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A GEOGRAPHICAL PERSPECTIVE ON TICKS AND ASSOCIATED DISEASE RISK IN BELGIUM

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ABSTRACT

Nature provides many benefits for human health. However, they also harbour zoonoses, diseases that usually circulate in animals and may be transmitted to humans. Many tick-borne pathogens, such as the causative agent of Lyme borreliosis, are transmitted by the bite of an infected tick. They constitute a complex system involving multiple agents that interact in the environment: pathogens, hosts, ticks, and humans. The interconnectedness between humans, animals and the environments and their influence on health calls for an integrated collaborative framework such as One Health.

The general objective of the thesis was to assess tick-borne disease risk in Belgium using a One Health approach. To account for the complexity of tick-borne disease ecology, we used datasets covering all components of the system, and methods ranging from mixed-effect regressions to multi-criteria decision analyses and Bayesian spatial models. We studied environmental determinants of tick abundance and infection at the forest scale in the Bois de Lauzelle in Louvain-la-Neuve. Our tick collections allowed investigating spatial and temporal variability in tick abundance and tick infection prevalence, and identify tick-associated micro-bacterial communities. We also used surveillance serological data for Walloon cattle to create a spatial index identifying the risk of Anaplasma phagocytophilum in pastures. Finally, by combining environmental predictors of tick bite risk, we created a risk map for Belgian municipalities and compared the results with crowd-sourced tick bite incidence data. This dissertation underlined the advantages of using an integrated multi-level One Health approach to assess the risk of tick-borne disease in Belgium.

RÉSUMÉ

La nature offre de nombreux avantages pour la santé humaine. Cependant, on y trouve aussi des zoonoses, c'est-à-dire des maladies qui circulent généralement chez les animaux et qui peuvent être transmises à l'homme. De nombreux agents pathogènes transmis par les tiques, comme l'agent responsable de la maladie de Lyme, sont transmis par la morsure d'une tique infectée. Les maladies à tiques constituent des système complexes impliquant de multiples agents qui interagissent dans l'environnement : les pathogènes, les hôtes, les tiques et les humains. L'interconnexion entre les humains, les animaux et l'environnement, ainsi que leur influence sur la santé nécessitent un cadre de collaboration intégré tel que One Health (Une Seule Santé).

L'objectif général de la thèse est d'évaluer le risque de maladie transmise par les tiques en Belgique en utilisant une approche One Health. Pour tenir compte de la complexité de l'écologie des maladies transmises par les tiques, nous avons utilisé un ensemble de données couvrant les composantes du système de maladies à tiques, ainsi que des méthodes allant des régressions à effets mixtes aux analyses de décision multicritères et aux modèles bayésiens spatiaux. Nous avons étudié les facteurs environnementaux associés à la présence et de l'infection des tiques dans le Bois de Lauzelle à Louvain-la-Neuve. Nos collectes de tiques ont permis d'étudier la variabilité spatiale et temporelle de l'abondance des tiques et de la prévalence de l'infection par les tiques, et d'identifier les communautés micro-bactériennes (microbiote) des tiques. Nous avons également utilisé les données sérologiques de surveillance du bétail wallon pour créer un indice spatial du risque d'infection avec *Anaplasma phagocytophilum* dans les pâturages. Enfin, en combinant les prédicteurs environnementaux du risque de morsure de tique, nous avons créé une carte de risque pour les communes belges, et comparé les résultats avec les données d'incidence de morsure de tique fournies par une application. Cette thèse a souligné les avantages de l'utilisation d'une approche intégrée à plusieurs niveaux de One Health dans l'évaluation du risque de maladie transmise par les tiques en Belgique.

SAMEVATTING

Natuur biedt veel voordelen voor de menselijke gezondheid. Er komen echter ook zoönosen, ziekten die gewoonlijk bij dieren voorkomen en op mensen kunnen worden overgedragen. Veel door teken overgedragen ziekteverwekkers, zoals de verwekker van Lymeziekte, worden overgedragen door de beet van een besmette teek. Ze vormen een complex systeem met meerdere ziekteverwekkers die in de omgeving op elkaar inwerken: ziekteverwekkers, gastheren, teken en mensen. De onderlinge verbondenheid tussen mensen, dieren en de omgeving en hun invloed op de gezondheid vereisen een geïntegreerd samenwerkingskader zoals One Health.

De algemene doelstelling van dit proefschrift is om het risico op tekenoverdraagbare ziekten in België te evalueren door gebruik te maken van een One Health benadering. Om rekening te houden met de complexiteit van de ecologie van tekenoverdraagbare ziekten, hebben we datasets gebruiken, die alle componenten van het tekenziektesysteem omvat, evenals methoden variërend van gemengde-effectregressies tot multicriteria-beslissingsanalyses en ruimtelijke bayesiaanse modellen. We bestudeerden omgevingsfactoren van tekenabundantie en infectie op de schaal van het bos het “Bois de Lauzelle” in Louvain-la-Neuve. Onze tekenverzamelingen maakten het mogelijk om de ruimtelijke en temporele variabiliteit in de overvloed aan teken en de prevalentie van tekeninfecties te onderzoeken en om microbacteriële gemeenschappen die met teken geassocieerd zijn te identificeren. We gebruikten ook serologische surveillancegegevens voor het Waalse vee om een ruimtelijke index te creëren die het risico op *Anaplasma phagocytophilum* in weilanden identificeerde. Tot slot creëerden we door het combineren van omgevingsvoorspellers van het risico op tekenbeten een risicokaart voor Belgische gemeenten en vergeleken we de resultaten met crowd-sourced gegevens over de incidentie van tekenbeten. Dit proefschrift benadrukte de voordelen van het gebruik van een geïntegreerde One Health-benadering op meerdere niveaus om het risico op tekenoverdraagbare ziekten in België te beoordelen.

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NOTICE

Parts of this thesis (two chapters) are published in peer-reviewed journals, as indicated hereafter:

- **Chapter III**

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- **Chapter IV**

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CONTENTS

CHAPTER 1

INTRODUCTION

1. BACKGROUND

1.1. EMERGING INFECTIOUS DISEASES AND THE ENVIRONMENT

Nature and ecosystems are essential for sustaining human life, providing various services, such as climate regulation, water regulation and supply, pollination and cultural and recreation activities (Costanza et al., 1997). Exposure to natural environments has also been associated to numerous positive effects on human health, including reduced stress, lower blood pressure, and better mental health and social interactions (Fuller et al., 2007; Hartig et al., 2011; Maas et al., 2006; Shinew et al., 2004). Additionally, natural environments provide opportunities for physical activities that promote good health (Bodin and Hartig, 2003). However, nature is also a source of infectious parasites (e.g., viruses, bacteria, protozoans) (Jones et al., 2008; Patz et al., 2000). Although they circulate among animals and are part of ecosystem functioning, they can also infect humans and cause diseases called zoonoses (Jones et al., 2008). Emerging infectious diseases are considered among the major challenges for health, with 60-80% of them having an animal origin (Jones et al., 2008; Wilcox and Ellis, 2006).

Vector-borne diseases are infectious diseases transmitted by a vector, of which ticks represent the majority in temperate regions (Randolph, 2001). Many vector-borne pathogens have emerged over the past decades, in relation to their introduction in new regions and through an increase in incidence in endemic areas (Kilpatrick and Randolph, 2012). Environmental changes, whether natural and/or anthropogenic, can trigger the emergence or re-emergence of infectious zoonotic diseases by changing

the distribution of species and their interactions (Kilpatrick and Randolph, 2012; Patz et al., 2008). These changes can have complex and interconnected effects on the prevalence and distribution of vector-borne diseases, operating at different spatial and temporal scales (Jore et al., 2014; Lambin et al., 2010; Patz et al., 2008, 2000). For example, global changes affect the distribution of *Ixodes ricinus* ticks, that are currently expanding at the northern limit of their range (Jore et al., 2014). Vector distribution and the associated pathogen transmission are also influenced by the composition and configuration of the landscape (Lambin et al., 2010). Peri-urbanization, the tendency for people to move from large urban centres towards the periphery, created new types of residential areas and interfaces with wildlife, leading to increased human-wildlife interactions and exposure to infectious agents. This interconnectedness between humans, animals and the environments and their mutual influence on health at different spatial and temporal scales has led to a new approach: One Health.

1.2. A ONE HEALTH FRAMEWORK FOR TICK-BORNE DISEASES

Humans have been practicing integrative thinking in environmental, animal, and human health since ancient times. Hippocrates' essay "On airs, waters and places", written 2500 years ago in Ancient Greece, argued that the health of humans was directly related to the atmosphere and the elements they were exposed to. In China, the Zhou Dynasty (11-13th century) had one of the earliest integrated public health systems with medical doctors and veterinarians (Zinsstag et al., 2011). In the late 1900s, the epidemiologist Calvin Schwabe introduced the concept of One Medicine, recognizing the close interactions between people and animals regarding nutrition, livelihood and health (Schwabe, 1984). During the 2000s the one medicine approach grew in popularity and the "Manhattan principles on One World, One Health" (www.oneworldonehealth.org) were published, offering a framework for improving collaborations among actors from the medical, veterinarian, and environmental sectors. These actors quickly realized the benefits of improving collaborations. The One Medicine concept progressively evolved into One Health, that highlighted the ecological and societal dimensions of health beyond clinical

medical issues (Zinsstag et al., 2011). However, One Health was associated with different practices and scopes and translated into various actions and interpretations (OHHLEP et al., 2022).

In May 2021, four international organizations, the World Health Organization (WHO), the Food and Agriculture Organization (FAO), the World Organisation for Animal Health (OIE) and the United Nations Environment Programme (UNEP) inaugurated the interdisciplinary One Health High-Level Expert Panel (OHHLEP) (2022) (https://www.who.int/groups/one-health-high-level-expert-panel). Their aims are to 1) develop consensus between stakeholders on a common definition of One Health, 2) enhance cross-sectoral collaborations, and 3) promote worldwide awareness for the urgency and complexity of One Health problems. (OHHLEP et al., 2022). The proposed framework to promote One Health from theory to practice, follows "the 4 Cs": communication, coordination, collaboration, and capacity building (Figure 1.1).

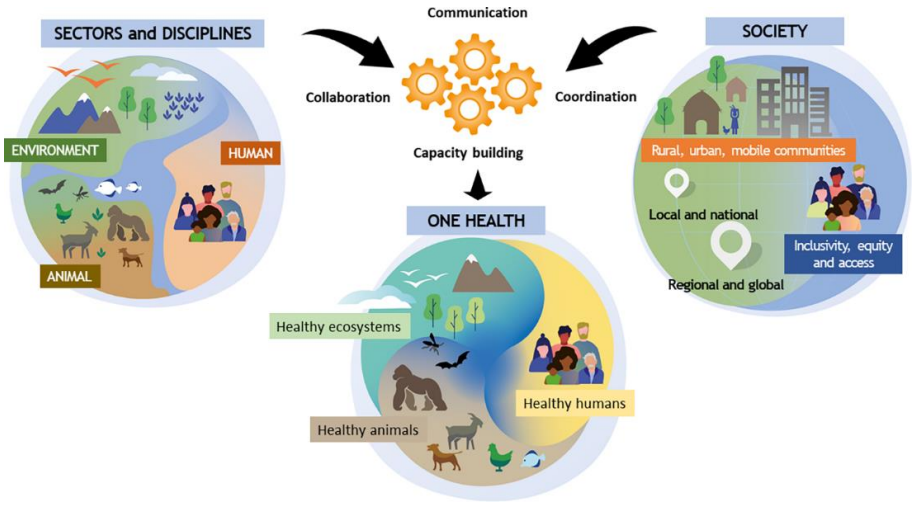


Figure 1.1. One Health toward a sustainable healthy future as developed by the One Health High-Level Expert Panel (OHHLEP). Source: https://doi.org/10.1371/journal.ppat.1010537.g001

One Health is a unifying approach that integrates the closely linked and inter-dependent health of people, of animals and of ecosystem to promote sustainable health

(OHHLEP et al., 2022). This approach mobilizes multiple sectors, disciplines, and communities to address global health challenges and to mitigate health risks arising at the environment-animal-human interface. It also promotes communication and mobilization of multiple sectors and communities at different levels of society (Dantas-Torres et al., 2012). One Health has been implemented in various contexts, such as climate change (Zinsstag et al., 2018), antimicrobial resistance (Queenan et al., 2016) or emerging zoonotic diseases (Cunningham et al., 2017).

Our understanding of the complex and hidden nature of zoonotic diseases, including tick-borne diseases, can greatly benefit from a One Health approach (Dantas-Torres et al., 2012). Reported cases provides only a partial understanding of the true burden of zoonotic diseases as non-reported cases and asymptomatic infections but also the circulation of pathogenic agents in nature or animals matter to the persistence and circulation of the infection. The concept of the zoonotic iceberg (Randolph and Sumilo, 2007) sums this up by considering the number of recognized and reported disease cases as the tip of an iceberg, while the submerged part of the iceberg corresponds to the hidden reservoir of zoonotic diseases.

In this thesis, we assess TBD risk following an integrated One Health approach that integrates the closely linked and inter-dependent health of people, of animals and of ecosystem. The dissertation offers new methods and tools to build new capacities for risk mitigation from the local to the national scale. Collaboration between multiple sectors, disciplines and communities need to be mobilized to address and mitigate TBD risks (OHHLEP et al., 2022). In this thesis, we present work of combined efforts of geographers with microbiologists (**chapters 2 and 3**), veterinarians (**chapter 4**) and epidemiologists and public health scientists (**chapter 5**).

1.3. TICK-BORNE BACTERIAL DISEASES

Ticks are the most important vectors of pathogens in domestic and wild animals. They are also, after mosquitoes, the second group of vectors for pathogens responsible of human disease worldwide, with more than 100 000 cases of illness reported (Dantas-Torres et al., 2012; de la Fuente, 2018; Estrada-Peña and de la Fuente, 2014). Tick-

borne diseases (TBD) have been described in the second half of the 19th century but the spectrum of TBD affecting domestic animals and humans and their incidence have been increasing recently (Dantas-Torres et al., 2012; Pfäffle et al., 2013).

Tick-borne pathogenic microorganisms include viruses, bacteria or protozoa (de la Fuente et al., 2008). The focus of this thesis is on the range of bacteria with significant public health and veterinary concerns. Lyme borreliosis (also known as Lyme disease), caused by bacteria of the *Borrelia burgdorferi* s.l. complex, is the most common TBD in Europe and North America (Stanek et al., 2012). Other bacteria with possible tick-transmission include *Anaplasma phagocytophilum*, *Francisella tularensis* and *Coxiella burnetii*, the causative agents of human granulocytic anaplasmosis in humans, bovine ehrlichiosis in cattle, of tularaemia and Q fever respectively.

Lyme borreliosis

Lyme borreliosis (LB) is the most prevalent and widely distributed vector-borne disease in the temperate zones of the United States and Europe (Gray, 1998; Stanek et al., 2012; Stanek and Reiter, 2011). Its symptoms were first described in Europe in 1883 (Gray, 1998), but its causal agent, *Borrelia burgdorferi* was discovered in *Ixodes* ticks in Old Lyme, Connecticut in 1982 (Pfäffle et al., 2013). Around 300 000 new cases are estimated per year in the world (Diuk-Wasser et al., 2021).

This inflammatory zoonosis is caused by *Borrelia burgdorferi* sensu lato (s.l.) spirochetes (Stanek et al., 2012; Strle and Stanek, 2009). Ticks acquire these spirochetes by feeding on an infected host or from an inherited infection, although the latter is limited (Matuschka et al., 1998). The bacteria are first colonizing and persisting in the tick midgut (Schwan et al., 1995). Blood feeding causes a rapid bacterial multiplication during the first hours of the blood meal, and they are transmitted to the salivary glands. After 24-36 hours, they start to infect the susceptible host. The probability of transmission increases to approximately 10% by 48 hours, and 70% by 72 hours (Eisen, 2018). Transmission being not immediate,

quick removal of the ticks in the early hours of attachment greatly reduces the risk of infection (Piesman and Dolan, 2002).

The *B. burgdorferi* s.l. complex contains at least 20 genospecies, from which eight are known human pathogens (Wolcott et al., 2021). They are often associated with different clinical symptoms (Rizzoli et al., 2011; Stanek et al., 2012; Strnad et al., 2017; van Dam et al., 1993). *Borrelia burgdorferi* sensu stricto (s.s.) is associated with arthritis and neuroborreliosis. It is the only pathogenic species in the north-eastern United States, and is also found in Europe (Steere et al., 2016). *Borrelia garinii* and *Borrelia afzelii* are the most common species causing LB circulating in Europe, the first one being associated with most typical Lyme neuroborreliosis cases, and the latter to less specific clinical symptoms and skin manifestations (Stanek and Reiter, 2011; van Dam et al., 1993). The role of other genospecies, e.g., *Borrelia lusitaniae*, *Borrelia valaisiana*, and *Borrelia bissetii* is unclear. They rarely cause disease in humans (Rauter and Hartung, 2005a; Strle and Stanek, 2009). The prevalence of *B. burgdorferi* genospecies may vary locally, but no significant differences were detected between ticks from different European countries (Strnad et al., 2017).

LB is a rarely fatal multisystemic disease that can cause severe debilitation in untreated chronic cases. There are three main stages in human clinical manifestations. Early Lyme borreliosis is often characterized by a typical erythema migrans and more rarely by a *Borrelia*l lymphocytoma, a small bluish red skin induration often located at the earlobe in children or at the breast in adults (Maraspin et al., 2016). An erythema migrans typically starts 7 to 14 days after a tick bite and is present in 70-80% of LB cases (ECDC, 2015). The second stage (early disseminated Lyme borreliosis) starts weeks to months after the tick bite and sees the dissemination of the spirochetes. The main manifestations are usually non-specific but include early neuroborreliosis, Lyme arthritis, multiple erythema migrans (Piesman and Gern, 2008; Steere et al., 2016). If untreated, late manifestations can occur months to years after the infection: acrodermatitis chronica atrophicans, Lyme arthritis, neuroborreliosis and possible autoimmune phenomena (Stanek et al., 2012; Stanek and Reiter, 2011). LB occurs

also in cattle, causing symptoms like fever, swollen joints, decreased milk production and abortion (Parker and White, 1992).

A vaccine for LB, LYMErix™, existed in 1999 but was withdrawn from the market by the manufacturer three years after amidst media coverage, fears of vaccine side-effects, and declining sales (Kilpatrick et al., 2017; Nigrovic and Thompson, 2007). No other vaccine is currently available, although developments of potential new vaccines are currently investigated. Infected patients can be treated with appropriate antibiotics. Early treatment can prevent the development of late complications. The drug type, dose, route (oral or intravenous) and duration vary with the symptoms and the stage of the disease (Bleyenheuft et al., 2015). Effective prevention for Lyme borreliosis is highly desirable because symptoms are unspecific, and the erythema migrans is not always present. This includes avoiding tick bites and early removal of attached ticks. Higher LB risk is associated with residency in rural and peri-urban areas, and with outdoor leisure (e.g., hunting, mushroom, flower picking) and professional activities (e.g., forestry work, farming) (De Keukeleire et al., 2016; Hall et al., 2017; Jore et al., 2020; Rizzoli et al., 2011).

Human granulocytic anaplasmosis and bovine ehrlichiosis

The gram-negative bacterium *Anaplasma phagocytophilum* is an emerging obligate intracellular rickettsial pathogen causing human granulocytic anaplasmosis and bovine ehrlichiosis, also called tick-borne fever (de la Fuente et al., 2005; Lillini et al., 2006; Stuen et al., 2013; Woldehiwet, 2006). *Anaplasma phagocytophilum* is naturally maintained in enzootic cycles in ticks and wild animals (Jahfari et al., 2014). It has been detected in various wildlife species, including ruminants, rodents, insectivores, carnivores, birds or reptiles, but their respective roles in the enzootic life cycle of *A. phagocytophilum* has not been fully elucidated (Stuen et al., 2013). Wild ruminants (red deer and fallow deer) often present higher rates of seropositivity of antibodies against *A. phagocytophilum* than domestic animals (45% versus 5-15%) (Ebani et al., 2008; Fabri et al., 2022). However, several 16S rRNA *A. phagocytophilum* variants, with ecologically distinct transmission cycles, have been found in mammals and ticks, and may exist within the same herd or in the same animal

simultaneously (Stuen, 2007). The strains differ in vectors, host preferences, geographical distribution and pathogenicity (Nahayo et al., 2014a). In North America, there are at least two strains circulating: Ap-ha, pathogenic to humans and whose principal reservoir host is the white-footed mouse, and Ap-variant 1, associated with the white-tailed deer as principal reservoir host but not associated with human infection (Massung et al., 2005; Nelder et al., 2019). Four ecotypes, identified based on the genetic differences in the groEL gene, are found in Europe, with no geographic clustering (Jahfari et al., 2014). They are also associated to distinct ranges of vertebrate hosts. Ecotype 1 was found in various host species, including humans and livestock, and Ecotypes 2, 3, and 4 in roe deer, rodents, and birds, respectively (Jahfari et al., 2014). *A. phagocytophilum* strains harboured in European cervids are not associated with humans cases (Nahayo et al., 2014a; Polin et al., 2004).

Human granulocytic anaplasmosis was first identified in 1990 in Wisconsin (USA) and the first European case in 1995 in Slovenia (Dumler et al., 2005; Jahfari et al., 2014). It is often asymptomatic or presents flu-like illness symptoms such as fever, chills or headache. Severe complications are possible, even though they are rare in Europe (Cochez et al., 2011; Dumler et al., 2005). The infection is suspected to be underdiagnosed in many European countries because of the discrepancy between seroprevalence and symptomatic rates (Cochez et al., 2011; Dumler et al., 2005). Bovine ehrlichiosis is one of the most widespread tick-borne diseases in animals worldwide, with babesiosis (Aguiar, 2017; Atif, 2015; Bock et al., 2006; Stuen et al., 2013). It has major health and economic impacts on livestock productivity. Its main clinical symptoms include anorexia, pyrexia, immunosuppression, cough, decreased milk production, and abortions, but asymptomatic infections are possible (Atif, 2015; Stuen, 2007; Woldehiwet, 2006).

Q fever

First described in the 1930s in Australia, Q fever is found across the world (Boarbi et al., 2016; Porter et al., 2011). Q fever is caused by the intracellular gram-negative bacterium *Coxiella burnetii*, a gamma(γ)-Proteobacteria of the Legionella order (Körner et al., 2021; Porter et al., 2011; Woldehiwet, 2004). This pathogen infects a

wide range of hosts, mainly small ruminants such as cattle, goat or sheep (Cutler et al., 2007; Mori et al., 2017). *Coxiella burnetii* may develop dessication-resistant forms and therefore survive for long periods in the soil (Körner et al., 2021). Infections generally occur through inhalation of *C. burnetii* contaminated aerosols in milk, faeces, urine, and birth products or abortions from infected ruminants (Anderson et al., 2013; Cutler et al., 2007; Duron et al., 2015). However, *C. burnetii* was also identified in 40 tick species (Anderson et al., 2013; Körner et al., 2021), and sometimes with high prevalences. In Argentina, Pacheco et al. (2013) found 41 out of 92 (44.6%) *Amblyomma* ticks infected with *C. burnetii*, using PCR that targeted the pyrG gene. Tick bites may thus constitute another route of transmission, though probably less important than the airborne route. However, it is still controversial because of the rare detection of *C. burnetii* in *Ixodes* ticks (less than 0.2% in *Ixodes* ticks after a Q fever outbreak in the Netherlands, Sprong et al., 2012) and the difficult differentiation with *Coxiella*-like endosymbionts (Anderson et al., 2013; Duron et al., 2015; Körner et al., 2021). Beaman and Hung (1989) reported a case of Q fever associated with pericarditis that occurred after multiple tick bites.

Human and animal infections are frequently under-reported due to the asymptomatically and sub-clinical forms of the disease (Porter et al., 2011; Saegerman et al., 2022). After one to three weeks of incubation, a human infection with Q fever may cause acute or chronic illness (Porter et al., 2011). In the acute form, most infections cause flu-like symptoms like fever or headache. In the chronic form, lung and liver complications are possible (Körner et al., 2021). The mortality rate from acute Q fever is estimated at 1-2% (Porter et al., 2011). Most cases recover without specific treatment, but chronic Q fever can be treated with antibiotics for several months (Anderson et al., 2013). In ruminants, the main clinical manifestations are reproduction disorders, stillbirth and abortions (Boarbi et al., 2016; Mori et al., 2013; Saegerman et al., 2022). Q fever can persist in herds, causing economic losses for the farmers (Porter et al., 2011). Domestic ruminants are also considered as the main reservoir for human infections (Cutler et al., 2007).

Tularaemia

Tularaemia is a disease with initial flu-like symptoms which can evolve towards serious clinical manifestations and significant mortality if untreated. Clinical suspicions are difficult because of the non-specificity of the early clinical symptoms (Maurin, 2020). Tularaemia occurs mainly in the Northern Hemisphere (Carvalho et al., 2014; WHO, 2007). Tularaemia is extremely infectious, so it was considered as a Tier 1 select agent and a potential biological weapon by the Centre for Disease Control (CDC) and the American Working Group on Civilian Biodefense (Dennis et al., 2001; Larson et al., 2020). The lethal rate for infection with the *F. tularensis* is 5–15% without antibiotic treatment (aminoglycosides, fluoroquinolones, or tetracyclines) but most patients recover completely under these treatments (Carvalho et al., 2014).

The agent of tularaemia is the gram-negative intracellular bacterium *Francisella tularensis,* a gamma-Proteobacteria of the Francisellaceae family (Carvalho et al., 2014). It has a large range of hosts, but their knowledge is still incomplete(Carvalho et al., 2014; Hestvik et al., 2015). *Francisella tularensis* is distributed worldwide and tularemia is particularly important in Europe: its range expanded in the last 25 years and it is considered as an emerging or re-emerging infection in multiple locations (Hestvik et al., 2015; Keim et al., 2007). Four subspecies have been identified: *tularensis* (type A), highly virulent and present in North America, *holarctica* (type B), causing less severe illness in the whole northern hemisphere, *mediasiatica*, poorly studied and sparsely found in Central Asia, and *novicida,* almost avirulent in humans and controversial (Akimana and Kwaik, 2011; Hestvik et al., 2015; Larson et al., 2020). *Francisella tularensis* can survive for weeks in moist environments including water, soil or decaying animal carcasses. Several routes of infection exist, including skin contact with infected animals, ingestion of contaminated water, and arthropod bites (ECDC, 2017; WHO, 2007). The route of infection and strain virulence lead to the different forms and clinical manifestations (WHO, 2007). The ulceroglandular and glandular clinical forms are the most common in Central Europe and Belgium (Litzroth et al., 2017) and they frequently result from arthropod bites (ticks and flies)

or animal contacts (Akimana and Kwaik, 2011; Keim et al., 2007). *F. tularensis* prevalence in ticks was estimated at between, 0 and 3% (Hestvik et al., 2015).

Tick-borne diseases in Belgium

With a population of 11 697 557 inhabitants on January 1, 2023, and a surface area of 30 689 km², Belgium is a densely populated (377.4 inhabitants/km²) federal state of Western Europe (Figure 1.2). The country is divided into three regions: the Flemish Region (Flanders), the Walloon Region (Wallonia), and the Brussels-Capital Region (Figure 1.2). 45% of its land surface is occupied by agricultural lands, 20% by forests. residential lands account 9% of the territory (StatBel, 2023).

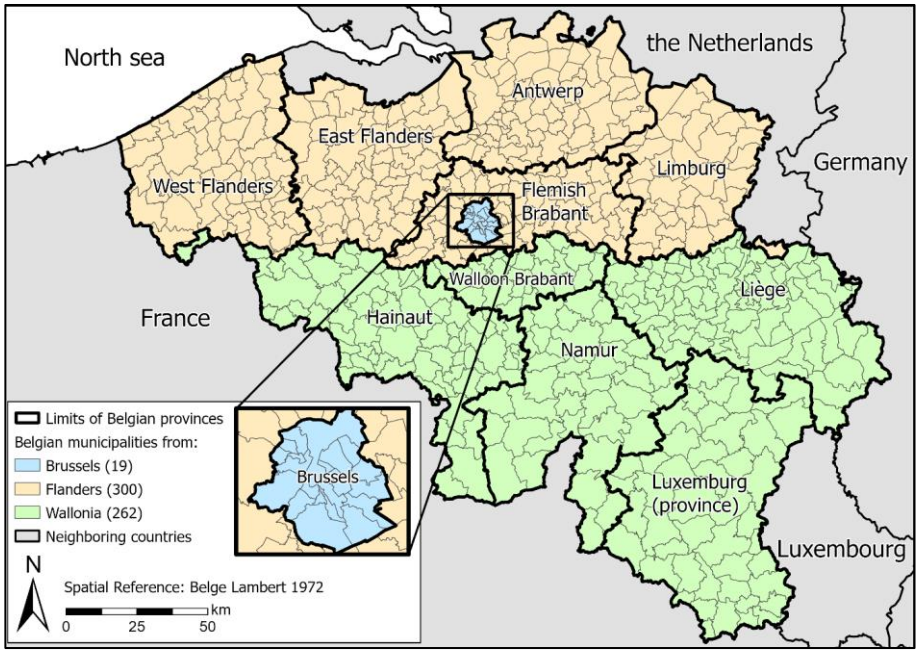


Figure 1.2. Political map of Belgium.

Sciensano is the Belgian federal institute for health and results from the fusion of the former Veterinary and Agrochemical Research Centre (CODA-CERVA) and Scientific Institute of Public Health (WIV-ISP). Emerging zoonotic diseases are one of their main priority topics, with antimicrobial resistance and health risks due to exposure to different environmental sources (Sciensano, 2023). In Belgium, *I. ricinus*

is the main human-biting tick species and it will therefore be the focus in this thesis. The epidemiological monitoring of zoonoses and tick-borne diseases in Belgium is based on surveillance data from three networks of microbiology laboratories (sentinel laboratories, reference laboratories and national reference centres), from notifications of reportable diseases and from a network of sentinel general practices (Lernout et al., 2023).

LB is the main human tick-borne disease in Belgium and its health burden and costs are assessed through different sources. The network of sentinel laboratories reports weekly the number of positive serological tests for *B. burgdorferi* s.l. (Bleyenheuft et al., 2015). They reported 1546 positive tests in 2019, which was below the average of ~2000 cases for the previous decade (Lernout et al., 2023). However, because data on clinical information is missing, active and past infections are hardly differentiable (Stanek and Reiter, 2011). 200 persons were also hospitalized for LB in 2019 (Lernout et al., 2023). In addition, the Belgian nationwide network of sentinel general practices voluntarily reports incidences of consultations for erythema migrans (Vanthomme et al., 2012). Geebelen et al. (2019) estimated incidences of 97.6 erythema migrans cases and 103 LB cases / 100 000 inhabitants. No Belgian data sources or studies indicate a significant increase of LB in the Belgian population over the past years (Bleyenheuft et al., 2015; De Keukeleire et al., 2017; Geebelen et al., 2019; Rebolledo et al., 2017). The number of hospitalizations and positive tests for Lyme borreliosis reach their peak around August, two months after the peak of questing *I. ricinus* in May-June (Bleyenheuft et al., 2015). This may be related to the onset of the first early disseminated complications after a tick bite and the increasing number of people practicing outdoor activities in that period. In Belgium, *B. burgdorferi* prevalence was estimated at 10.2% and 13.9% in ticks that fed on cats and dogs (Claerebout et al., 2013), and humans (Lernout et al., 2019) respectively. *Borrelia burgdorferi* prevalence in questing ticks was estimated in several studies, ranging between 9.1% and 24.7% (Adjadj et al., 2023; Jahfari et al., 2017; Kesteman et al., 2010; Misonne et al., 1998; Ruyts et al., 2016; Tack et al., 2012).

Other human infections with tick-borne bacteria are limited in Belgium, although probably underdiagnosed (Geebelen et al., 2022; Lernout et al., 2023). **Human granulocytic anaplasmosis** was first identified in 2000 in Belgium (Guillaume et al., 2002). In a 10-*year A. phagocytophilum* surveillance (2000–2009) 418 serum samples out of 1672 (25%) were positive (Cochez et al., 2011) and the establishment of *A. phagocytophilum* in Belgium has been confirmed (Hing et al., 2019). 25 probable human granulocytic anaplasmosis cases (only one confirmed) were detected in 2019-2021 (Lernout et al., 2023). The disease is probably underdiagnosed, due to unspecific symptoms. The first case of bovine ehrlichiosis in Wallonia, the southern region of Belgium, was detected in 2005 (Guyot et al., 2011). The high seroprevalence found in local herds and the widespread distribution of its main vector in Europe, *Ixodes ricinus*, confirm that the disease is endemic in Wallonia, but may be underdiagnosed (Lempereur et al., 2012a). High seroprevalence to *A. phagocytophilum* (> 45%) were found in roe deer in Flanders (Tavernier et al., 2015). Two studies found prevalence of 1.8% and 0.5% for *A. phagocytophilum* in ticks sent by citizens (Lernout et al., 2019) and in questing ticks from areas with high infection in cattle (Adjadj et al., 2023) respectively.

Tularaemia is a notifiable disease in Belgium. Only a limited number of cases are diagnosed annually, but the number of detected cases is increasing. 28 cases were diagnosed between 1950 and 2021, of which 25 were reported between 2012 and 2021, and at least one case annually since 2012, except in 2018 (Litzroth and Mori, 2021). Four, one and nine cases were diagnosed in 2019, 2020 and 2021 respectively (Lernout et al., 2023; Litzroth and Mori, 2021). The source of these contaminations was unknown for the majority of cases. In 2021, one case was contracted while gardening, one after contacts with animal carcasses, and one after a walk and sleep in the forest (Litzroth and Mori, 2021). No *F. tularensis* prevalence in ticks has been measured in Belgium.

Q fever is a notifiable disease in Belgium. In 2021, the national reference centre reported 15 cases, 6 confirmed (Litzroth et al., 2021). This was in line with the previous years, but the disease is probably underdiagnosed due to unspecific

symptoms. The source of these contaminations was unknown. Q fever is also widespread in domestic ruminants (Tomaiuolo et al., 2021), but cattle vaccination against Q fever is infrequent (Saegerman et al., 2022). Tavernier et al. (2015) found low antibody prevalence of *C. burnetii* in roe deer compared to domestic ruminants, indicating roe deer may not be a reservoir. Moreover, *C. burnetii* prevalence has not been measured in ticks in Belgium.

1.4. TBD COMPLEX

TBD constitute a complex ecological system involving multiple agents interacting in the environment: pathogens (e.g., viruses, bacteria), tick vectors, animal hosts (competent or not), and eventually humans (Killilea et al., 2008; Vanwambeke et al., 2019; Vu Hai et al., 2014). Competent (or reservoir) hosts are vertebrates that can become infected by a pathogen after a tick bite and maintain, amplify and transmit it to other non-infected ticks (Estrada-Peña and de la Fuente, 2014; Wolcott et al., 2021). In some animals, no systemic infection or pathogen replication is observed. They are called incompetent hosts. Each species of the TBD complex has its own habitat requirements, to find the resources needed to fulfil its ecological functions (Lambin et al., 2010; Medlock et al., 2013). These observations were formalized by Hartemink et al. (2015), in a concept based on the specific resources requirements of the different agents: the resource-based habitat concept (RBHC). Vanwambeke et al. (2016) illustrated the RBHC from Hartemink et al. (2015) for *B. burgdorferi* s.l. (Figure 1.3). This model assumes that organisms must perform several necessary functions (e.g., feeding or mating) required to complete their life cycle. These functions are associated to specific resources found in the environment (e.g., reproduction sites and/or suitable microclimate), resulting in specific functional habitats for the organisms.

