School of Doctoral Studies in Biological Sciences

University of South Bohemia in České Budějovice

Faculty of Science

Spatial Distribution of Tick-Borne Pathogens as a Consequence of Vector-Host-Pathogen Interactions with Environment

Ph.D. thesis

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České Budějovice 2015

This thesis should be cited as:

Hönig, V., 2015: Spatial Distribution of Tick-Borne Pathogens as a Consequence of Vector-Host-Pathogen Interactions with Environment. Ph. D. Thesis Series, No. 6. University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 192 pp.

Annotation:

The proposed thesis contributes to the basic knowledge in tick (*Ixodes ricinus*) and tick-borne pathogens (*Borrelia burgdorferi* sensu lato, tick-borne encephalitis virus) ecology in particular studying the spatial distribution, host associations and its causes and consequences in Central European habitats.

Declaration [in Czech]:

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Mgr. Václav Hönig

This thesis originated from a partnership of Faculty of Science, University of South Bohemia, and Institute of Parasitology, Biology Centre of the ASCR, supporting doctoral studies in the study programme Parasitology.

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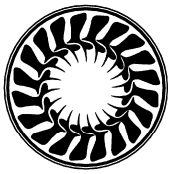

Parazitologický ústav,
Biologické centrum AV ČR

Institute of Parasitology
Biology Centre, AS CR

Financial support:

The research presented in this Ph. D. thesis was supported by following financial sources:

project "Ticks and tick borne infectious diseases in the conditions of South Bohemia and Bavaria", (financed by the European Development Fund of the European Union by the cross-border cooperation program: Objective 3 – Czech Republic – Free State of Bavaria, co-financed by the Region of South Bohemia; University of South Bohemia, Faculty of Science; P.I.: Libor Grubhoffer)

project 278976 "ANTIGONE" (the European Union FP7 HEALTH, Biology Centre AS CR, Institute of Parasitology; international consortium, co-ordinator: Thijs Kuiken, P.I. at BC ASCR: Libor Grubhoffer)

research project MSM 6007665801 (Ministry of Education, Youth, and Sports of the Czech Republic; University of South Bohemia, Faculty of Science; P.I.: Hana Šantrůčková)

research project Z60220518 (Ministry of Education, Youth, and Sports of the Czech Republic; Biology Centre AS CR, Institute of Parasitology; P.I.: Tomáš Scholz)

project AKTION 53p19 "Spatial mapping of ticks and tick-borne infectious diseases of the region of South Bohemia and Upper Austria" (Ministry of Education, Youth, and Sports of the Czech Republic; University of South Bohemia, Faculty of Science; P.I.: Libor Grubhoffer)

research centre LC06009 (Ministry of Education, Youth, and Sports of the Czech Republic; Biology Centre AS CR; University of South Bohemia, Faculty of Science; P.I.: Jan Kopecký, Libor Grubhoffer)

grant SP/2010184 (VSB-Technical university, Ostrava; P.I.: Pavel Švec)

grant GA206/09/H026 (Grant Agency of the Czech Republic; University of South Bohemia, Faculty of Science; P.I.: Václav Hypša)

grant GA310/06/1546 (Grant Agency of the Czech Republic; Správa Krkonošského národního parku Vrchlabí; P.I.: Jan Materna)

grant 2R44AI077156 (NIAID, NIH, USA; BIOSCIENCE DEVELOPMENT, INC; P.I.: Steven Schutzer)

grant CZB1-2963-CB-0 (NIAID, NIH, USA; P.I.: Maryna Golovchenko)

co-authors of the papers were supported by:
project “Postdok_BIOGLOBE” (CZ.1.07/2.3.00/30.0032) (co-financed by the European Social Fund and the state budget of the Czech Republic; Ján Štěrba)

Feodor Lynen Research Fellowship: (Alexander von Humboldt Foundation; Alexandra Schwarz)

AKTION scholarship (Ministry of Education, Youth, and Sports of the Czech Republic; Václav Hönig)

Acknowledgement:

I would like to thank Libor Grubhoffer for inviting me back to work with his research group and giving me the opportunity to work in a welcoming, inspiring, friendly group of people. Libor offered me a great chance to continue with the research in tick-borne ecology with a high level of independence on one hand but equally high level of support on the other. It was a big challenge but also a big experience. I would like to mention and thank also other personalities, who have inspired and influenced my professional life like: Nataliia Rudenko, Marina Golovchenko, Ryan Rego, Dr. Vlasta Danielová, Dr. Milan Daniel, Prof. Hubálek and the whole research group in Valtice, Dr. Lise Gern and Caroline Burri (thank you for the great experience in Neuchatel), Milan Stehlik (thank you for the great experience in Linz).

I would like to thank all the co-authors of the papers, for their contribution to our common work. Also, I would like to mention the closest collaborators and friends from the lab and the Institute of Parasitology: Janko Štěrba, Hanka Tykalová, Patrik Kilian, Zuzka Vavrušková, Vendy Vetišková, Martin Palus, Veronika Dorňáková, Gábi Loosová and from VSB-Technical University Ostrava: Pavel Švec, Dana Szturcová. Thank you for your help, support, inspiring discussions and personal friendship. It was a great time spent with you. I would also like to thank Honza Erhart for introduction to and help with tick sampling, help with tick feeding in other projects and for permission to use some of his tick photographs.

Last but not least I would like to thank my parents for supporting me in my endless studies and Helča for her support, understanding and acceptance of the weekends, evenings and nights spent by writing and not being always in a particularly good mood.

List of papers and author's contributions
The Ph. D. thesis is based on following papers and manuscripts**:**

I. Daniel, M., Materna, J., Honig, V., Metelka, L., Danielova, V., Harcarik, J., Kliegrova, S., Grubhoffer, L., 2009. Vertical distribution of the tick *Ixodes ricinus* and tick-borne pathogens in the northern Moravian mountains correlated with climate warming (Jeseniky Mts., Czech Republic). Cent. Eur. J. Public Health 17(3):139–145. (IF: 0.533, assigned 2014)

Václav Hönig participated on the design of the methods of pathogen detection, carried out the pathogen detection and identification in tick samples and processing of the primary data. Also, he participated on the drafting of the manuscript (methods of pathogen detection) and corrections.

II. Schwarz, A., Honig, V., Vavruskova, Z., Grubhoffer, L., Balczun, C., Albring, A., Schaub, G.A., 2012. Abundance of *Ixodes ricinus* and prevalence of *Borrelia burgdorferi* s.l. in the nature reserve Siebengebirge, Germany, in comparison to three former studies from 1978 onwards. Parasit. Vectors 5:268. doi:10.1186/1756-3305-5-268. (IF: 3.43)

Václav Hönig has contributed in following ways: design of the methods for tick sample testing (samples from period 2007 – 08), detection and identification of B. burgdorferi s. l. genospecies (samples from period 2007 – 08), comments and corrections of the manuscript, participation in the discussions with reviewers.

III. Crowder, C.D., Carolan, H.E., Rounds, M.A., Honig, V., Mothes, B., Haag, H., Nolte, O., Luft, B.J., Grubhoffer, L., Ecker, D.J., Schutzer, S.E., Eshoo, M.W., 2014. Prevalence of *Borrelia miyamotoi* in *Ixodes* Ticks in Europe and the United States. Emerg. Infect. Dis. 20(10):1678-1682. doi:10.3201/eid2010.131583 (IF: 6.75)

Vaclav Hönig has carried out collection of ticks from the Czech Republic and also performed the processing of these ticks for subsequent PCR-ESI/MS analysis.

IV. Honig, V., Svec, P., Halas, P., Vavruskova, Z., Tykalova, H., Kilian, P., Vetiskova, V., Dornakova, V., Sterbova, J., Simonova, Z., Erhart, J., Sterba, J., Golovchenko, M., Rudenko, N., Grubhoffer, L., 2015. Ticks and tick-borne pathogens in South Bohemia (Czech Republic) - Spatial variability in *Ixodes ricinus* abundance, *Borrelia burgdorferi* and tick-borne encephalitis virus prevalence. Ticks Tick-Borne Dis. 6(5):559-567. doi:10.1016/j.ttbdis.2015.04.010 (IF: 2.72)

Contributions IV and V are from a major part results of the project "Tick and Tick-borne Diseases in the Conditions of South Bohemia and Bavaria". Václav Hönig was the person responsible for the technical realization of the project including design and structure of the whole project, methods of sampling, pathogen detection and data processing, he also participated in the co-ordination of the international and interdisciplinary team (tick-borne pathogen biology – geographic information system - statistics - project management).

Concerning particularly paper nr. IV, Václav Hönig contributed to the field sampling, carried out a part of the laboratory analysis, performed most statistical analysis, drafted the manuscript of the paper, performed revisions and discussions with reviewers.

V. Honig, V., Svec, P., Mrkvicka, T., Zubrikova, D., Vögerl-Wittmann, M., Masar, O., Szturcova, D., Pfister, K., Grubhoffer, L., Estimation of acarological risk of exposure to Lyme borreliosis or tick-borne encephalitis infected ticks in the border area of the Czech Republic (South Bohemia) and Germany (Lower Bavaria and Upper Palatinate) and its presentation in a form of map portal.
Submitted.

Concerning particularly paper nr. V, Václav Hönig contributed to the field sampling, carried out a part of the laboratory analysis, performed a part of statistical analysis, drafted the manuscript.

VI. Honig, V., Vavruskova, Z., Kilian, P, Grubhoffer L. *Ixodes ricinus* tick host species spectrum and its association with genospecies of *Borrelia burgdorferi* sensu lato complex.
Unpublished results

Vaclav Hönig has designed the study, contributed to tick sampling, detection of pathogens and host identification. He contributed to the processing and statistical analysis of the results and drafted the manuscript.

Table of Contents

1 Preface

Different species of organisms enter various types of relationships. Based on the level of profit or harm the two species get, the interactions are described by the ecological terms mutualism, commensalism, parasitism or predation. In nature these relationships are in a very complex manner weaved into a stable network. From this perspective the existence of human vector-borne diseases may be seen not only as a significant health threat (for *Homo sapiens* species), but also as an example of a very sophisticated and well balanced natural system of species co-existence.

Even in the very basic concept including only pathogen-vector-host system, we may observe a wide range of interspecies relationships. Tick-borne encephalitis virus, in some cases lethal for human, circulates mainly among rodents, where it produces significant viremia with no serious impact on their health, and ticks, where the virus amplifies in the cells without any apparent harm. Transmission of *Borrelia burgdorferi* by a competent tick requires a relatively complex interaction of specific bacterial surface proteins and tick receptors. Some tick proteins are known to support borrelia infection in a vertebrate host. In return borrelia infected ticks seem to be more resistant to unfavorable conditions than infected ticks. In the real natural conditions, the pathogen, vector and host species enter numerous interactions not only with other species but also with abiotic factors of the environment.

In the case of the two pathogens in the main focus of this thesis – Lyme borreliosis spirochetes *Borrelia burgdorferi* and tick-borne encephalitis virus, all the three components of the natural cycle (i.e. pathogen, host and vector) display a huge intrinsic variability and also variability in interactions they may produce with each other, other species in their natural habitats and environmental factors. It is a bit of challenge to try to explain the mechanisms of these complex networks. Such information, however will bring us not only new approaches in protection from vector-borne diseases, but also a closer insight in the role a particular species plays in an ecosystem.

2 General introduction, literature review

In Europe the diseases transmitted by ticks are considered the most important vector-borne diseases. Concerning impact on human health the two most wide-spread are Lyme borreliosis (LB) and tick-borne encephalitis (TBE) (Charrel et al., 2004; Parola and Raoult, 2001). LB is caused by bacteria of *Borrelia burgdorferi* sensu lato complex and TBE by tick-borne encephalitis virus (TBEV).

Both the diseases are vector-borne zoonoses with natural nidality. Vector-borne diseases are transmitted from one vertebrate host to another by other organisms (vectors) (Braks et al., 2011). In the case of the agents of LB and TBE, different species of "hard" ticks of the family *Ixodidae* play the role of a vector.

Zoonotic diseases (zoonoses) are transmitted between vertebrate animals and humans (WHO, 1967) (but rarely from human to human) (Hubalek, 2003). Furthermore, the causative agents of both diseases circulate among their vectors and hosts in defined geographical areas (natural focus) and humans are infected while entering this area, hence both diseases display natural nidality (focality) (Rosicky and Weiser, 1952).

To add some more terminology, both diseases are used to be referred to as emergent, emerging or re-emerging, which means new, newly described, or (re-)occurring in a new context (Institute of Medicine, 1992). Although still being emergent, LB and TBE have already earned great amount of interest from the professional (research and medical) and general public. Despite all the efforts, blank spaces still remain even in the basic ecological principles ruling the survival, spread and extinction of the tick-borne pathogens in their natural foci of occurrence.

Furthermore, with increasing the amount of information the initially seemingly simple systems start to be more complex: *Borrelia burgdorferi* has fallen into many genospecies possessing variable ecologic and pathogenic features; new relapsing-fever borrelia species transmitted by Ixodid ticks are discovered; spatial distribution of TBE cases is shifting rapidly with no simple single explanation of cause; number of human TBE cases is not well corresponding with the prevalence of the virus in ticks; human attached ticks show higher TBE virus prevalence than field collected ticks from the same area; *Ixodes ricinus* previously thought to be a genetically homogenous throughout the area of occurrence displays host associated genetic

structure etc.). There are many exciting questions still remaining unanswered. Despite the two pathogens in the focus of the thesis share common vector species and, to some extent, also the vertebrate hosts, at the same time they display striking differences in the strategy of survival in their natural cycles (which than influences also the epidemiology of the diseases they cause). Therefore, these two pathogens provide a suitable bases for comparison. The aim of this thesis is to add some new puzzle pieces into the complex picture of pathogen – tick – host – environment interactions ruling the ecological background of the two most important vector-borne diseases in Europe.

2.1 Specific terms and mechanisms associated with vector-borne diseases

Vector-borne (tick-borne) diseases associated terms and their meanings as used in this thesis are presented in Table 1. Some of the definitions were adopted from or inspired by (Bush et al., 1997; Haydon et al., 2002; Margolis et al., 1982; Oliver et al., 2003).

