Eco-Epidemiology and Treatment of Babesiosis in Cervids

by

Ellie L. Milnes

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Doctor of Veterinary Science

in
Pathobiology

Guelph, Ontario, Canada

© Ellie L. Milnes, September, 2018
ABSTRACT

ECO-EPIDEMIOLOGY AND TREATMENT OF BABESIOSIS IN CERVIDS

Ellie L. Milnes
University of Guelph, 2018
Advisor(s):
Nicole Nemeth
Dorothee Bienzle

*Babesia odocoilei*, a protozoan hemoparasite of white-tailed deer (*Odocoileus virginianus*) transmitted by *Ixodes scapularis* ticks, is an increasingly recognized cause of disease in cervids in North America. Following an outbreak of babesiosis in reindeer (*Rangifer tarandus tarandus*) and wapiti (*Cervus canadensis*) at the Toronto Zoo in Ontario, Canada, we utilized a prospective postmortem survey to investigate the prevalence of *B. odocoilei* in wild, farmed, and zoo cervids in Ontario (*n*=270; 2016-2018) by polymerase chain reaction (PCR) and sequencing of spleen sample extracts. *Babesia odocoilei* was identified in 1.4% (2/142) of farmed red deer (*Cervus elaphus*), 4.4% (3/68) of wild white-tailed deer, and 3.4% (1/29) of captive wapiti. Wild white-tailed deer are the candidate wildlife reservoir for *B. odocoilei* in Ontario. Additionally, we designed a study to investigate the hypothesis that birds can disperse *B. odocoilei*-infected ticks along migratory flyways. Birds (*n* = 1,102) were captured during spring migration; the prevalence of *I. scapularis* infestation was 3.2% in 2016 and 6.7% in 2017, and 0.2% of birds carried one or more *I. scapularis* ticks that tested PCR-positive for *B. odocoilei*. Blanket dragging for questing ticks in southern Ontario revealed a minimum infection prevalence for *B. odocoilei* of up to 4.1% in ticks found in environments used by wild cervids. *Babesia odocoilei* can cause acute hemolytic crisis...
in susceptible cervids, thus evidence-based drug treatment protocols are needed to manage the disease. A single intramuscular injection of the anti/protozoal drug imidocarb dipropionate at 3.0 mg/kg may be useful for treatment of cervid babesiosis. To investigate this claim, a pharmacokinetic study of imidocarb was performed in 10 white-tailed deer. Plasma concentrations of imidocarb were determined using high-performance liquid chromatography. The disposition of plasma imidocarb was best characterised by a two-compartment open model, with rapid distribution and slow elimination. The mean ± SD maximal imidocarb concentration was 824.92 ± 1.55 ng/mL at 36.47 ± 1.38 minutes post injection. Plasma imidocarb concentrations were comparable to those effective for the treatment of babesiosis in domestic cattle. Clinical efficacy studies are needed to confirm the appropriate dosage regimen in cervids.
ACKNOWLEDGEMENTS

This work was generously supported by the American Association of Zoo Veterinarians Wild Animal Health Fund; the British Veterinary Zoological Society Zebra Foundation Scholarship; the Toronto Zoological Foundation; the Natural Sciences and Engineering Research Council of Canada; the Wilson Ornithological Society Research Grant; and the Canadian Foundation for Innovation. My DVSc stipend was provided by the Toronto Zoological Foundation.

For assistance with deer and tick sample collection, field work, and laboratory work I would like to thank: Alex Léveillé, Alexandra Reid and staff of the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA), Antonia Dibernardo, the hunters of Caldwell First Nation, Christina Lawrence, Don Thornton, Dorothee Bienzle, Doug Campbell, Erin Harkness, Grace Thornton, Hannah Bagnall, Janessa Price, John Barta, Mark Conboy and the staff and volunteers of Long Point Bird Observatory and Bird Studies Canada, OMAFRA, Kent Charlton, Lenny Shirose, Malika Ladak, Marg Stalker, Mary Ellen Clark, Robin Lindsay, Stephanie Sparling and the staff of Toronto Animal Services, Samantha Allen, Sarah Brisson, Simon Hollamby, Stu Mackenzie, Tami Sauder, Tarra Degazio, Tammy Dobbie and the staff of Parks Canada at Point Pelee National Park, Thisuri Eagalle, Rob Kirkpatrick, and keepers and staff of the Toronto Zoo.

For assistance with the pharmacokinetic study I would like to thank: Murray Woodbury, Adam Hering, Brian Tapscott, Claire Janse van Rensburg, Jeff Miedema and the staff of Townsend Butchers, Karen Gesy, Mitch Cooper, Patrick Robertson, Rob Stevens, Ronette Gehring, Ron Johnson, Saad Enouri, Sam Lee, Shannon Toy, Shelly Lefler, Tami Sauder, Todd Shury, Yu Gu, and the staff of the University of Saskatchewan Specialized Livestock Research Facility.

At the Toronto Zoo I would like to thank Andrea Dada, Cassia Devison, Chris Dutton, Cédric Larouche, Dawn Mihailovic, Graham Crawshaw, Julie Digianomenico, Michelle Lovering, Pauline Delnatte, Simon Hollamby, Steph Fleming, Adriana Pastor, and Tasha Long.

Thank you to my advisors Nicole Nemeth, Dale Smith, and Dorothee Bienzle, and my advisory committee John Barta, Pauline Delnatte, and Ron Johnson.
TABLE OF CONTENTS

Abstract ................................................................................................................................. ii
Acknowledgements .............................................................................................................. iv
Table of Contents ................................................................................................................. v
List of Tables ......................................................................................................................... ix
List of Figures ......................................................................................................................... x
List of Abbreviations ........................................................................................................... xi
List of Appendices ............................................................................................................... xii

1  Literature Review.............................................................................................................. 1
  1.1  Introduction to parasites of the genus Babesia.............................................................. 1
    1.1.1  Taxonomy of the genus Babesia............................................................................... 1
    1.1.2  Life cycle of Babesia species parasites..................................................................... 1
    1.1.3  Identification and characterization of Babesia species............................................. 3
    1.1.4  Immune response of the vertebrate host................................................................. 5
    1.1.5  Pathogenesis of clinical babesiosis......................................................................... 6
    1.1.6  Zoonotic Babesia species...................................................................................... 7
    1.1.7  Parasites of the genus Theileria............................................................................. 7
  1.2  Babesiosis in cervids...................................................................................................... 8
    1.2.1  Introduction to babesiosis in cervids ..................................................................... 8
    1.2.2  Laboratory diagnosis of cervid babesiosis............................................................ 20
    1.2.3  Pathology of cervid babesiosis.............................................................................. 22
    1.2.4  Cervid theileriosis............................................................................................... 24
  1.3  Treatment and prevention of cervid babesiosis............................................................ 24
1.3.1 Babesiacidal drugs.................................................................24
1.3.2 Supportive treatment for acute hemolytic crisis.................................26
1.3.3 Acaricidal treatment and prevention of tick infestation........................26
1.4 Ticks as vectors of cervid babesiosis in North America............................27
  1.4.1 Ticks belonging to the genus *Ixodes*............................................27
  1.4.2 Range expansion of *Ixodes scapularis* into Canada..........................29
  1.4.3 Diversity of *Babesia* species infecting questing Ixodid ticks..............30
1.5 Study rationale and objectives................................................................31

2 Molecular detection of *Babesia odocoilei* in wild, farmed, and zoo cervids in Ontario, Canada .................................................................32
  2.1 Abstract .........................................................................................32
  2.2 Introduction .......................................................................................32
  2.3 Materials and Methods .......................................................................33
    2.3.1 Study area and sample sources......................................................33
    2.3.2 Sample collection .........................................................................34
    2.3.3 Laboratory analyses ......................................................................34
  2.4 Results ..............................................................................................37
    2.4.1 PCR of spleen samples ..................................................................37
    2.4.2 DNA sequencing of PCR-positive spleen samples..........................38
  2.5 Discussion ..........................................................................................41

3 *Babesia odocoilei* and zoonotic pathogens identified from *Ixodes scapularis* ticks in southern Ontario, Canada .........................................................44
  3.1 Abstract ............................................................................................44
  3.2 Introduction ........................................................................................44
  3.3 Materials and methods .........................................................................46
3.3.1 Bird sampling and study area ................................................................. 46
3.3.2 Tick dragging and opportunistic collection of questing and host-feeding ticks ................................................................. 46
3.3.3 Taxonomic identification of ticks .......................................................... 47
3.3.4 Polymerase chain reaction (PCR) and DNA sequencing ....................... 47
3.3.5 Molecular analysis of tick DNA extracts for Babesia odocoilei and DNA sequencing ................................................................. 48
3.3.6 Molecular analysis of ticks for zoonotic pathogens ................................ 48
3.4 Results ................................................................................................. 49
3.4.1 Bird-borne ticks ................................................................................... 49
3.4.2 Tick dragging and opportunistic collection of questing and host-feeding ticks .............................................................................. 50
3.4.3 Babesia odocoilei PCR test results ....................................................... 50
3.4.4 Zoonotic pathogen PCR test results .................................................... 51
3.4.5 DNA sequencing to confirm Babesia odocoilei in PCR positive ticks .... 51
3.5 Discussion ............................................................................................ 58

4 Pharmacokinetics of imidocarb dipropionate in white-tailed deer (Odocoileus virginianus) after single intramuscular administration ................................................................. 62
4.1 Abstract ................................................................................................. 62
4.2 Introduction ........................................................................................... 62
4.3 Materials and Methods .......................................................................... 63
4.3.1 Animals .............................................................................................. 63
4.3.2 Experimental design .......................................................................... 64
4.3.3 Reagents and chemicals ..................................................................... 64
4.3.4 Assay method validation ..................................................................... 64
4.3.5 Assay analytical method ..................................................................... 65
4.3.6 Pharmacokinetic data analysis

4.4 Results

4.4.1 Imidocarb pharmacokinetics in deer

4.5 Discussion

5 Conclusions and Future Directions

References

Appendix: Supplementary Data for Chapter 3
LIST OF TABLES

Table 1.1 Babesia spp. organisms reported to cause clinical disease in cervids, and their pathogenicity in bovids and humans..............................................................11

Table 1.2 Literature review of all Babesia species identified globally in cervids. ..........12

Table 2.1 Polymerase chain reaction amplification primers and sequencing primers for nuclear 18S rDNA loci used in the identification of Babesia odocoilei from cervid tissue samples. ........................................................................................................36

Table 2.2 Wild, farmed, and zoo cervids sampled between May 2016 and January 2018 in Ontario, Canada, and tested for Babesia odocoilei infection by PCR using piroplasm-specific primers on DNA extracted from spleen samples.......................................................39

Table 3.1 Bird species that harbored ticks in spring 2016 and 2017 at Long Point Bird Observatory, Ontario, Canada, and the results of pathogen testing of Ixodes scapularis ticks. .............................................................................................................................53

Table 3.2 Ixodes scapularis ticks collected from Long Point Bird Observatory (LPBO), Point Pelee National Park (PPNP), and Toronto Zoo (TZ) in 2016 and 2017 and results of PCR testing for selected pathogens. ...............................................................................................57

Table 4.1 Pharmacokinetic parameters (mean ± SD) of imidocarb dipropionate in white-tailed deer (Odocoileus virginianus) after intramuscular administration at a dose of 3.0 mg/kg body weight........................................................................................................67

Table 5.1 Bird species investigated for ticks and found to be uninfested in spring 2016 and spring 2017 at Long Point Bird Observatory, Ontario, Canada.............................93
LIST OF FIGURES

Figure 1.1 Life cycle of parasites of the genus *Babesia* in the mammalian host and the tick vector. Redrawn from Hunfeld et al. (2008) .......................................................... 3

Figure 1.2 Geolocations of documented *Babesia odocoilei* infections in cervids in the United States and Canada ........................................................................................................ 16

Figure 1.3 *Babesia odocoilei* infected erythrocytes in a peripheral blood smear from a Toronto Zoo reindeer (*Rangifer tarandus tarandus*) with clinical cervid babesiosis ....21

Figure 2.1 Locations of all cervids that underwent postmortem *Babesia odocoilei* testing by PCR of spleen samples from 2016-2018 in Ontario, Canada (n=270) .................. 40

Figure 3.1 In 2016-2017, questing and host-feeding ticks were collected from three field sites in southern Ontario, Canada, to investigate the prevalence of *Babesia odocoilei*, *Babesia microti*, *Borrelia burgdorferi*, *Borrelia miyamotoi*, and *Anaplasma phagocytophilum* by polymerase chain reaction .......................................................... 52

Figure 4.1 Semi-logarithmic plot of mean plasma concentration vs. time curve of imidocarb dipropionate in white-tailed deer (*Odocoileus virginianus*) after single-dose intramuscular injection at 3.0 mg/kg body weight .................................................. 68
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rDNA</td>
<td>18S ribosomal DNA</td>
</tr>
<tr>
<td>AHL</td>
<td>Animal Health Laboratory</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>CHWC</td>
<td>Canadian Wildlife Health Cooperative</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IFA</td>
<td>immunofluorescence assay</td>
</tr>
<tr>
<td>LPBO</td>
<td>Long Point Bird Observatory</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>NML</td>
<td>National Microbiology Laboratory</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>TZ</td>
<td>Toronto Zoo</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PK</td>
<td>pharmacokinetic</td>
</tr>
<tr>
<td>PPNP</td>
<td>Point Pelee National Park</td>
</tr>
</tbody>
</table>
LIST OF APPENDICES

Appendix: Supplementary data for chapter 3.........................................................93
1 Literature Review

1.1 Introduction to parasites of the genus Babesia

1.1.1 Taxonomy of the genus Babesia

Babesiosis has long been recognised as a clinical entity in domestic animals, and the taxonomy of the genus Babesia (Family Babesiidae, Order Piroplasmida, Class Aconoidasida) has been repeatedly discussed and revised in the scientific literature (Levine 1971). Future taxonomic revisions are likely to occur with further phylogenetic analyses based on molecular criteria (Penzhorn 2006).

Traditionally, Babesia spp. were grouped according to their morphology in erythrocytes, the clinical course of infection, and host- and vector-specificity; however, Babesia species identifications derived from these criteria are unreliable and often inconsistent with phylogenetic analyses based on molecular data (Gray et al. 2010). The best method of discriminating between different Babesia species is characterization at the molecular level (Malandrin et al. 2010).

The term piroplasmosis is sometimes used to describe the disease caused by phenotypically similar apicomplexan protozoan parasites that have a life cycle stage within mammalian erythrocytes; namely, Babesia, Theileria, and Cytauxzoon spp. (Yabsley and Shock 2013). A detailed discussion of these is beyond the scope of this review; however, infection with Theileria spp. can cause hemolytic anemia and is a differential diagnosis for cervid babesiosis, and thus is reviewed briefly below.

1.1.2 Life cycle of Babesia species parasites

Similar to other members of the phylum Apicomplexa, Babesia undergoes a complex life cycle that involves both an arthropod vector and a mammalian host (Hunfeld et al. 2008). All species of Babesia are naturally transmitted by the bite of an infected Ixodid tick, with transstadial transmission (from larva to nymph, or nymph to adult) within the tick vector (Uilenberg 2006). Transovarial transmission within the tick vector occurs in some Babesia species (e.g., B. divergens and B. capreoli) and not in others (e.g., B. microti); infections that are transmitted transovarially persist at least to the adult stage of the next generation (Nikol’skii and Pozov 1972). There is evidence that Babesia infection may persist over several tick generations even without new infections derived from blood meals (Uilenberg 2006).
The life cycle of *Babesia* spp. is highly complex (Figure 1.1) and may be divided into three stages (Levine 1985):

(i) **Gametogony**: sexual reproduction, occurring within the tick vector (definitive host). Gametocytes develop into gametes and fuse within the tick gut, forming a zygote \( Z \) that develops into a kinete \( K \). Kinetes invade and replicate in the hemolymph of the tick.

(ii) **Sporogony**: occurs when kinetes invade the salivary glands of the tick vector, resulting in the production of sporozoites \( Sz \). The mammalian (intermediate) host becomes infected when the tick takes a blood meal, and sporozoites are transferred into the mammalian host’s blood stream. Sporozoites invade erythrocytes and develop into trophozoites \( T \).

(iii) **Merogony**: occurs within the erythrocytes of the mammalian host. Trophozoites divide by binary fission and grow to a large size while the nucleus divides repeatedly (merogony), producing a meront filled with merozoites \( M \); the meront eventually ruptures, destroying the host erythrocyte and liberating the merozoites, which reinitiate the infective cycle in the host by invading new erythrocytes and becoming trophozoites that again divide by binary fission. *In vitro*, the one cycle of *B. divergens* asexual intraerythrocytic reproduction is completed in eight hours (Valentin et al. 1991). A small percentage of merozoites do not divide but develop into non-dividing gametocytes \( G \) within the erythrocyte. Gametocytes are ingested when an Ixodid tick takes a blood meal from the mammalian host and undergo gametogony in the tick gut.

The maintenance of *Babesia* spp. within an ecosystem is dependent on the presence of both an invertebrate definitive host and vector (e.g., an Ixodid tick) and a mammalian intermediate host (Hunfeld et al. 2008).
1.1.3 Identification and characterization of *Babesia* species

1.1.3.1 Morphological features

Prior to the development of molecular techniques, *Babesia* spp. infections were diagnosed by parasite morphology on blood smears. *In vitro* culture of *Babesia* spp. in erythrocytes has been used to amplify the number of organisms for diagnostic purposes when there is low parasitemia in the host (Thomford et al. 1993). The differential diagnosis of *Babesia* spp. from other parasites with intraerythrocytic life cycle stages (e.g. *Plasmodium* and *Theileria* spp.) can be difficult, especially in geographic regions where more than one endemic hemoparasite species occurs in the same mammalian
host, although parasite morphology is sufficient for diagnosis at the genus level in many cases; for example, Babesia spp. do not form pigment within erythrocytes, unlike Plasmodium spp. (Uilenberg 2006). Co-infection of the same vertebrate host with Babesia spp. and other genera of hemoparasites is reported such as B. odocoilei and T. cervi in wapiti (Cervus canadensis)\(^1\) in Indiana, USA (Gallatin et al. 2003).

In general, Babesia spp. fall into two morphologic groups based on the size of the intraerythrocytic forms when viewed by light microscopy in stained blood smears: “small” babesias measuring less than 2.5 μm in length such as B. divergens, B. microti, and B. odocoilei, and “large” babesias measuring 2.5 to 5.0 μm diameter such as B. bovis and B. caballi (Samuel et al. 2001).

1.1.3.2 Molecular characterization

Polymerase chain reaction (PCR) and sequencing of PCR products allows direct molecular detection and identification of Babesia spp. organisms at the species level. The nuclear small subunit ribosomal DNA (18S rDNA) is the most frequently used target in phylogenetic studies of Babesia spp. Published protocols are designed with a primer pair such as BAB-GF2/BAB-GR2 framing the V4 hyper-variable region of the 18S rDNA gene: this internal fragment is highly conserved and can amplify a wide variety of Babesia and Theileria species (Zanet et al. 2014). Complete sequencing of the 18S rDNA gene suggests that the piroplasms should be divided into five distinct clades (Criado-Fornelio et al. 2003):

(i) Group 1: Archaeopiroplasmids (B. microti group). This group includes Babesia spp. that are morphologically small and are more closely related to Theileria spp. than to the large Babesia spp. Examples are B. rodhaini, B. leo, and B. microti.

(ii) Group 2: Prototheilerids (western US Theileria-like group), including B. conradae.

(iii) Group 3: Theilerids (Theileria group), containing Theileria and Cytauxzoon spp. e.g. T. orientalis (Yabsley and Shock, 2013).

---

\(^1\) Wapiti are also known as North American elk and are often described as such in the literature (Cervus canadensis, previously C. elaphus canadensis).
(iv) Group 4: Babesids (first Babesia sensu stricto group). This includes the morphologically 'small' Babesia that are not closely related to B. microti (e.g., B. divergens, B. odocoilei, B. capreoli, and B. venatorum), and the canid isolates B. canis and B. gibsoni. This group contains all of the Babesia species known to cause clinical disease in cervids.

(v) Group 5: Ungulibabesids (second Babesia sensu stricto group), comprised mainly of Babesia isolates from ungulates (e.g. B. caballi, B. bigemina, B. ovis, and B. bovis).