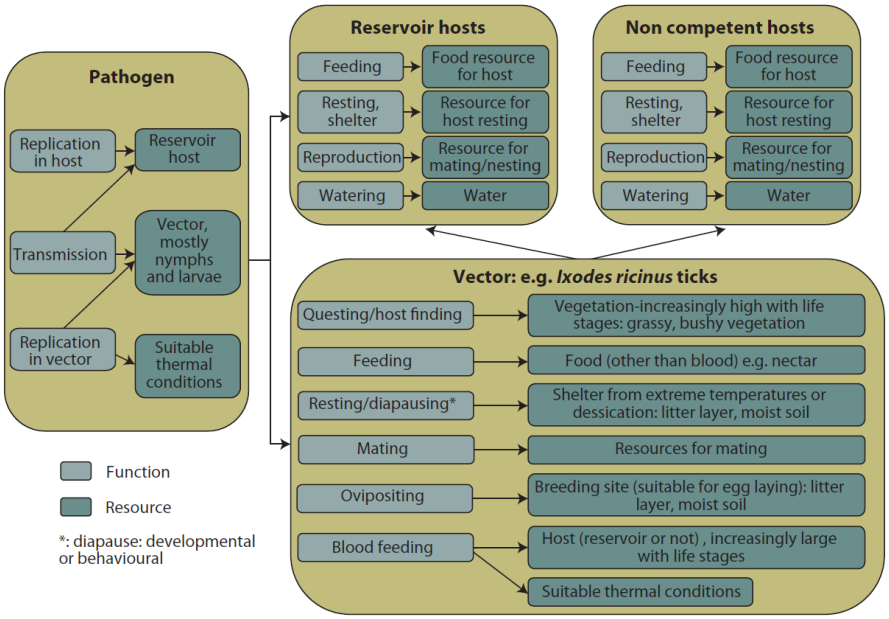


Figure 1.3. Application of the resource-based habitat concept (RBHC) to tick-borne pathogens. Identification of the functions (light grey boxes) and resources (dark grey boxes) is first done at the level of the pathogen and then at the level of both the vector and hosts. Source: Vanwambeke et al. (2016).

Ticks

Ticks are blood-feeding arthropods from the class Arachnida (subclass Acari). To date, there are almost 900 tick species listed in the world divided in three families, the Argasidae, the Ixodidae and the Nutalliellidae (Guglielmone et al., 2010). The latter is a monotypic family of minor importance. The former two families are commonly known as soft ticks and hard ticks respectively. Ixodidae are constituted by different genera, including *Amblyomma*, *Dermacentor*, *Hyalomma* or *Ixodes* (Guglielmone et al., 2010). 33 species only are known to commonly feed on people (Anderson and Magnarelli, 2008), out of which 28 are known vectors of human pathogens. Therefore, the identification of tick species is important for public health because they are not all biting humans and/or they may have different population dynamics, life cycles, and hosts preferences. Four species are the most important vectors of human pathogens in the northern hemisphere: *Ixodes persulcatus* and *Ixodes ricinus* in Eurasia, *Ixodes*

pacificus in western North America, and *Ixodes scapularis* in eastern and midwestern North America (Gray, 1998; Gray et al., 2016).

The development cycle of *Ixodes ricinus* ticks spans multiple stages (Figure 1.4). Their active life stage typically lasts from 2 to 6 years. Eggs hatch into larvae. After feeding, larvae molt into nymphs. Nymphs feed on another host, and moult into adults (female or male). Adult females feed on a third host, mate, and lay thousands of eggs on the ground in sites with enough relative humidity to protect against desiccation (Gray, 1998; Hillyard, 1996). Life cycle duration is variable and depends on factors such as temperature, relative humidity, questing success and the energy obtained during the blood meals (Estrada-Peña and de la Fuente, 2014).

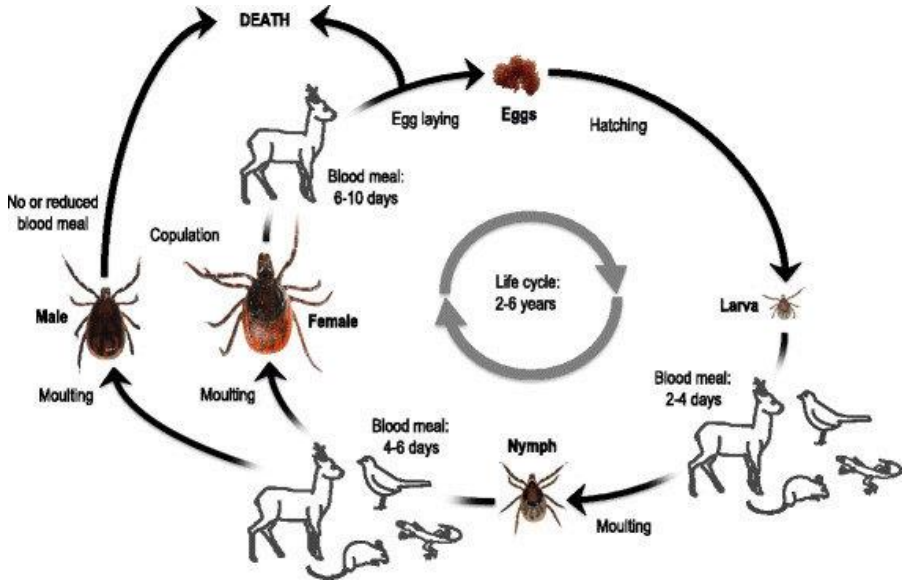


Figure 1.4. Life cycle of *Ixodes ricinus*. Source: Herrmann and Gern (2015).

Ixodes ricinus has its own functions (e.g., questing, feeding, diapausing), that may be associated to different resources. Two processes are particularly important: development of the moulting stage for immature stages, oviposition for females, and questing for a host (Estrada-Peña and de la Fuente, 2014). Newly moulted ticks usually become active within 1–2 weeks and start questing, a process in which active

ticks search for a host (Gray, 1998). The beginning of *I. ricinus* questing activity is based on climatic and photoperiod factors and it can quest from weeks to several months (Belozerov, 1982).

Due to its low intrinsic mobility, *I. ricinus* relies on host movements for their dispersion (Heylen et al., 2022; Lees and Milne, 1951). After feeding, ticks drop off hosts on the vegetation to start their development phase. The factors controlling the critical drop-off decision of the engorged tick are complex and caused by multiple factors (Gray et al., 2021). *Ixodes ricinus* and *I. frontalis* usually detach during the day, contrary to *I. arboricola*, which often detaches at night (Heylen et al., 2022). The exophilic *I. ricinus* detaches in the vicinity of brushes within seven days following the blood meals, increasing its dispersal from tick-dense areas. Tick movements are costly in energy but are needed for seeking two main resources: host blood and water vapour. Crooks and Randolph (2006) found that most *I. ricinus* movements respond to intrinsic and extrinsic stimuli. Ticks with lower energy reserves were less likely to walk horizontally. They were also more likely to walk towards saturated air when they were slightly dehydrated, and towards odour secreted by host skin, when the atmosphere was wet.

Ixodes ricinus may spend up to 95-99% of its time off the host, where it is highly vulnerable to dessication (Anderson, 2009; Gray, 1998). During questing activity, *I. ricinus* needs to regain water by frequently descending from the vegetation to reabsorb water from sub-saturated air (Estrada-Peña and de la Fuente, 2014; Lees, 1948; Lees and Milne, 1951). They require a minimum relative humidity of 80% for long periods to maintain a stable water balance (Estrada-Peña and Venzal, 2006; Gray, 1998). Below this threshold, *I. ricinus* cannot absorb atmospheric water, increasing its mortality rate. *Ixodes ricinus* thus seek areas with a good vegetation cover that retains humidity during the driest times of the year and protects them against wind and direct sunlight by sheltering behind its supporting stem (Lees and Milne, 1951).

Ixodes ricinus is an "opportunist" species that is questing on the vegetation to ambush passing hosts (Gray, 1998). They respond to non-specific stimuli, including presence of carbon dioxide, vibrations, changes in temperature, and probably to the odours of

some animals (Lees, 1948). Intrinsic (e.g., host-finding success, tick energy reserves) and extrinsic (e.g., temperature, sunshine, rainfall, saturation deficit) factors influence *I. ricinus* questing behaviour but their relative importance vary between different habitats (Gray et al., 2016; Kiewra et al., 2014; Perret et al., 2000). *Ixodes ricinus* has a low degree of host specificity and has been recorded on more than 300 vertebrate species, including humans (Gray et al., 2021; Pfäffle et al., 2013). However, the process of host selection for *I. ricinus* is still not fully understood (Gray et al., 2021) and the number of important hosts must be more limited (Hofmeester et al., 2016). This lack of host specificity in *I. ricinus* makes control of its population difficult. In Europe, deer species, the main hosts for adults, seem to play a role in limiting tick-borne diseases (Gilbert et al., 2012; Mysterud et al., 2016). Badgers and foxes appeared less suitable for ticks, despite their low body posture that favour exposure to questing ticks (Mysterud et al., 2021). Humans are considered an accidental host and their presence is not needed for the completion of the tick life cycle.

Ixodes ricinus usually feeds once in each of its three active stages, ingesting energy for molting or oviposition (Estrada-Peña and de la Fuente, 2014). Larvae and nymphs usually feed on small animals, e.g., rodents or birds, although deer can feed large number of immature ticks, while adults feed on large sized animals, e.g., deer, or livestock (Gray et al., 2021; Hofmeester et al., 2016; Stanek et al., 2012). After finding a suitable feeding site on a host, ticks use their external mouthparts to incise the host' skin (with a pair of barbed chelicerae) and to introduce saliva and suck blood and inflammatory exudate (with the hypostome) (Gray and Kahl, 2022). Blood meals generally last for 2-10 days, with adults feeding longer than immature stages. After the ingestion of large volume of blood, *I. ricinus* larvae can weight up to 10-20 times their unfed weight, the adults up to 100 times (Anderson and Magnarelli, 2008). *Ixodes ricinus* developed mechanisms to facilitate the success of feeding and, similarly, some animals developed antibodies to the proteins of ticks, decreasing the feeding success of a tick after the bite (Steele and Randolph, 1985). This feeding success is not completely understood. Blood feeding in competent hosts is one of the most efficient method for a vector to acquire and transmit pathogens (de la Fuente et

al., 2017; Pietzsch et al., 2005). Ticks can acquire pathogens also through cofeeding. In cofeeding, simultaneous infected and uninfected ticks are feeding on the same host in close proximity (Gern and Rais, 1996; Randolph et al., 1996). The host must not necessarily be competent and does not necessarily develop systemic infections, but serves as a "bridge" allowing direct infections between ticks (Estrada-Peña and de la Fuente, 2014). Ticks have a tendency to be aggregated in the environment and on the host, which benefits cofeeding transmission (Pfäffle et al., 2013), although the role of cofeeding varies between pathogens and is minor for *Borrelia burgdorferi* s.l. (Hartemink et al., 2008).

Pathogens

Hartemink et al. (2015) identified three main functions for the pathogens: replication in the host, replication in the vector and transmission. Pathogen replication functions will be developed in the subsections related to the vector and the hosts.

The efficiency of transmission of a specific pathogen by a tick is called the vectorial capacity (de la Fuente et al., 2017). It quantifies the capacity of a tick 1) to acquire a pathogen from blood meals in infected hosts or from co-infection 2) to maintain the pathogen through the different life stages after moulting (the trans-stadial or horizontal transmission) 3) to transmit the pathogen to the cohort of eggs by the engorged female tick (the transovarian or vertical passage) and 4) to successfully transmit it to another host in the next blood meal(s). This vectoral capacity is influenced by environmental, behavioural, and genetic factors (Beerntsen et al., 2000; Vayssier-Taussat et al., 2015).

Ixodes ricinus harbours a rich community of symbiotic, commensal, and parasitic microorganisms, through millions of years of co-evolution (de la Fuente et al., 2017). They are forming a complex microbiota of endosymbionts or parasites, engaged in relation with their tick hosts, influencing their fitness, vector capacity and co-infections with pathogenic agents (Bonnet et al., 2017; Pollet et al., 2020). Pathogenic agents may have positive and negative effects on ticks. In Wisconsin (United States), male *I. scapularis* ticks had lower rates of *Borrelia burgdorferi* infection when they

were infected by rickettsial endosymbionts than symbiont-free males (Steiner et al., 2008). Neelakanta et al. (2010) demonstrated that *I. scapularis* ticks infected with *A. phagocytophilum* induce the expression of antifreeze protein gene, resulting in increasing cold tolerance and survival. Large variety of microorganisms continue to be identified in tick microbiota. Yet, many microorganisms and their interactions in tick microbiota are unknown or unidentified (Bonnet et al., 2017; Rudolf et al., 2009). Microbes are vertically transmitted or acquired from the environment, suggesting an influence of the local environment on tick microbiota (Bonnet and Pollet, 2021).

Hosts

Competent hosts are essential for the maintenance and persistence of the pathogen. If these animals are not present, ticks may feed on other species, which limits the circulation of the pathogen. Vertebrate hosts may develop defences against ticks, by acquiring protective immune and allergic responses to exposure to tick antigens, including inflammation and immune responses (Randolph, 2009). Some rabbits, guinea pigs or bank voles developed resistance that results to premature detachment, or less blood conversion to eggs for female ticks (Gray et al., 2021). The lack of resistance from a specific host may suggest a long co-evolutionary history with *I. ricinus*, that suppresses the host's resistance responses (Gray et al., 2021; Heylen et al., 2010).

The availability of tick feeding opportunities is one of the parameter regulating tick population (Gray et al., 2021). Red deer and roe deer are common and widespread cervid species in Europe and are the main *I. ricinus* reproduction host (Levi et al., 2012; Pfäffle et al., 2013). Tick abundance is often associated with deer presence or abundance (Pfäffle et al., 2013). In areas of high deer density in New-York, Ostfeld et al. (2006), found that variations in deer abundance do not affect tick abundance, probably because adults aggregate in the remaining deer. Hofmeester et al. (2017b) found that, in Dutch forests, deer presence rather than abundance determines *I. ricinus* population density but extrapolations of this observation is not straightforward, as the specific composition of reservoir hosts and vectors seems more important than patterns of species biodiversity for TBD risk (Salkeld et al., 2013). However, deer

presence is also not essential to maintain tick population. In its absence, ticks may feed on other animals, such as sheep (Gray, 1998) or hares (Tälleklint and Jaenson, 1996; Gray, 1998). Moreover, deer are incompetent hosts for *B. burgdorferi* s.l. but not for at least one of *A. phagocytophilum* ecotypes in Europe (Mysterud et al., 2013; Stuen et al., 2013).

Small-size hosts, including rodents and songbirds, are not particularly important for the maintenance of tick population but have high levels of competence for many pathogens, including different subspecies of the *B. burgdorferi* s.l. complex (Gray et al., 2021). Small mammals have lower infection prevalence than birds but feed a larger proportion of larvae, indicating that these two population participate to the transmission and maintenance of *Borrelia* spp. through two different strategies (Hofmeester et al., 2016).

The abundance and composition of hosts population may affect tick infections due to their differences in host competence for human pathogens. Feeding on chipmunks, mice or shrews is associated to higher tick abundance but also higher infection by *B. burgdorferi* in nymphs and adults, while tick feedings on opossums and squirrels are associated to higher larvae mortalities (Keesing et al., 2009). The effects of host diversity on vector-borne diseases are still debated. The existence of a so-called dilution effect of host species diversity on LB risk has been discussed. The main assumptions of this concept are that an increase of host diversity may decrease Lyme borreliosis risk if fewer competent species are found in the environment and that competent hosts proliferate in poor biodiverse habitats (Keesing et al., 2009; Ostfeld and Keesing, 2012). Some scientists supported the idea that increasing diversity lead to a dilution and therefore to lower infection prevalence (Ostfeld, 2013; Ostfeld and Keesing, 2000). For example, LoGiudice et al. (2003) demonstrated that reduced host diversity results in an increase in *Borrelia* prevalence in *I. scapularis*. Other scientists are questioning the theoretical basis and assumptions of the inverse relationship between host diversity and TBD risk (Randolph and Dobson, 2012; Wood and Lafferty, 2013). Ruyts et al. (2018) found no relation between host diversity and the abundance of infected ticks in Belgian forests. A meta-analysis found no or weak

effects in favor of the dilution effect (Salkeld et al., 2013). The effects of biodiversity on disease risk are nuanced and depend on how disease risk is measured, the composition and interactions of the host species, the measures of fragmentation used, and the scale at which the host/tick prevalence effect is measured (Diuk-Wasser et al., 2021; Salkeld et al., 2013).

1.5. SURVEILLANCE METHODS TO ESTIMATE TBD RISK

Over the last decades, numerous studies have sought to establish connections between elements of the TBD complex and environmental factors, aiming to identify areas at risk or predict future population distributions (Vu Hai et al., 2014). Public health surveillance refers to the systematic acquisition, analysis and interpretation of health data in order to plan, implement and evaluate public health practices (Eisen and Paddock, 2020). Collecting health data is costly, constrained by ethical issues and often limited in scope to local areas. Nonetheless, by leveraging existing knowledge about the abiotic and biotic factors affecting tick distribution and TBD cases, TBD risk can be predicted in non-studied areas, larger regions, or in the future (Vu Hai et al., 2014).

Given the complex nature of TBD, no single dataset comprehensively covers the phenomenon. Moreover, an absence may be an environmental absence (the phenomenon is not present because of specific conditions), a contingent absence (the phenomenon is not present despite suitable conditions) or a methodological absence (there are bias in data collection) (Lambin et al., 2010). Therefore, only indirect indicators (proxies) can be considered and offer incomplete yet complementary perspectives on TBD, each presenting distinct opportunities and challenges (Randolph and Sumilo, 2007; Vanwambeke et al., 2019). The development of geographic information system has greatly assisted TBD risk assessment in identifying areas associated with TBD risk, pinpointing specific environmental factors that affect TBD risk and overlaying spatially TBD risk indicators and predictors (Daniel et al., 2004). A multitude of methods exists for collecting data on TBD, each capturing different facets of the TBD complex and offering varying interpretations. In this dissertation,

we will use different entry points, that are ways of measuring and modelling, to assess TBD risk in Belgium (Figure 1.5).

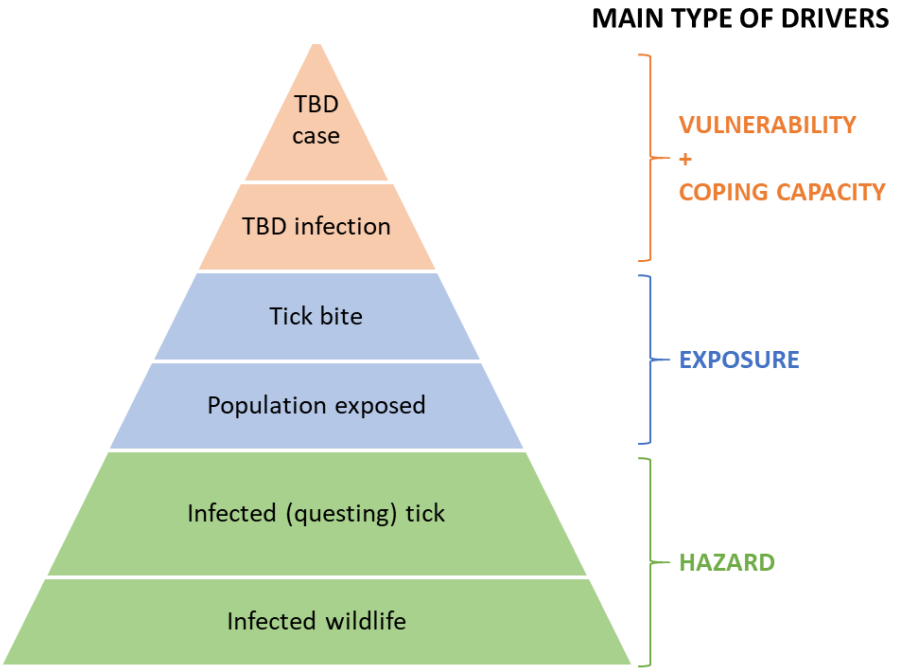


Figure 1.5. Surveillance pyramid for tick-borne diseases (TBD), and the main type of drivers affecting each layer (hazard, exposure and vulnerability and coping capacity drivers). Figure adapted from Braks et al. (2016).

The primary approach to preventing TBD in humans or livestock involves avoiding areas inhabited by infected ticks. A common entry point for TBD studies is therefore to identify areas or habitats associated with the abundance of (infected) ticks in the environment (Figure 1.5, in green). Many studies collect questing ticks by dragging or flagging a cloth on the vegetation from various habitats and conditions (e.g., Boyard et al., 2011; Richter and Matuschka, 2011; Schwarz et al., 2012; Van Gestel et al., 2021). These techniques simulate the passage of a host by dragging or sweeping a cloth (often white or pale for easier tick detection) across leaf litter or vegetation to capture questing ticks (Dantas-Torres et al., 2013; Nyrhilä et al., 2020; Salomon et al., 2020). Tick dragging is cost-efficient in terms of equipment, but labour-intensive and very sensitive to spatially and temporally localized factors, that are still poorly

quantified (Estrada-Peña and Venzal, 2006; Johnson et al., 2022; Jore et al., 2011). For example, ticks from different plots within the same area may exhibit different questing patterns, resulting in between-year variations in phenology. Moreover, the seasonality of tick questing behaviour in temperate regions may also exhibit severe variations between years, which may be overlooked in studies where ticks are sampled on one or two occasions corresponding to the estimated period of peak tick activity (Dobson, 2013). Other methods have occasionally been used to sample ticks from the vegetation, such as carbon dioxide traps (Gray, 1985) or by combining CO2 with sweep flags (Gherman et al., 2012) but they are more costly and their efficiency varies by tick species, developmental stage and host-seeking behaviour (Dantas-Torres et al., 2013). In complement to flagging carried out by trained technicians and scientists, volunteers from the community are increasingly involved in tick flagging in their gardens or large-scale projects (Lewis et al., 2018; Porter et al., 2019; Tran et al., 2021). Citizen involvement in data collection, compilation and analyses in collaboration with scientists offers opportunities for large-scale tick abundance estimations and public involvement, but strict protocols must be established to ensure data quality (Eisen and Eisen, 2021).

The number of ticks collected can be standardized by reporting the area sampled and converting the count of nymphs and adults into the density of nymphs (DON) and density of adults (DOA) respectively. Ticks collected can be identified, and tested for the presence of pathogens using a range of molecular techniques (Johnson et al., 2022). Multiplying DON or DOA by the infection prevalence results in a measure of the density of infected nymphs (DIN) or adults (DIA). Most epidemiological studies focus on DIN because nymphs are more numerous than adults, more difficult to detect in humans, and may already have been infected during their first blood meal (Gray et al., 1999). DIN is attractive as it avoids the complexities of human behaviour and is not affected by the ethical constraints of human health data (Dobson, 2013) or animal trapping and sampling. However, it offers a limited view on the TBD system, as it lacks exposure and DIN is not always correlated with the number of TBD cases

(Dobson, 2013; Vanwambeke and Schimit, 2021). In **chapters 2** and **3**, we collected questing *Ixodes* ticks in a peri-urban forest.

Ticks can also be collected directly from hosts, either from humans (Lernout et al., 2019), wild animals (Carpi et al., 2008; Heylen et al., 2010; Lempereur et al., 2012b), domestic animals and livestock (Abdullah et al., 2016; Claerebout et al., 2013) or from reports of veterinarians (Jore et al., 2011). This relies on the probability of contact between the ticks and human or animal hosts (Figure 1.5, in blue). This also offers some context to the tick-host-pathogen interactions by considering feeding or engorged ticks rather than questing ticks, and it may contribute to determining host specificity of different species (Carpi et al., 2008; Lernout et al., 2019). However, it is difficult to assess if the tick was infected before the blood meal, or acquired the pathogen while feeding. The location where the tick was encountered is also difficult to obtain, and is often a probable location, especially without travel and activity history of the humans and/or animals (Eisen and Eisen, 2021).

Tick bites is an interesting entry point to assess exposure to tick bite. Ticks-hosts contacts may be estimated through tick bites reported from surveys (Jore et al., 2011) or for different populations at risk (De Keukeleire et al., 2015) (Figure 1.5, in blue). For example, De Keukeleire et al. (2015) associated tick bites reported by scouts during their summer camps with the characteristics of the environment of the camp. Exposure to tick bites may also be complemented by a survey related to the probable place of infection, the characteristic of the individual, activity during the infection, previous knowledge on TBD (Vu Hai, 2014). Various set-ups also allow to collect tick bite reports from citizens, either through apps or through mail (Garcia-Marti et al., 2018; Lernout et al., 2019; Nieto et al., 2018). In this dissertation, we will create a map of tick bite risk from citizens self-reports on **Chapter 5**.

Serological tests offer insights of the presence and circulation of tick-borne pathogens. It indicates a present or past contact, even in the absence of clinical symptoms (Figure 1.5, in orange) (Bleyenheuft et al., 2015; Vu Hai et al., 2014). Therefore, a positive test does not specially indicate the presence of an active infection, considering the duration of antibodies persistence (Bleyenheuft et al., 2015). For Lyme borreliosis and

human granulocytic anaplasmosis, serological tests are recommended 6 to 8 weeks and 2 to 4 weeks after onset respectively (Bleyenheuft et al., 2015). Different groups of persons can be compared with serological tests to evaluate their levels of exposure (Vu Hai et al., 2014). In Belgium, workers professionally exposed to tick habitats (veterinarians, farmers, hunters, and gamekeeper) show higher seroprevalence with *B. burgdorferi*, *A. phagocytophilum* and *F. tularensis* than the general population (De Keukeleire et al., 2017). Exposure of domestic (e.g., Bock et al., 2004; Saegerman et al., 2022) and wild animals (e.g., Massung et al., 2005; Tavernier et al., 2015) to tick-borne pathogens may also be tested with serological tests. In **chapter 4**, we created a spatial index for bovine ehrlichiosis infection in cattle based on the presence of seropositive cows.

Finally, at the tip of the pyramid, human or animal clinical cases can be recorded from hospitals, veterinarian centres, or GP consultations (e.g., Bleyenheuft et al., 2015; Linard et al., 2007; Zeimes et al., 2014) (Figure 1.5, in orange). The number of cases jointly reflect hazard, exposure and vulnerability/coping capacity and constitute. We did not use any disease case record data in this thesis

1.6. SPATIAL RISK OF TICK-BORNE DISEASES

The risk of contracting an infection is not homogenous through space. Linard et al. (2007) found that most of the spatial variation in LB risk can be explained by environmental and socio-economic factors. Disease risk mapping is one of the most common tools to help public health surveillance and decision-making (Bouchard et al., 2023). They provide a rapid summary of the situation and help to detect spatial pattern in the phenomenon. Creating tick bite risk maps is a challenging exercise as it relies on 1) mapping the distribution of the vectors, reservoir, or disease cases, 2) characterizing the environmental variables influencing their distribution or abundance and 3) use this information to guide the implementation of intervention measures or to predict disease risk in another area or in the future (Ostfeld et al., 2005).

Assessing TBD risk is crucial for public health in order to implement appropriate prevention methods and, eventually, to prevent future outbreaks. Defining TBD risk

is not straightforward and depends on the combination of various aspects: risk assessment, risk management and risk communication) (Sedda et al., 2014). Moreover, a comprehensive understanding of the various factors involved in the TBD complex is often limited and varies over space and time. In this dissertation, TBD risk was defined as the probability of developing an infection when exposed to the enzootic hazard, considering individual vulnerability and coping capacity (Figure 1.6).

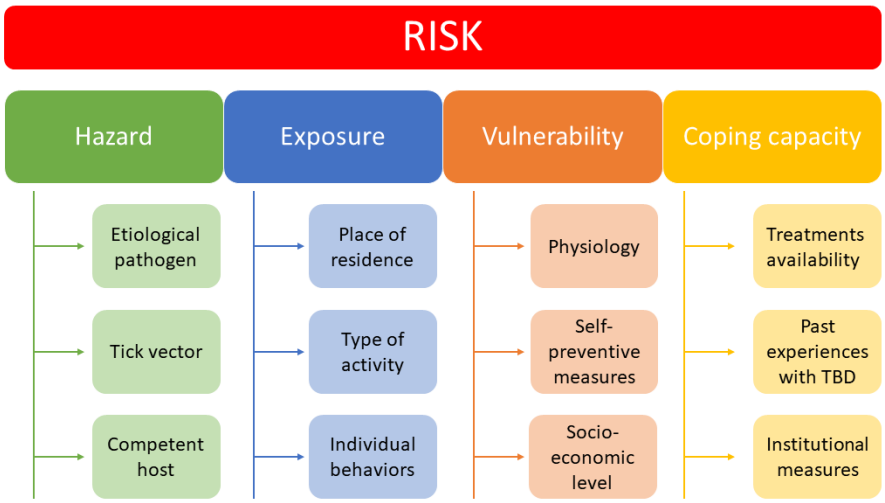


Figure 1.6. Components of tick-borne disease risk (hazard, exposure, vulnerability and coping capacity) and the types of factors infleuencing the risk.

Hazard

Hazard corresponds to a potential source of harm for individuals. For TBD, it corresponds to the potential of a tick to transmit the pathogen, which directly depends on the presence or abundance of infected ticks. TBD enzootic hazard is determined by the overlap between the distributions of the etiological agent, the tick vector and the reservoir and competent hosts. The spatial and temporal aspects of *I. ricinus* distribution is the first aspect of the TBD to assess (Gilbert et al., 2017; Gray, 1998; Jore et al., 2014; Medlock et al., 2013). At the European scale (Figure 1.7), tick presence is mainly constrained by climatic factors, defining its ecological niche (Estrada-Peña and de la Fuente, 2014; Gray et al., 2009). The ecological niche of a

species corresponds to the range of environmental and climatic conditions necessary for its persistence, considering the distribution of resources and the pressure of competition (Hutchinson, 1957). During its off-hosts periods (the questing, developmental and diapausing phases), *I. ricinus* is highly sensitive to temperature and humidity, which impact its survival and development. The development of *I. ricinus* is accelerated by temperature, causing an extension of its questing season through winter (Gray et al., 2009; Medlock et al., 2013). The northern limit of *I. ricinus* distribution in Europe is delimited by factors of temperature and length of daylight (Gray et al., 2009; Jaenson and Lindgren, 2011; Jore et al., 2014).

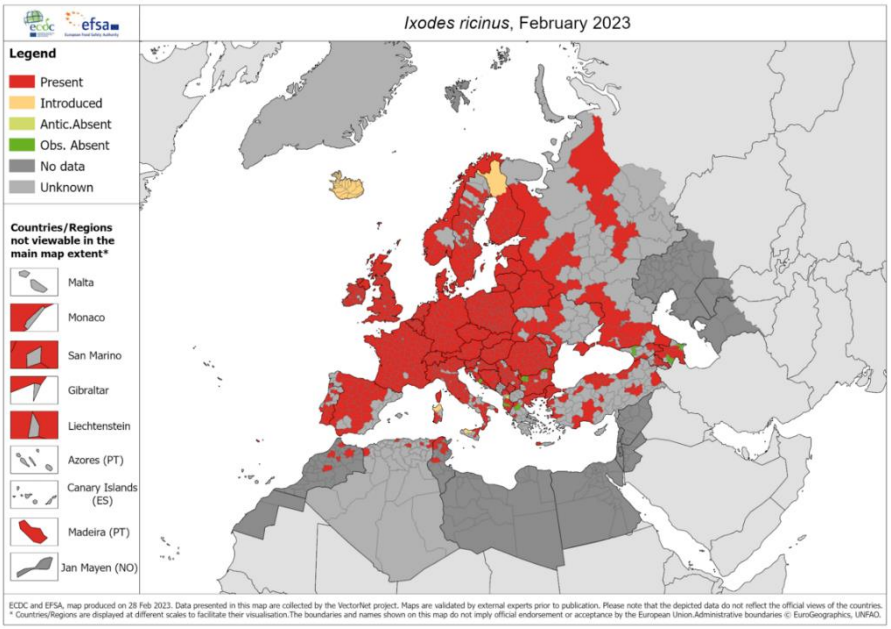


Figure 1.7. Current known distribution of *I. ricinus* in Europe at 'regional' administrative level, as of February 2023. Present: observed to be present. Introduced: no confirmed establishment. Anticipated Absent: never reported, high probability of absence. Confirmed Absent: never reported during studies carried out within the last 5 years. No Data: No sampling performed and no data is available. Unknown: The status is unknown. Source: European Centre for Disease Prevention and Control and European Food Safety Authority. Available in: https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/tick-maps.

Within its climatic envelope, *I. ricinus* presence is determined by biotic interactions and habitats and is not necessarily positively associated to climatic factors like precipitations and temperature (Killilea et al., 2008; Pfäffle et al., 2013). Forests provide suitable microenvironmental resources for ticks: they harbour significant numbers of large animals, such as deer, the main reproduction host of *I. ricinus*. Fragmented forests are associated with higher hazard and/or LB incidence because forest ecotones favour rodents population and human exposure (Allan et al., 2003; Brownstein et al., 2005; Jackson et al., 2006; Killilea et al., 2008). *Ixodes ricinus* is not restricted to deciduous forests and they are also present in other habitats, such as suburban forests (Paul et al., 2016), pastures (Boyard et al., 2011, 2008), meadows (Richter and Matuschka, 2011), urban green spaces (Hansford et al., 2017) or gardens (Jore et al., 2020; Richter et al., 2023). These habitats must also be considered when assessing public health risk for tick-borne diseases (Garcia-Martí et al., 2017). These habitats often have generally lower vegetation cover and tick densities but may be subject to intense human exposure or, in some areas, they may represent the best habitat available (Garcia-Martí et al., 2017; Zeimes et al., 2015).

Exposure

The second element of TBD risk, exposure, corresponds to the likelihood of an individual to encounter an infected tick or nymph. It relies on the factors that favour or discourage human or domestic animal/tick contacts in space and time (e.g., human behaviours, activities, land ownership) (De Keukeleire et al., 2015; Hall et al., 2017; Jore et al., 2020; Linard et al., 2007). Exposure therefore corresponds to the degree that individuals get in contact with tick-infested areas during the tick activity period (Zeimes et al., 2014). Behaviours leading to higher and lower exposure are driven by individual factors and the presence of working and/or recreational amenities.

Integrating exposure in risk modelling is not straightforward, as the same resources may be associated to different functions in humans and animals, making it difficult to disentangle their respective effects. For example, forest ecotones have been associated with TBD risk as they are popular places for competent hosts habitats (e.g., rodents), but also because human recreative presence in forests is higher at its edge (Garcia-

Marti et al., 2018). Recent studies tried to create integrated risk maps, by adding the human component (Bouchard et al., 2023; Hassett et al., 2022; Zeimes et al., 2014). Bouchard et al. (2023) integrated human exposure indexes (a global preventive behaviour score, a global knowledge score, and a global risk perception score) in spatially-explicit risk maps for prioritization of Lyme borreliosis interventions in the Estrie region of Québec (Canada).

Vulnerability and coping capacity

The last, and often neglected parameters in risk assessment are the degree to which individuals or communities are susceptible to harm resulting from a hazard (vulnerability) and the capacity to cope with it (coping capacity) (Vanwambeke and Schimit, 2021). Individual may develop and/or resist infections, in relation to biological components of immune status and health condition, and socio-economic factors, such as the financial barrier to seek diagnostic and receive adequate treatment.

