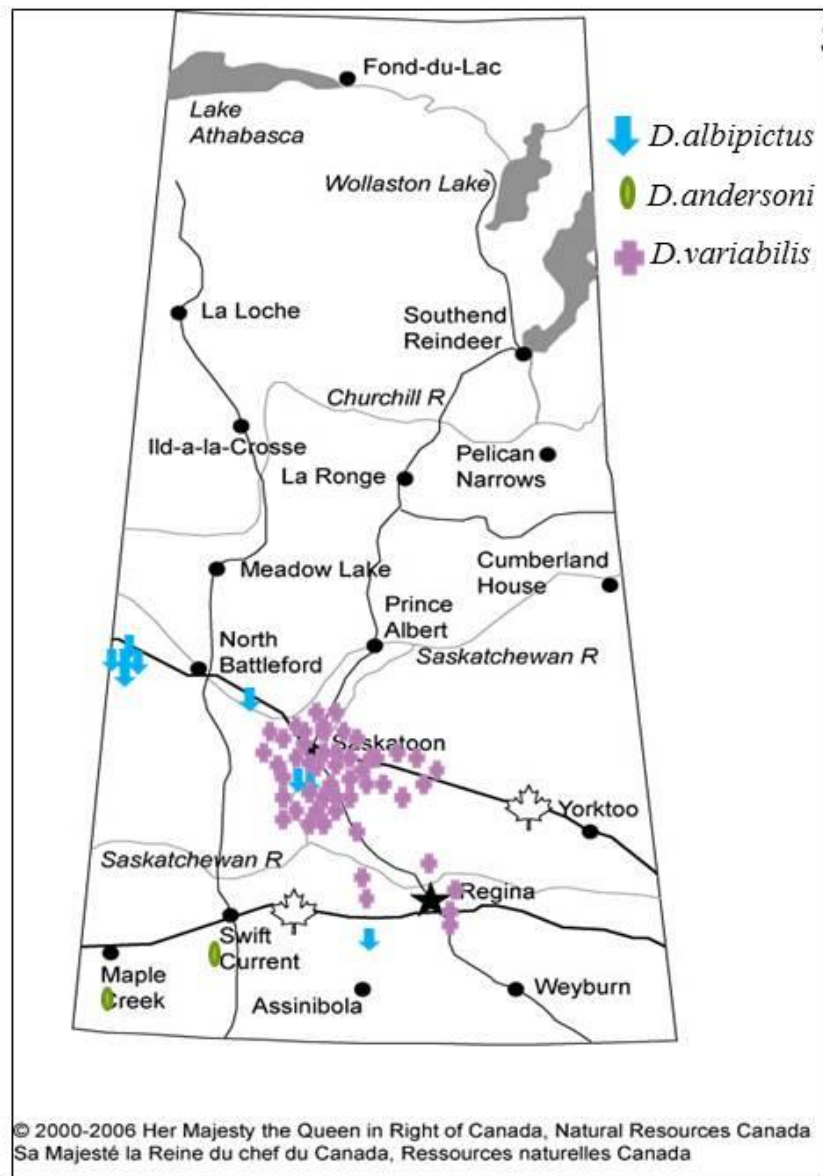**Figure 5.5.** Geographic distribution of tick submissions in SK (February-August 2012). Each symbol represents one submission. The average number of ticks per submission was 9 (range 1-94). There were 60 submissions originating from over 60 horses; 2 submissions contained ticks collected from 3 or more horses.