Table 1: Vector-borne disease associated terms and their meaning in the sense they are used in this PhD. thesis

competent vector	a (blood-feeding) arthropod species able to transmit a pathogen (the pathogen is ingested by the vector, it multiplies or develops in the vector (biological transmission, it is able to infect another competent host during vectors feeding)
maintenance vector	a type of competent vector which maintains the pathogen in the natural circulation by transmitting it among competent natural hosts; it rarely participates in transmission to human
bridge vector	a type of competent vector, which feeds on natural hosts of a pathogen and among others also attacks human; it serves as a „bridge" for the pathogen allowing it to cross from natural cycles to human
biological transmission	type of vector mediated transmission when the pathogen multiplies or undergoes development in the vector environment
mechanical transmission	type of vector mediated transmission when the pathogen does not multiply or undergo development in the vector and is transferred to a new host

host	vertebrate species serving as a blood-meal source for ticks (or other blood-feeding parasites) vertebrate species in which and from which the pathogen may be transmitted to vector or another vertebrate individual directly
competent host	type of host supporting transmission of the pathogen to vector or directly to another vertebrate host individual
reservoir host	crucial competent host species with a special significance for the maintenance of the pathogen; maintains the pathogen even in the periods of low activity of the natural circulation
accidental host	a host with low importance for the general natural circulation of the pathogen (e.g. due to low infestation by the competent vector)
prevalence	portion of positive samples (infested hosts, ticks carrying pathogen(s), hosts carrying a pathogen(s)) out of total samples examined
incidence	number of new disease cases in a particular area and time period divided by the number of uninfected hosts in the beginning of the time period
density	number of individuals of a particular species per unit of area
activity of host-seeking ticks or density of host-seeking ticks	number of questing ticks (ticks sampled by flagging) per unit of area or alternatively per time unit of sampling
relative abundance	mean number of individuals of a particular parasite species per examined individuals of a host species
mean intensity of infestation	mean number of individuals of a particular parasite species on infested individuals of a particular host species
co-feeding	tight aggregation of ticks while feeding on a vertebrate host
co-feeding transmission	pathogen transmission from an infected tick to another within a co-feeding formation – systemic infection of the host (viremia, bacteremia, parasitemia) is not required
aggregation level	rate of parasite clustering in time and space

2.2 Historical remarks

2.2.1 Revelation of Lyme borreliosis

Cutaneous symptoms of the disease, later named Lyme borreliosis, were recorded in Europe already in the 18th century (Buchwald, 1883) and the association with ticks was proposed (Marcus, 1910). In the 70's in Connecticut, USA, unusual incidence of juvenile rheumatoid arthritis was reported. Later it was realized that arthritis is only one of the signs of a multi-systemic, probably tick-associated, disease (Steere et al., 1977). The causative agent of Lyme borreliosis was identified in 1981, as the bacterium later named *Borrelia burgdorferi* (Johnson et al., 1984), was isolated from a specimen of an *I. scapularis* tick (Barbour, 1984; Burgdorfer et al., 1982). Later as more *B. burgdorferi* isolates were available from various sources and geographical origins including Europe, remarkable variability was revealed in their serological, pathogenic and ecological properties. Based on nucleotide sequence analysis individual genospecies of *B. burgdorferi* were defined. Nowadays, there are 20 genospecies recognized within the *B. burgdorferi* sensu lato complex.

2.2.2 Tick-borne encephalitis in Asia and Europe

In 1933 – 1939 several expeditions were organized to elucidate the occurrence of severe infections of central nervous system in several areas of far eastern USSR (The Union of Soviet Socialist Republics). The viral character of the disease was proved, the vector (*Ixodes persulcatus* tick) and the natural hosts were identified. The disease was referred to as Russian spring-summer encephalitis (RSSE). Later other areas with clinically similar cases were identified including the western part of the USSR (Blaskovic, 1967).

In Central Europe the disease was probably first noticed in Neunkirchen (Austria) by Dr. Schneider in 1931. He described a seasonally occurring aseptic meningitis (referred to as “epidemische akute Meningitis serosa”) (Dumpis et al., 1999). In the Czech Republic (and in Europe apart from the USSR) the virus was for the first time isolated in 1948 by Dr. Gallia from a patient hospitalized in Beroun (south of Prague) (Gallia et al., 1949) and in the same year by Dr. Krejčí from a patient hospitalized in Vyškov (proximity of Brno) (Krejci, 1949). The virus strain reacted with antibodies against RSSE virus and *Louping ill virus*. Finally, in 1949 the virus was isolated from *I. ricinus* ticks collected in areas where previous human encephalitis cases were reported (Rampas and Gallia, 1949) hence the presumable west-European vector

was identified. Thereafter, clinical cases of TBE were reported in many other European countries (reviewed by Blaskovic, 1967).

2.3 Ticks – potential disease vectors

2.3.1 Classification

Ticks are geographically widespread hematophagous acarine ectoparasites of a wide range of vertebrates. Taxonomically they belong to three families: *Argasidae* (186 species), *Nuttalliellidae* (1 genus, 1 species) and *Ixodidae* (692 species) (Nava et al., 2009).

Concerning the transmission of LB and TBE the genus *Ixodes* has the utmost importance, more specifically the species of *Ixodes persulcatus* complex. The genus *Ixodes* belongs to a group of prostriate ticks in the family *Ixodidae*. Several species of the genus are able to transmit the pathogens causing LB (e.g. *I. ricinus*, *I. persulcatus*, *I. scapularis, I. pacificus*) and TBE (*I. ricinus*, *I. persulcatus*) to human, others may be involved in the natural cycles of the pathogen circulation but do not feed on humans. Some species may harbor the pathogens only accidentally by feeding on a bacteremic or viremic host (Gray, 1998; Suss, 2003).

In Europe *I. ricinus* is the most important vector of human diseases. Based on phylogeographic data, it was assumed that the European population of this tick species is (despite of its low mobility and huge area of occurrence) genetically mostly homogeneous (Casati et al., 2008; Noureddine et al., 2011). Later, a host associated population structure was described (Kempf et al., 2011) and a phylogeographic genetic structure was revealed by use of mitochondrial genes multi-sequence analysis at least in the case of geographically distant populations (Dinnis et al., 2014).

2.3.2 Ixodes ricinus

I. ricinus (Fig. 1) is an exophilic tick with a three-host developmental cycle. The development takes 2-6 years depending on environmental conditions (in central Europe mostly about 3 years) (Cerny, 1972). The life cycle consists of three developmental stages (larval, nymphal and adult). *I. ricinus* ticks (except of adult males) have to obtain a single blood meal per stage to molt or to oviposit

successfully. The feeding takes 2-4 days for larvae, 3-5 days for nymphs and 6-10 days for adults (Suss, 2003). The developmental rate, host seeking period and diapause are strongly influenced by climatic factors. Therefore, seasonal climatic changes have major effects on the composition and activity of tick populations (Gray et al., 2009; Randolph et al., 2002).

Fig. 1 Female, male and nymphal *Ixodes ricinus* tick

2.3.2.1 Geographic distribution

Geographic distribution of *I. ricinus* spreads from Ireland (Gray et al., 1999) and the UK (Pietzsch et al., 2005), throughout Europe to the Baltic's and Russia, where it overlaps with *I. persulcatus*. The southeastern projection of *I. ricinus* distribution area reaches as far east as the Aral sea (Blaskovic, 1967; Yasyukevich et al., 2009). The northern border stretches through Scandinavia up to 66° N to the northern coastline of the Baltic Sea in Sweden (Jaenson et al., 2012; Lindgren and Jaenson, 2006). Moreover, populations of *I. ricinus* are found in the Mediterranean region although mostly restricted to areas with sufficient humidity of sub-Mediterranean bioclimatic zone (Colebrook and Wall, 2004; Vatansever et al., 2008). *I. ricinus* ticks are recorded even in the northwestern parts of Africa including Tunisia (Younsi et al., 2001), Morocco (Sarih et al., 2003) or Algeria (Dib et al., 2009). *I. ricinus* as a species with limited mobility is transported mainly by its hosts. Obviously, the flying bird species may transport the ticks (containing tick-borne pathogens) for large distances (Franke et al., 2010; Smith et al., 2006; Vollmer et al., 2011; Waldenstrom et al., 2007).

For a long time, 700-800 m a.s.l. was considered the altitudinal limit of *I. ricinus* in Central Europe. This limit was confirmed in field experiments as the ticks were not able to complete the developmental cycle in higher altitudes (Daniel et al., 2003). Since the 90´s, higher numbers of ticks were recorded in areas above 700 m a.s.l. (Burri et al., 2007; Daniel et al., 2003; Stunzner et al., 2006) and subsequent observations have proven stable local tick population as high as 1100 m a.s.l., although the tick density and development success decreases and the developmental rate slows down with rising altitude (Daniel et al., 2003; Materna et al., 2008). The maximum altitude where *I. ricinus* was found in Central Europe is 1350 m a.s.l. in Styria (Austria) (Stunzner et al., 2006). Obviously, the factor that restricts the distribution of the ticks is not directly altitude, but local microclimatic conditions that are closely correlated with altitude. According to long-term climatic data, the expansion of ticks can be explained by increasing average annual temperature associated with global climatic changes (Daniel et al., 2009; Materna et al., 2008).

2.3.2.2 Microclimatic conditions and seasonality

I. ricinus is an exophilic tick. In contrast to nidicolous ticks, which spend their life in close association with the host (and their nests and burrows), exophilic ticks seek for their hosts in open landscape, where they are exposed to environmental conditions, including climate. The crucial climatic factors influencing tick activity are temperature and relative air humidity, although significant influence of soil and air temperatures or solar radiation was also reported (Hubalek et al., 2003; Jensen, 2000; Perret et al., 2000; Vassalo et al., 2000).

In the Central Europe, *I. ricinus* ticks are generally active approximately from April to November (Nosek et al., 1967b). However, the questing activity of *I. ricinus* nymphs begins at temperatures as low as 2.5 – 4.4 °C (Hubalek et al., 2003; Kiewra and Sobczyñski, 2006; Walker, 2001), which may in some seasons result to occasional or continual winter activity even in the temperate climate zone (Dautel et al., 2008). The limits of relative humidity suitable for *I. ricinus* questing activity are approximately 24.5 – 95% (Kiewra and Sobczynski, 2006). In conditions close to the lower limit, the ticks may spend only a very limited time period and rehydration at the base of vegetation must follow (Gray, 1998). The limit of relative humidity in the microhabitats close to soil is approximately 85 % (Daniel et al., 1998).

Concerning the seasonal dynamics, two different patterns of tick activity are generally recognized in Europe: either a bimodal pattern with one peak of activity in spring and another in autumn (Kubiak and Dziekonska-Rynko, 2006; Talleklint and Jaenson, 1996) or a unimodal pattern with only one maximum in spring (Craine et al., 1995; Ferquel et al., 2006; Hubalek et al., 1994; Talleklint and Jaenson, 1996). The seasonal patterns are obviously influenced by local climatic and microclimatic conditions and possibly also by host availability. According to consecutive sampling experiments, these fluctuations are, at least to a certain level, due to changes in the tick activity itself than in fluctuations in the population size. The precise mechanism is lies in the variable length of retention period (time which a tick spends under vegetation between active questing periods) driven probably by changes in microclimatic conditions (Jensen, 2000). Ticks are forced to interrupt the host questing and spend some time in the lower levels of vegetation to rehydrate. On the other hand the increase in tick activity in autumn is, at least in a part, driven by newly emerged nymphs (Perret et al., 2000). Importantly, from the epidemiological point of view, the fluctuations in tick activity correspond with fluctuations in incidence of tick-borne diseases (Hubalek et al., 1991; Hubalek et al., 2003b).

The seasonal population dynamics and occurrence of questing ticks is markedly influenced by timing and duration of diapauses. Questing ticks enter behavioral diapause preventing host-seeking activity in extremely unfavorable conditions. Developmental diapause (arrested development) is entered by engorged ticks and enables them to survive winter and start a quick development in the next summer (Gray, 1998). Diapause timing may influence the *I. ricinus* populations structure and overall tick activity (level of synchronous emergence of different stages of ticks, co-occurrence of different cohorts) and also may give rise to annual fluctuations and geographical variability in the timing of the tick season (Gray, 1998; Randolph et al., 2002; Walker, 2001). All the above-mentioned facts may influence the circulation of tick-borne pathogens.

2.3.2.3 Ixodes ricinus hosts

Ixodes ricinus ticks feed on a wide range of hosts – more than 300 different species of mammals, birds and reptiles. Different developmental stages prefer different type of hosts. Small vertebrates like rodents (*Apodemus* spp., *Myodes glareolus* (Figure 2), *Microtus* spp., *Sciurus* spp.), insectivores (*Sorex* spp., *Talpa europaea*, *Erinaceus* spp.), birds (*Turdus* spp., *Erithacus rubecula*, *Parus* spp., *Sylvia* spp., *Sitta europaea*,

Emberiza citrinella, *Fringilla coelebs*, *Passer montanus*, *Garrulus glandarius*) and reptiles are parasitized mostly by larval and (occasionally) by nymphal *I. ricinus.* Medium sized and large mammals like lagomorphs (*Lepus europaeus*), carnivores (*Mustella spp., Meles meles, Vulpes vulpes*), artiodactyls (*Sus scrofa*, *Cervus* spp., *Capreolus capreolus* (Figure 3)*, Dama dama, Alces alces*) are parasitized mostly by adult stages of *I. ricinus* (Estrada-Pena et al., 2005; Humair et al., 2007; Kiffner et al., 2010; Kjelland et al., 2011; Matuschka et al., 1991a; Moran Cadenas et al., 2007; Nosek et al., 1967b; Wodecka et al., 2013).

Fig. 2 Frequent tick host – bank vole (*Myodes glareolus*)

The differences in host preference by individual tick developmental stages might be associated with different height of questing level. While adults seek for host on the vegetation up to 60-79 cm high, nymphs prefer lower vegetation and larvae quest near the ground level (Mejlon and Jaenson, 1997).

Nevertheless, large mammals also host high numbers of larvae and nymphs (Carpi et al., 2008; Kiffner et al., 2010; Vor et al., 2010). The importance of deer and other large mammals for *I. ricinus* populations is confirmed by positive correlation between population density of deer and density of questing ticks reported from different habitats and geographical locations (Gilbert, 2009; Ruiz-Fons and Gilbert, 2010). One of the reasons for this correlation is that large mammals are able to support huge amounts of ticks compared to rodents (which in turn reach higher population densities) and therefore, may support a relatively high portion of the tick population. The second reason is that large and medium sized mammals are the main source of

blood-meal for adult ticks. Thus they are extremely important for the wholetick population as by feeding the females (and allowing them to mate with males) the new generation of ticks is produced (Talleklint and Jaenson, 1994).

Fig. 3 Roe deer (*Capreolus capreolus*)

In case of such host generalist like *I. ricinus*, the estimation of the host fauna composition and relative proportion of the individual host species is not easy to perform. The most direct method is trapping of potential hosts and their examination for parasitizing ticks. This method brings data not only on host species, but also on host sex, age, parasite prevalence, abundance, aggregation etc. It also allows the monitoring of nidicolous tick species co-infesting the vertebrate hosts. On the other hand, it is laborious, time consuming and might be complicated by the legislative agenda associated with nature protection. Most importantly, this method is biased by trapping effort and trapping efficiency and does not offer a full undistorted picture of the host fauna of a particular tick species.

Techniques that do not require animal trapping are based on the host identification from blood remnants in the questing ticks. First, serologic methods were used for hematophagous Diptera, later polymerase chain reaction (PCR) based methods (reverse line blotting, restriction fragment length polymorphism, sequencing) were developed detecting the DNA of the hosts in hematophagous arthropods (as reviewed by Humair et al., 2007; Kent, 2009). Since engorged ticks can be collected very rarely by standard flagging method, blood-meal remnants in questing ticks have to be analyzed. In this case only a very limited amount of a relatively old material is available. Therefore, the sensitivity of the detection method is crucial. In reverse line blotting, PCR amplification of a conserved region of the host genome is followed by

hybridization with host-species specific probes. Various targets like 18S rDNA, 12S rDNA or cytochrome B are used for the PCR amplification (Estrada-Pena et al., 2005; Humair et al., 2007; Moran Cadenas et al., 2007; Pichon et al., 2003). The efficiency of the host identification methods varies greatly from one technique to another, but also from the season of the tick sampling, stage of the tick, time post mounting and type of host DNA. The methods are generally able to identify to a certain level approximately 50 % of the samples (Leger et al., 2015; Moran Cadenas et al., 2007). Apart from DNA based techniques, new methods based on proteomic or isotope analysis are developed (Schmidt et al., 2011; Wickramasekara et al., 2008).

