Aus dem Bereich Biologie, der Medizinischen Fakultät der Universität des Saarlandes, Homburg/Saar und dem Institut für Immunologie/Centre de Recherche Public de la Santé, Luxemburg

Prävalenz und Diversität durch Zecken übertragener Pathogene in Mittel- und Osteuropa sowie Westafrika

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Prevalence and Diversity of Tick-Borne Pathogens from Central and Eastern Europe as well as West Africa

DISSERTATION

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Anna Lena Reye

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List of Abbreviations

ACA	Acrodermatitis chronica atrophicans
ANOVA	Analysis of Variance
ASTA	Administration des services techniques de l'Agriculture
BCIP	5-Bromo-4-Chloro-3'-Indolyphosphate
BLAST	Basic Local Alignment Search Tool
CCHFV	Crimean-Congo Hemorrhagic Fever Virus
CNER	Comité National d'Ethique de Recherche
CNPD	Commission nationale pour la protection des données
CON	Consecutively
CRP	Centre Recherche Publique
CS	Collection Site
D	Deer
DC	Distinct Cluster
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
ddNTP	Dideoxynucleotide Triphosphate
DRF	Dermacentor reticulatus Francisella like endosymbiont
DTT	Dithiothreitol
DVF	Dermacentor variabilis Francisella like endosymbiont
ELISA	Enzyme Linked Immunosorbent Assay
ESRI	Environmental Systems Research Institute
F	Female
FLE	Francisella Like Endosymbiont
FSME	Frühsommer-Meningoenzephalitis
GFX	Pearson's goodness of fit chi-square
GIS	Geographic Information System
GNSS	Global Navigation Satellite System
GPS	Global Positioning Systems
HAF	Hyalomma aegyptium Francisella like endosymbiont
HC	Habitat Category
HGA	Human Granulocytic Anaplasmosis
HME	Human Moncytic Ehrlichiosis
HMF	Hyalomma marginatum marginatum Francisella like endosymbiont
IgG	Immunoglobulin G

IgM	Immunoglobulin M
LNS	Laboratoire National de Santé
LNVL	Letzebuerger Natur- a Vulleschutzliga
M	Male
N	Nymph
NA	Nucleic acid
NBT	Nitro-Blue Tetrazolium
NCBI	National Center for Biotechnology Information
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
R	Rodent
RAL	Rickettsia africae like
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Rounds per minute
RRG	Rickettsia rickettsii Group
rRNA	Ribosomal RNA
RS	Remote Sensing
SFG	Spotted Fever Group
SIM	Simultaneous
ST	Serotype
TBE	Tick-Borne Encephalitis
TBEV	Tick-Borne Encephalitis Virus
TD	Tick Density
TIBOLA	Tick-Borne Lymphadenopathy
TIR	Tick Infection Rate
TMB	Tetramethylbenzidine
TNA	Total Nucleic Acid

Zusammenfassung

Zecken zählen zu den wichtigsten Überträgern von Krankheitserregern die ernsthafte Infektionen bei Mensch und Tier auslösen können. Sie haben eine weltweite Verbreitung und sind in tropischen, subtropischen und gemäßigten Klimaten beheimatet. Nach Stechmücken sind Zecken die wichtigsten Überträger zoonotischer Infektionen des Menschen und verursachen jährlich mehr als 100.000 Erkrankungen weltweit (42). Zudem sind sie die wichtigsten Überträger von Infektionskrankheiten auf Wild- und Haustiere mit jährlichen wirtschaftlichen Verlusten in Milliardenhöhe (160, 226).
In Europa sind die wichtigsten von Zecken übertragenen Infektionskrankheiten des Menschen die Lyme Borreliose und Frühsommer-Meningoenzephalitis (FSME), die zusammen im Jahr mehrere zehntausend Neuinfektionen hervorrufen (46, 94, 127). Auf dem afrikanischen Kontinent stellen Zecken insbesondere für den Viehbestand eine große Bedrohung dar. Insbesondere die vier Infektionskrankheiten Anaplasmose, Herzwasserkrankheit, Babesiose und Theileriose verursachen dabei die größten wirtschaftlichen Verluste (10).

Im Rahmen der vorliegenden Dissertation untersuchten wir die Prävalenz von Krankheitserregern in Zecken aus Westeuropa (Luxemburg), Osteuropa (Weißrussland, Bulgarien und der russischen Enklave Kaliningrad) und Westafrika (Nigeria).
In Luxemburg sammelten wir im Laufe der Jahre 2007, 2008 und 2009 in der Aktivitätsphase der Zecken insgesamt 8.104 Larven, Nymphen und Adulttiere des Gemeinen Holzbockes

(*Ixodes ricinus*) von der Vegetation. Von Mai bis Oktober wurden dazu insgesamt 33 Probestellen einmal monatlich nach Zecken abgesucht. In den drei Untersuchungsjahren konnten wir signifikante Unterschiede in der Zeckendichte von Jungstadien, nicht jedoch von Adulttieren feststellen. Wir vermuten, dass das Zusammenspiel abiotischer und biotischer Faktoren ausschlaggebend für die beobachtete Populationsdynamik von *Ixodes ricinus* ist. Insgesamt wurden 5.638 Nymphen und Adulttiere auf das Vorhandensein aller in Europa relevanten, durch Zecken übertragenen Humanpathogene mittels spezifischer PCRs untersucht. Die durchschnittliche Infektionsrate der Zecken lag bei 16,3% für *Borrelia burgdorferi* sensu lato, 6,7% für Fleckfieber-Rickettsien, 1,8% für *Babesia*-Arten, 0,9% für *Anaplasma phagocytophilum* und 0,1% für *Bartonella*-Arten. Zusätzlich detektierten wir in einer Zecke das nicht-endemische Pathogen *Hepatozoon canis*, welches beim Hund die Hepatozoonose auslösen kann. Dieser Erreger wurde möglicherweise versehentlich durch einen infizierten Hund aus dem südlichen Europa nach Luxemburg eingeschleppt (6). In allen drei Jahren wurden weder das FSME-Virus, noch *Coxiella burnetii* oder Unterarten von *Francisella tularensis* in Zecken aus Luxemburg detektiert. Koinfektionen wurden in 3,3% der Zecken nachgewiesen und setzten sich hauptsächlich aus den vorherrschenden *Borrelia*- oder *Rickettsia*-Arten zusammen. Saisonale Veränderungen in der Zeckeninfektionsrate durch *Borrelia*-Arten konnten für jedes Jahr für nachgewiesen werden, obwohl diese am deutlichsten im Jahr 2007 ausfielen. Die von uns aufgestellte Hypothese, dass der in den Sommermonaten beobachtete Rückgang der Zeckeninfektionsrate auf Verhaltensänderungen infizierter Zecken zurückzuführen ist, scheint von den Ergebnissen einer aktuellen Studie zur Überlebensrate von *Borrelia*-infizierten *Ixodes ricinus* Zecken unterstützt zu werden (89). Des Weiteren konnten wir eine positive Korrelation zwischen dem Urbanisierungsgrad der Probestellen und der dortigen Zeckeninfektionsrate mit Borrelien zeigen, was auf einen möglichen etablierten Zyklus urbaner Zoonosen schließen lässt.

Die signifikanten jahresübergreifenden, saisonalen und regionalen Unterschiede sowohl in der Zeckendichte als auch der Zeckeninfektionsrate deuten darauf hin, dass nachfolgend auch signifikante Unterschiede des Zeckenstich- und Infektionsrisikos für den Menschen zu erwarten sind. Obwohl diese beobachteten Veränderungen durch abiotische und biotische Faktoren bedingt zu sein scheinen, sind jedoch weitere Studien notwendig, um die Populationsdynamiken von Zecken und Krankheitserregern aufzudecken.

Zur Abschätzung des Gesundheitsrisikos durch Lyme Borreliose in beruflichen Risikogruppen führten wir eine Seroprävalenzstudie an 280 Waldarbeitern aus Luxemburg durch und konnten in über 35% der untersuchten Waldarbeiter spezifische IgG

Serumantikörper gegen Borrelien nachweisen. Die Seroprävalenz von Borrelien stieg mit zunehmendem Alter, längerem berufsbedingtem Aufenthalt im Freien, sowie höherer Anzahl an Zeckenkontakten und Zeckenstichen. Die Anwendung von Präventivmaßnahmen wie Zeckenrepellentien, regelmäßige Körperinspektion und frühzeitiges Entfernen von saugenden Zecken schien keine Auswirkung auf die Seroprävalenzrate zu haben, nur das Tragen schützender, heller Kleidung schien einen leicht positiven präventiven Effekt auszuüben. Die regionale Verbreitung der Seroprävalenzrate bei Waldarbeitern stimmte bis zu einem gewissen Grad mit der regionalen Verbreitung der Borrelienprävalenz in Zecken überein, wobei ein direkter Vergleich jedoch dadurch erschwert ist, dass IgG Antikörper über Jahre hinweg persistieren können und die Infektion nicht notwendigerweise am Arbeitsplatz erfolgte.

In Nigeria wurden insgesamt 836 Zecken von der Vegetation und von Rindern gesammelt, die vier Zeckenarten zugeordnet werden konnten (*Rhipicephalus* (*Boophilus*) *annulatus*, *Amblyomma variegatum*, *Hyalomma impeltatum*, *Rhipicephalus evertsi*). Vorherrschende Krankheitserreger waren eine Rickettsienart, die mit der humanpathogenen Art *Rickettsia africae* verwandt zu sein scheint, sowie das Rinderpathogen *Anaplasma marginale*. Als weitere Krankheitserreger von Wiederkäuern wurden *Theileria mutans* und *Coxiella burnetii* in saugenden Zecken detektiert, wohingegen eine potentielle neue Borrelienart aus der Gruppe *Borrelia burgdorferi* s.l. nur in Zecken aus der Vegetation nachgewiesen werden konnte. Basierend auf unseren Ergebnissen einer erhöhten Diversität von Krankheitserregern in saugenden als in lauernden Zecken, vermuteten wir, dass entweder Rinder wichtige Reservoirwirte dieser Erreger darstellen könnten. Die Bedeutung dieser durch Zecken übertragenen Infektionskrankheiten für die Gesundheit von Mensch und Tier in Nigeria und die daraus resultierenden wirtschaftlichen Verluste müssen zukünftig ermittelt werden.

In Weißrussland führten wir eine Studie an insgesamt 553 *Ixodes ricinus* und *Dermacentor reticulatus* Zecken durch und stellten eine hohe Prävalenz von Rickettsien (24,4%) fest. Die vornehmlich in Zecken der Gattung *Dermacentor* gefundene, nicht näher identifizierbare Rickettsienart aus der *Rickettsia rickettsii*-Gruppe (43,8%) ließ auf eine hohe Rate transovarialer Transmission schließen. Borrelien wurden in 9,4% der Zecken gefunden, während andere Pathogene wie *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Bartonella henselae*, Unterarten von *Francisella tularensis* und *Babesia*-Arten seltener detektiert wurden. In unserer Studie konnten wir zeigen, dass lauernde und saugende Zecken in Weißrussland zu einem hohen Maße mit Krankheitserregern infiziert sind und ein Infektionsrisiko für Mensch und Tier darstellen. Die von uns identifizierten Hotspots mit hohen Zeckeninfektionsraten

sollten in zukünftige Surveillance-Studien eingebunden werden, insbesondere bei Beteiligung der hochinfektiösen, auch durch Aerosole übertragenen Bakterien *Coxiella burnetii* und *Francisella tularensis*.

In einer weiteren Studie wurden aus in Bulgarien gesammelten Zecken Endosymbionten isoliert, die eine große Ähnlichkeit mit humanpathogenen *Francisella*-Arten haben. Durch weitere molekulare Charakterisierung konnte gezeigt werden, dass diese isolierten Bakterien fakultative sekundäre Endosymbionten von Zecken zu sein scheinen. Nichtsdestotrotz ist eine Bestimmung des pathogenen Potentials dieser neuen Arten angebracht.

Um die Rolle von Zugvögeln auf die Ausbreitung von Fleckfieber-Rickettsien und *Babesia*-Arten zu untersuchen, wurden 126 Zecken von Zugvögeln aus der Kurischen Nehrung in der Enklave Kaliningrad, Russland gesammelt. Unsere Funde von Rickettsien in 15,1% und Babesien in 1,6% der Zecken zeigen, dass auch Zugvögel für die Verbreitung dieser Krankheitserreger verantwortlich sein könnten. Weitere Studien sind jedoch nötig, um die Auswirkungen des Vogelzugs auf die Verbreitung dieser beiden von durch Zecken übertragenen Krankheitserregern zu ermitteln.

Die von uns durchgeführten Untersuchungen heben die Notwendigkeit andauernder Surveillance-Studien zur Prävalenz, Verbreitung und Ausdehnung von Zeckenarten und Krankheitserregern hervor. Im Zuge des klimatischen Wandels könnten permanente Veränderungen abiotischer Faktoren wie Temperatur und relative Luftfeuchtigkeit die Überlebenschancen nicht-endemischer Zeckenarten positiv beeinflussen. Da eine zeitnahe Entdeckung invasiver Zeckenarten möglicherweise die Etablierung exotischer Zoonosen verhindern könnte, ist die Beobachtung der Zeckenbestände von großer Bedeutung. Zu diesem Zweck sollten zukünftig einheitliche Richtlinien zum Studiendesign etabliert werden.

Abstract

Ticks are important vectors of human and animal pathogens and are endemic in tropical, subtropical and temperate regions throughout the world. After mosquitoes, ticks are the second most important vector of human diseases worldwide, responsible for more than 100,000 cases of tick-borne diseases annually (42). Furthermore, ticks are the main arthropod vector of disease in wild and domestic animals throughout the world with estimated minimal economic losses of US$ 7 billion annually (160, 226).
In Europe the most important tick-borne diseases in humans are Lyme Borreliosis and Tick-Borne Encephalitis (TBE), both causing tens of thousands of severe infections in humans annually (46, 94, 127). In Africa, the most important health threat caused by tick-borne pathogens is to livestock animals and major economical losses have been associated with the four tick-borne diseases anaplasmosis, heartwater, babesiosis and theileriosis (10).

In the framework of the present dissertation we analysed the prevalence of tick-borne pathogens in questing and feeding ticks from Western Europe (Luxembourg), Eastern Europe (Belarus, Bulgaria and the Kaliningrad enclave) and sub-Saharan Africa (Nigeria).
In Luxembourg, 8,104 larval, nymphal and adult *Ixodes ricinus* ticks were collected from the vegetation during the activity period of ticks in the years 2007, 2008 and 2009. A total of 33 collection sites were visited on a monthly basis from May to October. Significant variations of density of immature tick instars were observed in the three consecutive years, whereas the

density of adult ticks remained similar. We hypothesized that the interplay of abiotic and biotic parameters is responsible for the observed population dynamics of *Ixodes ricinus*. Altogether 5,638 nymphal and adult ticks were analysed for the presence of all human tick-borne pathogens relevant in Europe using specific detection PCRs. The mean infection rate of ticks were 16.3% for *Borrelia burgdorferi* sensu lato, 6.7% for Spotted Fever Group (SFG) *Rickettsia* species, 1.8% for *Babesia* species, 0.9% for *Anaplasma phagocytophilum* and 0.1% for *Bartonella* species. We also found the non-endemic dog pathogen *Hepatozoon canis* in a female *Ixodes ricinus* tick, which possibly was introduced accidentally by the import of an infected dog from Southern Europe (6). Neither Tick-Borne Encephalitis (TBE) virus, nor *Coxiella burnetii* nor *Francisella tularensis* subspecies were detected in Luxembourgish ticks. Mixed infections were detected in 3.3% of all ticks and mainly involved the predominant *Borrelia* and *Rickettsia* species. Seasonal variations of tick infection rates were observed for the different *Borrelia* species each year, albeit most clearly in 2007. Our hypothesis that the observed decrease of the tick infection rate during summer months reflects a behavioural adaptation strategy of infected questing ticks seems to be supported by a recent study on the influence of *Borrelia* infection on the survival rate of ticks (89). Furthermore, we observed a positive correlation between the grade of urbanization and the *Borrelia* infection rate of ticks, suggesting an established urban zoonotic cycle. The significant interannual, seasonal and regional variations in the density of ticks and the prevalence of *Borrelia burgdorferi* s.l. and the other tick-borne pathogens entail that likewise significant changes in the risk of tick bites and infection are to be expected. The observed variations seem to be linked to abiotic and biotic factors, but further studies on the dynamics of ticks and tick-borne pathogens are warranted.

A seroprevalence study was conducted on samples from 280 forestry workers from Luxembourg and 35.4% displayed specific anti-*Borrelia burgdorferi* s.l. IgG antibodies. Evaluation of questionnaires revealed that age, hours spent outdoors, the number of tick encounters and the number of tick bites per year were important risk factors for exposure to spirochetes. The use of preventive measures like tick repellents, frequent body inspection and early removal of ticks did not seem to reduce the risk of exposure, whereas protective clothing seemed to have a slight beneficial effect. The regional variations in the seroprevalence rate of forestry workers matched to a certain extent with the *Borrelia* infection rate of questing ticks. However, comparison of the two distributions is difficult, as IgG antibodies can persist for years and infection was not necessarily obtained during working hours. According to our

findings, Lyme Borreliosis is a major health concern in professional risk groups in Luxembourg.

In Nigeria, 836 ticks were collected from the vegetation and cattle. Four tick species were found to infest cattle (*Rhipicephalus* (*Boophilus*) *annulatus*, *Amblyomma variegatum*, *Hyalomma impeltatum*, *Rhipicephalus evertsi*), whereas only the latter species was collected from the vegetation. Predominant pathogens were the *Rickettsia* species closely related to the human pathogenic *Rickettsia africae* and the cattle pathogen *Anaplasma marginale*. *Theileria mutans* and *Coxiella burnetii*, which both are known to infect ruminants, were detected only in feeding ticks, whereas a potentially new member of the *Borrelia burgdorferi* s.l. group was detected only in questing ticks. Based on our finding that the diversity of pathogens was significantly higher in feeding than in questing ticks we hypothesized that Nigerian cattle may serve as an important reservoir for at least some of the detected pathogens. The impact of tick-borne infections on human and animal health and the resulting economic losses need to be further assessed.

In Belarus, 553 *Ixodes ricinus* and *Dermacentor reticulatus* ticks were collected from the vegetation and cattle and the pathogen diversity was found to be higher in questing than in feeding ticks. *Rickettsia* species were detected in 24.4%, predominantly a species related to the *Rickettsia rickettsii* group were found. This species was responsible for the high *Rickettsia* infection rate of 43.8% in *D. reticulatus* ticks, suggesting high rates of transovarial transmission. *Borrelia burgdorferi* s.l. was detected in 9.4% of ticks, whereas the other pathogens *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Francisella tularensis* subspecies, species of *Babesia* and *Bartonella* were less frequently detected. We identified hotspots with high tick infection rates, which should be included in future surveillance studies, especially when *Francisella tularensis* and *Coxiella burnetii* are involved. Our survey revealed a high burden of tick-borne pathogens in questing and feeding *I. ricinus* and *D. reticulatus* ticks in different regions in Belarus, indicating a potential risk for humans and animals.

We also reported on the identification of two new *Francisella*-like endosymbionts (FLE), found in three different tick species from Bulgaria. The FLEs were molecularly characterized and seem to be facultative secondary endosymbionts of ticks. However, further studies are necessary to determine the pathogenic potential of these species.

In order to investigate the role of migratory birds in the spread of SFG Rickettsiae and *Babesia* species, 236 wild birds comprising of 8 species of Passeriformes were collected at Curonian Spit in Kaliningrad enclave of North-Western Russia. In total, 126 ticks were removed and analysed for the two pathogens. *Rickettsia* species were detected in 15.1% and

Babesia species in 1.6% of ticks. The survey indicates that migratory birds may become a reservoir for *Babesia* spp. and SFG Rickettsiae and are involved in their geographic dispersal. Future investigations need to characterize the role of birds in the epidemiology of these human pathogens in the region.

Our studies highlight the need for continuous surveys on the prevalence, distribution and spread of tick species and tick-borne pathogens. In the course of climatic change permanent changes in abiotic prerequisites like temperature and relative humidity may enhance survival of non-endemic tick species. Timely detection of invasive tick species and application of countermeasures can possibly prevent the establishment of exotic zoonoses. Tick surveillance is an important measure for the better understanding of the epidemiology of tick-borne pathogens and the population dynamics of the main vector ticks. However, the establishment of guidelines for the design of future studies is warranted.

1 Introduction

1.1 Ticks and Diseases

Ticks are important vectors of human and animal pathogens and are endemic in tropical, subtropical and temperate regions throughout the world. After mosquitoes, ticks are the second most important vector of human diseases, responsible for more than 100,000 cases of tick-borne diseases annually (42). Furthermore, ticks are the main arthropod vector of disease in wild and domestic animals throughout the world with estimated minimal economic losses of US$ 7 billion annually (160, 226). Due to the enormous amounts of blood that ticks can ingest, heavy tick infestation in livestock reduces live-weight gain, milk production and causes anemia (41, 159, 169). In addition, tick bites can lead to severe allergic reactions, toxicoses and even lethal paralysis (53, 126, 228). Skin lesions at the tick feeding sites cause major damage to the hide and commonly these are also loci of secondary infections by fungi, bacteria and macroparasites (170). More importantly, ticks are competent vectors of a variety of pathogens including eubacteria, viruses and protozoans causing serious human and animal diseases (7, 102, 170). Especially in tropical and subtropical countries the impact of ticks on livestock production is severe and control programs concentrating on tick eradication and vaccines against tick-borne diseases have been initiated (217).

In Europe the most important tick-borne diseases in humans are Lyme Borreliosis and Tick-Borne Encephalitis (TBE), both causing tens of thousands of clinical cases annually (46, 94, 127). Other human tick-borne diseases like spotted fever rickettsioses, anaplasmoses and babesioses have more or less been neglected in Europe due to the relatively low prevalence of the causative agents in ticks. Currently human vaccines are available only against TBEV; a vaccine against Lyme Borreliosis was withdrawn from the market due to poor market performance (148). In Africa, the most important health threat caused by tick-borne pathogens is to livestock animals and major economical losses have been associated with the four tick-borne diseases anaplasmosis, heartwater, babesiosis and theileriosis (10). Tick-borne infections affecting humans are spotted fever rickettsioses, Q-fever and tick-borne relapsing fever, but so far only little is known about the prevalence of the causative agents in West African ticks (231).

In the past years, the awareness of medical doctors and scientists for tick-borne diseases increased especially in western Europe due to reports on a potential spread of tick vectors as a result of climatic change (79, 87, 88, 213). It is hypothesized that an increase of the annual mean temperature in Europe might lead to a spread of non-endemic tick species further to the North, thus at the same time introducing exotic tick-borne diseases. As most tick-borne

pathogens are highly adapted to a certain vector tick species, the maintenance of exotic pathogens in non-endemic areas highly depends on the survival and fecundity of its competent vector species (7, 170).

1.2 The tick vector

Taxonomically ticks are classified as arachnid arthropods of the order Acari. Three families are currently recognised, the hard ticks Ixodidae, the soft ticks Argasidae and the Nutalliellidae. More than 900 tick species have been described worldwide, all of which are obligatory haematophagous ectoparasites of vertebrates (7). Ixodid or hard ticks are characterised by the hard shell-like scutum that covers the anterior (females, immature instars) or the complete part (males) of the dorsal surface. The more flexible alloscutum posterior to the scutum expands during feeding, enabling immature and female ticks to dramatically increase their body weight by 200 to 600 times, whereas in males the expansion of the alloscutum is limited by the full scutum (169).

1.2.1 Life cycle

The hemimetabolous life cycle of ixodid ticks is characterised by three developmental stages of larva, nymph and adult. In order to develop into the next stages all instars of ixodid ticks need to feed once and take up large blood meals. Most commonly ticks display a three host life cycle in which all stages quest for a suitable vertebrate host (Figure 1). Questing larvae attach to a suitable host and drop off after engorgement. Moulting into nymphs takes place in a sheltered spot on the ground. Nymphal ticks quest and feed in the same way as larvae and will moult into a male or female adult tick. Female ticks take up large quantities of blood during their final feeding, whereas males rarely feed at all. Mating usually occurs on the final blood-meal host and engorged females will lay between 2,000 and 20,000 eggs in a single batch on the ground. Female ticks die after depletion while male ticks can mate repeatedly (58). Depending on climatic conditions the life cycle is completed in 6 months to several years, but generally three-host ticks spend more than 90% of their life span off-host (36). Some tick species display a modified life cycle which is characterised by feeding on either one or two individual hosts. Larval ticks find their host by questing and after engorgement they will remain attached to the host, where moulting occurs. Nymphs feed on the same host and either drop off to moult into adult ticks on the ground (two-host life cycle) or remain attached (one-host life cycle). The life cycle of these tick species is shorter and usually completed within few months.

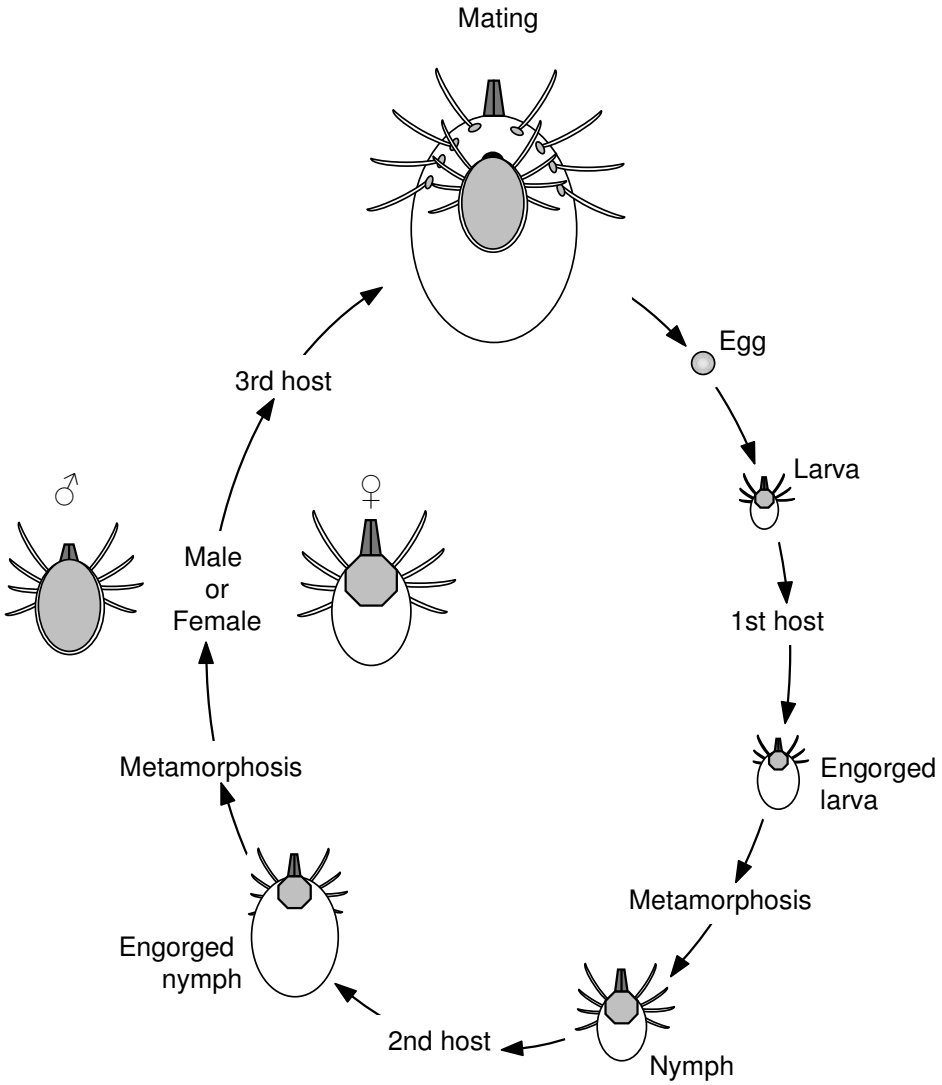

Figure 1: Three-host life cycle of *Ixodes ricinus*. Illustrated after Estrada-Pena et al, 2004 (58).

1.2.2 Host seeking

Ticks have developed different host finding strategies. Exophilic species seek hosts in the open environment, either by questing passively on the vegetation for passing hosts or by actively foraging the ground (36). Host recognition is triggered by body heat, mechanical stimuli and chemical compounds such as CO_2, ammonia, H_2S, butyric acid, lactic acid and urea which are detected by chemosensilla localised in the sensory fields of the palps and the Haller's organ on the tarsi of the front legs (26, 120, 201).

Ticks displaying questing behaviour are usually separated vertically according to their developmental stage. Larval ticks are found close to the ground, e.g. on leaf litter, where they

are likely to attach to small vertebrate hosts like mice and where microclimatic conditions are more stable. Nymphs quest at heights of up to 50cm and adults can climb the vegetation as high as 150cm. The questing period is interrupted when high temperature and low relative humidity create an unfavourable microclimate. Some tick species prevent desiccation by retreating periodically to the base of the vegetation, where they restore their lost body water. A hygroscopic salt solution is secreted by the salivary glands and the atmospheric water condensing on the mouthparts is ingested (17, 201). Other, more desiccation-tolerant tick species are able to rehydrate during the cool and humid periods of the night without having to retreat to the basis of the vegetation (201). In contrast, endophilic or nidicolous ticks complete their life cycle in the burrows and nests of vertebrates, where microclimatic conditions are relatively stable throughout the year (36). Some ticks display a so called domestic behaviour and have adapted to housings of humans, where they mainly feed on domestic animals (234).

1.2.3 Feeding

The mouthparts of the tick consist of chelicerae, hypostome and palps. The chelicerae are used to cut the dermis and break the capillary blood vessels close to the skin to form a feeding lesion. The toothed hypostome serves as an anchor to attach to the host, but it also forms a feeding channel for the uptake of blood. Saliva is released into the wound via the same route immediately after tick attachment to overcome the host's defensive reaction, e.g. haemostasis, cellular and humoral responses of the immune system (70, 112). Many tick species produce and excrete cement by the salivary glands to glue hypostome and palps to the dermis of the host. The feeding process is slow as the alloscutum needs to grow accordingly. Larval ticks generally feed for three to five days, nymphs for four to eight days and adult females can remain attached for five to twenty days (234). During the feeding process, nutrients are filtered from the blood and excess water is excreted back into the host by the salivary glands. In the midgut, the blood meal is taken up into digestive cells by endocytosis or phagocytosis (whole cells) and digested intracellularly. The midgut lumen can absorb amino acids, monosaccharides, free fatty acids, water and salts directly. Digestion is slow and can take weeks (71).

1.2.4 Seasonal Activity

Exophilic ticks display seasonal activity which is highly influenced by temperature, relative humidity and day length (48, 78, 171). Larval activity usually peaks in summer months and nymphs moulting from fed larvae emerge in late summer or early fall. Normally these new

nymphs overwinter, feed in the following spring and moult into adults during the summer. Adults begin questing in the autumn months of the same year, but can also be found on the vegetation in the following spring (171). In temperate regions diapausing ticks occasionally re-emerge during warm (non-freezing) winter days and can be found questing on the vegetation (48).

1.2.5 Role as vector

Ticks are ideal vectors of pathogens as all instars are obligatory haematophagous parasites feeding on different vertebrate hosts. Pathogen transmission is facilitated by the long duration of the blood meal, the continuous release of tick saliva and excess fluids into the host and the modulation of the host's immune response by tick saliva (16, 70). In addition, the long-term co-evolution with pathogens and the relatively long life span of ticks further enhance transmission of pathogens (71). A variety of pathogens is transmitted by ticks which usually are acquired from reservoir hosts during the first blood meal, transstadially maintained during development into the next instar and horizontally transmitted to other vertebrate hosts during subsequent blood meals. Some pathogens have the ability to be vertically (transovarially) transmitted from females to offspring (15, 130). In order to be successfully transmitted, pathogens need to prevent digestion, escape the tick's innate immune system and pass from the midgut into the salivary glands. These adaptations of tick-borne pathogens are oftentimes tick species-specific and it was shown that immunotolerance to pathogens is a crucial feature of competent tick vectors, whereas incompetent vectors are immunocompetent (139). Even competent tick vectors may suffer from the infection with a tick-borne pathogen and negative effects on the survival rate of ticks, fecundity and feeding time have been described (139). The prevalence of pathogens in ticks of a given region depends on the density of competent tick vectors, reservoir hosts as well as the availability of larger mammals which serve as a final blood meal host of female ticks and are crucial for successful reproduction.

1.2.6 Important Tick Species

Ticks of the genus *Ixodes* are the predominant vectors of human diseases in the temperate zones of the Northern hemisphere. In Western Europe, the sheep tick or castor bean tick *Ixodes ricinus* is the main vector of Lyme Borreliosis and Tick-Borne Encephalitis, whereas in Eastern Europe these diseases are mainly transmitted by *I. persulcatus* (92, 212). In addition, *I. ricinus* is a competent vector for several human pathogens such as *Anaplasma*, *Ehrlichia* and *Rickettsia* species. Host seeking behaviour of *I. ricinus* is exophilic and

therefore these ticks are often found to infest humans. The nidicolous *I. hexagonus* is predominantly parasitizing on wild, nest-building animals like the European hedgehog, but it also feeds on foxes, martens or pet animals like cats and dogs (162). Tick bites in humans are scarcely reported (100, 125). Another nidicolous tick species is the highly specialised *I. frontalis*, which exclusively feeds on birds (136).

The ornate dog tick *Dermacentor reticulatus* is distributed in Western and Eastern Europe and although local populations display a high focality, its range seems to be expanding (39, 150). Only adults are found on the vegetation questing for large blood meal hosts (e.g. deer, dogs), the immature stages feed on rodents (79). *D. reticulatus* is a competent vector of *Coxiella burnetii* and certain *Babesia* and *Anaplasma* species (21, 113, 238).

The most wide-spread tick species throughout subtropical and tropical regions is the kennel tick or pan-tropical dog tick *Rhipicephalus sanguineus* as it is highly adapted to domestic dogs (58). *Rh. sanguineus* transmits the agents of canine ehrlichiosis (*Ehrlichia canis*), canine babesiosis (*Babesia canis*, *B. gibsoni*) and hepatozoonosis (*Hepatozoon canis*) to dogs (58). It is also a competent vector *Rickettsia conorii*, the causative agent of tick typhus or boutonneuse fever in humans (38). *Rhipicephalus* (subgenus *Boophilus*) *annulatus* is mainly distributed in West and North Africa and predominantly infests cattle causing damage to hide and probably reducing cattle growth (234). It can transmit the agents of bovine babesiosis (*Babesia bigemina*, *Babesia bovis*) and bovine anaplasmosis (*Anaplasma marginale*) (234). Another tick species commonly infesting cattle is *Rhipicephalus evertsi*. The saliva of this tick species is toxic and heavy infestation causes paralysis in calves, lamb and adult sheep (75). Diseases transmitted by this tick species mainly affect horses (equine piroplasmosis caused by *Babesia caballi* and *Theileria equi*) and cattle (bovine anaplasmosis) (234).

The tropical bont tick *Amblyomma variegatum* is one of the most widespread tick species parasitizing on livestock in Africa. This tick is a competent vector for the causative agents of heartwater (*Ehrlichia ruminantium*), bovine ehrlichiosis (*Ehrlichia bovis*) and benign bovine theileriosis (*Theileria mutans*, *Theileria velifera*) (234). Heavy infestations can reduce live-weight gain and promote secondary bacterial infections like dermatophilosis (206). The main human diseases transmitted by *A. variegatum* are rickettsioses (21).