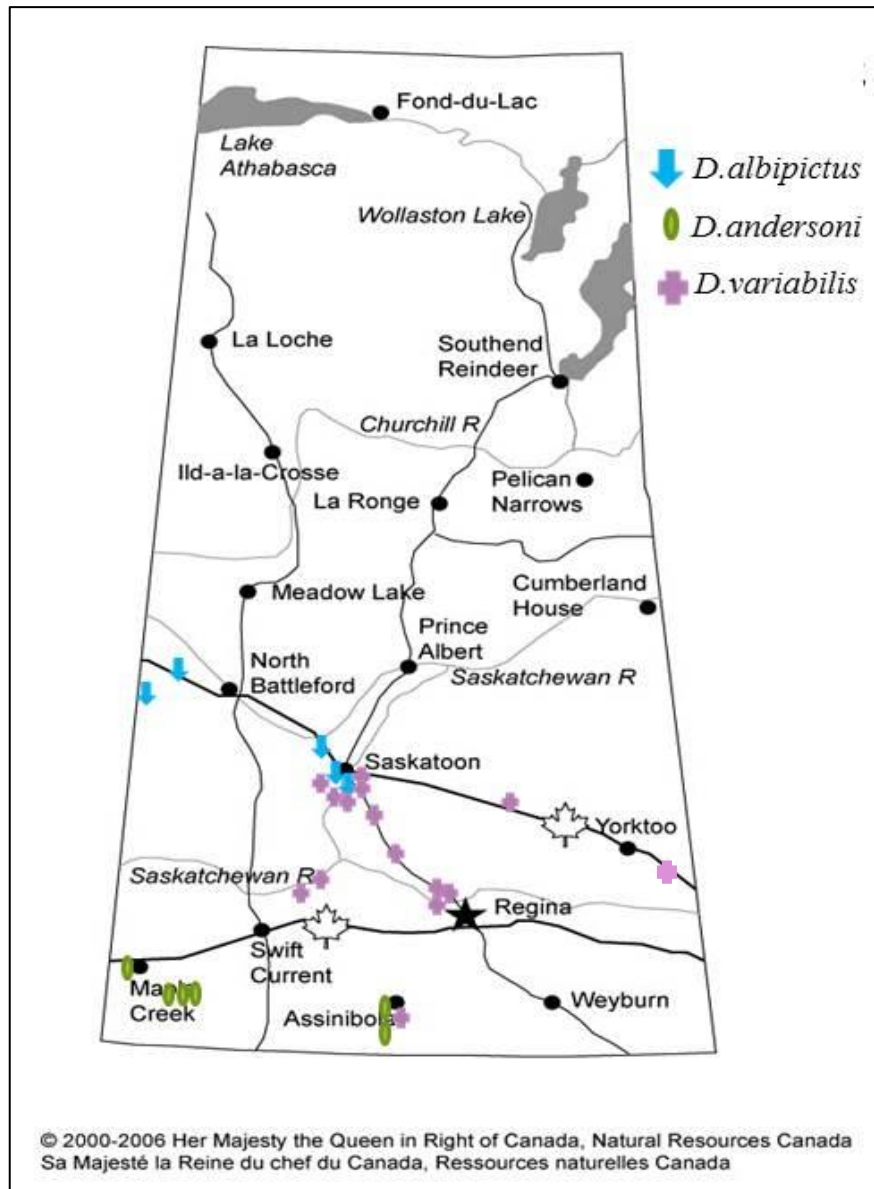
Figure 5.6. Geographic distribution of tick submissions from horses in SK (February-July 2013). Each symbol represents one submission. The average number of ticks per submission was 11 (range 1-78). There were 26 submissions originating from over 26 horses; 1 submission contained ticks collected from 2 or more horses.