The following example illustrates the importance of molecular methods in the characterization of Babesia species. The European species B. capreoli and B. divergens are indistinguishable by morphology and serology, but have distinct epidemiology, pathology, and host specificity (Malandrin et al. 2010). Babesia capreoli is a parasite of roe deer (Capreolus capreolus) that causes clinical disease in reindeer and is not known to be zoonotic or infectious to cattle, whereas B. divergens is an important zoonosis and cattle pathogen (Gray et al. 2010). The full sequences of the V4 region of the 18S rDNA genes of B. capreoli and B. divergens differ from each other at only three nucleotide positions: positions 631, 663 and 1,637 (Malandrin et al. 2010). Consequently, amplification and sequencing of the region that includes these polymorphic positions is required to reliably discriminate between these two species. In addition to 18S rDNA sequences, the use of molecular targets other than 18S rDNA may be used to better establish relationships between species and strains of apicomplexan parasites, for example the mitochondrial cytochrome c oxidase subunit I (COI) gene (Barta 2001).

1.1.4 Immune response of the vertebrate host

Both humoral and cellular factors are involved in the immune response of the intermediate host to infection with Babesia spp. The spleen removes infected cells from circulation and performs an essential function in protection against uncontrolled replication of Babesia spp. parasites, which may otherwise lead to acute hemolytic disease, as shown by the association between splenectomy and increased risk of clinical babesiosis in humans (Herwaldt et al. 2003). Humoral immunity is thought to be of limited importance, because the parasite is only vulnerable to antibody attack during the times in its life cycle when it is not within a host erythrocyte (i.e., when sporozoites are free in the bloodstream immediately after inoculation by a tick bite, and when an infected erythrocyte ruptures and releases free merozoites that then invade new erythrocytes) (Homer et al. 2000). Seroconversion in naturally-exposed white-tailed deer fawns (Odocoileus virginianus) in B. odocoilei-endemic areas of Texas coincides with the seasonal peaks of vector activity, but the degree to which seroconversion is
protective against the development of clinical disease in cervids is unknown (Waldrup et al. 1992).

Domestic cattle less than six months of age have a strong innate immunity against *B. divergens*, *B. bovis*, and *B. bigemina* that is independent of passive antibody transfer from the mother and can be eliminated by splenectomy (Zintl et al. 2005). The underlying mechanism of inverse age-related immunity in cattle is poorly understood, and it does not seem to occur with *Babesia* spp. infection in cervids, small ruminants, or dogs (Zintl et al. 2005). Even in animal species that are not resistant to *Babesia* spp. infection at a young age, a state of enzootic stability occurs in *Babesia*-endemic regions: animals tend to develop a non-clinical but persistent low-level parasitemia (i.e., less than 0.01% of erythrocytes parasitized), in which the parasite appears to exist in a state of balance with the host immune system and no clinical disease is seen (Penzhorn 2006). Parasitized erythrocytes are removed due to the action of CD4+ T helper cells that produce IFN-gamma and promote macrophage-mediated killing of infected erythrocytes, which are then removed from circulation in the spleen (Homer et al. 2000).

### 1.1.5 Pathogenesis of clinical babesiosis

The pathogenicity of *Babesia* spp. varies with parasite species, host species, and host immune status. Some host-parasite combinations result in highly pathogenic infections, and immunocompromised individuals are at a greater risk of presenting with severe acute disease than healthy immune competent individuals (Gray et al. 2010).

The primary pathologic process resulting from infection is hemolysis and anemia. Direct lysis of erythrocytes is due to parasite replication (i.e., the growth of trophozoites and merozoites induces erythrocyte rupture, liberating new parasites into circulation, which subsequently invade and destroy other erythrocytes) and erythrophagocytosis by phagocytic cells (Homer et al. 2000). Acute hemolytic disease is most likely to occur in immunocompromised animals, or in individuals that are exposed to the parasite as immunologically-naïve adults. Stressful situations that may predispose to the development of acute disease include malnutrition, reproductive stress (rut, calving), and concurrent disease (Gallatin et al. 2003). Thrombocytopenia and disseminated intravascular coagulation may result from tumor necrosis factor-mediated inflammatory responses (Homer et al. 2000). Hemoglobinuric nephrosis may occur in severe disease (Andrews et al. 2008).
1.1.6 Zoonotic Babesia species

The majority of human babesiosis cases are due to infection with *B. microti* (several hundred cases to date reported in North America) or to *B. divergens* (approximately 40 cases total reported in Europe) (Gray et al. 2010). The Centers for Disease Control and Prevention declared human babesiosis a nationally notifiable condition in the US in 2011, which subsequently increased the annual number of infections reported in the literature (Yabsley and Shock 2013). Transmission occurs by tick bite, or via blood transfusion from subclinically infected blood donors. The clinical features are nonspecific and range from subclinical infections or a mild flu-like illness with *B. microti*, to a fatal malaria-like hemolytic syndrome for *B. divergens* (Gray et al. 2010). Immunosuppression, particularly previous splenectomy, is a major risk factor. *Babesia duncani* (formerly known as *Babesia* sp. WA1), *Babesia* sp. CA1-CA4, *B. divergens*-like MO1, and other unnamed *Babesia* species have all been described as the cause of recent sporadic cases of human babesiosis in the US (Hunfeld et al. 2008). The wildlife reservoirs of these *Babesia* species are unknown. In Europe, several cases of *B. venatorum* have occurred in immunosuppressed patients (Herwaldt et al. 2003).

1.1.7 Parasites of the genus Theileria

*Theileria* spp. are tick-transmitted apicomplexan parasites (Family Theileriidae, Order Piroplasmida) that are closely related to *Babesia* spp. As described above, the life cycles of parasites in these genera are similar in their complexity; however, *Theileria* spp. differ from *Babesia* spp. in that *Theileria* spp. have a pre-erythrocytic life stage during which sporozoites initially penetrate leukocytes in which they develop into schizonts (Garner et al. 2012). Like *Babesia* spp., *Theileria* spp. are also transmitted by ixodid ticks: competent vectors include *Amblyomma americanum* in North America, and *Rhipicephalus*, *Hyalomma*, and *Haemaphysalis* spp. worldwide (Bishop et al. 2004). Worldwide, several *Theileria* species are known to be highly pathogenic to cattle (e.g., *T. orientalis* in New Zealand), and there are two case reports of hemolytic anemia in reindeer attributed to the cervid parasite *Theileria cervi* in the United States (Garner et al. 2012). The intraerythrocytic stage of *Theileria* spp. may be morphologically indistinguishable on blood smear from small *Babesia* species; molecular diagnostic testing is therefore recommended to differentiate *Theileria* and *Babesia* spp. organisms in geographic regions where both parasites occur, and co-infections in the same mammalian host are described (Gallatin et al. 2003). No *Theileria* spp. are known to be zoonotic (Penzhorn 2006).
1.2 Babesiosis in cervids

1.2.1 Introduction to babesiosis in cervids

Recently, the taxonomy of the true deer (family Cervidae) and their relatives the chevrotains (family Tragulidae) and the musk deer (family Moschidae) has changed due to advances in molecular phylogeny. All three deer families are classified as ruminant members of the order Artiodactyla (Masters and Flach 2015). In this review, the term 'cervid' refers to members of the family Cervidae. To date, babesiosis has not been reported in the Tragulidae or Moschidae.

The earliest documented cases of hemolytic anemia in cervids associated with piroplasmosis were in Russia in the early twentieth century: a fatal disease of domestic reindeer (*Rangifer tarandus tarandus*) characterised by icterus, pallor, and splenomegaly was reported by Chambers in 1921. The disease occurred in the Arctic tundra in late summer and was suspected to be vectored by ticks. Intraerythrocytic organisms were observed on blood smears from clinical cases, and the parasite was named *Piroplasma tarandi rangiferus* (Chambers, 1921). Yakimoff and Kolmakoff reported more cases of reindeer piroplasmosis in 1929 (cited by Nilsson et al. in 1965) and named the organism *Franciella tarandi rangifer*. These reports predate molecular diagnostics, so the identity of the causative organism in these cases remains unknown.

Currently, three species of *Babesia* are known to cause clinical disease in cervids (Table 1.1): *B. odocoilei* in North America (Holman et al. 2000), and *B. capreoli* and *B. venatorum* in Europe (Wiegmann et al. 2015). The role of *B. divergens* as an agent of hemolytic anemia in cervids is currently under debate, but current molecular evidence indicates that *B. capreoli* was misidentified as *B. divergens* in historic case reports (Malandrin et al. 2010).

1.2.1.1 Babesia species identified in cervids globally

In addition to the three *Babesia* species known to cause clinical disease in cervids, many subclinical *Babesia* spp. infections have been identified in different wild and captive cervid species in Europe and North America, with distinct geographic distributions based on the presence of competent mammalian hosts and invertebrate vectors (Table 1.2). The true species identity of some of these attributions is questionable; it is not always clear whether or how the *Babesia* species attributed by the authors were confirmed, and consequently the historical literature prior to the advent of
PCR and genetic sequencing should be interpreted with caution (Penzhorn 2006). Molecular sequencing is the key to resolving these uncertainties (Gray et al. 2010).

### 1.2.1.2 Cervid babesiosis in North America

*Babesia odocoilei* was historically endemic in the southeastern United States (Texas, New Mexico, and Oklahoma) in clinically healthy wild white-tailed deer (Spindler et al. 1958), the presumptive natural reservoir host (Emerson and Wright 1968, 1970). Clinical disease is rarely reported in white-tailed deer, although it can be induced experimentally in immunosuppressed, previously subclinically-infected individuals (Perry et al. 1985; Holman et al. 2000). Natural infection rates have not been extensively investigated and appear to vary widely with cervid species and geographic location. Figure 1.2 shows the currently reported distribution of *B. odocoilei* infection in wild white-tailed deer in North America. Surveillance for this parasite in wildlife is rarely performed and reported cases of *B. odocoilei* infection in wild deer in the literature are usually found in epidemiological investigations of disease outbreaks in captive animals (Schoelkopf et al. 2005). Evaluation of blood smears from white-tailed deer in Texas found a *B. odocoilei* parasitemia prevalence of 2% (Waldrup et al. 1989). In contrast, serosurveillance for prevalence of *B. odocoilei* exposure in wild white-tailed deer in Oklahoma and Texas varied from absent to 100% per sample site (Waldrup et al. 1992). In a captive wapiti herd in Indiana, 58% of animals screened had been exposed to *B. odocoilei* herds (Gallatin et al. 2003). The vector and definitive host of *B. odocoilei* in North America is the black-legged tick, *Ixodes scapularis* (Waldrup et al. 1990).

*Babesia odocoilei* is the only identified etiologic agent of clinical babesiosis in North American cervids to date. Overt hemolytic disease due to *B. odocoilei* infection has been reported in reindeer, caribou (*R. tarandus caribou*), and wapiti (Table 1.1). *B. odocoilei*-associated hemolytic anemia in captive cervids was first reported in the United States in 1993 but has only recently emerged in Canada with cases reported since 2012 (Pattullo et al. 2013). Figure 1.2 illustrates the geolocations of clinical babesiosis cases reported in the literature.

Molecular diagnostic techniques have yielded the discovery that clinically silent *Babesia* spp. infections occur in a wide range of endemic and exotic ruminants belonging to families Cervidae and Bovidae in North America (Table 1.2). *Babesia odocoilei* has been isolated from clinically healthy zoo markhor (*Capra falconeri*), muntjac (*Muntiacus reevesi*), and yak (*Bos grunniens*), and wild desert bighorn sheep (*Ovis canadensis nelsoni*) (Holman et al. 2000; Holman et al. 2003; Bartlett et al. 2009; Schoelkopf et al. 2005). Fulminant hemolytic anemia resulting from natural infection with *B. odocoilei* is
reported in musk oxen (*Ovibos moschatus*); this is the only report of *B. odocoilei*-associated clinical disease in a bovid (Schoelkopf et al. 2005). *Babesia odocoilei* has never been identified in domestic Bovidae.
Table 1.1 *Babesia* spp. organisms reported to cause clinical disease in cervids, and their pathogenicity in bovids and humans.

<table>
<thead>
<tr>
<th><em>Babesia</em> species</th>
<th>Geographic Location</th>
<th>Tick Vector</th>
<th>Major Reservoir Host</th>
<th>Cervids</th>
<th>Bovids</th>
<th>Zoonotic Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Clinical Disease</td>
<td>Sub-clinical Infection</td>
<td>Clinical Disease</td>
</tr>
<tr>
<td><em>Babesia capreoli</em></td>
<td>Europe</td>
<td>Ixodes ricinus</td>
<td>Roe deer (Capreolus capreolus)</td>
<td>Reindeer</td>
<td>Red deer (Cervus elaphus)</td>
<td>Chamois (Rupicapra rupicapra)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reindeer (Rangifer tarandus tarandus)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Roe deer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Babesia odocoilei</em></td>
<td>North America</td>
<td>Ixodes scapularis</td>
<td>White-tailed deer (Odocoileus virginianus)</td>
<td>Reindeer</td>
<td>Caribou (Rangifer tarandus caribou)</td>
<td>Desert bighorn sheep (Ovis canadensis nelsoni)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wapiti (Cervus canadensis)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>White-tailed deer (rare)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wapiti</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Muntjac (Muntiacus reevesi)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Babesia venatorum</em></td>
<td>Europe</td>
<td>Ixodes ricinus</td>
<td>Roe deer</td>
<td>Reindeer</td>
<td>Reindeer</td>
<td>Reindeer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.2 Literature review of all *Babesia* species identified globally in cervids.

<table>
<thead>
<tr>
<th>Vertebrate Host of Origin</th>
<th><em>Babesia</em> spp. as identified by authors</th>
<th>Geographic Location (Country or Region)</th>
<th>Wild or Captive Animal</th>
<th>Host Status</th>
<th>Diagnostic Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>European reindeer (Rangifer tarandus tarandus)</td>
<td><em>Babesia odocoilei</em></td>
<td>Canada</td>
<td>Captive</td>
<td>Acute babesiosis</td>
<td>PCR and sequencing</td>
<td>Mathieu et al. (2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>USA</td>
<td>Captive</td>
<td>Acute babesiosis</td>
<td>PCR and sequencing</td>
<td>Holman et al. (2003)</td>
</tr>
<tr>
<td></td>
<td><em>Babesia</em> odocoilei-like</td>
<td>Northern Europe</td>
<td>Captive</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Wiegmann et al. (2015)</td>
</tr>
<tr>
<td></td>
<td><em>Babesia</em> divergens&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Northern Europe</td>
<td>Captive</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Wiegmann et al. (2015)</td>
</tr>
<tr>
<td></td>
<td><em>Babesia</em> venatorum</td>
<td>Northern Europe</td>
<td>Captive</td>
<td>Acute babesiosis</td>
<td>PCR and sequencing</td>
<td>Robert et al. (2008)</td>
</tr>
<tr>
<td></td>
<td><em>Babesia</em> capreoli</td>
<td>Netherlands</td>
<td>Captive</td>
<td>Acute babesiosis</td>
<td>PCR and sequencing</td>
<td>Bos et al. (2017)</td>
</tr>
<tr>
<td></td>
<td><em>Babesia</em> sp.</td>
<td>USA (California)</td>
<td>Captive</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Kjemtrup et al. (2000)</td>
</tr>
<tr>
<td></td>
<td><em>Babesia</em> jakimovi</td>
<td>Russia</td>
<td>Semi-domesticated</td>
<td>Acute babesiosis</td>
<td>Parasite morphology</td>
<td>Holman et al. (2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nikof'skii et al. (1977)</td>
</tr>
<tr>
<td>Woodland caribou (Rangifer tarandus caribou)</td>
<td><em>Babesia odocoilei</em></td>
<td>USA</td>
<td>Captive</td>
<td>Acute babesiosis</td>
<td>Protozoal culture</td>
<td>Holman (1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PCR and sequencing</td>
<td>Holman et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Petri et al. (1995)</td>
</tr>
</tbody>
</table>

<sup>2</sup> The earliest reference to cervid babesiosis in each species and geographic location is listed.
<table>
<thead>
<tr>
<th>Vertebrate Host of Origin</th>
<th>Babesia spp. as identified by authors</th>
<th>Geographic Location (Country or Region)</th>
<th>Wild or Captive Animal</th>
<th>Host Status</th>
<th>Diagnostic Method</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wapiti (Cervus canadensis)</td>
<td>Babesia odocoilei</td>
<td>Canada</td>
<td>Captive</td>
<td>Acute babesiosis</td>
<td>PCR and sequencing</td>
<td>Pattullo et al. (2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Parasitemia with chronic debilitating disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>USA</td>
<td>Captive</td>
<td>Acute babesiosis</td>
<td>Protozoal culture</td>
<td>Holman et al. (1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IFA</td>
<td>Gallatin et al. (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>USA (south and southeast)</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Canadian Wildlife Health Cooperative (2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Parasitemia with chronic debilitating disease (rare)</td>
<td>Perry et al. (1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Parasite morphology</td>
<td>(Spindler et al. 1958)</td>
</tr>
<tr>
<td></td>
<td>Babesia bigemina</td>
<td>USA (Texas)</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Holman et al. (2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mexico</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Cantu et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Babesia cf. bovis</td>
<td>USA (Texas)</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Ramos et al. (2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mexico</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Cantu et al. (2007)</td>
</tr>
<tr>
<td>Roe deer (Capreolus capreolus)</td>
<td>Babesia capreoli</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Tampieri et al. (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Captive</td>
<td>Acute babesiosis</td>
<td>Parasite morphology</td>
<td>Dorrenstein et al. (1996)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Babesia venatorum</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Zanet et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Babesia divergens</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Duh (2005)</td>
</tr>
<tr>
<td></td>
<td>Babesia bigemina</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Zanet et al. (2014)</td>
</tr>
<tr>
<td>Vertebrate Host of Origin</td>
<td>Babesia spp. as identified by authors</td>
<td>Geographic Location (Country or Region)</td>
<td>Wild or Captive Animal</td>
<td>Host Status</td>
<td>Diagnostic Method</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------------------------------</td>
<td>----------------------------------------</td>
<td>-----------------------</td>
<td>-------------</td>
<td>-------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Red deer (Cervus elaphus)</td>
<td>Babesia capreoli</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Hoby et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>Babesia divergens²</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Duh (2005)</td>
</tr>
<tr>
<td></td>
<td>Babesia pceorum</td>
<td>Europe</td>
<td>Captive</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Jouglin et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Babesia bigemina</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Zanet et al. (2014)</td>
</tr>
<tr>
<td>Moose (Alces alces)</td>
<td>Babesia capreoli</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Pūraitė et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>Babesia odocoilei-like</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Pūraitė et al. (2016)</td>
</tr>
<tr>
<td>Fallow deer (Dama dama)</td>
<td>Babesia capreoli</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Rehbein et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Babesia bigemina</td>
<td>Mexico</td>
<td>Captive</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>García-Vásquez et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>Babesia bovis</td>
<td>Mexico</td>
<td>Captive</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>García-Vásquez et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>Babesia sp.</td>
<td>USA (California)</td>
<td>Captive</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Kjemtrup et al. (2000)</td>
</tr>
<tr>
<td>Siberian roe deer (Capreolus pygargus)</td>
<td>Babesia jakimovi</td>
<td>Russia</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>Parasite morphology</td>
<td>Nikof'kii et al. (1977)</td>
</tr>
<tr>
<td>Pampas deer (Ozotocerus bezoarticus)</td>
<td>Babesia bigemina</td>
<td>Brazil</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Silveira et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Babesia bovis</td>
<td>Brazil</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Silveira et al. (2013)</td>
</tr>
<tr>
<td>Brown brocket deer (Mazama gouazoubira)</td>
<td>Babesia bigemina</td>
<td>Brazil</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>da Silveira et al. (2011)</td>
</tr>
<tr>
<td>Marsh deer (Blastocerus dichotomus)</td>
<td>Babesia bovis</td>
<td>Brazil</td>
<td>Captive</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>da Silveira et al. (2011)</td>
</tr>
</tbody>
</table>

3 Babesia divergens has been claimed to occur in asymptomatic roe deer, red deer, and reindeer in Europe, but the species identity based on partial 18S rDNA sequences is questionable as stated by Malandrin et al. (2010).
<table>
<thead>
<tr>
<th>Vertebrate Host of Origin</th>
<th>Babesia spp. as identified by authors</th>
<th>Geographic Location (Country or Region)</th>
<th>Wild or Captive Animal</th>
<th>Host Status</th>
<th>Diagnostic Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mule deer (Odocoileus hemionus)</td>
<td>Babesia sp.</td>
<td>USA (California)</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>Parasite morphology, In vitro culture, PCR and sequencing</td>
<td>Thomford et al. (1993), Kjemtrup et al. (2000)</td>
</tr>
<tr>
<td>Muntjac (Muntiacus reevesi)</td>
<td>Babesia odocoilei</td>
<td>USA</td>
<td>Captive</td>
<td>Asymptomatic carrier</td>
<td>PCR and sequencing</td>
<td>Schoelkopf et al. (2005)</td>
</tr>
<tr>
<td>Hybrid Sika deer (Cervus nippon) x Red deer (Cervus elaphus)</td>
<td>Babesia capreoli</td>
<td>Europe</td>
<td>Wild</td>
<td>Asymptomatic carrier</td>
<td>Parasite morphology</td>
<td>Gray et al. (1990)</td>
</tr>
</tbody>
</table>
Figure 1.2 Geolocations of documented *Babesia odocoilei* infections in cervids in the United States and Canada. Dark grey shading indicates states and provinces where *Babesia odocoilei* has been reported in wild white-tailed deer (*Odocoileus virginianus*), the presumptive natural reservoir host: Massachusetts, Minnesota, New Mexico, Oklahoma, Saskatchewan, Tennessee, Texas, and Virginia. Black points represent clinical cases of babesiosis in captive cervids (Table 1.2)
1.2.1.3 Cervid babesiosis in Europe

In wild and captive cervids in Europe, three Babesia species are of interest: B. divergens, the causative agent of cattle babesiosis and of sporadic human cases, B. capreoli, which mainly infects cervids but also causes fatal hemolytic anemia in free-ranging alpine chamois (Rupicapra rupicapra), and B. venatorum, which is pathogenic to reindeer (Kik et al. 2011) and is a rare disease of humans (Herwaldt et al. 2003). These three species are morphologically and serologically indistinguishable and share the same tick vector, Ixodes ricinus. All three species belong to the Babesia sensu strictu clade and are genetically very closely related, especially B. divergens and B. capreoli, which only differ by three nucleotides on the 18S rDNA gene (Malandrin et al. 2010). Current molecular data suggest that historic reports of B. divergens and B. divergens-like organisms in European cervids (Langton et al. 2003) were likely misidentifications based on incomplete 18S rDNA sequences; the true species identity is probably B. capreoli (Malandrin et al. 2010).