2.3.3 Tick-host interactions with possible impact on the efficiency of tick-borne pathogen circulation

The pattern of host infestation by a potential vector may have major influence on pathogen circulation. As in most other parasites, also the distribution of ticks on hosts closely approximates negative binomial model (Craine et al., 1995; Perkins et al., 2003; Randolph et al., 1999; Stanko et al., 2007). It means that most hosts are infested by a small number of parasites, whereas a few individuals of host population feed the majority of parasites.

The reasons for parasite aggregation are still not fully understood. In case of ticks, the main reason is probably spatial aggregation of questing ticks (Perkins et al., 2003). It is obvious for larval stages – larvae hatch from a large batch of eggs and as a rather motion restricted organisms they seek for host in the place of hatching. Moreover, it was proven that immature *I. ricinus* ticks detach more often from resting small mammals than from active ones and diurnal regularities in detachment patterns were also observed (Matuschka et al., 1991b, 1990). This could result in aggregation of ticks in animal resting places thus forming high-risk areas for hosts to get infested. This seems a paradox in the case of *I. ricinus* which has different favored hosts in each developmental stage (nymphs rarely feed on mice whereas larvae do so and adults prefer larger animals). A question arises: How does the tick as a rather motion restricted species reach an ecologically distinct host (Matuschka et al., 1991b)? It may be the reason why ticks prefer heterogenous habitats with numerous ecotones enabling the contact between hosts of different ecological preference.

Attempts were made to find common features for the most infested individuals. Correlation between the body size and abundance of ticks was observed (Craine et

al., 1995; Ruiz-Fons et al., 2013; Talleklint and Jaenson, 1997). The most heavily infested members of a specific host species are frequently adult males (Perkins et al., 2003; Ruiz-Fons et al., 2013; Talleklint and Jaenson, 1997). In case of micro-mammals, the males are usually bigger and have bigger home ranges and thus higher probability of infestation. Immunosuppressive effect of testosterone may also contribute to the sex-specific differences (as discussed by Perkins et al., 2003).

Abundance and distribution of parasites is strongly affected by the abundance and species composition of the hosts. In case of *I. ricinus* ticks and small mammals, negative correlation was observed between the abundance of hosts and abundance, prevalence and aggregation of parasites. The correlation is explained on the basis of "n effect" – the number of ticks is spread over all available hosts (Krasnov et al., 2007). Differences in size of host populations and species richness affecting co-feeding transmission could be one of the explanations of spatial variability in the distribution of tick-borne pathogens (see chapters 2.6.4.1 Co-feeding transmission of *B. burgdorferi* and 2.7.4 Tick-borne encephalitis virus natural cycles).

The infestation levels undergo seasonal fluctuations associated not only with tick activity but also with population abundance of the target host species and alternative hosts. The highest number of ticks per host (*Apodemus flavicollis* main rodent species trapped) was reached approximately a month after the activity of questing ticks had reached its maximum (estimated by flagging) (Craine et al., 1995). High variability in the peak of infestation of bank voles by *I. ricinus* was recorded in Sweden (Talleklint and Jaenson, 1997). Craine et al. (1995) showed that squirrels are the main blood source for larvae in early spring when rodent populations are low but beginning July mice become much more important. Most larvae were fed in late summer and autumn on mice whereas most nymphs were fed in early spring on squirrels. A similar role was suggested for insectivores such as moles and shrews, which may ensure the circulation in early spring months, when rodent populations are low (Kozuch et al., 1967b, 1966; Nosek and Grulich, 1967).

Infestation patterns are to a certain level host-specific. For example, *A. flavicollis* mice are generally more infested than *M. glareolus*. The probable reasons are larger home range of *Apodemus* mice (Kurtenbach et al., 1995; Talleklint and Jaenson, 1997) and immunological resistance of repeatedly infested hosts, which was proven for bank voles but not for mice (Dizij and Kurtenbach, 1995).

Abundance, prevalence and aggregation of ticks on their hosts have apparent consequences for transmission of tick-borne pathogens in relation to viremic/bacteremic and especially non-viremic/non-bacteremic (co-feeding) transmission.

2.3.4 Ticks and human

Human biting ticks are of major medical importance. Apart from causing paralysis, bruises, allergic and toxic reactions, they are vectors of a variety of viral, bacterial and protozoan pathogens (reviewed by Estrada-Pena and Jongejan, 1999). In Europe the impact of ticks on human health is mostly due to pathogen transmission. The most common source of human infection is the above described *I. ricinus*. Apart from the agents of LB and TBE, this species may transmit *Anaplasma phagocytophilum,* an intracellular bacterium causing human granulocytic anaplasmosis (Stuen et al., 2013), pathogenic for immunocompromised patients *Candidatus* Neoehrlichia mikurensis (Derdakova et al., 2014; Fehr et al., 2010), spotted fever rickettsiae *(Rickettsia helvetica*, *R. monacensis*, *R. aeschlimanii*) (Parola et al., 2013), relapsing fever causing Borrelia miyamotoi (Crowder et al., 2014; Platonov et al., 2011), protozoan pathogens of the genus *Babesia* (*B. divergens* mainly of veterinary significance but able to cause infection at least in immunocompromised humans (Zintl et al., 2003), *Babesia venatorum,* a newly defined species with two human disease cases reported in asplenic patients (Herwaldt et al., 2003), *Babesia microti* pathogen of human previously assumed rare in Europe, but recently receiving more attention (Hildebrandt et al., 2007; Overzier et al., 2013; Rizzoli et al., 2014).

In Europe, *I. ricinus* is far the most important vector tick species transmitting diseases of humans. Nevertheless, recently there have been changes recorded in the distribution and abundance of other tick species with a potential to attack and transmit diseases to humans. Changes in the occurrence of *Dermacentor* sp. (*D. reticulatus*) ticks were reported from various European countries including Germany (Dautel et al., 2006), Poland (Mierzejewska et al., 2015; Zygner et al., 2009), Slovakia (Bullova et al., 2009), Belgium (Cochez et al., 2012), Hungary (Sreter et al., 2005) and the Czech Republic (Siroky et al., 2011).

Fig. 4 *I. ricinus* female crawling on human leg (© Jan Korbel)

The shift in the distribution of the vectors is associated with increased exposition to the pathogens they transmit (Butler et al., 2012; Cochez et al., 2012). *D. reticulatus* as a known vector of *B. canis, B. caballi*, *Theileria equi* (Jongejan and Uilenberg, 2004) has importance especially in the veterinary medicine. Nevertheless, this tick species may also attack human and be involved in transmission of *R. slovaca* and *R. raoultii* (Parola et al., 2013; Parola and Raoult, 2001), *B. burgdorferi* (Hubbard et al., 1998) and TBEV (Biernat et al., 2014; Kozuch and Nosek, 1971).

Other medically relevant tick species occur in some parts of the European continent. *Rhipicephalus sanguineus* is a vector of *R. conorii* causing Mediterranean fever, *R. conorii* subsp. *israelensis* causing clinically similar Israeli spotted fever, *D. marginatus* (and *D. reticulatus*) transmit *R. slovaca*, the causative agent of SENLAT - “scalp eschars and neck lymphadenopathy following tick bites syndrome” (previously TIBOLA and DEBONEL) (Parola et al., 2013; Parola and Raoult, 2001). In southern Europe, human cases of relapsing fever (caused by *B. hispanica*) or infections by *R. sibirica mongolotimonae* (Edouard et al., 2013; Psaroulaki et al., 2005) associated with *Ornithodoros erraticus* and *Hyalomma* sp. respectively were reported (Parola and Raoult, 2001). In Eastern Europe, the distribution areas of *I. ricinus* and *I. persulcatus* overlap. Both species contribute to transmission of several pathogens including several genospecies of *B. burgdorferi* s.l., different subtypes of TBEV, *A. phagocytophilum*, *Ehrlichia muris, Babesia* spp. (Alekseev and Dubinina, 2003; Katargina et al., 2013).

2.4 Epidemiology of Lyme borreliosis and tick-borne encephalitis

2.4.1 Lyme borreliosis and tick-borne encephalitis in Europe

With an annual average number of more than 85,000 (65,000 according to Hubalek, 2009) cases of LB and almost 2,900 cases of TBE, these two diseases are the vector-borne diseases with largest impact on human health in Europe (ECDC meeting report, 2012). Since the numbers are estimated from individual national or regional surveillance systems that differ largely (obligatory/voluntary reporting, case definition, methods of laboratory confirmation), the data have a character of a rough estimate rather than a precise number (ECDC meeting report, 2012). The situation is more complicated in the case of LB due to the multi-symptomatic character of the disease, confusing situation regarding chronic or post-lyme syndrome associated conditions, variable laboratory test performance and result interpretation (Hubalek, 2009; Rizzoli et al., 2011). Epidemiological situation in Europe and North America was reviewed by Hubalek, (2009).

In contrast, disease case based surveillance of TBE seems to be much more precise thanks to the relatively conservative clinical picture, reliable laboratory diagnostic tools and hence case definition. In the study concerning the EU member states (Stefanoff et al., 2011) (excluding Luxembourg, including Norway and Iceland, TBE cases were reported from 64 % 18 countries of the countries. TBE surveillance systems exist in 61 % of the countries (17). The reporting systems are in general mandatory and usually only laboratory confirmed (mostly by serological tests including ELISA, western blot, virus neutralization test) cases are recorded. Moreover, in the majority of the TBE positive countries, endemic areas are recognized, mostly based on incidence or number of TBE cases. Based on data from the countries with mandatory reporting, the incidence of TBE in 2007 ranged from 0.03 (Italy) to 10.4 (Estonia) cases per 100,000 inhabitants (Stefanoff et al., 2011). From a long-term perspective, the highest incidence is recorded in the Baltic area (Estonia, Latvia, Lithuania) and Slovenia (WHO-CISID). With regards to the pronounced focal distribution of TBE cases, it seems important to have relevant information with appropriate spatial resolution on a regional level.

More or less dramatic changes in the epidemiology of TBE are observed in many European countries (Randolph, 2001). Climatic and socio-economic factors (affecting the distribution, activity and population dynamics of ticks as well as the activity of human) are considered the main cause of the increase. Socioeconomic factors

include changes in agriculture and land use (reduced use of pesticides, increased landscape fragmentation, increase in game populations), human population structure (increase in elderly population more prone to complicated TBE disease course), increase of outdoor (leisure) time activities, etc. (Jaenson et al., 2012; Kriz et al., 2004; Medlock et al., 2013; Randolph, 2001; Sumilo et al., 2007; Zeman and Benes, 2014). Climatic factors have an obvious impact on shift of tick populations, including tick-borne pathogens, to higher altitudes (Burri et al., 2007; Daniel et al., 2009, 2003; Materna et al., 2008, 2005) and latitudes (Jaenson et al., 2012; Jaenson and Lindgren, 2011; Lindgren, 1998; Lindgren and Jaenson, 2006). Also the activity of the previously recognized TBE foci may undergo fluctuations in time (Daniel et al., 2011; Frimmel et al., 2014; Suss et al., 2004).

2.4.2 Epidemiological situation in the Czech Republic

Since several studies included in the Ph. D. thesis focus on the Czech Republic and the region of South Bohemia, this area will be described in more detail. In the Czech Republic, an annual average of 3263 LB disease cases (31.73 cases per 100 000 inh.) was registered for the time period of 1990-2006 (Hubalek, 2009).

Concerning TBE, 664.7 cases (6.5 cases/100,000 inh.) were registered annually in average (data for 2001-2010) (Kriz and Benes, 2011). A rising tendency is observed since the beginning of the 90´s. In 2006, a long-term maximum of more than 1000 clinical cases was reported (Kriz et al., 2012). In recent study comparing seroprevalence of anti-TBEV antibodies in human in 80's and in 2001, it was found that the seroprevalence was almost two times higher in 2001 (Kriz et al., 2015) documenting the rising trend in the contact of human with TBEV.

2.4.3 Specific epidemiological situation in South Bohemia

South Bohemia is in a long term a region with highest incidence of TBE in the Czech Republic (Kriz et al., 2013; Kriz and Benes, 2011). The average annual number of TBE cases registered in South Bohemia reached 146.3 (data for 2001-2010) giving incidence rate of 23.3 disease cases/100,000 inh. In comparison, the mean annual incidence calculated for the whole Czech Republic reached 6.5 cases/100,000 inh. for the same time period) (Kriz and Benes, 2011).

Interestingly, no such pattern is observed for Lyme borreliosis, although the causative agents share the common vector species and to some extent also competent hosts. A total of 1385 confirmed human cases were registered in South Bohemia in 2001-2009 reaching the average annual incidence of 24.4 cases per 100,000 inh. (data provided by the National Institute of Public Health, Prague Czech Republic; Regional Hygienic Station, Ceske Budejovice) compared to 35.7 per 100,000 inh. for the whole country (WHO-CISID, NIPH-EPIDAT). Thus, it seems, South Bohemian region provides extremely suitable environment for TBEV survival and/or high TBEV-human contact rate and that the difference is not associated with increased vector activity.

2.5 Lyme borreliosis and tick-borne encephalitis as diseases with natural focality

The theory of natural focality of infectious diseases was first presented by J.N. Pavlovsky in 1939. The concept is based on an observation that some agents, that are able to cause disease in humans, circulate among its natural hosts (and vectors) in nature and are entirely independent on the presence of humans. Humans become infected accidentally after entering the natural focus of the disease. A natural focus is a more or less geographically delimited area characterized by certain abiotic and biotic conditions suitable for the host species, vector species and the pathogen itself. To understand the mechanisms of the disease circulation and distribution in time and space, it is necessary to encompass all the components of the natural focus and their interactions (Rosicky et al., 1989; Rosicky and Weiser, 1952). TBE and LB are diseases with natural focality. The virus circulates between the tick vector and various natural hosts species. The epidemiological situation in a natural focus, successfulness of the pathogen survival and distribution is influenced by numerous interacting factors intrinsic properties of the pathogen (e.g. virulence), population dynamics and activity of the vectors (Hubalek et al., 1991; Hubalek et al., 2003 b), population dynamics of the hosts (Zeman and Januska, 1999), type of vegetation (Randolph et al., 2002; Schwarz et al., 2012) and climatic conditions (influencing all the other components of the natural focus directly or vicariously) (Danielova, 1990; Hubalek et al., 2003a; Jensen, 2000).

It was suggested that the focus has a specific structure consisting of a microfocus, where the actual intense circulation of the virus among ticks and natural hosts occurs and a larger area (macrofocus) surrounding the microfocus in which infected ticks may be disseminated by hosts (Dobler et al., 2011; Nosek et al., 1978). The

estimated size of a TBEV focus ranges from 2500 m^2 to 50,000 m^2 (Dobler et al., 2011; Pretzmann et al., 1963).

2.6 *Borrelia burgdorferi* sensu lato

The causative agent of Lyme borreliosis, bacteria from *Borrelia burgdorferi* sensu lato complex, are members of the genus *Borrelia*. Morphologically, they represent a cork-screw shaped Gram-negative eubacteria with mean length of 20 μm and 0.2 in diameter, with 7–14 periplasmic flagella (Barbour and Hayes, 1986; Merilainen et al., 2015). Apart from the spirochaetal form, *B. burgdorferi* may produce morphologically diverse forms according to cultivation or natural conditions in the environment (Merilainen et al., 2015).