5.7 Acknowledgments

6.   GENERAL DISCUSSION AND CONCLUSIONS

The studies presented in this thesis evaluated different aspects of horse exposure to the tick-borne diseases equine granulocytic anaplasmosis (EGA) and Lyme borreliosis (LB) in Canada. As evidence for the expansion of the geographic range of their common vector is increasing (38,64,75,85), and EGA and LB have been reported in horses in Canada (4–8), a better understanding of the current risk of exposure for horses in at least parts of Canada was desirable.

In the first study, it was hypothesized that seroprevalence of EGA and LB in SK, MB and ON is low.   A total of 376 equine serum samples from SK (n=202), MB (n=140) and ON (n=34) were tested using a point-of-care ELISA. For EGA, seropositive horses were found in SK and MB, and for LB, seropositive horses were found in SK, MB and ON. While a low seroprevalence for EGA and LB supported our first hypothesis, the small sample size did not allow statistical analysis to assess whether seroprevalence differed among provinces and additional studies are needed to answer this question. Yet, the finding of seropositive horses for EGA and LB in this study and previously reported cases of EGA in Canada support the need to consider EGA and LB in veterinary practice in Canada.

As part of the first study, test results of the point-of-care ELISA were compared to the results obtained when re-testing the same samples by laboratory-based serologic tests. The hypothesis was that the assessment of a sample as seropositive or seronegative for EGA or LB, respectively, will not differ between the tests. A lack of agreement between the point-of-care ELISA and an IFA for EGA, and only fair agreement between the point-of-care ELISA and an ELISA confirmed with WB for LB were found. Thus, our second hypothesis was rejected. The fact that seropositive horses were found in this study, together with the increasing concern over expansion of the geographic range of the vector, emphasizes the need for practical and reliable diagnostic tests for tick-borne diseases in horses. As varying levels of agreement were found between the point-of-care ELISA and laboratory-based serologic tests in the first study, further investigation of the agreement between available serologic tests was needed.

In the second study the hypothesis was that the assessment of a sample as seropositive or seronegative for EGA or LB, respectively, is independent of the specific testing method used. A new set of equine serum samples (n=50) was tested by point-of-care ELISA and the samples were also tested for antibodies against *A. phagocytophilum* by IFA in two referral

laboratories, and for antibodies against B. *burgdorferi* by IFA, ELISA confirmed with WB and Equine Lyme multiplex assay in three referral laboratories. A lack of agreement was found between all serologic tests for EGA. Agreement between serologic tests for LB ranged from poor to fair. Thus our third hypothesis was rejected. It is suggested that differences in test results for the same samples were due to the different test methods and antigens used; for example, some assays use whole cell organisms while others use a variety of specific surface antigens. Generally speaking, laboratory-based serologic tests in referral laboratories yielded a higher number of positive results compared to the point-of-care ELISA. As this study was designed to detect seropositive, but not necessarily actively infected horses, it is possible that the point-of-care ELISA yielded false negative results for LB as it may not detect all antibodies that are produced during different stages of infection or post exposure. The point-of-care ELISA is designed to only detect antibodies produced during active LB (against VlsE antigens) (28), and is therefore not optimal for detection of antibodies in cases of very acute infection or in exposed but not actively infected animals. However, it is also possible that false positive results were obtained in referral serologic tests for EGA and LB due to cross-reactivity with antibodies against similar organisms. Interestingly, 14% of the samples that were tested for this study were seropositive for both EGA and LB, suggesting that co-exposure in horses in Canada should be taken into consideration.

As historical or clinical data for the tested horses were not available, it is not possible to draw any conclusions about the clinical usefulness of the evaluated serologic tests. Although the aim was not to compare test performance for detection of antibodies in clinical cases, it is important to note that test interpretation in the context of clinical diagnosis of EGA and LB requires consideration of the clinical signs and history of exposure. Future studies should aim to standardize serologic tests for EGA and LB in horses, and establish approaches for screening horses with regards to methodology and interpretation. This is particularly important in non-endemic areas (86) where the positive predictive value of serologic testing is low, such as is the case when assessing seropositivity in horses in Canada where tick-borne diseases are currently uncommon.