At-risk individuals can also integrate individual and community-level strategies to cope with enzootic hazards or to recover from its negative effects. It can be achieved by mitigating the hazard (e.g., by reducing tick populations or controlling hosts), exposure (e.g., by avoiding tick infested areas or using repellents) or vulnerability (e.g., by performing regular body checks and quick tick removals, or by public health campaigns). The use of protective measures against tick bites remains relatively low and uneven in the population (Slunge and Boman, 2018). Counter-intuitive results recently came out regarding TBD knowledge and the use of preventive measures in relation with the perceived risk of TBD. In a survey in Denmark and Norway, Slunge et al. (2019) noticed that TBD risk perception was higher than scientific estimates in the surveyed population, but knowledge was low, especially in men and the 18-29 years-age group. Information about ticks and TBD remained a relevant public health strategy to make better associations between risk perceptions and the actual risk (Slunge et al., 2019). Despite the recent emergence of studies on these complex factors (e.g., Eisen and Stafford, 2021; Jore et al., 2020; Slunge et al., 2019; Vanwambeke and Schimit, 2021), they have been overlooked and require further investigation.

1.7. APPROACHES IN SPATIAL RISK MODELLING

Public health risk governance is a complex framework including risk assessment, risk management and risk communication (Sedda et al., 2014). VBD have been neglected compared to other diseases (e.g., cancers or cardiac diseases), probably due to their smaller importance in industrialized countries (Lippi et al., 2021; Sedda et al., 2014). Among VBD systems, quantitative analyses have long been dominated by insect systems (Lippi et al., 2021; Randolph, 1998). Because of its complexity and the number of actors involved (at least three species: the pathogen, host and tick vector), TBD, and more generally vector-borne diseases (VBD) cannot be defined by a single metric (Sedda et al., 2014).There are two main approaches in disease modelling: the empirical-statistical approach and the mechanistic-biological approach.

Empirical-statistical models

Empirical-statistical models use statistical methods to find robust associations between a dependent variable representing TBD risk (e.g., disease case counts or DON metrics) and a set of independent variables. These independent variables are either direct causal drivers or proxies of risk, and sometimes only correlated with risk. Regressions or odds ratio models are commonly employed in empirical-statistical models (Sedda et al., 2014). Some vector distribution models try to identify the distribution of the vector of a disease, based on presence (sometimes also absence) records and to extrapolate it based on environmental abiotic predictors (e.g., rainfall, annual temperatures, winter temperatures) that present the best spatial concordance with presence(/absence) records.

However, in these models, the relationships between the species and the environmental predictors remain mainly correlative. Moreover, specific challenges come with modelling disease cases, especially managing disparate data sources, integrating data at multiple scales and underreporting of diseases cases (Heesterbeek et al., 2015).

In this thesis, we used empirical-statistic models to associate tick abundance and infection prevalence (**chapter 2**), tick microbiota (**chapter 3**) and BE infection in

cattle (**chapter 4**) with environmental and landscape predictors. The predictors were selected based on the RBHC, aiming to extend associations beyond mere correlational relationships.

Mechanistic models

Mechanistic or process-based models capture one or several components of the biology of the tick-borne infection pathway (Sedda et al., 2014). These models try to establish causality by including the known biological and epidemiological aspects of the system. However, due to the complexity of the biology of the TBD system, incorporating previous knowledge into risk mapping can be challenging, particularly when dealing with poorly understood phenomena (Sedda et al., 2014).

The basic reproduction number (R0) is an example of mechanistic model in epidemiology. It represents the average number of secondary cases generated by a primary infectious case in a susceptible population (Heesterbeek et al., 2015; Randolph, 1998). R0 has been adapted for tick-borne infections by Hartemink et al. (2008). These infections are influenced by the biology of the infectious system, which is characterized by several transmission routes (systemic and non—systemic horizontal transmission and transovarial vertical transmission). In another example, Tardy et al. (2023) used mechanistic movement models to predict geographic range expansions of *I. scapularis* and *Amblyomma americanum.*

Knowledge-based models

A third approach, based on expert or other experience opinions, has expended in TBD models (Sedda et al., 2014). Experts may decide specific thresholds for certain environmental variables to create risk maps (e.g., Hill, (2012) for yellow fever). Stochastic method, mainly in a Bayesian framework, may use previous knowledge for the predictors estimates, and are increasingly used in vector-borne disease risk assessment (Sedda et al., 2014). One example of expert-based model is the multi-criteria decision analysis (MCDA) combined with geographic information system (GIS). This approach combines values of several predictive criteria according to user-defined weights, that can be derived from expert consensus (Malczewski, 2011,

2000a; Saegerman et al., 2023). MCDA has been developed in different contexts in TBD risk studies, e.g., to rank intervention for LB prevention intervention (Aenishaenslin et al., 2013), or to compare acceptability of LB prevention intervention between countries with different epidemiological contexts (Aenishaenslin et al., 2015). The distribution of *I. ricinus* habitat suitability has also been mapped with GIS-MCDA In Ireland (Rousseau et al., 2017) and in France (Lebert et al., 2022). In this thesis, the GIS-MCDA method was tested for tick bite risk mapping in **chapter 5**.

2. OBJECTIVES AND OUTLINE OF THE THESIS

The general objective of the thesis was to assess tick-borne disease risk in Belgium at different spatial scales with a One Health approach (Figure 1.8). Many health studies are either ecological studies, investigating the spatial and temporal factors influencing tick abundance and infection prevalence, or epidemiological studies, focusing on TBD cases / serologies in human or animals. Studies integrating the different components of risk and of the TBD system are expanding, in line with the One Health approach.

In this thesis, we decided to adopt a One Health approach, considering the interconnectedness of the interactions between environment, humans, and animals. We studied elements of the TBD system at different spatial scales. This dissertation is organized in six chapters, including this introduction, where we briefly introduced the subject and the main concepts of the thesis (**Chapter 1**).

Forests are the primary habitat of *I. ricinus* ticks and as such are often considered as a homogeneous habitat for ticks in the population. In line with recent studies (e.g., Dyczko et al., 2022; Kiewra et al., 2017; Van Gestel et al., 2021), **chapter 2** questioned this assumption and assessed the fine scale determinants of local heterogeneity in tick abundance and infection prevalence in a forest and neighbouring vegetated areas. We selected the Bois de Lauzelle, a periurban forest between Wavre, Louvain-la-Neuve and Ottignies, as a case study, as it presents suitable conditions for ticks and is frequently visited. Nymphs and adult ticks were collected during two years in several locations representing specific microhabitats in and around a peri-urban

forest. We tested the presence of three pathogenic bacteria, *Borrelia burgdorferi*, *Coxiella burnetii* and *Francisella tularensis*. We also discussed the spatial diversity in *B. burgdorferi* genospecies, and the efficiency of the classical method used for tick sampling to produce comparable estimations of abundances.

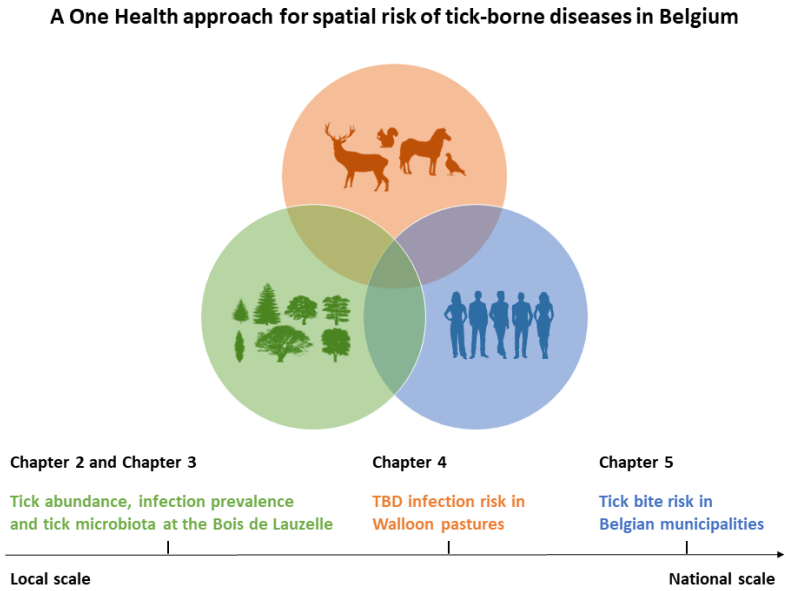


Figure 1.8. Graphical summary of the dissertation.

Ticks have a complex, diverse, largely unknown microbiota, including pathogenic bacteria. However, other microorganisms coexist as endosymbionts that may affect tick fitness and pathogen transmission. In **chapter 3**, we described the presence of non-pathogenic bacteria in ticks and analysed their diversity between different sites and different local environments. It was the first study identifying and describing the composition and diversity of the culturable bacteria found in ticks collected in Belgium in association with the habitats where the ticks were collected.

In **chapter 4**, we used livestock seroprevalence to understand spatial variation in risk at the regional scale. We used a kernel density method to create a spatial index of bovine ehrlichiosis infection in Walloon cattle. It is an underdiagnosed TBD affecting cattle in Belgium. This is mainly caused by a lack of awareness and therefore in a lack

of systematic data. Our dataset was the result of serological tests from one random cow by herd, in a large sample of herds. The precise location of infection remains uncertain as the samples were geolocated at the farm level. Based on the serologies, we created a continuous measure of the risk of infection in pastures at the landscape level. The resulting indicator was associated to important TBD predictors: the presence of large hosts, wild or domestic, and the presence and configuration of forests around the pasture. This index may help veterinary practitioners to quickly target areas at risk of BE.

Risk maps are a useful tool to understand the distribution of risk. **Chapter 5** aims to classify human tick bite risk in Belgium municipalities. Tick bites result from human contacts with ticks and are influenced by environmental and human factors. In this study, we used a spatial MCDA to create a risk map for Belgium. MCDA is a knowledge-based method that does not rely on a set of records but combines important predictors with user-defined weights. Two risk maps were generated. The first one is addressed to citizens, and identifies areas where hazard is higher. The second one incorporated human exposure variables and targets public health decisions makers. The aim of the later map is to highlight areas where intervention could be prioritized. The resulting map was compared with tick bites self-reports from Belgian citizens in the TiquesNet/TekenNet app (https://tiquesnet.sciensano.be/). We discussed the advantages and drawbacks of both approaches and highlighted the benefits of their integration in risk mapping.

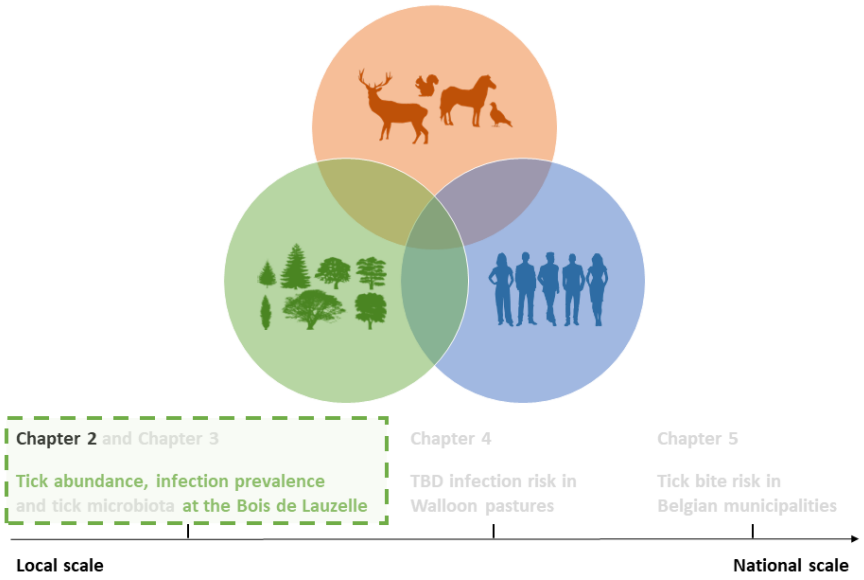
In **chapter 6,** we sum up, highlight, and discuss the main findings of chapters 2-5 and their implications for future research.

3. LIST OF ABBREVAIATIONS FOR CHAPTER 1

GIS	geographic information system
LB	Lyme borreliosis
MCDA	multi-criteria decision analysis
RBHC	resource-based habitat concept
TBD	tick-borne diseases

CHAPTER 2

HETEROGENEITY OF TICK ABUNDANCE AND INFECTION WITH THREE ZOONOTIC BACTERIA IN A BELGIAN PERI-URBAN FOREST



This chapter is based on the following working paper, currently in preparation for publication. Rousseau R., Mori M., Kabamba B., Vanwambeke S.O. Heterogeneity of tick abundance and infection with three zoonotic bacteria in a Belgian peri-urban forest.

ABSTRACT

Ixodes ricinus is vector of several pathogens Forests are their primary habitats, although, tick and infected tick densities are variable also within forests. This study assesses the spatio-temporal variations in tick abundance and their infection with three pathogens in a peri-urban forest where human exposure is high. Ticks were sampled in 2016 and 2018in several locations including areas within the forest with a diversity of undergrowth, and areas in a vegetated but not forested environment. Three zoonotic pathogens were screened for, Borrelia burgdorferi s.l., Coxiella burnetii, and Francisella tularensis. The influences of season, type of site and micro-environmental factors on tick abundance were tested with negative binomial generalized linear mixed-effects models. 1642 nymphs and 181 adult ticks were collected. Forest undergrowth type and height were good indicators of the level of tick abundance in a forest but poor at describing precise abundance as it is influenced by local factors. The within-forest tick heterogeneity was estimated with at least three consecutive drags. This method offers better estimations of tick abundance, as capture probability is influenced by the undergrowth complexity. Borrelia burgdorferi s.l. prevalence was estimated from pooled ticks at 5.33%, Coxiella burnetii was detected in six pools and F. tularensis was not detected. Borrelia afzelii was the dominant genospecies. Tick abundance and Borrelia burgdorferi s.l. infection prevalence were lower than estimates in other Belgian forests, when it was estimated with the ticks sampled during the first drag.

1. INTRODUCTION

Ticks are important vectors of pathogens in Western Europe (Dantas-Torres et al., 2012; de la Fuente et al., 2008). These pathogens include microorganisms (e.g., bacteria, viruses or protozoa) transmitted through the bite of infected ticks. Lyme borreliosis (LB) is the most widespread tick-borne disease in Western Europe (Gray, 1998; Stanek and Reiter, 2011). If untreated, an infection may result in skin, neurological, musculoskeletal, or cardiac complications (Stanek and Reiter, 2011; Strle and Stanek, 2009). This multi-systemic inflammatory disease is caused by spirochetes from the species complex *Borrelia burgdorferi* sensu lato At least five genospecies from this complex are responsible for human diseases: *Borrelia afzelii*, *Borrelia garinii*, *Borrelia burgdorferi* sensu stricto, *Borrelia bavariensis*, *Borrelia spielmanii* and *Borrelia valaisiana* (Stanek and Reiter, 2011; Strle and Stanek, 2009). In Belgium, the prevalence of *B. burgdorferi* s.l. in ticks collected from humans was estimated at 13.9% (Lernout et al., 2019), and the incidence of Lyme borreliosis at 103 per 100 000 inhabitants (Geebelen et al., 2019).

In Western Europe, the main tick vector, *Ixodes ricinus*, also transmits *Francisella tularensis* and *Coxiella burnetii*, the causative agents of respectively tularaemia and Q fever. Tularaemia starts with flu-like symptoms but can evolve towards serious clinical manifestations and significant mortality if untreated, occurring mainly in the Northern Hemisphere (WHO, 2007). Routes of infection include skin contact with infected animals, ingestion of contaminated water, and arthropod bites (ECDC, 2017; WHO, 2007). Its causative agent, the gram-negative intracellular bacterium *Francisella tularensis* has as its main reservoirs lagomorphs and rodents (Carvalho et al., 2014). Tularaemia is rare in Belgium, but the number of detected cases is increasing, similarly to the number of serological tests: 28 cases between 1950 and 2021, of which 25 were reported between 2012 and 2021 with an unknown source of contamination for the majority of cases (Litzroth and Mori, 2021).

Coxiella burnetii is a gamma(γ)-proteobacteria of the Legionella order (Körner et al., 2021; Woldehiwet, 2004) that infects a wide range of hosts, including dogs, bovine, deer, rodents, birds and ticks (Cutler et al., 2007; Mori et al., 2017). In humans, it can

cause acute or chronic illness, but the diagnosis is challenging because most human infections are sub-clinical (Anderson et al., 2013; Cutler et al., 2007; Mori et al., 2017). In Belgium, 15 cases of Q fever were reported in 2021 (Litzroth et al., 2021). Infections generally occur through inhalation of *C. burnetii* contaminated aerosols in milk, faeces, urine and birth products or abortions from infected ruminants (Anderson et al., 2013; Cutler et al., 2007; Duron et al., 2015). *Coxiella burnetii* was found in 40 tick species, and tick bites may constitute another route of transmission for human infections, probably less important than the airborne one (Anderson et al., 2013; Duron et al., 2015; Sprong et al., 2012).

Tick-borne diseases (TBD) occurrence depends on the probability of contact between pathogens, ticks and their hosts (Lambin et al., 2010). The life cycle of *I. ricinus* spans over two to six years and it takes a single blood meal by active stage (larva, nymph, and adult) on a broad range of vertebrates, including mammals, birds, and reptiles (Estrada-Peña and de la Fuente, 2014). This generalist species spends extended periods off-host in the environment, for questing, rehydrating, moulting or diapausing. These activities are strongly seasonal (Dobson, 2013; Dobson et al., 2011). *Ixodes ricinus* is found in various vegetated environments, but forests are their primary habitats and are often associated with high tick abundance at regional (e.g., Li et al., 2012; Ruiz-Fons et al., 2012; Vanwambeke et al., 2010) and local scales (e.g., Tack et al., 2012; Van Gestel et al., 2021; Vourc'h et al., 2016). Peri-urban forests are of particular interest, for both providing suitable habitats for ticks, that is, having a high level of hazard, and being intensively visited by humans, that is, generating high exposure (Dobson et al., 2011; Ruiz-Fons and Gilbert, 2010; Zeimes et al., 2014). In Belgium, 35% of tick bites were reported in forests (https://epistat.sciensano.be/ticks/, last access: 7th of August, 2023). Variations in tick abundance and infection in endemic areas are key components of tick-borne disease risk assessment (Horobik et al., 2006; Mysterud et al., 2013) .

Tick abundance in forests is often estimated by collecting ticks by flagging or dragging on a delimited area and counting the number of ticks collected (Nyrhilä et al., 2020). Estimates of hazard drawn from ideal tick habitat are sometimes used to

understand risk but do not necessarily represent well areas of intense human exposure in forests, as people may avoid bushy areas or be prevented from leaving paths as per forest regulations as often apply in periurban forests. Variability in tick density and pathogen prevalence is substantial in a forest, as indicated by Tack et al., (2012) and Van Gestel et al. (2021). In this study, we repetitively collected ticks throughout the period of tick activity. We assessed the spatio-temporal heterogeneity of tick density within and around a peri-urban forest, also accounting for varying degrees of potential human exposure. We hypothesize that tick density and infection prevalence are not homogeneous within a forest and are affected by within-forest heterogeneity in undergrowth. The prevalences of the three above-mentioned pathogens (*B. burgdorferi* s.l., *F. tularensis* and *C. burnetii*) in ticks were also assessed.

2. MATERIAL AND METHODS

2.1. STUDY AREA

The Bois de Lauzelle is a peri-urban forest of nearly 200 hectares located 30 kilometres south of Brussels (Figure 2.1). This peri-urban region has a high and growing population and has a higher incidence of erythema migrans than the Belgian average (Geebelen et al., 2019). The Bois de Lauzelle is bordered by a golf course, the city of Louvain-la-Neuve, and high-speed roads. The forest belongs to and is managed by the university (UCLouvain) since 1970. It presents a diversity of local conditions, with an alternation of loamy and sandy-loamy soils and altitudes ranging from 45 to 153 meters around the Blanc Ri River. The forest is dominated by deciduous trees, mainly beech and oak (Wallonie, 2021), and is legally protected by several statutes (e.g., Natura 2000, code BE31006) (EEA, 2020). The mean annual temperature and rainfall of the region are 10.6°C and 849.4mm / year (Royal Meteorological Institute, 2023).

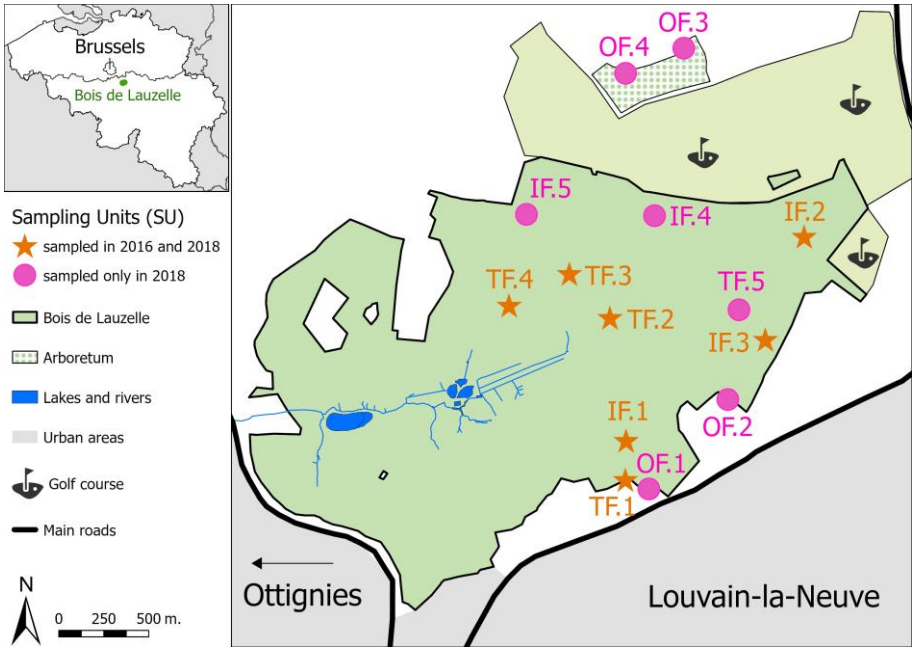


Figure 2.1. Location of the sampling units (SU) in the Bois de Lauzelle. SU sampled in 2016 and 2018 are represented by orange stars, those only in 2018 by rose circles. IF, TF and OF stand for interior-forest, trail-forest, and outside-forest.

2.1. TICK SAMPLING

Ticks were sampled every three weeks between March and November 2018 in fourteen (Figure 2.1, black and grey) sampling units (SU)., resulting in 11 sampling events (SE). Each SU was a delimited area of 10m², using a 1m²-white flannel over the leaf litter, woodland scrub, or low vegetation. The SU represented three types of sites. Interior-forest (IF) sites (Figure 2.1, circles) were forested sites with dense undergrowth and away from the trails (36±9m). Trail-forest (TF) sites (Figure 2.2, stars) were forested sites close to the trails (17±13m) on low undergrowth. Outside-forest (OF) sites (Figure 2.2, squares) were in a vegetated environment outside but close to the forest. A description of the 14 SU is provided in Figures S2.1 – S2.14.

Samplings were performed on dry and non-windy days between 8:00am and 1:00pm. SU were sampled in a random order during each SE to account for daily fluctuations in humidity and temperature with the time of the day. During each SE, we dragged a

flannel on each SU six times consecutively in a rapid total sequence of 9 ± 4 minutes for the six drags. The flannel was changed whenever it became dirty or humid and was examined for ticks at the end of each drag. Nymph and adult ticks were counted and collected after each drag, and ticks from the same SU were stored together in a vial with 70% ethanol. Larvae, found aggregated in the environment and rarely infesting humans, were not systematically sampled, and not analysed further.

2.2. TICK IDENTIFICATION AND PREPARATION

Tick life stage and species were identified under a Leica EZ4 binocular (X35), based on two conventional morphological identification keys (Estrada-Pena et al., 2004; Hillyard, 1996). We also used Heylen et al. (2014a) to distinguish between three similar species found in Belgium, *Ixodes ricinus*, *Ixodes frontalis* and *Ixodes arboricola*. Ticks were washed in three consecutive baths with 70% ethanol and sterilized water and crushed individually with a sterilized loop in Eppendorfs containing 200 µl of Dulbecco's modified Eagle's medium (DMEM) cell culture medium. Then, they were grouped in pools of four tick (50 µl/each, for a total volume of 200 µl) unless only a smaller pool was possible. Nymph and adult ticks sampled in 2018 were pooled separately. Pooled ticks were from the same SU and season. SE occurring between mid-March and mid-June were classed as Spring, between mid-June to mid-September as summer, and between mid-September to the end of October as fall.

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2.4. DNA EXTRACTION, SEQUENCING, AND PCR

DNA was extracted from 100 µl pool medium. The complete methods for DNA extraction and sequencing are described elsewhere (Rousseau et al., 2021). Tick genus (*Ixodes* versus *Dermacentor*) was confirmed and DNA extraction validated using SYBR Green real-time PCR on 5S and ITS2 genes. Ticks were screened for *B. burgdorferi* s.l., *F. tularensis,* and *C. burnetii* using: (i) two qPCR targeting the Outer Surface Protein A gene (*OspA*) and the *Borrelia* flagellin gene (*Fla*). This method was adapted from (Kesteman et al., 2010), (ii) TaqMan real-time (Light-Cycler® TaqMan® Master, Roche Diagnostics GmbH, Germany) targeting Francisella (ISFtu primers) (Versage et al., 2003) and Francisella-like (FopA primers) (Michelet et al., 2014), (iii) TaqMan real-time (Light-Cycler® TaqMan® Master, Roche Diagnostics GmbH, Germany) targeting *C. burnetii* insertion element IS1111 with previously described primers (Mori et al., 2013). The real-time PCR was done (Light-Cycler 480® SYBR Green I Master, Roche Diagnostics GmbH, Germany) instead of an end-point PCR and the identification of species was performed by Sanger sequencing on the Genetic Analyzer ABI 3730XL (Applied Biosystems, Invitrogen Life Technologies, Carlsbad, CA, USA), with the BigDye Terminator kit (Applied Biosystems). To achieve more consistent sequencing results, we used the universal M13 tailed primer attached to the locus-specific primers described by Kesteman et al. (2010). The obtained consensus sequences were analysed with the Basic Local Alignment Search Tool (BLAST) for species determination.

2.5. TICK VARIABLES

The density of nymphs (DON) and of adults (DOA) were computed from the number of nymphs and adults sampled in each SU and at each SE (Figure 2.2). We also computed drag-specific indices, DON_i and DOA_i, the cumulative number of nymphs

and adults captured during the first *i* consecutive drags. Nymph (NIP) and adult (AIP) infection prevalences were calculated by maximum likelihood with the PoolTestR R package (McLure et al., 2021). Finally, the densities of infected nymphs (DIN) and adults (DIA) were calculated by multiplying DON and DOA by NIP and AIP respectively.

2.6. PREVIOUS SAMPLING EVENTS

Ticks were also collected in 2016. Seven SU (Figure 2.1, black) were sampled every two weeks between March and November, resulting in 17 SE. The sampling design was the same as in 2018, but (i) 10 consecutive drags were performed on each SE instead of six, resulting in higher DON estimates, but challenging the assumption of sampling a closed population; (II) the numbers of nymphs and adults collected in each consecutive drag were not reported;(iii) nymphs and adults were pooled together, so we could only compute tick infection prevalence, which may overestimate DIN and underestimate DIA because adults had more blood meals and usually show higher infection rates (Gray, 1998).

2.7. ENVIRONMENTAL VARIABLES

We investigated the effect of environmental predictors on DON measured in 2018. *Ixodes ricinus* retreats in the leaf layer for transstadial development and when questing conditions are unfavourable, and uses vegetation as a support for questing for a bloodmeal (Gray, 1998; Van Overbeek et al., 2008). For each SU, we measured the undergrowth height throughout the sampling period and categorized it as low (< 20 cm), medium (20 to 40 cm), and high (> 40 cm). Forest edge habitats are suitable for several tick hosts (Allan et al., 2003; Brownstein et al., 2005; Tack et al., 2012). In peri-urban environnements, (Hansford et al., 2017) found more nymphs at the woodland edge than in other habitats. We measured, for each SU, the distance to the closest forest edge. Guerra et al. (2002) found more *Ixodes scapularis* on sandy and loamy-sand soils, compared to clay and loamy soils. Soil texture was extracted from the digital soil map of Wallonia (SPW, 2005).

Ixodes ricinus is vulnerable to desiccation, and temperature and relative humidity influence its survival, development rates and activity periods (Brownstein et al., 2005; Diuk-Wasser et al., 2010; Medlock et al., 2013; Ruiz-Fons et al., 2012). We controlled these parameters with measures of temperature and relative humidity over the course of the study period using a HOBO U23 Pro v2 Temperature/Relative Humidity Data Logger placed at 130 cm above the ground close to SU TF.3. Temperatures measured at 130 cm and 5 cm in a forest are often correlated (Ruyts et al., 2018). The recording interval was 15 minutes. Temperatures during sampling events ranged from 5.2 to 25.3°C and relative humidity from 41.6 to 99.5%. Mean temperatures and relative humidity for the 14 days preceding the samplings ranged from 7.5 to 21.7°C and from 68.6 to 89.4%.

2.8. STATISTICAL ANALYSES

Statistical analyses were conducted in R.4.2.1 (R Core Team, 2022). The dataset has multiple observations from the same locations and thus required a multi-level approach. DON, DOA, AIP, NIP, TIP, DIN, and DIA were tested for spatial autocorrelation with Moran's I test. Due to the multilevel structure, we performed repeated measures correlation between these indicators with the rmcorr R package (Bakdash and Marusich, 2023). This technique does not violate the assumption of independent observations and has a greater statistical power because no aggregation is necessary (Bakdash and Marusich, 2017).

First, we used generalized linear mixed models (GLMM) to investigate differences in DON and DOA, with the type of SU (IF, TF and OF), season (spring, summer or fall), year (2016 and 2018) and their one-way interaction, with SU as a random effect. We used DON_6 and DOA_6 for 2018, and DON_{10} and DOA_{10} for 2016, as we consider they were the best proxies of true tick abundances. They were tested at SE level (resulting in 273 observations for 2016 and 2018). We had too few observations for NIP, AIP, DIN to adjust a GLMM. To assess overdispersion, we compared Poisson distributions with negative binomial and their zero-inflated versions, using Vuong's non-nested tests (pscl R package, Jackman, 2020).

DON is usually estimated with the number of nymphs captured during a single drag. We created six GLMMs exploring the effects of season and type of sites on six measures using the number of nymphs collected using different number of drags for 2018.

Negative binomial mixed models were used to assess the effects of environmental variables on DON_6 in 2018, which had a complete environmental record. After standardization of the environmental predictors following Gelman (2008). the complete model included all non-collinear predictors. Colinear predictors were identified using a variance inflation factor < 3. We also computed the best-AIC model, based on Akaike's Information Criterion (AIC). The most parsimonious of models with differences of AIC smaller than |2|, considered equivalent, was chosen. All GLMMs were performed using the glmmTMB R package (Brooks et al., 2017). Residuals were controlled for dispersion, zero-inflation, heteroscedasticity and spatial autocorrelation with the DHARMa R package (Hartig, 2022).

Finally, we calculated the diversity of *Borrelia* genospecies with the R vegan package (Oksanen et al., 2020). We used the exponential back-transformation of the Shannon-Wiener Index to compute the "effective number of species", to facilitate interpretability and comparison and interpretability (Jost, 2006).

3. RESULTS

3.1. TICK ADUNDANCE AND TICK-BORNE PATHOGEN PREVALENCE

1642 *Ixodes* nymphs and 181 adults were sampled (Figure 2.3 and Figure S2.15). 1804 ticks were identified as *I. ricinus*, 17 *I. frontalis* and three *I. ventalloi*. 786 nymphs and 81 adults were collected in 2016, resulting in DON of 6.61 ± 8.39 and DOA of 0.68 ± 1.11 ticks per 10 m². 856 nymphs and 100 adults were collected in 2018, resulting in DON of 5.56 ± 9.06, and DOA of 0.65 ± 1.44 ticks per 10 m². All sites reached their maximum abundance before July. In 2018, DON was higher in forested (7.06 ± 10.14 nymphs / 10m²) than in non-forested sites (1.80 ± 3.38).

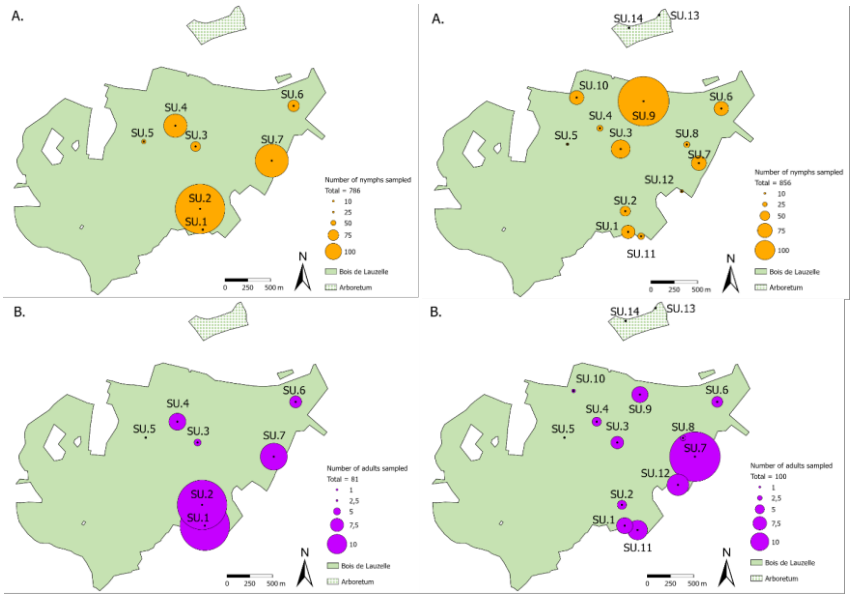


Figure 2.2. Number of nymphs (A, in orange) and adult ticks (B, in purple) sampled in 2016 (left) and in 2016 and 2018 (right) by Sampling Unit (SU).

Nymphs and adults were grouped in 499 pools (221 for 2016 and 278 for 2018). *Borrelia burgdorferi* s.l. was detected in 90 pools (31 for 2016 and 59 for 2018). We found *Borrelia* positive pools in all SU, except TF.3, TF.4, OF.3. The mean TIP was 5.33%, 95%-CI [4.32-6.47], higher in 2018 (6.82%, 95%-CI [5.26-8.63] than in 2016 (3.77%, 95%-CI [2.61-5.21]. TIP increased through seasons: 4.64% (95%-CI [3.42-6.10] in spring, 5.60% (95%-CI [3.95-7.62] in summer, and 9.04% (95%-CI [4.94-14.7] in fall. In 2018, NIP varied from 0 to 19% (IF.2) and AIP from 0 to 33% (IF.1 and OF.1). DIN varied from 0 to 1.25 infected nymphs per 10 m² in IF.2, and DIA from 0 to 0.20 in IF.3.

Spatial autocorrelation was only detected for DOA in 2016 (p-value = 0.04) (Table S2.1). We found significant repeated measures correlations between DON and DIN, DOA and DIA and DON and DOA, but not between tick prevalences and densities of ticks and infected ticks (Table 2.1). The strongest correlations were between DON and DIN (0.63, p-value < 0.0001) and DOA and DIA (0.45, p-value < 0.0001).

Table 2.1. Repeated measures correlations between tick indicators for 2016 and 2018. Upper part: coefficient [95% - confidence intervals]. Lower part: significance based on p-values. Degrees of freedom = 258. N.S. stands for not significant. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Significant correlations at p-value < 0.05 are in bold.