Spirochetes from *B. burgdorferi* s.l. complex posses a remarkable genome, consisting of a linear chromosome (approximately 911 kbp) and a variable number of linear and circular plasmids (ranging sizes from 9000 to 56,000 bp) (Fraser et al., 1997). The chromosome contains a constant part (~903 kbp) and a length variable section consisting of a plasmid-like structure. Approximately 815 putative protein-coding genes were identified in the constant region (account for 93 % of the constant region sequence). The annotation of the genes is still in progress (Casjens et al., 2012). The unique organization of rRNA coding sequence of *B. burgdorferi* (consisting of two tandem repeats of 23S and 5S rDNA placed apart from 16S rDNA sequence) is useful for its discrimination from relapsing fever borreliae (Schwartz et al., 1992). The 200-250 bp non-coding sequence of 5S (rrf) - 2S (rrl) intergenic spacer is frequently used for genospecies identification using PCR - restriction fragment length polymorphism (Postic et al., 1994), qPCR (Strube et al., 2010) or reverse-line blotting (Alekseev et al., 2001; Rijpkema et al., 1995) techniques.

While the chromosome is notably stable (clonally evolving), the plasmid content of borrelial genome is significantly variable, experiences frequent horizontal transfer and recombination and presents with high frequency of paralogs (Baranton and De Martino, 2009; Casjens et al., 2012; Glockner et al., 2004). A very important feature, when working with in vitro maintained strains, is that loss of linear plasmids occurs during passaging borrelial strains in vitro as early as in the 5th passage (Biskup et al., 2011). Nevertheless, some of the plasmids (lp54, cp26, cp32s) are significantly more conserved and are essential for borrelia survival and infectivity (Casjens et al., 2012; Glockner et al., 2004). Apart from cp26 which carries genes coding for metabolism

related proteins (nucleotide metabolism, small molecule transporters, telomere creating enzymes), the plasmids contain only few housekeeping genes (Casjens et al., 2012). Genes associated with *B. burgdorferi* s.l. pathogenicity are plasmid encoded, including nucleotide sequences coding for major outer surface proteins (OspA, OspC), nicotineamidase or adhesins (DbpA, B, BBK32) (reviewed in (Baranton and De Martino, 2009).

2.6.1 *B. burgdorferi* sensu lato classification and variability

B. burgdorferi belongs to the phylum Spirochaetes, class Spirochaetes, order Spirochaetales, genus *Borrelia*. Recently, it was proposed to separate the relapsing fever borrelia and Lyme disease borrelia into two genera *Borrelia* and *Borreliela* (Adeolu and Gupta, 2014).

Borrelia burgdorferi sensu lato complex currently constitutes of 20 named genospecies listed in Table 2. As indicated, the genospecies differ in their geographic distribution, affinity to certain groups of vertebrate hosts, presumable key vectors and also in ability to induce disease in human.

Table 2: List of currently established *B. burgdorferi* s.l. species their typical geographic distribution, pathogenicity to human, preferred host species and vectors

Genospecies	Distribution	Hosts species	Presumable key vectors*	Pathogenicity	Reference
B. burgdorferi s.s.	Europe, N. America	rodents, birds	***I. ricinus***, ***I. scapularis***, ***I. pacificus***, *I. spinipalpis, I. minor, I. affinis, I. uriae*	yes	Burgdorfer et al., 1982; Johnson et al., 1984
B. afzelii	Europe, Asia	rodents	***I. ricinus, I. persulcatus***, *I. granulatus*	yes	Canica et al., 1993
B. garinii	Europe, Asia	birds	***I. ricinus, I. persulcatus***, *I. granulatus, Haemaphysalis bispinosa*	yes	Baranton et al., 1992
B. bavariensis	Europe, Asia	rodents	***I. ricinus, I. persulcatus***	yes	Margos et al., 2009
B. spielmanii	Europe?	dormice	***I. ricinus***	yes	Richter et al., 2006
B. bissettii	N. America, Europe	rodents	***I. scapularis***, *I. spinipalpis*, *I. minor*, *I. affinis*, (***I. ricinus***)	yes	Postic et al., 1998
B. valaisiana	Europe	birds	***I. ricinus***, *I. granulatus*	yes/no	Wang et al., 1997
B. lusitaniae	Europe, Africa	reptiles	***I. ricinus***	yes/no	Le Fleche et al., 1997
B. japonica	Asia		*I. ovatus*		Kawabata et al., 1993; Postic et al., 1993
B. tanukii	Asia		*I. tanukii*		Fukunaga et al., 1996
B. turdi	Asia		*I. turdus*		Fukunaga et al., 1996
B. sinica	Asia	rodents?	*I. ovatus, I. granulatus*		Masuzawa et al., 2001
B. yangtzensis	Asia	rodents?	*I. nipponensis*		Chu et al., 2008; Margos et al., 2015
B. andersonii	N. America	rabbits	*I. dentatus*		Marconi et al., 1995)
B. americana	N. America	birds, rodents	*I. minor*, *I. pacificus*		Rudenko et al., 2009
B. carolinensis	N. America		*I. minor*		Rudenko et al., 2011
B. californiensis	N. America		*I. pacificus*, *I. spinipalpis*		Postic et al., 2007
B. kurtenbachii	N. America		*I. pacificus*, *I. spinipalpis,*		Margos et al., 2010
B. finlandensis	N. America		*I. ricinus*		Casjens et al., 2011
B. chilensis	S. America	long-tailed rice rats	*I. stilesi*		Ivanova et al. 2014

* species in bold are considered important vectors transmitting borrelia to human

All the features of the individual genospecies in Table 2 are assigned as general observations. Exceptions may and do occur: *B. garinii* was reported to be found in *I. uriae* in Canada (Smith et al., 2006). Similarly, the level of importance of different vector species may vary geographically, some of the listed species may be involved

in natural cycles only and do not play a significant role in transmission of the pathogen to human. The host association also works more in the sense of tendency than a strict rule.

2.6.2 *B. burgdorferi* hosts

From the broad range of the possible *I. ricinus* host species (compare with 2.3.2.3 *Ixodes ricinus* hosts), a significant part is competent for transmission of *B. burgdorferi* s.l. (Gern et al., 1998). *B. burgdorferi* genospecies display variable level of host specificity (Table 2). While *B. burgdorferi* s.s. is a host generalist, *B. spielmanii* seems to be strictly associated with dormice. *B. garinii* and *B. valaisiana* are associated with birds and *B. afzelii* and *B. bavariensis* are rodent-associated species and *B. lusitaniae* is linked with lizards (reviewed by Margos et al., 2009). The association of different genospecies with specific hosts is probably mediated by difference in the complement systems of the host species (Kurtenbach et al., 2002a, 2002b, 1998b). It was shown that complement reaction of incompetent spirochete hosts may even clear the particular genospecies in ticks (Kurtenbach et al., 2002b). From recent studies it seems that the host associations have rather a character of tendency than a strict rule (Franke et al., 2010; Khanakah et al., 2006; Moran Cadenas et al., 2007; Wodecka et al., 2013). Thus there might be individual differences in the immune reaction or protective mechanisms of *B. burgdorferi* s.l., also co-feeding transmission may contribute (Voordouw, 2015). Genospecies associated host preference resembles in the frequency and efficiency of multiple genospecies co-infection in ticks. Genospecies preferring same host species are more frequently found in co-infections than genospecies associated with different hosts (Herrmann et al., 2013).

On the other hand, some hosts may harbor several genospecies of *B. burgdorferi* s.l. simultaneously (Gern et al., 1997). Although, the co-occurrence was reported only for skin biopsies and it is not known how permanent the co-infection is, it may be the source of multiple genospecies infections in ticks.

Large ungulates like deer are generally considered incompetent hosts of *B. burgdorferi* s.l. (Kjelland et al., 2011; Matuschka et al., 1993; Talleklint and Jaenson, 1994). The probable mechanism is again the complement-mediated killing of borrelia (Nelson et al., 2000). Since live *B. burgdorferi* s.l. spirochetes were detected in the skin of roe deer relatively long time after tick feeding (Pichon et al., 2000) and in the ticks feeding on sika deer (Kimura et al., 1995), transmission of *B. burgdorferi* s.l. to

co-feeding ticks might be possible. Survival of borrelia in the skin indicates, that spirochetes might be able to protect against complement reaction, although they are not able to cause systemic infection. The crucial question is whether the spirochetes are in the long term able to survive also in the tick being exposed directly to the host's complement. Comparisons of prevalence rates in host-seeking and large ungulates feeding ticks indicate, these hosts are able to clear borrelia from the tick while feeding (Kjelland et al., 2011). On the other hand, successful co-feeding transmission of *B. burgdorferi* s.l. was described in sheep (Ogden et al., 1997), although most *B. burgdorferi* genospecies are at least partially sensitive to sheep complement (Bhide et al., 2005). It seems likely that the general findings are affected by unconsidered or even unknown variability on the side of host, vector (particularly host associated genetic structure) or pathogen.

Another abundant tick host species is wild boar (*Sus scrofa* Figure 5) (Wodecka et al., 2013). Seroprevalence studies indicate that wild boar migth be infected with spirochetes (Juricova and Hubalek, 2009). Borrelial DNA was detected in the sera of the host (Faria et al., 2015) and in the ticks fed on wild boar (Estrada-Pena et al., 2005; Moran Cadenas et al., 2007; Wodecka et al., 2013). Thus, *S. scrofa* may be a competent transmission hosts of borrelia.

Fig. 5 Potential *B. burgdorferi* host wild boar (*Sus scrofa*)

Apart from the host species with apparently high abundance in tick habitats, some species with minor representation, which may be heavily infested by ticks also support transmission of multiple *B. burgdorferi* s.l. genospecies. These species remain infective for a significant period of time and may have crucial importance for LB spirochetes circulation in nature. Such host species may be hedgehogs

(*Erinaceus europaeus* and *E. roumanicus*) (Gern et al., 1997; Skuballa et al., 2012), squirrels (*Sciurus vulgaris*) (Craine et al., 1997; Moran Cadenas et al., 2007), foxes (*Vulpes vulpes*) (Moran Cadenas et al., 2007) pheasants (*Phasianus colchicus*) (Craine et al., 1997; Kurtenbach et al., 1998a) and many other.

2.6.3 *B. burgdorferi* vectors

The most important vectors of *B. burgdorferi* s.l. transmitting the spirochetes to humans are the species of *Ixodes ricinus* (*persulcatus*) complex. Namely *I. ricinus* is the major vector in Europe, *I. persulcatus* in Asia and *I. scapularis* and *I. pacificus* in North America (Gray, 1998).

Although the above mentioned species are also frequently involved in the natural enzoonotic circulation of *B. burgdorferi,* in some areas, there are other species of ticks, rarely attacking humans which are contributing to the maintenance of the infection in nature. Such enzoonotic cycles were described in California with *I. spinipalpis* as the maintenance vector and *I. pacificus* as the bridge vector transmitting the spirochetes to human (Brown and Lane, 1992). Similar situation was later described for the south coast of USA (Oliver et al., 2003).

In Europe, there are several nidicolous species of ticks, which may play the role of a maintenance vector, like *I. hexagonus* associated with hedgehogs, *I. trianguliceps* frequently infesting rodents and insectivores, or bird associated *I. uriae*, *I. arboricola* and *I. frontalis* (Gern et al., 1997; Hubbard et al., 1998). These tick species co-infest their hosts with *I. ricinus* which is known too be a bridge vector to human. Nevertheless, the transmission experiments have shown that although it was possible to infect *I. arboricola* and *I. frontalis* larvae and the spirochetes survived tick molting, none of the xenodiagnostic *I. ricinus* larvae was infected by systemic infection or by co-feeding (Heylen et al., 2014). This example may be an appropriate illustration on how complicated the vector-host-pathogen relationships may be. There have been numerous records of *B. burgdorferi* s.l. in various species of tick and non-tick blood-feeding arthropods. Nevertheless, simple detection of the pathogen does not prove vector competence. As shown by Heylen et al. (2014), even successful survival in almost naturally infected tick vector does not necessarily mean that the candidate vector is able to transmit the pathogen to the next host.

Some incompetent vectors similarly to incompetent vertebrate hosts may clear spirochetes by reactions of their innate immunity (Johns et al., 2001).

2.6.4 Specific vector-host-pathogen interactions influencing *B. burgdorferi* transmission

2.6.4.1 Co-feeding transmission

As indicated in previous chapter, borrelia may be apart from classical bacteremic way transmitted from one tick to another locally, within co-feeding formation (Gern and Rais, 1996; Randolph et al., 1996). Particular mechanisms and consequences of co-feeding transmission will be discussed in more detail in chapters devoted to TBE (2.7.4 Virus circulation). The impact of co-feeding transmission on *B. burgdorferi* was recently reviewed by (Voordouw, 2015). Although co-feeding does not probably contribute significantly to the intensity of *B. burgdorferi* s.l. circulation among ticks and their hosts, it may be involved in several ecological and evolutionary mechanisms influencing the *B. burgdorferi* populations. It may contribute to the rate of co-infection of ticks by different genospecies (even host-incompatible combinations) and it may mediate higher frequency of contact between different genospecies and strains of borrelia giving an opportunity for horizontal gene transfer. Furthermore, Voordouw (2015) shows there might be striking differences in the efficiency of co-feeding transmission among different tick species and borrelia genospecies.

2.6.4.2 Transovarial transmission

Based on currently available data, there is still a high level of uncertainty about the ability of *B. burgdorferi* s.l. spirochetes to be passed from a female to her progeny via eggs. Because of the sympatric occurrence of undoubtedly transovarially transmitted *B. miyamotoi* in the same species of ticks, Rollend et al. (2013) propose, that the contradictory evidence for and against transovarial transmission of *B. burgdorferi* may be results of misidentifications of *B. burgdorferi* s.l. and *B. miyamotoi* (Rollend et al., 2013).

Contrary to that, a relatively high prevalence rate of *B. burgdorferi* s.l. in questing larval ticks was estimated by *B. burgdorferi* species - specific method. Nevertheless, this might be also a result of high frequency of interrupted feeding (Tappe et al., 2014). Considering the overall prevalence and relatively broad spectrum of transmission competent hosts, potential transovarial transmission does not play a very important role in the circulation of *B. burgdorferi* in nature.

Interestingly, a possibility of sexual transmission of borrelia between male and female mating ticks was proposed by (Alekseev and Dubinina, 1996).

2.6.4.3 Dilution effect

In ecology of zoonotic diseases this term has been introduced by Norman et al. (1999), defining the dilution effect as reduction of disease transmission in a natural cycle (involving reservoir) species due to addition of non-competent host species. The particular mechanism lies in reduction of transmission frequency between infected and susceptible host of the competent species via introduction of transmission events between infected and susceptible but less competent reservoir hosts (Keesing et al., 2006). Later, the theory was linked to a measure of biodiversity (in the sense of combination of species richness and evenness) (Keesing et al., 2006; Ostfeld and Keesing, 2000).

The theory of dilution effect has been applied in the ecology of tick borne diseases. Natural cycles of Lyme disease in the USA may serve as a perfect example, because the main competent reservoir hosts are well defined, as well as the alternative tick host species.

It is essential to notice that the intensity of circulation of a tick-borne pathogen is more closely associated with density of infected ticks rather than with infection prevalence, and changes in host biodiversity influence both. For instance, increased species richness by introduction of new, although less competent, host may not result in density reduction of infected ticks in case of simultaneous increase in total tick population.