As a fair number of seropositive samples were found in the first two studies, it was of interest to investigate whether seropositive horses were likely exposed within Canada and to identify potential risk factors for exposure of horses to EGA and LB in Canada. In the third study, it was hypothesized that management factors do not differ between horses that are seropositive or seronegative for EGA or LB, respectively. As samples for the first two studies

were obtained without identifying information, a mail-out survey was designed to contact horse owners. 392 surveys were sent out to the owners of the tested horses; to maintain confidentiality, surveys had to be mailed out via the laboratories that supplied the samples and the veterinary practices that originally submitted the samples. Surveys pertained to signalment of the horses, timing of pasture access, tick infestation history, province of residence and history of travel as well as previous diagnosis of EGA or LB by a veterinarian. Unfortunately, response rate was low at 11.5% and we could not conduct statistical analysis to assess differences in management factors between seropositive and seronegative horses. Thus, we could not truly test our fourth hypothesis. However, several interesting potential differences were noted and may serve to generate hypotheses for future studies. It was noticed that the majority of seropositive horses were pastured in the fall months and, according to their place of residence and lack of recent travel history, were likely to have encountered the diseases within Canada. The median age of the seropositive horses for EGA was higher (12 years) than the median age of the seronegative horses (3 years). While older horses were previously reported to present with more severe clinical signs, potential differences in the rate of infection with EGA in adult horses have not yet been reported. None of the seropositive horses were reported to be infested with ticks, which was surprising although it could be explained by a lack of recognition of horse ticks by the owners. The data indicated trends related to the exposure of horses to the causative organisms of EGA and LB within Canada, which will be important to further investigate in future studies.

The fourth study addressed the fact that, in general, data about ticks infesting horses are rare in the literature and lacking in Canada. It was surprising that tick infestation was not reported for seropositive horses in the previous study and thus, the aim was to describe the ticks infesting horses. The focus was on horses from SK, where a case of EGA had been reported (6) although *Ixodes* species are not known to be endemic in this province. The relative ease of recruiting participants and the direct applicability to horse owners in SK were important considerations in the design of the fourth study. It was hypothesized that tick species known to be established in SK, as well as adventitious ticks, can be found on horses in SK. A passive surveillance study of horse ticks took place in 2012 and 2013. Ticks were received and identified with the collaboration of Dr. Neil Chilton's parasitology laboratory at the Department of Biology (U of S). Species, sex, life stage and stage of engorgement were recorded. A total of 833 ticks, collected from over 86 horses, were received between February and August 2012 and between February and July 2013. All ticks were *Dermacentor* species,

which were previously reported to occur in SK (1,3). Timing of submission of specific tick species and life stages was compatible with the ticks' reported life cycle (1,3). A future study, preferably involving active surveillance of ticks on horses in SK, would likely help to further characterize and understand patterns of ticks infesting horses in SK. Although none of the ticks received for this study were expected to put horses at risk for tick-borne diseases, the possible clinical relevance of the infestation with these *Dermacentor* species may warrant further investigation. As evidence for exposure of horses in SK to adventitious *Ixodes* exists, and given the published predictions for expansion of tick habitats, studies to monitor changes in tick habitats in SK may further be warranted.

6.1. Future studies

Seropositive horses for EGA and LB can be found in Canada. According to a point-of-care test, seroprevalence of EGA and LB was low. Continuous assessment of seroprevalence in horses in Canada is warranted in order to monitor changes in level of exposure.

Despite the low seroprevalence of tick-borne diseases in horses at the present time, there is a need to establish and standardize approaches to serologic testing and screening for EGA and LB, as the risk for exposure to the tick vectors in Canada is expected to increase (40,64,77,81,82,95). Future studies should address the lack of gold standard serologic tests for EGA and LB in horses. Validation should include confirmation with bacteriological and molecular methods, or pathology, of known or experimentally infected animals. Standardization of test methods and interpretation should ideally be implemented.

According to the survey study, horses seropositive for EGA and LB were most likely exposed to the causative organisms within Canada. Future studies should address possible risk factors, specifically pasture access in the fall, for increased exposure in horses in Canada.

Horses in SK were found to be infested with 3 *Dermacentor* species that are known to be established in SK. *Ixodes* species were not received from horses in SK although evidence exists for the exposure of at least 2 horses in SK to adventitious *Ixodes*. Ongoing active and passive surveillance efforts are needed to monitor changes in tick infestation in horses in SK.

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