	DON	**DOA**	**TIP**	**DIN**	**DIA**
DON	-	**0.28 [0.16 - 0.39]**	-0.01 [-0.13 - 0.11]	**0.63 [0.55 - 0.70]**	-0.02 [-0.14 - 0.10]
DOA	***	-	-0.03 [-0.15 - 0.10]	**0.24 [0.12 - 0.35]**	**0.45 [0.35 - 0.55]**
TIP	N.S.	N.S.	-	**0.23 [0.11 - 0.34]**	0.05 [-0.07 - 0.17]
DIN	***	***	***	-	-0.02 [-0.14 - 0.11]
DIA	N.S.	***	N.S.	N.S.	-

3.2. GENERALIZED LINEAR MIXED MODELS WITH SITE TYPE, SEASON AND YEAR

For all DON_i indicators, the negative binomial distribution was preferred (Table S2.2). GLMMs expressed variations of the density of nymphs (Table 2.2) and of adults (Table 2.3) by year, season, and type of SU. The highest estimation of DON was for spring 2016 and in IF sites: 18.62 nymphs per 10 m². It decreased in summer and in fall and in TF sites and OF sites. There were no differences between the two years and the first-level interaction between year and season was not significant. No pattern was detected in the residuals. The estimated DOA in spring 2016 and in IF sites was 0.99 adults per 10 m². There were no differences between years, seasons and types of SU, except a lower tick density in fall. The first-level interaction between year and summer was also significant. No pattern was detected in the residuals (Dharma residuals plots presented in Figure S2.16 for the models with interaction).

Table 2.2. Negative binomial generalized linear mixed model estimates for density of nymphs (DON) and adults (DOA). The estimates are expressed as the exponential of the log coefficients of the models. The AIC for the null models were 1500.7 and 595.4 for DON and DOA respectively. N.S. stands for not significant. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Significant estimates at p-value < 0.05 are in bold.

	Estimate (standard deviation)	
Fixed Effects	**DON**	**DOA**
Intercept	**18.62 (1.30) *** **	0.99 (1.50) N.S.
Year (baseline = 2016)		
Year 2018	0.95 (1.25) N.S.	1.37 (1.39) N.S.
Season (baseline = spring)		
Summer	**0.60 (1.25) ***	1.01 (1.40) N.S.
Fall	**0.13 (1.28) *** **	**0.22 (1.56) *** **
SU Type (baseline = IF sites)		
TF sites	**0.37 (1.35) *** **	0.56 (1.58) 0.1961
OF sites	**0.16 (1.43) *** **	0.57 (1.70) 0.2973
Interactions		
Year 2018 * Season Summer	0.78 (1.34) N.S.	**0.36 (1.58) ***
Year 2018 * Season Fall	1.01 (1.41) N.S.	0.93 (1.82) N.S.
Random Effects	**Estimate (standard deviation)**	
SU	0.99 (1.50)	1.01 (1.76)

3.3. MIXED MODELLING FOR DIFFERENT ESTIMATES OF DON

In 2018, 34% of the nymphs (Table S.2.3). and 51% of the adults (Table S.2.4) were collected in the first drag. The lowest rates for both nymphs and adults were in IF sites. GLMM using DON estimated with various numbers of drags in 2018 show that the estimates of DON in spring and in IF-SU increased from 4.10 nymphs per $10m^2$ for DON_1 to 16.64 for DON6 (Table 2.3). As in Table 2.2, DON decreased from spring to fall and was higher in IF compared to OF and TF-SU, but the effect of site type was only detected when using at least three consecutive drags.

Table 2.3. Negative binomial generalized linear mixed model estimates for the number of nymphs sampled by consecutive drags in 2018 (DON_i, with *i* from 1 to 6). The estimates and standard deviations are expressed as the exponent of the log coefficient of the model. N.S. stands for not significant. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001.

	DON1	**DON2**	**DON3**	**DON4**	**DON5**	**DON6**
Fixed Effects						
			Estimates (standard deviations)			
Intercept	4.10 (1.44) ***	8.04 (1.36) ***	10.80 (1.35) ***	12.95 (1.36) ***	14.5 (1.34) ***	16.64 (1.34) ***
Season - baseline = Spring						
Summer	0.60 (1.27) *	0.51 (1.26) **	0.48 (1.24) ***	0.51 (1.24) **	0.50 (1.23) **	**0.46 (1.23) *** **
Fall	0.17 (1.37) ***	0.16 (1.33) ***	0.15 (1.31) ***	0.13 (1.31) ***	0.14 (1.30) ***	0.13 (1.29) ***
SU Type - baseline = IF sites						
TF sites	0.57 (1.63) N.S.	0.52 (1.48) N.S.	0.45 (1.48) *	0.43 (1.49) *	0.41 (1.45) *	**0.39 (1.45) ***
OF sites	0.21 (1.72) **	0.2 (1.54) ***	0.18 (1.53) ***	0.17 (1.54) ***	0.17 (1.51) ***	0.17 (1.50) ***
Random Effects						
			Estimates (standard deviations)			
SU	1.00 (1.93)	1.00 (1.63)	1.63	1.00 (1.68)	1.00 (1.63)	1.00 (1.64)

3.4. GENERALIZED MIXED MODEL FOR THE ENVIRONMENTAL PREDICTORS OF TICK DENSITY IN 2018

When estimating the effects of the environmental variables on 2018-DON estimators, we found an estimate of 20.10 nymphs per 10 m² in SU with high undergrowth, loamy sands and excessive drainage (Table 2.4). DON was also negatively affected by relative humidity and temperature of the 14 previous days and positively by temperature during the sampling. Undergrowth height was significant and included in the best-AIC model, but no other variables describing sites. Dispersion, zero-inflation, heteroscedasticity, and spatial autocorrelation were not detected in the residuals of these models.

Table 2.4. Negative binomial estimates for the generalized linear mixed models of the density of nymphs by the environmental variables. The estimates and standard deviations are expressed as the exponential of the log coefficients of the models. The AIC for the null model was 812.9. N.S. stands for not significant. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001.

	Complete model	**Best-AIC model**
AIC	784.2	778.7
Fixed Effects	**Estimates (standard deviations)**	
Intercept	20.10 (1.91) ***	14.86 (1.50) ***
Relative humidity - 14 days	0.73 (1.24) N.S.	-
Temperature - 14 days	0.34 (1.37) ***	0.40 (1.33) **
Relative humidity - sampling	0.56 (1.26) *	0.50 (1.24) **
Temperature - sampling	2.05 (1.36) *	1.91 (1.35) *
Undergrowth height (baseline = high)		
Medium	0.21 (1.45) ***	0.21 (1.45) ***
Low	0.14 (1.71) ***	0.14 (1.66) ***
Texture (baseline = loam)		
Sand	1.12 (1.82) N.S.	-
Sandy loam	0.65 (1.79) N.S.	-
Drainage (baseline = excessive)		
Favourable	0.79 (1.80) N.S.	-
Distance to forest edge	0.50 (1.64) N.S.	-
Random Effects	**Estimates (standard deviations)**	
SU	3.63 (2.10)	1.00 (2.23)

3.1. *BORRELIA* GENOSPECIES DIVERSITY

Six genospecies were identified: *B. afzelii* was dominant (54 isolations), followed by *B. garinii* (24), *B. burgdorferi* s.s. (13) and *B. valaisiana* (5). *Borrelia bavariensis* and *B. spielmanii* were only isolated once, in 2016 (Figure 2.3). The effective diversity number for *Borrelia* genospecies was 3.21, higher in 2016 (3.42) compared to 2018 (2.93). It was also higher in forested sites (4.00 for TF and 2.71 for IF) than in non-forested sites (1.51). The SU TF.2, TF.1, IF.4, IF.2 and IF.3 presented the highest diversity in the number of genospecies (5, 4, 3, 3 and 3 respectively), and of isolations (14, 17, 16, 13 and 14 respectively) (Table 2.5). SU OF.3 had the lowest diversity with no genospecies isolated while TF.4, TF.5 and OF.2 had only one (*B. spielmanii* for the former and *B. afzelii*, for the last two). *Coxiella burnetii* was detected in six pools, three in 2016: one from SU TF.1 in spring and two from IF.1 in summer and three in 2018: one from IF.2 in spring and two from IF.4 in spring and summer. Due to the low prevalence, these results were not analysed further. *Francisella tularensis* and *Francisella*-like bacteria were not detected.

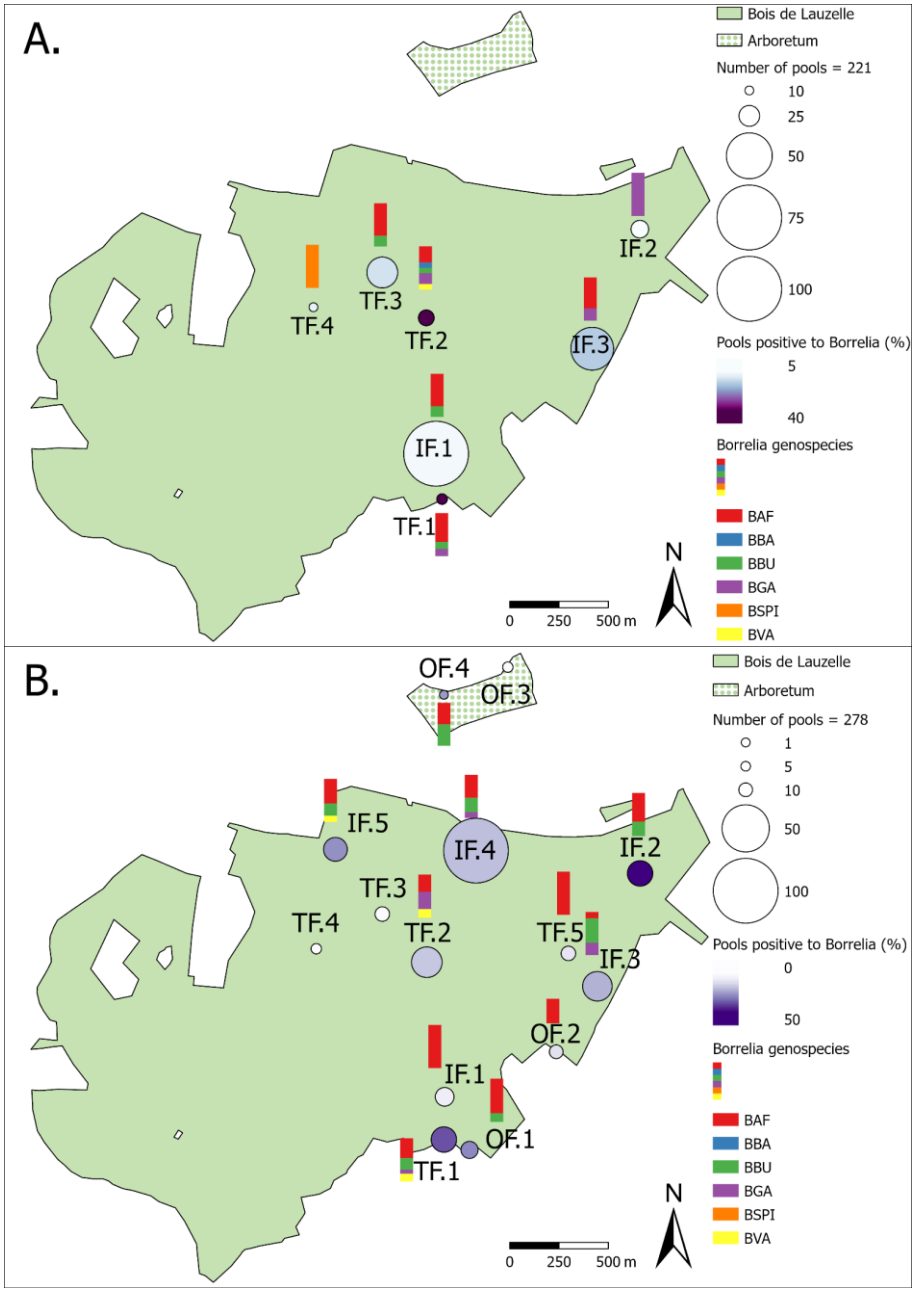


Figure 2.3. *Borrelia burgdorferi* s.l. infections in pooled ticks and genospecies composition by sampling unit in a) 2016 and b) 2018.

Table 2.5. *Borrelia burgdorferi* s.l. genospecies diversity by sampling unit (SU). BOR stands for *Borrelia burgdorferi* s.l., BAF for *B. afzelii*, BBA for *B. bavariensis*, BGA for *B. garinii*, BSPI for *B. spielmanii* and BVAL for *B. valaisiana*.

SU (pools)	BOR effective number	BOR species richness	BOR species abundance						
			BOR	BAF	BBA	BBU	BGA	BSPI	BVAL
TF	**2.71**	**4**	**54**	**32**	**0**	**14**	**7**	**0**	**1**
TF.1 (39)	3.26	4	17	9	0	4	2	0	2
TF.2 (50)	4.11	5	13	5	1	1	4	0	2
TF.3 (46)	1.75	2	4	3	0	1	0	0	0
TF.4 (19)	1	1	1	0	0	0	0	1	0
TF.5 (11)	1	1	1	1	0	0	0	0	0
IF	**4**	**6**	**35**	**18**	**1**	**6**	**6**	**0**	**4**
IF.1 (85)	1.65	2	5	4	0	1	0	0	0
IF.2 (46)	2.36	3	13	8	0	4	1	0	0
IF.3 (76)	2.94	3	14	6	0	4	4	0	0
IF.4 (71)	2.58	3	16	9	0	5	2	0	0
IF.5 (22)	1.57	2	6	5	0	0	0	0	1
OF	**1.51**	**2**	**7**	**6**	**0**	**1**	**0**	**0**	**0**
OF.1 (14)	1	1	4	4	0	0	0	0	0
OF.2 (10)	1	1	1	1	0	0	0	0	0
OF.3 (6)	0	0	0	0	0	0	0	0	0
OF.4 (4)	2	2	2	1	0	1	0	0	0
Total	**3.21**	**5**	**97**	**56**	**1**	**21**	**13**	**1**	**5**

4. DISCUSSION

This study investigated the heterogeneity of tick density and infection prevalence in a peri-urban forest. Our results indicated that the acarological indicators were heterogeneous within a forest, as previously reported by Vourc'h et al. (2016) in France. The mean densities of questing forested nymphs, estimated at 6.61 and 7.07 nymphs per 10 m² in 2016 and 2018 respectively, were in the upper range of other studies in Belgium. DON was estimated at of 4.05, 6.1 and 6.4 nymphs per 10 m² in

2013, 2018 and 2019 respectively (Ruyts et al., 2016; Van Gestel et al., 2021). However, direct comparison with our estimations is not straightforward, as their DON estimates were measured by dragging the vegetation once. DON estimated from a single drag at the Bois de Lauzelle were 2.93 and 2.38 nymphs per 10m² in 2016 and 2018 respectively.

Dragging is the most common method for tick sampling for its cost-effectiveness and its ease of implementation and replication among field workers (Nyrhilä et al., 2020). However, it tends to underestimate absolute tick abundance, and its efficiency also fluctuates, i.e., with the time of the day or the vegetation sampled (Bord et al., 2014; Boyard et al., 2007). Vegetation modifies the contacts between the drag and questing ticks (Tack et al., 2011). With varying capture probabilities, comparable abundance estimates are difficult to achieve (Mackenzie et al., 2002). We estimated DON based on consecutive drags, performed in fast succession to collect individuals from a closed population, which gives better proxies of the true abundances (Bord et al., 2014). Within forest heterogeneity was only significant with DON estimated with three consecutive drags. Sampling the same transect repeatedly increases the probability of tick/drag contact in complex undergrowth. We thus believe our estimate to be reliable and recommend performing DON estimations based on at least three consecutive drags when comparing areas with different vegetation types.

Tick samplings were regularly performed throughout the year to capture the temporal variation of tick density, as recommended by Dobson (2013) and Salomon et al. (2020). This limits the biases of occasional samplings caused by a variety of factors influencing questing, sometimes locally (e.g., passage of hosts before the sampling). In both years, ticks were active during the entire period of sampling, but their abundance was higher in spring over summer, over fall, as observed in other peri-urban areas of Western Europe (e.g., Hansford et al., 2017). Tick density also varied within seasons. Body inspection should therefore be recommended to forest users throughout the season of tick activity.

The density of sampled ticks was influenced by temperature and relative humidity during the sampling and the 14 previous days. Temperature and humidity affect

desiccation and therefore *I. ricinus* survival and activity (Perret et al., 2000). DON was positively associated to temperature at sampling time but negatively to the 14 previous day average. The influence of temperature is complex and not easily reproducible (Boyard et al., 2011). In Belgium, temperature is likely not a limiting factor for tick survival and persistence, but may affect tick activity at sampling time (Medlock et al., 2013). DON was negatively influenced by relative humidity during the samplings as observed in other studies (e.g., Hubálek et al., 2006; Kiewra et al., 2014; Li et al., 2012; Schwarz et al., 2009). These results stem from records at a single forest location, above the litter, not where *I. ricinus* shelter. Results by Boehnke et al. (2017) suggest that one site measurement may explain differences between sites, however, our results indicate that overall conditions also significantly affect tick activity. This would be relevant if wanting to use general weather observation as part of risk assessment.

Borrelia burgdorferi infection prevalence in *I. ricinus* ticks was estimated here at 5.3%, 95%-CI [4.3-6.5], lower than prevalences in questing ticks in Belgium: 17.8% (Heylen et al., 2019), 15.6% (Ruyts et al., 2016), 12% (Kesteman et al., 2010) and 9.1% (Tack et al., 2012). the low *B. burgdorferi* prevalence found at the Bois de Lauzelle may also relate to the lack of connections for wildlife with other forests, or to the presence of specific hosts and host composition (Ruiz-Fons et al., 2012). To analyse the effects of hosts composition on tick abundance and *B. burgdorferi* prevalence and genospecies composition, further studies should consider including methods like live-trapping for small mammals or camera for large-sized mammals (Pérez et al., 2012; Ruyts et al., 2018).

We screened ticks in pools, a conventional method for arthropod vector screening. This methods presents several challenges: the exact number of infected ticks in a positive pool cannot be determined and there may be a dilution effect for pools with high number of ticks (Fracasso et al., 2023). We grouped ticks in pools of four ticks, by SU, season, and stage (in 2018). We estimated prevalences with the maximum-likelihood estimate of pooled prevalence, which is less influenced by pool size and infection rate of ticks than the pool positivity rate and minimum infection rate methods

(Fracasso et al., 2023). This method produces robust estimates and confidence intervals of *B. burgdorferi* infection prevalence.

Tick infection prevalence with *Borrelia burgdorferi* was variable, but the small number of pools and the broad confidence intervals prevented us from analysing the differences in space and time. *Borrelia burgdorferi* infection prevalence was also found to be highly variable in Belgium and the Netherlands, ranging from 0-2% to 20-25%, over short distances (Hartemink et al., 2021; Kesteman et al., 2010). Tick infection prevalence should therefore not be estimated from a single location in a forest. Hartemink et al. (2021) and Ruyts et al. (2017) did not find inter-annual variation in NIP and considered that DIN is mostly determined by DON. Forests present a diversity of micro-environments influencing tick density and infection prevalence.

We found stronger correlations between DON and DIN (0.61) than between DON and TIP (0.23). A weak or absence of correlation between density of nymph and nymph infection prevalence is common in endemic areas (Randolph, 2001), and has been reported elsewhere (James et al., 2013; Jouda et al., 2004; Ruyts et al., 2017; Vourc'h et al., 2016).

Borrelia afzelii was the dominant genospecies, followed by *B. burgdorferi* s.s. *and B. garinii*, which was consistent with other studies in Belgium (online supplementary materials). A recent meta-analysis of *Borrelia* prevalence in *I. ricinus* questing ticks in Western Europe also identified a composition of 46.6% for *B. afzelii*, 23.8% for *B. garinii*, 11.4% for *B. valaisiana*, 10.2% for *B. burgdorferi* s.s., and *B. bavariensis*, *B. spielmanii* rarely detected (Strnad et al., 2017). The effective number of *Borrelia* species was higher in forest, especially in sites with open areas and under-developed undergrowth. *Borrelia bavariensis* and *B. spielmanii*, identified once in this study, are rare genospecies in Belgium. *Borrelia afzelii* and *B. spielmanii* are commonly associated with small mammals, while *B. garinii* and *B. valaisiana* are hosted by sea birds and songbirds. *B. burgdorferi s.s.* is generalist (Comstedt et al., 2006; Gray, 1998; Hanincova et al., 2003; Heylen et al., 2014b; Kurtenbach et al., 2002; Pedersen et al., 2020). The dominance of *B. afzelii* over *B. garinii* may suggest that rodents

(e.g., *Apodemus sylvaticus*) are the most important feeding hosts for larval ticks in the Bois de Lauzelle.

Francisella tularensis and *Francisella*-like bacteria were not detected in this study. Their prevalence in ticks is usually low. In the Sénart Forest (France), *F. tularensis* was found in one out of 69 ticks (1.45%) in 2008, and not detected between 2009 and 2014 (Paul et al., 2016). *Coxiella burnetii* was detected in three pools in 2016 and three in 2018 and its prevalence in questing *I. ricinus* is also usually low, 0.2% in a study from the Netherlands (Sprong et al., 2012). The prevalence of these pathogenic agents in ticks is currently not well known in Belgium. However, the *C. burnetii* Nine Mile strain (RSA 493), originally isolated from the tick *Dermacentor andersoni*, was recently isolated in Belgium (Tomaiuolo et al., 2021). The public health significance of pathogens, present at very low prevalence, is difficult to assess with the number of pools tested in this study.

5. CONCLUSION

We detected ticks in all locations sampled in and around a periurban forest. Tick abundance displayed two peaks: one biggest peak in late spring and a second, lower peak in late summer. DON was assessed with the consecutive dragging method, which offers a representative and comparable estimate of tick abundance in areas with different undergrowth. DON was higher within than outside the forest and heterogeneous within the forest. Forest undergrowth type and height are good indicators of the level of tick abundance in a forest, but are poor describing precise abundance, which depends on other local factors including presence of hosts. Tick abundance variability was associated to two types of factors: (i) the micro-environment of the sampling site providing suitable habitats for ticks and their hosts. (ii) the sampling method and weather at the time of sampling that influence tick capture efficiency and tick activity respectively. *Borrelia* infected ticks were also everywhere, but at relatively low prevalence compared to other studies in Belgian forests. Six different *Borrelia* species were identified, with a typical composition for Western European forests. *Coxiella burnetii* was present but rare, and *F. tularensis*

was not detected. Further understanding of tick abundance variability and its determinants may help identify high-hazard areas and, in heavily visited forests, assess the feasibility of managing vegetation to limit tick abundance in the most visited areas.

6. LIST OF ABBREVIATIONS FOR CHAPTER 2

AIP	adult infection prevalence
DOA	density of adults
DON	density of nymphs
DOT	density of ticks
GLMM	generalized linear mixed models
IF	sites located deep in the forest, in a dense undergrowth
NB	negative binomial
NIP	nymph infection prevalence
OF	sites located out of the forest
RE	random effect
SU	sampling unit
SE	sampling event
TF	sites located in the forest close to the trails in small undergrowth
TIP	tick infection prevalence
ZI	zero-inflated
OF	sites located out of the forest
RE	random effect
SU	sampling unit
SE	sampling event
TF	sites located in the forest close to the trails in small undergrowth.
TIP	tick infection prevalence
ZI	zero-inflated

7. SUPPLEMENTARY MATERIALS

I. Sampling units (SU) from the Bois de Lauzelle in 2016 and 2018.
Credits: Celia Chaiban Raphaël Rousseau and Elliott Wolter.

Figure S.2.1. TF.1 in 2016 (left) and 2018 (right). It was at an entrance of the forest, close to a cub scout's meeting place. The transect was located on loamy soil covered by grass and dead leaves.

Figure S.2.2. IF.1 in 2016 (left) and 2018 (right). IT was deep in the forest, away from the trails on dense undergrowth of brambles, ferns, and dead leaves

Figure S.2.3 TF.2 in 2016 (left) and 2018 (right). It was at the junction of two trails, close to a bench and an information panel. The undergrowth was not high or abundant but easily accessible to visitors.

Figure S2.4 : TF.3 in 2016 (left) and 2018 (right). It was at the edge of a small clearing, composed by middle-height grass. This area is often used for picnics

Figure S2.5. TF.4 in 2016 (left) and 2018 (right). It was in an area where trees had recently been cut. The undergrowth was poor, mainly composed of a thin layer of moss.

Figure S2.6. IF.2 in 2016 (left) and 2018 (right). It was close to a barbecue facility, but away from the trails, with a dense undergrowth composed of brambles ferns, and dead leaves.


Figure S2.7. IF.3 in 2016 (left) and 2018 (right). It was at the edge between coniferous and deciduous tree stands, away from the trails and with a dense undergrowth.

Figure S2.8. TF.5 in 2018. It was at the junction of two trails, close to an information panel. The undergrowth was not high or abundant and easily accessible to visitors.

Figure S2.9 : IF.4 in 2018. It was deep in the forest, away from the trails on dense undergrowth of brambles, ferns, and dead leaves.

Figure S2.10. IF.5 in 2018. It was at the edge between coniferous and deciduous tree stands, away from the trails and with a dense undergrowth.

Figure S2.11. OF.1 in 2018. It was at a busy entrance of the forest, close to the forest edge.

Figure S2.12. OF.2 in 2018. It was at the edge of the forest, close to a path for entering the forest. The undergrowth was composed of grass whose height varied through the year.

Figure S2.13. OF.3 in 2018. It was at the Arboretum of UCLouvain on a grassy area.

Figure S2.14. OF.4 in 2018. It was at the Arboretum of UCLouvain on a grassy area

II. Supplementary statistics and tables

Throughout this section, DON and DOA stand for density of nymphs and adults respectively. TIP, NIP and AIP stand for tick, nymph and adult *Borrelia burgdorferi* infection prevalence respectively. DIN and DIA stand for density of infected nymphs and adults respectively.

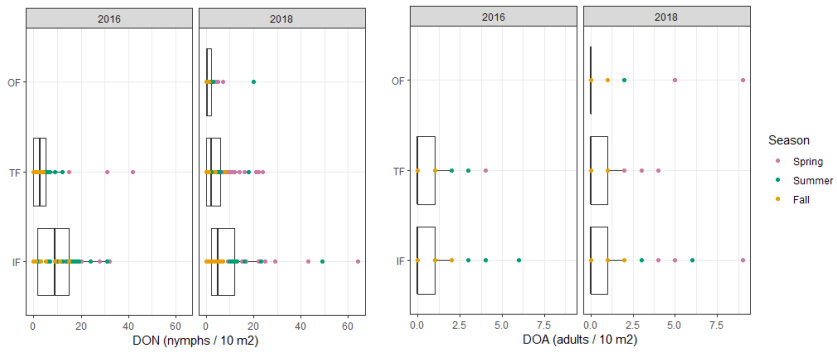


Figure S 2.15. DON (left) and DOA (right) by year, season and type of sampling unit. IF stands for Interior-forest, TF for trail-forest, and OF for non-forest.

Table S2.1. Moran's I index for the tick indicators. p-value < 0.05: '*'.

Variables	2016			2018		
	Observed	SD	p-value	Observed	SD	p-value
DON	-0.40	0.14	0.09	-0.09	0.05	0.71
DOA	0.14	0.15	0.04(*)	-0.02	0.05	0.25
TIP	-0.30	0.15	0.38	-0.09	0.07	0.77
NIP	-	-	-	-0.14	0.07	0.35
AIP	-	-	-	-0.11	0.05	0.51
DIN	-0.24	0.14	0.61	-0.13	0.07	0.46
DIA	-0.24	0.15	0.64	-0.01	0.07	0.34

Table S2.2. Repeated measures correlations between tick indicators for 2016 only. Upper part: coefficient [95% - confidence intervals]. Lower part: significance based on p-values. Degrees of freedom = 111. N.S. stands for not significant. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Significant correlations at p-value < 0.05 are in bold.

	DON	**DOA**	**TIP**	**DIN**	**DIA**
DON	-	**0.39**	**-0.22**	**0.51**	0.14
		[0.23 - 0.54]	**[-0.39 - 0.04]**	**[0.36 - 0.63]**	[-0.05 - 0.31]
DOA	***	-	-0.10	0.26	**0.68**
			[-0.28 - 0.08]	[0.08 - 0.43]	**[0.57 - 0.77]**
TIP	*	N.S.	-	0.16	0.05
				[-0.02 - 0.34]	[-0.13 - 0.24]
DIN	***	N.S.	N.S.	-	0.34
					[0.17 - 0.50]
DIA	N.S.	***	N.S.	N.S.	-

Table S2.3. Repeated measures correlations between tick indicators for 2018 only. Upper part: coefficient [95% - confidence intervals]. Lower part: significance based on p-values. Degrees of freedom = 139. N.S. stands for not significant. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Significant correlations at p-value < 0.05 are in bold.

	DON	**DOA**	**NIP**	**AIP**	**DIN**	**DIA**
DON	-	**0.23**	0.08	-0.03	**0.78**	0.02
		[0.07 - 0.38]	[-0.09 - 0.24]	[-0.20 - 0.13]	**[0.70 - 0.83]**	[-0.15 - 0.18]
DOA	**	-	0.08	-0.07	**0.29**	**0.50**
			[-0.09 - 0.24]	[-0.24 - 0.09]	**[0.13 - 0.43]**	**[0.36 - 0.61]**
NIP	N.S.	N.S.	-	**-0.19**	**0.37**	-0.02
				[-0.35 - -0.03]	**[0.22 - 0.51]**	[-0.18 - 0.15]
AIP	N.S.	N.S.	*	-	-0.12	**0.40**
					[-0.28 - 0.04]	**[0.25 - 0.53]**
DIN	***	***	***	N.S.	-	-0.04
						[-0.20 - 0.13]
DIA	N.S.	***	N.S.	***	0.6674	-

Table S2.4. Vuong test (Z) for the comparison between the negative binomial distribution, and, respectively the Poisson, zero-inflated (ZI) Poisson and ZI negative binomial distributions for DON and the DOA calculated with data from 2016 and 2018. When there was no significant z-test score, we choose the simplest distribution. P-value < 0.001 '***'; P-value < 0.01 '**'; P-value < 0.05 '*'.

Negative binomial distribution preferred over:	**DON**		**DOA**	
	Z	**P**-value	**Z**	**P**-value
Poisson distribution	6.5796	< 0.0001 (***)	2.8261	0.0024 (**)
ZI Poisson distribution	5.2715	< 0.0001 (***)	2.3499	0.0094 (**)
ZI negative binomial distribution	0.4494	0.3266	1.6459	0.0499 (*)

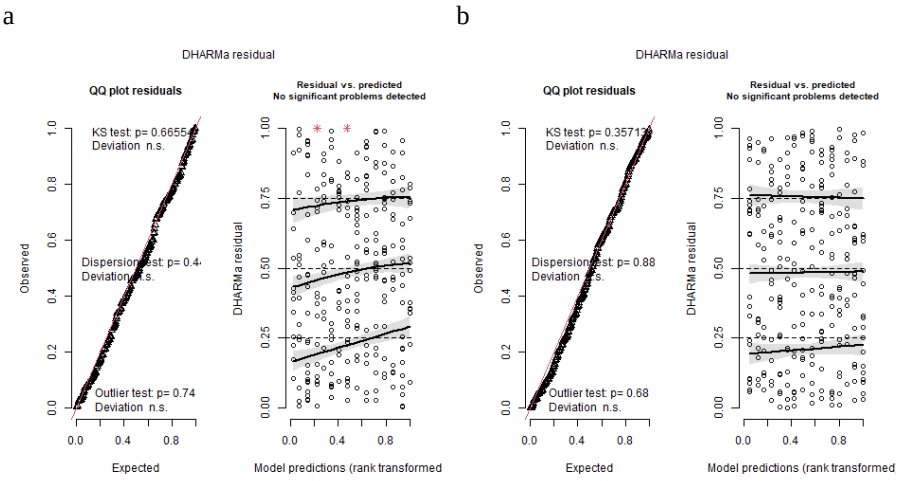


Figure S2.16. DHARMA residuals for the Year-Season-Site models, with interaction between Year and Season of a) DON (AIC = 1383.9), and b) DOA (AIC = 572.6). QQ-plot to detect overall deviations from the expected distribution, KS, dispersion and outlier tests (left panel). Plot of the residuals against the predicted value (right panel).

Table S2.5. Cumulative number (%) of nymphs collected by drag in 2018 based on the type of Sampling Unit (SU).

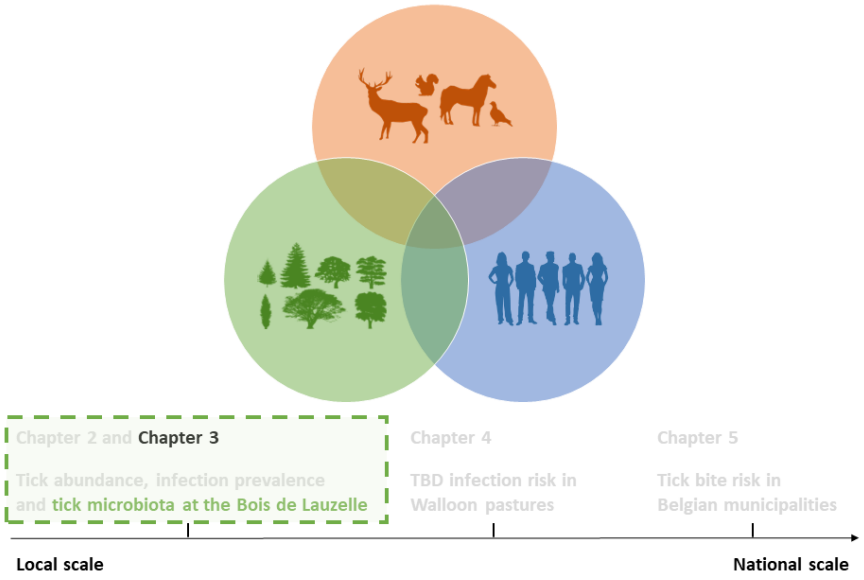
SU type	Ticks - 1 drag (%)	Ticks - 2 drags (%)	Ticks - 3 drags (%)	Ticks - 4 drags (%)	Ticks - 5 drags (%)	Ticks - 6 drags (%)
All	292 (34%)	483 (56%)	604 (71%)	722 (84%)	787 (92%)	856 (100%)
IF	150 (28%)	272 (51%)	358 (68%)	440 (83%)	484 (91%)	530 (100%)
TF	112 (45%)	165 (67%)	193 (78%)	219 (89%)	234 (95%)	247 (100%)
OF	30 (38%)	46 (58%)	53 (67%)	63 (80%)	69 (87%)	79 (100%)

Table S2.6. Cumulative number (%) of adults collected by drag in 2018 based on the type of Sampling Unit (SU).

SU type	Ticks - 1 drag (%)	Ticks - 2 drags (%)	Ticks - 3 drags (%)	Ticks - 4 drags (%)	Ticks - 5 drags (%)	Ticks - 6 drags (%)
All	51 (51%)	70 (70%)	83 (83%)	89 (89%)	97 (97%)	100 (100%)
IF	22 (44%)	33 (66%)	39 (78%)	43 (86%)	48 (96%)	50 (100%)
TF	14 (56%)	17 (71%)	21 (84%)	22 (88%)	24 (96%)	25 (100%)
OF	15 (60%)	20 (68%)	23 (92%)	24 (96%)	25 (100%)	25 (100%)

CHAPTER 3

THE ISOLATION OF CULTURABLE BACTERIA IN *IXODES RICINUS* TICKS OF A BELGIAN PERI-URBAN FOREST UNCOVERS OPPORTUNISTIC BACTERIA POTENTIALLY IMPORTANT FOR PUBLIC HEALTH



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ABSTRACT

Most bacteria found in ticks are not pathogenic to humans but coexist as endosymbionts and may have effects on tick fitness and pathogen transmission. In this study, we cultured and isolated 78 bacteria from 954 Ixodes ricinus ticks collected in 7 sites of a Belgian peri-urban forest. Most isolated species were non-pathogenic environmental microorganisms, and were from the Firmicutes (69.23%), Actinobacteria (17.95%) and Proteobacteria (3.84%) phyla. One bacterium isolate was particularly noteworthy, Cedecea davisae, a rare opportunistic bacterium, naturally resistant to various antibiotics. It has never been isolated from ticks before and this isolated strain was resistant to ampicillin, cefoxitin and colistin. Although cultivable bacteria do not represent the complete tick microbiota, the sites presented variable bacterial compositions and diversities. This study is a first attempt to describe the culturable microbiota of ticks collected in Belgium. Further collections and analyses of ticks of different species, from various areas and using other bacterial identification methods would strengthen these results. However, they highlight the importance of ticks as potential sentinel for opportunistic bacteria of public health importance.