Different scenarios were tested by Ogden and Tsao (2009). The study involved both the direct dilution effect, indirect dilution mechanisms (host species competition),

different tick acquisition and host survival rates on different hosts etc. Complex mechanistic model, including seasonality of hosts and vectors, has shown that the simple addition of a less competent host is more likely to result in amplification of density of infected nymph rather than in reduction. On the other hand, when competition among the host species, survival and acquisition rates were included, the reduction was more probable. Hence, the effect on frequency of infected ticks occurrence is strongly dependent on the detailed vector competent host / incompetent host interactions.

2.6.5 Lyme borreliosis in human

Four genospecies of *B. burgdorferi* s.l. complex commonly infect people, i.e. *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. bavariensis*. They cause disease with a wide spectrum of clinical manifestations ranging from minor erythema migrans to severe cutaneous manifestations (acrodermatitis chronica atrophicans), arthritis, neurological manifestations, endocarditis (Balmelli and Piffaretti, 1995; Stanek and Reiter, 2011; Steere et al., 2004). Furthermore, the presence of *B. valaisiana* (Rijpkema et al., 1997; Ryffel et al., 1999), *B. lusitaniae* (Collares-Pereira et al., 2004), *B. spielmanii* (Fingerle et al., 2008; Strle and Stanek, 2009) and *B. bissettii* (Rudenko et al., 2008; Strle et al., 1997), has been confirmed by direct or indirect methods in samples of patients with symptoms of LB.

B. burgdorferi s.l. genospecies present a certain level of tissue preference when infecting human. The cutaneous manifestations are usually associated with *B. afzelii*, whereas *B. garinii* prevails among infections of central nervous system and *B. burgdorferi* s.s. seems to be associated with arthritis (Strle and Stanek, 2009). Erythema migrans caused by *B. afzelii* presents with a slightly different clinical signs than the same manifestation caused by *B. garinii* (Logar et al., 2004). Similar observations were recorded for *B. garinii* vs**.** *B. afzelii* induced neuroborreliosis (Strle et al., 2006). It seems evident that there is significant variability in the pathogenic potential within the individual genospecies as documented on dissemination abilities associated with specific OspC genotypes (Lagal et al., 2003; Wormser et al., 2008)

It was frequently stated that the transmission of *B. burgdorferi* may be prevented if an infected tick is removed in 24 - 48 hours interval after attachment. This information probably comes from animal model transmission experiments (*B. burgdorferi* s.s., mice, *I. scapularis*) (Piesman et al., 1987). Nevertheless, even in this study, one animal was infected in less than 24 hours of tikc attachment. Another study using *I.*

ricinus, European *B. burgdorferi* species and gerbils proved transmission to 50 % of the experimental animals in less than 16.7 hours (shortest feeding duration tested post attachment (Kahl et al., 1998)). As later shown by Crippa et al. (2002) different genospecies may require different duration of feeding to be successfully transmitted. The phenomenon of feeding duration associated risk of transmission was thoroughly reviewed by (Cook, 2014), pointing out the incorrect interpretation of the results of previous studies, variability among genospecies of *B. burgdorferi* s.l. complex and indications of systemic infection of tick by LB spirochetes. Interestingly, there is also some evidence for probable transmission of *B. burgdorferi* s.l. to human by *Ixodes* ticks earlier than 24 hours after attachment (Hynote et al., 2012).

2.6.6 Prevalence rate of *B. burgdorferi* in ticks

Estimation of the pathogen prevalence in vectors is one of the most common surveillance approaches in tick-borne diseases. According to a metaanalysis of published data, the mean prevalence rate of *B. burgdorferi* in *I. ricinus* reached 13.7 % and was statistically significantly higher in adult ticks (18.6 %) than in nymphs (10.1 %) (Rauter and Hartung, 2005).

Concerning the proportions of *B. burgdorferi* genospecies in *I. ricinus* in Europe, in *B. afzelii* and *B. garinii* generally prevail (each accounting for more than 1/3 of the genospecies identified samples), followed by *B. burgdorferi* s.s. and *B. valaisiana*. *B. lusitaniae* is in general less frequently detected (Rauter and Hartung, 2005). Obviously, the proportions of different genospecies are influenced by the newly recognized species like *B. bavariensis* (previously included in *B. garinii*) or *B. spielmanii*. Nevertheless, it seems that these two genospecies mostly occur with low prevalence (Glatz et al., 2014; C. Herrmann et al., 2013; Honig et al., 2015; Richter and Matuschka, 2011; Wilhelmsson et al., 2010).

2.6.7 Isolation and detection methods – *Borrelia burgdorferi* s.l.

B. burgdorferi spirochetes are very fastidious organisms requiring a nutrient rich cultivation media. The cultivations are based on different modifications of Barbour-Stoener-Kelly (BSK) or modified Kelly Pettenkofer (MKP) media (Pollack et al., 1993; Ruzic-Sabljic et al., 2014). Cultivation media as well as specific cultivation conditions may significantly influence the biological properties of the cultivated strains (Biskup et al., 2011; Wang et al., 2004).

Currently, the polymerase chain reaction (PCR) techniques are methods of choice for detection of *B. burgdorferi* in tick or animal samples. Numerous primers and probes were described for the use in conventional and quantitative PCR (Cerar et al., 2015; Chan et al., 2013; Demaerschalck et al., 1995; Hojgaard et al., 2014; Rijpkema et al., 1995; Schwaiger et al., 2001). Concerning the number of genospecies, the ability to detect all genospecies of borrelia is crucial. The genospecies identification was previously based on restriction fragment length polymorphism (Postic et al., 1994), genospecies specific PCR (Demaerschalck et al., 1995) and other laborious techniques. Currently, either methods allowing simultaneous borrelia detection and genospecies identification are employed (Eshoo et al., 2012; Gern et al., 2010; Rijpkema et al., 1995) or the borrelia positive samples are sequenced (Cerar et al., 2015).

2.6.8 *Borrelia miyamotoi* – *Ixodes* associated relapsing fever *Borrelia*

Generally, the members of *B. burgdorferi* s.l. species complex are associated with "hard" ticks of the family *Ixodidae*, whereas relapsing fever (RF) borrelia were considered to be transmitted by Argasid species. *B. miyamotoi* is a member of the RF group of species but is found in ticks of the genus *Ixodes*. First specimens of the novel bacterium were obtained from *I. persulcatus* and *Apodemus argenteus* in Japan (Fukunaga et al., 1995). Later, the presence of this species was confirmed in other ticks of *Ixodes persulcatus* complex: *I. ricinus* (Crowder et al., 2014; Fraenkel et al., 2002; Hulinska et al., 2007; Pichon et al., 2005; Richter et al., 2003), *I. scapularis*, *I. pacificus* (Barbour et al., 2009; Crowder et al., 2014; Mun et al., 2006; Scoles et al., 2001). Apart from *B. miyamotoi*, another species of relapsing fever *Borrelia* – *B. theileri*, *B. lonestari* (Barbour et al., 1996) and *B. tursica* (Takano et al., 2010) are probably associated with Ixodid ticks.

B. miyamotoi may infect human causing mostly a mild febrile influenza-like illness (Chowdri et al., 2013; Krause et al., 2013; Platonov et al., 2011), although also cases of meningoencephalitis were described in immunocompromised patients (Gugliotta et al., 2013; Hovius et al., 2013).

B. miyamotoi reservoir hosts are probably rodents (Barbour et al., 2009; Cosson et al., 2014), however it was also found in ticks detached from birds (Lommano et al.,

2014). As proposed by Barbour et al. (2009), the Argasid species usually associated with RF spirochetes are nidicolous, therefore it is assumed that also RF borreliae transmitted by Ixodid ticks will have a narrow spectrum of hosts.

The transovarial transmission promoting the natural circulation of the pathogen in nature is a generally accepted mechanism in case of RF borrelia including *B. miyamotoi* (Rollend et al., 2013; Scoles et al., 2001). Compared to LB spirochetes, *B. miyamotoi* is also able to reach much higher level of bacteremia in the natural hosts probably rising efficiency of systemic transmission (Barbour et al., 2009).

2.7 Tick-borne encephalitis virus

2.7.1 Classification of tick-borne encephalitis virus

The causative agent of tick-borne encephalitis is a virus belonging to the family *Flaviviridae*, genus *Flavivirus*. Viruses in this genus were originally classified on the basis of cross-neutralization tests into 12 serogroups (Calisher et al., 1989). Later as complete genome sequences were available, phylogenetic relationships were analyzed in detail. The sequence data are generally concordant with the previous classification based on antigenic properties. Presently, a generally accepted classification scheme of the genus correlates with ecological properties of the viruses such as main vector and main reservoir host species. The genus consists of a large group of mosquito-borne flaviviruses (including West Nile virus, Yellow fever, Dengue), a group of tick-borne viruses and a group of viruses with no known vector (e.g.: Rio Bravo, Modoc) (Gaunt et al., 2001).

Tick-borne flaviviruses are further sub-divided into two groups according to preferred host: avian tick-borne flaviviruses (e.g. *Tyulenyi virus*, *Saumarez Reef virus*, *Kadam virus*) and mammalian tick-borne flaviviruses including: *Tick-borne encephalitis virus* (TBEV), *Louping ill virus* (LIV), *Powassan virus* (POWV), *Kyasanur forest disease virus* (KFDV), *Omsk hemorrhagic fever virus* (OHFV), *Langat virus* (LGTV), *Gadgets Gully virus* (GGYV), *Royal Farm virus* (RFV) (Gaunt et al., 2001).

Among TBEV strains three virus subtypes can be differentiated: European subtype (TBEV-Eu) (type strain Neudoerfl), Far Eastern subtype (TBEV-FE) (type strain Sofjin) and Siberian subtype (TBEV-Sib) (type strain Vasilchenko). Although high degree of similarity in antigenic properties as well as in nucleotide sequence (maximum 5.6 % difference in deduced amino acid sequence of the E-protein) was

revealed, the subtypes differ in the severity of the disease they cause, their main vector species and in the geographic distribution (Ecker et al., 1999).

TBEV-Eu is distributed widely throughout eastern, central and western Europe (excluding Iberian Peninsula, UK, Ireland and Benelux) and in parts of southern Europe and Scandinavia. TBEV-FE occurs from eastern Europe through Russia and China to Japan and TBEV-Sib is distributed mainly in western Siberia (Gritsun et al., 2003b; Stefanoff et al., 2011; Süss, 2003). In the Baltic's and parts of Russia, where both vectors occur, TBEV-Eu and TBEV-FE co-circulate (Golovljova et al., 2004; Katargina et al., 2013; Lundkvist et al., 2001). Occasionally, strains of Siberian subtype were detected in Latvia (Mavtchoutko et al., 2000), Estonia (Golovljova et al., 2004) and Finland (Jaaskelainen et al., 2006).

The main vector of TBEV-Eu is *I. ricinus*, while TBEV-FE and TBEV-Sib are mostly transmitted by *I. persulcatus* (Charrel et al., 2004).

Strains of the European subtype of TBEV cause usually a milder disease with lower mortality rate (1-5 %) compared to TBEV-FE (5-35 %). Infection with TBEV-Sib as well has milder course than TBEV-FE (Charrel et al., 2004; Gresikova and Kaluzova, 1997; Gritsun et al., 2003b). Persistent infections reported from Siberia were probably associated with TBEV-Sib (Gritsun et al., 2003a). Differences in the disease severity could be alternatively explained by factors other than biological properties of the virus – by different case definition or level of medical care (e.g. Ecker et al., 1999; Mandl, 2005).

Based on the antigenic and whole coding sequence analysis of TBEV and TBEV-related viruses, some authors propose rearrangement of the genera LIV and TBEV and formation of one genus Tick-borne encephalitis including four subtypes: TBEV-Eu, TBEV-FE, Louping ill Tick-borne encephalitis virus and Turkish sheep Tick-borne encephalitis virus (Grard et al., 2007; Hubalek et al., 1995). Contrary to the molecular data, there are remarkable differences in the ecology and epidemiology of LIV compared to TBEV (Gilbert et al., 2000; Gould et al., 2006).

2.7.2 Genome and structural characteristics of tick-borne encephalitis virus

TBEV has a spherical virion of approximately 50 nm in diameter containing an icosaedric capsid surrounded by a host cell-derived lipid bilayer (Chambers et al., 1990).

2.7.2.1 Genome

The genome consists of approximately 10-11 kb of single stranded RNA of positive polarity carrying type 1 cap on the 5´end (Chambers et al., 1990). 3´ polyA is present only in some of the strains (Asghar et al., 2014; Frey et al., 2013; Mandl et al., 1991). The genome contains single open reading frame (ORF) flanked on both sides by untranslated regions (UTR). UTRs are probably included in the regulation of replication, translation or packaging of the virus (Mandl et al., 1998).

2.7.2.2 Proteins

The ORF is translated into a single polyprotein (approximately 3400 amino acids) and subsequently cleaved by cellular and viral proteases into 3 structural proteins (capsid protein C, pre-membrane protein prM, envelope protein E) and 7 nonstructural proteins (glycoprotein NS1, subunits of viral protease NS2A, NS2B, viral protease/helicase NS3, NS4A, NS4B, RNA dependent RNA polymerase NS5). Protein C builds up the capsid, proteins M (cleaved prM protein) and E are integrated in the lipid envelope (Gritsun et al., 2003b).

2.7.3 Tick-borne encephalitis pathogenesis and virus replication

2.7.3.1 Pathogenesis

After inoculation of the virus into the skin by the vector tick, the virus is transported via Langerhans cells to lymphatic system and lymphatic nodules. Subsequent virus replication is followed by viremic phase and the virus is spread hematogenously to different organs. The virus is highly neurotropic, however particular mechanism directing the virus into central nervous system (CNS) is not known yet. Neurons represent the main target cells for the virus. The damage to CNS is partly due to the virus action itself and partly due to the host immune response (Haglund and Gunther, 2003; Mandl, 2005; Ruzek et al., 2009).

Additionally, alimentary infection, so called “biphasic milk fever”, can occur after ingestion of unpasteurized goat, sheep or cow milk or unpasteurized milk products can occur – . No differences were found between the TBEV strains transmitted alimentary and vectored by ticks (Gritsun et al., 2003b). A major outbreak of milk-borne TBE was recorded in 1951 in Rožňava (south-eastern Slovakia). Neverthelss, similar outbreakswere described in other countries as well (Gresikova and Kaluzova, 1997; Suss, 2003). Alimentary transmission has its importance in so-called pasture-type natural foci – partially deforested areas used as grazing land (Blaskovic, 1967). More recently, 6 cases of alimentary acquired of TBEV were recorded in Austria. Interestingly, the virus was transmitted by milk from goats which were probably infected in the pasture in altitude of 1500 m a.s.l. (Holzmann et al., 2009). Surprisingly high prevalence of TBEV (11-22 %) in sheep, goat and cow unpasteurized milk was demonstrated in Poland (Cisak et al., 2010). Laboratory infections were also recorded (Avsic-Zupanc et al., 1995; Gallia et al., 1949).