Hyalomma impeltatum ticks are predominantly found in Mediterranean, steppe and desert climates of Africa, mainly parasitizing on cattle and camels (56, 185). Its role as a disease vector is only partially understood, but it seems to be transmission competent for *Theileria annulata* to cattle and Crimean-Congo Haemorrhagic Fever Virus (CCHFV) to humans (234).

Hyalomma marginatum subspecies *marginatum* is widely distributed in the humid Mediterranean climate of northern Africa, southern Europe and in steppe climates further eastwards (234). It feeds on ungulates like cattle, sheep, goats, camels and horses and is a competent vector of *Babesia caballi* and *Theileria annulata*. *Hy. marginatum marginatum* also transmits CCHFV to humans and may be the main European vector of this virus (234).
Hyalomma aegyptium is distributed in the Mediterranean area and feeds on a variety of vertebrates, the main host for adult ticks are tortoises. Its vector competence has been experimentally proven for *Coxiella burnetii* (195).
Most of these tick species have a three-host life cycle except for the one-host tick *Rh.* (*Boophilus*) *annulatus* and the two-host ticks *Rh. evertsi* and *Hy. marginatum marginatum*.

1.2.7 Tick collection techniques

The most commonly used technique for tick collection is the so called cloth dragging method or flagging, which targets questing exophilic ticks. Generally, a white towel is dragged over the vegetation, mimicking the movements of a potential blood meal host and attached ticks are removed regularly. Modifications of this method are strip blankets, leggings and foot flags (48, 221). CO_2 traps which attract ticks questing in close proximity are rarely used (76). Another method is the direct removal of feeding ticks from domestic and wild animals or humans. Also nests and burrows of nest building animals can be searched for presence of nidicolous ticks. The main difference between these collection methods is that questing ticks are unfed and pathogens detected in these ticks will originate from the tick. Thus, the prevalence of a pathogen in a questing tick is a direct measure of the risk of infection after a tick bite for humans and animals, given that the tick is a competent vector. Feeding ticks, however, have already ingested blood of their current host and molecular detection of pathogens in these ticks does not yield information about the origin of infection (host or tick). Nevertheless, analysis of pathogen prevalence in feeding ticks can give valuable information on the presence of pathogens and their contact to vertebrate hosts. Moreover, the tick species diversity generally is higher when collecting ticks from wild animals and therefore also the diversity of pathogens may be higher in these ticks.

1.3 Tick-borne pathogens

The prevalence of tick-borne pathogens can be analysed in various ways, e.g. by the detection of pathogens in the tick vector or in the blood of vertebrate hosts using PCR or culture. Additionally, pathogens can indirectly be detected by measuring the specific antibody

response of the host using ELISA or immunoblots. Pathogen detection by PCR is more sensitive than culture, although it does not give information about the vitality and viability of the agent. Investigation of blood samples by PCR and culture can yield false negative results when the concentration of pathogens is too low or when the infection has manifested itself in other tissues. Measuring the serum antibody prevalence in hosts gives information about previous contacts with this agent, yet distinction between acute and past infections cannot be made with confidence. Important tick-borne pathogen species, the associated diseases, tick vector and reservoir host species and the geographical distribution are shown in Table 1.

1.3.1 Distribution and spread of tick-borne pathogens

Ticks play no direct role in the geographic distribution of a pathogen, as all vector species are quite immobile. The short-range distribution of pathogens is determined by the territory size of its respective reservoir host or its vector tick. Wild birds are believed to be responsible for the wide geographic distribution of various pathogenic microorganisms (104), which they carry either as a reservoir host or by dispersing infected arthropod vectors (93). Migratory birds are of special importance, since they cross national and intercontinental borders and can become long-range vectors for any pathogen they carry. Avian mobility is a crucial epizootiologic factor, since even sedentary birds sometimes move as far as 50-100km and migratory birds can transport pathogens to extremely distant sites, e.g. the arctic tern *Sterna paradisaea*, travels 50,000km between Antarctica and northern Scandinavia (93, 104). New endemic foci of disease can be established along the migration routes of birds given the availability of suitable vectors and reservoir hosts (177).

1.3.2 Borrelia burgdorferi s.l.

Lyme Borreliosis is the most commonly reported tick-borne disease in Europe. It was first described in 1970 in the US-American town Lyme, Connecticut. Twelve years later, Willy Burgdorfer discovered the causative agent of the disease: a gram-negative bacterium belonging to the Spirochaetaceae, subsequently named *Borrelia burgdorferi*.

Currently eighteen *Borrelia*-species are recognized as members of the *Borrelia burgdorferi* sensu lato group, including at least three human pathogenic species, namely *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (211). There are four more species, *B. valaisiana, B. lusitaniae, B. bissettii* and *B. spielmanii* suspected to be pathogenic in humans (65). *B. valaisiana* and *B. lusitaniae* were detected in patients with suspected Lyme Borreliosis.

Table 1. Tick-borne pathogens, associated disease, vectors, reservoir host species and distribution (modified from (40))

Pathogen	Disease	Tick Vector	Main End Hosts	Geographical Distribution
Bacteria: Borrelia				
B. afzelii	Lyme Borreliosis	*Ixodes* species	Human	Europe, Asia, Northern Africa
B. burgdorferi sensu stricto	Lyme Borreliosis	*Ixodes* species	Human	Europe, Asia, Northern Africa, North America
B. bissettii	Suspected pathogenicity	*Ixodes* species	Human	Europe, USA
B. garinii	Lyme Borreliosis	*Ixodes* species	Human	Europe, Asia, Northern Africa
B. lusitaniae	Suspected pathogenicity	*Ixodes* species	Human	Europe
B. spielmanii	Suspected pathogenicity	*Ixodes* species	Human	Europe
B. valaisiana	Suspected pathogenicity	*Ixodes* species	Human	Europe, Asia
Bacteria: Rickettsia				
R. aeschlimannii	Unnamed disease	*Hyalomma* species, *Rhipicephalus* species	Human	Europe, Africa
R. africae	African tick-bite fever	*Ambylomma* species	Human	Africa, Reunion Island, West Indies
R. helvetica	Suspected pathogenicity	*Ixodes ricinus*	Human	Europe
R. massiliae	Unnamed	*Rhipicephalus* species	Human	Europe, Asia, Argentina, USA
R. monacensis	Unnamed	*Ixodes ricinus*	Human	Europe
R. slovaca	TIBOLA	*Dermacentor* species	Human	Europe, Asia
Bacteria: Anaplasma				
A. phagocytophilum	Human Granulocytic Anaplasmosis	*Ixodes* species, *Dermacentor* species	Human	Europe, USA
A. marginale	Bovine Anaplasmosis	Various	Cattle	Worldwide
A. centrale	Bovine Anaplasmosis	Various	Cattle	Worldwide
Bacteria: Ehrlichia				
E. chaffeensis	Human Monocytic Ehrlichioses	*Amblyomma* species, *Dermacentor* species	Human	USA, Africa
E. ewingii	Human Ehrlichiosis, Canine Granulocytic Ehrlichiosis	*Amblyomma* species	Human, dogs	USA, Africa
E. ruminantium	Heartwater	*Amblyomma* species	Cattle	Africa, Caribbean
Bacteria: Bartonella				
B. henselae	Cat Scratch Disease	*Ixodes* species	Human, Cats	Worldwide
Bacteria: Coxiella				
C. burnetii	Q-Fever	(*Ixodes* species, *Dermacentor* species, *Hyalomma* species)	Human, ruminants	Worldwide
Bacteria: Francisella				
F. tularensis	Tularaemia	(*Ixodes* species, *Dermacentor* species)	Human, rodents	Europe, Asia, North America
Protozoa: Babesia				
B. divergens	Cattle Babesiosis	*Ixodes* species, *Dermacentor* species	Human, cattle	Europe
B. microti	Unnamed	*Ixodes* species	Human, rodents	Europe, Northern America
Babesia sp. EU1	Unnamed	*Ixodes* species	Human	Europe
Protozoa: Theileria				
T. mutans	Benign Theileriosis	*Amblyomma* species	Cattle	Africa
Protozoa: Hepatozoon				
H. canis	Hepatozoonosis	*Rhipicephalus* species, *Haemaphysalis* species	Dogs	Southern Europe, Middle East, Far East, Africa
Virus: Flavivirus				
Tick-Borne Encephalitis Virus	Tick-Borne Encephalitis	*Ixodes* species, *Haemaphysalis* species	Human	Europe, Asia

TIBOLA, Tick-Borne Lymphadenopathy; Tick species in brackets are not the main vectors of these pathogens.

Pathogenicity of *B. bissettii* was not proven, although isolates from erythema migrans of patients with high phenotypic and genotypic similarities to *B. bissettii* were cultured. (45). *B. spielmanii* represents a new species which was first described in 1993 (229) and classified in 2004 (182).

Lyme Borreliosis is a multisystem disorder that can affect skin, heart, nervous system, and to a lesser extent the eyes, kidneys and liver, but may also remain asymptomatic. During the early course of the disease an expanding skin lesion (erythema migrans, EM) develops around the tick bite site in about 60% of cases. Unspecific symptoms, such as headache, chills, fever and fatigue, have also been reported during this stage. As many tick bites remain unnoticed the early stage of Lyme Borreliosis is not recognized occasionally and the disease may progress to the next phase. The haematogenous dissemination of the bacteria can cause secondary skin lesions (multiple EM, Acrodermatitis chronica atrophicans) and extracutaneous manifestations, such as Lyme Arthritis and Neuroborreliosis (e.g. encephalitis, polyradiculitis, facial palsy). In most cases, treatment with antibiotics successfully prevents progression of the disease but the diagnosis can be complicated (212). The known three pathogenic *Borrelia*-species appear to be responsible for different symptoms in the late phase of the disease, but firm evidence is still lacking. *B. burgdorferi* s.s. is associated with Lyme Arthritis, *B. garinii* with Neuroborreliosis, and *B. afzelii* is suspected to cause Acrodermatitis chronica atrophicans (ACA) (45, 235).

The mean prevalence of *Borrelia*-infested ticks is about 10-20% in Europe, although the tick infection rates vary extremely between the different European countries and also within a country itself (25, 219). Seroprevalence studies have been performed in some European countries and a high prevalence of anti-*Borrelia* antibodies has been observed in occupational risk groups like forestry workers. Since the seroprevalence of *Borrelia burgdorferi* s.l. in humans depends on the density of infected ticks, significant regional variations are observed. In Europe, the seroprevalence in the general population ranges from 2.6% to 13.7% and in the risk groups from 8% to 25% (23, 109, 115, 175, 224).

1.3.3 Spotted Fever Group Rickettsiae

Rickettsiae belong to the order Rickettsiales and are mostly obligate intracellular, gram-negative alpha-proteobacteria. They have a global distribution and cause several diseases in humans and animals (98). *Rickettsia* species are transmitted by several arthropod vectors like ticks, mites and blood feeding insects. However, ixodid ticks seem to be competent vectors only for the Spotted Fever Group (SFG) Rickettsiae (203). The two main human pathogenic

SFG *Rickettsia* species in Europe are *R. helvetica* and *R. slovaca.* The first of these species is suspected to cause non-specific fever and endocarditis (47), whereas the latter causes tick-borne lymphadenopathy (TIBOLA) (20). Prevalences of *Rickettsia* species in field ticks vary extremely from 4% to 66% for *R. helvetica* (83, 166, 178, 203, 210, 237).

In Africa, several human pathogenic tick-borne *Rickettsia* species have been found including the causative agents of Mediterranean spotted fever (*Rickettsia conorii conorii*), Astrakhan fever (*Rickettsia conorii caspia*), rickettsial pox (*R. akari*), African tick bite fever (*R. africae*) and three emerging species *R. aeschlimannii, R. massiliae,* and *R. sibirica mongolotimonae* (69, 140, 158).

1.3.4 Anaplasma and Ehrlichia species

Ehrlichiosis is a generic name for infections caused by obligate intracellular, gram-negative bacteria of the family Anaplasmataceae. Affected by these emerging, potentially fatal infectious diseases are humans (human ehrlichiosis or human anaplasmosis), livestock (heartwater and bovine anaplasmosis), canines (canine ehrlichiosis) and horses (Potomac horse fever) (108). Five members of the Anaplasmataceae are known to infect humans: *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *E. ewingii*, *E. canis*, and *Neorickettsia sennetsu* (52, 143, 145). Of these, only the first 3 species have been studied in detail. The respective diseases are often named according to the leukocyte type infected by the different species. The human monocytic ehrlichiosis (HME) is caused by *E. chaffeensis*, which infects monocytes, while *A. phagocytophilum* targets granulocytes, causing the human granulocytic anaplasmosis (HGA) (51).

Studies in Northern and Southern Europe showed a prevalence of *Anaplasma* species of 6.6% to 25% in *I. ricinus* nymphs (64). In Africa, the most recognized diseases caused by *Anaplasma* and *Ehrlichia* species are infections of livestock. Bovine anaplasmosis is caused by the highly pathogenic species *Anaplasma marginale* sensu stricto and the naturally attenuated *A. marginale* subspecies *centrale* (2, 44). Heartwater is a fatal disease of livestock in sub-Saharan Africa and the eastern Caribbean and is caused by *Ehrlichia* (*Cowdria*) *ruminatium*, which infects endothelial cells (161). Its distribution is directly linked to that of its main vector *Amblyomma* sp. and the prevalence in ticks can be as high as 25% (22, 227).

1.3.5 Bartonella species

Eight species of *Bartonella*, gram-negative and fastidious bacteria, have been isolated from humans as the causative agents of several diseases (124). In Europe, two species are of

medical importance. *B. henselae* causes the Cat Scratch Disease and is usually transmitted by infected cats (232) and *B. quintana* is causing trench fever, a disease with symptoms like relapsing febrile illness, headache, leg pain, endocarditis and, in HIV-infected persons, bacillary angiomatosis (155).

Little is known about the prevalence of *B. henselae* and *B. quintana* in European ticks. In Italy, ticks were found to have an infection rate of 1.4% for *B. henselae* (186). In a Dutch study, more than 70% of the ticks investigated carried *Bartonella* species or species closely related, but the human pathogens *B. henselae* and *B. quintana* could not be detected (188).

1.3.6 Coxiella species

Coxiella burnetii, an obligate intracellular bacterium, is the causative agent of Q fever in animals and humans. *Coxiella burnetii* has a worldwide distribution, with the exception of New Zealand (37). Usually the infection is acquired via milk, faeces, urine, and birth products from infected ruminants or via aerosols. The acute state presents as a febrile illness with headache, chills, and respiratory symptoms. Recovery usually appears between 1-4 weeks, although 5% of the patients develop a chronic course of disease (215). In most cases the infection remains sub-clinical, but sometimes can cause severe and life-threatening complications, predominantly through endocarditis (37).

Approximately 100 human cases are reported in Britain yearly, most cases in man follow exposure to livestock, rather than tick exposure (37). The role of ticks in the transmission of the pathogen is probably minimal, which corresponds to the low prevalence found in European ticks, ranging from 0.09% to 0.4% (167, 200, 216). Seroprevalence studies show that *Coxiella burnetii* is widely distributed in West Africa; however, due to the close contact of humans with potential reservoir hosts the role of ticks in the transmission of this pathogen to humans is unclear (13, 132).

1.3.7 Francisella species

Tularemia is a pneumonic or ulceroglandular disease caused by the highly infectious bacterium *Francisella tularensis*. The mortality rate in humans may be as high as 30 to 60% without treatment (8). Several subspecies of *F. tularensis* have been identified. Of these *F. tularensis* ssp. *tularensis*, occurring mainly in North America, and *F. tularensis* ssp. *holarctica*, occurring throughout the Northern Hemisphere, are the most important (29). Humans contract the disease most commonly from arthropod bites, consumption of contaminated food or water, contact with infected animals or via aerosols (30, 114). In the

Czech Republic and neighbouring Austria, a prevalence ranging between 0.6 and 3.5% for *Francisella*-infected ticks (*Dermacentor reticulates*) was found (95). *Francisella* species do not seem to be endemic in Africa.

1.3.8 Babesia, Theileria and Hepatozoon species

Babesiosis is a malarialike illness, caused by a parasitic protozoan of the genus *Babesia*, an intraerythrocytic piroplasm. At least two *Babesia* species, namely *B. divergens* and *B. microti*, are known to infect humans. *Babesia sp*. EU1 was first described in 2003 after being isolated from two patients in Austria and Italy, and since then has been detected in *Ixodes ricinus* in Slovenia and Switzerland (14). In Europe, the prevalence of *Babesia* species in questing *I. ricinus* ticks is highly variable, ranging from 1% to 16.3% (86, 164, 198). The most important African piroplasms are the causative agents of bovine babesiosis (*Babesia bigemina*) and theileriosis (*Theileria velifera*, *Theileria mutans*) (10). The prevalence of these pathogens in questing African ticks remains unclear.

1.3.9 Tick-Borne Encephalitis Virus

The tick-borne encephalitis virus (TBEV) is a RNA-virus of the family Flaviviridae and the causative agent of the tick-borne encephalitis, an infection of the central nervous system. TBEV is endemic in wide parts of the northern hemisphere covering Europe, northern Asia, China and Japan (151). TBEV is a generic expression, which refers to three different subtypes: the European (FSME, Frühsommer-Meningoencephalitis), the Siberian (RSSE, Russian spring-summer encephalitis), and the Far-Eastern subtype (233). The European subtype is transmitted by *Ixodes ricinus*, while the Siberian and Far-Eastern subtypes are carried by *Ixodes persulcatus* (196). These three subtypes differ in their pathogenicity, with the Far Eastern subtype being the most virulent leading to mortality rates of up to 50% (151). While there is no causative treatment for the tick-borne encephalitis, it can easily be prevented by vaccination (117). TBEV is believed to cause at least 3,000 human cases in western Europe annually, the prevalence of infected ticks in endemic areas in Europe usually varies from 0.5 – 5% (179).

1.4 Objectives of this study

Despite the growing interest in ticks and tick-borne diseases, the underlying dynamics regulating the prevalence, distribution and spread of the vectors and pathogens remain largely unknown. A variety of pathogens can be transmitted by many tick species throughout the world, complicating surveillance, risk assessment and diagnosis of tick-borne diseases. Research mainly focuses on the main tick-borne diseases like Lyme Borreliosis and TBE for humans and Babesiosis and Anaplasmosis for livestock. However, studies on neglected pathogens are important in order to monitor changes in their prevalence and distribution in ticks. A central aspect of disease control is prevention of infection, which can be achieved by control of ticks, reservoir and blood meal hosts, but also by informing the public how to effectively avoid tick bites.

Surveillance of seasonal and annual variations in the prevalence of ticks and pathogens provide important aspects for a better understanding of the short-term impact of climatic conditions on disease dynamics. The impact of climate on ticks has been investigated under experimental and natural conditions for some tick species; however, the lack of reliable quantitative data makes it difficult to predict the long-term effects of climatic changes in the different ecologies of the world. Furthermore, the establishment of exotic species of ticks and tick-borne pathogens in new areas depends not only on abiotic conditions, but also on the availability of suitable blood meal hosts for ticks and reservoir hosts for pathogens. In order to measure changes in the abundance of ticks and the prevalence of pathogens, baseline prevalence data of vectors and pathogens in different ecological niches of a country is crucial.

The aim of this project was to study the diversity and prevalence of ticks and tick-borne pathogens known to cause disease in humans and animals in European and African countries and provide thorough baseline data for future surveillance projects. Specifically the following points were investigated:

Luxembourg

- Regional distribution of ticks and tick-borne pathogens
- Influence of habitat structures on tick abundance and infection rate
- Annual and seasonal variations in the prevalence of ticks and pathogens
- Determination of the rate of coinfections
- Seroprevalence of *Borrelia burgdorferi* s.l. in a risk cohort
- Identification of risk factors for *Borrelia* infection
- Implications for human health

Nigeria

- Infection status of questing and feeding ticks
- Implications for human and animal health

Belarus

- Regional distribution of ticks and tick-borne pathogens
- Infection status of questing and feeding ticks
- Implications for human health

Bulgaria

- Prevalence of *Francisella* species in ticks
- Genetic characterization of a new *Francisella*-like endosymbiont

Kaliningrad enclave

- Tick infestation status of migratory birds
- Role of birds in spread of ticks and tick-borne pathogens

2 Materials

2.1 Chemicals

Compound	Company
Agarose	Lonza
Ampicillin	Sigma
Dithiothreitol (DTT) 0.1 M	Invitrogen
Dimethylsulfoxide (DMSO)	Sigma
Ethanol 96-100%	Merck
Ethidium bromide	Invitrogen
Ethylendiaminetetraacetic Acid (EDTA)	Biorad
Kanamycin	Sigma
Luria Broth Base (LB)	Invitrogen
Magnesium Chloride ($MgCl_2$)	Invitrogen
Nucleotides (dNTPs)	Invitrogen
Oligonucleotides/Primers	Eurogentec
Orange G	Invitrogen
PCR Buffer without $MgCl_2$ (10x)	Invitrogen
Sodium Acetate	Merck
Sucrose	Sigma
SYBR®Green™ nucleic acid stain (10,000x)	Molecular Probes
SYBR®Safe™ DNA Gel Stain (10,000x)	Invitrogen
Tris(hydroxymethyl)aminomethane (Tris)	Sigma

2.2 Buffers and solutions

Buffer/solution	Reagent	Volume/concentration
DNA loading dye (6x)	Orange G	25mg
	Sucrose (40%)	4g
	ddH_2O	fill up to 10ml
	store at 4°C	
TAE-Buffer (50x)	Tris	2M
	Sodium Acetate	25mM
	EDTA	0.5M
	Adjust to pH 7.8	
S.O.C. Medium (Invitrogen)	Tryptone	2%
	Yeast Extract	0.5%
	NaCl	10mM
	KCl	2.5mM
	$MgCl_2$	10mM
	$MgSO_4$	10mM
	Glucose	20mM
Phosphate-Buffered-Saline (PBS)	NaCl	8g
	KCl	0.2g
	Na_2HPO_4	1.44g
	KH_2HPO_4	0.24g
	ddH_2O	fill up to 800ml
	Adjust pH to 7.4 with HCl	
	ddH_2O	fill up to 1l

2.3 DNA markers

1kb plus DNA ladder™ Life Technologies

2.4 Enzymes

Enzyme	Company
OneStep® RT-PCR Enzyme Mix (Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, HotStarTaq DNA Polymerase)	Qiagen
Platinum® Taq DNA polymerase	Invitrogen
RNaseOUT™ (Recombinant Ribonuclease Inhibitor)	Invitrogen
SuperScript™ III Reverse Transcriptase	Invitrogen

2.5 Commercial Kits

Kit name	Company
Big Dye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
B. afzelii + VlsE IgG Europe ELISA Testkit	Genzyme Virotech
Borrelia Europe plus TpN17 LINE IgG Immunoblot	Genzyme Virotech
InviMag® Tissue DNA Mini Kit/KF 96	Invitek
Jet Quick PCR Purification Spin Kit	Genomed
MagMAX™-96 Total Nucleic Acid Isolation Kit	Ambion
MagMAX™-96 Viral RNA Isolation Kit	Ambion
NucleoSpin® RNA II	Macherey&Nagel
NucleoSpin® RNA/DNA Buffer Set	Macherey&Nagel
QIAamp® viral RNA Mini kit	Qiagen
QIAamp® DNA Blood Mini Kit	Qiagen
QIAamp® RNeasy Mini Kit	Qiagen
QIAGEN® OneStep RT-PCR Kit	Qiagen
QIAprep® Spin Mini Kit	Qiagen
QIAquick® Gel Extraction Kit	Qiagen
TOPO TA Cloning® Kit	Invitrogen
TURBO DNA-free™ Kit	Applied Biosystems

2.6 Primers used for screening of pathogens in European ticks

Pathogen	Primer name	Primer orientation	Target gene	5'-3' Sequence	Ref.	Primer concentration	$MgCl_2$ concentration	Annealing step	Elongation step
Anaplasma phagocytophilum	EL(569)F	forward	groEL gene	ATGGTATGCAGTTTGATCGC		0.8 µM	2 mM	61°C 30s	72°C 45s
	EL(1193)R	reverse	groEL gene	TCTACTCTGTCTTTGCGTTC					
	EL(569)F	forward	groEL gene	ATGGTATGCAGTTTGATCGC		0.8 µM	2 mM	56°C 30s	72°C 45s
	EL(1142)R	reverse	groEL gene	TTGAGTACAGCAACACCACCGGAA	(3)				
Babesia sp.	BJ1	forward	18S rRNA	GTCTTGTAATTGGAATGATGG		0.8 µM	3 mM	61°C 30s	70°C 60s
	BN2	reverse	18S rRNA	TAGTTTATGGTTAGGACTACG	(28)				
Bartonella sp.	321s	forward	16S-23S	AGATGATGATCCCAAGCCTTCTGC		0.8 µM	1.5 mM	60°C 30s	72°C 45s
	983as	reverse	16S-23S	TGTTCTYACAACAATGATGATG	(124)				
Borrelia burgdorferi s.l.	Outer1	forward	flaB gene	AARGAATTGGCAGTTCAATC		0.8 µM	2 mM	59°C 30s	72°C 30s
	Outer2	reverse	flaB gene	GCATTTTCWATTTTAGCAAGTGATG					
	Inner1	forward	flaB gene	ACATATTCAGATGCAGACAGAGGTTCTA		0.8 µM	2 mM	59°C 30s	72°C 30s
	Inner2	reverse	flaB gene	GAAGGTGCTGTAGCAGGTGCTGGCTGT	(31)				
	V1a	forward	OspA gene	GGGAATAGGTCTAATATTAGC		0.8 µM	2 mM	50°C 45s	72°C 60s
	V1b	forward	OspA gene	GGGGATAGGTCTAATATTAGC					
	R2	reverse	OspA gene	CATAAATTCTCCTTATTTTAAAGC					
	R37	reverse	OspA gene	CCTTATTTTAAAGCGGC					
	V3a	forward	OspA gene	GCCTTAATAGCATGTAAGC		0.8 µM	2 mM	52°C 45s	72°C 60s
	V3b	forward	OspA gene	GCCTTAATAGCATGCAAGC					
	R2	reverse	OspA gene	CATAAATTCTCCTTATTTTAAAGC					
	R37	reverse	OspA gene	CCTTATTTTAAAGCGGC	(134)				
Coxiella sp.	Q5	forward	htpB gene	GCGGGTGATGGTACCACAACA		0.4 µM	1.5 mM	58°C 30s	72°C 30s
	Q3	reverse	htpB gene	GGCAATCACCAATAAGGGCCG					
	Q6	forward	htpB gene	TTGCTGGAATGAACCCCA		0.8 µM	2 mM	56°C 30s	72°C 30s
	Q4	reverse	htpB gene	TCAAGCTCCGCACTCATG	(225)				
Francisella tularensis ssp.	Fr153F0.1	forward	16S rRNA	GCCCATTTGAGGGGGATACC		0.4 µM	2 mM	60°C 30s	72°C 60s
	Fr1281R0.1	reverse	16S rRNA	GGACTAAGAGTACCTTTTTGAGT	(8)				
Rickettsia sp.	Rr17k.1p	forward	17-kDa	TTTACAAAATTCTAAAAACCAT		0.8 µM	2 mM	55°C 30s	72°C 45s
	Rr17k.539n	reverse	17-kDa	TCAATTCACAACTTGCCATT					
	Rr17k.90p	forward	17-kDa	GCTCTTGCAACTTCTATGTT		0.8 µM	2 mM	54°C 30s	72°C 45s
	Rr17k.539n	reverse	17-kDa	TCAATTCACAACTTGCCATT	(98)				

Tick-borne Encephalitis Virus	283F1	forward	E protein	GAGAYCAGAGTGAYCGAGGCTGG	(196)	0.4 µM	2 mM	57°C 30s	72°C 45s
	827R1	reverse	E protein	AGGTGGTACTTGGTTCCMTCAAGT					
	349F2	forward	E protein	GTCAAGGCGKCTTGTGAGGCAA		0.8 µM	2 mM	58°C 30s	72°C 30s*
	814R2	reverse	E protein	TTCCCTCAATGTGTGCCACAGG					
	Pp1	forward	C protein	GCGTTTGCTTCGGACAGCATTAGC		0.8 µM	1.5 mM	64°C 30s	72°C 30s
	Pm1	reverse	C protein	GCGTCTTCGTTGCGGTCTCTTTCG					
	Pp2	forward	C protein	TCGGACAGCATTAGCAGCGGTTGG		0.4 µM	2 mM	58°C 30s	72°C 30s
	Pm2	reverse	C protein	TGCGGTCTCTTTCGACACTCGTCG					

* only 25 PCR cycles

2.7 Primers used for screening of pathogens in African ticks

Pathogen	Primer name	Primer orientation	Target gene	5'-3' Sequence	Ref.	Primer concentration	$MgCl_2$ concentration	Annealing step	Elongation step
Anaplasmataceae	EHR1	forward	16S rRNA	GAACGAACGCTGGCGGCAAGC	(174)	0.4 µM	2 mM	63°C 30s	72°C 45s
	newEHR2*	reverse	16S rRNA	CACGCTTTCGCACCTCAGTGTC					
	EHR3	forward	16S rRNA	TGCRTAGGAATCTRCCTAGTAG		0.8 µM	2 mM	59°C 30s	72°C 45s
	newEHR2*	reverse	16S rRNA	CACGCTTTCGCACCTCAGTGTC					
Borrelia species	newLDf*	forward	16S rRNA	GTAAACGATGCACACTTGGTG	(128)	0.4 µM	2 mM	61°C 30s	72°C 30s
	newLDr*	reverse	16S rRNA	TCCGRCTTATCACCGGCAGTCT					
Coxiella	Q5	forward	htpB	GCGGGTGATGGTACCACAACA	(225)	0.4 µM	1.5 mM	58°C 30s	72°C 30s
	Q3	reverse	htpB	GGCAATCACCAATAAGGGCCG					
	Q6	forward	htpB	TTGCTGGAATGAACCCCA		0.8 µM	2 mM	56°C 30s	72°C 30s
	Q4	reverse	htpB	TCAAGCTCCGCACTCATG					
Rickettsiaceae	Rr17k.1p	forward	17-kDa	TTTACAAAATTCTAAAAACCAT	(98)	0.8 µM	2 mM	55°C 30s	70°C 45s
	Rr17k.539n	reverse	17-kDa	TCAATTCACAACTTGCCATT					
	Rr17k.90p	forward	17-kDa	GCTCTTGCAACTTCTATGTT		0.8 µM	2 mM	54°C 30s	72°C 45s
	Rr17k.539n	reverse	17-kDa	TCAATTCACAACTTGCCATT					
Piroplasmidae	BJ1	forward	18S rRNA	GTCTTGTAATTGGAATGATGG	(28)	0.8 µM	3 mM	61°C 30s	72°C 60s
	BN2	reverse	18S rRNA	TAGTTTATGGTTAGGACTACG					

* primer sequence modified

2.8 Bacterial strain

E. coli OneShot® TOP10 (Provided with the TOPO TA Cloning® Kit)	Invitrogen

2.9 Vector

pCR® 4-TOPO® cloning vector	Invitrogen

2.10 Random Primers

Random Hexamers	Invitrogen

2.11 Bioinformatics

Software	Reference
BioEdit	http://www.mbio.ncsu.edu/BioEdit
BLAST	http://ncbi.nlm.nih.gov/BLAST
CFX Manager	BioRad
CLUSTAL W	Thompson, JD et al, 1994 (223)
FastPCR v.3.7.8	R. Kalender, University of Helsinki, Finland
MEGA 3.1	http://www.megasoftware.net/mega3/mega.html (116)
MEGA 4.0	http://www.megasoftware.net/mega4/mega.html (222)
Opticon Monitor™ v3.1	BioRad
SeqScape	Applied BioSystems

2.12 Additional Software

Software	Reference
Adobe Photoshop and Illustrator	Adobe Systems Incorporated
ArcGIS 9.3.1 with ArcGIS Spatial Analyst	ESRI Inc.
Google Earth	Google Inc.
Microsoft® Office Suite 2003	Microsoft Corporation
SigmaPlot	Systat Software, Inc.
SigmaStat	Systat Software, Inc.

2.13 Instruments

Instrument	Reference
Balance	SARTORIUS Precision Balance
Centrifuges	Pico 17, Heraeus®
	Biofuge Stratos, Heraeus®
	UNIVAPO 150H, UniEquip
Electroporation apparatus	Pulse Controller Plus, Capacity Extender Plus, Gene Pulser II Plus, Biorad
Electrophoresis power supply	E835, Consort
Fluorescence reader	GENios Plus, Tecan
Gel Tank and Casting Form	Biozyme
Gel Documentation System	InGenius, Syngene
GPS Navigator eTrex	Garmin (Europe) Ltd
Heating Block	Thermomixer Comfort, Eppendorf
Incubator	HERAcell® 150, Heraeus
	Binder Incubators, Binder
KingFisher Flex	ThermoScientific®, Thermo Fisher Scientific
NanoDrop ND-1000 Spectrophotometer	Isogen
PCR Machine	Mastercycler® Gradient, Eppendorf
PRO200 Rotor-Stator Homogenizer	PRO Scientific Inc.
Real Time PCR Machines	Opticon® 2 DNA Engine, Chromo4™, CFX, MiniOpticon, Biorad
Safe Imager™ 2.0	Invitrogen
Sequencer	ABI PRISM® 3130xl Genetic Analyzer, Applied Biosystems
Shaker	Multitron 2, INFORS-HT
Stereomicroscope	Eschenbach
TissueLyser II	Qiagen
Vortex	Vortex-Genie® 2, Scientific Industries

3 Methods

3.1 Determination of Collection Sites

Sites for tick collection throughout Luxembourg were chosen using maps (scale 1:50.000) and the online mapping tool Google Earth. On the field, the precise location of each collection site was chosen based on criteria of suitability of habitat (regarding vegetation and relative humidity) and accessibility. Different habitats were chosen such as broad leaved forest, coniferous forest, mixed forest, agricultural and urbanized areas in order to represent the landscape structure of Luxembourg. Each of the 33 collection sites was mapped and GPS coordinates were taken using a GPS navigator. Coordinates were downloaded and used for positioning of the collection sites in Google Earth and ArcView 9.3.1.
Collection sites in Belarus, Bulgaria and Nigeria were selected by our collaborating partners based on similar criteria and GPS coordinates were taken. Collection of ticks was coordinated by our collaborating partners.

3.2 Tick collection

3.2.1 Collection of questing ticks

In Luxembourg, each of the 33 collection sites was visited once per month from May to October in the years 2007, 2008 and 2009. Questing ticks were collected from the vegetation using the cloth dragging method. In the process a white towel was dragged over the lower vegetation (up to 1m in height), mimicking the movements of a potential vertebrate host for ticks. The towel was frequently checked for attached ticks. Each tick was removed with forceps and stored separately in a 1.5ml Eppendorf tube containing fresh grass to provide sufficient humidity. Tubes were labelled with collection site, date and serial number. At each collection site about $100m^2$ were flagged and in case of unsuccessful flagging the area was extended to up to 1000 m^2. Abiotic parameters (temperature, relative humidity) were recorded at each visit.