1. INTRODUCTION

Ticks are important vectors of pathogens affecting humans and animals worldwide (Kmet' and Čaplová, 2019; Lejal et al., 2020; Murrell et al., 2003). These pathogens attract great public heath interest, and many studies tried to estimate the influence of human, environmental, and climatic factors on tick abundance and pathogen prevalence (Medlock et al., 2013; Pollet et al., 2020; Vanwambeke et al., 2016b). However, tick bacterial composition is not restricted to pathogenic agents. A bigger and richer community of symbiotic, commensal, and parasitic microorganisms coexists in *Ixodes* ticks, forming a complex microbiota. Pollet et al. (2020) defined tick microbiota as "the assemblage of all microorganisms present in and on ticks" (p. 3). Tick microbiota often consists of endosymbionts, engaged in relation with their tick hosts, influencing their fitness, vector capacity and co-infections with pathogenic agents (Bonnet et al., 2017; Pollet et al., 2020). In Wisconsin (United States), male *Ixodes scapularis* ticks had lower rates of *Borrelia burgdorferi* infection when they were infected by rickettsial endosymbionts (Steiner et al., 2008). Considering the potential importance of microbiota in *Ixodes* ticks and tick-borne pathogens (TBP), as well as the development of new metagenomic approaches, interest has steadily grown in recent years.

Ixodes ricinus is the most widespread tick species in Western Europe and is mainly found in forests, parks, and semi-natural habitats (Medlock et al., 2013). Its presence and abundance are affected by broad-scale characteristics, e.g., temperature, vegetation type or elevation, and fine-scale characteristics, e.g., undergrowth or the presence of specific hosts (Van Overbeek et al., 2008). Its microbiota is also highly variable between micro-climates, regions, and habitats (Aivelo et al., 2019; Bonnet et al., 2017; Carpi et al., 2011; Estrada-Peña et al., 2018; Van Overbeek et al., 2008; Vayssier-Taussat et al., 2013). The relative importance of these factors and their interactions on *Ixodes* microbiota is not well understood but is currently attracting a growing interest (Aivelo et al., 2019).

An increasing number of studies on tick microbiota have been published over the past years (Stewart and Bloom, 2020). Yet, many microorganisms and interactions in tick

microbiota are unknown or unidentified (Bonnet et al., 2017; Rudolf et al., 2009). Bacteria were classically identified with individual strains cultivated in the laboratory. However, they may be unculturable or difficult to culture. Recently, new molecular metagenomic approaches, such as Next Generation Sequencing (NGS), have been developed. This method has been explored for *I. ricinus* by Carpi et al. (2011) and has expanded since (e.g., Barbosa et al., 2017; Vayssier-Taussat et al., 2015, 2013). The very large number of microorganisms identified by NGS suggests that some have an environmental origin and may be unable to survive and develop in ticks (Stewart and Bloom, 2020). The composition of tick microbiota consists of microbes vertically transmitted or acquired from local environments (Bonnet and Pollet 2021).

Tick microbiota remains largely unknown but the presence of bacteria of environmental origin may suggest that the local environment influences the presence, abundance, and diversity of bacterial communities in *I. ricinus*. In this study, *Ixodes* ticks were collected by dragging in seven sites in a Belgian peri-urban forest throughout the season of tick activity. These sites represent several aspects of the forests in terms of tree covers and undergrowth. We developed a method to isolate and identify culturable bacteria from ticks, and we analysed their diversity between different sites and different local environments.

2. MATERIAL AND METHODS

2.1. STUDY AREA

Ticks were collected at seven locations in the Bois de Lauzelle, a periurban forest of 200 hectares located in the municipalities of Wavre and Ottignies-Louvain-la-Neuve in the Walloon Brabant province of Belgium (Figure 3.1). The forest is delimited by another forest and a golf facility in the north, and by high speed roads in the east, south, and west. This private forest is the property and under the management of the neighbouring university (UCLouvain) since 1968. The forest has loamy and sandy-loamy soils and is also a Natura 2000 site since 2002 (code BE31006). It has a mean

altitude of 103 meters (range: 45-153 m) and its hilly topography is influenced by the Blanc-Ry River.

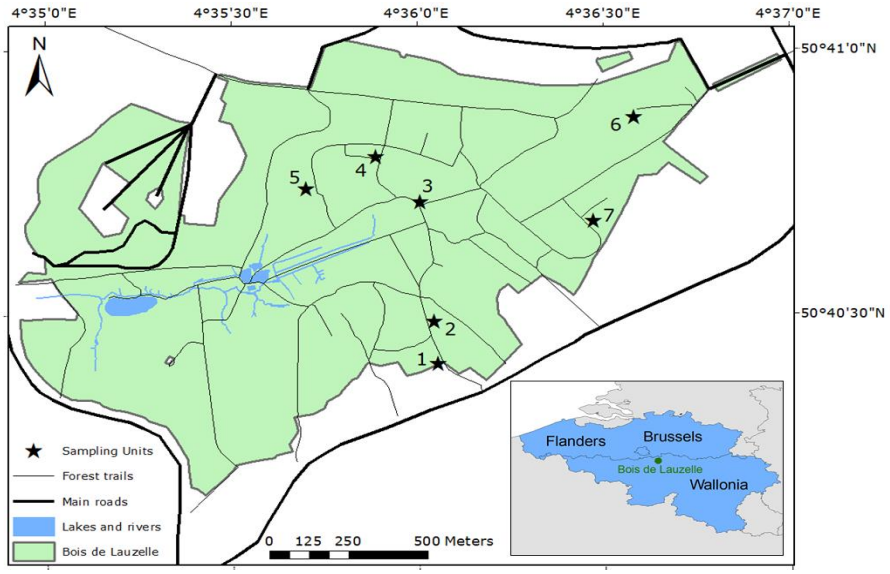


Figure 3.1. Location of the seven sampling sites in the Bois de Lauzelle.

Seven sites were selected for sampling (Figure 3.1). In each site, we sampled one transect of 10 m with a 1 m x 1 m white flannel. The mean distance between two sites is 632 meters (range: 146-1080 m). These sites presented diverse characteristics of the forest (Table 3.1). The western part of the forest was not accessible. Site 1 was at an entrance of the forest, close to a cub scout's meeting place. The transect was located on a loamy soil covered by grass and dead leaves. Site 2 was deeper in the forest, away from the trails on dense undergrowth of brambles and ferns, and dead leaves. Site 3 was at the junction of two trails, close to a bench and an information panel. The underground vegetation was not high or abundant. Site 4 was at the edge of a small clearing, composed by middle-height grass. Site 5 was in an area where trees had recently been cut. The underground vegetation was poor, mainly composed by a layer of moss. Site 6 was close to a barbecue facility, but away from the trails, with a dense undergrowth composed of brambles and ferns, and dead leaves. Site 7 was at the edge

between coniferous and deciduous tree stands, away from the trail and with dense undergrowth.

Table 3.1. Characteristics of the seven sites sampled.

Sites	Latitude (Degrees)	Longitude (Degrees)	Forest Type	Soil Vegetation	Soils
Site 1	4.6021	50.6729	Deciduous	Grass	Loamy
Site 2	4.6018	50.6742	Deciduous	Brambles	Loamy-Sand
Site 3	4.6014	50.6781	Deciduous	Grass	Loamy
Site 4	4.5994	50.6794	Coniferous	Grass	Sandy
Site 5	4.5963	50.6784	Deciduous	Moss	Sandy
Site 6	4.6111	50.6807	Deciduous	Brambles	Sandy
Site 7	4.6089	50.6772	Coniferous	Brambles	Loamy-Sand

2.2. DATA COLLECTION AND PREPARATION

Ticks were sampled in each site every two weeks between March and November 2016, by dragging a 1 m²-white flannel 10 times over an area of 10 m². Ticks were removed and collected after each drag and stored at -80°C until processed for DNA extraction. Larvae were not systematically sampled.

Tick life stage and species were identified using a Leica EZ4 binocular (X35), based on two morphological identification keys (Heylen et al., 2014a; Hillyard, 1996). After identification, ticks were washed in 3 successive baths for two min each, the first one with 70 °C-alcohol to kill bacteria on the surface and the last two with sterilized water. The water was screened to control sterilization. We made culture from the washing water after the ethanol bath resulting in an absence of bacterial isolation. Then, they were individually smashed in Eppendorfs containing 200 µL of Dulbecco's modified Eagle's medium (DMEM) cell culture medium. Vortex mixed homogenate of ticks were grouped in pools of four (50 µL/each, for a total volume of 200 µL) based on the site and season of collection. Larvae were pooled by four, separately from nymphs and adults. A total of 100 µL of each pool were placed in culture. Moreover, 50 µL in a selective medium based on Agar bacteriological Amersco and antibiotic (Vancomycin, Polymyxin, Trimethoprim and Cephalothin-with the scope of isolation

of *Francisella tularensis*), and 50 µL in a non-selective medium. The remaining 100 µL were used for DNA extraction.

2.3. DNA EXTRACTION AND HIGH-RESOLUTION MELTING ANALYSIS FOR *IXODES* SPP. CONFIRMATION

Initially, 5 µL of lysozyme (10 mg/mL) were added to the 100 µL pool medium for DNA extraction. After incubation at 37 °C for 30 min, 200 µL of lysis buffer from the MagMax™ Isolation Kit and 25 µL of proteinase K were included to continue with an incubation at 56 °C for 1 h. The homogenate product was then centrifuged at 11,000 rpm for 3 min and the supernatant processed with the MagMax™ Isolation Kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Next, 1/10th of the eluted DNA was used to confirm the tick genus by analysis of polymorphisms in 5S and ITS2 genes. This assessment was achieved with the high-resolution melting analysis (HRMA) using a SYBR green based real-time PCR run on a Light cycler® 480 Instrument II (Roche Molecular Systems, Inc., Pleasanton, CA, USA). The cycle run consisted of 1x cycle of 10 min at 95 °C followed by 50 cycles of 8 sec at 95 °C, 5 sec at 62 °C and 5 sec at 72 °C. The last step consisted of a melting curve assessment. The results were expressed as melting temperature of the corresponding amplicon. The DNA extracted from the ticks was available for molecular detection of pathogens and will be the subject of further studies. The DNA of isolated bacterial colonies used for whole genome sequencing (WGS) was obtained with the silica-based column method of the DNeasy Blood & Tissue Kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions.

2.4. BACTERIAL IDENTIFICATION

Once the colony was isolated in hard medium, the bacterium was firstly identified with the Bruker MALDI Biotyper IVD MSP Identification Standard Method 1.1. All the colonies present on the plate were collected for identification. Only scores above 2 were considered. Alternatively, ribosomal 16S DNA was amplified by standard PCR and sequenced using universal primers 27F and 1492R (Frank et al., 2008) (list of

primes in Figure S3.1). One strain was analysed by WGS and identified with Kraken (Galaxy Version 2.1.1). A representative diagram of the entire workflow is provided in Figure S3.1.

2.5. ANTIMICROBIAL SUSCEPTIBILITY TESTING

The minimum inhibitory concentration (MIC) was determined for 20 antimicrobials with the broth microdilution method using EUVSEC and EUST plates (SensititreTM, Thermo Fisher Scientific, Waltham, MA, USA). Following an 18–24 h incubation period, plates were read with a SensititreTM VizionTM instrument (Thermo Fisher Scientific, Waltham, MA, USA) using Sensivision software (MCS Diagnostics BV, Swalmen, The Netherlands). MICs were interpreted according to EUCAST breakpoints defined for Enterobacterales (https://www.eucast.org/clinical_breakpoints, accessed on 1 January 2021) or epidemiological cut-off values (ECOFF).

2.6. WGS AND IN SILICO ANALYSIS OF RESISTANCE GENES

WGS was performed on MiSeq platform (Illumina) using protocols defined elsewhere (Tomaiuolo et al., 2021). Briefly, short read sequencing libraries were prepared using a Nextera XT kit (Illumina) and sequenced with a 250-bp paired-end protocol (MiSeq v3 chemistry) according to the manufacturer's instructions. Raw sequencing data of *C. davisae* isolated in this study were submitted to NCBI (https://www.ncbi.nlm.nih.gov/ access on 12 November 2021) and are available under the accession number SAMN22562744. For the analysis of genome data, read quality control and trimming were performed with FastQC (Galaxy Version 0.72) and Trimmomatic (Galaxy Version 0.38.0). Genome reference-based assembly was achieved with Bowtie 2 (default settings in Galaxy Version 3.12.0). using the genome of *C. davisae* DSM 4568 (PRJNA30753) as reference. Derived contigs were analysed in KEGG Automatic Annotation Server (KAAS, https://www.genome.jp/kegg/kaas/, access on 12 November 2021) Ver. 2.1, which provided indication on the antimicrobial resistance determinants. *De novo* assembly coupled to *Resfinder*

(Galaxy Version 0.2) and KAAS analyses did not retrieve results on resistance determinants, and it was not further used.

2.7. BACTERIAL DIVERSITY

Bacterial diversity between the sites was estimated based on alpha and beta diversity indexes (Leung et al., 2018; Whittaker, 1960). All of the analyses were performed with the vegan package (Oksanen et al., 2020) in R 3.6.3 (R Core Team, 2022). Only pools of nymphs and adults were considered for diversity analyses, as larvae were not systematically sampled. Alpha diversity describes the diversity in each site separately and was measured by three indexes. The first index is the abundance, which is the total number of bacteria isolated in ticks by site, regardless of their species. The second, species richness, is the number of different species of bacteria by site, regardless of their abundance. The last, species diversity, incorporates both the number of species and their abundance. It was measured here with the Shannon Diversity Index (H) (Shannon, 1948). H is one of the most commonly used diversity indexes in ecology (Hill, 1973). It characterizes diversity based on the number of species present and the number of organisms per species. The values vary generally between 1 and 4, with low values reflecting low diversity. The number of ticks by site and alpha diversity indexes were tested for spatial autocorrelation using Moran's I.

Beta diversity considers differences in diversity between two sites and is represented by the presence or absence of different species between two or more sites (Koleff et al., 2003). Beta diversity indices were measured here as β_w from (Whittaker, 1960). Since this index may simply increase from an increase in the number of sites sampled, β_w was calculated from pairwise comparison of sites (Equation (3.1).

$$\beta_w = \frac{\frac{(a+b+c)}{(2a+b+c)}}{2} - 1 \qquad \text{(Equation 3.1)}$$

where a is the number of species shared in the two sites, b, the number of unique species in the first site, and c, the number of species in the second site. Based on this index, we calculated the Sorensen index of dissimilarity, which varies from zero,

indicating that the two sites share all their species, to one when the bacterial communities are totally different.

3. RESULTS

3.1. DESCRIPTION OF THE TICK CULTURABLE BACTERIA

954 *Ixodes* ticks were collected, 86 larvae, 787 nymphs, 37 males and 44 females (Table 3.2). All ticks captured were Ixodidae. One tick was identified as *Ixodes ventalloi*, 13 as *Ixodes frontalis*, and the remaining as *Ixodes ricinus*. 8.5% of the ticks were adults. It was relatively constant between the sites, except site 1 with a high percentage of adults (37.9%). Tick abundance was heterogeneous, ranging from sites with relatively low (sites 1, 3, 5 and 6) to high abundances (sites 2, 4 and 7).

Table 3.2. Number of ticks collected by life stage, sex, and site.

Site	Larvae	Nymphs	Females	Males	Ticks
Site 1	0	36	19	3	58
Site 2	23	248	10	12	293
Site 3	10	71	4	1	86
Site 4	38	131	3	6	178
Site 5	12	66	2	2	82
Site 6	0	60	1	5	66
Site 7	3	175	5	8	191
Total	86	787	44	37	954

Ticks were grouped in 242 pools. A total of 21 pools were made up exclusively of larvae. After culture, PCR amplification and sequencing, 76 strains were isolated from 63 pools. The list of bacteria isolated from the 242 pools is provided in the Supplementary Materials, (Table S3.1). Moreover, 26 species were isolated (Figure 3.2). Three bacteria were not identified neither by the MALDI-TOF MS method nor the ribosomal 16S DNA sequencing. Most strains were environmental bacteria, especially with soil or skin-mucosal origin. Except for the three *Proteobacteria*, all identified strains were Gram-positive (68/71). Three culturable bacteria were isolated from pools of larvae: *Bacillus megaterium, Micrococcus luteus* and *Staphylococcus hominis.*

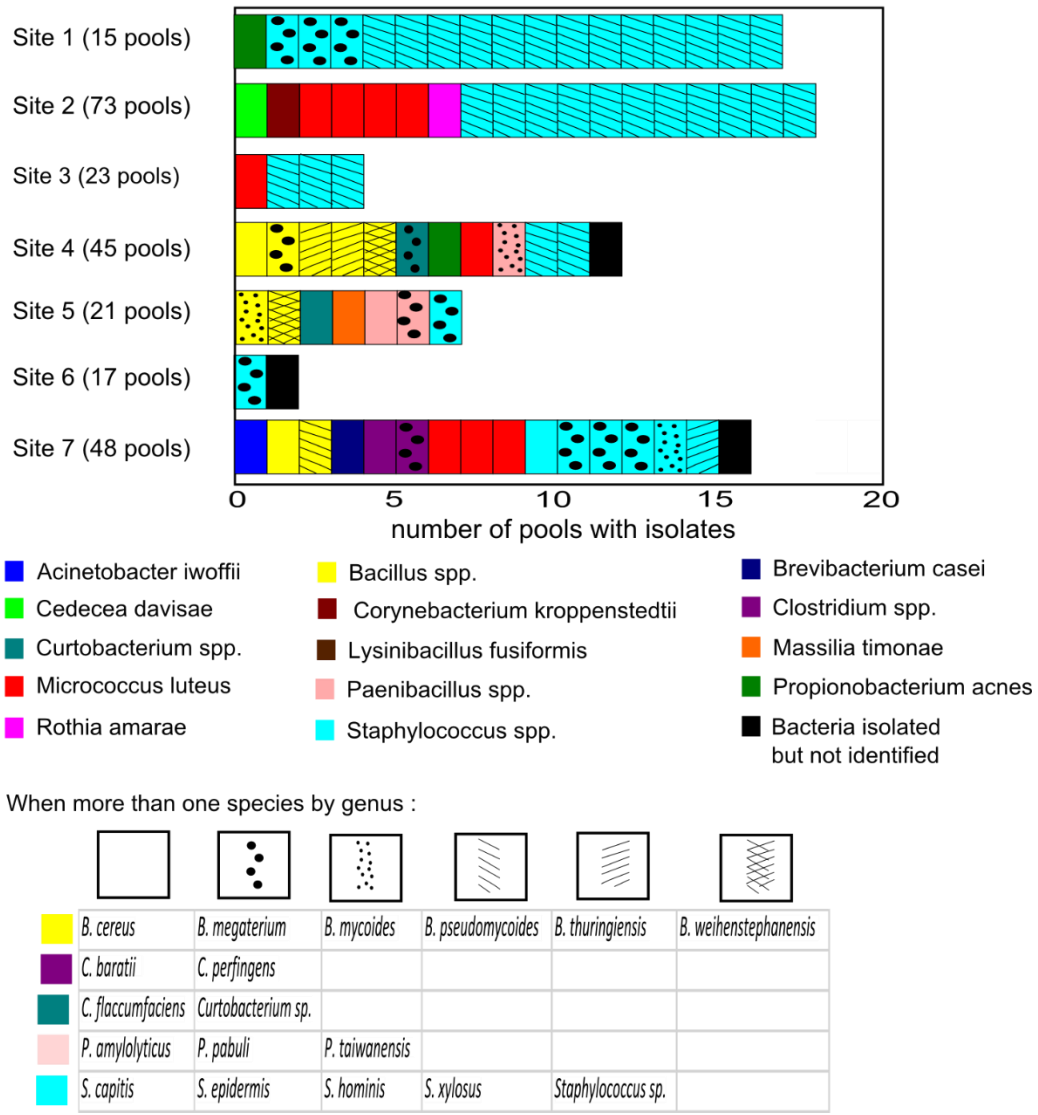


Figure 3.2. Number of pools with culturable bacterial isolates by site and species.

Most isolated culturable bacteria were from the Firmicutes phylum (55 isolates from 16 species), mainly from the *Staphylococcus* (40 isolates) and *Bacillus* (9 isolates) genera. *Staphylococcus xylosus* and *Staphylococcus epidermidis* were isolated 29 and 8 times, respectively (Figure 3.3). *Staphylococcus xylosus* was the dominant strain isolated in the most humid environments (sites 1–3). Other *Staphylococcus* species

were *S. capitis* and *S. hominis*. One *Staphylococcus* was not identified. These bacteria are common bacteria of the skin and mucous membranes of humans and animals (Otto, 2009). Six species of the genus *Bacillus* were isolated: *B. cereus, B. megaterium, B. mycoides, B. pseudomycoides, B. thuringiensis, and B. weihenstephanensis*. The last Firmicutes species isolated were *Clostridium baratii*, *Clostridium perfingens*, *Lysinibacillus fusiformis*, *Paenibacillus amylolyticus, Paenibacillus pabuli* and *Paenibacillus taiwanensis*.

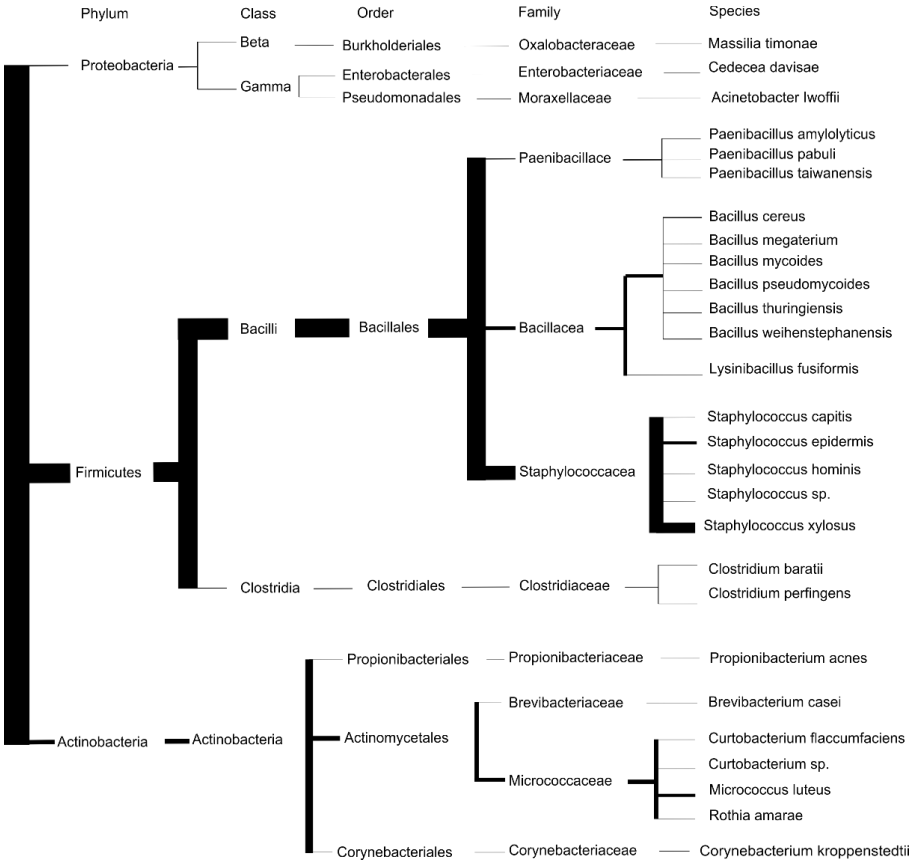


Figure 3.3. Dendrogram of bacterial species adapted from Bryksin and Matsumura (2010) with permission. Branch lengths do not represent evolutionary distance. Branch widths represent the number of bacteria isolated. Only species related to this study are shown.

One fifth of the culturable bacteria were from Actinobacteria phylum (15 isolates from 6 species). Nine *Micrococcus luteus* were isolated, from pools from five sites, making it the second most abundant species. Other Actinobacteria were *Curtobacterium flaccumfaciens, Rothia amarae, Brevibacterium casei, Corynebacterium kroppenstedtii* and *Propionibacterium acnes.* One *Curtobacterium* was isolated but not identified. Three culturable bacteria species were from the Proteobacteria phylum: *Acinetobacter lwoffii, Massilia timonae,* and *Cedecea davisae.*

3.2. DIVERSITY

We did not include the three unidentified bacteria, *Curtobacterium* sp., *Staphylococcus* sp., and the three bacteria isolated from pools of larvae for the diversity analyses. 68 bacteria were considered. Sites 1, 2 and 7 presented the highest abundance of bacteria, with respectively 18, 17 and 13 isolates. Sites 3 and 6 had the lowest, with four and one strains detected. The abundance of bacterial isolates by site was not correlated with the number of nymphs and adult ticks (Spearman's rho = 0.21, p-value = 0.66).

Table 3.3. Species diversity indices of the bacterial communities in ticks by sites. Pools made up of larvae were excluded. H refers to Shannon Diversity Index, and NA to not available.

Sites	Pools Tested	Species Richness	Abundance	H
Site 1	15	3	17	0.68
Site 2	69	5	18	1.11
Site 3	20	2	4	0.56
Site 4	35	6	8	1.73
Site 5	18	7	7	1.95
Site 6	17	1	1	0
Site 7	47	9	13	2.06

The richest sites in bacteria species were sites 7, 5 and 4, with respectively 9, 7 and 6 different species isolated, compared to the less rich sites (6, 2, 3 and 1) (Table 3.3).

Sites 7, 5 and 4 were the most diverse (H = 2.06, 1.95 and 1.73 respectively), and sites 3, 1 and 2, the least (H = 0.56, 0.68 and 1.11 respectively). We could not a compute diversity index for site 6, as only one strain was isolated. *Staphylococcus xylosus* was abundant in sites with low bacterial diversity (sites 1-3), where they constitute 60-76% of the strains isolated. In site 1, *S. xylosus* was isolated in 86.67% of the pools (13/15). Except in site 4, they were absent from sites with high bacterial biodiversity (sites 5 and 7).

There was no spatial autocorrelation between sites for the number of adult and nymphal ticks sampled (Moran's I p-value = 0.11), percentage of adults (Moran's I p-value = 0.85), abundance (Moran's I p-value = 0.23), species richness (Moran's I p-value = 0.46), and Shannon diversity index (Moran's I p-value = 0.64).

Beta diversity indices from the Sorensen matrix of dissimilarity between the seven sites were high (mean = 0.81, standard deviation = 0.16) indicating that the sites had relatively different communities (Table 3.4). Sites 2 and 3 were the most similar (index of dissimilarity = 0.43), sharing the two most abundant species identified, *S. xylosus* and *M. micrococcus*. Site 6 was the less similar site, especially due to the presence of a single identified isolate: *S. epidermidis*.

Table 3.4. Sorensen matrix of dissimilarity.

	Site 1	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Site 6**	**Site 7**
Site 1	-						
Site 2	0.75	-					
Site 3	0.60	0.43	-				
Site 4	0.78	0.82	0.75	-			
Site 5	0.80	1	1	0.85	-		
Site 6	0.50	1	1	1	0.75	-	
Site 7	0.83	0.86	0.82	0.87	0.88	0.80	-

3.3. ANTIBIOTIC RESISTANCE PATTERN AND GENOMIC CHARACTERISTICS OF C. DAVISAE

One bacterium was isolated both in selective and non-selective media, evidencing an intrinsic resistance to various antibiotics, *C. davisae*. Its bacterial identification was confirmed by MALDI-TOF MS, 16S rRNA sequencing and WGS. The phenotypic resistance profile of this *C. davisae* strain was characterized through MIC determination for 20 antimicrobials (Table 3.5). Phenotypic resistance was observed with cefoxitin (MIC of > 16 μg/ml), ampicillin (MIC of > 64 μg/ml) and colistin (MIC of > 16 μg/ml). To gain insight into the molecular features underlying the antimicrobial resistance pattern, WGS data were used to identify orthologs of resistance pathways in KAAS. Within the antimicrobial resistance genes categories, four gene sets were identified: (i) β-Lactam resistance, (ii) vancomycin resistance, (iii) cationic antimicrobial peptide (CAMP) resistance, including the LPS modification system associated with colistin resistance, and (iv) a miscellanea of genes implicated in multidrug resistance phenotype (complete list given in Table S3.2). In this strain, ampicillin resistance is mediated by genes of the mec family, the bla system and the ParR/ParS, CusR/CusS two-component systems. Colistin resistance is associated with lipopolysaccharide (LPS) modification via cationic substitution as the PhoQ/PhoP two-component system is involved. No mcr genes (1 to 10) were found excluding the possibility of acquisition of colistin resistance through horizontal gene transfer.

Table 3.5. MIC (µg/mL) values for the tick-derived *C. davisae* isolate as defined with the microdilution method. Interpretation is based on clinical breakpoints defined by EUCAST (http://www.eucast.org/clinical_breakpoints, access on 1 January 2021) or ECOFF (indicated by asterisks). Int. stands for interpretation, R. for resistant and S. for sensitive.
* when no clinical breakpoints available, interpretation was based on epidemiological cutoffs (ECOFF) values.

Antibiotic Class	Antibiotic Abbreviation	Antibiotic	*Cedecea davisae* (tick)		*Escherichia coli* ATCC 25922		*Enterobacterales* (EUCAST Clinical Breakpoints 1/01 2021) and ECOFF (*)	
			MIC (µg/mL)	Int.	MIC (µg/mL)	Int.	S≤	R>
Aminoglycosides	GEN	Gentamicin	<=0.5	S	<=0.5	S	2	2
	STR	Streptomycin	<=4	S *				>16 *
Carbapenem	MERO	Meropenem	0.12	S	<=0.03	S	2	8
Cephalosporins	FOT	Cefotaxime	<=0.25	S	<=0.25	S	1	2
	FOX	Cefoxitin	>16	R *			8 *	8 *
	TAZ	Ceftazidime	<=0.5	S	<=0.5	S	1	4
Diterpenes	TIA	Tiamulin	>4					
Fluoroquinolones	CIP	Ciprofloxacin	<=0.015	S	<=0.015	S	0.25	0.5
	NAL	Nalidixic Acid	<=4	S *	<=4	S *		>8
Macrolides, lincosamides and streptogramins	AZI	Azithromycin	16		4			
Penicillins	AMP	Ampicillin	>64	R	4	S	8	8
Tetracyclines	TET	Tetracycline	<=2	S *	<=2	S *		>8 *
	TGC	Tigecycline	<=0.25	S *	<=0.25	S *	1	>0.5
Miscellaneous agent	CHL	Chloramphenicol	<=8	S	<=8	S	8	8
	COL	Colistin	>16	R	<=1	S	2	2
	KAN	Kanamycin	<=4					
	MUP	Mupirocin	256					
	RIF	Rifampicin	>0.5					
	SMX	Sulfamethoxazole	>1024		64			
	TMP	Trimethoprim	<=0.25	S	0.5	S	4	4

4. DISCUSSION

Studying elements of tick microbiota is important because the bacterial flora may specifically influence tick fitness, reproduction, and competence as vectors. These bacteria may also facilitate or compete with tick-borne pathogens (Bonnet and Pollet, 2021). Bacterial phyla distribution and relative abundances found in this study were consistent with previous publications on culturable bacteria. *We found the same proportions of the 3 phyla, Firmicutes*, *Actinobacteria*, and *Proteobacteria, in the* 113 bacteria isolated from the gut of the haematophagous *Glossina pallidipes* (Malele et al., 2018). Six studies analysed culturable bacteria in Ixodidae ticks, one in *Ixodes scapularis* in the Unites States (Martin and Schmidtmann, 1998), one in *Ixodes holocyclus* in Australia (Murrell et al., 2003), and four in *Ixodes ricinus*, mostly in central Europe (Egyed and Makrai, 2014; Okła et al., 2012; Rudolf et al., 2009; Stojek and Dutkiewicz, 2004). Most bacterial genera isolated here had already been identified in ticks elsewhere, such as *Staphylococcus*, *Micrococcus*, *Bacillus*, *Paenibacillus*, *Acinetobacter*, *Propionibacterium*. A deeper comparison at the species level was not possible because of the absence of standard method to analyse culturable bacteria, as different culture media target different bacterial communities.

There is, to our knowledge, no scientific information about the composition of Ixodidae microbiota in Belgium, where ticks were mostly screened for known pathogenic agents (e.g., Lernout et al., 2019). This study is a first attempt to describe culturable bacterial composition in ticks from Belgium. We discovered a variable diversity of cultivable bacteria genus from ticks collected from the same forest stand. No bacteria species were found at all sites, and the most abundant, *S. xylosus*, was found in four sites (sites 1–3 and 7). *Staphylococcus xylosus* is a gram-positive bacterium that is generally not pathogenic, although a few strains are related to human infections (Dordet-Frisoni et al., 2007; Kloos et al., 1976). Another member of the *Staphylococcus* genus found in this study, *S. epidermidis* is an opportunistic pathogen widely spread in the environment and in human skins and mucosal surfaces where it can cause nosocomial infections (Namvar et al., 2014; Otto, 2009).

Forest floor structure is important for tick survival and may affect tick microbial composition (Van Overbeek et al., 2008). For example, soil bacterial communities are very diverse, and even if carbon mineralization rate can explain abundances of specific phyla, the ecological mechanisms explaining this diversity are not fully understood yet (Guizzo et al., 2020). The seven sites presented contrasted bacterial diversities in ticks, as indicated by the Sorensen matrix of dissimilarity (Table 3.5). The more similar sites with the more similar bacterial communities (Site 1 versus Site 3, Site 2 versus Site 3, and Site 1 versus Site 6) were not neighbours. No spatial autocorrelation was found for the microbial diversity. However, the presence of many isolates of *S. xylosus* in sites 1, 2 and 3, relatively close to each other, potentially suggest a localized environmental source. Sites 4, 5 and 7 had more abundant and diverse bacterial communities, but no relation with vegetation cover or the type of soils was highlighted, probably due to the small number of sites sampled. Although the number of bacterial strains isolated by site were not correlated with the number of ticks sampled, our indices of biodiversity must be moderated by the number of pools of ticks screened by site.

One of the Proteobacteria isolated from site 2, *Cedecea davisae* was particularly noteworthy because a common clinical presentation of infection is bacteraemia and it is inherently resistant to antibiotics (Abate et al., 2011; Thompson and Sharkady, 2020). It is a rare opportunistic bacterium, one of the five species in the genus *Cedecea*, with *C. neteri* and *C. lapagei* (Dalamaga et al., 2008). Its isolation in ticks needs to be further investigated to see if it can be transmitted through tick bite. Recent molecular techniques revealed an unexpected bacterial diversity in ticks and indicated that the majority of these bacteria are difficult to culture, or even unculturable (Bonnet and Pollet, 2021; Martiny, 2019). The culturable bacteria found in *Ixodes* ticks were typically from four bacterial phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Tanaka et al., 2017). Different techniques applied, such as culturing, cloning or species-specific PCR assays, may reveal different taxonomical groups in ticks (Barbosa et al., 2017; Van Overbeek et al., 2008). For example, using metagenomic approaches, the dominant bacteria phyla were Proteobacteria,

Actinobacteria and Firmicutes (Carpi et al., 2011). Proteobacteria was the dominant phyla detected in ticks by sequencing with an Illumina MiSeq machine, with 4 other phyla, Actinobacteria, Bacteroidetes, Firmicutes, and Spirochaetes represented by less than 5% of the total microbiota (van Treuren et al., 2015). The notion of culturable bacteria is also changing through time, as the culturing techniques evolve (Molina-Menor et al., 2021). Techniques subject to high-throughput sequencing are useful but may overestimate the number of members of the tick microbiota by including many contaminating DNA from environmental sources (Lejal et al., 2020; Stewart and Bloom, 2020; Van Overbeek et al., 2008). Cultures are therefore a useful complementary method for illumina techniques to describe the microbiota composition of ticks.