Roe deer (Capreolus capreolus) are the wildlife reservoir for B. venatorum (formerly called Babesia sp. EU1), a zoonotic pathogen that has caused fatal babesiosis in zoo reindeer in the Netherlands (Kik et al. 2011), Germany (Wiegmann et al. 2015), and Switzerland (Robert et al. 2008). Roe deer are also asymptomatic hosts of B. capreoli, the causative agent of fatal hemolytic anemia in zoo reindeer in the Netherlands (Bos et al. 2017) and in free-ranging chamois in Switzerland (Hoby et al. 2009). There is a single report of clinical hemolytic disease in a captive roe deer in the Netherlands that was attributed to B. capreoli based on parasite morphology, but no molecular diagnostics were performed in this case and the species identity cannot be verified (Dorrestein et al. 1996). Acute babesiosis can be induced experimentally in roe deer by immunosuppression of asymptomatic carrier animals (Enigk and Friedhoff 1962a).

Various Babesia species are found in wild European red deer (Cervus elaphus), moose (Alces alces), and (feral) fallow deer (Dama dama) (Table 1.2). Clinical disease has not been reported in these species. In northern Europe, sporadic cases of clinical babesiosis are reported in captive reindeer and are associated with B. venatorum and B. capreoli infection (Wiegmann et al. 2015). Subclinical infections may be common in reindeer: a PCR survey found that 23.6% of clinically healthy zoo reindeer surveyed in Germany were hosts of various Babesia species, including the two known known reindeer pathogens, B. capreoli and B. venatorum (Wiegmann et al. 2015).
1.2.1.4 Experimental transmission of Babesia odocoilei in cervids

Transfusion of blood containing B. odocoilei-infected erythrocytes from naturally infected clinically healthy white-tailed deer into a splenectomised white-tailed deer resulted in fulminant hemolytic crisis in the recipient (Emerson and Wright 1968). The results of experimental transfusion into spleen-intact white-tailed deer have been variable: one trial produced chronic disease characterised by emaciation and anemia six to 12 months following infection (Emerson and Wright 1968); another study reported no clinical disease, although a slight drop in PCV occurred between days seven and 13 following inoculation (Waldrup 1991), and in both studies, the deer became carriers as confirmed by in vitro culture of B. odocoilei in homologous erythrocytes.

Babesia odocoilei isolates were obtained separately from a wapiti and a caribou with clinical babesiosis, and both isolates were subsequently inoculated into red deer (Emerson and Wright 1968). The red deer became carriers of B. odocoilei with low parasitemia, but no clinical disease was seen (Emerson and Wright 1968). Domestic cattle appear to be completely resistant to both experimental and field-transmitted B. odocoilei infection (Emerson and Wright 1968).

1.2.1.5 Cross infection of Babesia species between domestic cattle and cervids

The most important causes of babesiosis (‘tick fever’) in domestic cattle are B. bovis, B. bigemina (both of which have a worldwide distribution), and B. divergens in Europe. Other species that can infect cattle include B. major, B. ovata in Japan, B. occultans in South Africa, and B. jakimovi in Siberia (Uilenberg 2006).

The wildlife reservoir of B. jakimovi is reportedly the Siberian roe deer (Capreolus pygargus). Natural infection with associated hemolytic anemia is described historically in reindeer, but no molecular diagnostics have yet been reported for B. jakimovi; thus, the true species identity and importance of field transmission between cattle and cervids remains ambiguous (Nikol'skii et al. 1977).

Experimental infections with cattle-origin B. divergens failed to produce clinical disease in splenectomised cervids, including roe deer (Enigk and Friedhoff 1962b), red deer, and fallow deer (Gray et al. 1990). An experimental study in 1965 reported that reindeer experimentally-infected with B. divergens developed fulminant hemolytic disease; however, the true identity of the isolate as B. divergens is questionable because the study pre-dates molecular diagnostics (Nilsson et al. 1965). An epizootic of hemolytic
anemia originally attributed to *B. divergens* in captive reindeer in Scotland (Langton et al. 2003) is now thought to have been due to *B. capreoli* based on 18S rDNA sequence analysis (Malandrin et al. 2010). To date, only two published surveys have reported *B. divergens* 18S rDNA sequences isolated from cervids that have 100% sequence identity to *B. divergens* cattle isolates available in GenBank, and neither of these was associated with clinical disease in the cervids from which they originated (Silaghi et al. 2011; Wiegmann et al. 2015). To the author’s knowledge, there are no reliable reports (i.e., supported by molecular diagnostic testing) of naturally-occurring (i.e., field-transmitted) *B. divergens*-associated clinical disease in reindeer or other cervids.

*Babesia bovis* and *B. bigemina* have been identified by PCR from the blood of wild white-tailed deer in northeastern Mexico (Cantu et al. 2007), and an isolate obtained from game-ranched white-tailed deer in Texas had 99% sequence homology to 18S rDNA gene sequences from a cattle *B. bovis* isolate (Ramos et al. 2010). The role of white-tailed deer in the epidemiology of bovine babesiosis is uncertain, and further research is required, although evidence to date suggests that deer do not play an important role in the epidemiology of tick fever in domestic cattle in North America (Waldrup et al. 1989).

### 1.2.1.6 Zoonotic potential of *Babesia* species hosted by cervids

*Babesia venatorum* (previously known as *Babesia* sp. EU1) causes a moderately severe malaria-like syndrome in immunosuppressed humans (Herwaldt et al. 2003). *Babesia divergens* is an important zoonotic agent in Europe, and the epidemiologic role of deer in the sylvatic cycle of *B. divergens* is the subject of ongoing research (Duh et al. 2005). *Babesia odocoilei* and *B. capreoli* have never been implicated in human illness, despite their close phylogenetic relationship with zoonotic *B. divergens* (Armstrong et al. 1998).

### 1.2.1.7 Clinical signs of babesiosis in cervids

The incubation period following *B. odocoilei* transmission to cervids by *I. scapularis* ticks is at least six to 10 days (Waldrup 1991). *Babesia* sporozoites transmitted in the tick’s saliva directly infect mammalian erythrocytes. Clinical manifestations of cervid babesiosis are typical of *Babesia* spp. infections in other mammals, and vary according to parasite species and host factors, including species, age, and immune status. Sporadic cases, epizootics, and clinically silent infections have all been described in captive cervids. Subclinical infection may progress to clinical disease in the face of concurrent stressors (Mathieu et al. 2018). In one case report, acute babesiosis was
seen two weeks following a stressful event in a wapiti herd (Gallatin et al. 2003). Presenting signs range from mild anemia to an acute hemolytic syndrome characterised by severe anemia, lethargy, hemoglobinuria, icterus, pyrexia, recumbency, and death (Pattullo et al. 2013). Sudden death may be the presenting sign (Mathieu et al. 2018). A chronic form of babesiosis characterised by wasting, mild anemia, and low parasitemia is reported rarely in white-tailed deer (Perry et al. 1985) and wapiti (Pattullo et al. 2013).

1.2.2 Laboratory diagnosis of cervid babesiosis

1.2.2.1 Microscopic examination of blood

The *Babesia* species of clinical significance in cervids (i.e., *B. odocoilei*, *B. venatorum*, and *B. capreoli*) are morphologically similar to *B. divergens* and to each other. Organisms generally appear in the blood as single, paired or tetrad pyriform and ring-shaped organisms, often located peripherally in the erythrocyte in the accolé position (Figure 1.3). Microscopic examination of Giemsa-stained thick and thin peripheral blood smears is adequate to detect acute infections with high numbers of circulating parasites, but not for identification of carriers in which parasitemia levels are very low.

1.2.2.2 Serological tests

Serological methods including immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) are useful to detect animals that have been exposed to *Babesia* spp. However, they do not provide information about active infection unless serial rising titres (i.e., acute and convalescent) are documented, and cannot differentiate asymptomatic carriers from animals that have cleared the parasite. Serology may be negative in the early stages of acute babesiosis before the animal mounts a detectable immune response. Strong cross-reactions occur between different *Babesia* species, so serology cannot be used for species identification (Malandrin et al. 2010), although the use of serologic testing for this purpose is widely reported in the literature (Petrini et al. 1995).
Figure 1.3 *Babesia odocoilei* infected erythrocytes in a peripheral blood smear from a Toronto Zoo reindeer (*Rangifer tarandus tarandus*) with clinical cervid babesiosis. Many erythrocytes contain paired *Babesia odocoilei* parasites in the accolé position at the periphery of the erythrocyte.

1.2.2.3 *In vitro* cultivation

*In vitro* culture in mammalian erythrocytes is used to demonstrate the presence of carrier animals, in which *Babesia* spp. parasitemia is generally very low (Malandrin et al. 2004). Blood culture is useful because of its high sensitivity but is an unsuitable technique for routine diagnosis because it is labour-intensive and vulnerable to contamination.
1.2.2.4 Animal inoculation (xenodiagnosis)

Prior to the development of molecular diagnostic methods, inoculation of gerbils (*Meriones unguiculatus*) and hamsters (*Mesocricetus auratus*) was one of the most sensitive methods for detection of *B. divergens* and *B. microti* infections (Gray and Kaye 1991). Xenodiagnosis is generally no longer used for diagnostic purposes (Homer et al. 2000).

1.2.2.5 Polymerase chain reaction (PCR) and sequencing

Polymerase chain reaction (PCR) is a highly sensitive and specific diagnostic method, permitting the precise identification of morphologically and serologically indistinguishable *Babesia* species, usually by sequencing of the 18S rDNA gene (Lockerbie et al. 2014) although other targets such as the mitochondrial COI gene may be useful (Barta 2001). At the time of writing, there are no commercially-available diagnostic tests for *B. odocoilei* offered by any veterinary laboratory in North America. In research laboratories, PCR and sequencing for molecular identification of *Babesia* isolates can be successfully performed on fresh, frozen, and formalin-fixed paraffin-embedded tissues (Lockerbie et al. 2014).

1.2.3 Pathology of cervid babesiosis

1.2.3.1 Clinical pathology

Cervids with clinical babesiosis typically have a normocytic, normochromic anemia with hemoglobinuria (Ameri et al. 2012). Severely affected animals may die before significant anemia has set in, perhaps due to circulatory shock (Valli et al. 2015). The number of parasites visible in blood smears from cases of acute babesiosis is extremely variable: organisms were observed in between five and 20% of wapiti erythrocytes (Ameri et al. 2012), and in up to 80% of reindeer erythrocytes (Bartlett et al. 2009) in fatal *B. odocoilei* infections. Intraerythrocytic *Babesia* parasites may also be identified on blood smears from clinically normal cervids with asymptomatic parasitemia (Wiegmann et al. 2015).

An inflammatory leukogram may be present (Gallatin et al. 2003). The serum of animals with acute babesiosis is often hemolyzed or icteric due to intravascular hemolysis, which can interfere with biochemistry analysis (Bartlett et al. 2009). No clinical chemistry changes are pathognomonic for clinical babesiosis (Ameri et al. 2012), but in severe acute cases, hyperbilirubinemia and bilirubinuria often occur secondary to extravascular hemolysis, and azotemia may occur secondary to hemoglobinuric nephrosis (Homer et
al. 2000; Bartlett et al. 2009). Metabolic acidosis may result from increased lactate generation secondary to tissue hypoxia (Homer et al. 2000).

1.2.3.2 Gross pathology

The gross postmortem findings in cases of acute cervid babesiosis are non-specific and typical of an acute intravascular hemolytic crisis (Valli et al. 2015). Lesions may include hepatomegaly, splenomegaly, dark red to brown urine (hemoglobinuria), icterus, subepicardial and subendocardial petechiation and ecchymoses, pulmonary edema and hemorrhage, pericardial and thoracic effusions, and diffuse dark red to black discoloration of the kidneys (Kik et al. 2011).

1.2.3.3 Histopathology and pathogenesis

Histologic tissue changes are variable and are generally consistent with hemolytic disease (Bartlett et al. 2009). In severe cases, hepatic centrilobular and midzonal hepatocellular vacuolar degeneration and necrosis occur secondary to decreased hepatic perfusion and oxygenation as a result of hemolytic anemia (Kik et al. 2011). Other microscopic changes in the liver may include centrilobular congestion, centrilobular and portal lymphoplasmacytic aggregates, and Kupffer cell hemosiderosis and hypertrophy (Valli et al. 2015). Intrahepatic cholestasis is a consistent finding in acute babesiosis of wapiti and reindeer (Mathieu et al. 2018). Splenic hemosiderosis and erythrophagocytosis and generalized lymph node erythrophagocytosis reflect extravascular hemolysis (Kik et al. 2011). Splenic white pulp and lymph node germinal centers may be necrotic with generalized splenic congestion. In the kidney, hemoglobinuric nephropathy (pigmentary nephrosis with acute tubular degeneration) with dilated renal tubules containing granular hemoglobin casts, proximal tubular necrosis, and tubular epithelial cell enlargement with cytoplasmic hemosiderin or hemoglobin may be observed in severe cases (Bartlett et al. 2009). Parasitized erythrocytes may be seen within capillaries of various tissues, particularly interstitial capillaries in the kidney and in skeletal muscle (Valli et al. 2015).

Impression smears of parenchymatous organs may reveal intraerythrocytic *Babesia* spp. (Kik et al. 2011). In *B. bovis*-infected domestic cattle, sludging of parasitized erythrocytes within cerebral blood vessels is commonly observed, but this has not been reported in *Babesia*-infected cervids (Petrini et al. 1995).
1.2.3.4 Electron microscopy

Electron microscopy has been used to characterize the ultrastructural appearance of *B. odocoilei* (Droleskey et al. 1993). This pathogen is characterised by its close proximity to the erythrocyte membrane, referred to as the accolé position. Intraerythrocytic *B. odocoilei* merozoites and trophozoites are limited by a single membrane that contains ribosomes, rough endoplasmic reticulum, vesicles containing entrapped erythrocytic cytoplasm, and a single nucleus. Merozoites contain rhoptries that form an apical complex (Droleskey et al. 1993).

1.2.4 Cervid theileriosis

*Theileria* spp. are morphologically similar to *Babesia* spp., which is evident on blood smears, and thus theileriosis is a differential diagnosis for babesiosis. The two organisms are reliably differentiated by PCR and sequencing (Garner et al. 2012). *Theileria cervi* is reported from white-tailed deer in North America and is typically asymptomatic in this species. Co-infections with *B. odocoilei* and *T. cervi* occur in wapiti (Gallatin et al. 2003). A case of fatal hemolytic anemia attributed to *T. cervi* were recently reported in captive reindeer in Alabama (Garner et al. 2012). A high-mortality outbreak of hemolytic anemia in semi-domesticated reindeer in Russia was attributed to *T. tarandirangiferis*, but the true identity of the causative organism is unknown because the report predates molecular sequencing (Tashkinov 1976).

1.3 Treatment and prevention of cervid babesiosis

1.3.1 Babesiacidal drugs

Successful treatment of clinical babesiosis depends on early diagnosis and prompt administration of babesiacides, as well as aggressive supportive treatment (Bartlett et al. 2009). Several chemical compounds have been reported to be effective against *Babesia* spp. infections in domestic animals, including imidocarb, diminazene diaceturate, amicarbalide, trypan blue, clindamycin, and the tetracyclines. Imidocarb is generally considered to be the safest and most effective of these treatments (Vial and Gorenflot 2006).

1.3.1.1 Clinical pharmacology of imidocarb

Imidocarb is a carbanilide derivative and is usually administered as the dipropionate salt or the dihydrochloride salt. The mechanism of action of imidocarb is not well understood; the drug may interfere with the production or use of polyamines, or block
inositol entry into infected erythrocytes, thereby starving the intraerythrocytic parasite (Mosqueda et al. 2012). Domestic cattle treated with imidocarb in endemic regions are protected from the development of clinical disease for four to six weeks following treatment. As drug levels start to decline, mild subclinical infections occur, allowing sufficient exposure of the host to the parasite that a robust immune response develops (Vial and Gorenflot 2006). So far, imidocarb is the only babesiacidal drug demonstrated to clear the host of the parasite and allow the host to develop immunity to disease challenge (Lewis et al. 1981).

In domestic cattle, the recommended treatment regime is 1.0-3.0 mg/kg imidocarb dipropionate administered by intramuscular or subcutaneous injection (Andrews et al. 2008). One treatment is usually effective, but a second treatment may be desirable after two weeks if animals are persistently parasitemic (Vial and Gorenflot 2006). Imidocarb may also affect the parasites within the tick vector: in one experimental trial, B. bovis-infected ticks appeared to lose their infectivity and failed to transmit B. bovis to the next tick life stage after they were placed on animals recently treated with imidocarb (Kuttler 1975). Domestic sheep infected with B. ovis that were given a single treatment of imidocarb dipropionate at a dose of 1.2 mg/kg body weight by intramuscular injection suffered severe recrudescence, which necessitated retreatment after 10-14 days (McHardy et al. 1986).

A commonly reported adverse effect of imidocarb treatment is pain on injection. Overdosage with imidocarb may result in transient liver and muscle damage, and signs of cholinesterase inhibition may be observed (Vial and Gorenflot 2006). In horses, adverse effects described after imidocarb dipropionate treatment include depression, colic and diarrhoea that are attributed to the muscarinic effects of cholinesterase inhibition; administration of glycopyrrolate as a pre-treatment reduced the occurrence and severity of imidocarb-associated adverse effects in a clinical trial (Donnellan et al. 2013).

### 1.3.1.2 Imidocarb as a treatment for cervid babesiosis

Imidocarb is not licensed or labelled in North America for use in cervids, and as for most therapeutics used in cervids, dosing and treatment schedules have not been determined (Bachtold et al. 2016). The administration of babesiacidal compounds at subtherapeutic dosages theoretically can lead to the development of drug-resistant parasites, although this has not yet been reported for imidocarb in any species (Mosqueda et al. 2012). Plasma concentrations of imidocarb that may be effective for treatment and prophylaxis of babesiosis in cervids are not known. In cattle, an *in vitro*
minimum inhibitory concentration of 0.027-0.034 μg/mL is described for \( B. \) \textit{divergens}, a “large” \textit{Babesia} species that is phylogenetically closely related to the cervid \textit{Babesia} spp. (Brasseur et al. 1998).

Most reports of clinical babesiosis in cervids describe cases that died before they could be treated, although successful treatment of infected caribou and wapiti using drug regimens developed for domestic cattle has been described (Mathieu et al. 2018). Imidocarb dipropionate at 2.2-3.0 mg/kg body weight by intramuscular injection has been used successfully in captive wapiti and reindeer to treat clinical disease and prevent the development of disease in asymptomatic carriers; the frequency of treatments reported to be effective in both clinically- and subclinically-infected cervids vary from a single treatment to repeated doses on days 1, 2, 6, 9, and 21 (Bartlett et al. 2009). No adverse effects of imidocarb treatment have been reported in cervids.