3.2.2 Collection of feeding ticks

In Luxembourg, feeding ticks were sent in by medical doctors, veterinarians, hunters, foresters, ornithologists, Centre de Soins pour la Faune de Sauvage (CdS-LNVL) and the general public who removed the parasites from various vertebrate hosts, e.g. humans, domestic and wild animals. A guide to the labelling and sending of ticks was available on the homepage of the Institute of Immunology, but also distributed together with collection tubes

to doctors, veterinarians and foresters. Required parameters were date of collection and host species. Only intact ticks with sufficient information were integrated into the tick data base.

3.2.3 Nigeria

In 2009, questing ticks were collected from the vegetation at seven locations (Elepo, Alowonle, Fuleni, Orisunbare, Lanlate, Maya, Igbo-Ora) and feeding ticks were obtained from cattle at four other locations (Moniya, Alakia, Bodija, Mokola) in southwestern Nigeria.

3.2.4 Belarus

In April and May 2009, ticks were collected from the vegetation and cattle at 32 collection sites in the regions Brest (n=8), Gomel (n=7), Grodno (n=2), Minsk (n=3), Mogilev (n=7) and Vitebsk (n=5). One tick was removed from a dog in Minsk region. Collection of questing ticks was performed as described in 3.2.1 and feeding ticks were removed with forceps.

3.2.5 Bulgaria

In the years 2005-2008, ticks were collected from the vegetation and human or animal hosts at rural or urban areas of nine major districts in Bulgaria (see also Figure 18).

3.2.6 Russia

In Russia, wild birds were caught in ornithological nets during the annual ringing season 2008 at the Rybachy Biological Station (Zoological Institute, Russian Academy of Sciences) at the Curonian Spit in Kaliningrad enclave. Any feeding ticks were removed from birds during the ringing procedure and stored individually in tubes.

3.3 Tick identification and storage

Ticks were identified using standard taxonomic keys for European and African tick fauna (58, 234), in ambiguous cases tick species was confirmed by mitochondrial 12S rDNA partial gene sequencing. Ticks from Luxembourg were directly identified and stored individually in sterile 1.5ml tubes at -80°C until further processing. Ticks from our collaborating partners were stored in 70% Ethanol at 4-8°C directly after collection, shipped to Luxembourg and identified to species level at the Institute of Immunology.

3.4 Serum Collection and Storage

Blood was drawn from forestry workers in April and May 2010 by the Inspection Sanitaire of the Ministry of Health in Luxembourg using 8ml Vacuette Serum Sep Clot Activator collection tubes (Greiner, Bio-One) and Vacutainer Safety Lock Blood Collection Set (BD Medical, Temse, Belgium). The blood was allowed to clot for 30 min at 37°C before centrifugation (3000 rpm for 10 min) and the serum was aliquoted and stored in individual tubes at -80°C. Questionnaires regarding place of work, age, gender, number of tick encounters, tick bites, protective measures and history of Lyme Borreliosis were completed by each participant on the day of blood drawing (see annex 7.1 and 7.2). This part of the study was approved by the competent ethical committee of Luxembourg (Comité National d'Éthique de Recherche, CNER). In addition a notification and authorisation was submitted and accepted by the Commission nationale pour la protection des données (CNPD).

3.5 Extraction Kit Comparison

An extensive kit comparison was performed on six commercial kits, namely KingFisher Total Nucleic Acid Kit, KingFisher Viral RNA Kit, QIAamp Viral RNA Mini Kit, QIAamp DNA Blood Mini Kit, QIAamp RNeasy Mini Kit, and the Macherey&Nagel Total RNA&DNA Kit. For each kit, single ticks (nymph, male, and female) and pools comprising of three ticks each (nymph, male, female) were tested. Samples were processed according to the manufacturer's protocol of the respective kits. The pooled ticks were disrupted in 750µl PBS and evenly distributed in different tubes, one for each kit. As a RNA extraction control a pool comprising of 20µl of each sample was spiked with Measles virus (RNA virus). DNA extraction was confirmed by using *Ixodes* specific primers. Negative controls were also included in the extraction comparison. Directly after total nucleic acid extraction a reverse transcription (RT) PCR was performed on the raw sample. On an aliquot of each sample a DNase I digestion using the TURBO DNA-free™ Kit was carried out to eliminate all DNA. This step was confirmed by specific PCR and RT-PCR was performed on these DNA-free samples. An *Ixodes* specific PCR on the cDNA on DNase I digested samples was performed as an additional RNA extraction control.

Of these kits, initially the QIAamp DNA Blood Mini kit was chosen based on its good cost/performance ratio and all questing ticks from 2007 were extracted with this kit. Due to the high number of ticks that were collected, the ticks of the following years were extracted using the automated KingFisher 96 Magnetic Particle Processor and either the KingFisher

Total Nucleic Acid Kit (ticks stored at -80°C) or the InviMag DNA Tissue kit (ticks stored in 70% Ethanol).

3.6 Total Nucleic Acid extraction with QIAamp® DNA Blood Mini Kit

The manufacturer's protocol was modified as follows: 300µl of lysis buffer AL was pipetted into a 2ml round-bottom safe-lock tube and the frozen tick was added. The tick was disrupted using the PRO200 Rotor-Stator Homogenizer with changeable probes or using the Tissue Lyser II with 5mm stainless steel beads at 25Hz for 6min. The lysates were centrifuged at 8,000rpm for 1min to remove any foam. 300µl of 96-100% Ethanol was added to each tick lysate, mixed thoroughly and centrifuged briefly. The lysate was applied to a spin column and centrifuged at 8,000rpm for 1min. The filtrate was discarded, 500µl of wash buffer AW1 was added and centrifuged at 8,000rpm for 1min. After discarding the filtrate, 500µl of wash buffer AW2 was added and centrifuged at full speed for 3min. The spin column was placed in a new collection tube and centrifuged dry at full speed for 1min. For elution, the spin column was placed in a 1.5ml Eppendorf tube, 200µl of elution buffer AE was added and incubated at room temperature for 1min. The final centrifugation step was carried out at 8,000rpm for 1min and each sample was split into four 50µl aliquots, which were stored at -80°C until further processing. The changeable probes were cleaned in several washing steps using bidistilled water, RNA/DNA Remover and 70% Isopropyl alcohol and autoclaved before next usage.

3.7 Total Nucleic Acid extraction with MagMAX™-96 Total Nucleic Acid Isolation Kit

Ninety-four samples were processed at a time and 2 RNA extraction controls were included on each plate. A mix of nucleic acid (NA) binding beads and lysis/binding enhancer at a ratio of 1:1 was prepared and 17.4µl was distributed onto a KingFisher 96 well processing plate. Two plates each with 150µl of either Wash Solution 1 or Wash Solution 2 in each well were prepared. Into the elution plate 100µl of Elution Buffer was dispensed. Tick disruption was performed in 66µl of Lysis/Binding Solution and 49µl of nuclease free water using the Tissue Lyser II as described in 3.6. For the RNA extraction control, 8µl of a 1:10 diluted Measles virus culture was added to 92µl of lysis buffer and nuclease free water mix. The tick lysates (100µl of the total volume) and the controls were transferred into the processing plate containing the magnetic bead mix. To each sample, 56.5µl of isopropyl alcohol was added

and all plates were placed into the KingFisher Flex (automated magnetic particle processor) and processed according to the program in Table 2.

After elution, samples were split into four aliquots (25µl each), transferred to sterile 96 well PCR plates and stored at -80°C until further processing.

Table 2: KingFisher 96 program for MagMAX™-96 Total Nucleic Acid Isolation Kit

Step	Procedure	Time
Binding	Automatically sample mixing	5min
	MAP separation	
	Moving MAP into Washing Plate 1	
Washing 1	Automatically sample mixing	50s
	MAP separation	
	Moving MAP into Washing Plate 2	
Washing 2	Automatically sample mixing	50s
	MAP separation	
	Moving MAP into Washing Plate 3	
Washing 3	Automatically sample mixing	50s
	MAP separation	
	Moving MAP into Washing Plate 4	
Washing 4	Automatically sample mixing	50s
	MAP separation	
	Drying the MAP outside the plate	2min
	Moving MAP into Elution Plate	
Elution	Incubation of MAP by mixing	3min
	MAP separation	
	Automatically remove MAP	

3.8 Total Nucleic Acid extraction with InviMag® Tissue DNA Mini Kit/KF96

Ticks that were stored in 70% ethanol were washed in PBS and RNase/DNase free water and were dried on sterile filter paper before DNA extraction. Homogenization and disruption of ticks was performed in 120µl of Lysis Buffer G using the Tissue Lyser II as described in section 3.6. A mix of Binding Buffer T (50µl) and MAP solution A (20µl) was dispensed into each well of the binding plate and 120µl of tick lysate was added. Three washing plates containing 150µl of Wash Buffer and one elution plate containing 100µl of Elution Buffer D were prepared. All plates were placed into the KingFisher Flex and processed according to the program in Table 3. After elution, samples were split into four aliquots (25µl each), transferred to sterile 96 well PCR plates and stored at -80°C until further processing.

Table 3: KingFisher 96 program for InviMag® Tissue DNA Mini Kit/KF96

Step	Procedure	Time
Binding	Automatically sample mixing	3min
	MAP separation	
	Moving MAP into Washing Plate 1	
Washing 1	Automatically sample mixing	50s
	MAP separation	
	Moving MAP into Washing Plate 2	
Washing 2	Automatically sample mixing	50s
	MAP separation	
	Moving MAP into Washing Plate 3	
Washing 3	Automatically sample mixing	50s
	MAP separation	
	Drying the MAP outside the plate	6min
	Moving MAP into Elution Plate	
Elution	Incubation of MAP by mixing	3min
	MAP separation	
	Automatically remove MAP	

3.9 Reverse Transcription

Reverse transcription was performed on total nucleic acid extracts from ticks collected in Luxembourg and on RNA extraction controls. Two mixes were prepared, one containing random primer, dNTPs, and RNAse free water, the other containing DTT, RNaseOUT, 5x First-Strand Buffer, RNase free water and SuperScript III (Table 4). After adding the template RNA to mix 1, nucleic acids were denaturated at 65°C for 5min and placed on ice. Mix 2 was added to mix 1 and incubated at 50°C for 80min. The reaction was heat inactivated at 70°C for 15min.

Table 4: Conditions for Reverse-Transcription PCR

Reagent	Mix	Volume (µl)	Concentration
Random primer	1	5	150ng
dNTP mix	1	1	10 mM each
RNA	1	5	10pg-5µg
RNase free water	1	1	
DTT	2	2	5mM
RNaseOUT™	2	0.5	20 units
5x First-Strand Buffer	2	4	1x
SuperScript® III	2	1	200 units
RNase free water	2	0.5	

3.10 PCR Optimisation

Primers for the pathogen detection PCRs applied in this study were taken from the literature and some published primers were modified in order to detect a broader range of species. Primer name, sequence, target gene, reference and any modifications are listed in section 2.6.

All primer pairs were subjected to an extensive optimisation using positive controls provided by research colleagues. Variable conditions in the optimisation PCRs were $MgCl_2$ concentration, primer concentration and annealing temperature of primers (Table 5). Optimal annealing temperatures were calculated using the software FastPCR 3.7.8 and three temperatures within the given range were tested. Based on cycle threshold (C_t) values, melting curve and agarose gel the best PCR conditions were chosen. The optimised PCR conditions are also given in section 2.6. For most detection PCRs, nested or semi-nested protocols were used in order to increase sensitivity and specificity.

Table 5: Variable and fix conditions for PCR optimisation

Reagent	Volume (µl)	Concentration
PCR Buffer (10x)	2.5	1x
MgCl2	0.5 - 1.5	0.99 - 2.98 mM
dNTP's	0.5	0.2 mM
primer fw	1 - 2	0.8 - 1.6 µM
primer rv	1 - 2	0.8 - 1.6 µM
SYBRGreen® (100x stock)	0.25	1x
Platinum Taq® polymerase	0.1	0.5 units
DNA template	5	
RNase/DNase free water	11.15 - 14.15	

3.11 Agarose Gel Electrophoresis

Amplified PCR products were size separated on a 1.5% agarose gel. This gel was prepared by completely dissolving 1.5g of powdered agarose in 100ml of 1x TAE buffer using a microwave oven. The mixture was cooled down to approximately 55°C, 10µl of SYBR® Safe was added, the hot gel was poured into a casting form (14x12cm) and combs were inserted. Also, big gels containing 300ml of TAE-buffer and 30 µl of SYBR® Safe were used in the respective casting form (25x25cm). After polymerization, the tray was placed into a gel chamber filled with sufficient 1xTAE buffer to cover the complete gel and the combs were removed. 5µl of amplified DNA was mixed with 1µl of 6x Loading Dye and loaded onto the gel. The outer two pockets of each row were loaded with a standardized size marker (1kb plus DNA ladder™). The gel was run at an electrical current of 130V for 40min. After completion, DNA bands were visualized under ultraviolet light (300nm) and pictures were taken with the InGenius Gel documentation System.

3.12 PCR Product Purification

PCR products were purified before subjecting them to downstream application such as cloning or sequencing in order to remove unspecific PCR products and/or residual primers

and non-incorporated nucleotides. The Jetquick PCR product Purification Spin kit (Genomed) was used for samples in which a specific DNA fragment was amplified (one clear band on gel). 20µl of PCR product were mixed with 175µl of buffer H1, transferred to a Jet Quick Spin® column placed in a 2ml collection tube and centrifuged at 10,000rpm for 1min. The filtrate was discarded; 500µl of buffer H2 was pipetted onto the spin column and centrifuged at 10,000rpm for 1min. The filtrate was discarded and the spin column was centrifuged dry at 10,000rpm for 1min. The spin column was placed into a sterile 1.5ml Eppendorf tube, 30µl of elution buffer TE was added and the column was incubated at room temperature for 1min. After centrifugation (10,000rpm for 1min) the eluted DNA was stored at -20°C until further processing.

In case of unspecific DNA amplification, the remaining 20µl of PCR product were mixed with 4µl of 6x Loading Dye and separated on a fresh 1.5% agarose gel. Bands of interest were excised using Safe Imager™ and a scalpel. Purification of DNA was performed using the QIAquick® Gel Extraction Kit. The gel slice was weighed and three volumes of Buffer QG were added to one volume of gel (100mg ~ 100µl). The gel was completely dissolved at 50°C for 10min in a heating block and one gel volume of isopropyl alcohol was added. After mixing the solution was applied to a QIAquick spin column and by centrifuging at 10,000rpm for 1min the DNA was bound to the membrane. The filtrate was discarded and 0.5ml of Buffer QG was added to remove all traces of agarose and centrifuged at 10,000rpm for 1min. Again, the filtrate was discarded and 0.75ml of Buffer PE was added to wash the sample. The spin column was placed in a new 2ml collection tube and centrifuged dry at 13,000rpm for 1min. To elute the purified DNA, the spin column was placed in a clean 1.5ml Eppendorf tube, 30µl of Buffer EB was added to the center of the membrane and incubated at room temperature for 1min before centrifugation (10,000rpm for 1min).

3.13 Cloning and M13 PCR

Purified PCR products were cloned into TOPO® vector and transformed into TOP10 *E. coli* electrocompetent cells using the TOPO TA Cloning® Kit for Sequencing. Briefly, 4µl of fresh PCR product are mixed with 1µl of diluted salt solution (1:10) and 1µl of TOPO® vector, incubated for 5min at room temperature and placed on ice. Electrocompetent *E. coli* were diluted with 50µl of sterile water and 50µl were pipetted into a pre-chilled electro-cuvette. Carefully 2µl of the cloning reaction were added to avoid formation of bubbles and samples were electroporated at 2,25kV, 200 Ohm and 25uF. Warm S.O.C. medium (37°C) was added directly after the electroporation the solution was transferred into a 2ml Eppendorf

tube and incubated at 37°C for 1 hour. For blue and white screening of the colonies, 40µl of X-Gal was spread onto LB agar plates containing 30µg/ml Kanamycin. These plates were also incubated at 37°C for 1 hour. After incubation, 60µl and 200µl of the same S.O.C. and bacteria solution were spread on two agar plates and incubated at 37°C overnight. White colonies were picked with sterile pipette tips and transferred on a new agar plate (master plate) and into a M13 PCR reaction (25µl reaction containing 2.5mM MgCL2, 200µM dNTPs, 0.8µM of each M13 primer, 0.5 units Platinum Taq Polymerase). Standard PCR parameters were used with an annealing temperature of 58°C and an elongation time of 1min for 35 cycles. PCR products were checked on a 1.5% agarose gel and only clones with right insert size were subjected to PCR product purification and sequencing.

3.14 Plasmid Mini Prep

In order to prepare large quantities of plasmids containing inserts of DNA fragments used as positive controls, liquid bacterial cultures were grown. A single white colony from the cloning plate (see section 3.13) was picked with a sterile pipette tip and used to inoculate 5ml of liquid LB medium containing 30µg/ml Kanamycin. The culture was incubated at 37°C overnight (maximum of 16 hours) with vigorous shaking. Cells were harvested by centrifuging at 1,500rpm for 10min. The pellet was resuspended in 250µl of Buffer P1 and transferred into a 1.5ml Eppendorf tube. 250µl of Buffer P2 was added and the solution mixed thoroughly by inverting the tube 5 times before adding 350µl of Buffer N3 and mixing again. The solution was centrifuged at 13,000rpm for 10min and the supernatant was transferred to a QIAprep spin column. After centrifuging at 13,000rpm for 1min, the filtrate was discarded and the membrane washed with 0.75ml of Buffer PE by centrifuging at 13,000rpm for 1min. The filtrate was discarded and the column was centrifuged dry at 13,000rpm for 2min. The purified plasmid was eluted with 50µl of Buffer EB (incubation 1min at room temperature, centrifugation at 13,000rpm for 1min) and stored at -20°C. Before usage, the plasmids were checked for correct insert by subjecting them to PCR following the pathogen detection protocol and sequencing the obtained DNA fragment.

3.15 Sequencing

Chain termination sequencing PCR was performed with the BigDye Terminator® v3.1 Cycle Sequencing kit using a mixture of dNTPs and fluorescently labelled, chain terminating dideoxynucleotides ddNTPs (Table 6). The PCR reaction was initially denaturated at 96°C for

1min, followed by 25 cycles of denaturation at 96°C for 10s, annealing at 50°C for 5s and elongation at 60°C for 2min.

Table 6: Sequencing PCR using the BigDye Terminator® v3.1 Cycle Sequencing kit

Reagent	Volume (µl)	Concentration
BigDye Terminator® Mix	1	1x
TE buffer	1.5	0.75
Primer fw	1	0.5µM
Primer rv	1	0.5µM
DNA template	5 (maximum)	10ng

The final PCR products were purified using 5µl of 125mM EDTA and 10mM of distilled water per sample. After mixing, 60µl of 96-100% Ethanol was added, the samples were mixed and incubated in the dark for 15min at room temperature. The samples were centrifuged at 3,000rpm for 30min (4°C) and the solution was removed. Washing was repeated as before using 70% ethanol. The samples were dried for 15min in a UNIVAP 150 H and stored at 4°C until further processing. Sequencing was performed on the capillary sequencer ABI PRISM® 3130xl Genetic Analyzer with 80cm capillaries. Samples were heated to 95°C for 5min, supplemented with 10µl HI-DI and incubated at 95°C for 5min before loading onto the capillary sequencer.

3.16 Phylogenetic Analysis

The generated electropherograms were imported into the SeqScape® program and aligned to a reference sequence that was downloaded from NCBI. The quality of each sequence was checked individually and in case ambiguous nucleotides suggested mixed infections, the samples were cloned and 16 clones per sample were sequenced and analysed. Further reference sequences were integrated and the alignment was exported. Quality control and further editing of the alignment was done with BioEdit software. Phylogenetic trees were constructed by applying bootstrap test of phylogeny with Neighbor-Joining algorithm, Kimura 2-parameter model and 1,000 bootstrap replicates using the MEGA 3.1 and 4.0 software. Neighbour-Joining is a simplified method based on the minimum evolution criterion, in which the topology with the minimum total branch length is used to estimate the correct tree. The widely used Kimura 2-parameter model takes transitional and transversional substitution rates into account while assuming that the substitution rate of all nucleotides is the same. Generally, the number of nucleotide substitutions reflects the evolutionary distance of sequences and is a valuable tool for molecular species identification. Bootstrap values give an estimate of the reliability of the topology of an inferred phylogenetic tree by calculating

multiple topologies based on a sub-sample of the alignment and comparing these to the original phylogenetic tree. Bootstrap values express the percentage of times in which the re-calculated trees display exactly this interior branch node. The higher the bootstrap value of a branch node the more reliable the resulting topology of the tree. Generally, nodes with values above 70% are considered reliable and those with values above 95% as correct.

3.17 Enzyme-Linked-Immuno-Sorbent-Assay (ELISA)

Serum samples from forestry workers were analysed for the presence of IgG serum antibodies against *Borrelia burgdorferi* sensu lato using the B.afzelii + VlsE IgG Europe ELISA IgG Test Kit on all samples. The tests were performed according to manufacturer's instructions. Each test run included blank, positive, negative and two cut-off controls. Serum samples were prepared in a 1:100 dilution. 100µl of each control and sample were pipetted into the antigen coated 96-well plate and the closed plate incubated at 37°C for 30min. The liquid was aspirated and the plate was washed four times with 400µl of diluted washing solution and the plate was completely dried on a cellulose paper. 100µl of Conjugate IgG containing anti-human antibodies linked to horseradish peroxidase was added to each well and the closed plate incubated in the dark at 37°C for 30min. The conjugate incubation was stopped by washing four times with diluted washing solution. As an enzyme substrate 100µl of 3,3',5,5'-Tetramethylbenzidine (TMB) was added, which reacted in the following incubation period (37°C, 30min) with the peroxidase, resulting in a colour change. The reaction was stopped using 50µl of Citrate Stopping Solution leading to another colour change from blue to yellow. The extinction of each sample is measured at 450nm/620nm; optical density (OD) value of the blank control was deducted from each sample. Test results were validated using the test specific validation criteria.

3.18 LINE Immunoblot

The more specific LINE immunoblot is used to validate the obtained equivocal and positive ELISA results. Two-tier testing is generally used to reduce the costs as pre-screening is performed with low-cost ELISA and only a subset of samples is submitted to the expensive immunoblot analysis. The Borrelia Europe plus TpN17 LINE IgG Line Immunoblot kit provides nitrocellulose strips that are coated with antigens of the pathogen. A test strip was placed into a channel of an incubation tray and soaked with 1.5ml of dilution/wash buffer for 1min. 100µl of positive, negative or cut-off controls or 15µl of patient serum were added to each strip and incubated for 30min on a rocking platform. Liquid was aspirated, strips were

washed three times in 1.5ml of dilution/wash buffer for 5min and the liquid was aspirated. The conjugate dilution containing anti-human antibodies linked to alcaline phosphatase was added to the dry strips and incubated for 30min on a rocking platform. Afterwards the liquid was aspirated; the strips were washed three times in 1.5ml of dilution/washing buffer for 5min and rinsed with deionised distilled water for 1min. Dry strips were incubated with 1.5ml of substrate solution containing BCIP/NBT for 10min on a rocking platform. The reaction was stopped by aspiration and the strips washed three times with deionised distilled water without incubation. After drying, the strips were analysed according to manufacturer's instructions.

3.19 Weather Data

Temperature and relative humidity data from 16 weather stations in Luxembourg was obtained from the Administration des Services Techniques de l'Agriculture (ASTA) for the years 2006 to 2009. Parameters were recorded automatically every ten minutes. Monthly mean temperature and relative humidity were determined for each weather station. In order to measure the drying power of the atmosphere, the saturation deficit as a function of temperature and relative humidity was calculated according to Randolph and Storey (172).

3.20 Statistical Analysis

For statistical analyses one way Analysis of Variance (ANOVA) tests (SigmaStat3.1, Systat Software, Erkrath, Germany) were performed. Kruskal-Wallis one-way analysis of variance on ranks was performed when Normality Test or Equal Variance Test failed using either the suggested Tukey Test or the Holm-Sidak method as an All Pairwise Multiple Comparison Procedure. Fisher's exact test or Pearson's goodness of fit chi-square (GFX) test were used for two by two analyses.

3.21 Habitat Categories

The habitat of each collection site from Luxembourg was characterized by the percentage of forest, agricultural plains, water bodies and urbanised areas (buildings, sealed surfaces) in a $1km^2$ area with the collection site as centroid using aerial photographs (Google Earth). Thus, four ecological categories were defined (category I: 0-4%, II: 5-9%; III: 10-24%; IV: 25-60% of urbanised area) with 6-11 collection sites per category Table 7.

3.22 Geographic Groups

In order to test for differences in the tick infection rates among geographic regions in Luxembourg, the data of 4-8 collection sites in the North, Northeast, East, South, West and Centre were pooled Table 7. In 2008 and 2009, these geographic groups were changed according to the administrative regions of the Eaux et Forêts of Luxembourg in order to match the seroprevalence data from Luxembourgish forestry workers.

In Belarus, the 32 collection sites were grouped according to the respective administrative regions of Brest (n=8), Gomel (n=7), Grodno (n=2), Minsk (n=3), Mogilev (n=7) and Vitebsk (n=5).

3.23 Georeferencing Tools

Geographic Information Systems (GIS) are tools for analysing, manipulating and visualizing georeferenced data in their geographic context. Spatial information is obtained from Global Positioning Systems (GPS) which use the space-based Global Navigation Satellite System (GNSS) to provide information about geographic location and time. The GPS receives time signals from satellites that are transmitted by radio and calculates its exact position based on the signal propagation delay. Remote Sensing (RS) provides surface information of the Earth in images taken from satellites, aircrafts, helicopters and ships. These images give valuable information on e.g. landscape features, vegetation types, land surface temperature, atmospheric moisture and rainfall. As a GIS tool for mapping the spatial distribution of *Borrelia burgdorferi* s.l. in ticks and its seroprevalence in forestry workers, ArcGIS 9.3.1 (ESRI) and ArcGIS Spatial Analyst extension was used. GPS coordinates were uploaded and each collection site was mapped using shape files provided by ESRI. Point data from collection sites was interpolated using the inverse distance weighted model and a cell size of 100m.

4 Results and Discussion

4.1 Prevalence and seasonality of tick-borne pathogens in questing *Ixodes ricinus* ticks from Luxembourg

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Prevalence and seasonality of tick-borne pathogens in questing Ixodes ricinus ticks from Luxembourg

A. L. Reye, J. M. Hübschen, A. Sausy, C. P. Muller

A. L. Reye contributed significantly to the study design and was in charge of tick collection, experimental setup, experimental work, data analysis and writing of the manuscript.

In Western Europe, the hard tick *Ixodes ricinus* is the main arthropod vector of various human and animal pathogens, causing several tens of thousands severe infections in humans every year (94, 127). The most common tick-borne infection is Lyme borreliosis. This multi-systemic disorder is caused by spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex which is comprised of at least 12 species worldwide (181). Among the 6 European species, only *B. garinii*, *B. afzelii* and *B. burgdorferi* sensu stricto (s.s.) are known as human pathogens, whereas the significance of *B. valaisiana, B. spielmanii* and *B. lusitaniae* for human health is not clear (91). In a metaanalysis of 154 European studies, a mean of 13.7%

of ticks were found to be infected with *Borrelia* sp., predominantly with *B. afzelii* and *B. garinii*. However, the prevalence of *Borrelia* species varies from 2 to 49% between different regions (176).

Other tick-borne bacteria which cause disease in humans are *Rickettsia* sp., *Anaplasma phagocytophilum*, *Bartonella henselae* and *B. quintana*, *Coxiella burnetii* and *Francisella tularensis* ssp., all of which show only relatively low prevalence rates of 0.1-4.8% in European ticks (63, 86, 95, 167, 192, 200). In addition, three species of the parasitic protozoan *Babesia* are known to infect humans, namely *B. divergens, B. microti* and the newly described *Babesia sp*. EU1 (11). In Western Europe, also *Tick-borne encephalitis virus* (TBEV) has a relatively low prevalence; however, this pathogen deserves special attention because of the severe disease it causes in humans. Tick-borne encephalitis affects at least 10,000 humans in Europe annually (46, 127) and up to 5% of ixodid ticks are infected in endemic areas (179, 213).

As a result of climatic changes and human impact on the environment, the prevalence of ticks and tick-borne infections in Central Europe are expected to be increasing (79, 232). Nevertheless, recent studies on human pathogens are rare in Central Europe (49, 61, 65, 110, 135, 192, 193) and comprehensive surveys to assess risks to human health are warranted.

Here we present such a comprehensive study in Central Europe which investigates all relevant human tick-borne pathogens in questing nymphal and adult ticks from 33 representative collection sites throughout the 2007 season.

4.1.1 Results

Tick numbers. A total of 1,500 ticks including 106 larvae, 752 nymphs and 642 adults (320 males, 322 females) were collected. All ticks belonged to the species *Ixodes ricinus*. Tick density ranged from 3.7 ticks per $100m^2$ in the West to 9.3 in the East. Higher densities were found in habitats of categories II and III (8.7 and 7.8 ticks) in comparison to the others (Table 7). The nymphal and adult tick activity was highest in May and June (Figure 2A). Larvae showed their main activity in August (9 sites, 1-56 larvae/site). Despite considerable variability in tick numbers per collection site (14-134 ticks/site, mean 46), geographic region and habitat category, highest numbers of nymphs and adults were always observed in spring (data not shown).

Tick infection rates. Of the 1,394 adult and nymphal ticks, a total of 19.5% (n=272) were infected with at least one pathogen. Nymphs had a significantly lower overall infection rate (16.4%) than adults (23.2%, $p<0.01$), with females showing a significantly higher infection rate (26.7%) than males (19.7%, $p<0.05$). A comparison of the infection rates by geographic regions and habitat categories revealed considerable variations (Table 7).

Borrelia. *B. burgdorferi* s.l. was the predominant pathogen group and was detected in 11.3% (n=157) of all ticks. As expected, the tick infection rate was significantly higher in adults (15.0%; males 14.7%; females 15.2%) than in nymphs (8.1%) ($p<0.01$). *Borrelia* infection rates were highest in the Northeast (21.9%) and lowest in the West (2.8%) (Table 7). The

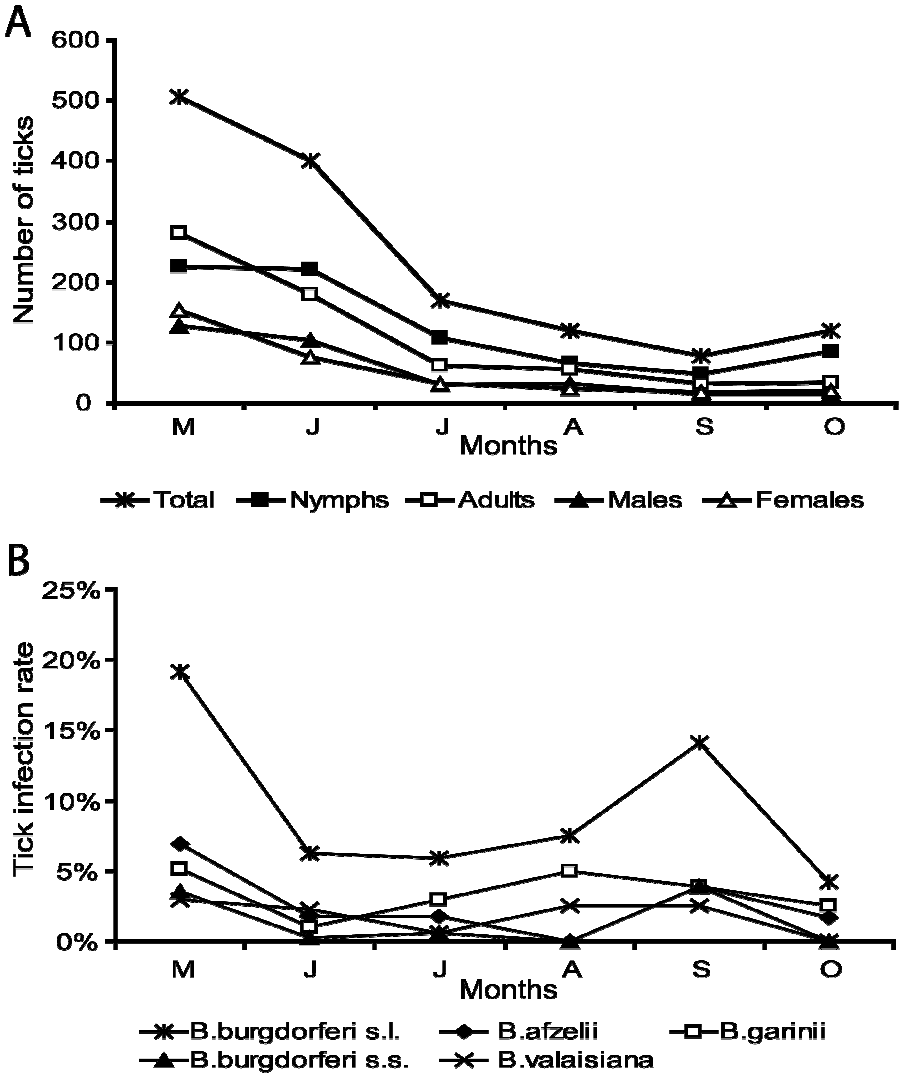

Figure 2: Overall seasonal activity of the developmental stages of *I. ricinus.* Number of ticks collected at 33 collection sites from May to October 2007 (A) and *Borrelia* infection rates of ticks (B).

habitat classification showed a positive correlation between infection rates and the extent of urbanisation, ranging from 8.9% in category I to 14.6% in category IV (Table 7).