Most studies focus on bacteria detection from entire ticks (Pollet et al., 2020). However, the main interest is on the microbiota present in the tick gut because it represents the entry point for tick-borne pathogens (Bonnet et al., 2017). Guizzo et al. (2020) found that the microbiota of the tick midgut was less abundant and more diverse than the rich but poorly diverse ovarian microbiota, dominated by *Midichloria* sp. Carpi et al. (2011) also observed variation in the occurrence of bacteria among individual ticks. The present study identified a limited and variable number of bacteria in ticks, that were closely related to those found previously in the tick midgut (Guizzo et al., 2020). Their transmission through tick bites requires further functional studies. Bacteria detected in cultures from ticks may not necessarily be part of the tick microbiota; they might indicate temporal contamination from an environmental source. Some bacteria we identified are also found in plants, soils, or the skin of other animals and could be part of the tick exoskeleton. The detection of culturable bacteria in three larvae pools may reflect contamination. The presence of contamination cannot therefore be excluded but is limited in this study for the following reasons. Tick skin bacteria were probably removed, due to the storage at −80 °C between the samplings and the analyses. A strict surface washing with ethanol and sterilized water was also performed on each tick before DNA extraction. For further studies, Binetruy et al. (2019) recently indicated that bleach should be used over ethanol for tick washing, as

the latter method may impact internal bacterial diversity in metagenomics sequencing-based studies, as DNA can easily stick to tick cuticle. Kmet' and Čaplová (2019) identified the same species of *Staphylococcus*, *Bacillus*, *Micrococcus* and *Brevibacillus* in ethanol sterilized ticks. The bacteria identified here were with distinct homogeneous prints, which is normally excluded in bacteria isolated from outside the ticks.

The spatial and temporal scales are important in studies of tick microbial communities (Bonnet and Pollet, 2021; Pollet et al., 2020)]. Interactions between microorganisms and the environment depend on the scale at which we consider these interactions. The density of specific animals, with variable reservoir competences, is also important to understand variations in tick microbiota at the local scale (Randolph, 2001). Differences in microbial communities between large areas, climatically and environmentally varied is not surprising. However, in this study, ticks were sampled from seven sites, distant of 632 m on average, in the same forest stand. Wildlife movements are not limited in this forest, which presents contrasted micro-climates in relation to different flora composition and topography. The sites sampled had contrasted culturable bacterial communities.

5. CONCLUSION

It is now clear that TBP are not the only microorganisms present in *Ixodes* ticks, which harbour larger, and still poorly known microbiota communities. They are particularly important in the TBP complex system as they may influence tick fitness and behaviour and interact with other pathogenic and non-pathogenic agents. Recent identifications of tick microorganisms based on several methods and technologies, such as NGS, identified an increasing number of bacteria. It remains questionable if these bacteria are part of tick microbiota or environmental contaminant, unable to physiologically survive and develop in ticks. Using cultures of ticks from the same forest stand, we found complex and variable bacterial communities.

The culturable bacteria found in this study were variable across the seven sites, but consistent with those found in the literature for *Ixodes* ticks. Bacteria from the Firmicutes phylum were the most abundant, followed by the Actinobacteria and the Proteobacteria phyla. Most strains were only isolated once or two times, except *S. xylosus*, *S. epidermidis* and *M. luteus*. The isolation of a strain of *C. davisae* naturally resistant to antibiotics was surprising as it has never been isolated from ticks before, and the transmission of this bacteria through tick bite needs confirmation from other studies. It may suggest a potential tick-mediated transmission of this opportunistic pathogen. The identified culturable bacteria here did not represent the complete tick microbiota, and some isolates might originate from environmental sources, even if this risk was limited with prior washings. It was the first attempt to describe a part of the tick microbiota in Belgium. The bacteria identified were consistent with the literature, but the results need to be confirmed with ticks from other areas, other species, other cultures, and other bacterial identification methods. This information is crucial to help understanding tick-bacteria interactions and detecting the presence of new bacteria in tick microbiota.

6. SUPPLEMENTARY MATERIALS

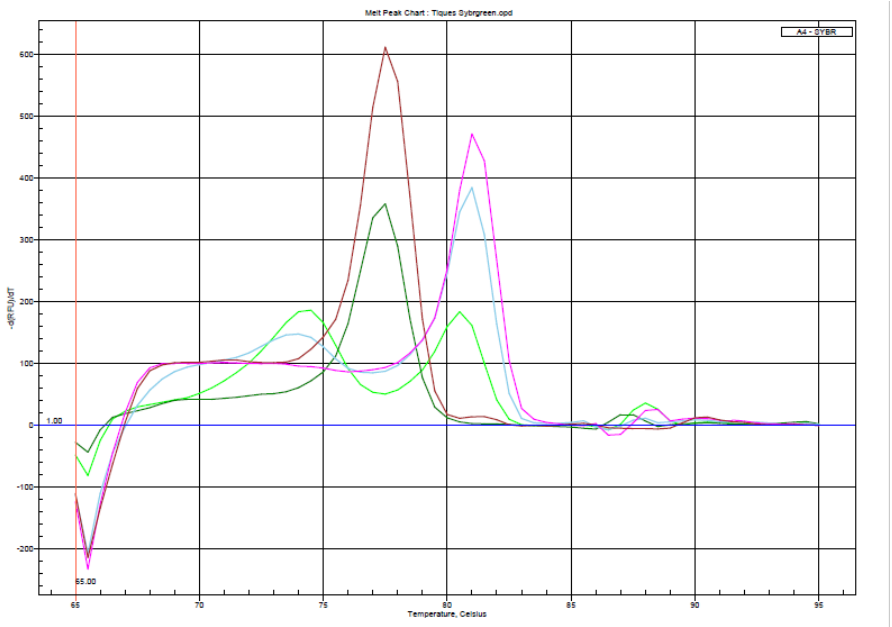


Figure S3.1. Melting curves derived from analysis of the 5S and ITS2 sequence polymorphisms in *Ixodes* and *Dermacentor* spp.by HRMA. Derivative fluorescent emission data recorded during the melting step. Two main profiles were observed with different Tms, the *Ixodes* spp. group (dark green and brown lines) and the *Dermatocentor* spp. group (green, light blue and fuchsia lines). Primers designed for the analysis: primers on 5S for *Ixodes ricinus:* FW: gtcgtagccttccgtcagtc, RV: acggcattcccctactggat; primers on ITS2 for *Dermacentor* spp: FW: cggacacctgcagggaaag, RV: cttccgactctctcgcaaac.

Table S3.1. Pools tested for the isolation and identification of cultivable bacteria by site, season, and tick development stage. Excel file available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8625411/bin/ijerph-18-12134-s001.zip.

Table S3.2. List of resistance genes and of their orthologs present in the tick-derived *C. davisae* genome as defined with the KAAS analysis.

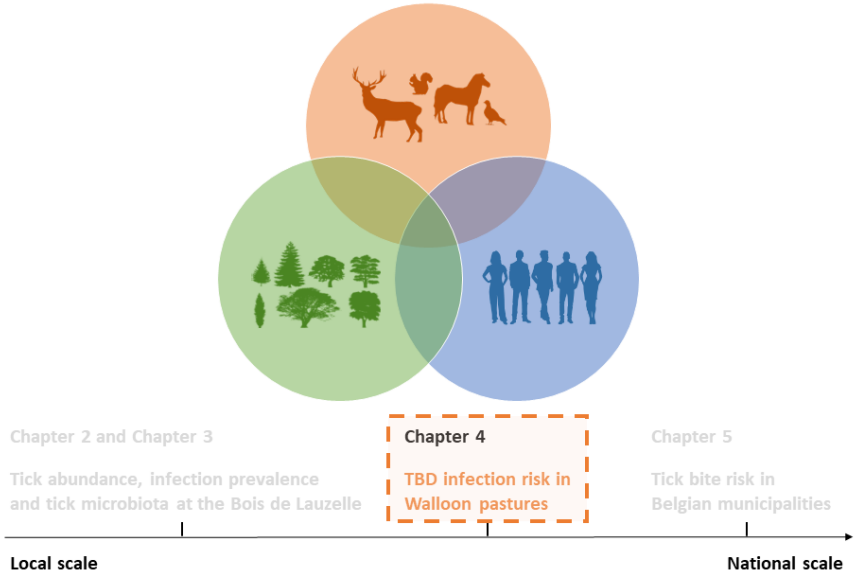
Resistance	**Resistance genes encoded in *C. davisae* genome**	**Orthologs**
β-Lactam		
Methicillin	mecR1; methicillin resistance protein	K02547
	mecI; methicillin resistance regulatory protein	K02546
	mecA; penicillin-binding protein 2 prime	K02545
Bla system	blaR1; bla regulator protein blaR1	K02172
	blaI; BlaI family transcriptional regulator, penicillinase repressor	K02171 K18766
	blaZ; beta-lactamase class A BlaZ	K17836
	penP; beta-lactamase class A	
Imipenem		K18073/ K18072
	ParR/ParS two-component system	K07665/ K07644
	CusR/CusS two-component system	K18297
	mexT; LysR family transcriptional regulator	K18093
	oprD; imipenem/basic amino acid-specific outer membrane pore	
Vancomycin		
D-Ala-D-Ser type	vanRE/vanSE; two-component system, OmpR family	K18349/K18350
	vanT; serine/alanine racemase	K18348
	vanC; D-alanine---D-serine ligase	K18856
	vanXY; D-alanyl-D-alanine dipeptidase/carboxypeptidase	K18866 K18344/K18345
D-Ala-D-Lac type	vanRB/vanSB; two-component system, OmpR family	K07260 K18346
	vanY; D-alanyl-D-alanine carboxypeptidase	K18352/K18351
	vanW; vancomycin resistance protein	K18354
	vanRAc/vanSAc; two-component system, OmpR family	K18353 K18347
	vanK; vancomycin resistance protein VanK	K15739 K08641
	vanJ; vancomycin resistance protein VanJ	K18906
	vanH; D-specific alpha-keto acid dehydrogenase	K08168
Other	vanB; D-alanine---(R)-lactate ligase	
	vanX; D-alanyl-D-alanine dipeptidase	
	mgrA; MarR family transcriptional regulator, multiple gene regulator MgrA	
	tetB; MFS transporter, DHA2 family, metal-tetracycline-proton antiporter	

Table S3.2 (continued). List of resistance genes and of their orthologs present in the tick-derived *C. davisae* genome as defined with the KAAS analysis.

Resistance	**Resistance genes encoded in *C. davisae* genome**	**Orthologs**
CAMP		
dltABCD operon	graR:grasS; two-component system, OmpR family	K19078/K19077
	dltA; D-alanine--poly(phosphoribitol) ligase subunit 1	K03367
	dltB; membrane protein involved in D-alanine export	K03739 K14188 K03740
	dltC; D-alanine--poly(phosphoribitol) ligase subunit 2	K14205 K19079/ K19080
VraFG transporter	dltD; D-alanine transfer protein	K07660/K07637
protease	mprF, fmtC; phosphatidylglycerol lysyltransferase	
PgtE	vraF/vraG; cationic antimicrobial peptide transport system ATP-binding protein	
	PhoP/PhoQ two-component system, OmpR family	
Miscellanea		
Efflux pumps	MexAB-OprM, MexCD-OprJ,,MexEF-OprN, MexJK-OprM, MexXY-OprM, MexPQ-OpmE, AdeABC, AcrEF-TolC, MdtEF-TolC, BpeEF-OprC, AbcA, NorB, QacA, MepA, repression of porin OmpF	K18131/ K03585/ K18138/ K18139/K18294/ K18295/ K18296/ K08721/ K18297/ K18298/ K18299/ K18300/ K18301

CHAPTER 4

ENVIRONMENTAL DETERMINANTS OF *ANAPLASMA PHAGOCYTOPHILUM* INFECTION IN CATTLE USING A KERNEL DENSITY FUNCTION



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ABSTRACT

The study of vector-borne zoonotic diseases often relies on partial data, because of the constraints associated with observing various elements of the transmission cycle: the pathogen, the vector, the host – wild or domestic. Each angle comes with its own practical challenges, leading to data reflecting poorly either on spatial or temporal dynamics, or both. In this study, we investigated the effect of landscape on the presence of bovine ehrlichiosis infection in Walloon cattle. This disease is transmitted to cattle through the bite of a tick infected by the bacterium Anaplasma phagocytophilum. The first case of bovine ehrlichiosis in the southern region of Belgium (Wallonia) was detected in 2005 and the high seroprevalence found in herds suggests that the disease is endemic.

The presence of antibodies of A. phagocytophilum in one cow selected in each of 1445 herds in 2010 and 2011 was detected using indirect immunofluorescence. Samples were geolocated at the farm. However, the precise location of infection remains uncertain. To account for the data sparsity, we elaborated a spatial index for the intensity of the presence of seropositive animals, based on a non-parametric kernel density estimation. We examined this index with the landscape surrounding the pastures, using multiple regressions. Landscape factors were selected using a conceptual framework based on the ecological resources needed for the transmission cycle of A. phagocytophilum.

Results suggest that our spatial index adequately reflected infection presence in cattle in Wallonia, which was highest in central regions, corresponding to more forested and fragmented landscapes. We noticed that the presence of large hosts, wild or domestic, as well as the composition and configuration of the landscape of the pasture, influenced the capacity of the pasture to support the presence of bovine ehrlichiosis in Walloon herds. This is consistent with the ecology of A. phagocytophilum and current knowledge about risk factors of tick-borne diseases in

cattle at the regional scale. The nature of the kernel density index, based on uncertainties over the location of cases positive to A. phagocytophilum, reflected the infectiousness profile at the landscape and not at the pasture level. Results also highlighted that the effects of some environmental variables remain, even when considering the different agro-geographic regions of Wallonia, which present contrasted landscapes and different levels of intensity of A. phagocytophilum infection. The kernel density index is a useful tool to help veterinary practitioner to quickly target areas where A. phagocytophilum infection is likely.

1. INTRODUCTION

Ticks constitute a challenge for human and animal health, being the second arthropod group transmitting disease worldwide after mosquitoes (Dantas-Torres et al., 2012; Halperin, 2011; Pfäffle et al., 2013; Piesman and Gern, 2008). *Anaplasma phagocytophilum* is an obligate intracellular bacterium transmitted by Ixodidae ticks and causing human granulocytic anaplasmosis in humans and bovine ehrlichiosis (BE), also called tick-borne fever, in bovines (Lillini et al., 2006; Stuen et al., 2013; Woldehiwet, 2006). BE is one of the most widespread tick-borne diseases in animals worldwide, with babesiosis (Aguiar, 2017; Atif, 2015; Bock et al., 2006; Stuen et al., 2013). Wild ruminants often present high percentages of seropositivity of antibodies against *A. phagocytophilum*, with 46.2% and 46.3% for red deer and fallow deer respectively (Ebani et al., 2008). Among domestic animals, cattle present the highest percentage (16.8%), compared to sheep (12.8%), dogs (8.8%) and goats (4.2%). This disease has major health and economic impacts on livestock productivity. Its main clinical symptoms include anorexia, pyrexia, immunosuppression, cough, decreased milk production, and abortions, but asymptomatic infections are possible (Atif, 2015; Stuen, 2007; Woldehiwet, 2006).

Detecting and understanding periods and places with high livestock exposure to infected tick are therefore important (Gilbert et al., 2017). Tick-borne diseases constitute a complex system, involving several agents, i.e., a pathogen, a vector, and wild or domestic hosts. Each species has its own habitat requirements, with resources needed to fulfil its ecological functions (Lambin et al., 2010; Medlock et al., 2013; Vanwambeke et al., 2016), which can be mapped to identify areas at higher risk of infection.

An extensive view of the intensity of tick-borne infections is difficult to reach because disease cases constitute only the emerged part of a zoonotic iceberg (Randolph and Sumilo, 2007). Data used to study tick-borne disease systems range from tick collections by flagging or counting off hosts (Boyard et al., 2011; Claerebout et al., 2013; Richter and Matuschka, 2011) to tick-bite counts (De Keukeleire et al., 2015),

serological tests (Chmielewska-Badora et al., 2012) or disease cases (Zeimes et al., 2015). However, data are often limited in time and space because of the cost of collection and analyses, limits in surveillance systems, or confidentiality. Moreover, many tick-borne diseases are emergent, and their dynamic nature requires other less costly and more adaptive methods, such as the creation of infection intensity indices. The first case of BE in Wallonia, the southern region of Belgium, was detected in 2005 (Guyot et al., 2011). The high BE seroprevalence found in local herds and the widespread distribution of its main vector in Europe, *Ixodes ricinus*, suggest that the disease may be endemic (Lempereur et al., 2012a; Obsomer et al., 2013). This recent detection, and therefore the lack of experiences with this disease in addition to multiple nonspecific flu-like symptoms of BE may cause underdiagnoses among veterinary practitioners. There is therefore a need to quickly identify areas where the infection may be important.

In this study, we used serological data to develop a spatially continuous index of seropositivity based on a kernel density function as an indication of *A. phagocytophilum* infection in cattle. Our first objective was to identify areas with high risks of this infection, using a sparse dataset. Our second objective was to investigate the effects of the landscape on this pattern.

2. MATERIAL AND METHODS

2.1. STUDY AREA

The southern region of Belgium (Wallonia) has a territory of 16,901 km² mainly composed of forests (28.5%), arable lands (25.6%), and grasslands (27.7%), heterogeneously spread in the territory. Nine agro-geographic regions are identified in Wallonia, reflecting specific biophysical conditions and historical agricultural practices (Figure 4.1). The population is mostly concentrated in the northern areas, following the industrial axis, between Mons and Liège (*Sillon Industriel*). Northern regions (*Hesbaye*, *Plateau limoneux brabançon*, *Plateau limoneux hennuyer*) are homogenous landscapes composed of large cultivated fields. Central and southern

regions (*Ardenne, Condroz, Fagne-Famenne, Lorraine*) have fragmented landscapes, with a higher proportion of forests (Table 4.1).

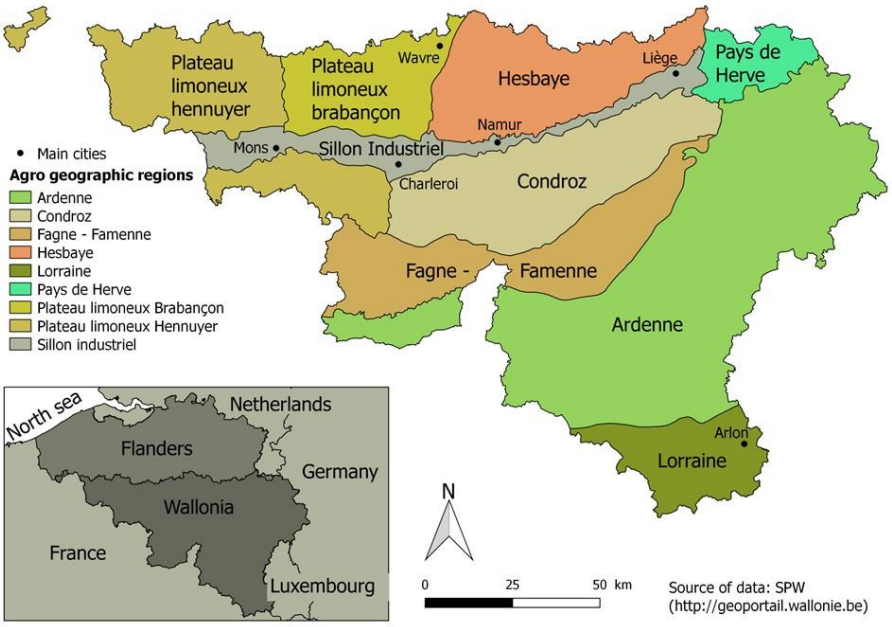

Figure 4.1. Agro-geographic regions of Wallonia.

Table 4.1: Landscape composition of the agro-geographic regions of Wallonia. Statistics were calculated based on the land cover map of Wallonia (2007), from Walloon Public Services.

Agro-geographic regions	Area (km²)	Artificial land (%)	Agricultural land (%)	Grasslands (%)	Forests semi-natural habitats (%)	Wetlands (%)	Water (%)	Unclassified (%)
Ardenne (ARD)	5577	7.55	5.82	31.18	51.14	2.73	0.73	0.84
Condroz (CON)	2178	14.04	30.80	28.69	24.36	1.28	0.82	0.01
Fagne-Famenne (FF)	1820	10.56	13.65	37.49	34.27	2.87	1.15	0.01
Hesbaye (HES)	1722	17.82	62.99	13.57	4.37	0.63	0.61	0.02
Lorraine (LOR)	928	11.96	12.39	35.75	36.38	2.50	0.76	0.26
Pays de Herve (PDH)	502	21.33	11.39	58.35	7.07	1.00	0.86	0.00
Plateau limoneux brabançon (PLB)	1148	23.37	47.93	18.12	8.52	0.86	1.12	0.08
Plateau limoneux hennuyer (PLH)	2084	16.73	51.95	21.05	8.40	0.81	1.05	0.02
Sillon Industriel (SIN)	942	47.07	20.71	14.43	8.95	5.35	3.18	0.30
Wallonia	**16901**	**14.81**	**25.61**	**27.73**	**28.47**	**2.06**	**0.98**	**0.32**

2.2. DEPENDENT VARIABLE

The Regional Association for Animal Registration and Health *(*ARSIA) is a non-profit organization in charge of animal traceability and animal health in Wallonia (Arsia, 2020). They receive blood samples collected from bovines in dry tubes by veterinarian practitioners for pathology screenings. Before analyses, 1 ml-serum samples were stored at -20°C. The sera were tested for several pathologies, including the presence of *A. phagocytophilum* Immunoglobulin G (IgG) antibodies. These antibodies were detected using an indirect immunofluorescence antibody (IFA) detection kit (VMRD, Pullman, USA) at a 1:40 dilution. A titer higher than 40 was considered positive. 1445

samples received between July and October 2010 and between June to September in 2011 were randomly selected from this database, covering the entire Walloon territory. When there were sera from multiple animals in the same herd, only one was selected. Herds were spatially assigned to the pastures used, as declared by the owners. 15.4% (222/1445) of the animals were tested positive. On average, the herds tested were associated to 15.4 (range 1-96) pastures. The mean distance between a pasture and the centroid of the pastures associated to the same herd was 3 km (SD = 4 km, range = 0-120 km). Details on effective use of the pasture was unavailable because herd owners generally report using multiple pastures. Furthermore, infection cannot be dated precisely based on serology. They were thus linked to the centroid of each pasture associated with their herd. Prevalence was estimated between 30.8% and 77% in eight Walloon farms in 2010 (Lempereur et al., 2012). An absence of detection in the serological tests thus cannot be interpreted strictly as an absence in the herd.

Considering uncertainties on geolocation and interpretation of negative results, data on bovines seropositive for *A. phagocytophilum* were pre-processed using a kernel density estimation (KDE) to compute an index of Intensity of Bovine Ehrlichiosis Infection (IBEI). A KDE is a non-parametric method that estimates the continuous distribution of a phenomenon based on places where its intensity is known or has been measured (Silverman, 1986). The kernel is a symmetrical bounded surface centered over each known or recorded event and reflects the estimated intensity of the event in its neighbourhood (Fotheringham et al., 2000). The intensity is not uniform under the kernel surface. It is highest at the location of the recorded event, and decreases following a distance decay function.

A kernel was built over each of the 4150 pastures associated to the 222 seropositive bovines (named hereafter “seropositive pastures”). As the shape of the kernel function is not critical (Kelsall et al., 1995; Loo et al., 2011), we chose the widely used isotropic Gaussian function (Baddeley et al., 2015; Guidoum, 2015). There exist numerous methods to choose the appropriate bandwidth value (Silverman, 1986). Based on the statistical properties of the data, the likelihood cross-validation bandwidth, which minimizes the Kullback-Leibler distance, was chosen (Horne and Garton, 2006;

Loader, 1999). It produces estimates with better fit and less variability than likelihood cross-validation bandwidths (Horne and Garton, 2006). The resulting bandwidth for the kernels is 7133 meters here. Edge effects at the limits of Wallonia were corrected with Diggle's estimator (Baddeley et al., 2015; Diggle, 1985). Diggle's correction is explained in detail in Diggle (1985).

The intensity weighting is illustrated in Figure 4.2, and explained hereafter. The intensities associated to each seropositive pasture (I_k) were calculated using Equation 4.1. A maximum intensity value of 1 was allocated to each seropositive bovine and this maximum value was divided equally between each pasture associated to this seropositive bovine ($\frac{1}{K_b}$). To avoid IBEI of simply reflecting areas with more pastures and possibly herds and bovines, we multiplied it by $(1 - NP_a)$ where NP_a represents the normalized local density of pasture and was calculated using Equation 4.2.

$$\mathrm{I_a} = \frac{1}{\mathrm{K_b}} * (1 - \mathrm{NP_a}) \qquad \text{(Equation 4.1)}$$

where *a* is a pasture associated with a seropositive bovine *b*, I_a is the intensity value of pasture *a*, K_b is the number of pastures reported used by the herd of bovine *b*, and NP_a is the normalized local density of pasture a.

$$NP_a = \frac{\mathrm{p_a} - \min \mathrm{p_A}}{\max \mathrm{p_A} - \min \mathrm{p_A}} \qquad \text{(Equation 4.2)}$$

where *A* is the total number of seropositive pastures, p_a is the number of pastures in the kernel of *a* divided by the area of the kernel corrected for the edge effects at the borders of Wallonia. Pastures were counted using their centroids. p_a was normalized with the minimum and maximum values of p_a for all seropositive pastures (A). The minimum was rounded to the nearest inferior whole number to prevent a value of 0 for the NP_a.

In Figure 4.2, the first seropositive bovine (blue) is associated to one pasture, the second (orange) at two pastures, and the third (pink) at one pasture. In this example, four kernels are built, each with an area of 1 km² (black circles). The numbers of pastures in the kernels are counted and divided by the area of the kernels. Pastures 1,

2, 3 and 4 have respectively a local density of pastures around them of 4, 2, 8 and 2 by km². However, half of the area of the kernel of pasture 4 is located in France, where no data on pasture was available. The local density of pastures for pasture 4 (p_4) was therefore 4 pastures by km².

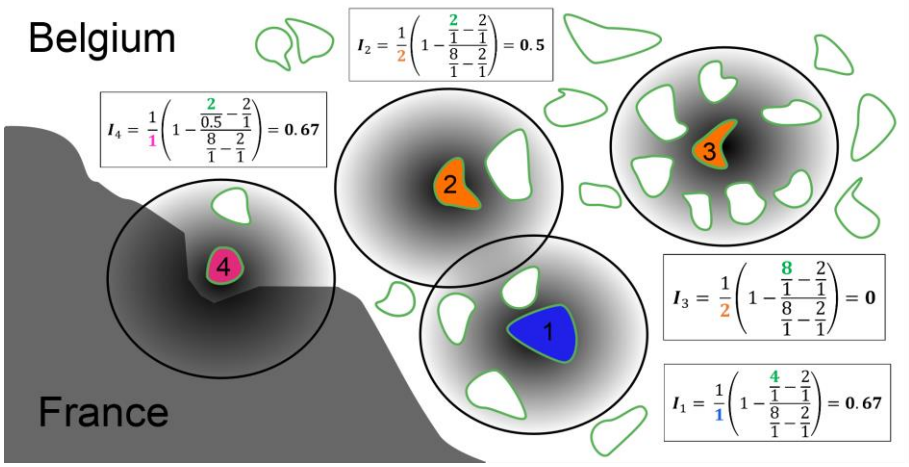

Figure 4.2. Example for the KDE intensity weighting. Seropositive pastures are represented by polygons outlined in green and the associated kernels by circles. Each pasture colour is used by a same herd.

KDE were implemented using the spatstats library in R 3.4. We created a continuous intensity surface (IBEI) by summing the intensities estimated by all the kernels at any location in Wallonia (Equation 4.3).

$$\mathrm{IBEI}(x, y) = \sum_{a=1}^{A} \mathrm{KDE}_a[\mathrm{Ia}, (x, y)] \qquad \text{(Equation 4.3)}$$

where $\mathrm{IBEI}(x, y)$ is the index of Intensity of Bovine Ehrlichiosis Infection at any location (x,y), $\mathrm{KDE}_a[\mathrm{Ia}, (x, y)]$ is the value of the KDE computed on seropositive pasture *a* and estimated at location (x,y), and I_a is the weighted intensity value associated to pasture a, as in Equation 4.1.

This surface was interpreted as a proxy of the local infectiousness of the environment for *A. phagocytophilum*. As we are mostly interested in the distinction between areas with high and low intensities of bovine ehrlichiosis infection, the continuous intensity surface IBEI was standardized.

2.3. INDEPENDENT VARIABLES

The explanatory environmental variables were selected following the resource-based habitat concept (RBHC) (Hartemink et al., 2015; Vanwambeke et al., 2016). This conceptual model identifies the resources needed by the pathogen, vectors, and hosts to fulfil their ecological functions (Figure 4.3). The resources used in this study are presented in Table 4.2.

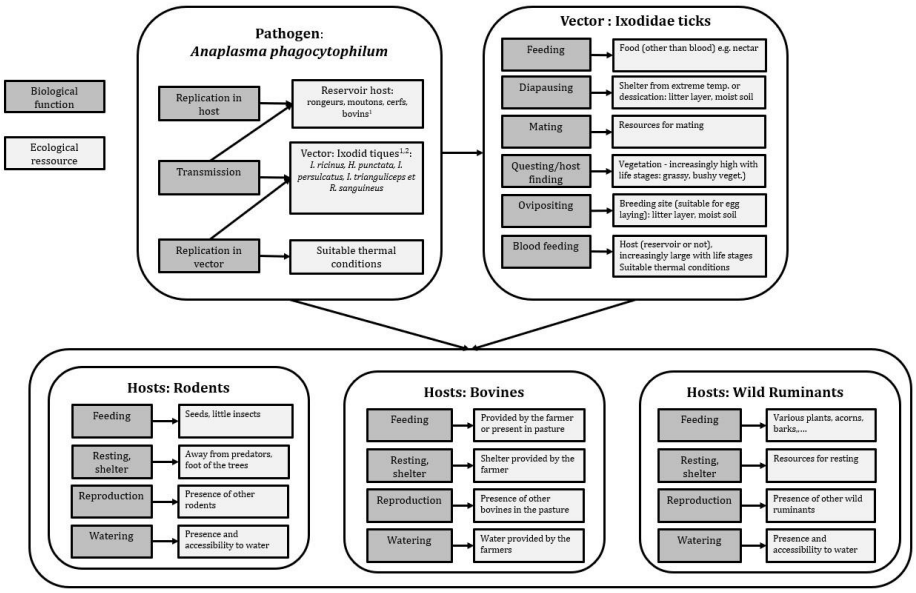

Figure 4.3. Application of the Resource Based Habitat Concept (RBHC) to bovine ehrlichiosis in Wallonia, figure adapted from Vanwambeke et al. (2016).

Pathogen level. We identified three functions for *A. phagocytophilum*: replication in the vector, replication in the reservoir host, and transmission (Stuen et al., 2013). Wallonia has suitable climatic conditions for the bacteria, so the two main resources here are vectors and reservoir hosts.

Table 4.2: Summary statistics of the independent variables and their connection with the resource-based habitat concept. All the values presented were calculated as the mean values for the 200 samplings.

	Descriptive statistics		Organism affected
Variables (unit)	**Mean (Range)**	**Mean (Range)**	**(function associated)**
	1 km buffer	**1 m buffer**	
Landscape variables			
% of grassland	37.09 (3.56-84.59)	48.02 (0.02-99.90)	Bovine (feeding, resting, reproduction)
% of forests	18.45 (0-82.54)	6.97 (0-71.95)	Tick (feeding, diapausing, mating, questing, ovipositing, blood-feeding) Rodent (feeding, resting, reproduction) Wild ruminant (feeding, resting, reproduction)
% of semi-natural habitats	1.81 (0-21.12)	1.70 (0-61.68)	Tick (feeding, diapausing, mating, questing, ovipositing, blood feeding) Rodent (feeding, resting, reproduction) Wild ruminant (feeding, resting, reproduction)
% of artificial lands	14.83 (1.14-69.16)	24.35 (0-94.11)	Mainly reflects the absence of resources provided by the other landscape classes
% of cropland	26.84 (0.08-82.80)	17.34 (0-94.74)	Rodent (feeding, resting, reproduction) Mainly reflects the absence of resources provided by the other landscape classes
Forest fragmentation variables			
Forest Patch Density (number / hectare)	0.04 (0-0.13)	-	Tick (feeding, questing, blood feeding) Rodent (feeding, resting, reproduction)
Forest Edge Density (m/ha)	43.13 (0-149.80)	-	Tick (feeding, diapausing, questing, blood feeding) Rodent (feeding, resting, reproduction)
Forest Shape Index	1.60 (0-2.81)	-	Tick (feeding, diapausing, questing, blood-feeding) Rodent (feeding, resting, reproduction)

Table 4.2 (continued): Summary statistics of the independent variables and their connection with the resource-based habitat concept. All the values presented were calculated as the mean values for the 200 samplings.

	Descriptive statistics		Organism affected (function associated)
Variables (unit)	**Mean (Range)**	**Mean (Range)**	
	1 km buffer	**1 m buffer**	
Forest Aggregation Index (%)	89.35 (0-99.22)	-	Tick (feeding, diapausing, questing, blood feeding)
Forest Fractal Dimension Index	1.25 (0-1.64)	-	Rodent (feeding, resting, reproduction) Rodent (feeding, resting, reproduction)
Deer Variables			
Density of deer (number / km²)	1.79 (0-19.73)	-	Wild ruminant (indicator of the presence of deer)
Density of roe deer (number / km²)	7.3 (0.51-29.45)	-	Wild ruminant (indicator of the presence of roe deer)
Pasture Variable			
Pasture Shape Index	1.54 (1.10-3.96)	-	Favours bovine contacts with other landscapes and animal species

Vector level. In Western Europe, BE is mainly transmitted by *I. ricinus* (Stuen, 2007; Stuen et al., 2013; Woldehiwet, 2010). *Ixodes ricinus* is a generalist species with three host life-stages, i.e., larvae, nymph, and adult. Each life-stage takes a single blood meal, parasitizing a large range of hosts. Its functions are: questing for hosts, feeding, resting, and mating, ovipositing, and blood-feeding (Vanwambeke et al., 2016). Forest litter provides the most suitable conditions for ticks to survive and develop, when not questing nor attached to a host (Dobson et al., 2011; Gray, 1998; Tack et al., 2012). Tick abundance is generally the highest there, but they are also present in heathland, moorland, rough pasture, and urban parks (Medlock et al., 2013; Pfäffle et al., 2013; Walker et al., 2001).

We measured landscape composition around pastures within two buffers: 1 km to represent the fraction of tick habitat around the pasture, and 1 m to represent the

environment directly in contact with the pastures. They were calculated using the land cover map of Wallonia ("Carte d'occupation du sol de Wallonie, COSW, 2007, Copyright-SPW-n°190517-0815), after conversion to raster at 10 m spatial resolution.