1.3.2 Supportive treatment for acute hemolytic crisis

The prognosis is grave for animals that are debilitated by acute hemolytic disease associated with \textit{Babesia} spp. infection (Vial and Gorenflot 2006). Aggressive supportive treatment is required in these patients and should include anti-inflammatory drugs and fluid therapy to minimize the secondary renal effects. Based on treatment results in clinical cases in domestic ungulates, blood transfusions may be life-saving in very anemic animals, although this technique has not yet been reported as part of the clinical management of babesiosis in cervids. A review of the literature found no published information on cervid blood groups.

Anemia may continue to worsen for up to seven days following treatment with babesiacidal drugs due to the continued removal of parasitized erythrocytes from circulation (Bartlett et al. 2009).

1.3.3 Acaricidal treatment and prevention of tick infestation

Complete eradication of tick vectors from the environment is rarely a feasible disease management strategy, and thus, consideration should be given to maintaining enzootic stability of \textit{Babesia} spp. in endemic regions (Pastor and Milnes 2018). A strategic tick control program should integrate pasture management (removal of bushes and long grass) with application of acaricides. In a captive setting, tick control programs should include considerations for reducing the risk of development of resistance to acaricides among tick populations.
Topical amitraz or permethrin at the recommended label dose for cattle have been used safely and effectively in zoo cervids to control ticks (Gallatin et al. 2003); ivermectin at 0.4 mg/kg body weight (i.e., double the cattle dose) may also be administered subcutaneously or orally (Masters and Flach 2015). Wild deer should be excluded from outdoor zoo exhibits. Strategies such as culling (Stafford et al. 2003) or treatment of wild deer with an acaricide (using a passive applicator that applies amitraz to the deer as they feed at a bait station) may be used to reduce environmental tick burdens (Pound et al. 2000).

1.4 Ticks as vectors of cervid babesiosis in North America

Ticks (Order Ixodida, Class Arachnida) are hematophagous, obligate ectoparasites of terrestrial vertebrates. Two families of ticks are of veterinary importance: Argasid or soft ticks (Family Argasidae), and Ixodid or hard ticks (Family Ixodidae). The Ixodid tick *I. scapularis*, also known as the black-legged or deer tick, is the only known competent vector for *Babesia odocoilei* in North America (Waldrup et al. 1990).

1.4.1 Ticks belonging to the genus *Ixodes*

*Ixodes* is the largest genus of Ixodid ticks. All known *Ixodes* spp. are three-host ticks and generally have a two- to four-year life cycle in temperate regions (Sonenshine 1991). Their preferred habitats are grassy meadows and forests. Host-seeking behaviour (‘questing’) is stimulated by carbon dioxide, heat, and movement. A recent experimental field study showed that *I. scapularis* ticks from populations in the northern United States were more likely to ascend vegetation while questing for a host as compared to ticks of southern origin, suggesting that host-seeking behaviour is determined by both tick genetics and environmental conditions (Arsnoe et al. 2015).

1.4.1.1 *Ixodes scapularis*

*Ixodes scapularis* ticks are distributed discontinuously throughout eastern and southern North America, with the largest endemic populations in the northeastern United States (Keirans et al. 1996). Much of the older scientific literature refers to *I. dammini*, which in 1993 was determined to be conspecific with *I. scapularis* (Oliver et al. 1993). *Ixodes scapularis* is the primary vector of many diseases of public health and veterinary importance, including human and canine granulocytic anaplasmosis (*Anaplasma phagocytophilum*), babesiosis (*B. microti*), Powassan virus (lineage II; deer tick virus),
and Lyme borreliosis (*Borrelia burgdorferi*). It is the only proven competent vector of *Babesia odocoilei* (Waldrup et al. 1990).

### 1.4.1.2 Other tick vectors

Several cases of cervid babesiosis due to *B. odocoilei* have been reported in the Canadian province of Saskatchewan (Figure 1.2), where there are no known established *I. scapularis* populations (Pattullo et al. 2013). There may be an alternative, unknown vector responsible for disease transmission in these cases, such as Ixodid ticks of the genus *Dermacentor*, which are endemic in this region. *Dermacentor albipectis* ticks were found to be feeding on wapiti with clinical babesiosis in one case report (Holman et al. 2000). This association remains unproven and to date *B. odocoilei* DNA has never isolated from *Dermacentor* spp. ticks (Mathieu et al. 2018).

### 1.4.1.3 Life cycle of *Ixodes scapularis*

This three-host tick exhibits a two-to four-year life cycle across most of its range, and develops from an egg through larval, nymphal, and adult life stages by simple metamorphosis. During each life stage, the tick takes one blood meal from a vertebrate host and remains attached for three to eight days until completely engorged. An off-host inactive period of weeks or months follows each blood meal, and the tick then moults to the next life stage and begins to quest for a new host. *I. scapularis* are host generalists, reported to feed from at least 125 different host species, including mammals, birds, and lizards (Keirans et al. 1996). Each life stage has a host preference that differs based on habitat type and geography. Larval and nymphal ticks prefer smaller mammalian and ground-foraging avian hosts and most commonly feed on the white-footed mouse (*Peromyscus leucopus*), whereas adults prefer to feed on white-tailed deer and other large mammals. However, all stages may occur on less-preferred hosts. *Ixodes scapularis* are most abundant in areas where deer are also numerous (Sonenshine 1991).

In the province of Ontario, Canada, adult female *I. scapularis* engorge mainly in the fall, but feeding may also occur in winter and early spring (Clow et al. 2017). Adults survive over winter, and eggs are laid in the early summer. Larvae hatch and feed during May to September, and moult approximately two months after feeding. Nymphs generally feed during spring and early summer of the following year, and adults feed again in the fall. Each life cycle stage (i.e., larva, nymph and adult) can overwinter and survive for at least one year if unengorged (Keirans et al. 1996).
1.4.2 Range expansion of *Ixodes scapularis* into Canada

Over the past two decades, the geographic range of *I. scapularis* has expanded substantially (Ogden et al. 2008). In 1991, there was only one established *I. scapularis* population at Long Point in southern Ontario; by 2008, there were numerous endemic areas in the provinces of Ontario, Manitoba, Quebec, New Brunswick, and Nova Scotia (Ogden et al. 2008).

Ticks are unable to move long distances during off-host periods, so range expansion and the invasion of new habitats is a function of host movement. Mechanisms of tick invasion may include movement of ticks by migratory birds, deer, and small mammals and lizards (Madhav et al. 2004).

1.4.2.1 Migratory birds

Birds are not competent hosts for any known mammalian *Babesia* species, but the large-scale seasonal movements of migratory birds provide opportunities for bird-associated ectoparasites to disperse rapidly over large geographic distances. It is estimated that 0.9 to 3.5% of migratory birds carry ticks during their northward migration to breeding grounds, which translates to between 50 and 175 million ticks being dispersed across Canada each spring (Ogden et al. 2008). Several studies have investigated the importance of birds as a route of introduction of the human Lyme disease agent (Cohen et al. 2015), but a review of the literature found no research assessing the role of bird-borne ticks in the spread of pathogens of veterinary importance.

Ground-foraging bird species are more likely to encounter questing ticks on low vegetation, and birds with reduced fat stores are more likely to carry ticks than are well-conditioned birds, as birds with reduced energy status are expected to expand their foraging heights and substrates (Cohen et al. 2015).

1.4.2.2 Terrestrial vertebrate hosts

Wild populations of white-tailed deer have relatively large territorial ranges (Nixon et al. 1991), and an individual deer may disperse adult female *I. scapularis* ticks (equating to thousands of larvae) up to 30 kilometres (Hamer 2010). Smaller terrestrial vertebrate hosts (e.g., small mammals and reptiles) have much smaller home ranges and are unlikely to be a major factor in *I. scapularis* range expansion.
1.4.2.3 Global climate change and altered land use patterns

It is possible that global climate change may precipitate the emergence of vector-borne diseases, such as babesiosis, including the expansion and establishment of populations of tick and other arthropod vectors into regions that were previously too cold to support them (Ogden et al. 2008). Altered land use and human activity patterns (e.g., deforestation and land conversion to agriculture, sport hunting, alterations in deer population density) alter both the number of vertebrate hosts and the amount of suitable habitat available to ticks (Yabsley and Shock 2013).

1.4.3 Diversity of Babesia species infecting questing Ixodid ticks

Molecular identification of the sporozoites infecting the salivary glands of questing ticks provides supportive evidence for the role of ticks in the transmission of various Babesia species; however, this technique is labour-intensive because it requires microscopic dissection of the tick salivary gland to confirm the presence of sporozoites by PCR and sequencing (Armstrong et al. 1998). Most studies that report the prevalence of Babesia species in questing ticks did not attempt to confirm the presence of sporozoites infecting the tick salivary gland, but instead submitted the entire tick for PCR and sequencing. The latter method is more cost- and time-effective, but because engorged ticks may contain Babesia spp. DNA if their last blood meal was taken from a Babesia-infected host, the presence of Babesia spp. DNA does not confirm the tick as a vector (Estrada-Peña et al. 2013).

A survey of I. scapularis in the northeastern (Maine and Massachusetts) and upper Midwestern (Wisconsin) United States revealed that 12% of adult ticks collected from the environment contained B. odocoilei DNA, and that ticks were rarely co-infected with both B. odocoilei and B. microti (Armstrong et al. 1998). In another study that found a similar prevalence of B. odocoilei DNA in adult I. scapularis collected in Michigan, rare co-infections with B. odocoilei and B. burgdorferi were reported (Hamer 2010). All of these studies used PCR to detect the 18S rDNA gene of Babesia-genus organisms, with purification and sequencing of all Babesia-positive amplicons for species identification. Babesia odocoilei-positive questing ticks have also been reported from Tennessee (Fritzen et al. 2014), Indiana, Pennsylvania (Steiner et al. 2008), and Mississippi (Goltz 2012).
1.5 Study rationale and objectives

This graduate research project was motivated by the deaths of several captive wapiti (Cervus canadensis) and reindeer (Rangifer tarandus tarandus) at the Toronto Zoo in southern Ontario, Canada, due to hemolytic anemia caused by the protozoan hemoparasite Babesia odocoilei. The deaths occurred from 2012 to 2015 and were the first reported cases of B. odocoilei in Ontario (Mathieu et al. 2018). The overall goals of this study were to acquire further knowledge about the epidemiology of babesiosis in cervids and of the tick vector Ixodes scapularis in Ontario using prospective and opportunistic surveillance methods, and to provide information on the pharmacokinetics of the anti-protozoal drug imidocarb in cervids.

Specific research objectives were:

1. To estimate the prevalence and host range of B. odocoilei in Ontario by conducting a prospective and opportunistic survey amongst wild and captive cervids using PCR on tissue samples (Chapter 2);

2. To survey ticks removed from birds captured by mist net during spring migration at Long Point Bird Observatory in southern Ontario (a high-density stopover point for northward-migrating birds) and to determine the prevalence of B. odocoilei-infection in these bird-borne ticks by PCR (Chapter 3);

3. To perform blanket dragging and PCR to determine the prevalence of B. odocoilei-infected questing I. scapularis ticks in environments used by cervids (Chapter 3); and

4. To develop recommendations for the treatment and prophylaxis of cervid babesiosis by determining the pharmacokinetic (PK) behaviour of the anti-protozoal drug imidocarb in captive white-tailed deer following a single intramuscular injection of imidocarb dipropionate at the dose commonly administered to treat Babesia spp. infections in domestic cattle (Chapter 4).
2 Molecular detection of *Babesia odocoilei* in wild, farmed, and zoo cervids in Ontario, Canada

2.1 Abstract

*Babesia odocoilei*, a tick-borne protozoan hemoparasite of white-tailed deer (*Odocoileus virginianus*), is an increasingly recognized cause of disease in captive cervids in North America. Historically endemic in white-tailed deer, the natural wildlife reservoir in the southeastern United States, *B. odocoilei* has been recently associated with hemolytic anemia in captive reindeer (*Rangifer tarandus tarandus*), wapiti (*Cervus canadensis*), and woodland caribou (*Rangifer tarandus caribou*) within the north-central and northeastern United States and several Canadian provinces. The emergence of *B. odocoilei* is likely related to the northward range expansion of the tick vector, *Ixodes scapularis*, and possibly to cervid translocations. Following a disease outbreak in reindeer and wapiti at the Toronto Zoo in Ontario, Canada, we utilized a prospective postmortem survey to investigate the prevalence of *B. odocoilei* in wild, farmed, and zoo cervids in Ontario (*n*=270; 2016-2018) by PCR and DNA sequencing of spleen samples. Zoo bovids have been suggested as potential hosts of *B. odocoilei* in zoos affected by cervid babesiosis, so we also collected postmortem samples from five species of bovids (*n*=7) at the Toronto Zoo that died or were euthanized during this time. We detected *B. odocoilei* in 1.4% (2/142) of farmed red deer (*Cervus elaphus*), as well as in 3.4% (1/29) of captive wapiti, and 4.4% (3/68) of wild white-tailed deer. Tissues from all zoo bovids and caribou, zoo and wild moose (*Alces alces*), and farmed white-tailed deer, wapiti-red deer hybrids, and fallow deer (*Dama dama*), tested negative for *B. odocoilei*. No clinical cases of babesiosis were encountered during this study. These findings suggest that white-tailed deer are a potential natural wildlife reservoir for *B. odocoilei* in Ontario, and that red deer and wapiti could serve as more localized reservoirs.

2.2 Introduction

*Babesia odocoilei* is a tick-borne protozoan parasite that is endemic in wild white-tailed deer (*Odocoileus virginianus*) in the southeastern United States (Waldrup et al. 1990). *Ixodes scapularis* ticks, the definitive hosts and vectors for *B. odocoilei*, are widely distributed along the Atlantic seaboard and across the southeastern United States (Keirans et al. 1996). Infections with *B. odocoilei* do not appear to cause severe disease in immunocompetent white-tailed deer (Perry et al. 1985). However, *B. odocoilei* infection can cause fatal hemolytic anemia in other members of the family Cervidae, namely wapiti (also known as North American elk: *Cervus canadensis*, previously *Cervus elaphus canadensis*), reindeer (*Rangifer tarandus tarandus*), and woodland caribou (*Rangifer tarandus caribou*) (Holman et al. 2000; Petrini et al. 1995). *Babesia odocoilei* has recently emerged as a cause of morbidity and mortality in captive Canadian cervids, and potentially threatens vulnerable wild populations of boreal
woodland caribou. For example, between 2012 and 2015, five wapiti and three reindeer died due to cervid babesiosis at the Toronto Zoo in southern Ontario, Canada (Mathieu et al. 2018).

The geographic range of *I. scapularis* is expanding northwards within Canada in association with global climate change and anthropogenic alterations in land use (Ogden et al. 2008). Exposure of susceptible cervids to *I. scapularis* infected with *B. odocoilei* may underlie the recent emergence of cervid babesiosis in Canada (Mathieu et al. 2018). The current geographic distribution of *B. odocoilei* in wild and captive Canadian cervids is unknown because surveillance has not yet been performed. Further, the role of non-cervid hosts in regional *B. odocoilei* epidemiology is uncertain. These knowledge gaps result in a poor understanding of the risk that this pathogen poses to captive and free-ranging cervids in Canada.

As cervid babesiosis is an emerging disease in Canada, improved knowledge of the geographic distribution, potential wildlife reservoirs, and host range of *B. odocoilei* is urgently needed. The objective of this research study was to survey wild, farmed, and zoo cervids in Ontario for *B. odocoilei* infection. In addition, surveillance of bovids housed at the Toronto Zoo was performed to assess for *B. odocoilei* infection in non-cervid hosts. Data gathered will aid in understanding the regional epidemiology of this emerging pathogen and help to inform management strategies for at-risk cervids.

### 2.3 Materials and Methods

#### 2.3.1 Study area and sample sources

Postmortem samples were collected opportunistically from wild, farmed, and zoo cervids in Ontario, Canada. Sources included diagnostic cases submitted to the Canadian Wildlife Health Cooperative (CWHC – Ontario/Nunavut) and the Animal Health Laboratory (AHL) at the University of Guelph (Ontario, Canada), as well as hunter-harvested animals, those killed on the road or by predators, farmed cervids sent for slaughter at Ontario abattoirs, and deaths at the Toronto Zoo. Additionally, postmortem samples were collected from animals of the family Bovidae that died naturally or were euthanized at the Toronto Zoo during the study.
2.3.2 Sample collection

Samples were collected from cervids and bovids from May 2016 to January 2018. The latitude and longitude of the animal’s location at the time of death was recorded when known; otherwise, the nearest street intersection was used. Fresh spleen samples collected within 8 hours of death were frozen to -20°C either prior to transport or immediately upon arrival to a research laboratory in the Department of Pathobiology, University of Guelph. Samples were subsequently thawed and a 1 cm$^3$ piece of spleen was placed into a sterile cryovial and frozen to -80°C until testing.

2.3.3 Laboratory analyses

2.3.3.1 DNA extraction

DNA isolation was performed on spleens from 270 cervids and seven bovids. From each spleen, 50 mg samples were minced with a sterile scalpel blade and lysed by repetitive pipetting in 1 mL of DNAzol® Reagent (Invitrogen Life Technologies, Carlsbad, California, USA). DNA was extracted from each spleen sample according to the manufacturer’s instructions for the tissue protocol. The DNA quality and concentration were determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) and stored at 4°C.

2.3.3.2 Primer design and polymerase chain reaction

A piroplasm-specific 18S rDNA primer (Piro_18S_300F, Table 2.1) was designed using an alignment of publicly available piroplasms 18S rDNA sequences that also included the 18S rDNA sequence (EU823286.1) of one of the target hosts, white-tailed deer (O. virginianus), so that amplification of host DNA was avoided. Primer design used Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012) executed from within Geneious® (version 6.1 or later, www.geneious.com; Kearse et al., 2012).

Extracted DNA was used as a template in a standard PCR assay designed to amplify 18S rDNA of Babesia spp. (Mathieu et al. 2018). Amplification primers (Table 2.1) were Piro_18S_300F and Piro_18S_1688R (Mathieu et al. 2018; similar to primer BN1700 of Ramos et al. 2010). PCR-positive samples produced a primary amplicon of 1290 bp. PCR was performed in a 25 µL reaction containing 3 mM MgCl$_2$, 0.4 mM dNTPs, 10× PCR buffer, 2U Platinum Taq polymerase (Invitrogen Life Technologies, Carlsbad, California, USA), 1.25 µM of each amplification primer, nuclease-free water, and 100 to 200 ng of sample DNA. Negative and positive controls were included in each run, with water as the negative control, and B. odocoilei DNA extracted from the spleen of a
Toronto Zoo reindeer that died from acute babesiosis as the positive control (Mathieu et al. 2018). Amplification was carried out in a T100 thermal cycler (Bio-Rad, Mississauga, Canada) after the following reaction conditions: initial melt of 94°C for 3 min, followed by 35 amplification cycles (denature at 94°C for 30 s, anneal at 60°C for 30 s, extend at 72°C for 90 s), and a final extension of 72°C for 5 min. The PCR products were separated by electrophoresis using a 2% agarose gel with 0.5 × TAE buffer (120 mL) and 12 µL of SYBR Safe DNA gel stain (Invitrogen Life Technologies) and visualized under ultraviolet light. The GeneRuler 1 kb Plus DNA size ladder (Thermo Scientific) was used to determine product fragment length. An additional re-amplification step was performed on samples that produced a band of appropriate size by subsequent agarose gel electrophoresis.

To obtain sufficient product for subsequent Sanger sequencing, the resulting primary amplicons were used as templates in a secondary, hemi-nested, PCR reaction using primers Cocci_18S_595F and Piro_18S_1688R (Table 2.1). This PCR was performed in a 20 µL reaction containing 10 µL HotStar Taq Master Mix (Qiagen, Toronto, Ontario, Canada), 1.25 µM of each amplification primer, and 9 µL of reaction product in a T100 thermal cycler using the above reaction conditions at an annealing temperature of 58°C and extension time of 60 s.
Table 2.1 Polymerase chain reaction amplification primers and sequencing primers for nuclear 18S rDNA loci used in the identification of Babesia odocoilei from cervid tissue samples.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Size (bp)</th>
<th>Primer names</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary PCR</td>
<td>1,393</td>
<td>Piro_18S_300F⁴</td>
<td>5'-GACGGTAGGGTATTGGCCTA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Piro_18S_1688R⁵</td>
<td>5'-CGACTTCTCCTCTTCTTAAGTGATAAG-3'</td>
</tr>
<tr>
<td>Hemi-nested Secondary PCR</td>
<td>1,147</td>
<td>Cocci_18S_595F⁶</td>
<td>5'-CCGCGGTAATTCCAGCTCCAAT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Piro_18S_1688R⁵</td>
<td>5'-CGACTTCTCCTCTTCTTAAGTGATAAG-3'</td>
</tr>
<tr>
<td>Sequencing Primer</td>
<td>n/a</td>
<td>Lank_18S_1278R⁷</td>
<td>5'-TCAAGAAAGAGCCTATCAATCT-3'</td>
</tr>
</tbody>
</table>

⁴ This study.
⁵ Mathieu et al. 2018; similar to primer BN1700 of Ramos et al (2010).
⁷ Barta Lab, unpublished.
2.3.3.3 DNA sequencing

The resulting primary or nested amplicons were purified using a QIAquick PCR Purification Kit (Qiagen). Direct sequencing of Babesia spp. amplicons was performed by the Genomics Facility Advanced Analysis Centre, University of Guelph using amplification or sequencing primers (Table 2.1). For one sample, multiple sequencing chromatograms were assembled into a consensus sequence using the Geneious® bioinformatics program. The identities of the other five PCR-positive samples were confirmed by direct-sequencing using the Lank_18S_1278R primer (Table 2.1). Sequences were trimmed to remove primers and then searched against public sequence databases using the basic local alignment search tool (BLAST) algorithm (Altschul et al. 1990).