Six different *Borrelia* species were identified based on the flagellin B (FlaB) gene (Figure 3A). *B. afzelii* (33.1%; n=52) and *B. garinii* (29.9%; n=47) were the most prevalent species, followed by *B. valaisiana* (19.1%; n=30), *B. burgdorferi* s.s. (14.6%; n=23), *B. spielmanii* (2.5%; n=4), and *B. lusitaniae* (0.6%; n=1). Sequences for the outer surface protein A (OspA) were obtained for 133 FlaB-positive samples, resulting in 9.3% *B. burgdorferi* s.s., 37.7% *B. afzelii*, 31.8% *B.garinii* (serotypes 3-7: 2.6%; 0%; 2.6%; 10.6%; 3.3% and 11.9% of sequences in three distinct clusters most closely related to *B. garinii* strains) (Figure 3B). OspA sequences of *B. valaisiana* and *B. spielmanii* formed distinct clusters. The OspA

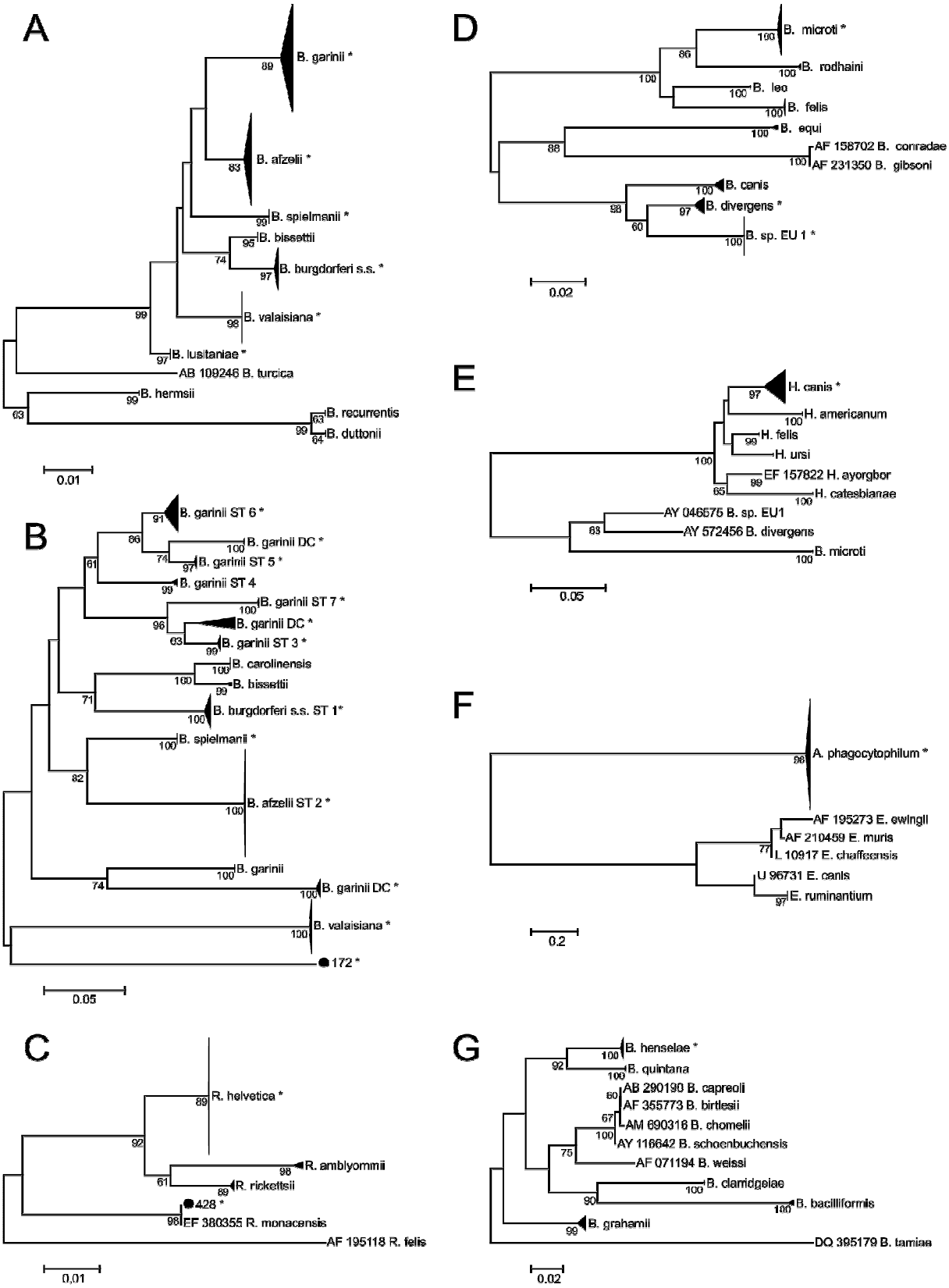

Figure 3: Phylogenetic trees for speciation of pathogens in Luxembourg. For detailed legend see next page.

Figure 3: Phylogenetic trees for speciation of pathogens in Luxembourg. Phylogenies are based on (A) 209 nucleotides of the FlaB gene of *B. burgdorferi* s.l. (nucleotides 151-359 of GQ918147.1) including 157 samples and 71 reference sequences; (B) 462-465 nucleotides of the OspA gene of *B. burgdorferi* s.l. (nucleotides 9441-9905 of CP001433.1) including 133 samples and 59 reference sequences; (C) 190 nucleotides of the 17kDa antigen gene of *Rickettsia* species (nucleotides 140-329 of GU292313.1) including 72 samples and 14 reference sequences; (D) 343-370 nucleotides of the 18rRNA of *Babesia* species (nucleotides 481-850 of EF413181.1) including 36 samples and 32 reference sequences; (E) 293 nucleotides of the 18rRNA of *Hepatozoon* species (nucleotides 171-461 of FJ608736.1) including 1 sample and 34 reference sequences; (F) 466 nucleotides of the groEL gene of *Anaplasma* species (nucleotides 45-510 of GQ988761.1) including 26 samples and 55 reference sequences; (G) 313 nucleotides of the 16S-23S region of *Bartonella* species (nucleotides 1782-2094 of AJ749669.1) including 4 samples and 26 reference sequences. Bootstrap values above 60 are shown. Stars represent (clusters including) our sequences.

Infection rates of adult ticks with *B. garinii* and *B. valaisiana* were significantly higher ($p<0.01$) than those for nymphs. A higher adult infection rate was found in habitat category II to IV, whereas in category I both infection rates were similar (data not shown).

***Rickettsia*.** In 5.1% (n=71) of ticks *Rickettsia* species were detected. These were identified as *R. helvetica* (n=70) and *R. monacensis* (n=1). No clear trend in the seasonal variation of infected tick activity was observed (data not shown). Highest tick infection rates were found in the South, lowest rates in the Centre (Table 7). All habitats had similar infection rates (Table 7). Nymphal and adult tick infection rates were similar (4.9% versus 5.3%), but the prevalence of *Rickettsia* infected females (7.8%) was significantly higher than that of males (2.8%) ($p<0.01$).

***Babesia*.** *Babesia* species were detected in 2.7% (n=37) of ticks, with *B.* sp. EU1 being predominant (59.5%) and *B. microti* being the second most common species (35.1%). *B. divergens* and *Hepatozoon canis* were each detected in a single tick only (2.7%). The highest prevalence was found in September (data not shown). Tick infection rates ranged from 1.4% in the South to 4.5% in the Northeast and from 1.1% to 3.0% in the different habitat categories (Table 7). *B. microti* infection rates were twice higher for adults (1.3%) than for nymphs (0.7%), whereas *B.* sp.EU1 was more prevalent in nymphs (1.9%) than in adults (1.3%).

***Anaplasma phagocytophilum*.** 1.9% (n=27) of ticks were infected with *A. phagocytophilum*. There was a clear unimodal seasonality for infected adult ticks with a peak in September, but no such pattern was found for nymphs (data not shown). The highest infection rate of *A. phagocytophilum* was found in ticks collected in the East (4.5%) (Table 7). Tick infection

sequence derived from tick #172 (FlaB sequence clustered with *B.lusitaniae*) clustered distinctly from all reference and sample sequences, suggesting the OspA sequence also to be from *B. lusitaniae*. 24 samples (1.7%) with nucleotide ambiguities were cloned and 19 mixed infections were confirmed. In 2 additional samples the FlaB- and the OspA-fragments corresponded to different *Borrelia* species. The most frequent combinations were *B. valaisiana* and *B. garinii* (10/21) (Table 8).

Seasonal evolution of *Borrelia* infections in ticks showed a bimodal seasonal activity beginning with high numbers in May and a second peak in September (Figure 2B). On a species level different patterns of seasonality were observed (Figure 2B).

Regional differences in the prevalence of *Borrelia* species were also observed. *B. afzelii* was predominant in the North (59.5%) and South (35.7%), whereas *B. garinii* was most prevalent (53.6%) in the East. *B. valaisiana* was the predominant species in the Northeast (29.4%). In the West, *B. garinii*, *B. afzelii* and *B. burgdorferi* s.s. were equally prevalent (33.3%).

Species composition varied between habitat categories (Figure 4): *B. afzelii* and *B. garinii* were equally prevalent in all categories except in IV, where *B. afzelii* was predominant. In category III, *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s. and *B. valaisiana* showed similar prevalence rates (23.4 to 25.5%). Tick infection rates of *B. garinii* were similar in all categories (3.0-4.0%), whereas *B. afzelii* seemed to prefer category IV (6.7%) to the others (2.7-3.8%). The 1.6-fold higher infection rates of categories III and IV (14.3%) in comparison to I and II (9.0%) are caused by *B. valaisiana* and *B. burgdorferi* (III) and *B. afzelii* (IV), respectively.

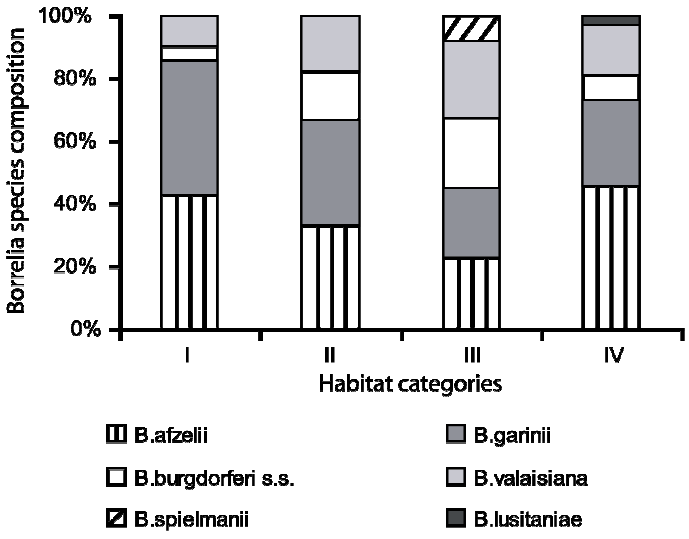

Figure 4: *Borrelia* species composition by habitat category.

rates were lowest in habitat category IV (0.8%) and highest in II (2.5%) (Table 7). Female ticks seem to be more often infected (3.4%) than male (1.6%) or nymphal ticks (1.5%).

***Bartonella*.** *B. henselae* was detected in 0.3% of all ticks and the 4 infected ticks were found at different collection sites and in different months (data not shown). *B. henselae* was only found in the North, East and South and in habitat categories I and II (Table 7).

Coxiella*, *Francisella tularensis* and *TBEV were not detected in any of the 1,394 ticks analysed.

Mixed infections. Infections with more than one pathogen occurred in 3.2% of all ticks (n=44), most of which were coinfections with 2 pathogens (n=42) (Table 8). Combinations of *B. burgdorferi* s.l. and *Babesia* sp. (22.7%) and *B. burgdorferi* s.l. and *R. helvetica* (18.2%), were most frequent. All coinfections involving *Babesia microti* were exclusively with *B. afzelii* (n=6). Additionally, no *Anaplasma* infected tick was coinfected with *B. afzelii*. Almost half of the observed coinfections (n=21) involved different *Borrelia* species (also see above). Two coinfections with 3 pathogens (*B. garinii*, *A. phagocytophilum* and *Babesia* sp. EU1 as well as *B. garinii* ST6, *B. valaisiana* and *B.* sp.EU1) were found in a male and nymph respectively. The adult coinfection rate (4.5%) was twice as high as the nymphal (2.0%) ($p<0.01$) and the great majority of multiple infected ticks (75%) were collected in May and June (data not shown).

Table 7. Tick infection rates for geographic groups and habitat categories in Luxembourg.

Geographic groups	CS	TD	*Total TIR*	*Borrelia*	*Rickettsia*	*Babesia*	*Anaplasma*	*Bartonella*
North	6	7.7	17.6%	12.1%	4.6%	3.9%	0%	0.7%
Northeast	4	5.9	27.1%	21.9%	6.5%	4.5%	0.6%	0%
East	6	9.3	20.1%	9.1%	7.8%	1.6%	4.5%	0.3%
West	4	3.7	10.3%	2.8%	4.7%	1.9%	2.8%	0%
Centre	8	8.1	15.6%	10.3%	2.4%	2.9%	1.3%	0%
South	5	4.4	20.7%	10.0%	7.9%	1.4%	2.1%	0.7%
Habitat categories								
I	6	5.5	16.9%	8.9%	4.7%	3.0%	1.3%	0.8%
II	11	8.7	18.1	9.0%	5.3%	1.1%	2.5%	0.4%
III	10	7.8	22.9	14.0%	5.1%	2.7%	2.4%	0%
IV	6	6.1	20.6	14.6%	5.1%	2.8%	0.8%	0%

CS, number of collection sites per group; TD, tick density; TIR, Tick infection rate. Note that for the total tick infection rates mixed infected ticks were counted only once.

4.1.2 Discussion

The present study is the most complete survey of all relevant tick-borne human pathogens in Central Europe. Additionally, it is among the very few studies with a monthly sampling of multiple collection sites.

The densities of *I. ricinus* at most collection sites (3.6 to 9.5 ticks/100m^2) correspond to the category "low tick abundance" (3-10 ticks/100m^2) according to Schwarz *et al.* (190). The observed infection rates of *B. burgdorferi* s.l. (11.3%), *R. helvetica* (5.1%), and *A. phagocytophilum* (1.9%) in Luxembourg are comparable to those reported from the neighbouring countries Germany (2002-2005: *Borrelia* 13.9-24%, *Rickettsia* 8.9%, *Anaplasma* 1.0% (86, 123, 152)), Belgium (1998: *Borrelia* 23% (135)) and France (2006: *Borrelia* 20.4%, *Anaplasma* 0.5%, *Rickettsia* 16% of tick pools, (63, 83)).

In Europe, the most prevalent *Borrelia* species are either *B. afzelii* (27, 101, 123) or *B. garinii* (12, 135). We observed marked differences in the prevalence of *Borrelia* species in both, the geographic regions and the habitat categories, which may be related to the specific host preference of the different *Borrelia* species. Based on their sensitivity to reservoir host complement, *Borrelia* species have been divided into three ecological groups (118). Thus, *B. afzelii* and certain *B. garinii* strains (OspA serotype 4) are mainly associated with rodents, *B. valaisiana* and other *B. garinii* strains (OspA serotypes 3,5,6 and 7) with birds, whereas *B. burgdorferi* s.s. is found in both rodents and birds (118). In categories III and IV, higher *Borrelia* infection rates are caused by *B. burgdorferi* s.s., *B. valaisiana* and *B. afzelii*, suggesting an established urban zoonotic cycle with synanthropic rodents and songbirds as main hosts. Urban zoonoses have been described for other arthropod- and tick-borne pathogens, e.g. *Bartonella*, *Coxiella*, *Ehrlichia* and *Rickettsia*, and their increasing incidence has been linked to various extrinsic and intrinsic factors (32).

The prevalence rates of *Babesia* species in our study are similar to reports from Germany (1%) (28, 86), but are much lower than in France (20.0%) (82). However, in Germany *B. divergens* is by far the most prevalent species (86), whereas in Luxembourg *B.* sp. EU1 and *B. microti* are predominant. We also detected *H. canis*, which has never been found in questing *I. ricinus* ticks from Central Europe before. The causative agent of canine hepatozoonosis is endemic in Southern Europe, Africa, and the Middle and Far East, where it is transmitted to dogs by oral uptake of infected *Riphicephalus sanguineus* ticks during grooming (6). International tourism including the importation of pet animals (6) may explain the introduction of non-endemic pathogens. The finding of *H. canis* in a questing female *I. ricinus*

suggests the successful transmission from an infected dog to a feeding instar in Luxembourg that maintained the infection transstadially. Whether *I. ricinus* is a competent vector and whether ecological factors favour the establishment and spread of this pathogen in Central Europe requires further attention.

Bartonella henselae (0.3%) has not been found in questing *I. ricinus* ticks in Central Europe before. This pathogen is commonly transmitted by infected cats and causes the Cat Scratch Disease in humans. Only recently the role of *I. ricinus* as a competent vector for *B. henselae* has been confirmed experimentally (33).

Although no TBEV infected tick was found in this study, findings from France and recently also from Luxembourg's two neighbouring German states Saarland and Rhineland-Palatinate (183, 224) suggest a further spread of this virus.

Since transovarial transmissions are rare, coinfections in *I. ricinus* ticks may shed some light on the route of infection, e.g. consecutive feedings, coinfected hosts or cofeeding. Interestingly, analysis of reservoir host preferences of each pathogen (Table 8) revealed that pathogen combinations which normally do not occur in the same host were about eight times more frequent in adults (0.8%) than in nymphs (0.1%). In contrast, pathogen combinations that occur in the same host had slightly higher rates for adults (1.4%) than for nymphs (0.8%). This suggests that coinfections of nymphs are acquired during larval feeding on coinfected hosts, while in adults consecutive feedings are the main source of coinfections.

Only few studies have taken the seasonal variations of tick infection rates into account. Intriguingly, tick infection rates of *Borrelia* sp. and *Babesia* sp. were low in summer (July and August) and significantly increased in September (6.7% to 14.1% ($p<0.05$) and 1.7% to 3.9% ($p<0.05$), respectively). For *A. phagocytophilum*, a similar pattern was observed (1.4% to 5.1%) that may reflect a behavioural adaptation strategy of ticks. Aridity can force ticks to undergo quiescence in order to avoid critical loss of energy, which may be exacerbated by pathogen infections (72, 111, 157, 197) and thus contribute to preferential collection of uninfected ticks and to the observed seasonal variations in the tick infection rates.

In conclusion, the habitat does not only influence tick densities and vertebrate host population, but also the prevalence of *Borrelia* species. The observed seasonality of *Borrelia*, *Anaplasma* and *Babesia* species has not been reported before and together with the possibility of urban zoonoses it has major implications for human health. In addition, imported or neglected pathogens like *H. canis* and *B. henselae*, as well as coinfections with various pathogen combinations may represent new potential threats to human and animal health.

Table 8. Coinfections of *Ixodes ricinus* in Luxembourg in 2007. Information on potential reservoir hosts and mode of acquisition is given.

Developm. stage/sex of ticks	Pathogen species					Reservoir host preferences	Acquisition of coinfection
	Borrelia	*Borrelia*	*Rickettsia*	*Babesia*	*Anaplasma*		
F	*afzelii*	-	-	*microti*	-	R + R	SIM
F	*afzelii*	-	-	*microti*	-	R + R	SIM
F	*afzelii*	-	*helvetica*	-	-	R + R/D	SIM or CON
F	*valaisiana*	-	*helvetica*	-	-	R + R/D	SIM or CON
F	*valaisiana*	-	*helvetica*	-	-	R + R/D	SIM or CON
F	*valaisiana*	-	*helvetica*	-	-	R + R/D	SIM or CON
F	*valaisiana*	-	-	-	*phagocytophilum*	R + B/D	CON
F	-	-	*helvetica*	-	*phagocytophilum*	R/D + B/D	SIM or CON
F	-	-	*helvetica*	*sp. EU1*	-	R/D + D	SIM or CON
F	*garinii* ST3	*garinii* ST7	-	-	-	B + B	SIM
F	*garinii* ST6	*valaisiana*	-	-	-	B + B	SIM
F	*garinii* ST6	*valaisiana*	-	-	-	B + B	SIM
F	*garinii* DC	*valaisiana*	-	-	-	unclear + B	SIM or CON
F	*burgdorferi* s.s.	species unclear	-	-	-	R/B + unclear	SIM or CON
M	*afzelii*	-	-	*Microti*	-	R + R	SIM
M	*afzelii*	-	-	*sp. EU1*	-	R + D	CON
M	*afzelii*	-	-	*sp. EU1*	-	R + D	CON
M	*garinii*	-	*helvetica*	-	-	R/B + R/D	SIM or CON
M	*garinii*	-	*helvetica*	-	-	B + R/D	CON
M	*garinii*	-	-	*sp. EU1*	*phagocytophilum*	B + D + B/D	CON
M	*afzelii*	*burgdorferi s.s.*	-	-	-	R + R/B	SIM or CON
M	*afzelii*	*spielmanii*	-	-	-	R + R	SIM
M	*afzelii*	*spielmanii*	-	-	-	R + R	SIM
M	*garinii*	*burgdorferi s.s.*	-	-	-	R/B + R/B	SIM or CON
M	*garinii*	*valaisiana*	-	-	-	R/B + B	SIM or CON
M	*garinii*	*valaisiana*	-	-	-	R/B + B	SIM or CON
M	*garinii* ST7	*valaisiana*	-	-	-	B + B	SIM
M	*garinii* DC	*valaisiana*	-	-	-	unclear + B	SIM or CON
M	*valaisiana*	*burgdorferi* s.s.	-	-	-	B + R/B	SIM or CON
N	*afzelii*	-	*helvetica*	-	-	R + R/D	SIM or CON
N	*afzelii*	-	*helvetica*	-	-	R + R/D	SIM or CON
N	*afzelii*	-	-	*microti*	-	R + R	SIM
N	*afzelii*		-	*microti*	-	R + R	SIM
N	*afzelii*	-	-	*microti*	-	R + R	SIM
N	*garinii* ST6	*valaisiana*	-	*sp. EU1*	-	B + B + D	CON
N	-	-	*helvetica*	-	*phagocytophilum*	R/D + B/D	SIM or CON
N	-	-	*helvetica*	-	*phagocytophilum*	R/D + B/D	SIM or CON
N	-	-	*helvetica*	*sp. EU1*	-	R/D + B/D	SIM or CON
N	*afzelii*	*garinii*	-	-	-	R + R/B	SIM or CON
N	*afzelii*	*burgdorferi* s.s.	-	-	-	R + R/B	SIM or CON
N	*garinii* ST3	*valaisiana*	-	-	-	B + B	SIM
N	*garinii* ST5	*garinii* ST6	-	-	-	B + B	SIM
N	*garinii* ST5	*garinii* ST6	-	-	-	B + B	SIM
N	*garinii* DC	*valaisiana*	-	-	-	unclear + B	SIM or CON

N, nymph; M, male; F, female; ST, serotype; DC, distinct cluster; R, rodents; B, birds; D, deer; SIM, simultaneously; CON, consecutively. Reservoir host preferences were taken from the literature (9, 43, 66, 107, 118, 203).

4.2 Interannual, Seasonal and Regional Variations in the Prevalence of Borrelia burgdorferi sensu lato and other Tick-Borne Pathogens in Ixodes ricinus Ticks

Manuscript in preparation.

A. L. Reye, J. M. Hübschen, C. P. Muller

A. L. Reye contributed significantly to the study design and was in charge of tick collection, experimental setup, experimental work, data analysis and writing of the manuscript.

Annual dynamics of questing activity of exophilic ticks are regulated by seasonal variation of temperature, interannual population dynamics of ticks are driven by abiotic and biotic factors (84). Suitable temperature and relative humidity are crucial for the survival of ticks. Especially dry and hot conditions during the summer months and very cold and dry conditions throughout the winter can lead to increased mortality rates of ticks (171, 172). In addition, blood meal host abundance and suitability of habitat are important biotic factors regulating the survival of ticks. The risk of contracting a tick-borne disease is closely linked to the survival rate of ticks, since the abundance of ticks determines the frequency of contact with humans.

The seroprevalence of tick-borne pathogens in humans is a marker for the rate of exposure to bites of infected ticks. In many cases infections will resolve without disease manifestation, thus the prevalence of serum antibodies against a pathogen is not a marker of disease. Forestry workers have been identified as a risk group for tick-borne pathogens as they spent most of their professional time working outdoors. It has been found that infection rates of forestry workers can be two to six times higher than in the general population (23, 109, 115, 175, 224).

In this study the interannual, seasonal and regional variations in the prevalence of tick-borne pathogens based on data from a three year survey conducted in Luxembourg are investigated. A combined questionnaire and seroprevalence study on forestry workers reveals risk factors for contracting Lyme Borreliosis in Luxembourg.

4.2.1 Results

2007. In 2007, 1500 *Ixodes ricinus* ticks comprising of 320 females, 322 males, 752 nymphs and 106 larvae were collected at 33 collection sites throughout Luxembourg. Details of this study are presented in chapter 4.1 and were published by Reye et al, 2010 (180).

2008. In 2008, 3578 *I. ricinus* ticks consisting of 343 females, 367 males, 1645 nymphs and 1223 larvae were collected at the same collection sites in Luxembourg. All nymphal and adult ticks (n=2355) were analysed for presence of tick-borne pathogens. The most prevalent pathogens belonged to the *Borrelia burgdorferi* s.l. complex (19.4%; n=458), with *B. afzelii* (6.8%; n=160) and *B. garinii* (5.0%; n=117) being the most prevalent, followed by *B. valaisiana* (3.6%; n=84), *B. burgdorferi* s.s. (2.7%; n=64) and *B. lusitaniae* (0.04%; n=1). Few pathogens could not be identified to species level (0.5%, n=12). The second most prevalent pathogen were Spotted Fever Group (SFG) Rickettsiae, which were detected in 6.5% of ticks. *R. helvetica* was the predominant species (6.2%; n=145); three other species were rarely detected, namely *R. monacensis* (0.3%; n=6), *R. slovaca* (0.04%; n=1) and a species most closely related to *Rickettsia* sp. "male killing" (AJ269517) (0.04%, n=1). *A. phagocytophilum* was detected in 0.2% (n=4) of ticks and not further identifiable *Babesia* and *Bartonella* species in 1.49% (n=35) and 0.2% (n=4), respectively. TBEV, *C. burnetii* and *F. tularensis* were not detected. The tick infection rate of adults was at least 1.1 times higher than that of nymphs, except for *Bartonella* and *Babesia* species (factor 0.8).

Coinfections were detected in 73 ticks (3.1%), mainly in adults (4.5%; n=32) but also in nymphs (2.5%; n=41; Table 9). The majority of these were formed by *Borrelia* and *Rickettsia*

species (53.4%, n=39), but also combinations of different *Borrelia* species, including different serotypes of *B. garinii* were frequently detected (30.1%; n=22).

2009. In 2009, 3027 *Ixodes ricinus* ticks comprising of 179 females, 166 males, 1543 nymphs and 1139 larvae were collected at the same collection sites as the years before. In addition, one female *Dermacentor reticulatus* tick was collected in southern Luxembourg. In total, 1889 nymphal and adult ticks were subjected to pathogen detection procedures. Predominantly members of the *Borrelia burgdorferi* s.l. complex were detected (16.0%; n=302) with *B. afzelii* (4.8%; n=90), *B. valaisiana* (3.8%; n=71) and *B. garinii* (3.4%; n=64) being the most prevalent species. *B. burgdorferi* s.s. (0.8%; 16), *B. spielmanii* (0.1%; n=2) and *B. lusitaniae* (0.1%; n=1) were only rarely detected. Few pathogens could not be identified to species level (1.3%, n=24). SFG Rickettsiae were detected in 8.1% (n=153) of ticks, predominantly identified as *R. helvetica* (7.1%; n=134). *R. monacensis* was detected in one tick (0.1%) and pathogens from 18 ticks (1.0%) could not be identified to species level. Low tick infection rates were found for *A. phagocytophilum* (1.2%; n=22), *Babesia* sp. EU1 (1.4%, n=26) and *Babesia microti* (0.1%; n=1). TBEV, *C. burnetii* and *F. tularensis* were not detected. Tick infection rate of adults was at least 1.4 times higher than that of nymphs, except for *A. phagocytophilum* (factor 0.9).

Coinfections were found in 71 ticks (3.8%) with higher rates in adults (7.2%; n=25) than nymphs (3.0%; n=46;Table 10). Combinations of different *Borrelia* species formed most of the mixed infections (47.9%; n=34) although double infections including *Borrelia* and *Rickettsia* species were also frequently detected (31.0%; n=22).

Comparison of tick numbers and tick infection rates of 2007-2009. In 2007, 57.2% of all collected ticks were immature stages (50.1% nymphs, 7.1% larvae). In 2008 and 2009, the percentage of immature ticks increased to 80.2% (46.0% nymphs, 34.2% larvae) and 88.6% (51.0% nymphs, 37.6% larvae), respectively. The overall tick density for Luxembourg was lower in 2007 (4.0 ticks/100m^2) than in 2008 (10.1 ticks/100m^2) and 2009 (14.2 ticks/100m^2), respectively (Figure 5). As the population of adult ticks remained somewhat stable at 1.8 ticks/100m^2 (+/-0.2) in all three years, immature instars were responsible for the significant increase in tick density ($p<0.05$).

The three year tick infection rates were 16.3% for *B. burgdorferi* s.l. (n=918), 6.7% for *Rickettsia* species (n=377), 1.8% for *Babesia* species (n=99), 0.9% for *A. phagocytophilum* (n=53) and 0.1% for *Bartonella* species (n=7). The total tick infection rate of *Borrelia burgdorferi* s.l. in 2007 was significantly lower than in 2008 ($p<0.01$) and 2009 ($p<0.01$) and also the difference between 2008 and 2009 was statistically significant ($p<0.01$). *B.afzelii* was

the predominant *Borrelia* species (mean prevalence rate of 5.4%), followed by *B. garinii* (mean rate 3.7%) and *B. valaisiana* (mean rate 3.3%). Infection rates of nymphs were significantly lower than that of adults for *B. garinii*, *B. burgdorferi* s.s. and *B. valaisiana* ($p<0.05$), but not for *B. afzelii* (nymphs 5.1%; adults 5.9%). Both, *Rickettsia* and *Bartonella* infection rates were significantly higher in 2009 than in the preceding years ($p<0.05$). Throughout the study, *R. helvetica* was always the predominant *Rickettsia* species (mean rate 6.2%). In 2008, significantly less ticks were infected with *A. phagocytophilum* than in 2007 or 2009 ($p<0.01$). Significantly more *Babesia* species were detected in ticks from 2007 than in the other two years ($p<0.05$). Predominantly *Babesia* sp. EU1 was identified (mean rate 0.9%).

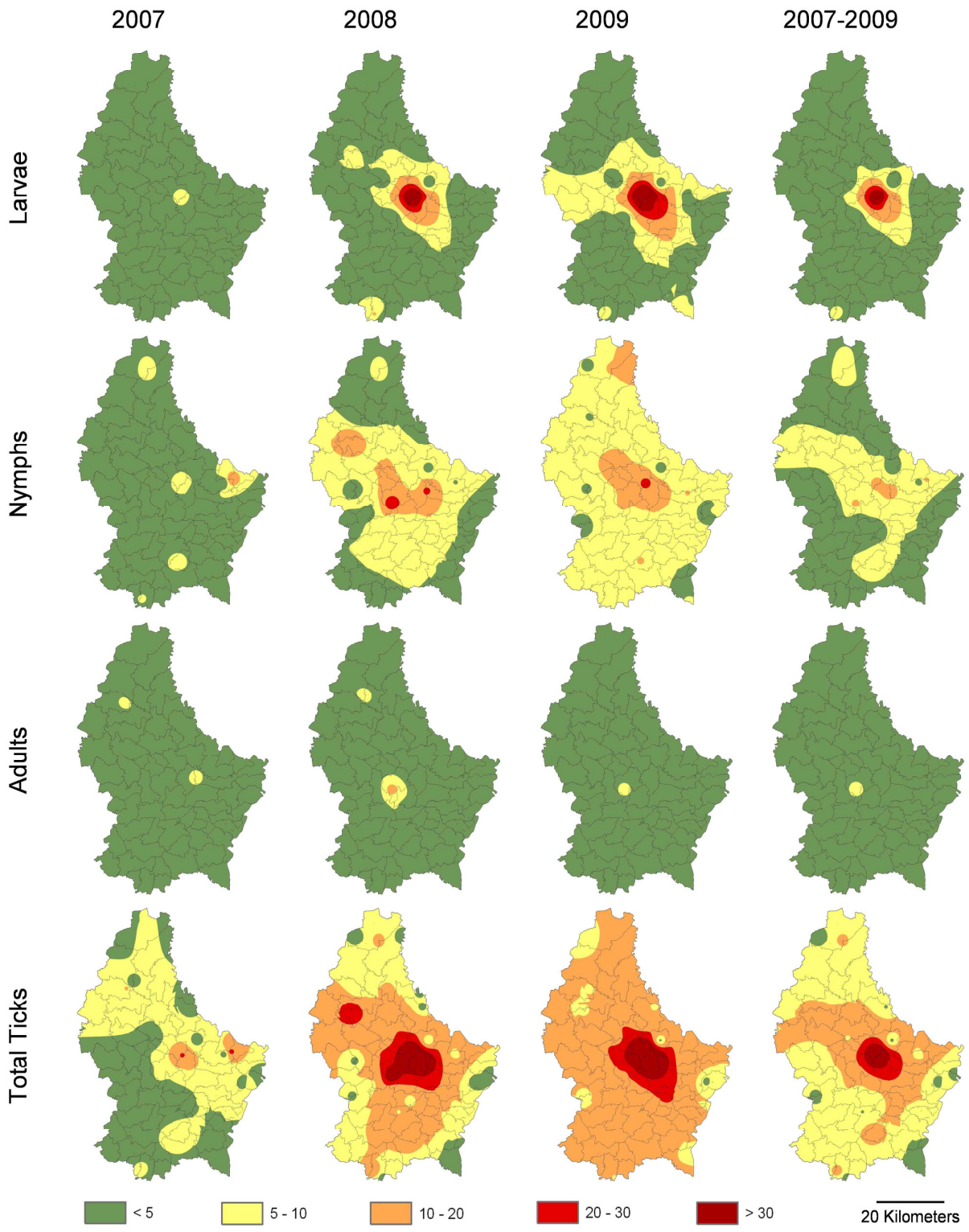

Figure 5. Interpolated density maps of questing ticks. Data derives from 33 collection sites that were visited monthly from May to October 2007, 2008 and 2009. Colours visualize the density of ticks per 100m2.

Seasonality of ticks and tick-borne pathogens 2007-2009. Aggregation of data from all years revealed seasonal changes in the numbers of ticks. Highest numbers of nymphal and adult ticks were collected in May and June, whereas larval ticks displayed a peak in August (Figure 6A). Throughout the season a similar ratio between female and male ticks was observed, while the ratio between nymphs and adults increased significantly over the months, displaying its peak in autumn (Figure 6B).

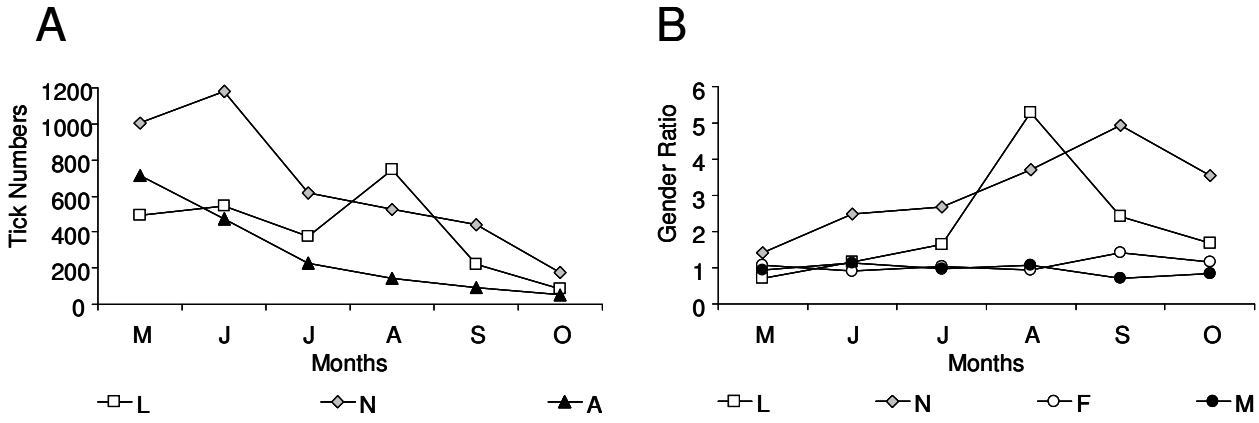

Figure 6. Seasonality of abundance and density of *Ixodes ricinus* instars. (A) Seasonal changes in the abundance of *Ixodes ricinus* instars and (B) in the ratio of immature to mature and female to male ticks.

In 2007 a bimodal seasonality of the ticks infected with *Borrelia burgdorferi* s.l., *Babesia* species and *A. phagocytophilum* was observed (see chapter 4.1).

In 2008, the *Borrelia* tick infection rates were stable around 20.0% (±1%) except in August, where it significantly decreased to 14.3% (Figure 7A). On *Borrelia* species level, the bimodal seasonality was again visible: ticks infected with *B. afzelii* and *B. burgdorferi* s.s. were frequently found during the early collection period, the number of which dropped in August/September, but increased again in October. Also *B. garinii* and *B. valaisiana* infected ticks were most often collected in September. For *Babesia* species the bimodal seasonality was also observed, whereas the overall *A. phagocytophilum* infection rates were too low to monitor seasonal changes.