Host level. As no transovarial transmission has been identified for *A. phagocytophilum* in ixodid ticks, reservoir hosts maintain the endemic cycle (Stuen et al., 2013). The whole range of reservoirs hosts for *A. phagocytophilum* is unknown (Nahayo et al., 2014b). Therefore, we considered three groups: small rodents, wild ruminants, and large domestic animals. They all need to fulfil the same four functions, i.e., feeding, resting, reproduction, and watering, but have different resources associated. Rodents mainly feed on seeds, fruits, and invertebrates, and inhabit grasslands, agricultural fields, and forests, especially the later in winter (Tellería et al., 1991). They prosper in fragmented forests, reaching high densities in small forest patches (Allan et al., 2003; Brownstein et al., 2005; Tack et al., 2012). As no rodent abundance data covering the entire study area exist, the following fragmentation indices were computed for forests: patch density, mean shape index of patches, mean patch fractal dimension index, edge density, and aggregation index (McGarigal et al., 2012) with the SDMtools package in R 3.4. Wild ruminants constitute an important group of reservoir hosts for *A. phagocytophilum*, and are represented by deer and roe deer in Wallonia (Rosef et al., 2009; Ruiz-Fons et al., 2012; Ruiz-Fons and Gilbert, 2010; Stuen, 2007; Walker et al., 2001). These animals are found in forests and semi-natural habitats, and especially in forest edges (Halos et al., 2010; Tufto et al., 1996). We calculated deer and roe deer densities in forests using the number of deer and roe deer shot in 2010 (from the Department of Hunting and Fishing of Wallonia) by forest management unit (i.e., "cantonnement") (Li et al., 2012). These statistics constitute a representative estimate of relative abundance in Wallonia. The last host group is mainly made of domestic bovines. They graze on pastures where they receive most of their resources. We calculated the shape index of each pasture, hypothesizing that less compact pastures increase contacts with surrounding tick habitats.

2.4. STATISTICAL ANALYSES

The dependent variable was the value of IBEI extracted at pasture centroids. Because the kernel density estimation is spatially correlated, 200 samples of around 215 (210-225) pastures were randomly selected. Pastures in a sample had to be more distant than the kernel bandwidth. Normality was not rejected using the Shapiro-Wilk test for the 200 samples at p-value < 0.01 (mean statistic = 0.99, mean p-value = 0.08 for the 200 samples). All independent variables were standardized. We first performed bivariate linear regressions. Five variables were subsequently selected as they were significant ($p < 0.05$) in all 200 samples and were often-used proxies of ecological resources of the pathogens, vectors, and hosts of the *A. phagocytophilum* cycle (Table 2). These variables were not collinear, as assessed with a Spearman's rank correlation rho lower than 0.8 and a Variance Inflation Factor < 5. We then performed multiple regressions, first with the five variables, then with the five variables and their first level interactions. The optimal model was selected following a backward stepwise selection based on Akaike's Information Criterion (AIC). The procedure continued until the lowest AIC was reached, considering that two models with a difference in AIC lower than 2 are considered equivalent. This procedure was repeated for the 200 samples.

The effects of the environmental variables may differ between agro-geographic regions (AGR). For example, in areas with less forest, the effect of grasslands or other semi-natural habitats may be more important, as they would serve as default habitat for some species. We tested the role of the AGR using multilevel models. We used the intraclass coefficient correlation (ICC) to measure the proportion of the total variance found at the group level (Snijders and Bosker, 1999). A high ICC indicates that a multilevel approach with mixed effects may be necessary. We followed the protocol developed by Zuur et al. (2009). The random effects and their structure were assessed based on AIC and likelihood ratio tests, using restricted estimated maximum likelihood (REML) estimators. The structure was either based on Fixed-Effects only (FEO), Random Intercepts (RI), or Random Slopes and Intercepts (RSI). The optimal fixed components were then determined by comparing the candidate models with a

stepwise procedure based on Akaike's Information Criterion (AIC). Maximum likelihood (ML) estimators were used, because REML does not allow comparison of models with nested fixed effects (Snijders and Bosker, 1999; Zuur et al., 2009). The final models were then estimated using REML estimators. This procedure was repeated for the 200 samples. Linear mixed regressions were performed with the nlme library in R 3.4.

3. RESULTS

The distribution of pastures associated to herds with an animal tested as positive (in blue) or negative (in grey) is presented in Figure 4.4. Because of the uncertainties related to the location of infection of the herds, we created the IBEI. Its spatial distribution is presented in Figure 4.5 and indicates that the highest IBEI values were found in the centre of Wallonia and the lowest in the northern areas.

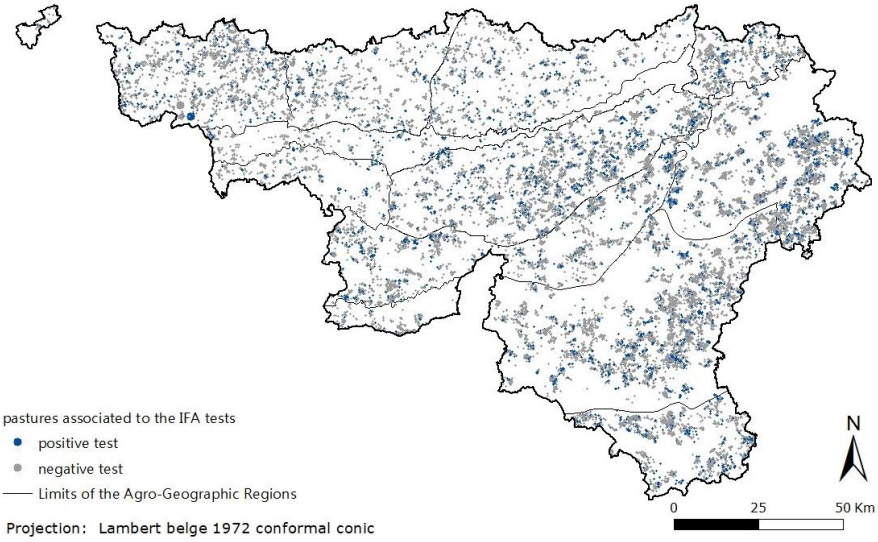

Figure 4.4. Location of the pastures associated with a positive (blue) and negative (black) result to the IFA test for the presence of *A. phagocytophilum* IgG antibodies. The exact locations were randomly moved spread at a maximum distance of 1 km from their actual position to ensure anonymity while preserving the spatial pattern.

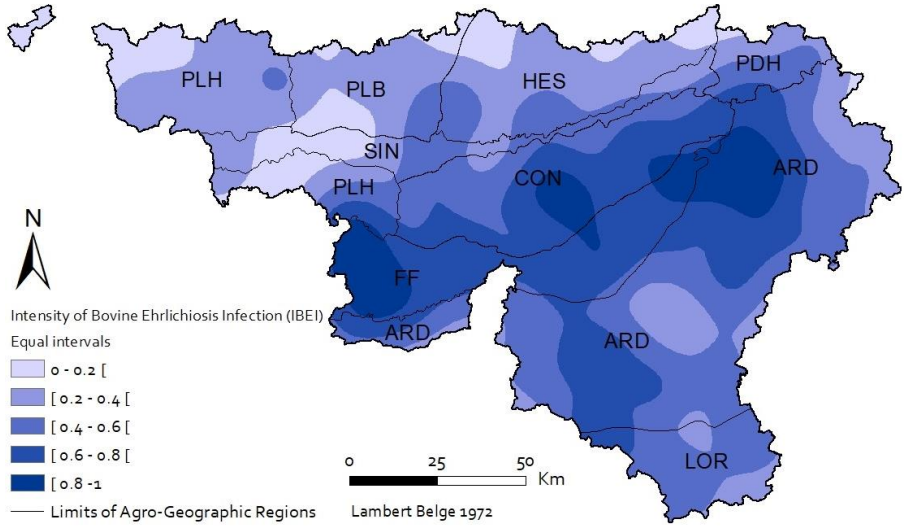

Figure 4.5. Intensity of Bovine Ehrlichiosis Infection (IBEI) in cattle.

The frequency of significant outcomes ($p < 0.05$), mean coefficients and p-values for the bivariate regressions for the 200 samples are summarized in Table 4.3. Landscape composition variables in a 1 km-buffer were always significantly associated with IBEI ($p < 0.05$), except for semi-natural habitats, less important in Wallonia. The IBEI was negatively associated with artificial lands and agricultural lands, and positively with forests, grasslands, and semi-natural habitats. Variables calculated at the 1m-buffer had the same signs, but with smaller and less often significant effects. Forest fragmentation variables and wildlife variables were significantly positively associated with IBEI, in 139 to 200 samples and 200 samples, respectively. Local characteristics of the pastures were significant in less than one-fourth of the 200 simulations.

Table 4.3: Bivariate linear regressions for the IBEI models, with no random effects. The first column indicates the number of simulations where a variable was significant ($p < 0.05$) and the mean estimated coefficients and p-values. * p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.001. The five variables selected for the multi-level modelling are in bold.

	Bivariate regressions (only sign. variables)			
Variables	**Frequency of significance (/200)**	**Mean coefficients**	**Mean p-value**	
Landscape composition variables (1 km-buffers)				
Proportion of artificial lands (%)	200	-0.07	< 10^-16	***
Proportion of croplands (%)	200	-0.11	< 10^-16	***
Proportion of grasslands (%)	**200**	**0.08**	**< 10^-16**	*******
Proportion of forests (%)	**200**	**0.08**	**< 10^-16**	*******
Proportion of semi-natural hab. (%)	58	0.04	0.02	*
Proportion of wetlands (%)	4	-0.03	0.04	*
Landscape composition variables (1 m-buffers)				
Proportion with artificial lands (%)	47	-0.04	0.02	*
Proportion with croplands (%)	173	-0.05	0.01	***
Proportion with grasslands (%)	135	0.04	0.01	*
Proportion with forests (%)	156	0.04	0.01	*
Proportion with semi-natural hab. (%)	13	0.04	0.02	*
Forest Configuration variables (1 km-buffers)				
Forest Patch Density (number/ha)	**200**	**0.06**	< 10^-16	*******
Forest Shape Index	190	0.05	< 10^-16	***
Forest Edge Density (m/ha)	**200**	**0.09**	**< 10^-16**	*******
Forest Aggregation Index	196	0.06	< 10^-16	***
Forest Fractal Dimension Index	139	0.04	0.01	*
Wildlife variables				
Density of deer (number/km²)	200	0.05	< 10^-16	***
Density of roe deer (number/km²)	**200**	**0.07**	**< 10^-16**	*******
Pasture variable				
Pasture Shape Index	8	0.03	0.03	*

The five variables selected for the stepwise regressions with Fixed Effects Only (FEO) were the proportion of grasslands, the proportion of forests, the forest patch density, the forest edge density, and the density of roe deer. The proportion of croplands and of artificial lands were also significant in the 200 regressions but were not included to avoid collinearity, and because they rather represent the absence of suitable habitats. Results are shown in Table 4.4 when considering only the main effects and in Table 5 for the main effects and their first-level interactions. For the FEO regressions, the mean adjusted R^2 calculated for the 200 best models was 0.36 (standard deviation = 0.02). All variables were positively associated with the IBEI. The percentage of grassland was always present in the best model and had the highest mean standardized coefficient (0.08, standard deviation = 0.008). The density of roe deer and percentage of forest were also present in most simulations (197 and 184 respectively). Forest edge density and the number of forest patches were present in 118 and 71 simulations respectively (Table 4). No heteroscedasticity was detected at p-value < 0.001 (mean Breush-Pagan statistic = 7.89, mean p-value = 0.39). Residuals were normally distributed (mean Shapiro statistic = 0.99, mean p-value = 0.32) and had a mean of zero (mean Student t Statistic = -2.66, mean p-value > 0.99).

Table 4.4: Multilevel regressions (no interactions) and the frequency of significance in the best models. The coefficients and p-values are expressed as the means and standard deviations (SD) for the 200 samples. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001.

Variable	Frequency of significance (/200)	Coefficient		P-value		
		Mean	SD	Mean	SD	
Intercept	200	0.48	0.004	<10^-16	<10^-16	***
Proportion of grasslands	200	0.08	0.008	<10^-16	<10^-16	***
Density of roe deer	197	0.03	0.006	0.025	0.002	*
Proportion of forests	184	0.06	0.018	0.018	0.002	*
Forest Edge Density	118	0.05	0.016	0.025	0.003	*
Forest Patch Density	71	0.03	0.011	0.026	0.003	*

Table 4.5: Multilevel regressions with first-level interactions, and the frequency of significance in the best models. The coefficients and p-values are expressed as the means and standard deviations (SD) for the 200 samples. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001.

Variable	Frequency of significance (/200)	Coefficient		P-value		
		Mean	SD	Mean	SD	
Intercept	200	0.50	0.014	<10^-16	<10^-16	***
Main terms						
Proportion of grasslands	200	0.07	0.011	<10^-16	<10^-16	***
Density of roe deer	197	0.05	0.009	0.003	0.006	**
Proportion of forests	191	0.07	0.023	0.027	0.091	*
Forest Edge Density	152	0.04	0.023	0.150	0.219	
Forest Patch Density	88	0.01	0.020	0.299	0.286	
Interaction terms						
Density of roe deer: Proportion of grasslands	144	-0.04	0.007	0.015	0.015	*
Density of roe deer: Proportion of forests	132	-0.04	0.016	0.009	0.012	**
Forest Edge Density: Proportion of forests	74	-0.04	0.009	0.016	0.017	*
Proportion of grasslands: Proportion of forests	48	0.05	0.014	0.016	0.017	*
Density of roe deer: Forest Edge Density	40	-0.02	0.048	0.014	0.015	*
Density of roe deer: Forest Patch Density	32	0.03	0.025	0.014	0.015	*
Forest Edge Density: Proportion of grasslands	32	-0.02	0.055	0.020	0.016	*
Forest Patch Density: Proportion of forests	23	0.03	0.035	0.024	0.018	*
Forest Patch Density: Proportion of grasslands	17	0.02	0.038	0.027	0.014	*
Forest Patch Density: Forest Edge Density	16	-0.03	0.027	0.015	0.012	*

Two first-level interactions between pairs of the five variables were more frequently significant: the density of roe deer with the percentage of grassland (144) and with the percentage of forest (132) (Table 4.5). Other interactions were significant between 16

and 74 times in the best models. No heteroscedasticity was detected in the 200 regressions, at p-value = 0.01 (mean Breush-Pagan statistic = 7.89, mean p-value = 0.56). Residuals were normally distributed (mean Shapiro statistic = 0.99, mean p-value = 0.34) and had a mean of zero (mean Student t Statistic = -5.96, mean p-value > 0.99).

The ICCs of the null models showed that 63% (range 59% - 67%) of the variance was at the level of the agro-geographic regions (AGR), indicating that multilevel models with random intercepts (RI), or random slopes and intercepts (RSI) are more appropriate. In the 200 simulations, the likelihood ratio test indicated that RI models performed better than FEO models (mean L = 62.15, range 41.59 – 94.85, df = 1, $p < 0.001$). In most RSI simulations, the algorithm did not converge for the variable treated as random slope and, when the convergence was reached, the likelihood ratio tests indicated that RI models performed better. The random intercepts were always significant in the RI models selected (Table 4.6). The explanatory variables were less often significant than in FEO regressions. The percentage of grassland appeared in 168 selected RI models, followed by the forest edge density, percentage of forests, and forest patch density (Table 4.6). The density of roe deer never appeared in the best RI models. The results were similar when the first-level interactions were included in the model selections (results not presented). Interaction terms had few significant occurrences in the 200 best models (from 13 to 56 occurrences), indicating an absence of similarities between the models for the interactions, and making their interpretation uncertain.

Table 4.6: Multilevel mixed regressions (no interactions) with the agro-geographic regions as random effect and the occurrences and frequency of significance (p-value < 0.05) of the variable in the best models. The coefficients and p-values are expressed as the means and the ranges for the 200 samples. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001.

Fixed Part

Variables	Frequency (200)	Frequency of significance (/200)	Model coefficients		P-values	
			Mean	SD	Mean	SD
(Intercept)	200	200	0.45	0.01	0.0000	<10^-16
Proportion of grasslands	168	162	0.04	0.01	0.0107	0.0146
Forest Edge Density	139	139	0.05	0.01	0.0033	0.0086
Proportion of forests	59	57	0.05	0.01	0.0118	0.0168
Forest Patch Density	27	25	0.02	0.02	0.0245	0.0183

Random part

	Coefficients		
	Mean	Min	Max
Fagne-Famenne	0.71	0.65	0.76
Condroz	0.64	0.62	0.67
Ardenne	0.49	0.42	0.55
Lorraine	0.45	0.40	0.50
Pays de Herve	0.40	0.33	0.48
Hesbaye	0.37	0.29	0.45
Sillon Industriel	0.36	0.25	0.47
Plateau limoneux hennuyer	0.33	0.27	0.40
Plateau limoneux brabançon	0.33	0.25	0.42

4. DISCUSSION

BE is likely to be present in bovine herds everywhere in Wallonia, indicating that it may be endemic (Delooz, 2014) . However, the spatial distribution of the intensity of the disease is not known. In this study, we used a kernel density to estimate the spatial distribution and environmental determinants of *A. phagocytophilum* infections in Walloon cattle at the landscape level. The IBEI map, resulting from a sparse dataset, reported high infection intensity in the central regions of Wallonia, i.e., Fagne-Famenne and Condroz, and low in the northern regions, i.e., Plateau limoneux brabançon, Plateau limoneux hennuyer and Hesbaye (Figure 4.4). Due to the absence of complementary data, a complete validation of the IBEI map is not possible. Knowledge over BE among farmers and veterinary practitioners has improved since the period of the data used in this study (2010). A new collection of samples in Walloon cattle may, therefore, be undergone, targeting several herds located in pastures with contrasting environmental characteristics. However, it was beyond the scope of this study. Nevertheless, this map was consistent with existing knowledge. Lempereur et al. (2012) measured the annual variation in BE prevalence in 2010 in cows from eight voluntary Walloon farms with known history of BE. The percentages of seropositive cows ranged from 30.8% in spring to 77% in summer, which were higher compared to previous publications in Europe (Ebani et al., 2008). These farms were in Fagne-Famenne and Ardenne, regions where the IBEI is high. Since 2014, *A. phagocytophilum* is systematically sought in bovine abortions in Belgium, as other pathogenic agents, e.g., *Brucella* sp. and *Coxiella burnetii* (Delooz, 2017). Except for the high IBEI values of the south-west of Wallonia, the spatial pattern of IBEI corresponds with the geographical distribution of herds with abortion cases caused by *A. phagocytophilum* in 2014 (Delooz, 2014). This disease is zoonotic and *A. phagocytophilum* may also cause human granulocytic anaplasmosis, although analyses on the major surface protein 4 (*msp4*) gene of *A. phagocytophilum* indicated that strains from humans could be different from strains infecting ruminants (de la Fuente et al., 2005). Farmers and veterinarians in particular are exposed to contacts with ticks due to their outdoor professions. De Keukeleire et al. (2017) found an *A.*

phagocytophilum seroprevalence of 8.1% for this group in Belgium, comparing to 5.7% and 6.2% for rural and urban blood donors.

The serological data used in this study presented several sources of uncertainties. Serological tests do not detect the infection per se, but an indication of a contact with *A. phagocytophilum* within the last 3-4 months, the bovine IgG half-life being estimated at 17 to 22 days (Pusterla et al., 1998). Therefore, serological data generally does not permit the knowledge of the exact period and place of contact. Lempereur et al. (2012) found that some bovines encounter double seroconversions for *A. phagocytophilum* in five months. Also, the sampling of a single animal in each herd, while allowing more herds being sampled in a limited-resource context, does not allow to interpret seronegative samples. We thus opted for the creation of an index based on a kernel density function. This method differentiated areas according to the intensity of seropositive tests. It thus has an important advantage for infectious diseases when the exact place of infection is unclear.

Results from the bivariate and multivariate regression were consistent with existing knowledge of the landscape determinants of tick-borne diseases. The IBEI in cattle was positively associated with the five selected variables that represented important resources for the pathogen system. These variables appeared in most of the best models of the 200 regressions. As the transmission cycle of *A. phagocytophilum* relies on multiple species, an environmental variable may proxy different resources. The percentage of grasslands was always present in the best models and had the biggest effects. The effect of this variable may be inflated because the IBEI was created with a kernel density function based on the locations of pastures associated with serological tests for *A. phagocytophilum*. Nevertheless, this possible inflation is not sufficient to explain its observed importance. Indeed, the percentage of grasslands was the only landscape variable directly associated with the presence of cattle, a reservoir host of *A. phagocytophilum.* Cattle are likely to play an important role in the persistence of the disease system. Lempereur et al. (2012) found that 26% of cattle still tested positive against *A. phagocytophilum* before returning to pastures in spring, and therefore had remained carriers through the winter. The presence of the percentage of

grassland in most of the best models is therefore not surprising. The second most important variable was the percentage of forests. Forests constitute the preferred habitat for ticks and harbour many animal species that serve as hosts for ticks and the pathogen. The occurrence of the variable density of roe deer in the best FEO models underlines the importance of wild hosts for *A. phagocytophilum* and ticks in Wallonia. A high seroprevalence against *A. phagocytophilum* of 46.2% was found in the northern part of Belgium, Flanders, in 184 roe deer collected between 2008 and 2013 (Tavernier et al., 2015). The effects of forest edge density were detected more often than those of the number of forest patches, underlying the importance of forest ecotones, which host many different animal species, especially small rodents (Allan et al., 2003; Brownstein et al., 2005). Interactions between explanatory variables were sometimes significant but not as often as their main effect. The interactions of the density of roe deer with the percentage of grassland and with the percentage of forest were the only important interaction terms. For low roe deer densities, the effect of high percentages of grasslands or of forests is more important than in situations with high roe deer densities. This effect is illustrated for one of the 200 samples in Figure 4.6. The percentage of grassland and of forest are proxies for the habitats of large domestic and wild reservoir hosts, respectively. The negative coefficient for the interaction indicates that the effect of one variable is less important for high values in the other variables. This could highlight the complementarity between reservoir hosts for *A. phagocytophilum*, with wild and domestic hosts sustaining pathogen transmission in their respective habitats. The persistence of *A. phagocytophilum* in cattle during winter may maintain BE cycles through the years. The high seroprevalences against *A. phagocytophilum* found in roe deer (Ebani et al., 2008; Tavernier et al., 2015) could also contribute to the persistence of the infection in pastures, especially in those in a forested and fragmented landscape.

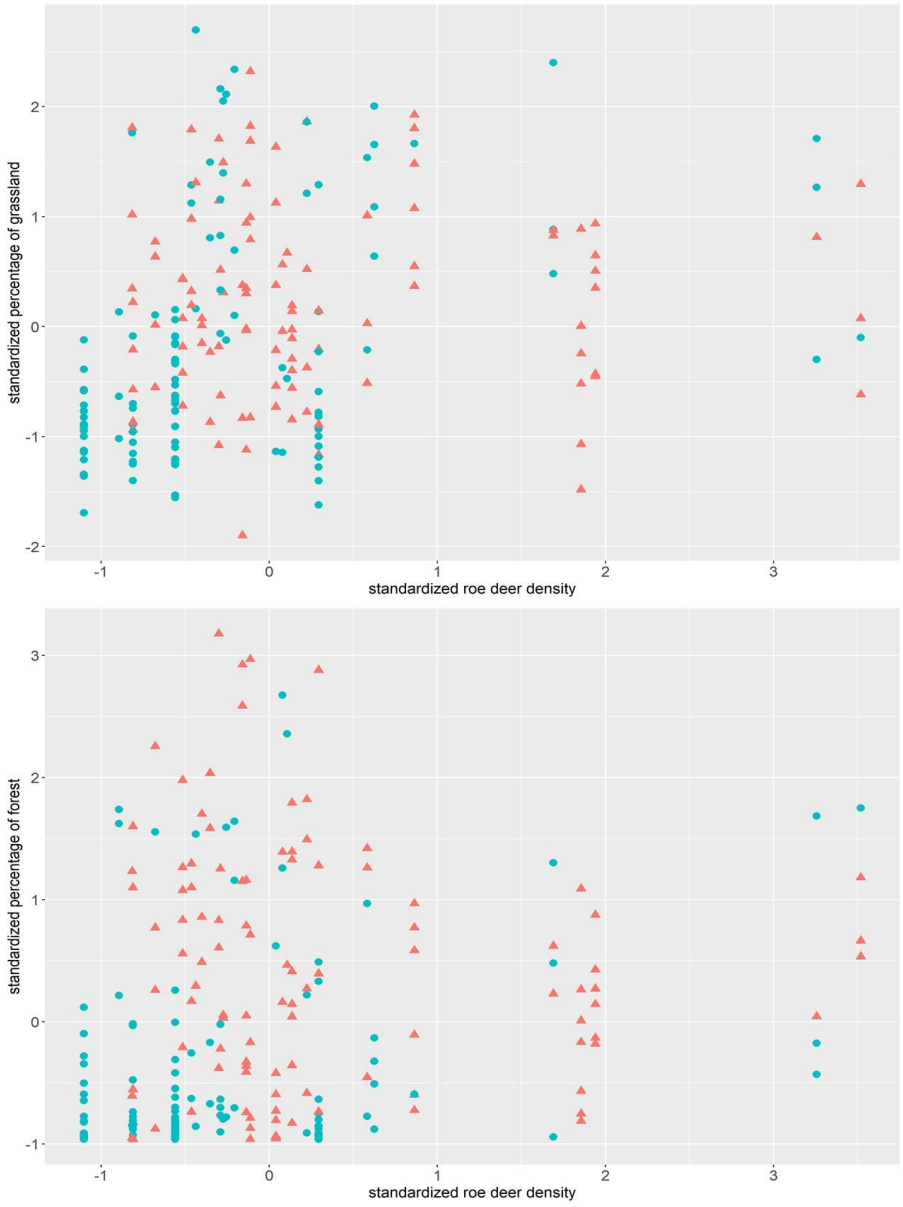

Figure 4.6. Interaction between the variables roe deer density and percentage of grassland (a) and percentage of forests (b) for the sample 1 of the IAI models. High risks (red triangles) represent IAI > 0.5, and low risk (blue circles), IAI < 0.5.

Models with random intercepts for the agro-geographic regions always performed better than FEO models. Regions composed by open field landscapes with few and not well-connected forests and semi-natural habitats had the smallest IBEI values. The effects of forest configuration remained quite important in the RI models, highlighting the importance of forest fragmentation independently to its abundance. Spatial variations in the risk of infectious disease are affected by the composition and configuration of habitats (Lambin et al., 2010). The density of roe deer did not appear in the best RI models. This may be related to the data source and its processing. Our variable approximated the density of roe deer by hectare of forests by forest management units, whose boundaries are overlapping those of the agro-geographic regions. Differences in roe deer densities were therefore represented by the differences in intercepts between the AGR and were not significant in the RI models. The effects of the five environmental variables were consistent throughout the AGR no matter the differences in anaplasmosis intensity, as indicated by the better performance of RI models rather than the random intercepts and slopes models.

The ecological variables used in disease ecology are often proxies for the resource needed by an organism to fulfil its ecological functions. Their biological interpretation can be challenging, because a single variable may proxy several resources, and, one resource represented by several variables. The RBHC (Hartemink et al., 2015) constitutes a promising conceptual framework to reflect the ecological meaning of these variables. The RBHC also highlights the need to collect more information on the spatial distribution of reservoir hosts. In this study, we limited the multiple regression to five variables well known for their connections to the transmission system of *A. phagocytophilum*, and whose effects in the bivariate regression were the most significant. In doing so, we avoided spurious variables or collinearity. Overspecified models tend to overestimate the effects of environmental variables (Galipaud et al., 2014).

The IBEI, created by a kernel density method, has some limitations. The creation of this index depended on the quality of the data used as the intensity in the kernel density function. We did not use the proportion of positive tests because most herds were

associated with multiple pastures, and only one animal was tested by herd. Instead, we used the locations of pastures associated with seropositive tests. Also, although we weighted the kernel density function using pasture density, the presence of seropositive tests remains influenced by the number of pastures. The kernel density estimation was also limited by the spatial extent of Wallonia. Despite our correction for edge effects, loss of information is inevitable as the distribution of *A. phagocytophilum* is not restricted to Wallonia. The IBEI reflected the variations of intensity of bovine ehrlichiosis infection in Wallonia at the landscape level but is not appropriate to represent local differences at the pasture level, directly influenced by its characteristics. When assessing the first cases of BE in Belgian cattle herds, Guyot et al. (2011) noticed that the infected herds where on pastures surrounded by wild vegetation, such as trees, hedges, cornfields and bushes that constituted a suitable biotope for ticks. Despite these uncertainties, we believe IBEI reflects well the seroprevalence of *A. phagocytophilum* in Walloon cattle. Many tick-borne disease studies focus on human health, with often a narrow focus on forests as the riskiest environments. However, pastures are where cattle get exposed to tick bites. Pusterla et al. (1998) noted an increase in seroprevalence in bovines after the move to the pastures, especially for those being grazed outside for the first time. IBEI was built at the landscape level and therefore did not account for the local characteristics of pastures that also influence the risk and exposure of cattle to bovine ehrlichiosis. Studies targeting pastures specifically thus have great potential use in veterinary risk assessment.

5. CONCLUSION

The study of vector-borne zoonotic disease often relies on partial data, i.e., presence or abundance of vectors, serological tests, reported cases. In multiple situations, the exact place of infection is unknown, complicating the efforts of medical and veterinary practitioners. In Wallonia, BE is mainly transmitted by *I. ricinus* ticks. Therefore, cattle infection with *A. phagocytophilum* is strongly affected by environmental conditions. In this study, we created an indicator reflecting the spatial

variability of the intensity of *A. phagocytophilum* infection, based on a kernel density estimation. In Wallonia, areas with the highest intensity of cattle infection with *A. phagocytophilum* were in central and southern regions, corresponding to more forested and fragmented landscapes. This is consistent with previous records of bovine ehrlichiosis in Wallonia. This method is easy to implement and can be updated when new data become available. It can help veterinary practitioners targeting more quickly and cost-effectively areas where *A. phagocytophilum* infection is likely. A rapid detection is crucial to assess veterinary risk in the herds, especially because cattle may remain carriers throughout the winter and the disease is still underdiagnosed.

6. LIST OF ABBREVIATIONS FOR CHAPTER 4

AGR	agro-geographic region
BE	bovine ehrlichiosis
FEO	fixed-effects only
IBEI	intensity of bovine ehrlichiosis index
IgG	immunoglobulin G
KDE	kernel density estimation
RI	random intercepts
RIS	random intercepts and slopes

CHAPTER 6

CONCLUSIONS

One Health issues are complex and multifaceted, involving interactions between humans, animals, and the environment (Stärk et al., 2015; Zinsstag et al., 2011). The risk of tick-borne disease transmission is affected by human or animal exposure to environmental hazards, and therefore by the overlap of ticks, pathogens, hosts, wild or domestic animals, and humans for zoonoses. As tick-borne diseases are complex systems, focusing only on one single aspect of the system may lead to incomplete results. Studying this risk relies on partial data, because of the constraints associated with observing the various species involved in the transmission cycle. Specific approaches are required because each element comes with its own practical challenges, leading to data often reflecting poorly either on spatial or temporal dynamics, or both. However, comparing studies on tick-borne diseases is challenging due to the heterogeneity and limitations of the different datasets.

Through this dissertation, we explored and combined different aspects of tick-borne disease risk at multiple scales in Belgium. There is a need for a unified approach between epidemiology, ecology, veterinary and human medicine as promoted by approaches like One Health. During this thesis, we collaborated with experts from different health-related disciplines. In this chapter, we place the main findings of the thesis in a broader perspective, identified limitations, and present plans and recommendations for future research.

1. TICK BORNE DISEASE RISK AT DIFFERENT SPATIAL SCALES

This thesis participated to the assessment of tick-borne disease risk in Belgium. Belgium is of particular interest, because it is in the endemic range of *I. ricinus,* and it is one of the countries with the highest incidence of Lyme borreliosis in Europe (Burn et al., 2023). However, many tick-borne diseases remain highly underdiagnosed, e.g., human granulocytic anaplasmosis and bovine ehrlichiosis, caused by *A. phagocytophilum* in humans and cattle respectively (Adjadj et al., 2023). Moreover, recent environmental changes may affect tick-borne disease risk, and their effects remain uncertain. For example, the effects of climate change on tick-borne disease incidence in Belgium are debatable, as warmer winters could lead to a longer period of tick activity, while warmer summers and longer could reduce tick activity and survival (Dantas-torres, 2015; Gray et al., 2009). These elements contribute to future uncertainties and call for continuous surveillance of tick-borne diseases.

2. LOCAL ESTIMATES OF THE ACAROLOGICAL RISK

Tick bites result from the contact between humans and ticks. The overall risk of developing Lyme borreliosis after a bite was estimated at 2.6% and increase with the duration of attachment (Hofhuis et al., 2017). Therefore, body inspection and prompt tick removal remain effective disease prevention measure for Lyme borreliosis, and tick abundance surveillance (Figoni et al., 2019). Tick-borne disease hazard is generally estimated by the density of infected ticks (also called the acarological risk). This is calculated by multiplying the density of ticks, obtained by counting the number of ticks collected in a delimited area, by the tick infection prevalence, i.e., the proportion of the number of the ticks infected by a specific pathogen. The absence of current licensed human vaccines for several tick-borne disease (e.g., Lyme borreliosis, human granulocytic anaplasmosis) makes studies on tick distribution, abundance, and infection prevalence imperative from a One Health perspective.

2.1. DENSITY OF TICKS

Ixodes ricinus has been established for a long time in Belgium, where it is the most widespread human-biting tick species (Fain, 1989). Estimating tick population size is challenging, and dragging or flagging a cloth over the low vegetation for a determined distance, has long been identified as a common method for quantifying tick density (Eisen and Paddock, 2020). This method offers a local estimate of tick abundance, that can be extrapolated to other locations presenting similar characteristics. Researchers often sample multiple transects in an area, to get a representative sample if the area and/or to cover different habitats, as we did in the Bois de Lauzelle (**chapter 2**). Dragging has commonly been used in studies estimating tick abundance because of its relative reliability, cost-efficiency and ease of implementation (e.g., Boyard et al., 2011; Richter and Matuschka, 2011; Schwarz et al., 2012; Van Gestel et al., 2021).

In this thesis, we regularly sampled *Ixodes* ticks in the Bois de Lauzelle, a Natura 2000 peri-urban forest of central Belgium (**chapter 2**). This forest belongs and is managed by the Université catholique de Louvain (UCLouvain) and offers opportunities for various experimental scientific works in forestry, ecology, botany, zoology, soil science and hydrology. As such, it offers opportunities for multidisciplinary studies on environmental, animal, and human health. There, ticks were sampled for two years (2016 and 2018). Estimates of tick abundance were similar between 2016 and 2018, but higher in the forest than in vegetated non-forested environments. Several studies, collecting ticks in different region of Belgium did not systematically report the total area sampled, preventing the measurement and comparison of tick abundance (e.g., Kesteman *et al.*, 2010; Lempereur *et al.*, 2012; Adjadj *et al.*, 2023). Only a few used particular design allowing to measure tick densities, and they were mainly undertaken in forest stands from the north-eastern part of the country (Heylen et al., 2019; Ruyts et al., 2016; Tack et al., 2012; Van Gestel et al., 2021). Another study analysed *I. ricinus* ticks collected in 125 events from 51 broad-leaved or mixed forest stands throughout Belgium (Li et al., 2012). They had one of the lower estimates of tick abundance for the Walloon Brabant province. The Bois de Lauzelle also had low tick abundance estimations and is in the Walloon

Brabant province, but these estimates are hardly comparable, as the abundance of Li et al. (2012) was estimated only from one sampling during spring. A nation-wide sampling collection with comparable designs may confirm these observations.