2.4 Results

From May 2016 to January 2018, 270 wild, farmed, and zoo cervids of six species and one hybrid species (wapiti-red deer) were sampled, as well as seven zoo bovids of five species (Table 2.2). In total, spleens from 2.2% (6/270) of cervids tested positive by PCR and were confirmed as B. odocoilei by sequencing of the 18S rDNA fragment. There was no clinical suspicion of babesiosis in any of the animals sampled. Cervids originated from across Ontario, but the number of submissions was highest in southern Ontario (Fig. 1).

2.4.1 PCR of spleen samples

Wild cervid samples were from 68 white-tailed deer and one moose (Alces alces). This included 39 hunter-harvested white-tailed deer culled for management purposes under advisory from the Ministry of Natural Resources and Forestry and Parks Canada at Point Pelee National Park in January 2017 and January 2018. Sixteen wild white-tailed deer and one moose were submitted to the CWHC for necropsy. Seven white-tailed deer were killed by vehicles in the Greater Toronto Area. Hunters submitted spleens from five white-tailed deer in the 2016 and 2017 hunting seasons. One wild white-tailed deer was killed by coyotes on the Toronto Zoo grounds. Three of these 68 wild white-tailed deer (4.4%), including two from the CWHC and one hunter-harvested, tested positive for B. odocoilei by PCR (Table 2.2). The wild moose tested negative for B. odocoilei.

Commercially farmed cervid samples originated from red deer (n=142), wapiti (n=28), wapiti-red deer hybrids (n=13), fallow deer (Dama dama) (n=12), and white-tailed deer (n=3) sent for slaughter at Ontario abattoirs. Among these, 1.4% (2/142) of farmed red deer tested PCR-positive for B. odocoilei. All farmed wapiti, wapiti-red deer hybrids,
fallow deer, and farmed white-tailed deer tested PCR-negative for *B. odocoilei* (Table 2.2). Zoo cervids sampled included two from the Toronto Zoo; one moose was euthanized due to age-related disease and one wapiti was euthanized following a traumatic injury. One caribou from a private zoo was submitted for necropsy to the AHL. Zoo bovids sampled included one barbary sheep (*Ammotragus lervia*), one chamois (*Rupicapra rupicapra*), one west Caucasian tur (*Capra caucasica*), three wood bison (*Bison bison athabascae*), and one yak (*Bos grunniens*) that died or were euthanized at the Toronto Zoo. Of the zoo cervids and bovids, only wapiti tested positive for *B. odocoilei* by PCR. Overall, 3.4% (1/29) of captive (zoo and farmed) wapiti were *B. odocoilei* positive (Table 2.2).

### 2.4.2 DNA sequencing of PCR-positive spleen samples

The partial 18S rDNA sequence (1,290 bp) from *B. odocoilei* infecting a white-tailed deer sampled in this study was submitted to GenBank under accession number MH366302. This partial 18S rDNA sequence had 100% identity over its entire length to numerous *B. odocoilei* sequences in GenBank from various hosts and geographic locations. These included wild white-tailed deer in Texas (U16369.2), farmed wapiti in Saskatchewan (KC460321), and the recent Toronto Zoo clinical babesiosis cases (Mathieu et al. 2018) in wapiti (MF357056) and reindeer (MF357057). The other five PCR-positive samples were direct-sequenced using primer Lank_18S_1278R generating 571bp reads that spanned the highly variable region (bp 609-645 of the *Babesia odocoilei* reference sequence - U16369.2) that discriminates this species from other closely related *Babesia* species; all samples had 100% sequence identity with the reference sequence over this 571 bp region.
Table 2.2 Wild, farmed, and zoo cervids sampled between May 2016 and January 2018 in Ontario, Canada, and tested for *Babesia odocoilei* infection by PCR using piroplasm-specific primers on DNA extracted from spleen samples.

<table>
<thead>
<tr>
<th>Species</th>
<th>Commercial farm</th>
<th>Zoo or private collection</th>
<th>Free-ranging</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>White-tailed deer <em>Odocoileus virginianus</em></td>
<td>3 (0); 0%</td>
<td></td>
<td>68 (3); 4.4%</td>
<td>71 (3); 4.2%</td>
</tr>
<tr>
<td>Red deer <em>Cervus elaphus</em></td>
<td>142 (2); 1.4%</td>
<td></td>
<td></td>
<td>142 (2); 1.4%</td>
</tr>
<tr>
<td>Wapiti <em>Cervus canadensis</em></td>
<td>28 (0); 0%</td>
<td>1 (1); 100%</td>
<td></td>
<td>29 (1); 3.4%</td>
</tr>
<tr>
<td>Wapiti-red deer hybrid</td>
<td>13 (0); 0%</td>
<td></td>
<td></td>
<td>13 (0); 0%</td>
</tr>
<tr>
<td>Fallow deer <em>Dama dama</em></td>
<td>12 (0); 0%</td>
<td></td>
<td></td>
<td>12 (0); 0%</td>
</tr>
<tr>
<td>Caribou <em>Rangifer tarandus caribou</em></td>
<td>-</td>
<td>1 (0); 0%</td>
<td></td>
<td>1 (0); 0%</td>
</tr>
<tr>
<td>Moose <em>Alces alces</em></td>
<td>-</td>
<td>1 (0); 0%</td>
<td></td>
<td>2 (0); 0%</td>
</tr>
<tr>
<td>Total</td>
<td>198 (2); 1.0%</td>
<td>3 (1); 33.0%</td>
<td>69 (3); 4.3%</td>
<td>270 (6); 2.2%</td>
</tr>
</tbody>
</table>

8 DNA sequencing was used to confirm the identity of all PCR-positive amplicons.
Figure 2.1 Locations of all cervids that underwent postmortem *Babesia odocoilei* testing by PCR of spleen samples from 2016-2018 in Ontario, Canada (n=270). The solid black circles represent collection sites with one or more *B. odocoilei*-negative animals, while the remaining grey symbols each represent a single *B. odocoilei*-positive animal.
2.5 Discussion

This study documents *B. odocoilei* infection in wild white-tailed deer in Ontario, supporting the hypothesis that this species could serve as a natural reservoir of infection in this geographic region as it does in the southeastern United States (Waldrup et al. 1989). An infection prevalence of 4.4% was found in wild white-tailed deer in Ontario; although no comparable PCR-based surveillance for *B. odocoilei* has been performed in wild cervids in the United States, examination of blood smears from wild white-tailed deer in Texas and Oklahoma found a prevalence of *B. odocoilei* parasitemia of 2% (Waldrup et al. 1989). Asymptomatic *B. odocoilei* infection was also identified in two species in the genus *Cervus* in Ontario, with an infection prevalence of 1.4% in red deer and 3.4% in wapiti. *Babesia odocoilei*-infected wapiti are known to be susceptible to acute hemolytic crisis, which may be triggered by stress or immunosuppression (Gallatin et al. 2003). The pathogenicity of *B. odocoilei* in red deer is unknown. Red deer, a European species, are farmed for venison in Ontario, and wapiti are present in zoos, on farms, and as reintroduced wild populations in Ontario. A *B. odocoilei*-like parasite was detected in blood samples from three wild red deer in Ireland, although the species of this parasite was not confirmed (Zintl et al. 2011). It is possible that *Cervus* species may serve as additional reservoirs of *B. odocoilei*. Samples were obtained only from captive Ontario wapiti, and thus no comment can be made regarding subclinical infections or disease in the wild wapiti population.

In the current study, all sequenced samples were 100% identical to each other and to *B. odocoilei* isolates from several different host species and geographic locations, including the partial 18S rDNA sequence isolated from a fatal case of babesiosis in a Toronto Zoo reindeer in 2012 (Mathieu et al. 2018). Comparison of nuclear 18S rDNA sequences is widely accepted as a method for resolving questions of relatedness and identity of *Babesia* spp. isolates (Holman et al. 2000). As the 18S rDNA fragment of *B. odocoilei* appears to be well-conserved among cervid species in Ontario, further molecular characterisation of the isolates using primers specific for a mitochondrial target such as the cytochrome c oxidase subunit I (COI) would be desirable to further elucidate the epidemiology of this parasite.

Prior to the first cases in 2012 of hemolytic anemia due to babesiosis in Saskatchewan farmed wapiti (Pattullo et al. 2013) and in reindeer and wapiti at the Toronto Zoo (Mathieu et al. 2018), *B. odocoilei* was not recognized as a clinical problem in Canadian cervids, and surveillance for this pathogen was not conducted in captive or free-ranging wildlife. While it is possible that the parasite was present in Canada prior to 2012, a lack of reports of disease in captive or wild cervids make it unlikely to have been widespread. Based on limited home ranges of white-tailed deer in many areas (Nixon et al. 1991) as well as the rapid emergence of clinical cases of cervid babesiosis in
several widely disparate locations across North America (Mathieu et al. 2018), the natural movements of free-ranging white-tailed deer are unlikely to have been a primary driver in the emergence of B. odocoilei in Canada. The underlying cause(s) of the likely incursion of B. odocoilei into Canada remain unknown, but possibilities include translocation of an asymptomatic infected zoo or farmed cervids or range expansion of B. odocoilei-infected ticks, perhaps through adventitious carriage on migratory birds (Ogden et al. 2008).

The locations of historic Canadian cervid babesiosis cases and of the B. odocoilei PCR-positive cervids in the present study coincide with the distribution of known I. scapularis populations, with the exception of the wapiti cases in Saskatchewan where I. scapularis is not known to be established (Pattullo et al. 2013). Dermacentor spp. ticks are present in Saskatchewan, but the vector competence of this tick species for B. odocoilei has not been investigated. Ixodes scapularis serves as both the definitive host and vector for B. odocoilei (Waldrup et al. 1990). Following gametogony in the tick gut, B. odocoilei gametes fuse and form a zygote that develops into a kinete, which subsequently invades and replicates in the tick hemolymph. Sporogony follows invasion of the kinetes into the tick salivary glands. Susceptible cervids are exposed to B. odocoilei by the bite of an infected tick that introduces infective stages into the cervid host’s blood stream (Homer et al. 2000). These stages invade the cervid erythrocytes and undergo merogony, primarily through binary fission. In a parasitemic cervid, B. odocoilei merogonic stages appear on a blood smear as single, paired, or tetrad pyriform and ring-shaped organisms usually in the accolé position at the periphery of the erythrocyte. The infected erythrocyte eventually ruptures, liberating merozoites, that invade new erythrocytes and either become trophozoites that undergo merogony again by binary fission, or alternatively develop into non-dividing gamonts that are infective to a tick when it feeds on the cervid intermediate host. Maintenance of B. odocoilei within an ecosystem is therefore dependent both on I. scapularis as the definitive host and a mammalian intermediate host.

We did not detect B. odocoilei infection in any bovids at the Toronto Zoo, but the significance of this finding is limited by small sample sizes and limited range of species. Subclinical B. odocoilei infection has been identified in endemic and exotic ruminants belonging to the family Bovidae, including zoo markhor (Capra falconeri), zoo yak (Bartlett et al. 2009), and wild bighorn sheep (Ovis canadensis nelsoni) in the United States (Schoelkopf et al. 2005).

In this study, B. odocoilei was identified in both captive and wild cervids. Transmission of disease between the two groups is well recognized, with the management of cervids
in captivity on private farms or in zoological institutions posing a recognized health risk to free-ranging wild cervids, and vice versa (Gerhold and Hickling 2016). Captive cervid facilities provide ample opportunities for transmission of pathogens and ectoparasites between captive and wild cervids, including direct contact through fence lines, escape of captive animals, and ingress of wild cervids into inadequately fenced enclosures. In the absence of reliable pre-movement disease testing, the translocation of captive cervids across provincial and international borders necessarily results in the simultaneous translocation of their pathogens including ticks. Babesia odocoilei requires a tick host to complete its life cycle, and therefore direct transmission of babesiosis between cervids does not occur, with the possible exception of iatrogenic transmission such as via blood transfusion (Pastor and Milnes 2018).

The lack of reliable commercially-available diagnostic tests for screening and pre-movement testing of cervids for hemoparasites such as babesiosis is a major problem for the captive cervid industry, and the development of a commercial PCR assay for blood testing of carrier animals would help to alleviate this problem (Holman et al. 2000). Molecular testing of postmortem spleen samples for B. odocoilei DNA through PCR, as used in this study, is a highly sensitive and specific method for detecting latently infected animals with low-level parasitemia that may not be evident on microscopic examination of blood smears (Holman et al. 2000). This method is also suitable for use on whole blood samples and has been used successfully to identify subclinically infected cervids in a zoo setting (Bos et al. 2017).

In conclusion, the results of this study suggest that B. odocoilei has an established wildlife reservoir in wild white-tailed deer in Ontario, Canada. Given the expanding range and increasing density of the arthropod host and vector, I. scapularis, continued expansion of transmission and thus infection among the while-tailed deer population is likely. The finding of B. odocoilei in apparently healthy captive cervids emphasizes the importance of proactive tick prevention and biosecurity measures (e.g., double fencing) in cervid facilities to prevent disease and vector transmission between captive and wild animals, and losses due to clinical disease in more susceptible species. In addition, viable options for prophylaxis and treatment of clinical disease in captive cervids should be explored. Cervid managers should be aware of the risks involved in translocating animals between herds.
3 Babesia odocoilei and zoonotic pathogens identified from *Ixodes scapularis* ticks in southern Ontario, Canada

3.1 Abstract

Cervid babesiosis, caused by the non-zoonotic protozoan hemoparasite *Babesia odocoilei* and transmitted by the black-legged tick *Ixodes scapularis*, is an emerging disease of Canadian cervids. Data are lacking on the role of migratory birds in the adventitious spread of *B. odocoilei*-infected ticks, as well as on the infection status of *I. scapularis* in environments used by susceptible wildlife hosts. Following a high-mortality outbreak of cervid babesiosis at the Toronto Zoo (TZ), the present study was initiated to investigate *B. odocoilei* and other tick-borne pathogens of veterinary and public health importance in *I. scapularis* at three sites in southern Ontario, Canada. During the spring of 2016 and 2017, 1,102 birds were examined for ticks at Long Point Bird Observatory (LPBO). One or more *I. scapularis* were found on 3.2% of birds (n=595) in 2016, and 6.7% (n=507) of birds in 2017. Overall, 0.18% and 0.55% of birds carried one or more *I. scapularis* ticks that tested PCR-positive for *B. odocoilei* and *Borrelia burgdorferi*, respectively. Blanket dragging for questing ticks yielded *I. scapularis* from the three sites evaluated: TZ, Point Pelee National Park (PPNP), and LPBO. The minimum infection prevalence of *B. odocoilei* in *I. scapularis* was 1.24% (n=161) at the TZ and 4.1% (n=49) at LPBO. *Borrelia burgdorferi* was identified in *I. scapularis* at all three sites with the following minimum infection prevalences: TZ 0.6%, PPNP 4.9% (n=41), and LPBO 4.1%. *Anaplasma phagocytophilum* was identified in *I. scapularis* collected from the TZ with a minimum infection prevalence of 0.6%. These data indicate that *B. odocoilei*-positive *I. scapularis* are found in southern Ontario, suggest that bird-borne ticks may contribute to the range expansion of *B. odocoilei* and *B. burgdorferi*, and expand upon previous detections of the causative agents of Lyme borreliosis and granulocytic anaplasmosis in *I. scapularis* in Ontario.

3.2 Introduction

The black-legged tick (*Ixodes scapularis*) is the vector for many pathogens of medical and veterinary importance in North America. The range of this tick is expanding within Ontario, Canada as a result of anthropogenic influences on the environment and climate warming (Leighton et al. 2012). Ticks carrying the causative agents of medically important tick-borne zoonoses, such as Lyme borreliosis (*Borrelia burgdorferi*) and human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), may be transported over vast distances and introduced to new areas by birds in flight (Ogden et al. 2008). The role of migratory birds in the northward range expansion of non-zoonotic tick-borne
diseases of veterinary importance has received little research attention. One such disease is cervid babesiosis, which is caused by the protozoan hemoparasite Babesia odocoilei, for which I. scapularis ticks are the only known competent vector and the definitive host (Waldrup et al. 1990). Babesia odocoilei is endemic in the southeastern United States (Holman et al. 2000) where the natural reservoirs are wild white-tailed deer (Odocoileus virginianus). Disease associated with B. odocoilei (cervid babesiosis) has emerged in captive cervids within Canada since 2012 (Mathieu et al. 2018). In addition, the zoonotic pathogens Babesia microti, B. burgdorferi, Borrelia miyamotoi, and Anaplasma phagocytophilum share the same tick vector, I. scapularis, and are also emerging in Ontario (Dibernardo et al. 2014).

The Toronto Zoo (TZ) in southern Ontario, Canada, is situated within a natural area of forest and wetlands (the Rouge National Urban Park) with abundant free-ranging wildlife including white-tailed deer, which are the main blood meal hosts for adult I. scapularis (Rand et al. 2003). Such a setting presents a potential risk to the health of zoo animals and human inhabitants (staff and visitors) due to the presence of disease-carrying vectors. Tick parasitism of zoo animals and staff is occasionally reported, but the likelihood of tick-borne disease transmission has not been investigated. Following a high-mortality outbreak of cervid babesiosis in reindeer (Rangifer tarandus tarandus) and wapiti (Cervus canadensis) at the TZ from 2012 to 2015 (Mathieu et al. 2018), we investigated B. odocoilei and other tick-borne pathogens in I. scapularis at the TZ and two other sites in southern Ontario, Canada where I. scapularis is endemic (Barker and Lindsay, 2000). Further, we investigated the potential for bird-borne movement of ticks and tick-borne pathogens to contribute to the emergence of B. odocoilei in regions sensitive to climate change.

Our objectives were: 1) to survey northward-migrating birds captured at Long Point Bird Observatory (LPBO, Port Rowan, Ontario) for tick infestation; 2) to survey ticks by blanket dragging and opportunistic collections from three sites used by wild cervids (TZ, LPBO, and Point Pelee National Park PPNP); and 3) to determine the prevalence of B. odocoilei, B. microti, B. burgdorferi, B. miyamotoi, and A. phagocytophilum infection in host-feeding and questing I. scapularis obtained from these locations by polymerase chain reaction (PCR).
3.3 Materials and methods

3.3.1 Bird sampling and study area

Bird sampling took place during spring migration from 19 to 28 May 2016 and from 21 to 28 May 2017 at LPBO migration monitoring field station in southern Ontario. Mist nets and ground traps were used to capture birds daily from sunrise until noon, in accordance with protocols described by the North American Bird Banding Manual (Gustafson et al. 1997). Each captured bird was identified to species and leg banded with a unique bird identification number. All birds were checked for ticks; the additional time spent handling birds for this purpose was limited to 3 min per bird. Feathers along apterylae of the head, neck, and body were gently parted and the skin was inspected for ticks using binocular head loupes. All ticks were removed using fine forceps, placed in sterile labelled cryovials containing 70% ethanol. Within-year recaptures were excluded from analysis. Fieldwork was carried out with approvals from the Animal Care Committees of the University of Guelph and the TZ in compliance with the regulations of the Canadian Council on Animal Care.

3.3.2 Tick dragging and opportunistic collection of questing and host-feeding ticks

Tick collection was carried out at the TZ, based on the history of clinical B. odocoilei infection in zoo cervids, and at LPBO and PPNP because these sites host populations of wild white-tailed deer and are known endemic sites for I. scapularis (Barker and Lindsay, 2000).

Blanket dragging was used to sample questing ticks (i.e., ticks in the environment that are searching for a blood meal) at the TZ on 20 and 25 October 2016, 18 and 19 July 2017, 31 August 2017, and 20 October 2017; at PPNP on 9 and 10 November 2016; and at LPBO from 22 to 26 May 2016, and from 22 to 27 May 2017. Blanket dragging was conducted only on days when surface vegetation was dry to the touch. A 1m² white flannel drag cloth attached to a wooden pole was pulled across the surface of ground vegetation in parallel transects for at least 1.5 (and up to 6) person-hours per site. Field personnel checked each drag cloth and their clothing at 3 min intervals, during which time the timer was stopped. At each site, at least 1,000 m² of vegetation were sampled per visit.