2.7.3.2 Virus replication

The cell receptors of the virus as well as the mechanism of passing through the blood-brain barrier remain unknown (Haglund and Gunther, 2003). The virus is internalized by the means of receptor-mediated endocytosis. Protein E triggers the fusion with the membrane of endosome under low pH. After the viral coat is resolved the transcription begins. Whole genome copies of negative and positive polarity are produced. The genome RNA serves as well as mRNA and is translated into a single polyprotein, which is subsequently cleaved. Proteins are processed in association with the endoplasmic reticulum. Capsid and envelope are assembled and premature virions are produced. Maturation occurs in the Golgi apparatus by cleavage of the prM protein enabling the formation of E protein homodimers. A mature virion is able to leave the cell by the means of membrane fusion (Mandl, 2005).

2.7.3.3 Clinical course

Various clinical forms of the tick-borne encephalitis can be observed – febrile, meningeal, meningoencephalitic, poliomyelitic, polyradiculoneuritic or chronic. The course of severe forms with neurological symptoms is typically biphasic. First, influenza-like symptoms emerge 3-7 days post-infection. After a relapse of the first symptoms, 20-30 % of the patients develop second phase including neurological

symptoms of variable severity: meningeal signs, irritability, impaired concentration, confusion, cognitive disorders, altered consciousness, paralysis etc. Convalescence is long and long-lasting sequelae such as paralysis, paresis or psychiatric disorders may remain (Charrel et al., 2004; Gritsun et al., 2003b; Haglund and Gunther, 2003).

Inapparent infections probably occur quite frequently. Lunackova et al. (2003) estimated the ratio of 2:3 of manifest to inapparent infection in a highly active natural focus. High portions of inapparent infections are known for other flaviviruses indicating individual differences in susceptibility to particular virus infection (Chambers and Diamond, 2003). Cases of TBE with hemorrhagic syndrome were recorded in Novosibirsk region (Russia) (Ternovoi et al., 2003) and chronic TBE forms in Siberia (Gritsun et al., 2003a).

2.7.4 Tick-borne encephalitis virus natural cycles

TBEV is ecologically classified as an arbovirus – arthropod-borne virus. Arboviruses were defined as viruses transmitted by arthropod vector from a viremic vertebrate host to another. Viral replication occurs in both – vertebrate host and arthropod vector (World Health Organization, 1967).

Ixodes ricinus is the main vector of TBEV in Europe. The virus survives in the tick transstadially and is, although with relatively low efficiency, transmitted transovarially (Danielova, 2002; Danielova et al., 2002; Danielova and Holubova, 1991; Nosek and Grulich, 1967). In general, the virus circulates in natural foci between the developmental stages of *I. ricinus* and its hosts, however other vectors may contribute. Common way of arbovirus transmission involves ingestion of the blood of a viremic host by the tick and transfer of the virus during subsequent feeding to another host. TBEV-infected natural hosts usually undergo a very short viremic phase, which obviously affects the probability of the virus transmission. Rodents and insectivores are considered the most important hosts for TBEV because of their relatively short generation time ensuring supply of non-immune hosts, which develop viremia during the infection (Kozuch et al., 1981; Radda et al., 1969).

Apart from viremic transmission, virus may be transferred from one tick to another in the absence of viremia by the means of co-feeding. Co-feeding transmission occurs between ticks feeding in immediate vicinity to each other – localized transmission occurs without virus circulation in blood. This mechanism was first described for

Thogoto virus in 1987 (Jones et al., 1987), later it was confirmed also for TBEV and *I. persulcatus* (Alekseev and Chunikhin, 1990) and *I. ricinus* ticks (Labuda et al., 1996, 1997). Currently, this way of transmission is considered an important mechanism of TBEV maintenance in natural cycles. Interestingly, co-feeding transmission can occur even on immune hosts. This fact remarkably shifts the insight into TBEV circulation since the number of available hosts is much higher than previously estimated (excluding immune hosts). In other words, the basic reproductive rate including the possibility of non-viremic transmission on immune hosts (R_0 = 1.65) is much higher than previously estimated for viremic transmission only (R_0 = 0.98; R_0 > 1 - not sufficient for virus survival) (Labuda et al., 1997). Moreover, this way of transmission may be enhanced by immunomodulative factors present in tick saliva – so called “saliva activated transmission” (Labuda et al., 1993a). Co-feeding transmission is strongly supported by aggregated distribution of ticks within the host species population (preferred individuals in population) (Craine et al., 1995; Perkins et al., 2003; Stanko et al., 2007; Talleklint and Jaenson, 1997) and on the hosts (preferred body parts) (Craine et al., 1995). Infestation patterns of natural hosts will be further discussed in the next chapter.

Although the importance of co-feeding in TBEV transmission cycles is generally accepted, the precise mechanism is still under discussion. Some authors consider spring co-incidence of larvae and nymphs the fundamental prerequisite for the emergence of TBEV foci. Co-feeding transmission from infected nymphs to uninfected larvae (passing from one generation to another) is assumed the major way of virus survival. The theory is supported by statistical analysis of field data concerning infestation patterns of small mammals in TBE-endemic and non-endemic areas (Randolph et al., 1999). In TBE non-endemic areas, the co-infestation of small mammals with nymphs and larvae was found to be very rare compared to a TBE endemic focus. Interestingly, other authors show an example of a typical TBE focus with no co-occurrence of nymphs and larvae (Danielova, 2002). Generally, the number of nymphs feeding on small mammals is much lower than the number of larvae (Craine et al., 1995; Humair et al., 1993; Matuschka et al., 1991a; Sinski et al., 2006; Stanko et al., 2007). Therefore Danielova et al. (2002) propose a slightly different role of co-feeding. Their hypothesis is based mainly on co-feeding of larval ticks alone and on the role of transovarial transmission (TOT). The virus prevalence in larvae acquired by TOT is amplified by co-feeding (of larvae), which results in higher prevalence in subsequent nymphal stage (Danielova, 2002; Danielova et al., 2002). Highly aggregated distribution of larvae supports this theory. On the other hand, the frequency of TOT was proven to be very low (17.6 % of egg batches of

infected females were infected; 0.23 – 0.75 % of larvae from an infected batch were infected) (Danielova and Holubova, 1991). Obviously, both above mentioned mechanisms are included in the transmission cycles of TBEV, but he contribution of particular pathway remains unresolved. The real impact of co-feeding on TBEV transmission under natural conditions is technically difficult to estimate. Various mathematical models, which take in consideration co-feeding, aggregation, viremic and nonviremic hosts, were proposed (Foppa, 2005; Hartemink et al., 2008; Norman et al., 1999; Rosa et al., 2003).

Apart from inoculation of the virus by hematophagous arthropod, alimentary infection was described for some arboviruses. Insectivorous vertebrates may be infected with *Rift Valley fever virus, Yellow fever virus, Japanese encephalitis virus* after ingestion of infected arthropod (reviewed by Kuno and Chang, 2005). Similarly, *Louping ill virus* (LIV) is probably spread through ingestion of infected ticks by juvenile red grouse (*Lagopus lagopus scoticus)* (Gilbert et al., 2004). LIV is closely related to TBEV. Cases of milk-borne alimentary infection are well documented, but no experiments focused on infection acquired by ingestion of infected tick were done so far. Grooming activity and subsequent chewing or ingestion of ticks as defense mechanisms of the hosts are documented (Shaw et al., 2003). Alimentary infection of natural hosts could possibly induce a different course of virus amplification in the host and possibly affect the infectivity.

2.7.5 Tick-borne encephalitis virus hosts

A variety of vertebrates has been proven to have some interaction with TBEV. Nevertheless, it can be hardly ascertained what kind of interactions they were. Detection of specific antibodies indicates that particular host was in contact with TBEV, but does not provide any information on the transmission abilities. Virus isolation is a proof that virus is able to survive in the host, at least for certain period of time, but again does not say much about transmission from that host. The ability of infecting another vector can be proved by xenodiagnostic test. Even after that, some uncertainty remains whether this vector-host interaction really works under natural conditions. A list of tick host species and information available on their relationship to TBEV are summarized in Table 3.

Table 3: List of tick host species and their potential relationship with tick-borne encephalitis virus

Host species	Antibodies detected	Virus isolation	Experimental infection	References
Myodes glareolus	+	+	+	Ernek et al., 1963; Golovljova et al., 2004; Kozuch et al., 1995; Radda and Kunz, 1968
Apodemus flavicollis	+	+	+	Ernek et al., 1963; Kozuch et al., 1995, 1990, 1967a; Labuda et al., 1993b; Radda and Kunz, 1968
A. sylvaticus	0 – 16.3 %	+		Kozuch et al., 1995, 1967a; Radda and Kunz, 1968
Apodemus agrarius		TBEV-FE TBEV-Eu		Golovljova et al., 2004; Kim et al., 2008
Microtus arvalis	3.3 -10.5 %	0 (0/55)		Kozuch et al., 1995, 1990, 1967a; Radda and Kunz, 1968
Sorex araneus	2.8 %		*I. ricinus* *H. inermis*	Kozuch et al., 1990, 1967a, 1967b; Radda and Kunz, 1968
Erinaceus roumanicus	17 %	+		Kozuch et al., 1967a
Pitymys subterraneus	0 %			Radda and Kunz, 1968
Talpa europaea	26 %	+	*I. ricinus*	Kozuch et al., 1966
Vulpes vulpes	1.2 – 50 %			Radda and Kunz, 1968; Wurm et al., 2000
Capreolus capreolus	50 %			Radda and Kunz, 1968
Anas crecca	14.3 %			Ernek et al., 1967
Buteo buteo			+	Rehacek et al., 1963
Falco tinnunculus			+	Rehacek et al., 1963
Turdus merula	15.4 %			Ernek et al., 1968
Passer domesticus	6.2 – 13.8 %			Ernek et al., 1968

+ positive in particular test, numbers in parenthesis – number of positive/number of tested

Not all natural hosts of ticks have the same potential to transmit TBEV. Large mammals generating short and low level of viremia are generally considered incompetent hosts for transmission of TBEV. However, the incompetence for co-feeding transmission was probably never experimentally verified (Pettersson et al.,

2014). These accidental hosts which do not contribute to the (viremic) transmission of TBEV are also referred to as “indicator hosts”, because presence of TBEV specific antibodies in their blood may indicate TBEV circulation activity in nature (Charrel et al., 2004). Anyway, the abundance of large mammals was shown to be linked with total abundance of ticks and thus with total disease risk. Several studies suggest also direct link between large mammal abundance and TBEV incidence (Carpi et al., 2008; Hudson et al., 2001; Rizzoli et al., 2009) (compare chapters 2.3.2.3*Ixodes ricinus* hosts and 2.6.4.3 Dilution effect).

Although there are certain indications that birds may play some role in the circulation of TBEV, data on TBEV host competence of birds are scarce so far. Nevertheless, at least ground-foraging birds are an important blood-meal source for *I. ricinus* ticks. Of course, migrating birds are of a particular interest because they can transfer (infected) tick for long distances (Waldenstrom et al., 2007).

Rodents and insectivores are considered the crucial hosts for TBEV. These species exhibit high and long lasting viremia and co-feeding transmission was proven to be possible even on immune rodents (Labuda et al., 1997, 1996). Nevertheless, there are significant differences in the level of TBEV transmission among different species of rodents. Labuda et al. (1997) have shown a significantly lower efficiency of TBEV transmission by *Myodes glareolus* voles than by *Apodemus flavicollis* mice. Similar level of seroprevalence of anti-TBEV antibodies indicates similar contact rate with the virus (Kozuch et al., 1995, 1990). Instead of differences in the activity of the two host species, the difference may be more likely assigned to immune response to tick bite, which is triggered in *M. glareolus* but not in *A. flavicollis*, as mentioned above (Dizij and Kurtenbach, 1995). The tick-induced activation of cell-mediated immunity could prevent the transmission of TBEV to a certain degree (Labuda et al., 1997). Together with lower infestation levels of *M. glareolus*, this species can be assumed a less important species for TBEV circulation than *Apodemus* mice.

2.7.6 Tick-borne encephalitis virus vectors

TBEV is a typical arthropod-borne virus (arbovirus). One of the basic features of arboviruses is viral amplification in vertebrate hosts as well as in arthropod vectors. Therefore, the virus transmission is biological and not only mechanical. Biological transmission is much more efficient (e.g. because of lower level of viremia required) although a number of adaptations is needed to make amplification in two distinct

environments possible (Kuno and Chang, 2005). Therefore, simple detection of a pathogen in an arthropod species can not be considered a proof of a true vector competence. For example, Crimean-Congo hemorrhagic fever has been detected in a number of Ixodid tick species (*Dermacentor*, *Rhipicephalus*, *Hyalomma*) but only ticks of *Hyalomma* sp. are considered the principle vectors (Charrel et al., 2004). Different vectorial capacity of tick species may be caused by numerous physiological, ecological or behavioral factors (Kuno and Chang, 2005).

Apart from *Ixodes ricinus* and *I. persulcatus,* experimental transmission of TBEV from one animal to another was possible via *I. arboricola* (Lichard and Kozuch, 1967), *Haemaphysalis inermis* (Kozuch et al., 1967b), *H. concinna*, *H. spinigera*, *H. turturis* (Nosek et al., 1967a) and *Dermacentor marginatus* (Kozuch and Nosek, 1971). Even though the ability of TBEV transmission has been proven under laboratory condition, these ticks may, for various ecological or behavioral reasons, still not be included in the virus circulation in nature.

Moreover, TBEV was isolated from *I. hexagonus* (Krivanec et al., 1988) in the Czech Republic and TBEV RNA was detected in *D. reticulatus* ticks in Poland (Biernat et al., 2014). Survival of the virus in *I. hexagonus* tick from larva to adult and transmission from infected *I. hexagonus* to a hedgehog was proved under laboratory conditions (Nosek and Grulich, 1967).

The integration of *I. trianguliceps* in transmission cycles of TBEV seems quite probable as small mammals are frequently infested by *I. ricinus* and *I. trianguliceps* simultaneously (Stanko et al., 2007), and all three developmental stages of *I. trianguliceps* infest micro-mammals. Slight ecological differences can be found between *I. ricinus* and *I. trianguliceps*. *I. trianguliceps* ticks prefer higher altitudes and are nidicolous ticks (spend their life on the hosts or in their nests) preventing infestation of human. *I. trianguliceps* is far more frequently infesting micro-mammals in higher altitudes than *I. ricinus* and therefore could play a significant role in TBEV circulation in mountain areas. Under these conditions, it would be possible that TBEV had circulated in higher altitudes before the shift of *I. ricinus* occurred and after the shift *I. ricinus* has acted as a “bridge” vector from natural hosts to human (M. Daniel, V. Danielová personal communication).

2.7.7 Prevalence rate of tick-borne encephalitis virus in ticks

The average prevalence rate of TBEV in *I. ricinus* ticks does not usually exceed 2 % although occasionally numbers as high as 14 % were recorded (Casati et al., 2008). In a meta-analysis concerning prevalence reports for Scandinavia, an average MIR (minimum prevalence rate) of 0.28 % was obtained (Pettersson et al., 2014). As suggested by Petterson at al. (2014) the differences may be induced by the difference of sampling area and area of TBEV natural focus – sampling out of the focus of TBEV circulation may result in inclusion of low TBEV infected tick populations and hence lower prevalence rate.

Interestingly, much higher prevalence rate (about 4 times higher) was recorded in partially engorged ticks collected from humans than in questing ticks collected on vegetation (Bormane et al., 2004; Suss et al., 2004). Proposed causes of the difference are: virus amplification in infected ticks after obtaining another blood-meal and hence increased probability of successful detection (Suss et al., 2004) and/or, increased host-seeking activity of infected ticks. Belova et al. (2012) have recorded increased activity and resistance to chemical repellent in TBEV infected ticks (ticks were infected by injection) (Belova et al., 2012).