In 2009, *Borrelia* tick infection rate was more or less stable at 15.6% ±2.2%, with the highest rate in July (17.7%) and lowest in October (13.5%; Figure 7A). On species level, *B. garinii* and *B. valaisiana* displayed the bimodal seasonality observed in the preceding years. *B. afzelii* was predominantly detected in spring ticks, making up 39.0% of all detected *Borrelia* species, but tick infection numbers steadily decreased until October (0%). Infection rates of *B. burgdorferi* s.s. were too low to monitor seasonality. *A. phagocytophilum* infected ticks were predominantly found later in the season (August to October).

Table 9. Coinfections of *Ixodes ricinus* in Luxembourg in 2008. Information on potential reservoir hosts and mode of acquisition is given.

Developmental stage/sex of ticks	Pathogen species						Reservoir Host Preferences	Acquisition of Coinfection
	Borrelia	*Borrelia*	*Rickettsia*	*Babesia*	*Anaplasma*	*Bartonella*		
F	*afzelii*	-	*monacensis*	-	-	-	R + unclear	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	-	spec.	-	-	R + unclear	SIM or CON
F	*afzelii*	*burgdorferi* s.s.	-	spec.	-	-	R + R/B + unclear	SIM or CON
F	*afzelii*	*garinii*	*helvetica*	-	-	-	R + R/B + R/D	SIM or CON
F	*afzelii*	*valaisiana*	-	spec.	-	-	R + B + unclear	CON
F	*burgdorferi* s.s.	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
F	*burgdorferi* s.s.	*garinii* ST6, ST5, ST unclear	-	-	-	-	R/B + B + B + R/B	SIM or CON
F	*garinii* ST6	-	*helvetica*	-	-	-	B + R/D	CON
F	*garinii* ST6	*garinii* ST unclear	-	-	-	-	B + R/B	SIM or CON
F	*garinii*	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
F	*garinii* ST6	*garinii* ST3	*helvetica*	-	-	-	B + B + R/D	CON
F	*garinii* ST unclear	*valaisiana*	*helvetica*	-	-	-	R/B + B + R/D	CON
F	*garinii* ST unclear	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
F	*garinii* ST unclear	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
F	*valaisiana*	-	*helvetica*	-	-	-	B + R/D	SIM or CON
F	*valaisiana*	-	*helvetica*	-	-	-	B + R/D	SIM or CON
F	-	-	*helvetica*	spec.	-	-	R/D + unclear	SIM or CON
M	*afzelii*	-	-	spec.	-	-	B + unclear	SIM or CON
M	*afzelii*	-	-	spec.	-	-	B + unclear	SIM or CON
M	*afzelii*	-	-	-	*phagocytophilum*	-	B + B/D	SIM or CON
M	*garinii* ST6	*garinii* ST3	-	-	-	-	B + B	SIM
M	*garinii* ST6	*garinii* ST5, ST unclear	-	-	-	-	B + B + R/B	SIM or CON
M	*garinii* ST unclear	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
M	*garinii*	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
M	*valaisiana*	-	*helvetica*	-	-	-	B + R/D	CON
M	*valaisiana*	-	-	spec.	-	spec.	B + unclear + unclear	SIM or CON
N	*afzelii*	*garinii*	-	-	-	-	R + R/B	SIM or CON
N	*afzelii*	*garinii*	-	-	-	-	R + R/B	SIM or CON
N	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
N	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON

N	*afzelii*	-	-	spec.	-	-	R + unclear	SIM or CON
N	*afzelii*	-	-	spec.	-	-	R + unclear	SIM or CON
N	*afzelii*	-	-	spec.	-	-	R + unclear	SIM or CON
N	*afzelii*	-	-	spec.	-	-	R + unclear	SIM or CON
N	*afzelii*	-	-	spec.	-	-	R + unclear	SIM or CON
N	*afzelii*	-	-	spec.	-	-	R + unclear	SIM or CON
N	*afzelii*	-	-	spec.	-	-	R + unclear	SIM or CON
N	*afzelii*	-	-	spec.	-	-	R + unclear	SIM or CON
N	*afzelii*	-	*helvetica*	spec.	-	-	R + R/D + unclear	SIM or CON
N	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
N	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
N	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
N	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
N	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
N	*afzelii*	-	-	-	*phagocytophilum*	-	R + B/D	SIM or CON
N	*burgdorferi* s.s.	*garinii* ST6	-	-	-	-	R/B + B	SIM or CON
N	*burgdorferi* s.s.	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*burgdorferi* s.s.	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*burgdorferi* s.s.	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*burgdorferi* s.s.	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*burgdorferi* s.s.	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*garinii* ST6	*garinii* ST unclear	-	-	-	-	B + R/B	SIM or CON
N	*garinii* ST6	*garinii* ST5, ST7, ST unclear	-	-	-	-	B + B + B + R/B	SIM or CON
N	*garinii*	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
N	*garinii* ST unclear	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
N	*garinii* ST unclear	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
N	*garinii* ST unclear	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
N	*garinii*	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*garinii*	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*garinii*	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*valaisiana*	-	*helvetica*	-	-	-	B + R/D	SIM or CON
N	*valaisiana*	-	*helvetica*	-	-	-	B + R/D	SIM or CON
N	*valaisiana*	-	*helvetica*	-	-	-	B + R/D	SIM or CON
N	*valaisiana*	-	*monacensis*	-	-	-	B + unclear	SIM or CON
N	spec.	-	*helvetica*	-	-	-	unclear + R/D	SIM or CON
N	-	-	*monacensis*	-	-	spec.	unclear + unclear	SIM or CON
N	-	-	*helvetica*	spec.	-	-	R/D + unclear	SIM or CON

N, nymph; M, male; F, female; ST, serotype; R, rodents; B, birds; D, deer; SIM, simultaneously; CON, consecutively. Reservoir host preferences were taken from the literature (9, 43, 66, 107, 118, 203)

Table 10. Coinfections of *Ixodes ricinus* in Luxembourg in 2009. Information on potential reservoir hosts and mode of acquisition is given.

Developmental Stage/sex of ticks	Pathogen species						Reservoir Host Preferences	Acquisition of Coinfection
	Borrelia	*Borrelia*	*Rickettsia*	*Babesia*	*Anaplasma*	*Bartonella*		
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
F	*afzelii*	*burgdorferi* s.s.	-	-	-	-	R + R/B	SIM or CON
F	*burgdorferi* s.s.	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
F	*garinii* ST unclear	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
F	*garinii* ST6	-	*helvetica*	-	-	-	B + R/D	SIM or CON
M	*afzelii*	-	-	spec. EU1	-	-	R + D	CON
M	*afzelii*	-	-	spec. EU1	-	-	R + D	CON
M	*afzelii*	-	-	*microti*	-	-	R + R	SIM
M	*afzelii*	-	spec.	-	-	-	R + unclear	SIM or CON
M	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
M	*afzelii*	-	*helvetica*	-	-	-	R + R/D	SIM or CON
M	*afzelii*	*valaisiana*	-	-	-	-	R + B	CON
M	*burgdorferi* s.s.	*garinii* ST6	-	-	-	-	B + B	SIM
M	*burgdorferi* s.s.	*valaisiana*	-	-	-	-	B + B	SIM
M	*burgdorferi* s.s.	*valaisiana*	-	-	-	-	B + B	SIM
M	*garinii* ST unclear	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
M	*garinii*	-	*helvetica*	-	-	-	R/B+ R/D	SIM or CON
M	*garinii*	-	*monacensis*	-	-	-	R/B+ unclear	SIM or CON
M	*garinii*	-	spec.	-	-	-	R/B + unclear	SIM or CON
M	*garinii*	-	spec.	-	-	-	R/B + unclear	SIM or CON
N	*afzelii*	-	-	-	*phagocytophilum*	-	R + B/D	SIM or CON
N	*afzelii*	-	-	spec. EU1	-	-	R + D	CON
N	*afzelii*	-	-	spec. EU1	-	-	R + D	CON
N	*afzelii*	*burgdorferi* s.s.	-	-	-	-	R + R/B	SIM or CON
N	*afzelii*	*burgdorferi* s.s.	-	-	-	-	R + R/B	SIM or CON
N	*afzelii*	*burgdorferi* s.s.	-	-	-	-	R + R/B	SIM or CON
N	*afzelii*	*burgdorferi* s.s.	-	-	-	-	R + R/B	SIM or CON
N	*afzelii*	*burgdorferi* s.s.	-	-	-	-	R + R/B	SIM or CON
N	*afzelii*	*burgdorferi* s.s.	*helvetica*	-	-	-	R + R/B + R/D	SIM or CON
N	*afzelii*	*garinii*	-	-	-	-	R + R/B	SIM or CON

N	*afzelii*	*garinii*	-	-	-	-	R + R/B	SIM or CON
N	*afzelii*	*garinii*	-	-	-	-	R + R/B	SIM or CON
N	*afzelii*	*garinii*	-	-	-	-	R + R/B	SIM or CON
N	*afzelii*	*garinii* ST6	-	-	-	-	R + B	CON
N	*afzelii*	*valaisiana*	-	-	-	-	R + B	CON
N	*afzelii*	*valaisiana*	-	-	-	-	R + B	CON
N	*afzelii*	*valaisiana*	-	-	-	-	R + B	CON
N	*burgdorferi* s.s.	-	*rickettsii* group	-	-	-	R + unclear	SIM or CON
N	*burgdorferi* s.s.	*garinii* ST6	-	-	-	-	R/B + B	SIM or CON
N	*burgdorferi* s.s.	*garinii* ST6	-	-	-	-	R/B + B	SIM or CON
N	*garinii* ST5	*garinii* ST6, ST unclear	-	-	-	-	B + B + R/B	SIM or CON
N	*garinii* ST5	*garinii* ST6	-	-	-	-	B + B	SIM
N	*garinii* ST5	*garinii* ST6	*helvetica*	-	-	-	B + B + R/D	SIM or CON
N	*garinii* ST5	*garinii* ST7	*helvetica*	-	-	-	B + B + R/D	SIM or CON
N	*garinii* ST6	*garinii* ST unclear	-	-	-	-	B + R/B	SIM or CON
N	*garinii* ST6	*garinii* ST unclear	-	-	-	-	B + R/B	SIM or CON
N	*garinii* ST unclear	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
N	*garinii* ST unclear	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
N	*garinii* ST unclear	*valaisiana*	spec.	-	-	-	R/B + B	SIM or CON
N	*garinii* ST5	*valaisiana*	-	-	-	-	B + B	SIM
N	*garinii*	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
N	*garinii*	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
N	*garinii*	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
N	*garinii*	*valaisiana*	-	-	-	-	R/B + B	SIM or CON
N	*garinii*	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*garinii* ST unclear	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*garinii* ST unclear	-	*helvetica*	-	-	-	R/B + R/D	SIM or CON
N	*garinii* ST3	-	-	spec. EU1	-	-	B + B/D	SIM or CON
N	*valaisiana*	-	*helvetica*	-	-	-	B + R/D	SIM or CON
N	*valaisiana*	-	*helvetica*	-	-	-	B + R/D	SIM or CON
N	*valaisiana*	-	*helvetica*	-	-	-	B + R/D	SIM or CON
N	*valaisiana*	-	spec.	-	-	-	B + unclear	SIM or CON
N	-	-	-	spec. EU1	*phagocytophilum*	-	B/D + B/D	SIM or CON
N	-	-	*helvetica*	-	*phagocytophilum*	-	R/D + B/D	SIM or CON
N	-	-	*helvetica*	-	*phagocytophilum*	-	R/D + B/D	SIM or CON
N	-	-	*helvetica*	spec. EU1	-	-	R/D + B/D	SIM or CON

N, nymph; M, male; F, female; ST, serotype; R, rodents; B, birds; D, deer; SIM, simultaneously; CON, consecutively. Reservoir host preferences were taken from the literature (9, 43, 66, 107, 118, 203).

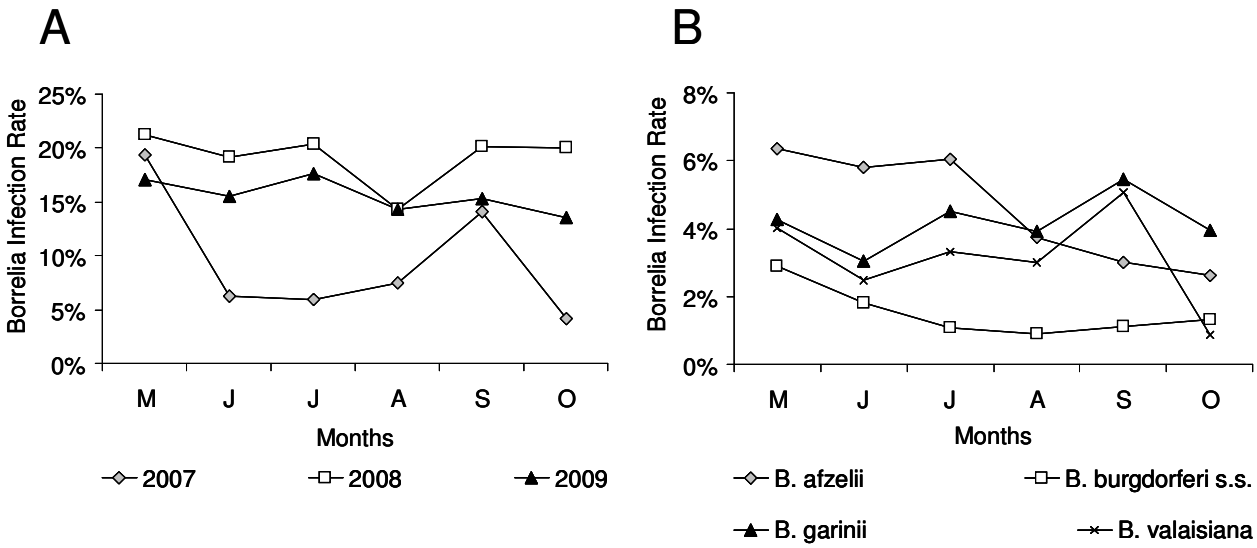

Figure 7. Seasonality of *Borrelia burgdorferi* s.l. infection rates in ticks. (A) Overall *Borrelia burgdorferi* s.l. infection rates in ticks collected in 2007, 2008 and 2009. (B) Mean seasonal changes in the tick infection rate of the four predominant *Borrelia* species.

Aggregation of the seasonal data revealed that *B. afzelii* and *B. burgdorferi* both display highest rates in spring months, which decrease towards autumn. *B. garinii* and *B. valaisiana* in contrast both show increased prevalence in ticks in September (Figure 7B).

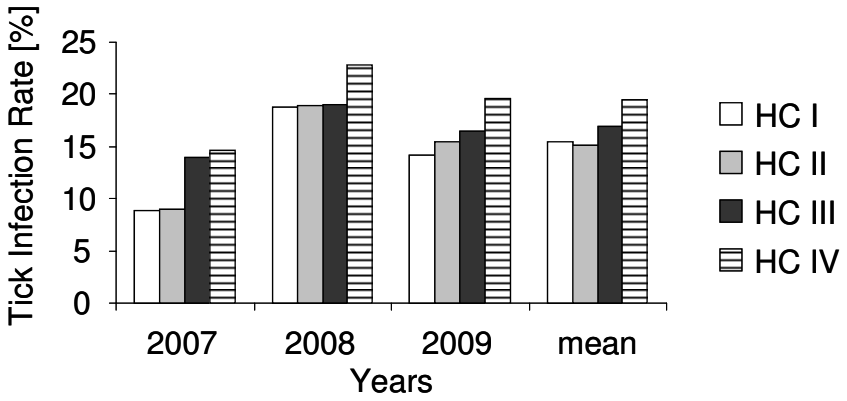

Figure 8. *Borrelia burgdorferi* s.l. infection rate of ticks in the habitat categories in the years 2007, 2008 and 2009.

Habitat categories 2007-2009. The mean tick densities were highest in habitat category (HC) I and II (9.1 and 9.6 ticks/100m^2) and lowest in HC IV (6.5 ticks/100m^2). In all years the *Borrelia* infection rate was highest in category IV (Figure 8). Overall, *B. afzelii* was the predominant species in all habitat categories, followed by *B. garinii*, *B. valaisiana* and *B. burgdorferi* s.s., however changes in the prevalence rates were observed during the years

(Figure 9). The habitat categories did not have a significant influence on the prevalence of *Rickettsia* species in ticks. *A. phagocytophilum* infected ticks were less prevalent at collection sites assigned to category IV. In 2007, the habitat categories did not have an influence on the prevalence of *Babesia* infected ticks; however in 2008 and 2009, ticks of HC III displayed lower rates of infection. Ticks from HC IV were not infected with *Bartonella* species.

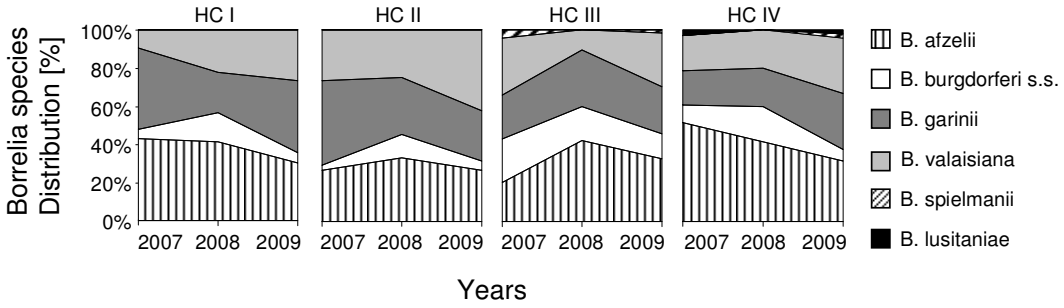

Figure 9. *Borrelia* species composition in the habitat categories in the years 2007, 2008 and 2009.

Regionality of ticks and tick-borne pathogens 2007-2009. Tick densities were highest in Central East (14.8 ticks/100m^2) and lowest in the East (4.2 ticks/100m^2) of Luxembourg. Adult tick densities were more stable throughout the collection period (1.0-2.6 ticks/100m^2) than nymphal (1.9-6.1 ticks/100m^2) or larval (0.6-6.8 ticks/100m^2) densities (see also Figure 6B).

Regional differences in the *Borrelia* infection rate were observed in all three years. In 2007, the north-eastern and south-eastern parts of Luxembourg displayed high prevalence rates of more than 20% in ticks (Figure 10A). In the following year, these regions expanded towards northern and central Luxembourg (Figure 10B) and in 2009, the North was still a region with high *Borrelia* prevalence in ticks, whereas in the south-eastern part moderate tick infection rates were observed (Figure 10C). Overall, the mean *Borrelia* tick infection rate was highest in the regions South (19.5%), North (19.0%) and East (18.8%) and significantly lower in Central East (13.8%; $p<0.01$) and Central West (14.4%; $p<0.05$; Figure 10D). *B. garinii* was the only species detected at all collection sites, *B. afzelii* and *B. valaisiana* were each absent from two and *B. burgdorferi* s.s. from six sites. *B. spielmanii* was found at three sites in the regions North and Central East and the prevalence of *B. lusitaniae* was restricted to a single site in the East. Ten hotspots of *Borrelia* infected ticks with rates above 20% were observed in all regions except the Central West with a peak value of 35.8% at a site in the Central East. Two collection sites with very low prevalence rates of *Borrelia* species in ticks (<5%) were found.

Rickettsia infected ticks were most prevalent in the East (10.3%) and least in the North (4.6%). All collection sites were populated by *Rickettsia* infected ticks and *A. phagocytophilum* were found at 22 collection sites located in all regions, but predominantly in East (1.7%) and Central East (1.5%). *Babesia* species were found at 28 sites, highest tick infection rates were found in the North (2.5%). *Bartonella* species were detected in ticks from seven sites located in all regions.

Gréngewald. In total 336 *Ixodes ricinus* ticks comprising of 29 females, 20 males, 177 nymphs and 110 larvae were collected in the Gréngewald. *Borrelia burgdorferi* s.l. was detected in 17.3% of ticks (females: 24.1%; males: 25.0%; nymphs: 15.3%) with *Borrelia garinii* being the most prevalent (10.2%), followed by *B. valaisiana* (3.1%), *B. afzelii* (2.7%), *B. burgdorferi* s.s. (0.9%) and *B. spielmanii* (0.4%). *Rickettsia helvetica* was detected in 6.2% of ticks (females: 13.8%; males: 10.0%; nymphs: 7.9%), *Anaplasma phagocytophilum* in 3.1% (females: 6.9%; males: 5.0%; nymphs: 4.0%) and Babesia sp. EU1 in 0.9% (nymphs: 1.1%). Four ticks were found to harbour mixed infections, all involving *R. helvetica* together with either *A. phagocytophilum*, *B. afzelii*, *B. burgdorferi* or *B. garinii*. The adult tick infection rate was found to be at least 1.2 times higher than the nymphal for all pathogens, except for *B. garinii* (factor 0.6).

Ticks from Hosts. In total, 1767 ticks were removed from eleven host taxa, namely humans (n=202), dogs (n=936), cats (n=309), deer (n=111), foxes (n=60), hedgehogs (n=58), horses (n=36), cattle (n=14), birds (n=39), marten (n=1) and shrews (n=1). Predominant tick species was *Ixodes ricinus* (89.8%; n=1587), followed by *Ixodes hexagonus* (9.2%; n=163), *Ixodes frontalis* (0.6%; n=10) and *Dermacentor reticulatus* (0.4%; n=7). *I. ricinus* was the only tick species found on humans, *I. hexagonus* was removed from cats (n=63), hedgehogs (n=50), foxes (n=42), dogs (n=7) and marten (n=1), *D. reticulatus* only from dogs (n=7) and *I. frontalis* only from birds (n=10). The first ticks from humans were collected in March (0.5%) and April (6.0%), but humans were predominantly at risk in May and June (53.2%). During summer, 30.8% of encounters were observed and in September and October 9.5%. Some of the ticks from humans (n=44) were analysed upon request for the presence of *Borrelia burgdorferi* s.l. and 15.9% (n=7) were positive.

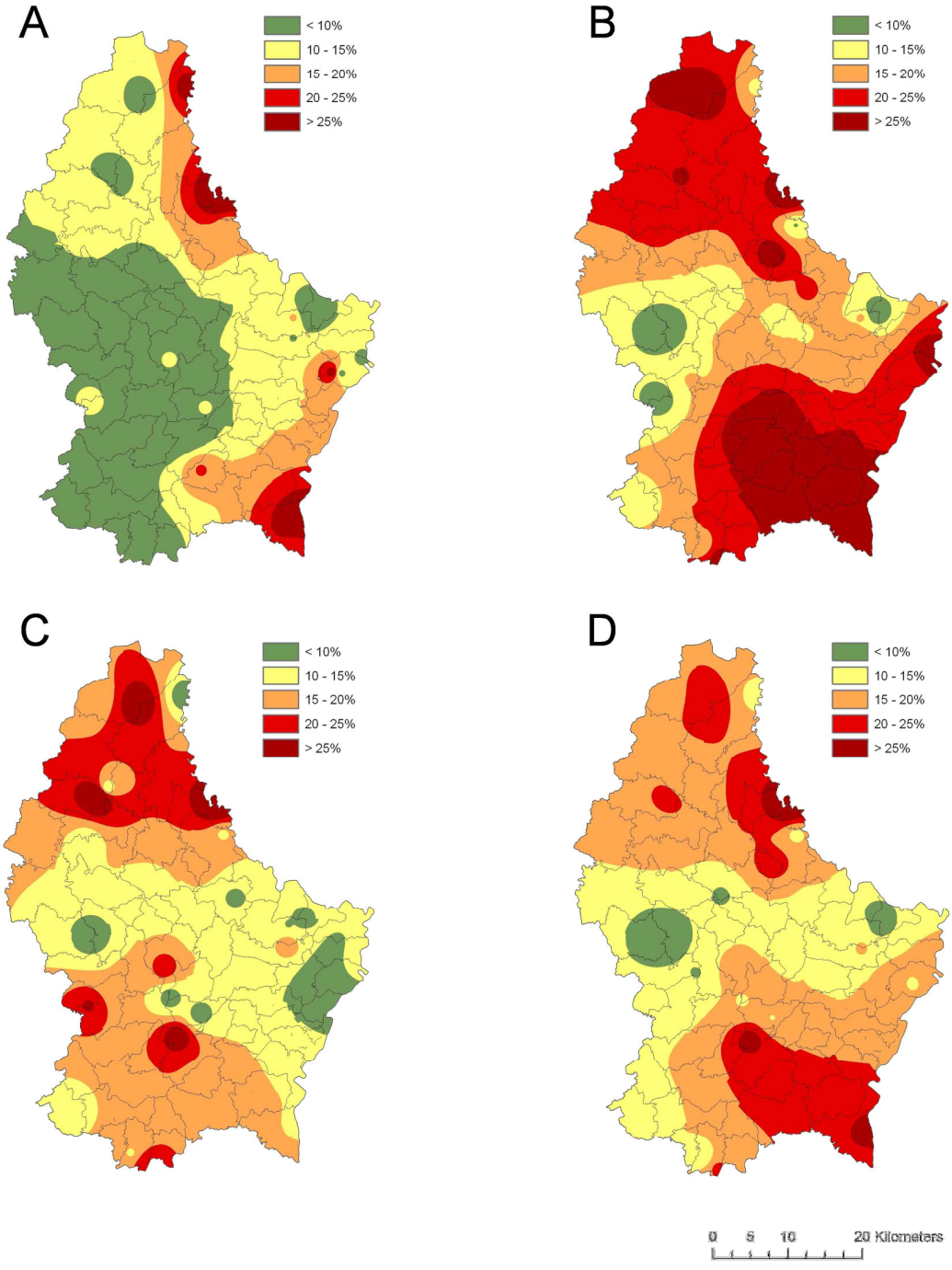

Figure 10. Interpolated tick infection rate of *Borrelia burgdorferi* s.l. (A) in 2007, (B) 2008, (C) 2009 and (D) for all three years.

Serology Forestry Workers. 280 forestry workers participated in the seroprevalence study on Lyme Borreliosis, consisting of 270 men and 10 women. The majority of forestry workers (42.9%; n=120) were between 30 and 39 years old, followed by the group of 40-49 years old (25.4%; n=71); only 2.9% (n=8) were 60 years or older (Figure 11A). 35.4% (n=99) of forestry workers were clearly anti-*Borrelia* antibody positive and 2.5% (n=7) were equivocal in the immunoblot. In total, 12.9% of forestry workers had a history of diagnosed and treated Lyme Borreliosis. Age was identified as a significant risk factor ($p<0.01$), as 68.9% of forestry workers older than 40 years were seropositive, as compared to 22.0% of those younger (Figure 11A). Spending eight or more hours working outdoors also increased the risk of infection, even though not statistically significant (Figure 11B). More than 60% of forestry workers had contact to five or more ticks per year (Figure 11C) and the majority of forestry workers (51.1%; n=143) reported one to five tick bites per year (Figure 11D). The seroprevalence rate of *Borrelia burgdorferi* s.l. was highest in the groups which reported contact to 21-50 mobile or attached ticks (55.3%; n=38; Figure 11C). With further increasing tick contact (more than 51 ticks per year), the seroprevalence decreased to 22.7% (n=22). In the group of forestry workers reporting between 11 to 20 tick bites, the seroprevalence rate was highest (50%; Figure 11D). A similar seroprevalence rate of 40.0-40.8% was observed in those reporting either 6-10, 21-50 or more than 100 tick bites per year. There were no beneficial effects of tick repellents, frequent body inspection or early removal of ticks on the seroprevalence rate (Figure 12A-D).

In fact the rates were slightly increased in the group applying these measures (repellent group: 38.1% vs. 35.2%; body inspection group: 36.8% vs. 33.7%; early tick removal group: 40.0% vs. 31.4%). A small beneficial effect was observed in the group wearing protective clothing (trousers into socks, long-sleeves, light colours): the seroprevalence was reduced by 9% (30.2% vs. 39.2%; Figure 12D).

On a regional level highest seroprevalence rates were observed in the East (43.6%; n=78), and North (41.2%; n=51) and significantly lower seroprevalence rates were observed in forestry workers from the South (23.6%; n=63; $p<0.05$; Figure 13). The mean age of forestry workers in the different regions ranged from 43 to 45 years (median: 42-47). In the North more than 80.4% of the forestry workers were older than 40 years, whereas in the Central East and South only 65.1% were older than 40 years. History of Lyme Borreliosis was reported by 21.8% of forestry workers from the East, 11.8% from the North, 11.1% from South, 9.3% from Central West and 4.5% from Central East.

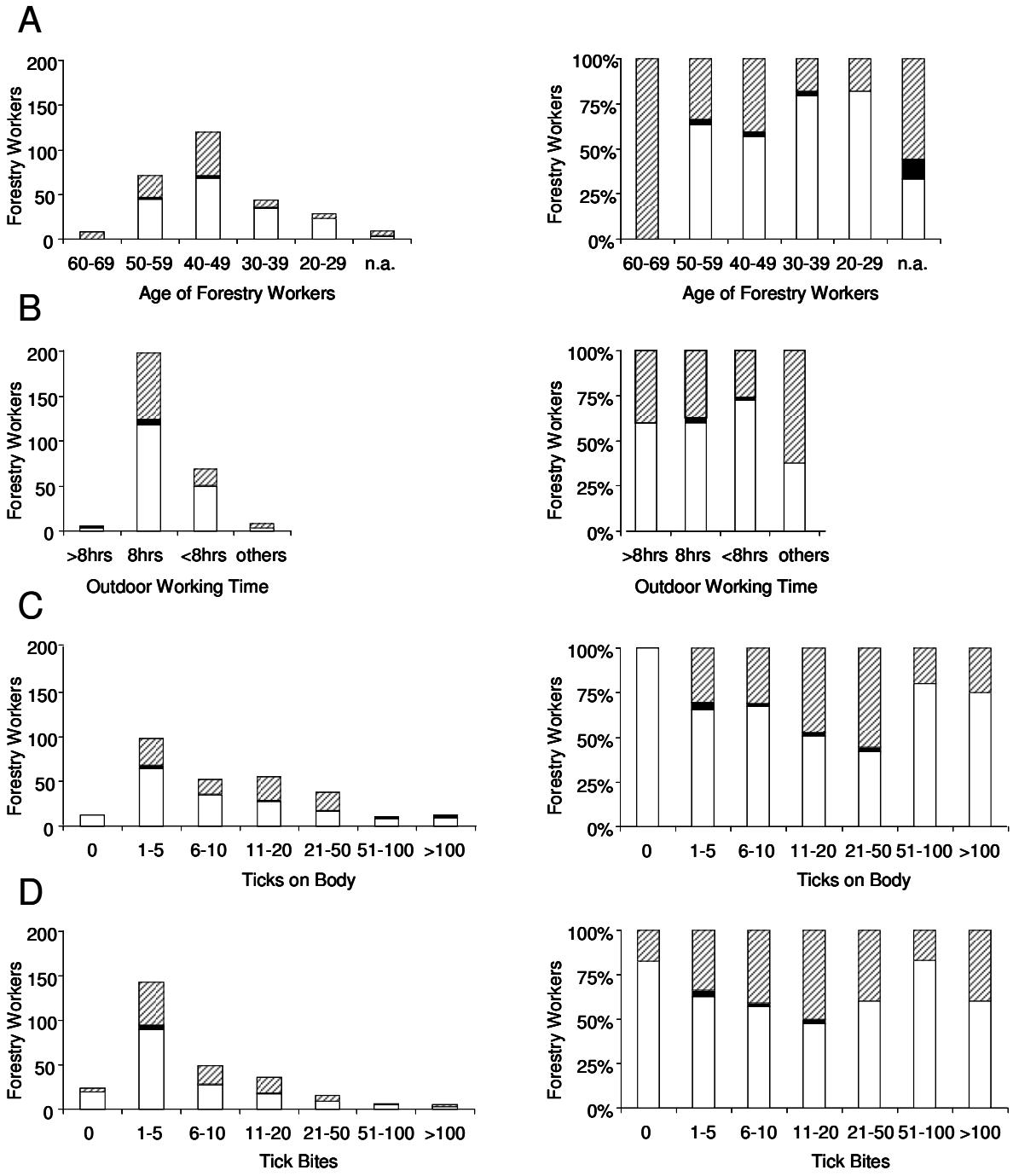

Figure 11. Seroprevalence of *Borrelia burgdorferi* s.l. in forestry workers. (A) Age distribution of forestry workers (left) and the seroprevalence rates of Borrelia burgdorferi s.l. according to the age groups (right). (B) Distribution of outdoor working time (left) and respective seroprevalence rates (right). (C) Distribution of total tick numbers (mobile and attached) found on body per year (left) and respective seroprevalence rates (right). (D) Distribution of numbers of tick bites observed per year (left) and respective seroprevalence rates (right). White bars indicate seronegative, shaded bars seropositive and black bars borderline immunoblot results.

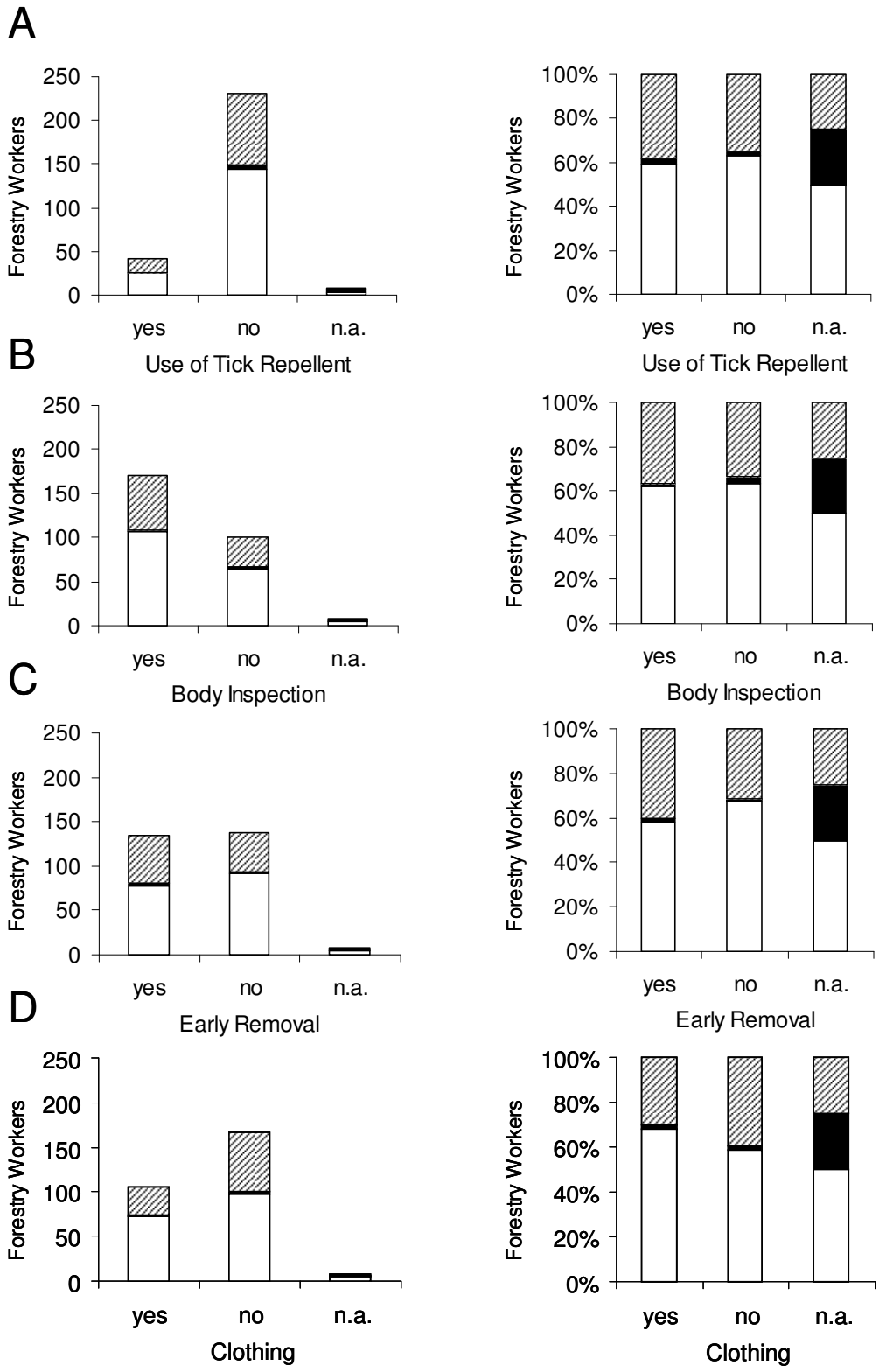

Figure 12. Protective measures against Lyme Borreliosis. (A) Usage of tick repellents (left) and the seroprevalence rates of *Borrelia burgdorferi* s.l. according to the groups (right). (B) Body inspection (left) and respective seroprevalence rates (right). (C) Early removal of attached ticks (left) and respective seroprevalence rates (right). (D) Usage of protective clothing (left) and respective seroprevalence rates (right). White bars indicate seronegative, shaded bars seropositive and black bars borderline immunoblot results.