Staff at the TZ were asked to submit ticks found on their clothing or on animals from May 2016 to November 2017. In addition, bird banders and volunteers at LPBO
submitted ticks found on their clothing from 19 May to 4 June 2017. All ticks were placed in sterile labelled tubes containing 70% ethanol immediately after collection.

3.3.3 Taxonomic identification of ticks

Ticks were identified morphologically to species and life stage using a stereomicroscope and taxonomic keys (Keirans and Litwak 1989) at the University of Guelph (Guelph, Ontario, Canada) and the National Microbiology Laboratory (NML; Winnipeg, Manitoba, Canada).

3.3.4 Polymerase chain reaction (PCR) and DNA sequencing

All ticks identified as *I. scapularis* were tested for the presence of *B. odocoilei, B. microti, B. burgdorferi, B. miyamotoi* and *A. phagocytophilum*. DNA extraction and conventional PCR testing for infection with *B. odocoilei* was performed at the University of Guelph. DNA was extracted from individual ticks, except when multiple *I. scapularis* were present on a given bird or were collected from the same environmental location of the same day. In these instances, ticks collected from the same bird or from the same environmental sampling location and date were pooled by life stage (i.e., adults, nymphs, or larvae) for DNA extraction. Minimum infection prevalence (i.e., number of positive pools divided by the total number of individual ticks tested), assuming one positive tick per pool, was used for the interpretation of results. The minimum infection prevalence assumes that a positive pool contains only one infected tick, an assumption that may be invalid when infection rates are high.

Ticks were bisected with a sterile scalpel blade and homogenized with sterile lysis beads (0.75 g of 2 mm dia. zirconia beads and 0.15 g of 0.1 mm dia. zirconia/silica beads, BioSpec Products, Bartlesville, Oklahoma, USA) in a cryovial containing 0.5 mL of DNAzol® Reagent (Invitrogen Life Technologies, Carlsbad, California, USA) for 30 minutes at 30 Hz with a TissueLyser II (Qiagen, Toronto, Ontario, Canada). For pools, one half of every tick in the pool was added to the same cryovial. After homogenization, the mixture was incubated overnight at room temperature on a rotator (Fisher Roto-Rack, Model 96, Fisher Scientific Co., Pittsburgh, Pennsylvania, USA), then centrifuged at 4,000 x g for 2 min. The supernatant was transferred to a sterile cryovial to which 0.25 mL of 100% ethanol was added. The cryovial was inverted 3-6 times, and the samples were incubated for 2 min at room temperature. The mixture was centrifuged at 4,000 x g for 2 min to pellet the DNA, and the supernatant was discarded. The DNA pellet was washed twice with 0.5 mL of fresh 75% ethanol by gently inverting the tube 3-6 times. Finally, the DNA pellet was air dried and resuspended in 0.1 mL of 8mM NaOH
by slowly passing the pellet through a pipette tip. Insoluble materials were removed by centrifugation at 12,000 x g for 10 min. The DNA quality was determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) after which sample aliquots were stored at -20°C until testing for B. odocoilei at the University of Guelph by PCR. Additional aliquots were then shipped overnight on ice packs to the NML for B. microti, B. burgdorferi, B. miyamotoi, and A. phagocytophilum PCR.

3.3.5 Molecular analysis of tick DNA extracts for Babesia odocoilei and DNA sequencing

Extracted DNA was used as a template in a standard PCR assay designed to amplify characteristic 18S rDNA fragments of Babesia (Ramos et al. 2010), with agarose gel electrophoresis followed by re-amplification and purification of positive samples as described previously (Chapter 2). Direct sequencing of Babesia amplicons was performed by the Genomics Facility Advanced Analysis Centre, University of Guelph. Primers used for sequencing were Piro_18S_1688R, Piro_18S_300F, and Lank_18S_1278R (Table 2.1). The sequences were assembled into consensus sequences using a bioinformatics program (Geneious® 11.0.5., Biomatters, Auckland, New Zealand). Thereafter, the primers were trimmed, and were searched against the public sequence databases using the basic local alignment search tool (BLAST) algorithm (Altschul et al. 1990).

3.3.6 Molecular analysis of ticks for zoonotic pathogens

Extracted DNA from all ticks identified as I. scapularis was submitted to the NML and tested for the presence of B. microti, B. burgdorferi, B. miyamotoi, and A. phagocytophilum using real-time PCR assays as described previously (Ogden et al. 2006; Dibernardo et al. 2014). A multiplex real-time PCR assay was used to screen for B. burgdorferi and A. phagocytophilum (Courtney et al. 2004), followed by an ospA real-time PCR to confirm B. burgdorferi infection in multiplex real time PCR-positive samples (Ogden et al. 2006). Analysis for B. microti was conducted using methods described by Bullard et al. (2014).
3.4 Results

3.4.1 Bird-borne ticks

Ticks were found on 7.2% of birds examined (79/1,102; combined data for 2016 and 2017), with five species of tick identified: *I. scapularis*, *Ixodes dentatus*, *Ixodes marxi*, *Haemaphysalis leporispalustris*, and *Amblyomma americanum* (Table 3.1 and Appendix Table 6.1). Ticks were found around the eyes, at the commissures of the beak, and within the ear canal. Tick-infested birds belonged to 24 species, and the diversity of bird species harboring ticks was less in 2016 (13 species) than in 2017 (21 species) even though the total number of species examined was similar in both years (2016, 67 species; 2017, 63 species). With the exception of the Carolina wren (*Thryothorus ludovicianus*), which is a year-round resident at LPBO, all tick-infested birds were migratory species that reside in the United States or further south during winter and migrate northwards into Canada for the breeding season (Sibley 2014). Across both years, 4.8% (53/1,102) of birds carried one or more *I. scapularis* ticks.

Of the 595 birds examined in 2016, ticks were found on 22 individuals of 13 species for an overall tick infestation prevalence of 3.7% (22/595). In total, 47 ticks were found in 2016; however, one tick from a grey catbird (*Dumetella carolinensis*) was not collected, and thus 46 ticks were submitted for identification. Of these, 84.8% (39/46; 32 nymphs and 7 larvae) were *I. scapularis*, 13.0% (6/46; one nymph and five larvae) were *I. dentatus*, and 2.2% (1/46; one nymph) were *Haemaphysalis leporispalustris*. *Ixodes scapularis* ticks were carried by 3.2% (19/595) of birds. The highest level of infestation was six *I. scapularis* nymphs on one blue jay.

In 2017, 507 birds were examined. A total of 57 individuals of 21 species harbored ticks, for an overall tick infestation prevalence of 11.2% (57/507). One tick from a Carolina wren was observed but not collected, thus 167 ticks from 56 birds were collected for identification. Most ticks were of two species: 76.0% (127/167; 81 nymphs and 46 larvae) were *I. scapularis*, and 22.2% (37/167; 30 nymphs and 7 larvae) were *H. leporispalustris*. A single *I. marxi* larva was removed from a Baltimore oriole (*Icterus galbula*), and one *A. americanum* larva was removed from each of a grey-cheeked thrush (*Catharus minimus*) and a red-eyed vireo (*Vireo olivaceus*). *Ixodes scapularis* ticks were carried by 6.7% (34/507) of birds, and *H. leporispalustris* ticks were carried by 3.7% (19/507) of birds. The highest level of infestation was 49 *I. scapularis* (21 larvae and 28 nymphs) on a blue jay (*Cyanocitta cristata*).
3.4.2 Tick dragging and opportunistic collection of questing and host-feeding ticks

In total, five species of ticks (*I. scapularis*, *Ixodes cookei*, *I. marxi*, *H. leporispalustris*, and *Dermacentor variabilis*) were found at the three field sampling sites (Table 3.2; Fig. 3.1). The only tick species collected at the TZ was *I. scapularis*, which was also the most prevalent tick at PPNP. *Dermacentor variabilis* was the most prevalent tick at LPBO. Eleven and 150 *I. scapularis* were collected at the TZ in 2016 and 2017, respectively. At LPBO, 20 ticks were collected in 2016 (16 *D. variabilis* and four *I. scapularis*, all adults), and 137 ticks were collected in 2017 (89 adult *D. variabilis*, 45 adult *I. scapularis*, and one nymph each of *H. leporispalustris*, *I. cookei*, and *I. marxi*). Over both years and all collection methods, the numbers of *I. scapularis* ticks collected from each site was 161 from the TZ and 49 from LPBO. PPNP was only sampled in 2016 when blanket dragging yielded 47 adult ticks, of which 41 were *I. scapularis*; three were *I. cookei*, and three were *I. marxi*.

3.4.3 Babesia odocoilei PCR test results

All bird-borne *I. scapularis* ticks collected from 2016 and 2017 (*n* = 166; 113 nymphs and 53 larvae) were tested by PCR for *B. odocoilei*, either individually or pooled by bird and life stage as described previously. A pool of larvae removed from a blue jay in 2016 and a pool of larvae removed from a Swainson’s thrush (*Catharus ustulatus*) in 2017 were PCR positive for *B. odocoilei* for a prevalence of 0.2% (2/1,102) among birds harboring *B. odocoilei* positive ticks (Table 3.1). Of the birds carrying one or more *I. scapularis* ticks, the prevalence of *B. odocoilei* infection was 3.8% (2/53). In addition, both birds were co-infested with *I. scapularis* nymphs, which tested PCR negative for *B. odocoilei*. The minimum infection prevalence of *B. odocoilei* in all bird-borne *I. scapularis* ticks over both years was 1.2% (2/166).

All *I. scapularis* ticks collected opportunistically and by blanket dragging from the TZ, PPNP, and LPBO in 2016 and 2017 were tested individually or pooled by date, location, and life stage (Table 3.2; Fig. 3.1). A total of 161 *I. scapularis* ticks were collected from the TZ in 2016 and 2017, of which two larval pools tested positive for *B. odocoilei* by PCR, giving a minimum infection prevalence for *B. odocoilei* of 1.2% (2/161). At LPBO, one adult and one pool of adult ticks were positive for *B. odocoilei*, with a minimum infection prevalence of 4.1% (2/49). All adult *I. scapularis* from PPNP tested negative for *B. odocoilei*. 
3.4.4 Zoonotic pathogen PCR test results

All bird-borne *I. scapularis* ticks collected in 2016 and 2017 were tested by PCR for selected zoonotic bacterial and protozoal agents. Overall, 0.5% (6/1,102) of birds carried a tick that was positive by PCR for *B. burgdorferi* (Table 3.1). Of the birds carrying one or more *I. scapularis* ticks, the prevalence of *B. burgdorferi* infection was 11.3% (6/53). All *B. burgdorferi* positive ticks were individual or pooled nymphs. The minimum prevalence of *B. burgdorferi* in all bird-borne *I. scapularis* ticks over both years was 3.6% (6/166). Co-infection of ticks of different life stages that were positive for different pathogens was identified in one blue jay, which carried both a *B. odocoilei*-positive larval pool and a *B. burgdorferi* positive nymphal pool. No bird-borne ticks tested positive for *A. phagocytophilum, B. miyamotoi, or B. microti*.

All *I. scapularis* ticks collected opportunistically from hosts and by blanket dragging at the TZ, PPNP, and LPBO in 2016 and 2017 were tested for zoonotic pathogens (Table 3.2; Fig. 3.1). The minimum infection prevalence of *B. burgdorferi* was 0.6% (1/161) at the TZ, 4.9% (2/41) at PPNP, and 4.1% (2/49) at LPBO. Three engorged adult *I. scapularis* were removed from the carcass of a wild white-tailed deer that was predated by coyotes on the TZ grounds in May 2016, and pooled DNA extracted from these ticks tested PCR positive for *A. phagocytophilum*, giving a minimum infection prevalence for *A. phagocytophilum* of 0.6% (1/161). None of these ticks tested positive for *B. microti* or *B. miyamotoi*.

3.4.5 DNA sequencing to confirm Babesia odocoilei in PCR positive ticks

With the one exception described below, all partial 18S rDNA sequences from *B. odocoilei* PCR positive ticks in this study had 100% identity to each other and to the sequences obtained from clinical cervid babesiosis cases in the TZ reindeer (GenBank accession number MF357057) and wapiti (MF357056) during the 2012-2015 outbreak (Mathieu et al., 2018). These samples also showed complete identity with *B. odocoilei* isolated from wild white-tailed deer in Texas (U16369.2). A representative sequence from this study (originating from a questing adult *I. scapularis* collected from LPBO in 2017) was submitted to GenBank with accession number MH366540.

The partial 18S rDNA sequence from a *B. odocoilei* infected larval tick pool removed from a Swainson’s thrush at LPBO in 2017 was submitted to GenBank under accession number MH366631. This sequence was 99.5% homologous to sequences obtained from *B. odocoilei* positive ticks in the present study and to the above cervid isolates.
In 2016-2017, questing and host-feeding ticks were collected from three field sites in southern Ontario, Canada, to investigate the prevalence of *Babesia odocoilei*, *Babesia microti*, *Borrelia burgdorferi*, *Borrelia miyamotoi*, and *Anaplasma phagocytophilum* by polymerase chain reaction.
Table 3.1 Bird species that harbored ticks in spring 2016 and 2017 at Long Point Bird Observatory, Ontario, Canada, and the results of pathogen testing\(^9\) of *Ixodes scapularis* ticks.

<table>
<thead>
<tr>
<th>Avian species on which ticks were identified</th>
<th>No. of birds examined</th>
<th>Total no. of birds carrying ticks</th>
<th>No. of birds carrying ticks of each species (no. of birds carrying <em>Ixodes scapularis</em> ticks PCR-positive for <em>Babesia odocoieli</em> / no. of birds carrying <em>Ixodes scapularis</em> ticks PCR-positive for <em>Borrelia burgdorferi</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>American robin</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Baltimore oriole</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Blue jay</td>
<td></td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Brown-headed cowbird</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Canada warbler</td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

\(^9\) All *Ixodes scapularis* ticks were PCR negative for *Babesia microti* and *Borrelia miyamotoi*.
Avian species on which ticks were identified | No. of birds examined | Total no. of birds carrying ticks | No. of birds carrying ticks of each species (no. of birds carrying *Ixodes scapularis* ticks PCR-positive for *Babesia odocoiel* / no. of birds carrying *Ixodes scapularis* ticks PCR-positive for *Borrelia burgdorferi*) | 2016 | 2017 | 2016 | 2017 | 2016 | 2017 | 2016 | 2017 | 2016 | 2017 | 2016 | 2017 | 2016 | 2017 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Carolina wren&lt;sup&gt;10&lt;/sup&gt;</td>
<td><em>Thryothorus ludovicianus</em></td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1 (0/0)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chipping sparrow</td>
<td><em>Spizella passerina</em></td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1 (0/0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Common grackle</td>
<td><em>Quiscalus quiscula</em></td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1 (0/0)</td>
<td>1 (0/0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Common yellowthroat</td>
<td><em>Geothlypis trichas</em></td>
<td>27</td>
<td>27</td>
<td>2</td>
<td>4</td>
<td>2 (0/0)</td>
<td>3 (0/1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>European starling</td>
<td><em>Sturnus vulgaris</em></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1 (0/0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grey catbird</td>
<td><em>Dumetella carolinensis</em></td>
<td>38</td>
<td>28</td>
<td>3</td>
<td>4</td>
<td>1 (0/0)</td>
<td>1 (0/0)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>10</sup> The Carolina wren is a winter resident at LPBO (i.e., non-migratory). Some birds carried more than one tick species.
<table>
<thead>
<tr>
<th>Avian species on which ticks were identified</th>
<th>Total no. of birds carrying ticks</th>
<th>No. of birds carrying ticks of each species (no. of birds carrying <em>Ixodes scapularis</em> ticks PCR-positive for <em>Babesia odocoiel</em> / no. of birds carrying <em>Ixodes scapularis</em> ticks PCR-positive for <em>Borrelia burgdorferi</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grey-cheeked thrush <em>Catharus minimus</em></td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Indigo bunting <em>Passerina cyanea</em></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Lincoln’s sparrow <em>Melospiza lincolni</em></td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>Mourning warbler <em>Geothlypis philadelphia</em></td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Northern waterthrush <em>Parkesia noveboracensis</em></td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Red-eyed vireo <em>Vireo olivaceus</em></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Red-winged blackbird <em>Agelaius phoeniceus</em></td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Avian species on which ticks were identified</td>
<td>No. of birds examined</td>
<td>Total no. of birds carrying ticks</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>---------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>Song sparrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Melospiza melodia</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Swainson’s thrush</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Catharus ustulatus</em></td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Swamp sparrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Melospiza georgiana</em></td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Veery thrush</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Catharus fuscescens</em></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>White-crowned sparrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Zonotrichia leucophrys</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Wood thrush</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hylocichla mustelina</em></td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>13 species (2016)</strong></td>
<td><strong>21 species (2017)</strong></td>
</tr>
</tbody>
</table>
Table 3.2 *Ixodes scapularis* ticks collected from Long Point Bird Observatory (LPBO), Point Pelee National Park (PPNP), and Toronto Zoo (TZ) in 2016 and 2017 and results of PCR testing for selected pathogens.11

<table>
<thead>
<tr>
<th>Date collected</th>
<th>Location</th>
<th>No. of <em>I. scapularis</em> and life stage13</th>
<th>Ticks fed or unfed14</th>
<th>Babesia odocoilei</th>
<th>Anaplasma phagocytophilum</th>
<th>Borrelia burgdorferi</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 2016</td>
<td>LPBO</td>
<td>4 A</td>
<td>Unfed</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>May 2016</td>
<td>TZ</td>
<td>3 A</td>
<td>Fed</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Oct 2016</td>
<td>TZ</td>
<td>3 L; 1 N; 4 A</td>
<td>Unfed</td>
<td>1 L</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nov 2016</td>
<td>PPNP</td>
<td>41 A</td>
<td>Unfed</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>May-June 2017</td>
<td>LPBO</td>
<td>45 A</td>
<td>Unfed</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>June 2017</td>
<td>TZ</td>
<td>24 A</td>
<td>Unfed</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Aug 2017</td>
<td>TZ</td>
<td>126 L</td>
<td>Unfed</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

12 All *Ixodes scapularis* ticks were PCR negative for *Babesia microti* and *Borrelia miyamotoi*. Ticks collected from the same environmental sampling location on the same date were pooled by life stage for testing.
13 L = Larva; N = Nymph; A = Adult.
14 Unfed ticks were collected from the environment by blanket dragging or were found questing but unattached on a vertebrate host; fed ticks were found actively feeding on the host.
3.5 Discussion

This study was initiated to investigate the eco-epidemiology of *B. odocoilei* in southern Ontario, Canada, following an outbreak of *B. odocoilei*-associated cervid babesiosis amongst reindeer and wapiti at the TZ in 2012-2015 (Mathieu et al. 2018). Our findings represent the first detections of *B. odocoilei* in *I. scapularis* ticks in Ontario, both in questing ticks at the TZ and in the endemic *I. scapularis* population at LPBO, as well as in ticks found on northward migrating birds sampled at LPBO. Given the geographic range and large population of wild white-tailed deer, the primary vertebrate host for *B. odocoilei*, in southern Ontario, the establishment of a wildlife reservoir for cervid babesiosis in this region seems likely.

Comparison of partial 18S rDNA sequences from tick-derived *B. odocoilei* in our study revealed that all but one isolate showed complete (100%) identity with *B. odocoilei* 18S rDNA sequences in GenBank from various hosts and geographic locations, including a wildlife reservoir in Texas, USA (Holman et al. 2000) and clinical cases in cervids from the TZ (Mathieu et al. 2018). This suggests that the strains of *B. odocoilei* occurring in southern Ontario are similar if not identical to those reported in the United States. However, one sequence derived from a bird-borne tick in our study showed only 99.5% identity with the other southern Ontario tick-derived sequences and the previously mentioned cervid isolates. This could indicate a different strain of *B. odocoilei*, and further molecular characterisation using primers specific for a mitochondrial target (e.g., the mitochondrial cytochrome c oxidase subunit I (COI) gene) would be required to investigate this further (Barta 2001).