Comparing tick abundance estimated from multiple habitats or areas requires that the different estimates are close to the real abundances or, at least, comparable across habitats. It is not feasible to ascertain the exact size of the tick population from an area, because the efficiency of dragging is assumed to be variable and low, with less than 10% of the total tick population sampled according to Nyrhilä et al. (2020) and Tälleklint-Eisen and Lane (2000). Only questing ticks may be captured by dragging, excluding rehydrating, feeding, digesting, and moulting ticks. Moreover, each drag only captures a fraction of the questing tick population, depending on various factors, such as the weather, the type of vegetation or differences in investigator efficiency (Bord et al., 2014; Dobson et al., 2011). In our opinion, tick abundance estimated with the classical "single drags" method, should be analysed with caution because of various dragging efficiencies.

To offset these limitations, we performed consecutive-drags sampling at the Bois de Lauzelle, as proposed by Bord et al. (2014).The same sampling units were dragged consecutively multiple times in fast succession. We found that one third of the total number of nymphs sampled were collected during the first of six consecutive drags. However, the sampling probability was lower in locations with complex undergrowth, probably because the drag can hardly cover various levels of vegetation in one drag. With this technique, we identified differences in tick abundance between different habitats in the forest only when tick abundance was estimated with a minimum of three consecutive drags, and not only by the number of ticks collected during the first drag. There are no common standard protocol for tick dragging, even though protocols have been recently proposed (e.g., Eisen *et al.*, 2019; Salomon, Hamer and Swei, 2020). Such protocols provide guidelines to analyse spatio-temporal patterns and trends of tick abundance, and for comparisons across studies. Based on our samplings at the Bois de Lauzelle, we suggest the following recommendations for further tick dragging collections:

i. Always report the area sampled to obtain comparable measures of tick abundance.
ii. Do not limit tick samplings on single dates assumed to correspond with activity peaks, and employ regular samplings throughout the season of tick activity (or throughout the peak activity season) to prevent extreme estimates resulting from occasional samplings, as suggested by Dobson (2013).
iii. We strongly advise future studies to perform three consecutive drags per transects for comparing tick abundances. Although this technique is less efficient for the capture of large numbers of ticks because less ticks are sampled per consecutive drag, it provides better estimates for tick abundance comparisons.

Identifying tick species prior to tick-borne pathogen detection is crucial to understand their roles as hosts and vectors for these pathogens because they are associated to different phenology and activities. *Ixodes ricinus* is bridge vector (vectors that feed on more than one host species) and is the main human-biting tick species in western Europe (Rizzoli et al., 2011). However, other *Ixodes* species may be involved in the transmission and maintenance of different tick-borne pathogens in natural cycles (Plantard et al., 2021). For example, several variants of *A. phagocytophilum* (ecotypes) have been detected in different *Ixodes* species, including *I. frontalis* and *I. ventalloi*. During this thesis, we learnt tick morphological identification, used it to identify the species of the ticks collecting in the Bois de Lauzelle and transmitted this skill for other ticks-related research.

2.2. TICK INFECTION PREVALENCE

Infection prevalence with different pathogens may be estimated from sampled ticks. Lernout *et al.* (2019) found a prevalence of 14% for *Borrelia burgdorferi* s.l. in human-biting ticks sent by citizens. Large variations in prevalence of tick-borne bacteria in *I. ricinus* has been observed between forested areas (e.g., Coipan et al., 2013; Halos et al., 2010; Kesteman et al., 2010). *Borrelia burgdorferi* infection prevalence in *Ixodes* ticks from the Bois de Lauzelle was estimated at 5.3% (**chapter**

2), lower than the mean tick infection prevalence of 13.7% calculated from questing ticks in Europe (Rauter and Hartung, 2005b). The low *Borrelia* prevalence from the Bois de Lauzelle was unexpected but is not uncommon, as a similar range has been found in *I. ricinus* from central France (Halos et al., 2010). It could be related to the lack of connections for wildlife between the Bois de Lauzelle and other forests, that may result in smaller or less diverse host populations and compositions (Ruiz-Fons et al., 2012).

We identified six different *B. burgdorferi* genospecies, with a composition similar to a recent meta-analysis in Western Europe (Strnad et al., 2017). *Borrelia afzelii* was the dominant genospecies isolated, followed by *B. garinii*, *B. burgdorferi* s.s. and *B. valaisiana*. *Borrelia afzelii* is commonly associated with rodents, which suggests that they are the main host for larvae in the Bois de Lauzelle as in other Belgian forests (Hofmeester et al., 2016; Ruyts et al., 2018). Rodents population have cyclic annual population that may affect inter-annual variations of the estimations of tick-borne pathogen infections in questing ticks (Hofmeester et al., 2016). In our study, we did not find differences between 2016 and 2018 in tick abundance, but tick infection prevalence was lower in 2016. Similarly to Halos *et al.* (2010), nymph infection prevalence was also lower in non-forested compared to forested habitats. The presence of high-speed roads and car traffic close to the non-forested sites may disturb animal populations, that remain in the forest. Accordingly, the forested sites harboured more diverse *B. burgdorferi* genospecies communities than outside of the forest. Pathogen infection prevalence in ticks for a specific area should not be estimated from ticks from a single location, as it is likely heterogeneous spatially and temporally.

Another pathogenic bacterium, *C. burnetii*, was detected from six out of 499 pools, while *F. tularensis* was not isolated. These pathogens are usually found in low prevalence in *I. ricinus*. Human transmission of *C. burnetii* from ticks is controversial because of its rare detection in *Ixodes* ticks and the difficult differentiation with *Coxiella*-like endosymbionts (Anderson et al., 2013; Duron et al., 2015; Körner et al., 2021; Sprong et al., 2012). In Belgium, most sources of infection with *C. burnetii*

were unknown but some cases have been related to forested areas (Litzroth et al., 2021). The different isolations of *C. burnetii* in the Bois de Lauzelle confirm its presence in *Ixodes* ticks in Belgium, but the tick bite transmission route to humans must still be confirmed. *Francisella tularensis* was not detected in the ticks of the Bois de Lauzelle. However, re-emergence has locally been observed in several European countries, suggesting continuing the surveillance of *F. tularensis* in ticks in Belgium (Hestvik et al., 2015).

2.3. TICK MICROBIOTA

To the best of our knowledge, **chapter 3** was the first attempt to describe the microbiota of *I. ricinus* collected in Belgium using cultures of bacteria from ticks of the same forest. Tick microbiota is constituted by a rich community of symbiotic, commensal, and parasitic microorganisms coexisting and interacting that is changing in time and scale (de la Fuente, 2018; Vayssier-Taussat et al., 2015). They have been transovarially transmitted or acquired from local environment or blood feeding (Bonnet and Pollet, 2021). Although cultivable bacteria do not represent the complete tick microbiota, they influence tick communities, as these microorganisms may interact with their tick hosts and/or other microorganisms, including pathogens. It is suggested that *I. scapularis*-associated microbiota impacts tick midgut colonization of *B. burgdorferi* (Narasimhan et al., 2014).

Tick microbiota has been understudied for a long time but interest has grown in the last decade with the increasing number of metagenomics studies using next-generation sequencing (NGS), that enabled rapid sequencing of large amount of microorganisms (Barbosa et al., 2017; Carpi et al., 2011; Vayssier-Taussat et al., 2015, 2013). Diverse and variable bacterial communities were found throughout the Bois de Lauzelle. Bacteria from the Firmicutes phylum were the most abundant, followed by the Actinobacteria and the Proteobacteria phyla. They were mostly common bacteria found in the environment or in the skin and mucous membranes of humans and animals. The presence of bacteria of environmental origin in *I. ricinus* indicates the close associations between the local environment and the presence,

abundance, and diversity of *I. ricinus* microbiota. However, further research is needed to differentiate environmental contaminants from tick endosymbionts.

Analysing tick microbiota may also generate evidence of unanticipated microbes potentially harmful for human and veterinary health. In this thesis, we made the first isolation of a strain of *Cedecea davisae* from *I. ricinus* in **chapter 3**. This bacterium is naturally resistant to antibiotics and may cause bacteraemia, so its potential presence in tick microbiota needs to be studied further. Vayssier-Taussat et al. (2013) also uncovered unexpected bacterial pathogens in ticks from France: *Bartonella henselae*, and *Riccetsia felis*, microorganisms that are typically contaminating humans from cat or flea bites.

3. MAPPING TICK BITE RISK AT THE LANDSCAPE SCALE

We assessed tick-borne disease risk by focussing on human exposure to tick bites. Local studies focusing on specific populations may identify individual factors or specific habitats associated with higher exposure to tick bites, as demonstrated by De Keukeleire et al. (2015) with scouts in Belgium. However, assessing tick bite risk at a larger scale is more challenging, because exposure factors, like accessibility to tick-infested areas and human behaviours heavily depend on individual or local factors and are not easily measured at broad scales (Garcia-Marti et al., 2018).

In **chapter 5**, we created risk maps of human exposure to tick bites at the municipality level with a multi-criteria decision analysis (MCDA). This method is not very common in tick-borne disease risk studies, but has already been used in other European countries to estimate tick habitat suitability (Lebert et al., 2022; Rousseau et al., 2017). Our methodology differs as we also included variables related to human exposure, and we worked at the municipality level. Results of the MCDA were compared with tick bite records by municipality from TiquesNet/TekenNet and we were able to identify and address differences between the two approaches in modelling tick bite risk.

4. SPATIAL PATTERN IN TICK-BORNE DISEASES FROM SPARCE SEROPREVALENCE DATA

The assessment of tick-borne disease risks may also consider animal exposure to tick-borne pathogen infection (**chapter 4**). It was represented by serological data consisting on bovines blood samples screened for the presence of antibodies specific to *A. phagocytophilum*. Lempereur et al. (2012) found long term persistence of antibodies against *A. phagocytophilum* in bovines indicating that positive results do not automatically indicate an active infection, especially in the absence of clinical symptoms. Rapid detection is therefore crucial to assess veterinary risk caused by *A. phagocytophilum* (Adjadj et al., 2023; Cochez et al., 2011), particularly in herds, because bovine ehrlichiosis is one of the most widespread tick-borne infection in animals (Stuen, 2007). In Belgium, livestock has a limited mobility, being restricted to their pastures, where they may be exposed to significant bovine ehrlichiosis risk.

We created an index of risk based on a kernel density method, to reflect the infectiousness profile of the landscape of the pasture permitting to differentiate areas according to the intensity of presence of seropositive tests. High risks of infection with *A. phagocytophilum* were identified in central regions of Wallonia, particularly in the province of Liège, while low infection areas were mainly in the north (**chapter 4**). Although no measure of seroprevalence incidence was possible in our study, the spatial pattern of our index of intensity of infection were consistent with a recent study analysing sera of 339 cattle selected from all over Belgium (Adjadj et al., 2023). Herds from the eastern province of Liège had high seroprevalence for *A. phagocytophilum*, in herds from Liège, the province where the first case of bovine ehrlichiosis has been identified in Belgium (Guyot et al., 2011). We thus believe that our kernel density index adequately reflected infection presence in cattle in Wallonia at the landscape level.

An index based on the kernel method could be applied to cattle infection with other tick-borne pathogens in contexts where the datasets present the same limitations, i.e., uncertain location, no systematic screening of the pathogens, and limited number of seroprevalence test by herd. This index can be a useful tool for helping veterinary

practitioners to quickly target areas with possible high infection. Moreover, identifying risk of infection for cattle also has implications for human health by exposing veterinarians and farmers to tick bites in pastures during their outdoor activities of their profession. De Keukeleire et al. (2017) found higher *A. phagocytophilum* seroprevalence for this group in Belgium, comparing rural and urban blood donors. The same strains of *A. phagocytophilum* have been identified in humans and bovines, and the zoonotic potential of bovine strains should be considered until additional data are collected. (Bianchessi et al., 2023).

5. MODELLING THE ASSOCIATIONS BETWEEN TBD INDICATORS AND ENVIRONMENTAL FACTORS

5.1. MAPPING TICK-BORNE DISEASE RISK

Maps are a major tool for visualizing spatial information on tick-borne infections and for communicating potential risk of exposure (Lippi et al., 2021; Vu Hai et al., 2014). Regardless of their complexity, each map has a specific purpose and relies on maps rely on the availability of georeferenced datasets. Advanced spatial techniques are still underused in the communication of spatial information for tick-borne disease. Over height different mapping techniques, mapping raw occurrence points of pathogens and vectors remains the most common method used, over risk mapping (projected a modeled output onto a continuous geographic area), endemicity mapping (mapping tick-borne disease occurrence based on reviews of previous published data), and ecological niche modelling (applying a species distribution algorithm to predict the presence of a species based on occurrences of ticks and environmental drivers) (Lippi et al., 2021).

In this thesis, we produced different types of maps reflecting different processes. In **chapter 2**, our maps simply displayed tick abundance and infection prevalence at the location of the sampling units of the Bois de Lauzelle. In **chapter 4,** we elaborated a spatial index for the intensity of bovine infections with *A. phagocytophilum*. This index was based on the location of the pastures associated with seropositive herds and

had a strong spatial pattern, represented by the non-parametric kernel density estimation. In **chapter 5**, tick-bite risk maps for Belgian municipalities were generated and displayed. These maps serve as valuable tools to detect a spatial pattern in the risk and for risk assessment communication with the public or policy makers.

5.2. MODELLING TOOLS

Nowadays, improvements in statistical techniques and modeling create complex models that include the effects of environmental variables on tick-borne disease risk at different scales. Throughout this thesis, several spatial modelling techniques have been developed, in relation to the specificities of the different datasets: i.e., abundance indexes, serological tests, and citizen self-reports. Multi-levels regressions are common methods in spatial epidemiology to model the effect of specific predictors of interests on tick-borne disease indicators that are most of the time nested. In **chapter 2**, ticks were sampled on several occasions through the year in the same sampling units. The type of sampling unit was included as a random effect in generalized linear mixed models to account for repeated measures and autocorrelation. In **chapter 4**, we performed multi-level regressions to test the effects of five environmental variables on our index of pasture infection. Adding the agro-geographic regions as a random effect identified if the effects of the environmental variables vary through regions with different habitats. When possible, multi-levels must be considered as a gold standard to consider the nested structure of the data.

The exact location of tick exposure may not be accurately assessed, especially for cattle. Surveillance data from blood sample from a single animal in each herd, while allowing more herds being analysed in a limited-resource context, does not allow to interpret seronegative samples. We thus created a continuous index based on a kernel density function that differentiated areas according to the intensity of infection based on seropositive animals. The kernel density method is useful to identify spatial variations in the risk of infection, when the exact place of infection is unclear.

Finally, we analysed tick bite risk with the spatial overlay of different environmental drivers with an MCDA. This is an interesting alternative to data-driven methods

because of its transparency, ease of implementation and reproducibility in different regions. The choices of the environmental drivers and of the variables used to represent these drivers are the main challenges, as a tick-borne disease system is complex, with different species interacting and using different resources. These resources can be represented by different variables, complicating the risk assessment.

5.3. FACTORS INFLUENCING TICK-BORNE DISEASE INDICATORS

Throughout this thesis, we tested the effects of environmental variables selected based on their role in the tick-borne disease system as identified by the RBHC (Hartemink et al., 2015). The ecological variables used in the different studies were proxies for the resources needed by an organism to fulfil its ecological functions, and their biological interpretation may be challenging, especially if one proxy variable may serve for different resources. Moreover, proxy environmental variables also present uncertainties in terms of resolution and precision, which may influence the outcome of the statistical analyses. For example, measuring forest fragmentation with land cover data of different spatial resolutions may provide different results smallest forest patches are not detected in land cover data with the coarser spatial resolution.

The effects of the explanatory factors are scale dependent. The associations found at a specific scale, cannot be extrapolated at another spatial level (ecological fallacy). Throughout the thesis and consistently across different scales, the presence of forests remains the most important predictor for tick-borne disease risk in Belgium. Highest abundances of ticks were found within the Bois de Lauzelle (**chapter 2**). Pastures close to forests are more susceptible for bovine ehrlichiosis infections independently of the agro-geographic region of Wallonia (**chapter 4**). The presence of forests remains an important factor differentiating relative risks of tick bites between Belgium municipalities (**chapter 5**). These studies also confirmed the within forest tick heterogeneity (similarly to e.g., Van Gestel et al., 2021) and the various range of habitats for *I. ricinus* (similarly to e.g., Mathews-Martin et al., 2020).

Evaluate the effects of an environmental variable on tick-borne indicators across different regions based on empirical studies is another important challenge, as a

significant association with an environmental variable may be (i) consistently associated with the phenomenon regardless of the study conditions (ii) associated through specific conditions (iii) the result of a type 1 error of the model, i.e., rejecting a null hypothesis when it is true (Boyard et al., 2011). We assessed the reproductive effect of the environmental variables on the index of intensity with bovine ehrlichiosis (**chapter 4**), by realizing 200 regressions based on different sets of pastures. The results show consistency, as the variables tested appeared most of the time in the best models. The repetition of studies in different areas, scales and specific conditions may assess the reproducibility of the effects of environmental variables on tick borne disease risk.

Zeimes et al. (2015) identified three response scenarios to an environmental factor tested on a large area: similar effects over the whole area, non-linear effects depending on the value of the variable encountered, or no clear signal that cannot be interpreted at another scale or in another area. The important risk factors for *A. phagocytophilum* infection in pastures from Wallonia were (**chapter 4)**:

- Forests, especially fragmented forests, the preferred habitat for *I. ricinus* harbouring host species for ticks and the pathogen.
- Red deer and roe deer for the maintenance of large populations of *I. ricinus*.
- Grassland, associated to the presence of cattle, for the role of cattle in the persistence of *A. phagocytophilum* in the pasture.

The effects of these variables were relatively robust across the different agro-regions of Wallonia, irrespective of their differences in anaplasmosis intensity levels. The environmental variables can be incorporated into country-wide models or in models specifically focused on a single region of Belgium. However, these effects of the environmental variables have not been tested at a smaller scale and may therefore not be appropriate to identify local differences in risk of infection at the pasture level.

6. LIMITATIONS

The main limitation of the work on the acarological risk was the limited number of sampling units from the Bois de Lauzelle. Tick sampling is a labour-intensive method and only fourteen sampling units were selected to represent three types of habitats. We were able to identify and quantify differences in tick abundance between these different forested habitats and the seasons of tick activity. Nevertheless, studies, considering more sampling units, may confirm these observations.

Estimating tick population is particularly challenging (Bord et al., 2018). The consecutive drag method used in this thesis is a removal sampling method, commonly used by ecologists to estimate animal abundance by capturing individuals and removing them from the population over successive samplings (Tälleklint-Eisen and Lane, 2000). This technique offers better estimates of the "true populations" than single drag dragging. However, extrapolating the "true" population size from these estimates with Bayesian or frequentist methods remain limited due to the uncertainties related to population abundance and sampling rate, especially when the sampling rate is small, i.e., less than 10% (Bord et al., 2018).

Variability in the estimations of tick abundance by dragging was associated with two types of factors: those representing the presence of suitable conditions and habitats for ticks and their hosts, and those influencing tick capture efficiency. As these factors are often represented by the same sets of variables, it is difficult to disentangle their respective effects. Tick abundance data are often associated with meteorological data from nearby official weather stations, that have a strong spatial measuring error with the conditions measured in forests (De Frenne and Verheyen, 2016). Occasional measures may also fail to record the periodicity in meteorological conditions in the area and the periodicity in tick activity (Boehnke et al., 2017). Therefore, at the Bois de Lauzelle, temperature and humidity were continuously measured from one location in the interior of the forest. However, this single location does not represent the fine scale heterogeneity in microclimatic conditions encountered by ticks in the forest. It illustrates the need for continuous in situ measurements to grasp the complex relative humidity conditions in forests. However, in term of resource allocation in scientific

studies, it is still an open question to what kind of meteorological data are necessary to answer specific questions in tick research (Boehnke et al., 2017).

Arthropod vector are commonly screened in pools, especially due to high cost of diagnostic and to optimise resources, when a high number of ticks are tested and for pathogens with low infection prevalence (McLure et al., 2021). Tick infection prevalence are then estimated by screening these pooled ticks by PCR targeting segments of specific genes (Frank et al., 2008). In **chapter 2**, tick infection prevalence with three pathogenic bacteria was estimated based on a limited number of pools (499) of four ticks from the same sampling unit and season. The low number of pools per sampling units did not permit identifying variations between different locations of the forest, due to the low number of pools per sampling units. The mean tick infection prevalence with *B. burgdorferi* was low: estimated at 3.77% in 2016 and 5.33% in 2018. This may be an underestimation of the true prevalence. First, the exact number of infected ticks in a pool tested positive cannot be determined. Tick infection prevalence was estimated with maximum-likelihood estimates, a method that is more reliable than the pool positivity rate and minimum infection rate methods (McLure et al., 2021). Second, pools with a high number of ticks and low infection prevalence or high engorgement status may decrease the sensitivity of the diagnostic (dilution effect) (Fracasso et al., 2023). Halos et al. (2010) also found similar low *B. burgdorferi* prevalence, but they tested pools composed by 5, 10 and 50 ticks. The dilution effect was less likely for the pools of ticks of the Bois de Lauzelle, as they were grouped by four or less. Third, pools may contain different life stages (as for the ticks from 2016-samplings but not from the 2018-samplings).

Contamination cannot be excluded in our analyses of tick microbiota, but it was likely limited. Most tick-skin bacteria were probably, due to the storage at -80°C between the samplings and the analyzes. Moreover, we used a strict surface washing on each tick before DNA extraction. The washings consisted in consecutive baths with sterile water and ethanol that also removed the microorganisms present on their surface. Sterilization protocols are highly important for tick screenings, as ticks are likely to harbour external microbes from the skin microbiome of their vertebrate hosts during

blood-feeding, and from the soil or plants during their they are “off-host” period (Binetruy et al., 2019). Most bacterial isolated in this study have already been identified in sterilized tick before (Kmet’ and Čaplová, 2019). Sterilization is a complex process. Although ethanol washing kills external microbes, but will not denature their DNA, that may remain present at the tick’s cuticle. This constitutes a potential source of contamination. Further studies should consider using bleach over ethanol for tick washing, as the latter method may greatly impact results found for the internal bacterial diversity (Binetruy et al., 2019). Finally, ticks were not dissected before crushing. Therefore we could not differentiate bacteria found in different parts of the tick (e.g., in the midgut or ovaries), which is important to understand their respective roles in tick fitness (Guizzo et al., 2020).

The effects of variables related to animal hosts on tick-borne disease indicators were tested throughout the thesis at different scales and with different datasets. We could not identify the precise presence of hosts at each sampling units at the Bois de Lauzelle (**chapters 2** and **3)**, and only used indications on the presence of specific animal species in the forest, assuming they were likely to be present everywhere. We could have used camera traps to detect the presence of vertebrates, as in the studies of Hofmeester et al. (2017) and Ruyts et al. (2018). They identified multiple species with camera traps and bait, including bank vole, wood mouse, roe deer, wild boar and common blackbird. In **chapters 4** and **5**, rodent habitat was estimated with land cover configuration variables, by using forest ecotones as a proxy. Accurate estimation of rodents over large areas is challenging because their populations are likely to fluctuate cyclically between years, particularly between years with different levels of production of tree seeds (Gray and Kahl, 2022). The presence or abundance of three wild large mammals (red deer, *Cervus elaphus*, roe deer, *Capreolus capreolus*, and wild boars, *Sus scrofa*) were estimated from shots animals over large areas. These estimations were measured at coarse resolution and did not indicate the distribution of these species within these large areas.

In **chapter 4**, we could not determine the different ecotypes of *A. phagocytophilum* from the blood samples of bovine. Domestic ruminants are commonly associated to

Ecotype 1, which is probably the only zoonotic ecotype in Europe (Bianchessi et al., 2023; Dugat et al., 2014). Ecotype differentiations of *A. phagocytophilum* is an ongoing research topic. The RBHC method applied to the predictors in chapter 4 need to be adapted if the ecotypes, associated to distinct hosts (rodents, birds and are birds for Ecotypes 2, 3 and 4 respectively) circulate in ecologically distinct transmission cycles (Jahfari et al., 2014).

7. OUTLOOK AND PERSPECTIVES

This work contributed to the understanding of tick-borne disease and identified different for future research.

7.1. MONITORING AT THE BOIS DE LAUZELLE

The Bois de Lauzelle may serve as an interesting study area to monitor annual variations in tick population and to examine human exposure within a forest because this forest (i) has regulations preserving biodiversity (e.g., Natura 2000). (ii) is located a few kilometers away from city centers and is frequently visited. (iii) has fine scale estimates of the entomological risk in different locations and habitats (**chapter 2**). Further tick samplings will help in tracking changes in tick abundance, distribution, and infection rates over time in relation with temporal variations in environmental and meteorological factors.

The factors affecting the adoption of risky behaviours for tick bites often have an individual-based nature. The decision of, e.g., walking out of the trails or neglecting preventive measures, may be influenced by past experiences and previous knowledge about ticks and tick-borne diseases (Bord et al., 2022). An exploratory study from a recent master thesis interviewed more than 150 visitors of the forest in 2021 (Couck et al., 2022).. Preliminary results indicated that these visitors acquired knowledge on tick bite born disease more often from internet (35.7%) than from the doctor (24%). These visitors also have a limited use of protection measures against ticks, with most of them never or rarely: used tick repellent (88%), put their pants in their socks (88%), wear long clothes (55%) and performed regular tick checks (48%). Aenishaenslin et

al. (2017) found similar results in Canada, with less than half of the persons who having adopted specific preventive behaviours toward tick bites, despite high Lyme borreliosis awareness. Understanding the factors influencing the adoption of preventive behaviour is of primary interest for public health. Moreover, identifying the frequentation of forest routes may provide useful information for forest managers to lead the flow of visitors through safer locations. Further investigations are needed to understand human behaviour regarding tick-borne disease risk in forest.

7.2. THE CONSECUTIVE DRAG TECHNIQUE TO SAMPLE TICKS FROM DIFFERENT TYPES OF HABITATS

It is now commonly accepted that *I. ricinus* is not an exclusive forested species and that tick exposure happens in a variety of semi-natural habitats (Garcia-Marti et al., 2018). In addition to tick exposure in forests, people may also be exposed in a large range urban and peri-urban settings, including, urban parks (Hassett et al., 2022; Mathews-Martin et al., 2020), private gardens (Drehmann et al., 2019; Richter et al., 2023), or urban green spaces (Heylen et al., 2019). These areas pose substantial risk, as they are generally associated with lower tick densities but higher human presence. More tick bites have been reported from gardens over forests in TiquesNet/TekenNet (https://epistat.sciensano.be/ticks/, last access, 23rd of August 2023). However, many citizens do not identify gardens, parcs or other grassy areas as at risk for tick-borne disease and do not systematically use prevention techniques when entering these habitats. Moreover, little is known about the characteristics of residential settings that support tick population and human exposure (Richter et al., 2023).

This situation creates opportunities to initiate long-term monitoring of tick populations throughout Belgium. Recently, new ongoing citizen science projects were launched to determine the levels of tick-borne disease risk, and the characteristics associated in residential settings. Examples of these projects are Teek a break (https://www.uantwerpen.be/nl/projecten/teek-a-break/, last access 23rd of August, 2023) in Flanders (Belgium) and TiquOJardin (https://www.citique.fr/tiquojardin/, last access 23rd of August, 2023) in France. A holistic approach, combining expertise from acarology, environmental science, human medicine and social sciences is needed

to continue the surveillance in urban settings. The consecutive drag technique implemented in **chapter 2** may be of primary interest, as these settings result in various habitats, and therefore different dragging efficiencies.

7.3. ROLE OF HOSTS COMMUNITIES ON TICK-BORNE PATHOGENS

Improving our understandings of the roles of vertebrate host communities in the maintenance, circulation and transmission of tick-borne pathogen is an important ongoing topic, as it may contribute to the identify areas with increased risks for tick-borne pathogens. There is a need to collect more information on the presence and spatial distribution of tick hosts, because many species are still understudied, including the Eurasian jay (*Garrulus glandarius*), Eurasian red squirrel (*Sciurus vulgaris*), European badger (*Meles meles*), or fallow deer (*Dama dama*) (Fabri et al., 2022; Hofmeester et al., 2016). The role of birds on tick-borne disease infection is another ongoing topic of research, for their implication in *Borrelia burgdorferi* genospecies transmission and their role in tick dispersion. For example, song birds have already been under investigation for the recent years by Heylen et al. (2016, 2014). The RBHC may offer theoretical background to incorporate different species in the TBD system.

Anaplasma phagocytophilum is characterized by a high degree of genetic diversity, resulting in different strains (ecotype) whose classification is an ongoing area of research (Fabri et al., 2022; Jahfari et al., 2014). To date, four ecotypes of *A. phagocytophilum* have been identified in Europe, based on the sequence of the groEL heat shock operon gene (Jahfari et al., 2014). These ecotypes might circulate in ecologically distinct transmission cycles in various geographic areas (Bianchessi et al., 2023). Ecotype 1 has been associated with many different hosts, including human and livestock, while Ecotypes 2,3 and 4 were mostly associated with roe deer, rodents and birds respectively (Jahfari et al., 2014). *Ixodes ricinus* is vector for all *A. phagocytophilum* ecotypes, but ecotypes 3 and 4 have also been identified in *Ixodes trianguliceps* for the former and in *Ixodes frontalis* and *Ixodes ventalloi* for the latter (Scharf et al., 2011). Further research is needed to confirm these observations and

understand the roles of the different tick species in the maintenance and circulation of the different ecotypes in natural cycles.

Most wild ungulates species are increasing in size and population in several parts of Europe (Hofmeester et al., 2016), including Belgium (Prévot and Licoppe, 2013) and may affect ticks and tick-borne pathogens dynamics. In **chapter 4**, roe deer was significantly associated with our index of bovine ehrlichiosis infection, but only when the agro-geographic regions were not included. It may relate to (i) data source and its processing, (ii) the different associations of roe deer with ecotype 2 of *A. phagocytophilum* compared to ecotype 1 for cattle. Therefore, the significant association between roe deer and the bovine ehrlichiosis index in the model excluding the agro-geographic regions may be spurious and caused by specific characteristics of the regions with high roe deer population rather than roe deer populations themselves. More research on the different ecotypes is needed to improve our understanding of the epidemiology of the diseases caused by *A. phagocytophilum* bacteria.

7.4. CHARACTERIZING TICK MICROBIOTA

Chapter 3 gave the first indications on *I. ricinus* microbiota in Belgium, which is useful for surveillance of tick microbiota composition. Further interdisciplinary studies on microbial ecology is still required to characterize tick microbiota communities, their source of acquisition, level of adaptation in the tick, and mutual interactions, as well as their interactions with the tick hosts. The addition of studies covering different areas and tick populations is still needed, as the microbiota of *Ixodes* ticks was variable with regard to geography, species and sex (van Treuren et al., 2015)

Determining elements of tick microbiota may also offer opportunities to identify microorganisms of veterinary or public health importance, preventing future potential diseases in domestic animals or zoonoses. The isolation of opportunistic bacteria of veterinary or public health importance, such as *Cedecea davisae*, from human-biting tick species constitutes only the first step in the surveillance of specific, potentially harmful, microorganisms. Experimental studies of *I. ricinus* vectorial competence for

all the potential pathogens detected must be performed before reclassifying them as tick-borne pathogens (Vayssier-Taussat et al., 2013). These microorganisms need to be detected in the ticks, but also to physiologically survive and develop in the tick, to survive molting, and to be transmitted to the next tick host.

7.5. MAPPING HUMAN EXPOSURE AT NATIONAL LEVEL

Our tick-bite risk map (**chapter 5**) included variables related to hazard and exposure. While hazard-variables at the national level are relatively well understood (forests, wild hosts, and suitable climatic conditions), those related to exposure are more difficult to assess. In our study, we used mainly variables related to the land (presence of parcs, gardens, recreative areas, and trails), and one related to the presence of tourists. Recent studies tried to integrate human behaviours into integrated socio-behavioural and ecological risk maps in Canada (Bouchard et al., 2023, 2018). Human behaviours were estimated with three indicators: and the human population density and two indicators based on the adoption of preventive behaviours (Bouchard et al., 2023). These elements but they require surveys to collect this information from the Belgian population.

7.6. CITIZEN SCIENCE DATA

Citizen-based data is increasingly used as a source to complement traditional scientific data collection, including in the health-related topics (e.g., Garcia-Marti et al., 2018; Porter et al., 2019; Tran et al., 2021; Zhang and Zhu, 2018). It offers multiple advantages, including data collection from broader geographic coverage and increase involvement and awareness of the public (Porter et al., 2019). There may also be reporting bias, including the uncertain location of tick bites (Zhang and Zhu, 2018). Participation bias may also be substantial in citizen sciences approaches, leading to a group of participants who does not correspond to the general population. Citizens from higher income and higher educated groups are more likely to participate in programs on a voluntary basis (Gamo and Park, 2022; Henderson and Chatfield, 2011). Although tick bite reports were free of charge in TiquesNet/TekenNet, there may be a participation bias in favour of citizens with high education, or high levels of

knowledge about tick-borne diseases. Collecting socio-economic information about the participants of TiquesNet/TekenNet could facilitate the identification of participation bias. For example, age has been associated to different activities and habitats of tick bite records in the Netherlands, with citizen aged from 0 to 60 being more likely to report tick bites during forest visits and hikes, whereas people older than 60 were mainly bitten in gardens (Mulder et al., 2013). However, asking personal information, even confidentially, may also discourage citizens' participation.

7.7. CITIZEN INVOLVEMENT IN TICK-BORNE DISEASE RISK

The involvement of citizens in tick-borne disease related projects remains mainly limited to the report of tick bites or the collection of ticks by flagging or by sending human-biting ticks (e.g., Eisen and Eisen, 2021; Garcia-Marti et al., 2018; Lernout et al., 2019). Studies assessing tick-borne disease risk could greatly benefit from the participation of citizens in multiple steps of the projects. Using non-scientist local knowledge may offer a better understanding of the tick-borne disease system locally; while focusing on issues directly identified by the population may strengthen their knowledge and acceptation of further public health measures, as encouraged by the One Health framework.

Spatial studies on ticks and tick-borne disease remain mostly limited to Europe and North America, that have relatively good surveillance programs. Globally, most tick-borne pathogens remain under-supervised and understudied, despite their threat for human and animal health and the influence of global environmental changes (Lippi et al., 2021; Medlock et al., 2013). According to a recent literature review, only 9 out of 27 important tick-borne pathogens were associated to more than 10 mapping studies, with the majority focusing on Lyme borreliosis (40.26%) and tick-borne encephalitis (15.51%), the main tick-borne pathogens of western countries (Lippi et al., 2021). The majority of tick-borne pathogens remain undermapped or not mapped.

Participatory-geographic information system is an option to development citizen empowerment over health-related issues (Orban-Ferauge et al., 2011). This bottom-up approach offers opportunities to combine their needs, visions and ideas with

geographic methods and techniques. It can serve as starting point for communication and discussion with experts of health-related fields, and with policy makers. The GIS-based MCDA approach, developed in **chapter 5** for tick bite risk mapping in Belgium, is a valid option to create risk maps involving citizens, especially in a data-scarce context (Aenishaenslin et al., 2016, 2015; Hongoh et al., 2011). It could be particularly appropriate for understudied tick-borne pathogens. Stakeholders may participate in the selection and weighting of relevant predictors, and in the validation of the MCDA risk maps. The RBHC also may serve as starting point for predictors' selection, as it provides an adaptable framework to identify key resources for different tick-borne disease system and regions.

Improving tick-borne disease surveillance and citizen awareness and involvement in this subject are, in my opinion, keys to offer a better health to humans and animals. The interdisciplinary work, the development of modelling techniques, and the increasing availabilities of open-sources dataset offer opportunities for scientists to progress in the understanding of disease systems, and, therefore, to improve the health of the environment, of animals, and of all humans.

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