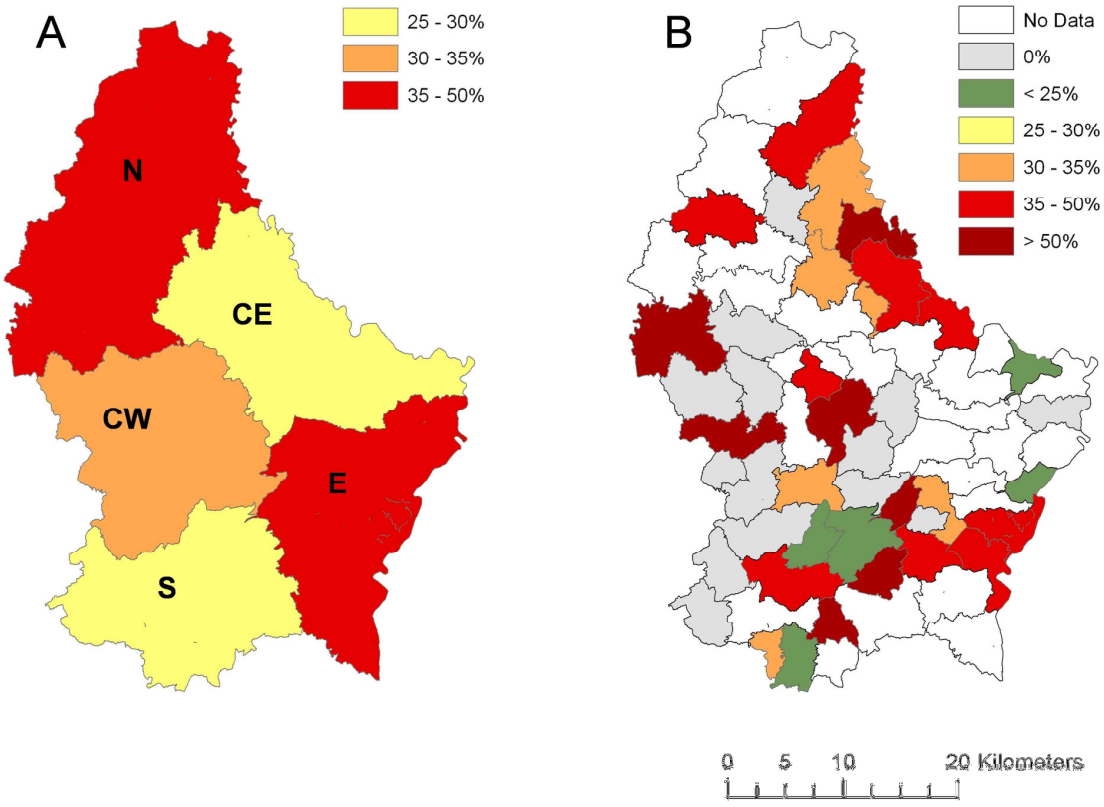

Figure 13. Interpolated seroprevalence rate of *Borrelia burgdorferi* s.l. in the five arrondissements (A) and triages (B). N, North; CE, Central East, CW, Central West, E, East, S, South.

4.2.2 Discussion

The most prevalent tick species in Luxembourg was the sheep tick *Ixodes ricinus*, making up 99.9% of ticks collected from the vegetation and almost 90% of ticks collected from hosts. Cloth dragging is a useful method to collect exophilic ticks like *I. ricinus*, while nidicolous ticks or species closely related to their hosts are neglected (163). Although the collection of ticks from hosts, especially wild animals, is deemed to better reflect tick diversity and density (76), the risk for humans to contract a tick-borne disease is best assessed by collecting ticks from the vegetation, as this resembles the typical human-tick-contact. In Luxembourg all of the ticks that were found on humans were *I. ricinus*, predominantly nymphs (73.8%), but also adults (22.3%) and larva (4.0%). The recorded tick bites in humans were primarily caused by nymphs (79.5%) but also females (20.5%). This is in line with reports from Western Europe, where mainly nymphal and female *I. ricinus* are responsible for tick bites in humans and only few infestations with other instars and tick species have been reported (99, 125). The high rates of contact between ticks and humans in May and June is in line with the high numbers of questing ticks observed at this time. In the summer months the tick-human contact rate is higher than the questing tick density implies, probably caused by an increase of recreational outdoor activities.

Borrelia burgdorferi s.l. was the most important tick-borne pathogen in Luxembourg, as it had a wide regional distribution, a high prevalence in questing *Ixodes ricinus* ticks and also a high seroprevalence in forestry workers. The *Borrelia* infection rate of ticks in the Gréngewald, a former secluded hunting area, was comparable to the rest of Luxembourg and also the significant predominance of *B. garinii* over *B. afzelii* had been observed at some sites outside of this forest. The second most prevalent pathogen in questing ticks from Luxembourg was the Spotted Fever Group Rickettsiae *R. helvetica*, a human pathogen known to cause non-specific fever, whereas its potential to cause endocarditis remains unclear (47). Other tick-borne human pathogens like *Rickettsia slovaca*, *Anaplasma phagocytophilum*, *Babesia* sp. EU1, *B. microti*, *B. divergens* and *Bartonella henselae* are prevalent in Luxembourg only at very low rates and therefore seem to be of minor threat to public health. The infection rates of nymphs (21.0%) were significantly lower than that of adults (30.0%; $p<0.01$), as questing adults have completed an additional blood meal as compared to nymphs. Interestingly, *B. afzelii* was similarly prevalent in nymphal and adult ticks (5.1% and 5.9% respectively), suggesting that nymphal ticks preferably feed on other blood meal hosts than larvae. In the three consecutive years, neither Tick-Borne Encephalitis Virus, nor *Coxiella burnetii* nor

Francisella tularensis were detected in questing ticks, suggesting that they are not endemic at least at the collection sites. Since an additional route of transmission of the two bacteria is via contaminated aerosols it is possible that these pathogens would be detectable in different biological sources (5, 67).

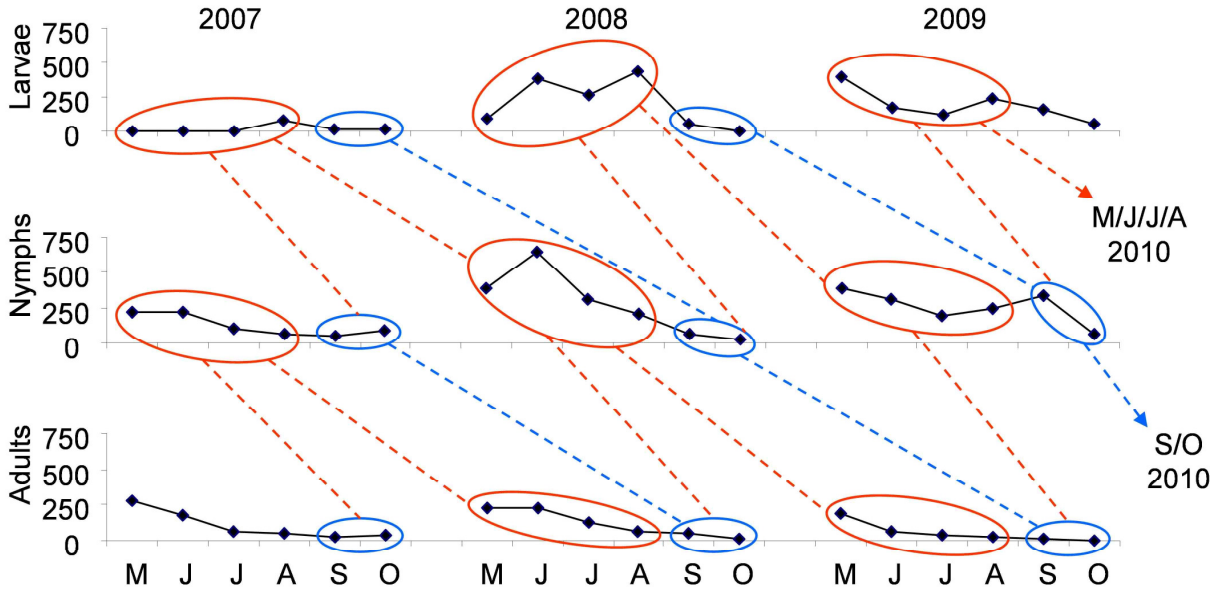

Figure 14. Interstadial periods of Ixodes ricinus based ticks collected from May to October in 2007, 2008 and 2009 in Luxembourg. Ticks feeding in spring/summer are either found questing as the next instar in autumn of the same year or in spring of the next year (red lines). Ticks feeding in autumn delay their development and are found questing in autumn of the following year (blue lines).

Our study revealed significant annual variations of the population dynamics of *I. ricinus*, visible in tick numbers and the ratio of developmental stages. In 2007, almost half of the ticks collected were adults, whereas in the following years it was less than 20%. Density calculations revealed that the numbers of adults per 100m^2 remained comparatively stable in the three years, while the number of immature instars increased significantly (Figure 5). The biological expectations of 100 larvae to 10 nymphs to 2 adults (48) were not matched, as immature stages were considerably underrepresented (3.5 : 5.3 : 2). Although cloth dragging yields highest numbers of nymphal and adult ticks, which are mainly involved in disease transmission, this method is not always representative of the actual abundance of larvae due to the vertical separation of questing positions (73, 133, 220). Even when assuming that the underrepresentation of immature instars is partially caused by sampling parameters, it should have introduced the same bias into the data of all years. The significant increase in counts of immature ticks in 2008 and 2009 is therefore likely to be caused by additional parameters. Abiotic factors (climate, landcover) play an important role for the survival of ticks during

their off-host phase. Significantly higher mean temperatures were recorded in the first four months of 2007, possibly triggering earlier questing activity in overwintering ticks and explaining the low numbers of collected ticks from May onwards. Although larvae usually display a single peak of questing activity in mid-summer (59, 77, 103, 171, 190), analysis of the inter-stadial periods between questing instars according to Randolph et al. (171) revealed that a proportion of the high nymphal counts in 2008 must derive from a larval population questing unusually early before the start of collection in 2007 (Figure 14).

April 2007 was marked not only by higher mean temperature but also by a significantly lower relative humidity, resulting in an unusually high saturation deficit (Figure 15). Although ticks have adapted to transitory unfavourable microclimatic conditions (172), high saturation deficits especially in the spring months after overwintering may cause increased mortality rates, especially in immature instars. This may explain the absence of larvae and the relatively low numbers of nymphs found on the vegetation during the early collection season of 2007. Regardless of the density of immature instars in preceding years we observed that the density of adult ticks remained stable throughout the three years, suggesting a regulation of the tick population independent of variations of annual climatic conditions. It has been reported for several animal species that in regularly infested hosts the immune response influences the efficacy of tick feeding, leading to reduced engorgement weights (153). After moulting, these ticks suffer from reduced energy reserves, making them more susceptible to unfavourable microclimatic conditions and possibly leading to increased mortality rates (48, 172, 214) and thus restricting the density of adult ticks. Interestingly, in mice increasing tick infestation does not reduce the efficacy of feeding (19). As small rodents are the main hosts of larval ticks, this might explain why the density of nymphs was not regulated in the same way as that of adult ticks. As the interplay between abiotic and biotic factors is extremely complex and cannot be explained by data deriving from a three year survey, this hypothesis needs further confirmation from laboratory and long-term population studies.

We also observed regional variations in the density of developmental stages of ticks. Some collection sites were characterized by higher numbers of immature instars than others, indicating the presence of suitable hosts of adult ticks (e.g. deer) in this area. The distribution of *I. ricinus* is directly linked to the movements of its host, meaning that hatched larvae will quest in proximity to the site of egg laying. The preferential hosts of larvae are small vertebrates, which usually have a limited territory also restricting the distribution of engorged larvae. Therefore, nymphs are likely to be found at sites with high numbers of larvae.

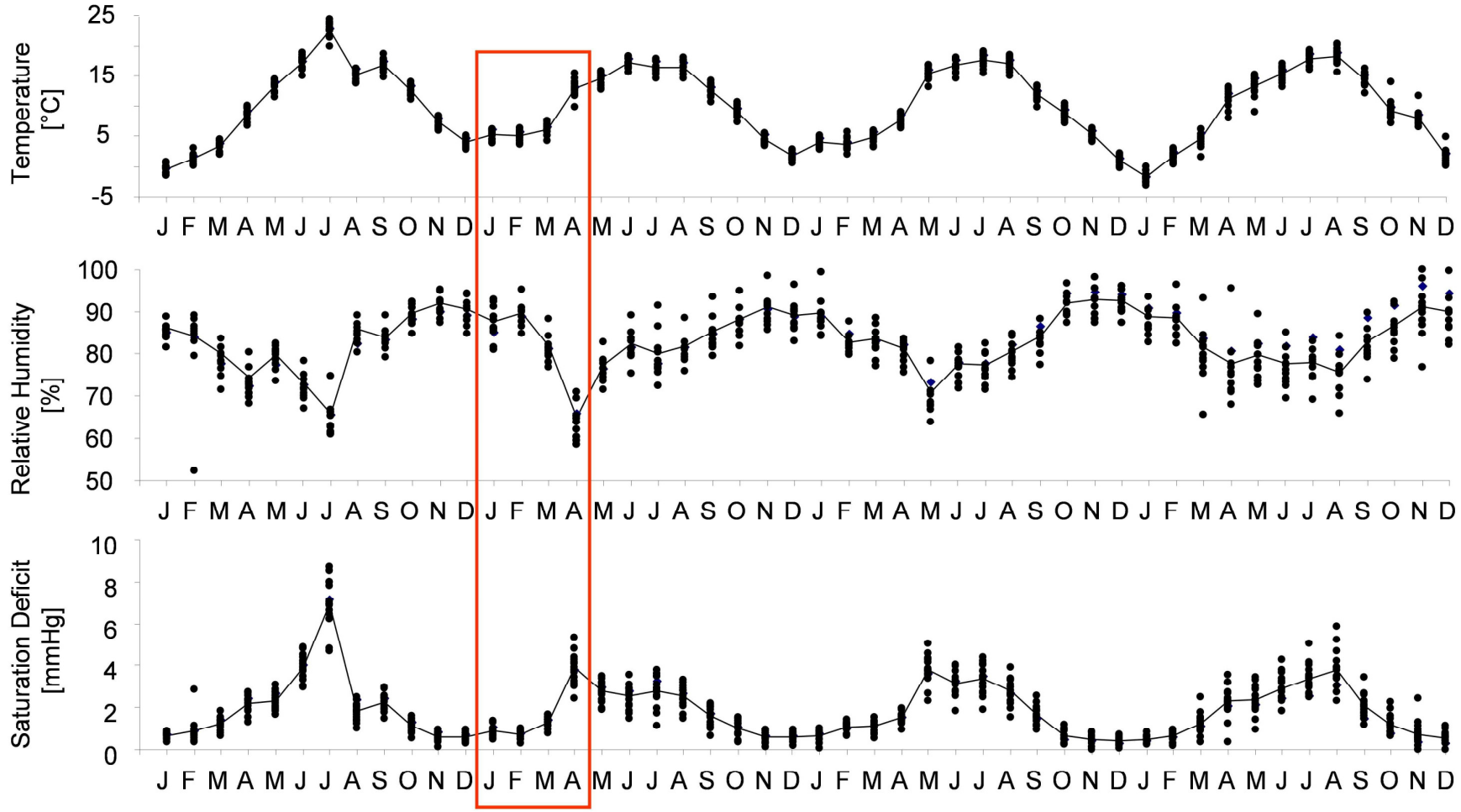

Figure 15. Monthly temperature, relative humidity and saturation deficit from 16 weather stations in Luxembourg for the years 2006 to 2009 (data obtained from ASTA). The red rectangle marks the significant increase of temperature measured in the first four months of 2007 and the significant decrease in relative humidity in April 2007. Saturation deficit as a function of temperature and relative humidity increased significantly in April 2007.

Interestingly, the adult tick density was not higher at these sites, suggesting that a proportion of feeding nymphs were transported to other areas by their hosts.

Annual, seasonal and regional variations could also be observed in the prevalence of tick-borne pathogens in questing ticks were observed, albeit most clearly for *B. burgdorferi* s.l., the predominant pathogen. *Borrelia*-infected ticks displayed a bimodal seasonality, with peaks in spring and autumn and lower tick infection rates during summer in all three years. In 2007 this pattern was more clearly visible than in the following years. We hypothesized that the presence of spirochetes may have a negative influence on the energy reserves of questing ticks, resulting in prolonged quiescence phases during unfavourable microclimatic conditions and leading to an increase of the infection rate in autumn, when conditions become less harsh. In a recent laboratory study, the survival rate of field collected ticks infected with *Borrelia* species was significantly higher under thermohygrometric stress conditions than that of uninfected (89). The authors hypothesized that the spirochetes might be able to modify physiology and/or metabolism of ticks as a response to unfavourable conditions, thus having a beneficial influence on tick survival. This is in line with our field observations and the assumption of behavioural adaptations to harsh conditions. The finding that *B. afzelii* infection in particular has a beneficial effect on the survival rate might explain the observed decrease of *B. afzelii* infections in questing ticks throughout the season. If *B. afzelii* infected ticks suffered less from unfavourable microclimatic conditions, they would be able to quest for longer intervals and thus have a higher rate of host-finding than those ticks undergoing early quiescence.

Although *Borrelia* infected ticks were found at each collection site, differences in the prevalence rate were observed on a regional level. In a small country like Luxembourg, the ecological characteristics of each collection site (e.g. presence of reservoir host species) will have a more important influence on the infection rate of ticks than its geographic location. This will apply also to other tick-borne pathogens and indeed highest *Anaplasma phagocytophilum* infection rates are found in ticks collected in areas with high deer density, a competent reservoir host.

However, as the same collection sites were visited during the study period the maps visualize the annual variations of *Borrelia* prevalence. In 2008, a higher *Borrelia* infection rate was observed together with an increase density of immature ticks, suggesting that infected blood meal hosts for larvae were more abundant in 2007 than in the year before, causing an amplification of *Borrelia* infection. This may also explain the decrease of *Borrelia* coinfections found in questing nymphs in 2008 (0.5%) as compared to the other years (0.9%

and 1.9%), although the rate of mixed infections did not vary significantly during the three years. The acquisition of coinfections in larvae mainly occurs by feeding on mixed infected hosts or cofeeding with infected ticks. An increase in blood meal host abundance is likely to reduce the number of cofeeding events, thus also reducing the chance of acquiring coinfections via cofeeding.

Forestry workers are considered a high risk group for tick-borne infections, as they spent most of their occupational time in tick infested areas. In our seroprevalence study, we found that 35.4% of forestry workers displayed IgG antibodies against *Borrelia* species. This rate seems high compared to other studies, where IgG seroprevalence rates between 8% to 25% were measured in forestry workers (23, 109, 115, 175, 224). These studies also showed that the seroprevalence rate of the general population was two to six times lower than in the professional risk groups, suggesting a seroprevalence rate of 5.9% to 17.7% in the general population of Luxembourg.

Of the seropositive forestry workers, only a proportion of these had previously been diagnosed for Lyme Borreliosis. This is in line with reports of several infections with borrelial spirochetes remaining asymptomatic (54, 60). The seroprevalence rate was found to increase expectedly with age as IgG antibodies can persist for years after an infection (34, 35). Potential risk factors of Lyme Borreliosis were identified, such as hours spent outdoors, number of tick encounter and the number of tick bites per year. Interestingly, some individuals reported extremely high numbers of tick encounters or bites but were found to be seronegative, suggesting either high awareness and early removal of any attached ticks or personal exaggeration of actual tick numbers. The benefit of certain protective measures was investigated, however application of tick repellents, frequent body inspection and early tick removal seemed to have rather a negative effect, as the seroprevalence rates in these groups were always slightly increased. We found that these protective measures are applied significantly more often by individuals with a history of Lyme Borreliosis, who seem to be more aware of the risk after a tick bite but already display IgG antibody titers. Only protective clothing is applied by those with and without a history of Lyme Borreliosis at similar rates and here a small beneficial effect is observed.

Comparison of the regional distribution of seroprevalence of *B. burgdorferi* s.l. and its prevalence in ticks revealed that in most cases the regions with high prevalence of infected ticks also displayed high seroprevalence rates in the forestry workers. In the central West of Luxembourg, higher seroprevalence rates were observed than suggested by the interpolated

tick infection rates, probably caused to some extent by low sampling numbers (n=3 and n=4, respectively) and high age of seropositive forestry workers in these regions. However, drawing the link between seroprevalence in humans and prevalence in ticks is difficult, as point data on the prevalence of *Borrelia* infected ticks cannot be used to predict the infection rate of ticks without taking landscape features and microclimatic conditions into account. More importantly, the origin of infection in forestry workers cannot confidently be linked to a certain region or time, as it is the case for infected ticks.

In conclusion, Lyme Borreliosis is a major health concern for professional risk groups in Luxembourg and also the impact on the general population may be high. The significant interannual, seasonal and regional variations in the density of ticks and the prevalence of *Borrelia burgdorferi* s.l. and the other tick-borne pathogens entail that likewise significant changes in the risk of tick bites and infection are to be expected. The observed variations seem to be linked to abiotic and biotic factors, but further studies on the dynamics of ticks and tick-borne pathogens are warranted.

4.3 Pathogen prevalence in ticks collected from the vegetation and livestock in Nigeria

This manuscript has been submitted as:

Pathogen prevalence in ticks collected from the vegetation and livestock in Nigeria

A. L. Reye, O. G. Arinola, J. M. Hübschen, C. P. Muller

A. L. Reye contributed significantly to the study design and experimental work and was in charge of experimental setup, training of the visiting scientist, data analysis and writing of the manuscript.

Ticks are important disease vectors that can cause considerable economic losses by affecting animal health and productivity, especially in tropical and subtropical regions (169, 205, 231). In Africa, the tick fauna is remarkably diverse with about 50 endemic tick species that are known to infest domestic animals (234). However, the highest impact on livestock health is caused by species belonging to only three genera, namely *Amblyomma*, *Hyalomma* and *Rhipicephalus* (169). Damage is either direct (skin lesions, impairment of animal growth) or indirect by transmission of a variety of pathogens (205). Major economical impact has been associated with the four tick-borne diseases anaplasmosis, heartwater, babesiosis and theileriosis, all of which are prevalent in Africa (10).

Bovine anaplasmosis is caused by the highly pathogenic species *Anaplasma marginale* sensu stricto and the naturally attenuated *A. marginale* subspecies *centrale* (2, 44). *Anaplasma*

species are commonly detected in cattle and seroprevalence rates between 4.6% (Kenya) to 98% (South Africa) from different sub-Saharan countries are reported (10, 138, 156, 194). The causative agents of bovine babesiosis and theileriosis have been frequently detected in blood smears of cattle in Ghana, with prevalences as high as 97% for *Theileria mutans*, 87% for *Theileria velifera* and 61% for *Babesia bigemina* (10). Tick-borne human ehrlichiosis of varying severity are caused by *E. chaffeensis* and *E. ewingii* (145). Several human pathogenic tick-borne *Rickettsia* species have been found in Africa including *Rickettsia conorii conorii*, *R. conorii caspia, R. africae, R. aeschlimannii, R. massiliae, R. akari* and *R. sibirica mongolotimonae* (69, 140, 158). Humans are frequently infected with *Rickettsia* species in Senegal, Burkina Faso, Cameroon, Mali and the Ivory Coast, where seroprevalence rates from 17-36% have been reported (131). *Coxiella burnetii* causes Q fever in humans and high serological prevalences have been reported from West African countries (132). Although transmission mainly occurs via contact with infected reservoir hosts (domestic goats, sheep and cows) also ticks transmit this bacterium. The most important borrelial infection in Africa is relapsing fever transmitted either by lice (louse-borne relapsing fever) or soft ticks (tick-borne relapsing fever, TBRF). TBRF is caused by at least 16 *Borrelia* species, of which *Borrelia crocidura* seems to be of increasing importance in West Africa (230). In Ghana, 15% of blood smears from cattle were positive for *Borrelia* species (10).

Pathogens belonging to the genera of *Anaplasma*, *Ehrlichia*, *Coxiella*, *Rickettsia*, *Babesia*, *Theileria* and *Borrelia* have been reported in ticks from some West African countries. In Mali, Niger, Mauretania and Cameroon feeding ticks from cattle were analysed for the prevalence of *Rickettsia* species (142, 158). In Cameroon the prevalence of *Ehrlichia* species was investigated in ticks removed from dogs (145). However it is important that these studies on feeding ticks are complemented by pathogen prevalence studies in naïve (questing) ticks collected from the vegetation to estimate the risk of infection after tick bites during the next blood meal. So far throughout West Africa only a single study investigated questing ticks in Burkina Faso for *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks (1).

Thus, studies on tick-borne pathogens in ticks are fairly limited in West Africa. Here we present the first comprehensive study on the diversity of bacterial and protozoal tick-borne pathogens in questing and feeding ticks from Nigeria.

4.3.1 Results

Tick numbers. The 836 analysed ticks comprised of four species. The predominant species on cattle were *Rhipicephalus* (*Boophilus*) *annulatus* (37.5%, n=51) and *Amblyomma. variegatum* (33.8%, n=46), followed by *Hyalomma impeltatum* (14.7%, n=20) and *Rhipicephalus evertsi* (13.9%, n=19). From the vegetation only *Rh. evertsi* (n=700) was collected. Mainly adult ticks were collected from both environmental sources (males: 45.1%, females: 53.5%, nymphs: 1.4%).

Anaplasmataceae. Members of the *Anaplasmataceae* were detected in 11% (15/136) of ticks removed from cattle, with *Anaplasma marginale* subspecies being the most prevalent (53.3%; 8/15 ticks). The second most abundant was a not further identifiable *Ehrlichia* species (33.3%; 5/15 ticks). Both *Ehrlichia ewingii* and *Ehrlichia chaffeensis* were detected in a single tick only (Figure 16A). All four tick species were found to harbour *Anaplasmataceae* (*Rh.* [*Bo.*] *annulatus*: *A. marginale* ssp. [n=7] and *E. ewingii* [n=1]; *Hy. impeltatum*: *A. marginale* ssp. [n=1], *E. chaffeensis* [n=1] and *Ehrlichia* sp. [n=1], *Am. variegatum*: *Ehrlichia* sp. [n=1] and *Rh. evertsi*: *Ehrlichia* sp. [n=3]). Ticks from the vegetation were not found to be infected with *Anaplasmataceae* bacteria. However, two sequences with highest similarity to an uncultured alpha proteobacterium (GenBank accession number AY254690) were recovered from two questing *Rh. evertsi* ticks collected in Lanlate (Figure 16A).

Rickettsiaceae. *Rickettsia* species were detected in 13.2% (18/136) of ticks from cattle and in 3.1% (22/700) of ticks from the vegetation. In feeding ticks, a *Rickettsia africae*-like species (RAL) was predominant (83.3%; 15/18) followed by *Rickettsia aeschlimannii* (16.7%; 3/18). In at least one tick of each species *Rickettsiaceae* was detected: *Am. variegatum* (RAL, n=11; *R. aeschlimannii*, n=1), *Rh.* (*Bo.*) *annulatus* (RAL, n=1; *R. aeschlimannii*, n=1), *Rh. evertsi* (RAL, n=1; *R. aeschlimannii*, n=1) and *Hy. impeltatum* (RAL, n=2). Questing *Rh. evertsi* ticks were mainly infected with *Rickettsia massiliae* (95.5%; 21/22). In one tick (4.5%) a *Rickettsia* species belonging to the *Rickettsia rickettsii* group was detected (Figure 16B).

Piroplasmidae. Only *Theileria mutans* was detected in 2.9% (4/136) of feeding *Rh.* (*Bo.*) *annulatus* (n=3) and *Hy. impeltatum* (n=1) ticks (Figure 17A). Questing ticks were not found to be infected with members of the *Piroplasmidae*.

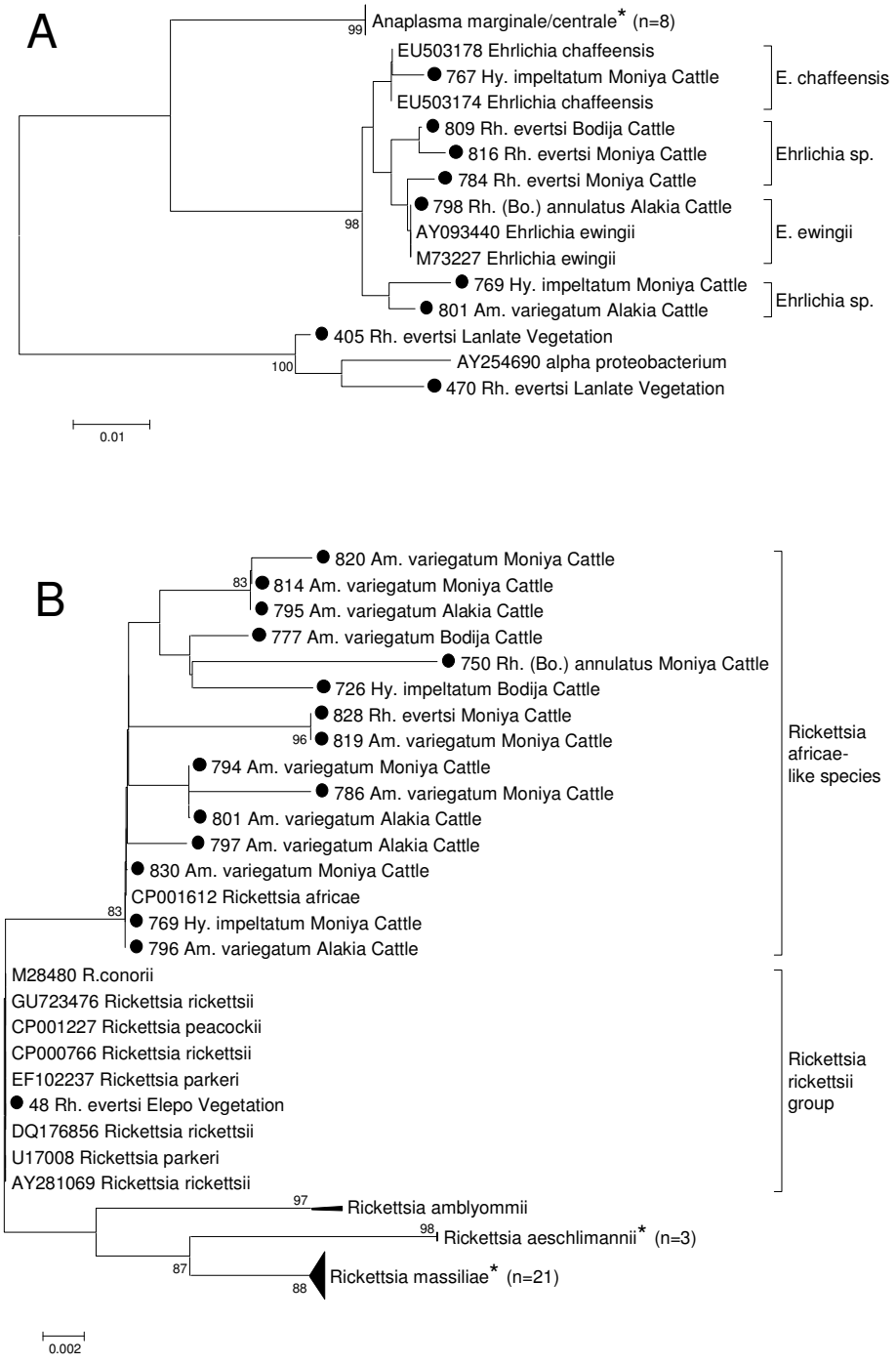

Figure 16. Phylogenetic trees for speciation of pathogens in Nigeria. Neighbour-Joining trees based on (A) a 263 nt fragment of the 16S rRNA gene of Anaplasmataceae (nt 246466 - 246728 of CP001079.1), (B) a 339 nt fragment of the 17-kDa gene of Rickettsiaceae (nt 1194686 - 1195024 of CP000766.2) Nigerian sequences are named with their unique identifier, tick species, geographic location and biological source. Compressed clusters containing sequences from Nigeria are marked with an asterisk. Only bootstrap values above 70 are shown.