2.7.8 Isolation and detection methods – tick borne encephalitis virus

Traditionally, TBEV (as other arboviruses) is isolated in suckling mice (1-4 days old) infected by intra-cerebral inoculation. Today most of the isolation attempts still use this technique. However, the virus can be isolated from various biological materials (blood, brain and other organs, ticks) (Golovljova et al., 2004; Gresikova and Nosek, 1967; Hayasaka et al., 2001; Jaaskelainen et al., 2006).

Alternatively, virus isolation and propagation can be performed on cultures of various cell lines (e.g. VERO, BHK-21, RH A549) (Charrel et al., 2004) and primary cultures (e.g. chick embryonic cells) (Gresikova and Kaluzova, 1997; Slonim, 1957). Porcine stable cells (PS) are widely used for such purposes (Danielova et al., 2002; Kozuch and Mayer, 1975). The number of living viral particles can be estimated by the plaque method (de Madrid and Porterfield, 1969).

Currently, the most frequently used methods for TBEV detection are reverse transcription polymerase chain reaction based methods, either as conventional PCR or as, usually more sensitive, quantitative PCR (Schwaiger and Cassinotti, 2003).

2.8 Spatial epidemiology and modeling

Spatial epidemiology studies distribution of disease cases in space and its associations with demographic, behavioral, socio-economic as well as infection risk factors (Estrada-Pena and Venzal, 2007). As tick-borne diseases exhibit high degree of spatial structuring, tools of spatial epidemiology seem to be very useful in analyzing the background causes and/or building predictive models.

Research in the eco-epidemiology of tick-borne diseases has accumulated a considerable amount of data describing vector ecology, host-vector interactions, associations with environmental variables etc. Development in the availability of spatially referenced environmental data together with powerful tools (geographic information systems – GIS) for their analysis allows integration of the knowledge into a wider context and draw conclusions with increased general applicability.

Vector presence or absence in an area is usually predicted based on habitat suitability modeling. Based on definition of cut-off values of certain environmental variables, the area is classified as suitable or unsuitable for tick population survival. Similar models predicting the quantitative information on tick abundance also use the relationship of environmental variables, this time to tick activity. For pathogens with variable distribution in the tick populations, the model accuracy is improved when modeling infected vector distribution.

Various approaches are used for construction of spatially predictive models in the tick and tick-borne diseases distribution. Although phenological studies focused on ecology of ticks exist (Alonso-Carne et al., 2015), the main purpose is usually devoted to prediction of the infection risk in space (Brownstein et al., 2003; Daniel et al., 1998; Diuk-Wasser et al., 2010; Eisen et al., 2010, 2006; Eisen and Eisen, 2008; Guerra et al., 2002; Rizzoli et al., 2002). Environmental data based modeling may also provide useful predictions of the disease risk distribution under different (climatic) future scenarios (e.g. climate changes) (Jaenson and Lindgren, 2011; Khatchikian et al., 2012; Leighton et al., 2012; Lindgren, 1998).

Generally, these models are based either on disease case distribution (Eisen and Eisen, 2008; Racz et al., 2006; Zeman, 1997) or (infected) vector distribution (Diuk-Wasser et al., 2010; Eisen and Eisen, 2008; Guerra et al., 2002; Rizzoli et al., 2002). The advantages and drawbacks of the two model types were well discussed recently

(Ostfeld et al., 2005). In general, incidence based models are able to integrate more of the human population derived characteristics, such as activity of human population, vaccination rate and other preventive measures, whereas the vector data based models are associated with the spatial distribution of the biological risk (probability of encounter of an infected vector). Frequently, the two approaches are combined (Daniel et al., 1998).

Various combinations of environmental data were used for the dependent variable prediction including: climatic data (temperature, air humidity, precipitations usually remotely sensed satellite data), vegetation cover data (vegetation indices - NDVI, classified satellite images – CORINE Land Cover, vegetation structure), physical geographical characteristics (altitude, exposition, soil type) and data on host activity (numbers of game animals, numbers of culled game, other evidence of game). Various statistical approaches are used for the predictor identification and parametrization (linear, logistic, log-linear regressions, classification and regression trees etc.) (Brownstein et al., 2003; Daniel et al., 1998; Eisen et al., 2010, 2006; Eisen and Eisen, 2008; Racz et al., 2006; Rizzoli et al., 2009, 2002).

Apart from prediction of spatial features, the models may also be used for population dynamics modeling (Dobson et al., 2011), pathogen host system modeling (Foppa, 2005; Hartemink et al., 2008; Labuda et al., 1997; Norman et al., 1999; Ogden and Tsao, 2009; Rosa et al., 2003) and temporal predictions (Daniel et al., 2006; Vassalo et al., 2000).

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3 Aims and objectives

The Ph. D. thesis is focused on analysis of the distribution of ticks and tick-borne pathogens, its causes and consequences (in the epidemiology of human tick-borne disease cases).

Specific aims:

1. to collect and analyze the data on incidence of human disease cases of Lyme borreliosis and tick-borne encephalitis

2. to evaluate the distribution of ticks, tick host-seeking activity and collect tick samples in type localities with regards to

    a) altitudinal gradient in a mountainous ecosystem up close to the altitudinal limit of *Ixodes ricinus* distribution

    b) temporal comparison of tick activities and pathogen prevalence rates

    c) spatial comparison of tick activities and pathogen prevalence rates among different habitat types

3. to estimate the prevalence of tick-borne pathogens of humans, namely *Borrelia burgdorferi* sensu lato (including genospecies differentiation) and tick-borne encephalitis virus in tick samples

4. to collect wide environmental data to be used as potential explanatory variables of variation in tick activity and pathogen prevalences

5. to use the environmental variables for construction of a risk model predicting the spatial distribution of the risk to encounter an (infected) tick

6. to analyze the spectrum of *Ixodes ricinus* host species in different types of habitats

4 Research papers and manuscripts

4.1 Paper I

Vertical Distribution of the Tick *Ixodes ricinus* and Tick-Borne Pathogens in the Northern Moravian Mountains Correlated with Climate Warming (Jeseniky Mts., Czech Republic)

Milan Daniel, Jan Materna, Václav Hönig, Ladislav Metelka, Vlasta Danielová, Josef Harčarik, Stanislava Kliegrová, Libor Grubhoffer

Central European Journal of Public Health, 2009, 17(3): 139-145

VERTICAL DISTRIBUTION OF THE TICK *IXODES RICINUS* AND TICK-BORNE PATHOGENS IN THE NORTHERN MORAVIAN MOUNTAINS CORRELATED WITH CLIMATE WARMING (JESENÍKY MTS., CZECH REPUBLIC)

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SUMMARY

A study of the vertical distribution of the common tick *Ixodes ricinus* and tick-borne pathogens – tick-borne encephalitis virus (TBEV) and genospecies of *Borrelia burgdorferi* s.l. – was performed in the highest part of the Jeseníky mountain area (the Hrubý Jeseník Mts. with the highest summit Praděd, 1,491 m above see level). Altogether 1,253 specimens of all tick stages (607 larvae, 614 nymphs, 8 females and 24 males) were collected at the altitude 990–1,300 m above sea level on 12 collection sites by the flagging method. Altogether 1,207 ticks (8 females, 24 males, 568 nymphs and 607 larvae) were examined for the presence of tick-borne encephalitis virus and *B. burgdorferi* s.l. None of the samples contained TBEV, 35 samples (6% of adult ticks, 5% of nymphs, 0.7% of larvae) were positive for *B. burgdorferi* s.l. The most prevalent genospecies were *B. afzelii* (44%), *B. garinii* (28%), less frequent were *B. burgdorferi* sensu stricto (5%), *B. valaisiana* (3%). The rather large number of ticks

(in abso-lute numbers as well as recounted to the index: average number of nymphs/worker/collection hour) and the presence of all developmental stages clearly demonstrate that there are viable local tick populations in all the sites, and that recorded ticks were not randomly individuals brought into higher altitudes by birds or game animals. The results are compared with the long-term (2002–2007) monitoring of the tick altitudinal distribution in the Krkonoše Mts. and the conditions, which allow ticks to establish local populations up to the timberline in both mountain areas, are discussed. Simultaneously, changes in climatic conditions (especially the air temperature) monitored at 3 meteorological stations in the area of the Jeseníky Mts. were compared with the records from another 8 stations in other mountain areas in the Czech Republic. A very similar statistically significant trend of increasing mean air temperatures during the last three decades is found at all analyzed stations. The trend is most pronounced in the spring and summer months with the highest activity of *I. ricinus* ticks.

Key words: Ixodes ricinus, *Borrelia burgdorferi* genospecies, tick-borne encephalitis virus, vertical distribution, climate warming

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4.2 Paper II

Abundance of *Ixodes ricinus* and Prevalence of *Borrelia burgdorferi* s. l. in the Nature Reserve Siebengebirge, Germany, in Comparison to Three Former Studies from 1978 Onwards

Alexandra Schwarz, Václav Hönig, Zuzana Vavrušková, Libor Grubhoffer, Carsten Balczun, Antje Albring, Günter A. Schaub

Parasites & Vectors 2012, 5: 268

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RESEARCH Open Access

Abundance of *Ixodes ricinus* and prevalence of *Borrelia burgdorferi* s.l. in the nature reserve Siebengebirge, Germany, in comparison to three former studies from 1978 onwards

Alexandra Schwarz[1,2*], Václav Hönig[1,3], Zuzana Vavrušková[1,3], Libor Grubhoffer[1,3], Carsten Balczun[2], Antje Albring[2] and Günter A Schaub[2]

Abstract

Background: During the last decades, population densities of *Ixodes ricinus* and prevalences of *Borrelia burgdorferi* s.l. have increased in different regions in Europe. In the present study, we determined tick abundance and the prevalence of different *Borrelia* genospecies in ticks from three sites in the Siebengebirge, Germany, which were already examined in the years 1987, 1989, 2001 and 2003. Data from all investigations were compared.

Methods: In 2007 and 2008, host-seeking *I. ricinus* were collected by monthly blanket dragging at three distinct vegetation sites in the Siebengebirge, a nature reserve and a well visited local recreation area near Bonn, Germany. In both years, 702 ticks were tested for *B. burgdorferi* s.l. DNA by nested PCR, and 249 tick samples positive for *Borrelia* were further genotyped by reverse line blotting.

Results: A total of 1046 and 1591 *I. ricinus* were collected in 2007 and 2008, respectively. In comparison to previous studies at these sites, the densities at all sites increased from 1987/89 and/or from 2003 until 2008. Tick densities and *Borrelia* prevalences in 2007 and 2008, respectively, were not correlated for all sites and both years. Overall, *Borrelia* prevalence of all ticks decreased significantly from 2007 (19.5%) to 2008 (16.5%), thus reaching the same level as in 2001 two times higher than in 1987/89 (7.6%). Since 2001, single infections with a *Borrelia* genospecies predominated in all collections, but the number of multiple infections increased, and in 2007, for the first time, triple *Borrelia* infections occurred. Prevalences of *Borrelia* genospecies differed considerably between the three sites, but *B. garinii* or *B. afzelii* were always the most dominant genospecies. *B. lusitaniae* was detected for the first time in the Siebengebirge, also in co-infections with *B. garinii* or *B. valaisiana*.

Conclusions: Over the last two centuries tick densities have changed in the Siebengebirge at sites that remained unchanged by human activity since they belong to a nature reserve. Abiotic and biotic conditions most likely favored the host-seeking activity of *I. ricinus* and the increase of multiple *Borrelia* infections in ticks. These changes have led to a potential higher risk of humans and animals to be infected with Lyme borreliosis.

Keywords: *Ixodes ricinus*, Tick density, *Borrelia* prevalence, *Borrelia lusitaniae*, Multiple infections, Siebengebirge

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Background

Ticks are obligate hematophagous ectoparasites and important vectors of infectious diseases transmitting parasites of livestock and humans, e.g. the etiologic agents of babesiosis, theileriosis or anaplasmosis and of human tick-borne encephalitis and Lyme disease [1,2]. The sheep tick *Ixodes ricinus* is the most common tick species and principal vector for various infectious diseases in Europe and some regions of Asia and North Africa [3]. The distribution and abundance of *I. ricinus* depends on various abiotic and biotic factors such as the microclimate, habitat (vegetation) and host cenosis [4]. The host-seeking activity of *I. ricinus* is favored by air temperatures between 7°C and 24°C and relative humidities of 45-100% due to the risk of desiccation [5,6]. Ecosystems that have a strong buffering capacity, for example, for humidity, such as mixed deciduous and coniferous forests with well-developed leaf and shrub layers are preferred tick habitats [7,8]. However, also forest biotopes differ in the abundances of *I. ricinus*, presumably correlated to the water content of the soil [7,8].

Since the development and survival of ticks strongly depends on climatic conditions, the distribution and abundance of ticks might also be influenced by global warming. In Sweden, the distribution of *I. ricinus* extended towards the north, and this was suggested to be caused by increased air temperatures which favored the survival, activity and development of ticks [4]. Similarly, in the Czech Republic *I. ricinus* spread towards higher altitudes, from 700m to 1100m above the sea level within the last 20 years [9].

In addition to climatic factors, the host cenosis also affects the distribution and abundance pattern of ixodid ticks. *I. ricinus* is an euryphage species that has a broad host spectrum and thus feeds on more than 300 vertebrate species [10]. It predominantly infests small rodents (mice), passerine birds and larger mammals such as hedgehogs, hares, squirrels, wild boar and roe deer [11]. Increased population densities of these hosts induce an increase in the densities of ticks (summarized by [8]). In addition, also the anthropogenic impact on habitat changes the temporal and spatial pattern of tick populations [12].

An increase in the abundance of *Ixodes* can increase the transmission risk of diseases, e.g. of the spirochete bacterium *Borrelia burgdorferi* sensu lato, the etiologic agents of Lyme disease which is endemic in Europe [13]. Prevalences of *B. burgdorferi* s.l. in *I. ricinus* range up to 11%, 43% and 58% in larvae, nymphs and adults, respectively [14]. During the last decades, *Borrelia* prevalences have increased in different regions in Europe, such as in Denmark and Germany [15,16]. Infection prevalences differ between different regions in Europe because the transmission of *Borrelia* depends on a complex zoonotic cycle between their reservoir hosts and their tick vectors. More than 50 avian and mammalian hosts are reservoir hosts for *B. burgdorferi* s.l. in Europe [17].

Different genospecies have been identified in the *B. burgdorferi s.l.* complex. In Europe, seven genospecies are prevalent, *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. bissettii, B. spielmanii*, *B. lusitaniae* [18,19], and *B. bavariensis,* a recently classified genospecies [20] that was previously described as the rodent-associated *B garinii* OspA serotype 4 [21]. Usually *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* (including *B. bavariensis*) are present in tissues of Lyme disease patients [22,23]. Spirochetes of *B. valaisiana* were isolated from a few patients who showed erythema migrans or acrodermatitis chronica atrophicans manifestations and an old man who showed strong clinical evidence of neuroborreliosis [23,24]. *B. bissettii* was detected in tissues from a few patients suffering from Lyme borreliosis in Slovenia and in the Czech Republic [25,26]. *B. spielmanii* was present in the skin of a few patients with erythema migrans [27]. Only once, *B. lusitaniae* was identified in a patient, but he showed symptoms that are untypical for the clinical manifestations of Lyme disease [28]. In *I. ricinus* from Slovakia, Latvia, Germany, Portugal and the United Kingdom, the most prevalent *Borrelia* genospecies were *B. afzelii*, *B. garinii* (including *B. bavariensis*) and *B. valaisiana* with overall prevalences of 39.3%, 21.2% and 12.8%, respectively [29].