We detected *B. odocoilei* in extracts from homogenized bisected ticks found both in the environment and on migratory birds. The source of this DNA could be either the salivary gland of the tick (i.e., indicating that the tick vector was truly infected with *B. odocoilei*), or blood in the tick gut derived from feeding recently on an infected vertebrate host (Estrada-Peña et al. 2013). *Ixodes scapularis* is a host generalist that has been reported to take blood meals from at least 125 different vertebrate host species including mammals, birds, and lizards (Keirans et al. 1996). However, the white-tailed deer is the preferred host for the adult life stage and consequently, *I. scapularis* are most abundant in regions where deer are also numerous (Rand et al. 2003). The ability of *B. odocoilei* to infect avian and other non-cervid wildlife hosts is unknown. Previous studies have demonstrated that wild birds are competent reservoir hosts for *B. microti* (Hersh et al. 2012), so it is possible that our positive results reflect ticks feeding on a *B. odocoilei*-infected avian or other wildlife hosts. Our finding of *B. odocoilei* PCR-positive larval pools collected using blanket dragging (i.e., unfed larvae) suggest that transovarial transmission of this parasite is possible; this has not yet been demonstrated experimentally for *B. odocoilei*, but is documented for the closely related species,
Babesia divergens. Transovarial Babesia infection may persist over several tick generations, even without new infections derived from blood meals (Uilenberg 2006). Ixodes scapularis are the definitive hosts for B. odocoilei, with a major part of the protozoan life cycle taking place within the arthropod. Future studies on the role of I. scapularis in the transmission of B. odocoilei should include blood sampling of avian and other wildlife hosts to survey for B. odocoilei infection, as well as laboratory studies of vector and reservoir competence.

We surveyed migratory birds to assess whether the transportation of B. odocoilei-infected ticks over long distances by avian hosts could be a factor in the recent emergence of cervid babesiosis in Canada (Mathieu et al. 2018). In the current study, we found an I. scapularis infestation prevalence of 3.2% in migrating birds captured in spring 2016, and 6.7% in birds captured in 2017. These values are considerably greater than those found in a study conducted during the spring of 2005 and 2006, where the prevalence of I. scapularis infestation of migratory birds was estimated at 2.2% at 12 bird observatories in eastern Canada, including LPBO where our investigation was also undertaken (Ogden et al. 2008). Undercounting of ticks on birds is a recognised problem in these studies, and our higher reported prevalence in 2017 may be due to sensitization of observers to finding ticks in the second year of the study. Alternatively, our findings may represent a true increase in tick infestation of migratory birds in the decade since the previous survey. There is an endemic I. scapularis population at LPBO that was first documented in the 1970s (Watson and Anderson 1976), and it is likely that birds acquired ticks at the site of capture as well as carrying ticks from the south to LPBO during their northward spring migration. As did Ogden et al. (2008), we found that the prevalence of I. scapularis infestation was highest in ground-foraging bird species such as thrushes. The combined data from both years of our study revealed that 0.2% of birds carried I. scapularis ticks that were PCR positive for B. odocoilei. Stable isotope analysis of tail feathers has been used to investigate the migration strategies of birds captured at LPBO, and revealed that some bird taxa, particularly thrushes, are disproportionately more likely to disperse I. scapularis ticks over long distances because they breed in the far northern regions of Canada (Ogden et al. 2015). Larval and nymphal I. scapularis feed for 2-4 days on their host, during which time a migratory bird may fly hundreds of kilometres northwards (Marra et al. 2005). In the present study, our finding of a B. odocoilei-infected tick attached to a Swainson’s thrush illustrates the potential threat of adventitious B. odocoilei-infected ticks to immunologically naïve wild cervids, such as the boreal population of woodland caribou (Rangifer tarandus caribou) found across the northern parts of all Canadian provinces (Thomas and Gray 2002).

In addition to our investigation of the veterinary pathogen B. odocoilei, the potential threat of selected zoonotic diseases vectored by I. scapularis was also assessed. Our
study identified *B. burgdorferi* in *I. scapularis* ticks collected from the TZ, LPBO and PPNP, and from migratory birds, consistent with previous reports from southern Ontario (Barker and Lindsay 2000). Ogden et al. (2008) reported that 18.1% (25/138) of *I. scapularis*-infested birds examined in 2005-2006 carried a *B. burgdorferi*-positive tick, and in 2016-2017, we found that 11.3% (6/53) of *I. scapularis*-infested birds carried a *B. burgdorferi*-positive tick. The nymph life stage of *I. scapularis* is likely to have been infected with *B. burgdorferi* by feeding on a reservoir-competent rodent during the larval life stage. However, an alternative explanation for these PCR-positive bird-borne ticks is that the tick gut contained *B. burgdorferi*-infected blood from the avian host. Numerous bird species are competent reservoirs for *B. burgdorferi*, including the Swainson’s thrush (Scott et al. 2010). Our results are consistent with previous studies that support the role of migratory birds in *I. scapularis* and *B burgdorferi* range expansion in Canada. Overall, however, avian species are considered much less important in the ecology of human Lyme borreliosis than small mammals such as the white-footed mouse (*Peromyscus leucopus*) (Giardina et al. 2000).

Clow et al. (2017) recently conducted active surveillance of questing *I. scapularis* across 104 sites in eastern, central, and southern Ontario, and did not detect *B. microti*, *B. miyamotoi*, or *A. phagocytophilum* at any sites. Similarly, we did not identify *B. microti* or *B. miyamotoi* in any ticks in our study. The small sample size of our study likely limited our ability to detect these pathogens, which are all present but at a very low prevalence (approximately 0.3%) in *I. scapularis* in Ontario (Nelder et al. 2014; Dibernardo et al. 2014). We did detect *A. phagocytophilum* in host-feeding *I. scapularis* adults collected from a wild white-tailed deer carcass at the TZ, giving a minimum infection prevalence for *A. phagocytophilum* of 0.6% in *I. scapularis* at this location, which is consistent with previous estimates of its prevalence in Ontario (Nelder et al. 2014). *Anaplasma phagocytophilum*-associated clinical disease is an emerging disease of humans (human granulocytic anaplasmosis) and domestic horses (equine granulocytic ehrlichiosis) in the United States (Dumler et al. 2005). Equine granulocytic ehrlichiosis is caused by the same pathogen and was recently reported in a captive herd of Przewalski’s horses (*Equus ferus przewalskii*) at a zoo in the mid-Atlantic United States (Sim et al. 2017). The TZ also houses a herd of Przewalski’s horses, which should be considered at high risk for this disease.

In conclusion, we investigated the role of migratory birds and environmental *I. scapularis* ticks in the eco-epidemiology of *B. odocoiel*, *B. microti*, *B. burgdorferi*, *B. miyamotoi*, and *A. phagocytophilum* in southern Ontario, Canada. Our study provides the first evidence of established local populations of *I. scapularis* ticks in southern Ontario that are infected with *B. odocoiel*. We found that *B. odocoiel*-infected ticks are harbored by migratory birds, providing a possible route by which *B. odocoiel* could be introduced to naïve cervid populations. Additionally, we demonstrated the presence of
A. phagocytophilum and B. burgdorferi in I. scapularis ticks at the Toronto Zoo, highlighting the need for public health and veterinary preventative health measures for zoo animals, members of the public, and zoo staff.
Pharmacokinetics of imidocarb dipropionate in white-tailed deer (*Odocoileus virginianus*) after single intramuscular administration

4.1 Abstract

Cervid babesiosis, a hemolytic disease caused by the tick-borne protozoan hemoparasite *Babesia odocoilei*, is an emerging cause of morbidity and mortality amongst North American cervids. Anecdotal reports from zoological institutions suggest that a single intramuscular injection of imidocarb dipropionate at the typical dosage used for *Babesia* spp. infections in domestic cattle (3.0 mg/kg) is safe and effective for both treating clinical disease and eliminating subclinical *B. odocoilei* infection in a variety of captive cervid species. The pharmacokinetics of imidocarb dipropionate were studied in white-tailed deer (*Odocoileus virginianus*) after a single intramuscular injection of 3.0 mg/kg. Imidocarb dipropionate was administered to 10 healthy adult female white-tailed deer, and blood samples were collected prior to drug administration and at regular intervals for 48 h after treatment. Plasma concentrations of imidocarb were determined using high-performance liquid chromatography. The disposition of plasma imidocarb was best characterised by a two-compartment open model. The mean ± SD maximal imidocarb concentration in deer was 824.92 ± 1.55 ng/mL at 36.47 ± 1.38 min post injection. The distribution phase had a half-life (*t*₁/₂ₐ) of 24.63 ± 1.36 min, and plasma imidocarb concentration slowly declined with an elimination half-life (*t*₁/₂ₐ) of 46.65 ± 1.36 min. Volume of distribution based on the terminal phase (*V*ₚ) was 9.2 ± 2.7 L/kg and total body clearance (*Cl*) was 15.97 ± 1.28 mL/min/kg. No adverse effects were observed in any animals during the course of this study. Clinical efficacy studies are needed to confirm the appropriate imidocarb dosage regimen for treatment and prophylaxis of babesiosis in deer.

4.2 Introduction

Cervid babesiosis, caused by the hemoprotozoan parasite *Babesia odocoilei*, is an emerging tick-borne disease of North American cervids. The parasite life cycle involves the black-legged tick (*Ixodes scapularis*) as the definitive host and only known vector of *B. odocoilei*. The geographic range expansion of *I. scapularis* into Canada is thought to have contributed to epizootics of cervid babesiosis in captive reindeer (*Rangifer tarandus tarandus*) and wapiti (*Cervus canadensis*) in several Canadian zoos and deer farms since 2012 (Mathieu et al. 2018). Wild white-tailed deer (*Odocoileus virginianus*) are the natural reservoir of *B. odocoilei* and rarely show clinical signs of disease.
A large number of chemical compounds have been reported to be effective against *Babesia* spp. in domestic livestock, including diminazene diaceturate, amicarbalide, clindamycin, and tetracycline. Imidocarb, a carbanilide derivative usually administered as the dipropionate salt by intramuscular or subcutaneous injection, is considered to be the safest and most effective of these treatments (Vial and Gorenflot, 2006). There are no anti/protozoal drugs licensed or labelled for use in cervids worldwide. Pharmacokinetic studies of imidocarb are widely reported in several domestic species, but nothing is known about its pharmacokinetic behavior following intramuscular injection in cervids. Although there are reports in the zoo medicine literature of imidocarb use in captive reindeer and wapiti for elimination of parasites both from animals that are suffering from acute babesiosis and from asymptomatic infected animals (Bartlett et al. 2009), a review of the published literature failed to find a reference to the pharmacokinetic study of imidocarb in any cervid species.

The objective of this study was to determine the plasma concentrations and pharmacokinetic behaviour of the anti/protozoal drug imidocarb in captive white-tailed deer following a single intramuscular injection of imidocarb dipropionate at the dose commonly administered to treat *Babesia* spp. infections in domestic ruminants (3.0 mg/kg). The results provide information that can be used to form a drug regimen specifically designed for the treatment and prophylaxis of *Babesia odocoilei*-associated hemolytic anemia in cervids. White-tailed deer were chosen for this study because they are the reservoir hosts for *B. odocoilei*, and they represent a suitable research model for other cervid species.

### 4.3 Materials and Methods

#### 4.3.1 Animals

Ten adult female white-tailed deer of mixed ages weighing 50 – 68 kg were used in this study. The animals were housed at the Specialized Livestock Research Facility at the University of Saskatchewan (Saskatoon, Saskatchewan, Canada) and had no history of imidocarb exposure or infection with *Babesia odocoilei*. Animals were maintained on pasture forage during the summer months, and baled forage with mineral supplements in the winter months. Fresh water was provided *ad libitum*. Routine preventative health care was provided, consisting of annual application of anthelmintic and a multivalent clostridial vaccine. All animals were determined to be clinically healthy based on routine veterinary examination and assessment of hematology and biochemistry parameters at the start of the study and did not receive any drug treatment for at least 8 weeks before the trial began. The study was carried out with approvals from the University of...
Saskatchewan’s Animal Research Ethics Board, the Toronto Zoo Animal Care Committee, and the University of Guelph Animal Care Committee, in compliance with the regulations of the Canadian Council on Animal Care.

4.3.2 Experimental design

Deer were individually weighed in a commercial white-tailed deer handling system (The DeerHandler™, 54423 R.R. 252, Sturgeon County, AB, Canada) to which they had been previously trained. A single deep intramuscular injection of imidocarb dipropionate (Imizol®, Merck Animal Health, Madison, NJ, USA) was administered in the lower third region of the neck. Nine deer were dosed at 3.0 mg/kg, and one deer was dosed at 2.4 mg/kg due to a technical error. Blood samples (10 mL) were collected into lithium-heparin vacutainer tubes by jugular venipuncture immediately prior to dose administration and at 10, 20, and 30 min, and 1, 2, 3, 4, 6, 8, 12, 24, and 48 h after dosing. Plasma was separated from the collected blood samples by centrifugation at 1,500 x g for 10 min within 1 h after collection, transferred to sterile cryovials, and stored at -20.0 °C until shipped for analysis. Three of the 10 deer vocalized during the intramuscular injection of imidocarb dipropionate, otherwise there were no adverse effects observed. All animals remained healthy throughout the study.

4.3.3 Reagents and chemicals

Imidocarb dipropionate standard, trifluoroacetic acid, 1-pentanesulfonic acid sodium, and triethylamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium hydroxide solution was purchased from Fisher Scientific (Oakville, ON, Canada). HPLC-grade acetonitrile and methanol were obtained from Caledon Laboratories Ltd. (Georgetown, ON Canada). Throughout the study, water was obtained from a Milli-Q™ system (Millipore, Bedford, MA, USA).

4.3.4 Assay method validation

A stock solution of imidocarb dipropionate was prepared in methanol/water (50/50, v/v) at a concentration of 1 mg/mL and stored at –80.0 °C. Calibration standards (10, 20, 40, 100, and 200 ng/mL), and quality controls (15 and 150 ng/mL) were prepared from a pool of blank white-tailed deer plasma collected from animals with no history of imidocarb exposure. Correlation coefficients ($r^2$) were > 0.99 for all calibration curves.
The mean percentage recovery of imidocarb from plasma was 90.0%. The observed retention time of imidocarb was 2.89 min. The limit of detection for this assay, based on three times the signal to noise ratio at the time of elution of the imidocarb, was 0.005 \( \mu \text{g/mL} \). The limit of quantification for the assay was 0.01 \( \mu \text{g/mL} \). The analytical method was repeatable and reproducible with average intra- and inter-day precisions of 10.6% and 9.3%, respectively.

### 4.3.5 Assay analytical method

HPLC analysis was performed on a Waters Alliance 2695 separations module coupled with a Waters 2696 photodiode array detector (Milford, MA, USA), according to the procedure described by Su et al. (2007), with modifications as follows. An aliquot of 1.0 mL was taken from each plasma sample, acidified with 1 mL of phosphoric acid (4%), and added to a Waters Oasis™ WCX (Waters, Dublin, Ireland) cartridge after the cartridge was conditioned with 2 mL of methanol and then equilibrated with 3 mL of water. The cartridge was first washed with 3 mL of ammonium hydroxide solution (2%) and then washed with 3 mL of a mixture of ammonium hydroxide (5%) and acetonitrile (50%). The analyte was eluted with 3 mL acetonitrile/methanol/trifluoroacetic acid (500/490/10, v/v/v), evaporated under a constant flow of nitrogen, reconstituted in 200 \( \mu \text{L} \) of the mobile phase, and 50 \( \mu \text{L} \) was injected onto the column. The conditions for the HPLC analysis were as follows: separation was performed on a 50 x 4.6 mm column (3.5 \( \mu \text{m} \); XBridge™ C18 column, Waters) equipped with a Security Guard C18 guard column (4 x 3 mm I.D., Phenomenex™, Torrance, CA, USA). The mobile phase consisted of acetonitrile, 1-pentanesulfonic acid sodium salt (5 mM), and 0.1% trimethylamine trifluoroacetic acid at fan 89:11 (v/v) ratio. The flow rate was fixed at 1.0 mL/min and the UV detector was set at 260 nm. Concentrations were derived by comparing peak areas of the samples to those of an external standard curve of imidocarb made from spiked plasma samples put through the same cleanup process as described above. Samples with imidocarb concentration greater than 200 ng/mL were diluted with blank deer plasma prior to extraction.

### 4.3.6 Pharmacokinetic data analysis

The pharmacokinetic parameters of imidocarb were calculated using Phoenix WinNonlin software (Certara™, Princeton, NJ, USA) and standard approaches to both non-compartmental and compartmental analysis conducted on individual animal data sets. Samples were weighted using \( 1/(Y*Y) \). The plasma pharmacokinetics of imidocarb after intramuscular administration best fit a two-compartment open model with first order absorption and elimination, with the following equation:
where \( C \) is the plasma drug concentration at time \( t \); \( A \) is the mathematical coefficient for the zero-time intercept of the distribution slope in the compartment model; \( B \) is the mathematical coefficient for the zero-time intercept of decline in plasma concentration of the drug; \( \alpha \) is the distribution rate constant; \( \beta \) is the rate constant for the terminal elimination phase; \( k_a \) is the first-order absorption rate constant; \( e \) is the natural log. The optimal model was determined based on visual inspection of goodness-of-fit plots (observed versus predicted and residuals), and Akaike’s information criterion.

The following pharmacokinetic parameters were derived from the noncompartmental analysis: the area under the curve (AUC) calculated by the trapezoidal method from the time of dosing and extrapolated to infinity (\( AUC_{0-\infty} \)), the AUC from the time of dosing to the last measurable time point for each deer (\( AUC_{last} \)), the volume of distribution based on the terminal phase (\( V_z \)), and the total body clearance (\( Cl \)). The noncompartmental analysis also provided the mean residence time (MRT) from the time of dosing to the time of the last measurable concentration (\( MRT_{last} \)).

### 4.4 Results

#### 4.4.1 Imidocarb pharmacokinetics in deer

The plasma concentration-time curve of imidocarb following intramuscular administration is presented in Fig. 1 with the corresponding pharmacokinetic parameters presented in Table 1. The imidocarb plasma concentration-time data best fit an open two-compartmental model with first-order absorption. Imidocarb was detectable within 10 min in the plasma of all treated animals. Peak plasma imidocarb concentration (\( C_{max} \); 824.92 ± 1.55 ng/mL) was reached at 36.47 ± 1.38 min. The distribution phase had a half-life (\( t_{1/2a} \)) of 24.63 ± 1.36 min. The plasma imidocarb concentration declined with a terminal phase half-life (\( t_{1/2b} \)) of 46.65 ± 1.36 min. Detectable imidocarb concentrations were maintained for 8 h in all deer, 12 h in 9 out of 10 deer, 24 h in 4 out of 10 deer, 36 h in 3 out of 10 deer, and 48 h in 2 out of 10 deer. Total area under the concentration-time curve (\( AUC_{0-\infty} \)) was 194.33 ± 15.06 (min-ng/mL).
Table 4.1 Pharmacokinetic parameters (mean ± SD) of imidocarb dipropionate in white-tailed deer (*Odocoileus virginianus*) after intramuscular administration at a dose of 3.0 mg/kg body weight.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>ng/mL</td>
<td>29140.88 ± 1.85</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>L/min</td>
<td>0.028 ± 1.36</td>
</tr>
<tr>
<td>$B$</td>
<td>ng/mL</td>
<td>144.36 ± 1.89</td>
</tr>
<tr>
<td>$\beta$</td>
<td>L/min</td>
<td>0.0022 ± 0.0014</td>
</tr>
<tr>
<td>$t_{1/2a}$</td>
<td>min</td>
<td>24.63 ± 1.36</td>
</tr>
<tr>
<td>$t_{1/2b}$</td>
<td>min</td>
<td>46.65 ± 1.36</td>
</tr>
<tr>
<td>$k_a$</td>
<td>L/min</td>
<td>0.03 ± 1.36</td>
</tr>
<tr>
<td>$t_{1/2Ka}$</td>
<td>min</td>
<td>22.90 ± 1.36</td>
</tr>
<tr>
<td>$t_{\text{max}}$</td>
<td>min</td>
<td>36.47 ± 1.38</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>ng/mL</td>
<td>824.92 ± 1.55</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$</td>
<td>min·ng/mL</td>
<td>194.33 ± 15.06</td>
</tr>
<tr>
<td>$AUC_{\text{last}}$</td>
<td>min·ng/mL</td>
<td>184.76 ± 13.39</td>
</tr>
<tr>
<td>$V_Z$</td>
<td>L/kg</td>
<td>9.2 ± 2.7</td>
</tr>
<tr>
<td>MRT$_{\text{last}}$</td>
<td>min</td>
<td>205.52 ± 43.43</td>
</tr>
<tr>
<td>$Cl$</td>
<td>mL/min/kg</td>
<td>15.97 ± 1.28</td>
</tr>
</tbody>
</table>

$A$, zero-time intercept of the distribution slope in the compartment model; $B$, zero-time intercept of decline in plasma concentration of imidocarb; $\alpha$, distribution rate constant; $\beta$, elimination constant; $k_a$, absorption rate constant; $t_{1/2Ka}$, absorption half-life; $t_{1/2a}$, half-life during distribution phase; $t_{1/2b}$, the half-life of elimination; $AUC_{0-\infty}$, total area under the concentration–time curve from zero to infinity; $AUC_{\text{last}}$, total area under the concentration–time curve from zero to the last measurable time point; $C_{\text{max}}$, peak drug concentration; $t_{\text{max}}$, time to $C_{\text{max}}$ from time zero; $V_Z$, volume of distribution based on the terminal phase; MRT$_{\text{last}}$, mean residence time from the time of dosing to the time of the last measurable concentration; $Cl$, total body clearance.
Figure 4.1 Semi-logarithmic plot of mean plasma concentration vs. time curve of imidocarb dipropionate in white-tailed deer (*Odocoileus virginianus*) after single-dose intramuscular injection at 3.0 mg/kg body weight. Error bars represent standard deviations.
4.5 Discussion

The treatment and prophylaxis of cervid babesiosis is a topical problem for zoos and deer farms in North America (Pastor and Milnes 2018). Anthropogenic land use change and climate warming have expanded the geographic range of the vector tick *I. scapularis*, thus increasing the number of cervids at risk of exposure to *B. odocoilei* (Chapter 2). Control of babesiosis in cervids requires a combination of anti-protozoal drugs to treat clinical cases and eliminate subclinical infections, and strategic tick control programs integrating pasture management with application of acaricides (Pastor and Milnes 2018). Determination of the pharmacokinetic behavior of antiPROTOZOAL drugs is required in order to develop recommendations for their use in cervid species.