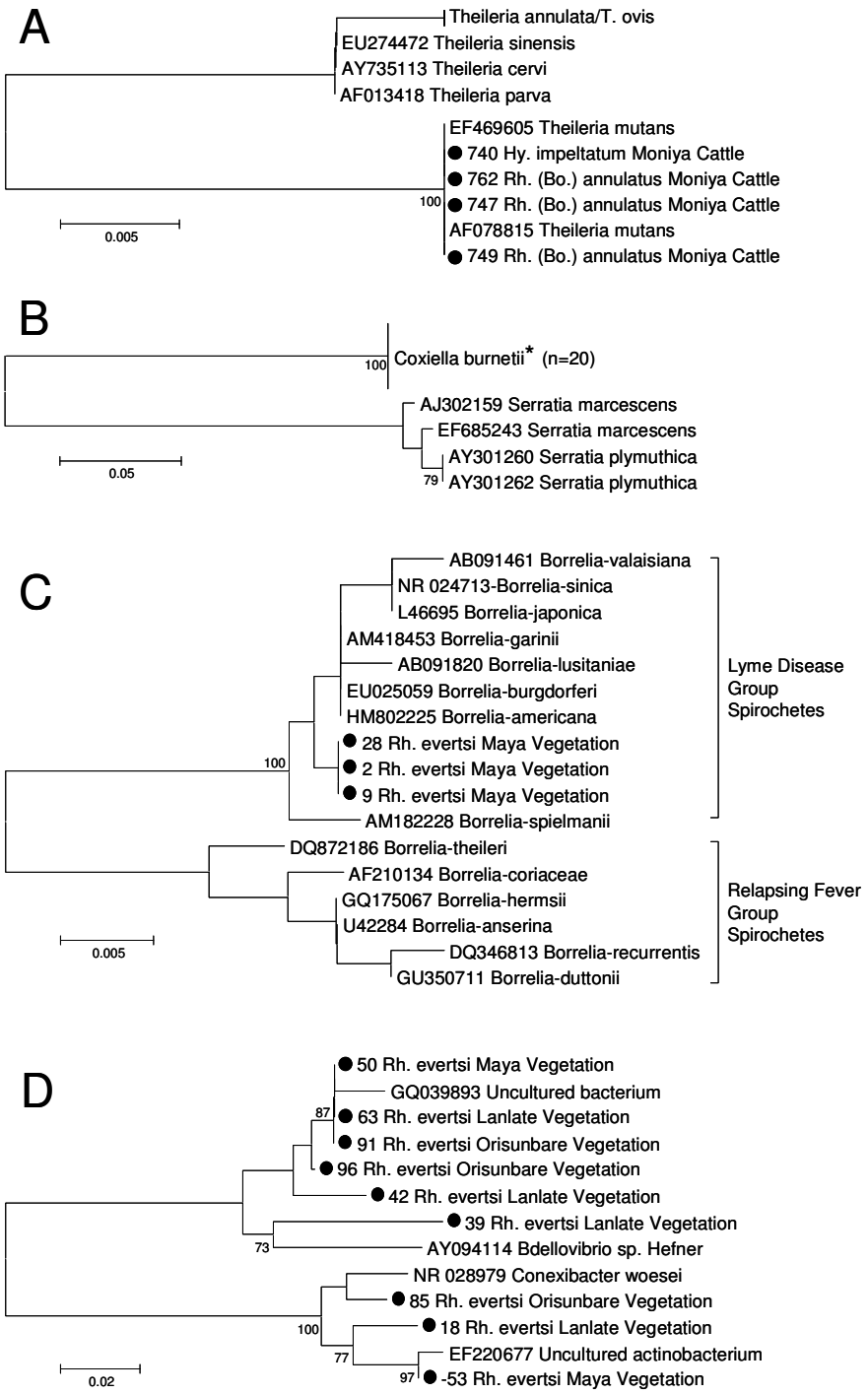

Figure 17. Phylogenetic trees for speciation of pathogens in Nigeria. Neighbour-Joining trees based on (A) a 226 nt fragment of the 18S rRNA gene of Piroplasmidae (nt 656 - 881 of HQ184411.1), (B) a 317 nt fragment of the htpB gene of Coxiella (nt 273435 - 273751 of CP000733.1), (C) a 323 nt fragment of the 16S rRNA gene of Borrelia species (nt 444099 - 443777 of CP002228.1) and (D) a 321 nt fragment of the 16S rRNA gene of Conexibacter woesei (nt 834 - 1151 of NR_028979.1). Nigerian sequences are named with their unique identifier, tick species, geographic location and biological source. Compressed clusters containing sequences from Nigeria are marked with an asterisk. Only bootstrap values above 70 are shown.

Coxiella burnetii. In 14.0% (19/136) of feeding ticks *Coxiella burnetii* was detected (Figure 17B). The only *Coxiella burnetii* infected questing tick (0.1%; 1/700) was collected in Orisunbare. Again, at least one tick of each species was found to harbour *C. burnetii* (*Am. variegatum,* n=9; *Rh.* (*Bo.*) *annulatus,* n=5; *Hy. impeltatum,* n=2; *Rh. evertsi,* n=4).

Borrelia species. *Borrelia* species were only found in questing *Rh. evertsi* ticks (0.4%; 3/700). *Borrelia* species identification was not possible with the sequence obtained from the 16S rRNA (Figure 17C). Further characterization of the *Borrelia* species using primers directed against the flagellar gene was also unsuccessful. In addition, 16S rRNA sequences from unknown organisms were detected in 9 questing *Rh. evertsi* DNA extracts. Three of which had highest sequence similarity to the soil bacterium *Conexibacter woesei*, the remaining six sequences formed a separate cluster (Figure 17D).

Mixed Infections. All mixed infections (1.3%; 11/836) were detected in feeding ticks and were predominantly formed by RAL and *C. burnetii* (36.4%; 4/11) as well as *T. mutans* and *A. marginale* ssp. (18.2%; 2/11). Pathogen combinations found only once were *E. chaffeensis* and *C. burnetii*, *Ehrlichia sp.* and *C. burnetii*, as well as *Ehrlichia sp.* and RAL. Two triple infections formed by *C. burnetii*, *T. mutans* and *A. marginale* ssp. as well as *C. burnetii,* RAL and *Ehrlichia* sp. were found. Involved tick species were *Am. variegatum* (n=5), *Hy. impeltatum* (n=3), *Rh.* (*Bo.*) *annulatus* (n=2) and *Rh. evertsi* (n=1).

4.3.2 Discussion

The tick species found in this study are known to commonly infest life stock in West African countries (234). Surprisingly *Rh. evertsi* was the only tick species collected from the vegetation, either suggesting a collection bias or different habitat requirements of other tick species. This is the first comprehensive study on the diversity of bacterial and protozoal tick-borne pathogens in both questing and feeding ticks not only in Nigeria but sub-Saharan Africa. All of the investigated pathogens are widespread throughout Africa and represent a threat to both human and animal health (10, 140-146, 158).

As expected the infection rate for most of the pathogens was significantly higher in feeding than in questing ticks (Table 11), suggesting that a number of these pathogens originated from the cattle blood ingested before tick collection rather than from transstadially maintained infections acquired during earlier blood meals. Therefore, the detection of pathogens in feeding ticks cannot establish vector competence whereas infected naïve ticks have maintained the pathogen transstadially. Although the latter are more likely to serve as vectors of live pathogens, detection of residual DNA from dead pathogens in the tick cannot be totally excluded.

Table 11. Infected ticks from the vegetation and cattle in Nigeria. Statistical significant differences are given. Fisher's exact test was performed on all except for the total infection rate, where Pearson's goodness of fit chi-square (GFX) test was used. Only P values smaller that 0.05 are shown.

Pathogens	Vegetation	Cattle	*P* value
Anaplasma marginale/centrale	0	8	<0.01
Ehrlichia chaffeensis	0	1	
Ehrlichia ewingii	0	1	
Ehrlichia sp.	0	5	<0.01
alpha proteobacterium	2	0	
R.aeschlimannii	0	3	<0.01
R.africae-like	0	15	<0.01
R.massiliae	21	0	<0.01
R.rickettsii group	1	0	
Borrelia sp.	3	0	
unknown bacteria	9	0	
T.mutans	0	4	<0.01
C.burnetii	1	19	<0.01
Total	37	43*	<0.01

*Note that the total number of infected ticks is lower than the sum of all pathogens detected as ticks with mixed infections were only counted once.

The only study on the prevalence of *Coxiella burnetii* in ticks was conducted in Senegal, where 0.7-6.8% of feeding ticks from cattle were found to be infected (132). This rate is considerably lower than the 14.0% of feeding ticks that we found to be infected. Interestingly, *C. burnetii* was frequently detected in multiple ticks collected from the same cow. This observation and the difference between infection rates in questing and feeding ticks (0.1% vs. 14%, $p<0.01$) may be a reflection of cattle as reservoir hosts of *C. burnetii*. In Nigeria, *C. burnetii* seems to represent a considerable risk factor for those in contact with cattle. In Senegal, where the prevalence of *C. burnetii* in cattle is relatively low (3.6%), seroprevalence rates in humans can be as high as 21.4-51.0% (105, 132) , suggesting even higher prevalence rates in Nigeria, where an estimated 27.4% (17/62) of cattle were infected. Thus both ticks and cattle must be considered as a considerable source of Q fever and a significant threat to human health in the region.

Eight *Anaplasma marginale/centrale* positive ticks were collected and the estimated prevalence in cattle was 4.4%. The available sequences did not allow to distinguish between the highly pathogenic bovine *A. marginale* sensu stricto and the naturally attenuated *A. marginale* subsp. *centrale*, which is sometimes used as a vaccine (44). As the cattle in this study were not vaccinated, they must have been naturally infected with either one of these subspecies but the risk of disease cannot be estimated.

Theileria mutans, the causative agent of benign bovine theileriosis, was detected in four *Rh.* (*Bo.*) *annulatus* and *Hy. impeltatum* ticks removed from three cows of the same herd in Moniya, but not from questing *Rh. evertsi*, suggesting that this tick species may not be a competent vector for this pathogen. It seems that the estimated prevalence rate in Nigerian cattle (4.8%; 3/62) is much lower than e.g. in Ghana, where 97.0% of cattle are infected with *T. mutans* (10).

Different SFG *Rickettsia* species have been reported in ticks from cattle in Mali (16.2%), Niger (16.3%), Mauretania (0%) and Cameroon (74.7%). We detected *R. massiliae* and a member of the *R. rickettsii* group only in questing ticks ($p<0.05$). This is compatible with the minor role of vertebrates in the perpetuation and survival of *R. massiliae* (130). In contrast, RAL and *R. aeschlimannii* were only detected in feeding ticks, indicating a potential role of cattle as hosts. All ticks infected with the predominant RAL were collected from 13 cows, corresponding to an estimated prevalence rate of 9.6% of infected cows (13/136).

Different *Borrelia* species have been described in Africa, most of which are transmitted by soft ticks (230). Ticks of the genus *Rhipicephalus* are known to transmit *Borrelia theileri* to

cattle, causing bovine borreliosis. The 16S rRNA sequences of the *Borrelia* species detected in this study differed at least in 3 nucleotide positions from all known *Borrelia* sequences. These new sequences form a separate cluster within the *Borrelia burgdorferi* s.l. group, possibly belonging to a so far unknown *Borrelia* species. Unfortunately, further characterization based on other genes was unsuccessful.

Several sequences from unknown bacteria were obtained in the *Anaplasmataceae* and *Borrelia* detection PCRs. They were most likely derived from bacteria from the outside rather than the inside of the ticks, as they were most closely related to a soil bacterium.

We also report here for the first time mixed infections in feeding ticks from West Africa involving mainly RAL and *C. burnetii*. Mixed infections involving *C. burnetii* may originate either from subsequent blood meals, co-feeding events, or feeding on co-infected hosts. In mixed infections involving *Rickettsia* species also transovarial transmission may play a role. Coinfections with multiple pathogens may complicate the diagnosis and treatment.

The diversity of tick-borne pathogens in Nigeria was higher in feeding than in questing ticks, suggesting that cattle serve as reservoirs for at least some of the studied pathogens in particular *Coxiella burnetii*. The impact of these infections on human and animal health and the resulting economic losses require further attention to assess the cost benefit of vaccination against *Anaplasma marginale* sensu stricto and other preventive measures.

4.4 Prevalence of Tick-Borne Pathogens in Ixodes ricinus and Dermacentor reticulates Ticks from Different Environmental Sources in Belarus

This manuscript has been submitted as:

Prevalence of Tick-Borne Pathogens in Ixodes ricinus and Dermacentor reticulates Ticks from Different Environmental Sources in Belarus

A. L. Reye, V. Stegniy, S. Velhin, S. Wolter, J. M. Hübschen, G. Ignatyev, C. P. Muller

A. L. Reye contributed significantly to the study design and experimental work and was in charge of experimental setup, training of the visiting scientist, data analysis and writing of the manuscript.

In Eastern Europe, Lyme Borreliosis is considered a major threat to human health, the annual incidence of this disease ranges from 4.8 to 35 cases per 100.000 population (121). In neighbouring countries of Belarus, the prevalence in questing ticks ranges from 3.3% to 37.6%. Other bacterial and protozoal tick-borne pathogens of interest are *Rickettsia* species with a prevalence from 2.9% to 15.1%, *Anaplasma* species (2.3%-20.8%), and *Babesia* (3%-11%; (57, 81, 129, 137, 168, 174, 200, 208, 236, 239). Not much is known about the prevalence of Coxiella, Francisella and Bartonella species in Eastern Europe (174, 200).
This is the first comprehensive study on the prevalence of bacterial and protozoal tick-borne pathogens of questing and feeding ticks in Eastern Europe and Belarus.

4.4.1 Results

Tick numbers. In total 553 ticks belonging to *Ixodes ricinus* (n=327; 59.1%) and *Dermacentor reticulatus* (n=226; 40.9%) were collected from the vegetation (n=453), cattle (n=99) and a dog (n=1). Most ticks were collected in Gomel (n=297), followed by Brest (n=82) and Minsk region (n=79). Only few ticks were collected in Grodno (n=45), Mogilev (n=31) and Vitebsk (n=19). Adults were the predominant instars (n=551; 59.5% females; 40.1% males), only two nymphal *I. ricinus* (0.4%) were collected from the vegetation. In Brest, Gomel and Minsk region, both tick species were equally prevalent (50 ±5%), whereas in Mogilev, Grodno and Vitebsk *I. ricinus* was predominant (67.7 - 97.8%). The total tick infection rate for Belarus (counting mixed infections only once) was 36.8%. On a regional level considerable differences in the total tick infection rates were observed, ranging from 10.5% in Vitebsk to 46.3% in Brest, and 34.4 ±3% in the other regions (Figure 18).

Rickettsiaceae. The most prevalent pathogen detected in 24.4% of ticks belonged to the Spotted Fever Group (SFG) *Rickettsia*. The *Rickettsia* infection rate was significantly higher ($p<0.01$) in *D. reticulatus* (43.8%, n=99) than *I. ricinus* (11.0%, n=36). In questing and feeding *D. reticulatus* ticks 99% of the infections were caused by a single *Rickettsia* species of the *R. rickettsii* group (RRG) (

Figure 19A). Interestingly, 57.1% of RRG-infected *D. reticulatus* derived from the same collection site in Gomel region. The only *D. reticulatus* tick harbouring *R. helvetica* was feeding on a dog. The infection rate of *I. ricinus* ticks was 8.9% for *R. helvetica* (n=29), 1.5% for a species belonging to the cluster *R. monacensis*/*R. tamurae* (n=5) and 0.6% for members of the RRG (n=2) (

Figure 19A). *R. helvetica* and *R. monacensis*/*R. tamurae* were only detected in questing, whereas RRG was only found in feeding *I. ricinus* ticks. On a regional level, *Rickettsia* species displayed highest prevalence rates in Brest (28%) and Gomel (26.6%), medium rates in Grodno (17.8%), Minsk (21.5%) and Mogilev (22.6%) and lowest rates in Vitebsk (5.3%).

***Borrelia* species.** *Borrelia burgdorferi* sensu lato was the second most prevalent pathogen and detected in 9.4% (n=52) of all ticks. The *Borrelia* infection rate was significantly higher ($p<0.05$) in *I. ricinus* (14.1%, n=46) than in *D. reticulatus* (2.7%, n=6). *I. ricinus* ticks were infected with *B. afzelii* (6.1%), *B. garinii* (3.4%), *B.valaisiana* (2.5%), *B. burgdorferi* s.s. (1.8%) and *B. lusitaniae* (0.3%) (

Figure 19B). In *D. reticulatus* ticks only *B. burgdorferi* s.s. (1.8%), *B. afzelii* (0.4%) and *B. valaisiana* (0.4%) were detected (

Figure 19B). The infection rate of ticks from the vegetation (10.4%, n=47) was significantly higher ($p<0.05$) than from cattle (5.1%, n=5). Interestingly, the *Borrelia* tick infection rate was significantly higher in

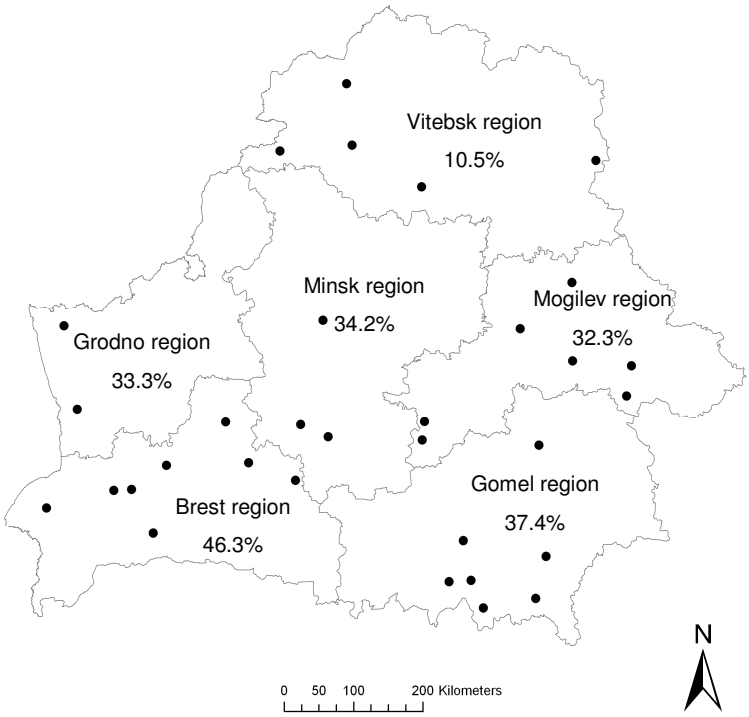

Figure 18. Administrative regions of Belarus showing the 32 collection sites and the tick infection rates.

Grodno (15.6%) and Brest (15.9%) than in Gomel (7.7%; $p<0.05$) and the remaining regions (5.3% - 9.7%; not significant). *Borrelia* species diversity was highest in ticks from Gomel (all 5 species detected) and lowest in Vitebsk region (only *B. garinii*).

Low prevalent pathogens. The other pathogens were exclusively detected in questing ticks. *Anaplasma phagocytophilum* (2.2%; n=12), *Coxiella burnetii* (0.9%; n=5), *Francisella tularensis* ssp. (0.7%; n=4), *Babesia* sp. EU1 (0.4%; n=2) and *B. microti* (0.5%; n=3) were only detected in *I. ricinus* ticks (

Figure 19C,

Figure 20A-D), whereas both tick species harboured *Bartonella henselae* (0.7%; n=4) (

Figure 20D). *A. phagocytophilum* was detected in three regions and the tick infection rate was significantly higher in Minsk (6.3%) than in Gomel (2.0%; $p<0.05$) and Grodno (2.2%, not significant). Ticks from Brest and Gomel region were infected with *B. henselae* and both *Babesia* species, whereas only ticks from Gomel harboured *F. tularensis* ssp. and *C. burnetii*.

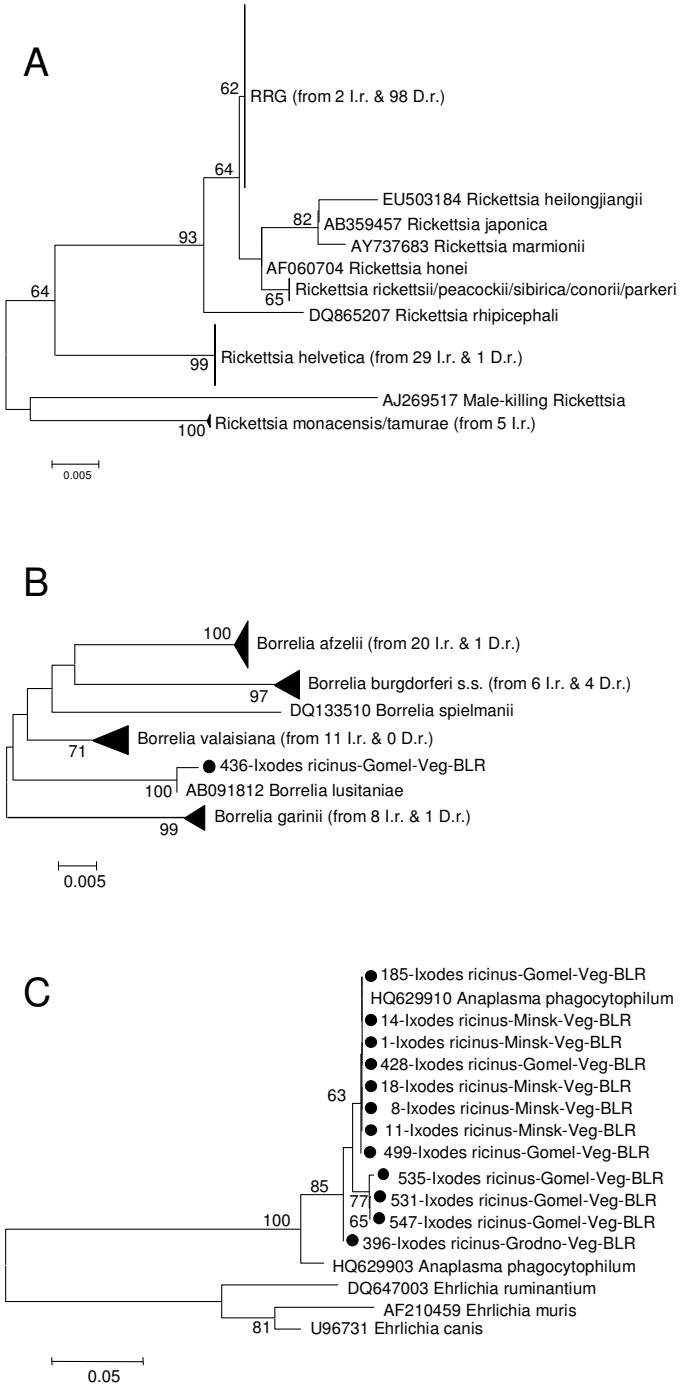

Figure 19. Phylogenetic trees for speciation of pathogens in Belarus. Neighbour-Joining trees based on (A) a 344 nt fragment of the 17-kDa gene of Rickettsia species (nt 1194706 - 1195039 of CP000766.2), (B) a 348 nt fragment of the FlaB gene of Borrelia burgdorferi s.l. (nt 97 - 444 of HM345909.1), (C) a 352 nt fragment of the groEL gene of Anaplasma species (nt 732 - 1083 of HQ629903.1). Sequences from Belarus are named with their unique identifier, tick species, geographic location, biological source and WHO country code. The number of pathogens from Belarus in a compressed cluster and the tick species are given in brackets. Veg = Vegetation. Solid circle = Ixodes ricinus. I.r. = Ixodes ricinus; D.r. = Dermacentor reticulatus. Only bootstrap values above 60 are shown.

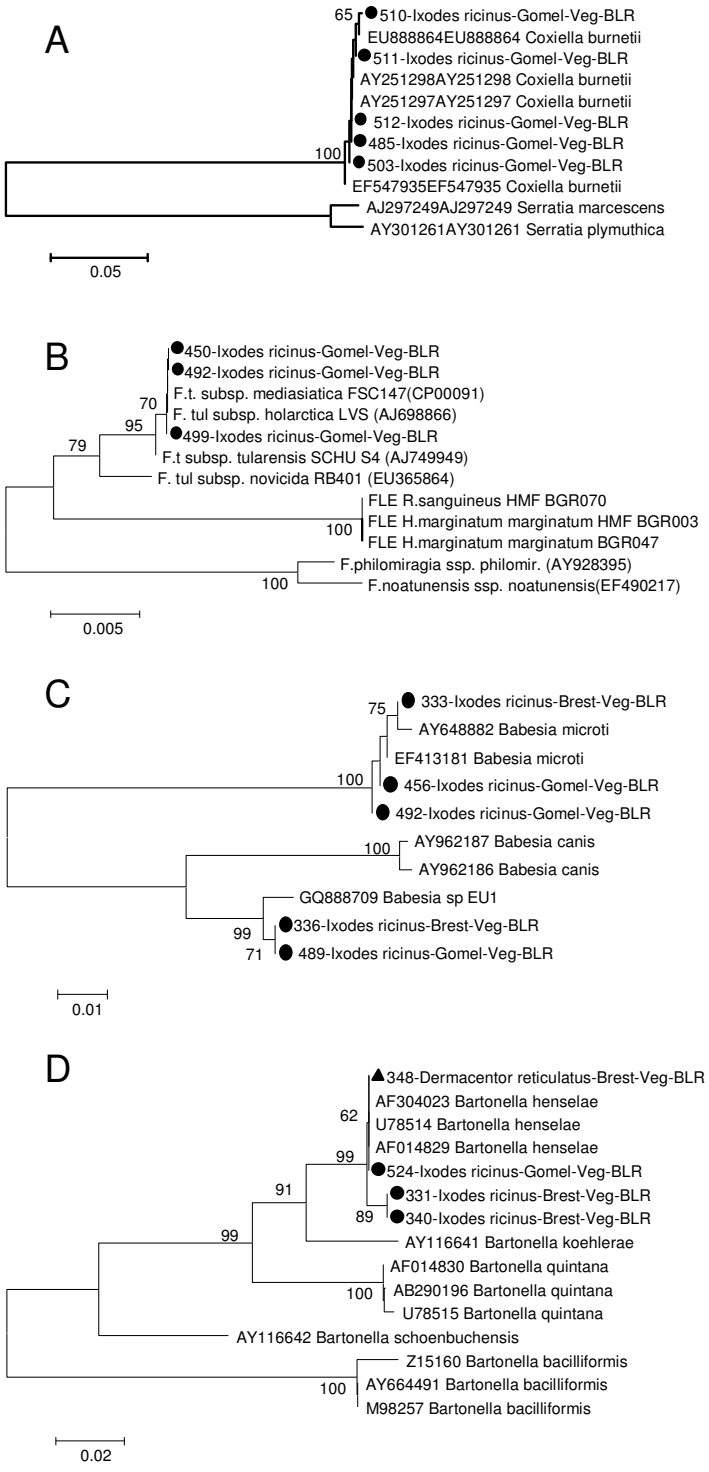

Figure 20. Phylogenetic trees for speciation of pathogens in Belarus. Neighbour-Joining trees based on (A) a 319 nt fragment of the htpB gene of Coxiella burnetii (nt 320 - 638 of EU888863.1), (B) a 894 nt fragment of the 16S rRNA gene of Francisella species (nt 8 - 898 of HM371361.1), (C) a 516 nt fragment of the 18S rRNA gene of Babesia species (nt 1 - 516 of GQ856653.1) and (D) a 320 nt fragment of the 16S-23S gene of Bartonella species (nt 14 - 333 of GU827129.1). Sequences from Belarus are named with their unique identifier, tick species, geographic location, biological source and WHO country code. The number of pathogens from Belarus in a compressed cluster and the tick species are given in brackets. Veg = Vegetation. Solid circle = Ixodes ricinus; solid triangle = Dermacentor reticulatus. Only bootstrap values above 60 are shown.

Questing and feeding ticks. Overall, the pathogen species composition was more diverse in questing as compared to feeding ticks (14 vs. 4 species) and in *I. ricinus* as compared to *D. reticulatus* ticks (13 vs. 5 species). Interestingly, the *I. ricinus* infection rate was significantly lower in feeding than in questing ticks (13.2% vs. 32.9%; $p<0.01$), whereas it was similar in *D. reticulatus* ticks (42.6% vs. 46.3%). Mixed infections were detected in 2.7% (n=14) of ticks, the majority of which were formed between members of the two most prevalent pathogen genera *Rickettsia* and *Borrelia* (n=8). Also, mixed infections occurred more often in *I. ricinus* than in *D. reticulatus* ticks (3.4% vs. 1.3%).

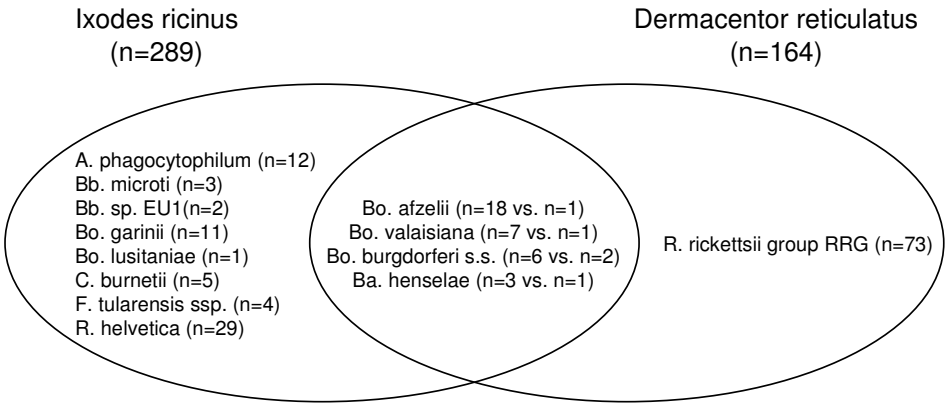

Figure 21. Venn diagram of pathogen diversity in questing *I. ricinus* and *D. reticulatus* ticks. Each tick species is represented by an oval. A, Anaplasma; Bb, Babesia; Bo, Borrelia; C, Coxiella; F, Francisella; R, Rickettsia; Ba, Bartonella.

4.4.2 Discussion

This is the first comprehensive study on tick-borne bacterial and protozoan pathogens of human and veterinary interest in Eastern Europe. We observed a higher total tick infection rate of 24.4% for *Rickettsia* species as compared to Russia (15.1%), Poland (2.9 - 8.7%) and Slovakia (8.8%) (137, 200, 208). The overall *Borrelia* infection rate of ticks was 9.4%, which is on the lower range of infection rates of 3.3-37.6% reported from Eastern Europe (57, 119, 174, 176, 209, 239). The low prevalent pathogens *Anaplasma*, *Babesia*, *Bartonella*, *Coxiella* and *Francisella* were found only at few sites in southern and central regions of Belarus with rates comparably low as in other Eastern European countries (95, 137, 168, 174, 200, 236, 239), although sometimes higher tick infection rates have been reported (80, 174, 239). Interestingly, hotspots of infection were discovered at sites in Minsk and Gomel region for *A. phagocytophilum* (12.5-17.2%), *F. tularensis* ssp. (5.5%) and *C. burnetii* (9.1%). As an important route of transmission of *F. tularensis* ssp. and *C. burnetii* is the inhalation of contaminated aerosols (5, 67), their focal finding suggests a rather high prevalence in reservoir hosts at these sites. Therefore, tick surveillance at the identified hotspots and neighbouring regions are warranted in order to predict and perhaps avoid outbreaks of tularemia and Q-fever.

The observed regionality of the low prevalent pathogens *Anaplasma*, *Bartonella*, *Babesia*, *Coxiella* and *Francisella* is likely to be influenced considerably by the numbers of ticks collected. Gomel region with highest tick numbers, displayed also the highest pathogen diversity in the country, since also low prevalent pathogens were detected. This suggests that Gomel region may best reflect the infection status of ticks in Belarus. Interestingly, this hypothesis only partially holds true as the *Borrelia* infection rate in this region was significantly lower than in the western regions Grodno and Brest. Even when focusing only on the known *Borrelia* competent vector species *I. ricinus*, Gomel region still displayed significantly lower infection rates. However, habitat features of each collection site are more likely to have a significant impact on the local prevalence of *Borrelia* species than regional aspects.

Questing ticks give more information about possible vector competence than feeding ticks, as an infection must have been at least transstadially maintained. We found that pathogen diversity in questing ticks was higher in *I. ricinus* than in *D. reticulatus* ticks (Figure 21). Also, the infection rate was significantly higher in questing than in feeding *I. ricinus* but similar in *D. reticulatus*. This is interesting as feeding adult ticks were removed during their

third blood meal, whereas questing adults only fed twice. Lower numbers of feeding tick (n=38 for *I. ricinus* and n=61 for *D. reticulatus*) could be responsible for this observation, as well as the limited host diversity. Removal of feeding ticks from a single host species is likely to bias the actual burden of tick-borne pathogens toward an adapted species. Since livestock herds are kept in defined areas, tick populations in these regions may predominantly feed on these hosts. Amplification or dilution of pathogen prevalence in ticks can occur depending on the reservoir competence of the involved vertebrates (154). The stable RRG prevalence in *D. reticulatus* seems to be caused by a high rate of transovarial transmission, which often occurs in *Rickettsia* species (130, 165), whereas cattle does not seem to play a role for pathogen maintenance. In congruence with our observation are findings from Poland, where 40.7% of *D. reticulatus* were infected with a *Rickettsia* species closely related to *R. slovaca*, *R. sibirica*, *R. honei* and other SFG rickettsiae (207). It indeed seems likely that the *Rickettsia* species from Poland and the RRG from Belarus represent the same species, which is highly adapted to *D. reticulatus*.

Our survey revealed a high burden of tick-borne pathogens in questing and feeding *I. ricinus* and *D. reticulatus* ticks in different regions in Belarus, indicating a potential risk for humans and animals. The pathogenic potential of RRG and the role of *D. reticulatus* as its arthropod vector require further attention. Identified hotspots of infected ticks especially when *Francisella tularensis* and *Coxiella burnetii* are involved should be included in future surveillance studies.

4.5 Detection of new Francisella-like tick endosymbionts in Hyalomma spp. and Rhipicephalus spp. (Acari: Ixodidae) from Bulgaria

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Detection of new Francisella-like tick endosymbionts in Hyalomma spp. and Rhipicephalus spp. (Acari: Ixodidae) from Bulgaria

I. N. Ivanov, N. Mitkova, A. L. Reye, J. M. Hübschen, R. S. Vatcheva-Dobrevska, E. G. Dobreva, T. V. Kantardjiev, C. P. Muller

A. L. Reye contributed to the experimental setup, supervision of the visiting scientist and writing of the manuscript.

Francisella is an expanding genus of closely related Gram-negative coccobacilli. During the past two years at least three new taxa have been described that are pathogens either in fish or humans (96). Yet the classification of many so called *Francisella*-like endosymbiotic (FLE) bacteria found both in hard and soft ticks remains unresolved (147, 191, 218).

FLEs seem to replicate intracellularly, they are transmitted transovarially and to date there is no evidence of horizontal transmission through tick bites. FLEs have been mainly found in the female's reproductive tissues (191) but recently a *Dermacentor variabilis* endosymbiont (DVF) was detected in the hemolymph, potentially suggesting colonization of the salivary glands (74). The pathogenic potential of FLEs remains unknown although sequences homologous to *iglC* and *mglA* genes of *F. tularensis* implicated in pathogenicity have been

detected (24, 122). Studies involving FLEs are hampered by their inability to grow on cell-free media. Hence most of the molecular studies have been performed with total DNA extracts from ticks or tissues rather than on FLE cultures. This together with the fact that FLEs have never been detected outside of ticks suggested that they represent secondary endosymbionts. FLEs seem to be widely distributed and during the last decade a number of diverse FLEs have been reported in various tick genera in at least four continents (122, 149, 191, 204, 218). To date, the only FLE ever reported from Europe is from *Dermacentor reticulatus* in Hungary (204), Portugal (40) and Serbia (GenBank accession numbers HM629448 and HM629449). The discrimination between FLEs and *F. tularensis* without gene sequencing is difficult and the validation of new specific molecular markers is important (114).

Here we report on the detection and molecular characterization of two new, so far undescribed FLEs in three different tick species that seem to lack RD1, an important molecular marker for the discrimination of pathogenic *F. tularensis* subspecies.

4.5.1 Results and Discussion

A total of 472 ticks removed from human (n=32) or animal (n=264) hosts or collected from the environment (n=176) during 2005-2008 were screened for the presence of *F. tularensis* and FLEs. The ticks originated from rural or urban areas of nine major districts in Bulgaria. In total, 12 tick samples or pools including *H. m. marginatum* (9 pools containing 16 ticks), *H. aegyptium* (1 tick), *R. sanguineus* (1 pool containing 2 ticks) and *D. reticulatus* (1 pool containing 3 ticks) were positive for *Francisella* spp. 16S rRNA amplicons. All 16S rRNA sequences clustered within the monophyletic clade of previously described FLEs rather than with *F. tularensis* (Fig.1A) (191).