To the best of the authors' knowledge, this is the first report of the pharmacokinetics of imidocarb dipropionate in white-tailed deer following intramuscular injection at the dose commonly used to treat babesiosis in domestic ruminants (3.0 mg/kg). The plasma pharmacokinetic profile was best described by a two-compartment open model. This is in agreement with previous pharmacokinetic observations in horses (Belloli et al. 2002), small ruminants (sheep and goats; Belloli et al. 2006), and pigs (Su et al. 2007). Peak plasma concentrations were reached rapidly after intramuscular administration, as expected for a lipid-soluble organic base. The mean plasma concentration showed a fast distribution phase ($t_{1/2a} = 24.63 \pm 1.36$ min), followed by a slower terminal phase ($t_{1/2b} = 46.65 \pm 1.36$ min). At 48 h after treatment, detectable plasma concentrations were recorded in only 2 out of 10 animals. The mean percentage recovery of imidocarb from white-tailed deer plasma was 90%, which is similar to pharmacokinetic data from horses (89%; Belloli et al. 2002), sheep and goats (88%; Belloli et al. 2006) and pigs (87%; Su et al. 2007).

Pharmacokinetic assessment of imidocarb in deer is an important component in understanding drug behavior and efficacy in these species. However, reports on plasma concentrations of imidocarb that may be effective for treatment and prophylaxis of babesiosis in cervids are not known, and the mechanism of action of imidocarb against *Babesia* parasites is poorly understood. The drug may interfere with the production or use of polyamines, or block inositol entry into infected erythrocytes, thereby starving the intraerythrocytic parasite (Mosqueda et al. 2012). *Babesia odocoilei* is morphologically similar to the “large” babesias such as *B. bigemina*, *B. caballi*, and *B. divergens* (Holman et al. 2002). In cattle, an *in vitro* minimum inhibitory concentration of imidocarb of 27.0-34.0 ng/mL is described for *B. divergens* (Brasseur et al. 1998). At 8 h after treatment in our study, imidocarb was detectable in all deer at a plasma concentration of $53.91 \pm 6.18$ ng/mL. Consequently, the imidocarb plasma concentration profile we describe in white-tailed deer following intramuscular injection at 3.0 mg/kg may be efficacious for the treatment of *B. odocoilei* in this species, and the rapid distribution and
slow elimination of imidocarb in deer may result in plasma concentrations remaining at therapeutic concentrations for at least 8 h.

The volume of distribution in our study was \(9.2 \pm 2.7\) L/kg, which is in agreement with values previously described in sheep (\(4.18 \pm 0.44\) L/kg) and goats (\(7.68 \pm 0.57\) L/kg) (Belloli et al. 2006). The finding of a very large volume of distribution is not surprising, because imidocarb is a lipid-soluble organic base that is subject to sequestration (ionic trapping) within cells. *Babesia odocoilei* is an intracellular parasite, so an effective anti-*Babesia* drug must be able to cross cell membranes to be effective. In ruminants, ion trapping within rumen fluid (pH 5.5-6.5) also contributes to very large volumes of distribution for basic drugs (Abdullah and Baggot 1983). *In vivo* residue studies on sheep and goats following intramuscular injection with 3.0 mg/kg imidocarb dipropionate found that high and long-lasting drug levels are found in the liver and kidney (Lai et al. 2002). Several studies in domestic animals suggest that imidocarb in the liver and kidney may have a “reservoir effect” that acts as a delivery system producing low plasma concentrations (i.e., undetectable by HPLC assay) for several weeks that may be prophylactic against babesiosis (Belloli et al. 2006). Studies of cattle treated with imidocarb in *Babesia*-endemic regions show that, following a single injection of imidocarb at 3.0 mg/kg, animals are protected from the development of clinical disease for four to six weeks (Vial and Gorenflot 2006). Total body clearance in our study was low (15.97 \(\pm\) 1.28 mL/min/kg), supporting the observed slow elimination values. Clinical trials in parasitemic cervids infected with *B. odocoilei* are required in order to evaluate the efficacy of this dose of imidocarb as a treatment for cervid babesiosis.

In cattle, it is recommended to give a single intramuscular or subcutaneous injection of imidocarb for treatment and prophylaxis of babesiosis (Andrews et al. 2008). Intravenous injection is not recommended because this has been reported to cause acute death from severe bronchoconstriction secondary to cholinesterase inhibition (Abdullah et al. 1984). Following intramuscular injection of imidocarb in deer in the present study, no adverse effects were seen other than vocalization during injection in 3 of 10 deer. Pain upon injection of imidocarb is reported commonly in domestic dogs (Vial and Gorenflot 2006).

The administration of babesiacidal compounds at subtherapeutic dosages could encourage the development of drug-resistant parasites, although this has not yet been reported for imidocarb in any species (Mosqueda et al. 2012). All imidocarb usage in cervids constitutes off-label use, and a conservative meat withdrawal period is recommended when considering the use of this drug in commercial cervids. Imidocarb remains detectable in edible bovine tissues for several months following dosing, due to
deposition in hepatocytes (Moore et al. 1996), and further research is warranted to gain insight into the possible residual concentrations of imidocarb in cervid products for human consumption.
5 Conclusions and Future Directions

The overall objectives of this research were two-fold. First, we sought to acquire further knowledge about the epidemiology of Babesia odocoilei in cervids and in the tick vector Ixodes scapularis in Ontario, Canada. The second objective was to determine the plasma concentrations and pharmacokinetics of the anti/protozoal drug imidocarb in white-tailed deer following a single intramuscular injection at the dose used to treat babesiosis in domestic ruminants (3.0 mg/kg), in order to inform an evidence-based treatment protocol for babesiosis in cervids.

Our prevalence survey revealed that 4.4% of free-ranging white-tailed deer sampled across southern Ontario were infected with a B. odocoilei strain that is very similar or identical to those isolated from captive cervids in acute hemolytic crisis across North America. White-tailed deer are thus a potential natural wildlife reservoir for B. odocoilei in Ontario. We identified B. odocoilei infection in a wapiti at the Toronto Zoo (TZ) with no clinical signs of babesiosis, consistent with previous data indicating that this species can be an asymptomatic carrier of B. odocoilei as well as being susceptible to acute hemolytic parasitemia. Farmed red deer were shown, for the first time, to be infected with B. odocoilei at a prevalence of 1.4%. The pathogenicity of B. odocoilei for red deer is unknown, and the finding of B. odocoilei-infected, apparently healthy red deer on two farms on Ontario is concerning for the health of commercially farmed cervids as well as providing another potential disease reservoir in this geographic region.

We surveyed two field sites in southern Ontario with known endemic I. scapularis populations (LPBO and PPNP) and one site with unknown I. scapularis status (the TZ). We found that I. scapularis are present at the TZ, and that questing ticks in this location are infected with B. odocoilei as well as the causative agents of human and equine granulocytic anaplasmosis (Anaplasma phagocytophilum) and Lyme borreliosis (Borrelia burgdorferi). Babesia odocoilei-infected ticks were also collected from LPBO, and B. burgdorferi-infected ticks were found at both LPBO and PPNP. Based on our findings, we recommend that preventative health measures continue to be undertaken for humans and animals at the TZ to prevent transmission of tick-borne diseases beyond cervid babesiosis.

Additionally, we investigated the role of migratory birds in the range expansion of B. odocoilei-infected I. scapularis ticks. We found that 3.2% and 6.7% of birds harbored one or more I. scapularis ticks in 2016 and 2017, respectively. Collectively across both years of the study, we found that 0.2% of birds examined hosted one or more I. scapularis ticks that were B. odocoilei-positive by PCR. Global climate change leading
to warmer environments in northern latitudes supports the range expansion of disease vectors, and the adventitious spread of ticks and tick-borne pathogens by birds represents a substantial disease risk to humans and animals across Canada. This research underscores the need for further information on the behavior of tick-infested birds during migration to better understand potential tick dispersal on birds, including the distance traveled while a tick is attached, and the length of time spent at stopover points such as LPBO where they are likely to encounter questing *I. scapularis* ticks infected with *B. odocoilei* and other pathogens of veterinary and public health significance.

In chapter 4, we described the pharmacokinetic behaviour of imidocarb dipropionate in white-tailed deer measured by HPLC in plasma following a single intramuscular injection at 3.0 mg/kg. No adverse effects were observed. The disposition of plasma imidocarb was best characterised by a two-compartment open model, with rapid distribution and slow elimination, reaching maximal plasma concentrations that exceed the *in vitro* minimum inhibitory concentration described for the treatment of a closely related *Babesia* species in domestic cattle. Thus, the imidocarb plasma concentration profile we describe in deer may result in therapeutic efficacy for cervid babesiosis. Pharmacokinetic studies of imidocarb in highly susceptible cervid species (reindeer and wapiti), as well as clinical trials in cervids infected with babesiosis, are needed to confirm the appropriate dosage regimen in these species.

Lastly, the potential for cervid babesiosis to emerge as a significant disease of wild cervids in Canada should not be overlooked. Wild caribou and reindeer are declining globally due to habitat loss and global climate change (Vors and Boyce 2009), and the boreal population of woodland caribou found across the northern parts of all Canadian provinces is listed as Threatened by the Committee on the Status of Endangered Wildlife in Canada (Thomas and Gray 2002). Climate change is predicted to increase ecto- and endoparasitism in *Rangifer* spp. in the sub-Arctic and Arctic because of more favorable environmental conditions to parasite survival (Mallory and Boyce 2018). Woodland caribou in zoos are known to be highly susceptible to acute fatal babesiosis following *B. odocoilei* infection (Petrini et al. 1995). The continued expansion of the northern geographic limit of *I. scapularis* could result in a *B. odocoilei* disease epidemic in immunologically naïve woodland caribou populations in northern Ontario.
REFERENCES


Current advances in detection and treatment of babesiosis. *Current Medicinal 
Chemistry, 19*(10), pp. 1504-1518.

Nelder, M.P., Russell, C., Lindsay, L.R., Dhar, B., Patel, S.N., Johnson, S., Moore, S., 
surveillance and detection of expanding foci of blacklegged ticks *Ixodes 
scapularis* and the Lyme disease agent *Borrelia burgdorferi* in Ontario, 

Nikol'skii, S.N., Pozov, S.A., 1972. *Ixodes ricinus* ticks as carriers of *Babesia capreoli* in 
the roe deer. *Veterinaryia, 4*, 62.


353-359.

tailed deer in an intensively farmed region of Illinois. *Wildlife Monographs*, pp.3-
77.

Ogden, N.H., Trudel, L., Artsob, H., Barker, I.K., Beauchamp, G., Charron, D.F., Drebot, 
*Ixodes scapularis* ticks collected by passive surveillance in Canada: analysis of


### APPENDIX: SUPPLEMENTARY DATA FOR CHAPTER 3

Table 5.1 Bird species investigated for ticks and found to be uninfested in spring 2016 and spring 2017 at Long Point Bird Observatory, Ontario, Canada

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>Family</th>
<th>2016</th>
<th>2017</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acadian flycatcher</td>
<td><em>Empidonax virescens</em></td>
<td>Tyrannidae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>American goldfinch</td>
<td><em>Spinus tristis</em></td>
<td>Fringillidae</td>
<td>6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>American redstart</td>
<td><em>Setophaga ruticilla</em></td>
<td>Parulidae</td>
<td>29</td>
<td>10</td>
<td>39</td>
</tr>
<tr>
<td>American robin</td>
<td><em>Turdus migratorius</em></td>
<td>Turdidae</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>American woodcock</td>
<td><em>Scolopax minor</em></td>
<td>Scolopacidae</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Baltimore oriole</td>
<td><em>Icterus galbula</em></td>
<td>Icteridae</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Barn swallow</td>
<td><em>Hirundo rustica</em></td>
<td>Hirundinidae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Bay-breasted warbler</td>
<td><em>Setophaga castanea</em></td>
<td>Parulidae</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Black-and-white warbler</td>
<td><em>Mniotilta varia</em></td>
<td>Parulidae</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Black-billed cuckoo</td>
<td><em>Coccyzus erythropthalmus</em></td>
<td>Cuculidae</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Blackburnian warbler</td>
<td><em>Setophaga fusca</em></td>
<td>Parulidae</td>
<td>8</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Black-capped chickadee</td>
<td><em>Poecile atricapillus</em></td>
<td>Paridae</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Blackpoll warbler</td>
<td><em>Setophaga striata</em></td>
<td>Parulidae</td>
<td>9</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Black-throated blue warbler</td>
<td><em>Setophaga caerulescens</em></td>
<td>Parulidae</td>
<td>6</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Black-throated green warbler</td>
<td><em>Setophaga virens</em></td>
<td>Parulidae</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Blue-headed vireo</td>
<td><em>Vireo solitarius</em></td>
<td>Vireonidae</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Brown-headed cowbird</td>
<td><em>Molothrus ater</em></td>
<td>Icteridae</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Brown thrasher</td>
<td><em>Toxostoma rufum</em></td>
<td>Mimidae</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Bird species</td>
<td>Scientific name</td>
<td>Family</td>
<td>No. of birds examined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>----------------------------------</td>
<td>-----------------</td>
<td>-----------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Common name</td>
<td></td>
<td></td>
<td>2016</td>
<td>2017</td>
<td>Total</td>
</tr>
<tr>
<td>Canada warbler</td>
<td>Cardellina canadensis</td>
<td>Parulidae</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Cape May warbler</td>
<td>Setophaga tigrina</td>
<td>Parulidae</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cedar waxwing</td>
<td>Bombycilla cedrorum</td>
<td>Bombycillidae</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Chestnut-sided warbler</td>
<td>Setophaga pensylvanica</td>
<td>Parulidae</td>
<td>9</td>
<td>19</td>
<td>28</td>
</tr>
<tr>
<td>Chipping sparrow</td>
<td>Spizella passerina</td>
<td>Emberizidae</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Eastern kingbird</td>
<td>Tyrannus tyrannus</td>
<td>Tyrannidae</td>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Eastern phoebe</td>
<td>Sayornis phoebe</td>
<td>Tyrannidae</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Eastern wood-pewee</td>
<td>Contopus virens</td>
<td>Tyrannidae</td>
<td>10</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Great crested flycatcher</td>
<td>Myiarchus crinitus</td>
<td>Tyrannidae</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Grey-cheeked thrush</td>
<td>Catharus minimus</td>
<td>Turdidae</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Hooded warbler</td>
<td>Setophaga citrina</td>
<td>Parulidae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>House sparrow(^{15})</td>
<td>Passer domesticus</td>
<td>Passeridae</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>House wren</td>
<td>Troglodytes aedon</td>
<td>Troglodytidae</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Indigo bunting</td>
<td>Passerina cyanea</td>
<td>Cardinalidae</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Least flycatcher</td>
<td>Empidonax minimus</td>
<td>Tyrannidae</td>
<td>26</td>
<td>7</td>
<td>33</td>
</tr>
<tr>
<td>Least sandpiper</td>
<td>Calidris minutilia</td>
<td>Scolopacidae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Magnolia warbler</td>
<td>Setophaga magnolia</td>
<td>Parulidae</td>
<td>69</td>
<td>58</td>
<td>127</td>
</tr>
<tr>
<td>Mourning dove</td>
<td>Setophaga magnolia</td>
<td>Columbidae</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Nashville warbler</td>
<td>Leiothlypis ruficapilla</td>
<td>Parulidae</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Northern cardinal(^{15})</td>
<td>Cardinalis cardinalis</td>
<td>Cardinalidae</td>
<td>1</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Northern parula</td>
<td>Setophaga americana</td>
<td>Parulidae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^{15}\) The house sparrow and northern cardinal are winter residents in Ontario (i.e., non-migratory)
<table>
<thead>
<tr>
<th>Bird species</th>
<th>Scientific name</th>
<th>Family</th>
<th>2016</th>
<th>2017</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange-crowned warbler</td>
<td>Vermivora celata</td>
<td>Parulidae</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ovenbird</td>
<td>Seirus aurocapillus</td>
<td>Parulidae</td>
<td>2</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Philadelphia vireo</td>
<td>Vireo philadelphicus</td>
<td>Vireonidae</td>
<td>5</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>Philadelphia warbler</td>
<td>Geothlypis philadelphia</td>
<td>Parulidae</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Red-bellied woodpecker</td>
<td>Melanerpes carolinus</td>
<td>Picidae</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Red-eyed vireo</td>
<td>Vireo olivaceus</td>
<td>Vireonidae</td>
<td>38</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td>Red-headed woodpecker</td>
<td>Melanerpes erythrocephalus</td>
<td>Picidae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Red-winged blackbird</td>
<td>Agelaius phoeniceus</td>
<td>Icteridae</td>
<td>9</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Rose-breasted grosbeak</td>
<td>Pheucticus ludovicianus</td>
<td>Cardinalida</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Ruby-crowned kinglet</td>
<td>Regulus calendula</td>
<td>Regulidae</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Savannah sparrow</td>
<td>Passerculus sandwichensis</td>
<td>Emberizida</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Scarlet tanager</td>
<td>Piranga olivacea</td>
<td>Thraupidae</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Sharp-shinned hawk</td>
<td>Accipiter striatus</td>
<td>Acciptridae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Summer tanager</td>
<td>Piranga rubra</td>
<td>Thraupidae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Tennessee warbler</td>
<td>Oreothlypis peregrine</td>
<td>Parulidae</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Traill’s flycatcher</td>
<td>Empidonax traillii</td>
<td>Tyrannidae</td>
<td>29</td>
<td>12</td>
<td>41</td>
</tr>
<tr>
<td>Tree swallow</td>
<td>Tachycineta bicolor</td>
<td>Hirundinidae</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Veery thrush</td>
<td>Catharus fuscens</td>
<td>Turdidae</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Warbling vireo</td>
<td>Vireo gilvus</td>
<td>Vireonidae</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>White-eyed vireo</td>
<td>Vireo griseus</td>
<td>Vireonidae</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>White-throated sparrow</td>
<td>Zonotrichia albicollis</td>
<td>Emberizida</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Wilson’s warbler</td>
<td>Cardellina pusilla</td>
<td>Parulidae</td>
<td>12</td>
<td>22</td>
<td>34</td>
</tr>
<tr>
<td>Common name</td>
<td>Scientific name</td>
<td>Family</td>
<td>2016</td>
<td>2017</td>
<td>Total</td>
</tr>
<tr>
<td>------------------------------</td>
<td>---------------------</td>
<td>---------------</td>
<td>------</td>
<td>------</td>
<td>-------</td>
</tr>
<tr>
<td>Wood thrush</td>
<td><em>Hylocichla mustelina</em></td>
<td>Turdidae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Yellow-bellied flycatcher</td>
<td><em>Empidonax flaviventris</em></td>
<td>Tyrannidae</td>
<td>27</td>
<td>12</td>
<td>39</td>
</tr>
<tr>
<td>Yellow-billed cuckoo</td>
<td><em>Coccyzus americanus</em></td>
<td>Cuculidae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Yellow warbler</td>
<td><em>Setophaga petechia</em></td>
<td>Parulidae</td>
<td>40</td>
<td>23</td>
<td>63</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td></td>
<td>421</td>
<td>273</td>
<td>694</td>
</tr>
</tbody>
</table>