Three distinct FLE genotypes were distinguished in total. Two of them were found to be without a homologue in GenBank. The 16S rDNA FLE sequences from the *H. m. marginatum* and *R. sanguineus* ticks were identical and comprised a distinct genotype (subsequently referred to as HMF). HMF was detected in both *H. m. marginatum* males (6 samples) and females (3 samples) that were removed from various domestic animals or collected from the environment as well as in one pool of three *R. sanguineus* males. The closest related GenBank entries were FLEs of the soft ticks *Ornithodoros moubata* and *O. porcinus* with 99% sequence identity, corresponding to 11 and 13 differing nucleotides, respectively (Fig. 1A).

As reported for other FLEs this new FLE was also detected in two different tick species supporting the hypothesis of an independent evolution of FLEs and tick hosts (122, 191). The prevalence of HMF in *H. m. marginatum* ticks ranged from 32% (assuming 1 positive tick per pool) to 57% (assuming 16 positive ticks in the 9 pools). In *R. sanguineus* the HMF prevalence ranged from 0.7% (1 positive tick) to 1.4% (2 positive ticks).

The second new FLE genotype was detected in a single female *H. aegyptium* tick (subsequently referred to as HAF) removed from a human. Phylogenetic analysis showed that HAF was more closely related to FLE of *O. moubata* (99% and seven nucleotide differences) than HMF (Fig.1A). As only one *H. aegyptium* tick was collected, the prevalence of HAF cannot be estimated with confidence.

The third FLE genotype (subsequently referred to as DRF) was found in a pool of three *D. reticulatus* males removed from an animal host. DRF differed in only two nucleotide positions from the previously reported *D. reticulatus* FLE from Hungary and Portugal (Figure 22) (40, 204). The prevalence of DRF in *D. reticulatus* ticks ranged from 5.8% (assuming 1 positive tick per pool) to 17.6% (assuming 3 positive ticks per pool).

The detected FLEs showed a specific geographic distribution. All HMF samples were from the same two neighboring regions in Central and South Bulgaria, whereas HAF and DRF originated from one Eastern and one Northern region, respectively. Interestingly, FLEs were only detected in a fraction of ticks collected in the same region suggesting that FLEs are facultative and non-essential for the survival of the tick host and probably have diverged from a transmissible ancestor in the recent geologic past (191).

To further characterize the new FLEs the two additional molecular markers RD1 and *tul4* were analyzed (18, 191). *tul4* was successfully amplified from six of the 12 FLE positive samples (Fig 1B). The *tul4* sequences of HMF and HAF clustered separately from all known FLEs and further supported the 16S rRNA assay results (Fig. 1B). Despite the high sensitivity of the assay (106) no RD1 amplicons were obtained from any of the FLE positive samples, suggesting that they lack this region or at least have a significantly different RD1 sequence. Our finding suggests that *F. tularensis* can be readily distinguished from the three FLEs described in this study by RD1. If RD1 is absent also in other FLEs, this marker could be of interest for a sequence-independent broad differentiation of *F. tularensis* from FLEs (114).

Further studies are needed to assess the pathogenic potential of FLEs, e.g. by comparing the genomes of non-pathogenic endosymbionts with their pathogenic relatives as was recently done for *Rickettsia* species (62). Symbionts that were previously considered non-pathogenic

may thus turn out to be pathogenic, as was shown for *Rickettsia helvetica* and *R. slovaca* (173).

In conclusion, our findings add two new FLEs, found in three different ticks namely in *Hyalomma marginatum marginatum*, *Hyalomma aegyptium* and *Rhipicephalus sanguineus* to an increasing diversity of *Francisella* species. These two new taxa seem to be facultative secondary endosymbionts of ticks.

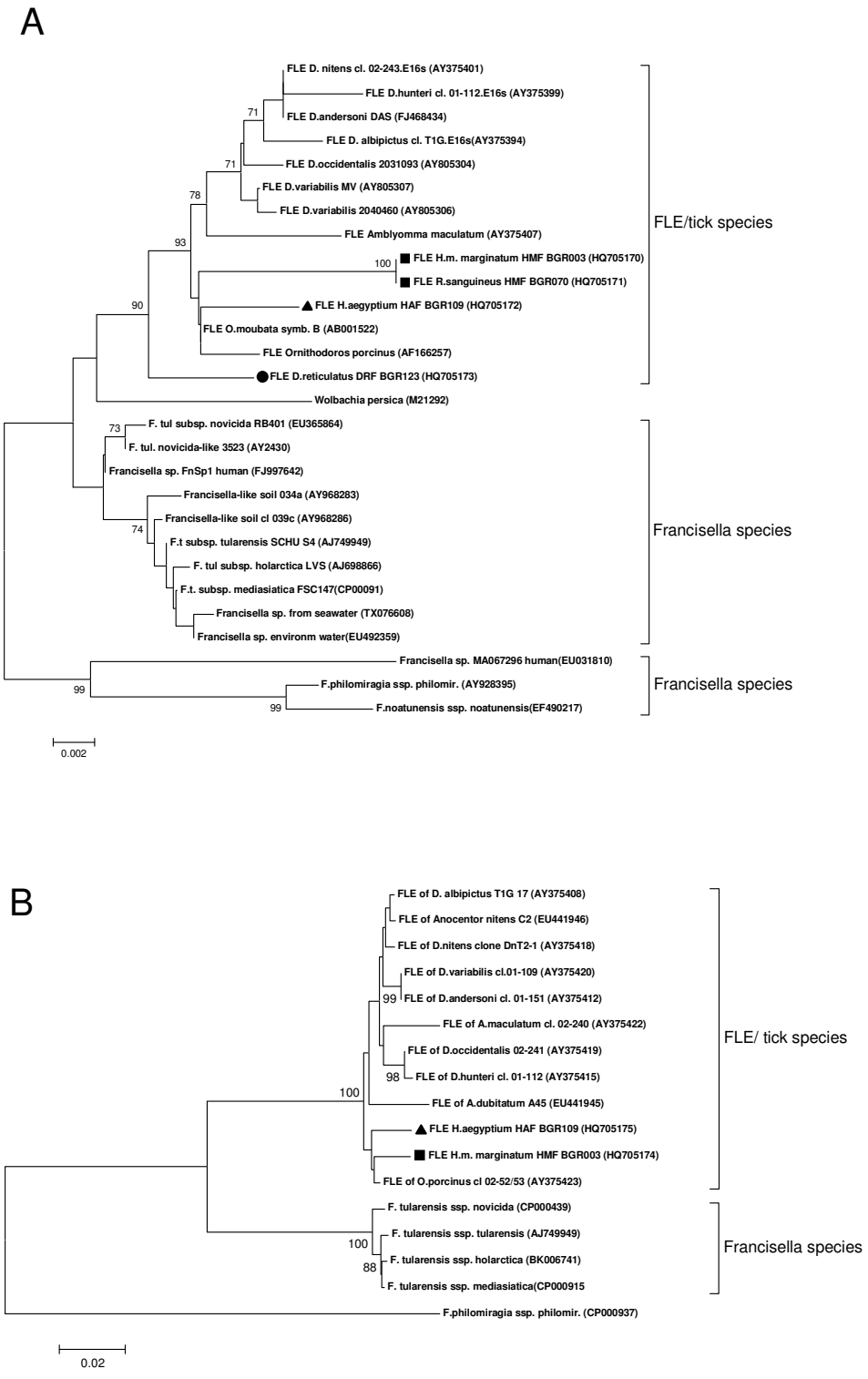

Figure 22. Phylogenetic trees for speciation of FLEs in Bulgaria. Neighbor-joining trees of the 16S rRNA (A) and tul4 (B) genes of various Francisella spp.. Bootstrap values (1000 replications) above 60 are shown. GenBank accession numbers are given in brackets. The sequences characterized in this study are designated with symbols (HMF=square, HAF= triangle and DRF= circle).

4.6 Detection of Babesia sp. EU1 and Members of Spotted Fever Group Rickettsiae in Ticks Collected from Migratory Birds at Curonian Spit, North-Western Russia

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Detection of Babesia sp. EU1 and Members of Spotted Fever Group Rickettsiae in Ticks Collected from Migratory Birds at Curonian Spit, North-Western Russia

A. Movila, A. L. Reye, H. V. Dubinina, O. O. Tolstenkov, I. Toderas, J. M. Hübschen, C. P. Muller, A. N. Alekseev

A. L. Reye contributed to the experimental setup, supervision of the visiting scientist and writing of the manuscript.

The tick *Ixodes ricinus* is a common ectoparasite in Europe. It is a well-known vector for many pathogenic human viruses, bacteria, and protozoa, causing zoonoses and circulating in the natural foci. Birds, mainly passerines (order Passeriformes), often host subadult ticks and represent a reservoir for human tick-borne pathogens (93). Migratory birds can act as long-distance vectors for several microbial agents of human disease. *Borrelia garinii* was detected in ground dwelling and sea birds in Eurasia, whereas *Borrelia valaisiana* and *Borrelia burgdorferi* sensu stricto were identified in different passerine birds in Europe (50). The

human pathogenic members of the family of Anaplasmataceae, spotted fever group (SFG) rickettsia, *Coxiella burnetii*, and Tick-borne encephalitis (TBE) virus have been detected in ticks from different species of migratory birds collected in Europe (4, 55, 97, 187, 202, 233). However, the involvement of birds in the ecology and epidemiology of babesiosis has so far been little studied. Skotarczak et al. (2006) investigated by PCR the prevalence of *Babesia* in ticks' and birds' blood from West-Central Poland (199).

In this study, the prevalence of SFG rickettsiae and *Babesia* sp. in *I. ricinus* ticks collected from the migratory birds at Curonian Spit, North-Western Russia, was investigated.

4.6.1 Results

Altogether, 236 birds were captured, representing 8 species of Passeriformes: *Turdus philomelos* (n=59), *Fringilla coelebs* (n=68), *Troglodytes troglodytes* (n=28), *Parus major* (n=17), *Sturnus vulgaris* (n=26), *Fringilla montifringilla* (n=18), *Sylvia borin* (n=6), and *Phylloscopus trochilus* (n=14). Eighty-six of the captured birds (36.4%) hosted 126 nymphs (Table 12). All ticks were identified as *I. ricinus*. The DNA was successfully isolated from all of the tested ticks. *Babesia* spp. were detected in two cases of 126 (1.6%) analyzed ticks collected from two specimens of *T. philomelos* (Table 12). The partial sequence of 18S rDNA had 100% similarity to human pathogenic *Babesia* sp. EU1. The SFG rickettsiae were detected in 19 of 126 (15.1%) ticks (Table 12). BLAST analysis of SFG rickettsiae gltA assigned sequences to human pathogenic *Rickettsia helvetica* (10.4%), *Rickettsia monacensis* (3.4%), and *Rickettsia japonica* (0.8%) with 98%–100% sequence similarity. The *R. helvetica* was detected in ticks detached from the three species of birds, *T. philomelos*, *P. major*, and *F. coelebs*, whereas *R. monacensis* and *R. japonica* were revealed in *F. coelebs* and *S. vulgaris*, respectively (Table 12).

Table 12. Prevalence of *Babesia* and SFG Rickettsiae ticks from Curonian Spit.

Bird species	Common name	No. of infested by ticks/no. of collected	Total no. of tested ticks	No. of positive ticks (%)	No. of ticks with pathogen (%)			
					Babesia sp. EU1	*Rickettsia* *helvetica*	*Rickettsia* *monacensis*	*Rickettsia* *japonica*
Turdus philomelos	Song thrush	46/59	78	10 (7.8)	2 (1.6)	8 (6.3)	0	0
Fringilla coelebs	Chaffinch	14/68	22	6 (4.8)	0	1 (0.8)	5 (3.9)	0
Parus major	Great Tit	11/17	11	4 (3.2)	0	4 (3.2)	0	0
Sturnus vulgaris	Starling	15/26	15	1 (0.8)	0	0	0	1 (0.8)
Total		86/170	126	21 (16.6)	2 (1.6)	13 (10.3)	5 (3.9)	1 (0.8)

4.6.2 Discussion

To the best of our knowledge, this is the first report describing SFG rickettsiae and *Babesia* sp. EU1 in ticks collected from the passerines in the North-Western part of Russia. It was previously shown that the tick *I. ricinus* represents a potential vector and natural reservoir of *R. helvetica* and *R. monacensis* in Russia (184); however, the SFG rickettsiae-infected ticks have never been found in birds captured in Russia. The *R. helvetica* had the highest prevalence among the above-listed pathogens and was found in 10.4% of infected ticks. The *R. helvetica*-infected ticks were found only in three of eight captured passerine species (*T. philomelos*, *F. coelebs*, and *P. major*). The *R. monacensis* was detected only in ticks collected from *F. coelebs*. Only one tick detached from starling was infected by R. japonica. The identification of *R. japonica* in bird-feeding *I. ricinus* ticks is perhaps the most significant finding. This member of SFG rickettsiae is commonly associated with the tick species *Dermacentor taiwanensis*, *Haemaphysalis flava*, and, perhaps, *Haemaphysalis longicornis* from parts of Asia and Japan (68). In North Europe, SFG rickettsiae were detected only in ticks collected from migratory birds in Sweden (55). *Babesia* sp. EU1 was found only in two ticks collected from the two exemplars of song thrush birds. *Babesia* spp. are piroplasmid protozoan parasites of human and animal red blood cells (28). In Europe, human cases of babesiosis have been reported over the past years and have been traditionally attributed to infections with the bovine parasite *Babesia divergens* transmitted by *I. ricinus* (28, 90). However, Herwaldt et al. (2003) reported the first molecular characterization of a new *Babesia* sp., *Babesia* sp. EU1, isolated from patients in Southern Europe (90). Until now, tick-transmitted *Babesia* sp. EU1 has only been detected in roe deer, sheep, goats, and humans (28, 90). Skotarczak et al.(2006) were the first who tried to reveal *Babesia* sp. in *I. ricinus* ticks collected from nine passerine bird species as well as from questing ticks (199). They additionally tested blood samples of 84 bird specimens, from which ticks were detached. Specific DNA was not detected in any samples of either ticks or birds' blood. The detection of *Babesia* sp. EU1 in tick species that is frequently found on humans and that have only fed on passerines suggests that some bird species may represent another reservoir with a potential risk for humans. Interestingly, only nymphs were detached from birds within a time of tick collection. This unusual situation was detected for the first time. Our previous studies indicated that larvae and nymphs are parasitizing together on birds in this region (4). From the

literature published to date, it seems that some birds such as *Parus caeruleus* and *Sitta europea* (199) or *F. coelebs* (85) can be the hosts only for *I. ricinus* nymphs, for at least within some periods of their migration. Dubska et al. (2009) reported about lower prevalence of larvae in song thrushes and dunnocks (50).

Our survey indicates that wild birds may play a significant role as a reservoir of babesiae and SFG rickettsiae and that ticks being infected by these pathogens may transmit them to humans. Future investigations are necessary to further characterize the role of birds in the epidemiology of these human pathogens.

5 Conclusion and Perspectives

Ticks are important vectors of human and animal pathogens throughout the world. We found that in Western and Eastern Europe, ticks were predominantly infected with members of the complex *Borrelia burgdorferi* sensu lato and of the Spotted Fever Group Rickettsiae. In Nigeria the predominant pathogen species were the cattle pathogen *Anaplasma marginale* and a *Rickettsia* species most closely related to the human pathogenic *Rickettsia africae*.

The current study shows that the prevalence of ticks and tick-borne pathogens is subjected to significant interannual, seasonal and regional variation, which seem to be tightly linked to abiotic (temperature, relative humidity) and biotic (vector and host density) parameters. These dynamics entail that likewise significant changes in the risk of tick bites and infection are to be expected. Habitat structure also influences the abundance of ticks and vertebrate hosts, consequently affecting also the prevalence of tick-borne pathogens, as we show for *Borrelia burgdorferi* sensu lato. In addition, we show that Lyme Borreliosis is a major health concern for risk groups who are highly exposed to ticks. Our findings suggest that also the general population is at risk of contracting Lyme Borreliosis as the prevalence of *Borrelia* species in ticks can be as high as 35.8% at certain sites and urbanized collection sites tend to have higher tick infection rates than natural sites. The occurrence of coinfections with two or more pathogens can complicate diagnosis after an infective tick bite and knowledge on the most frequent pathogen combinations may be of assistance. The surveillance of neglected or imported pathogens like *Anaplasma phagocytophilum*, *Bartonella henselae* and *Hepatozoon canis*, but also of those tick-borne pathogens that were not detected in questing ticks from Luxembourg (*Coxiella burnetii*, *Francisella tularensis* subspecies and Tick-Borne Encephalitis Virus) is of importance to monitor the potential impact of climate change on human health.

In Nigeria we found that the diversity of both, tick and pathogen species in feeding ticks was significantly higher than in ticks collected from the vegetation. Cattle seem to be an important reservoir host for many tick-borne pathogens and the infections are likely to be spread within animals of one herd. The impact of these infections on the Nigerian population as well as animal health and the resulting economic losses require further attention to assess e.g. the cost benefit of vaccination against the cattle pathogen *Anaplasma marginale* sensu stricto.

On the contrary we found that questing ticks in Belarus displayed a significant higher diversity of tick-borne pathogens than feeding ticks from cattle, suggesting that both dilution

and amplification processes determine the diversity and prevalence of pathogens in nature. The high overall tick infection rate in Belarus indicates a significant risk for humans and animals to contract a tick-borne disease. Especially the identified hotspots of ticks infected with *Francisella tularensis* and *Coxiella burnetii*, two highly human pathogenic agents, necessitate further surveillance.

The need for continuous surveys of tick-borne pathogens is also highlighted by the detection of potentially new species of the *Borrelia burgdorferi* sensu lato complex in Nigeria and the *Francisella*-like endosymbiont in ticks from Bulgaria. Also the detection of the exotic dog pathogen *H. canis* in Luxembourg further underlines this need. Exotic pathogens are often introduced to non-endemic areas by international tourism, importation of pet and domestic animals and also by natural dispersal of pathogens along flight routes of migratory birds, which are believed to play a major role as reservoirs for some *Borrelia* species. Our results indicate that they may also be reservoirs for other human pathogens like *Babesia* species and Spotted Fever Group Rickettsiae and therefore may be involved in their geographic distribution.

In order to enable establishment of exotic pathogens at new loci, various vector and reservoir host related criteria need to be met. In the course of climatic change permanent changes in abiotic prerequisites like temperature and relative humidity may enhance survival of non-endemic tick species. Timely detection of invasive tick species and application of countermeasures can possibly prevent the establishment of exotic zoonoses.

Tick surveillance is an important measure for the better understanding of the epidemiology of tick-borne pathogens and the population dynamics of the main vector ticks. However, guidelines for the selection of collection sites, collection methods, frequency of collection, tick species identification and molecular detection of pathogens are required, in which interannual, seasonal and regional variations as well as habitat characteristics are be taken into account.

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7 Annex

7.1 Fragebogen zur Ermittlung des Infektionsrisikos für Waldarbeiter nach einem Zeckenstich

Allgemeine Angaben

Geschlecht: ☐ männlich ☐ weiblich Geburtsjahr:_____
Wohnort (Kanton):_____ Arbeitsort + Kanton: _____
Beschäftigt seit:_____ als _____

1. Wurde seit Ausübung des Berufes das Revier oder der Wohnort gewechselt?

Jahr des Wechsels: _____
Vorheriger Arbeitsort (Kanton):_____
Vorheriger Wohnort (Kanton):_____

2. Durchschnittliche Aufenthaltsdauer im Freien von März bis Oktober (Zeckenaktivitätsperiode)

Dienstlich:_____ (Stunden pro Tag) Privat:_____ (Stunden pro Tag)

3. Wieviele Zecken sammeln Sie jährlich von sich ab? (inkl. derjenigen, die sich noch nicht festgebissen hatten)

☐ keine Zecke ☐ 1-5 Zecken ☐ 6-10 Zecken ☐ 11-20 Zecken ☐ 21-50 Zecken ☐ 51-100 Zecken
☐ >100 Zecken

4. Wieviele Zeckenstiche stellen Sie jährlich an sich fest?

☐ keine Zecke ☐ 1-5 Zecken ☐ 6-10 Zecken ☐ 11-20 Zecken ☐ 21-50 Zecken ☐ 51-100 Zecken
☐ >100 Zecken

5. Wie entfernen Sie die Zecke? (Mehrfachnennungen möglich)

☐ Entfernung durch einen Arzt

Wie entfernen Sie die Zecken, wenn sie keinen Arzt aufsuchen?
☐ von Hand ☐ mit Pinzette ☐ mit Zeckenzange ☐ mit Zeckenkarte ☐ mithilfe von Klebstoff oder Öl
☐ mit Drehbewegung beim Herausziehen ☐ ohne Drehbewegung beim Herausziehen

6. Ergreifen Sie regelmäßig Präventionsmassnahmen? (Mehrfachnennungen möglich)

☐ Zeckenabwehrmittel
☐ Körperinspektion nach Aufenthalt in Zeckengebieten
☐ Frühes Entfernen festgebissener Zecken
☐ Kleidung als Schutz (helle Farben, lange Hosen, Strümpfe über die Hosenbeine gestülpt)
☐ Andere (genaue Angaben)_____

7. Beobachten Sie die Einstichstelle nach Entfernung der Zecke?

☐ Ja, für ca. _____ Tage ☐ Nein

8. Sind Sie gegen Frühsommer-Meningoenzephalitis (FSME), Gelbfieber oder Japanische Enzephalitis geimpft?

☐ Ja, aufgrund einer Reise nach _____Impfung im Jahr_____
☐ Ja, aus anderen Gründen (genaue Angaben)_____Impfung im Jahr_____
☐ Nein

9. Wurde bei Ihnen bereits eine der folgenden Krankheiten diagnostiziert?

☐ Lyme Borreliose
☐ Frühsommer-Meningoenzephalitis
☐ Anaplasmose
☐ Rickettsiose
☐ Babesiose
☐ Q-Fieber
☐ Tularämie (Hasenpest)
☐ Katzenkratzkrankheit, Wolhynisches Fieber oder Fünftagefieber

10. Haben Sie oder ein Arzt folgende Symptome nach einem Zeckenstich festgestellt? Wenn ja, in wieviel Prozent der Zeckenstiche traten diese Symptome auf?

☐ Lokal beschränkte Rötung ____% der Zeckenstiche
☐ Lokale Entzündung ____% der Zeckenstiche
☐ Wanderröte (Erythema migrans) ____% der Zeckenstiche
☐ Kopfschmerzen ____% der Zeckenstiche
☐ Fieber ____% der Zeckenstiche
☐ Müdigkeit ____% der Zeckenstiche
☐ Gelenkschmerzen ____% der Zeckenstiche
☐ Hautveränderungen (ACA) ____% der Zeckenstiche
☐ Neuronale Schäden (halbseitige Gesichtslähmung, etc.) ____% der Zeckenstiche
☐ Andere Symptome (genaue Angaben) ____% der Zeckenstiche

11. Wurden Sie in der Vergangenheit nach einem Zeckenstich mit Antibiotika behandelt?

☐ Ja, nach einem Zeckenstich mit Symptomen
☐ Ja, nach einem Zeckenstich ohne Symptome (Postexpositionsprophylaxe)
☐ Nein, trotz Auftreten von Symptomen nach einem Zeckenstich
☐ Nein, es sind noch nie Symptome nach einem Zeckenstich aufgetreten

12. Falls Lyme Borreliose bei Ihnen diagnostiziert wurde und Sie daraufhin mit Antibiotika behandelt wurden, machen Sie bitte einige Angaben zur Therapie.

Aufgrund welcher Symptome wurde die Therapie begonnen? Bitte kreuzen Sie in der Tabelle die zutreffenden Symptome für den jeweiligen Zeckenstich (1.- 3.) an. Wenn bei Ihnen häufiger als 3 Mal Symptome für Lyme Borreliose diagnostiziert wurden, erweitern Sie die Tabelle bitte dementsprechend.

Symptome	**1.**	**2.**	**3.**
Lokal beschränkte Rötung			
Lokale Entzündung			
Wanderröte (Erythema migrans)			
Kopfschmerzen			
Fieber			
Müdigkeit			
Gelenkschmerzen			
Hautveränderungen (ACA)			
Neuronale Schäden			

13. Wie erfolgreich wurde die Lyme Borreliose behandelt?

☐ Vollständige Heilung (genaue Angaben zu Antibiotikum und Zeitraum)_____
_____

☐ Vollständige Heilung, jedoch erst nach Verlängerung der Antibiotikatherapie (genaue Angaben zu Antibiotikum und Zeitraum)_____
_____

☐ Vollständige Heilung, jedoch erst nach Wechsel des Antibiotikums (genaue Angaben zu den verabreichten Antibiotika)_____
_____

☐ Zunächst vollständige Heilung, später erneutes Auftreten von Symptomen (genaue Angaben zu Symptomatik und Zeitraum)_____
_____

☐ Keine vollständige Heilung, verbliebene Symptome sind (genaue Angaben zur Symptomatik)_____
_____

☐ Therapie schlug fehl, Symptome sind (genaue Angaben zur Symptomatik)_____
_____

Weitere Kommentare:_____
_____
_____
_____

14. Wenn Sie trotz Auftreten von einem oder mehreren Symptomen der Lyme Borreliose nicht mit Antibiotika behandelt wurden, machen Sie bitte folgende Angaben:

☐ Die Symptome klangen selbständig ab, es konnten keine neuen Symptome festgestellt werden (genaue Angaben zu Symptomatik und Zeitraum)_____
_____

☐ Die Symptome klangen zunächst selbständig ab, es wurden jedoch neue Symptome festgestellt (genaue Angaben zu Symptomatik und Zeitraum)_____
_____

☐ Die Symptome verstärkten sich und/oder es traten neue Symptome auf (genaue Angaben zu Symptomatik und Zeitraum)_____
_____

Wurde in diesem Fall eine Antibiotikatherapie begonnen? ☐ Ja ☐ Nein

7.2 Questionnaire pour la détermination du risque d'infections après une piqure de tiques pour les forestiers

Données générales

Sexe: ☐ masculin ☐ féminin Année de naissance:_____
Domicile (canton):_____ Lieu de travail + canton: _____
Occupé(e) depuis: _____ en tant que : _____

1. Le lieu de travail (district) ou le domicile ont-ils changé depuis le début de votre travail?

Année du changement: _____
Lieu de travail précédent (canton):_____
Domicile précédent (canton):_____

2. Durée moyenne de séjour à l'air libre pendant la période de mars – octobre (période actives des tiques)

Raisons professionnelles:_____ (heures par jour) Privé(e):_____ (heures par jour)

3. Combien de tiques avez-vous détecté par an sur votre corps ? (y compris les non fixés)

☐ Aucune tique ☐ 1-5 tiques ☐ 6-10 tiques ☐ 11-20 tiques ☐ 21-50 tiques ☐ 51-100 tiques
☐ >100 tiques

4. En moyenne, combien de piqures de tiques avez-vous eu par an?

☐ Aucune piqure ☐ 1-5 piqures ☐ 6-10 piqures ☐ 11-20 piqures ☐ 21-50 piqures ☐ 51-100 piqures
☐ >100 piqures

5. Par quelle méthode enlevez-vous la/les tiques(s) ? (plusieurs réponses sont possibles)

☐ enlèvement fait par un médecin
Si vous ne consultez pas un médecin, comment enlevez vous la/les tique(s) ?
☐ A la main ☐ A l'aide d'une pincette ☐ A l'aide d'une pince à tiques ☐ A l'aide d'une carte à tiques
☐ A l'aide d'huile ou de colle
☐ Avec mouvement rotatif lors de l'enlèvement ☐ Sans mouvement rotatif lors de l'enlèvement

6. Est-ce que vous adoptez régulièrement des mesures préventives? (plusieurs réponses sont possibles)

☐ Répulsifs de tiques
☐ Inspection corporelle après un séjour en pleine air
☐ Enlèvement précoce des tiques fixées
☐ Habits comme protection (couleurs claires, pantalons longs, retourner les bas sur l'inférieur du pantalon)
☐ Autres (indications précises)_____

7. Observez-vous l'endroit de piqure fait par la tique?

☐ Oui, pour environ _____ jours ☐ Non

8. Etes-vous vacciné(e)s contre la méningo-encéphalite à tiques (MET), la fièvre jaune ou contre l'encéphalite japonaise ?

☐ Oui, à cause d'un voyage en/au _____
Vaccination en (année) _____
☐ Oui, pour des raisons différentes (indications précises) _____
Vaccination en (année) _____

☐ Non

9. Est-ce qu'une des maladies suivantes a été dépistée chez vous?

☐ Borréliose de Lyme
☐ Méningo-encéphalite à tiques (MET)
☐ Anaplasmose
☐ Rickettsiose
☐ Babesiose
☐ Q-Fieber
☐ Tularämie (Hasenpest)
☐ Katzenkratzkrankheit, Wolhynisches Fieber oder Fünftagefieber

10. Après une piqure de tiques, les symptômes suivants ont-ils été détectés par un médecin ou vous même? Si oui, pour combien de cas (pourcentage) avez-vous eu un ou plusieurs de ces symptômes ?

☐ Rougeur locale et limitée ____% des piqures de tiques
☐ Inflammations locales ____% des piqures de tiques
☐ Érythème chronique migrant (Erythema migrans) ____% des piqures de tiques
☐ Maux de tête ____% des piqures de tiques
☐ Fièvre ____% des piqures de tiques
☐ Fatigue ____% des piqures de tiques
☐ Arthralgie ____% des piqures de tiques
☐ Manifestations cutanées (ACA) ____% des piqures de tiques
☐ Manifestations neurologiques (hémiplégie faciale, etc.) ____% des piqures de tiques
☐ Autres symptômes (indications précises) ____% des piqures de tiques

11. Dans la passé, avez-vous subit un traitement aux antibiotiques suite à une piqure de tiques?

☐ Oui, après une piqure de tiques accompagnée de symptômes
☐ Oui, après une piqure de tiques non accompagnée de symptômes (Postexpositionsprophylaxe)
☐ Non, malgré l'apparition de symptômes après une piqure de tiques
☐ Non, je n'ai encore jamais observé de symptômes après une piqure de tiques

12. Si la borréliose de Lyme a été diagnostiquée chez vous et si vous étiez traité à l'aide d'antibiotiques, précisez s'il vous plait la thérapie.

En vertu de quelles symptômes, la thérapie a-t-elle était lancée? Cochez les symptômes correspondants aux différentes piqures de tiques (1. – 3.). Si des symptômes étaient apparentes dans plus que 3 piqures de tiques, agrandissez le tableau ci-contre.

Symptômes	**1.**	**2.**	**3.**
Rougeur locale et limitée			
Inflammations locales			
Érythème chronique migrant (Erythema migrans)			
Maux de tête			
Fièvre			
Fatigue			
Arthralgie			
Manifestations cutanées (ACA)			
Manifestations neurologiques (hémiplégie faciale, etc.)			

13. Avec quel succès a-t-on traité la borréliose de Lyme?

☐ Guérison complète (indications précises sur l'antibiotique et la durée du traitement) _____
_____

☐ Guérison complète, mais seulement après une prise prolongée des antibiotiques (indications précises sur l'antibiotique et la durée du traitement) _____
_____

☐ Guérison complète, mais seulement après la prise d'un autre antibiotique (indications précises sur l'antibiotique et la durée du traitement) _____
_____

☐ Dans un premier temps, guérison complète, mais nouvelles survenances de symptômes ultérieurement (indications précises sur les symptômes et l'espace de temps)_____
_____

☐ Pas de guérison complète, les symptômes restants sont (indications précises sur les symptômes) _____
_____

☐ La thérapie a échouée, les symptômes sont (indications précises sur les symptômes) _____
_____

Commentaires supplémentaires: _____
_____

14. Si vous n'aviez pas subit de traitement antibiotique, malgré la manifestation d'un ou plusieurs symptômes de la borréliose de Lyme, veuillez répondre aux questions suivantes:

☐ Les symptômes ont décru indépendamment, de nouveaux symptômes n'ont pas pu être dépistés (indications précises sur les symptômes et l'espace de temps) _____
_____

☐ Les symptômes ont décru indépendamment, de nouveaux symptômes ont pu être dépistés (indications précises sur les symptômes et l'espace de temps) _____
_____

☐ Les symptômes se sont aggravées et étaient accompagnés ou non de nouveaux symptômes (indications précises sur les symptômes et l'espace de temps) _____
_____

Dans ce cas-ci, une thérapie sous antibiotiques a-t-elle été lancée? ☐ Oui ☐ Non

7.3 Conference Participations

2010 Reye AL, et al: Seroprevalence of Borrelia burgdorferi s.l. in forestry workers in Luxembourg; 11th Saar-Lor-Lux-Meeting on Virus Research. Homburg, Germany.

Reye AL, et al: Tick-Borne Pathogens in Luxembourg; Meeting of Clinical Pediatricians, Luxembourg

2008 Reye AL, et al: Prevalence of Borrelia burgdorferi s.l. and other tick-borne pathogens in Luxembourg; 11th International Conference on Lyme Borreliosis and Other Tick-borne Diseases, Irvine, California, USA.

Reye AL, et al: Lyme Borreliosis and other Tick-borne diseases in Luxembourg; 28ième Journée Nationale de la Biologie Clinique, Luxembourg.

Reye AL, et al: Prevalence of Borrelia burgdorferi s.l. and other tick-borne pathogens in Luxembourg; 12th Saar-Lor-Lux-Meeting on Virus Research. Remich, Luxembourg.

2007 Reye AL, et al: Prevalence of Borrelia burgdorferi s.l. and other tick-borne pathogens in Luxembourg; 11th Saar-Lor-Lux-Meeting on Virus Research. Homburg, Germany.

7.4 Publications

2011 Reye AL, Arinola OG, Minden S, Hübschen JM, Muller CP
'Pathogen prevalence in ticks collected from the vegetation and livestock in Nigeria'
Submitted

2011 Reye AL, Stegniy V, Velhin S, Wolter S, Hübschen JM, Ignatyev G, Muller CP
'Prevalence of Tick-Borne Pathogens in Ixodes ricinus and Dermacentor reticulates Ticks from Different Environmental Sources in Belarus'
Submitted

2011 Ivanov IN, Mitkova N, Reye AL, Hübschen JM, Vatcheva-Dobrevska RS, Dobreva EG, Kantardjiev TV, Muller CP
'*Detection of new Francisella- like tick endosymbionts in Hyalomma spp. and Rhipicephalus spp. (Acari: Ixodidae) from Bulgaria'*
Applied and Environmental Microbiology, 2011 Jun 24. [Epub ahead of print]

2011 Movila A, Reye AL, Dubinina HV, Tolstenkov OO, Toderas I, Hübschen JM, Muller CP, Alekseev AN
'Detection of Babesia Sp. EU1 and Members of Spotted Fever Group Rickettsiae in Ticks Collected from Migratory Birds at Curonian Spit, North-Western Russia'
Vector-Borne and Zoonotic Diseases, 2011 Jan;11(1):89-91

2010 Reye AL, Hübschen JM, Sausy A, Muller CP
'Prevalence and seasonality of tick-borne pathogens in questing Ixodes ricinus ticks from Luxembourg'
Applied and Environmental Microbiology, 2010 May;76(9):2923-31.

2007 Köhler HR, Kloas W, Schirling M, Lutz I, Reye AL, Langen JS, Triebskorn R, Nagel R, Schönfelder G
'Sex steroid receptor evolution and signalling in aquatic invertebrates'
Ecotoxicology. 2007 Feb;16(1): 131-43

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