Prevalence and the distribution of *Borrelia* genospecies strongly depend on the local host cenosis due to the host's reservoir competence [30]. *B. afzelii* is mainly associated with rodents and *B. garinii* and *B. valaisiana* with birds [31]. Both, rodents and birds are competent reservoir hosts of *Borrelia burgdorferi* s.s. [32]. Rodents do also serve as reservoir hosts of *B. bissettii* and *B. bavariensis* [21,33] and the garden dormouse seems to be the main reservoir host of *B. spielmanii* in Central Europe [34]. *B. lusitaniae* is associated with birds and lizards [35,36].

In the present investigation, we determined the abundance of *I. ricinus* and the prevalence of *Borrelia* and of the different genospecies in ticks in the Siebengebirge, a nature reserve and well visited local recreation area near Bonn, Germany. In 2007 and 2008, ticks were collected at three sites that represented different plant communities and possessed different population densities of *I. ricinus*. These sites have been already examined in previous investigations in the years 1987, 1989, 2001 and 2003 [8,15,37]. Thus, abundance and prevalence were compared with previous investigations.

Methods

Study area and tick collections of all studies since 1978

In 1987 and 1989, 2001, 2003, 2007 and 2008 host-seeking ticks (nymphs and adults) were collected in the

nature reserve Siebengebirge, a forested and hilly region located south-east of Bonn [8]. As presented in Table 1, ticks were collected throughout the seasonal tick activity period in each study year in the Siebengebirge. In all years, exactly at the same sites in three plant communities, the Fraxino-Aceretum pseudoplatani, the Luzulo-Fagetum milietosum (*Athyrium filix-femina* variant) and the Galio-Fagetum typicum (considered as Melico-Fagetum typicum by [15,37,38]) were chosen for the tick collections [8,15,37,38]. A detailed description of the examined plant communities including a map of the exact study sites was previously published in Schwarz *et al.* [8]. Briefly, study site 1 (N50°39'37.1",E 7°14'55.4", 298m) in the Galio-Fagetum typicum (considered as Melico-Fagetum typicum by [15,37] and study field 2 by Schwarz et al. [8]) possessed a highly developed herb layer and well-developed shrub layer (dominant plant species, e.g. *Melica uniflora*) and a dry to wet soil water capacity [8]. Study site 2 (N50°39'53.2", E7°13'05.8", 130m, site 3 by [8]) in the Fraxino-Aceretum pseudoplatani was a dry to fresh vegetation type with a poorly developed herb and shrub layer (dominant plant species, e.g. *Dentaria bulbifera*). The soil water capacity was moderate. The third study site (N50°39'51.5", E7°13'19.2", 123m, site 4 by [8]) located in the Luzulo-Fagetum milietosum was the richest plant community in species and plant densities (dominant plant species, e.g. *Athyrium filix-femina*) and it had a high soil water capacity. In our previous study in 2003, medium, low and high numbers of ticks were characteristic for the Galio-Fagetum typicum, the Fraxino-Aceretum pseudoplatani and the Luzulo-Fagetum milietosum, respectively [8].

In all Siebengebirge/s studies, ticks were collected monthly by repeated blanket dragging [8,37,38], with the exception of the study in 2001 when weekly tick collections were carried out [15] (Table 1). In all studies, a cotton flannel was used for blanket dragging, and the sites were repeatedly re-flagged until no more ticks were collected. According to Hubálek *et al.* [6], tick collections in 2003, 2007 and 2008 were carried out when air temperatures were between 7°C and 24°C and a relative humidity between 45% and 100% without rain and no strong winds occurred. The effective measured temperature and relative humidity ranges at the collection sites were listed for the three years in Table 1. In the years 1978, 1989 and 2001 ticks were collected when at least 16°C and 80% relative humidity was reached without rain and no strong winds [15,37,38]. All collected ticks were preserved in 70% ethanol and identified to species level in the laboratory [39].

DNA extraction

In a chronological arrangement of the methodologies, in 2008 all adults and up to about 60 nymphs/month/site (in total 61 adults and 641 nymphs) were homogenized each in 200 µl 20% Chelex 100 resin solution (Bio-Rad) using the TissueLyser II (Qiagen) and stainless steel beads (5 mm) [40,41]. For total DNA extraction, 120 µl of each homogenate were incubated at 56°C overnight, vortexed and incubated for 10 min at 96°C. After

Table 1 Overview of the methodological differences in the *Ixodes ricinus* studies in the Siebengebirge in 1987, 1989, 2001, 2003, 2007 and 2008

Methods	1987/89*	2001	2003	2007	2008
Months[1]	Apr-Oct	May, Aug-Oct	May-Nov	May-Nov	May-Nov
Frequency	monthly	weekly	weekly	monthly	monthly
Size	100m^2	100m^2	225m^2	100m^2	100m^2
Air temp.[2]	≥16°C	≥16°C	8°C-25°C	15°C-25°C	15°C-23°C
Humidity[2]	≥80%	≥80%	57%-74%	45%-85%	52%-85%
DNA extraction	n/a[3]	Ammonia solution	n/a	Ammonia solution	Chelex 100 resin solution
Borrelia detection	IFA[+]	Simple, modified PCR [45]	n/a	Nested PCR [44]	Nested PCR [44]
		Nested PCR [44]			
		Modified PCR [46]			
		IFA [15,38]			
Borrelia genotyping	n/a	Reverse line blotting[4] [44]	n/a	Reverse line blotting[5] [44,47]	Reverse line blotting[5] [44,47]

*The years 1987 and 1989 are listed together because the same methods were used in both study years for the study of *I. ricinus* abundances and *Borrelia* prevalences.
[1] Tick collections were carried out in the respective months (Apr = April, Aug = August, Oct = October, Nov = November).
[2]Air temperatures and relative humidities were measured 5cm above the ground at the study sites in all years.
[3]n/a = not applicable.
[4]*B. burgdorferi* s.l., *B. burgdorferi* s.s., *B. garinii, B. afzelii* and *B. valaisiana* were identified by reverse line blotting according to Rijpkema et al. [44].
[5]*B. burgdorferi* s.l., *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* were identified by reverse line blotting according to Rijpkema et al. [44] and DNA probes for *B. garinii, B. valaisiana*, *B. lusitaniae, B. spielmanii* and B. *bissettii* were designed according to Gern et al. [47].
[+] IFA = Immunofluorescence assay.

centrifugation at 15,000 g for 3 min, the supernatant was used directly for PCR.

In 2007, all adults and up to about 90 nymphs/month/site (in total 50 adults and 652 nymphs) were pestled each in 100 μl 1.25% ammonia [42]. The tick homogenates were boiled at 100°C for 20 min, cooled down briefly, centrifuged at 16,000 g for 5 min and the supernatants boiled again to evaporate the ammonia until 30 μl of DNA solution was left.

In 2001, Kampen *et al.* [15] randomly selected 366 nymphs and 179 adults in 2001 for *Borrelia* examination. Similar to our study in 2007, the DNA of these ticks was extracted by ammonia (Table 1).

The following *B. burgdorferi* s.l. genospecies served as positive controls in PCR and/or reverse line blottings: *B. burgdorferi* sensu stricto N40, B31 and CB53, *B. garinii* PSth, *B. afzelii* VS461, *B. valaisiana* VS116 and *B. lusitaniae* PotiB3. The strains N40 and PSth were provided by the Baden-Wuerttemberg State Health Office, Stuttgart, Germany, B31 by J. F. Anderson (Connecticut Experiment Station, New Haven, USA), CB53 by J. Kopecký (Institute of Parasitology, ASCR, České Budějovice, Czech Republic) and the strains VS461, VS116 and PotiB3 by Ian Livey (Baxter Innovations, Orth an der Donau, Austria). All bacteria were cultured in BSK-H medium (Sigma-Aldrich) at 34°C as described previously [43].

Total DNA of the *B. burgdorferi* s.s. strain N40 and *B. garinii* PSth was isolated using Chelex 100 (Bio-Rad) [40]. Briefly, 100 μl of each *Borrelia* culture was centrifuged and the pellet resuspended in 40 μl of a 20% Chelex 100 suspension. The suspensions were incubated at 56°C for 30 min and, after thoroughly mixing, boiled for 10 min. Chelex 100 was removed by a final centrifugation step and the supernatant stored at −20°C. DNA of the *B. burgdorferi* s.s. strains B31 and CB53, *B. afzelii* VS461, *B. valaisiana* VS116 and *B. lusitaniae* PotiB3 were prepared using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer/s instructions.

Detection of *B. burgdorferi* s.l.

In 2008 and 2007, ticks were tested for *Borrelia* DNA by nested PCR according to Rijpkema *et al.* [44]. The first PCR mix contained 5 μl total DNA and final concentrations of 200 nM *B. burgdorferi* s.l. specific primers targeting the 5S-23S rDNA intergenic spacer region (23SN1 and 23SC1; [44]), 100 μM dNTPs, 1.5 mM $MgCl_2$ and 1.25 U GoTaq Flexi DNA Polymerase (Promega). The following DNA amplification step using 35 cycles was set up: 94°C for 30 sec, 53°C for 30 sec and 72°C for 1 min. For the second PCR, 5 μl of the first PCR product using the same PCR mix and the specific primers 23SN1 and 5SCB without biotin label of the 5S-23S rDNA intergenic spacer region were used [44]. The same thermal cycling conditions of the first PCR were set up for the second PCR but using a primer annealing temperature of 55°C. Positive and negative controls were always included, and nested PCR products were screened for *B. burgdorferi* s.l. DNA by agarose gel electrophoresis.

In 2001 [15], *Borrelia* infection in ticks were analyzed by simple and nested PCR and immunofluorescence assay (IFA) according to Liebisch *et al.* [45] (with modifications), Rijpkema *et al.* [44] and Kurtenbach *et al.* [38]/Kampen *et al.* [15], respectively. A third, modified PCR protocol originally performed by Schwartz *et al.* [46] was additionally applied in 2001 if contradictory results between the simple and nested PCR approaches occurred.

In 1987, Kurtenbach *et al.* [38] examined 1189 nymphal and adult *I. ricinus* and 1050 nymphs and adults in 1989 for *Borrelia* infection. *Borrelia* prevalences were calculated without specification of the respective year; only prevalences of 1987/89 were published. The same IFA protocol as used by Kampen *et al.* [15] in 2001 was carried out for the tick examinations in 1987/89 [38].

Genotyping of *Borrelia* species

A total of 249 tick samples positive for *B. burgdorferi* s.l. in 2007 and 2008 were further identified to the genospecies level [47]. Briefly, *B. burgdorferi* s.l. DNA was amplified by PCR using the 5′-biotinylated *Borrelia* specific B-5SBor primer and the 23SBor primer [48]. A touchdown PCR with an annealing temperature starting from 60°C to 52°C (1°C decrease per cycle) was set up to minimize amplification of non-specific DNA products. After the final annealing temperature was reached a further amplification step of 40 cycles using 52°C was carried out [49]. Amplification products were hybridized to 14 *Borrelia* specific oligonucleotide probes detecting the following genospecies [47]: *B. burgdorferi* s.l. (SL probe), *B. burgdorferi* s.s. (SS probe), *B. garinii* (GA, GANE and GANE1 probe), *B. afzelii* (AF probe), *B. valaisiana* (VSNE probe), *B. lusitaniae* (LusiNE, LusiNE1 and LusiNE2 probe), *B. spielmanii* (SpiNE2 and SpiNE3 probe) and *B. bissettii* (BisNE1 and BisNE2 probe). *B. burgdorferi* s.l., *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* probes were designed according to Rijpkema *et al.* [44]. All other probes (including probes GANE and GANE1) were used according to Gern *et al.* [47]. Hybridized products were visualized by chemiluminescence using the ECL Detection Reagent and Hyperfilm ECL (GE Healthcare). Negative controls were included, and *Borrelia* DNA of the different genospecies served as positive controls. Additionally, *Borrelia* PCR products that hybridized with the probes GA, GANE1 and LusiNE1 were sequenced using OspA primers in order to distinguish

between *B. garinii* and *B. bavariensis* genotypes as described previously [47,50].

Additionally, *Borrelia* samples from 2001 were genotyped by Kampen *et al.* [15] also using reverse line blotting. Similar to the studies in 2007 and 2008, probes for *Borrelia* identification targeted the 5S-23S rDNA spacer region. Probes were designed for the detection of *B. burgdorferi* s.l., *B. burgdorferi* s.s., *B. garinii*, *B. afzelii* and *B. valaisiana* according to Rijpkema *et al*. [44]. *B. lusitaniae, B. spielmanii* and *B. bissettii* identifications were not carried out in 2001.

Data analysis

Tick densities and *Borrelia* prevalences were compared between the different studies in the Siebengebirge from 1987/89 to 2008. For tick density comparisons, only the months May to September were compared from each study year and the densities were calculated for $100m^2$ of study site. Therefore, the average monthly tick abundances from 2003 were recalculated from $225m^2$ to $100m^2$.

For the comparison of *Borrelia* prevalences throughout the different study years, IFA data from 1987/89 and 2001 were compared and nested PCR *Borrelia* data from 2001 with the results from 2007 and 2008 because in those years the same experimental protocols were used. In 2001, the same ticks were examined by IFA and nested PCR by Kampen *et al*. [15].

Statistical analysis of data was performed using Prism 4 (GraphPad Software). Differences in tick abundances, infection prevalences with *Borrelia* and *B. burgdorferi* s.l. genospecies between the three study sites and study years were analyzed by the chi square test or Mann–Whitney U test. Only *Borrelia* prevalences derived from more than 20 ticks were statistically compared. Climate parameters between different years of the last two centuries were compared by a one - way analysis of variance (One -Way ANOVA) with a pairwise multiple comparison procedure (Tukey test), the Kruskal-Wallis test or the Mann–Whitney U test. Correlations of tick densities with *Borrelia* prevalences were tested using the Spearman's Rho rank correlation test. *P*-values of 0.05 or less were considered statistically significant for all tests.

Results

Abundances of *I. ricinus* in 2007 and 2008

Exclusively *I. ricinus* ticks were captured by blanket dragging. In 2007, a total of 1046 host-seeking ticks (50 adults, 996 nymphs) were collected (Table 2). In the Fraxino-Aceretum pseudoplatani, the plant community representing a low abundance biotope, the number of host seeking ticks decreased from May to July and increased up to September (Figure 1). In the other two plant communities, the Luzulo-Fagetum milietosum and the Galio-Fagetum typicum, that possessed higher numbers of ticks, the densities increased from May to June, decreased until August and increased slightly or remained at the same level in September (Figure 1). In 2008, a total of 1591 host-seeking *I. ricinus* (61 adults, 1530 nymphs) were collected at the three sites (Table 2). In the Fraxino-Aceretum pseudoplatani plant community more ticks were collected than one year before, and the monthly abundance graph showed a peak in June and no ticks in September (Figure 1). This was also evident for the site with the highest abundances, the Galio-Fagetum typicum, but at a higher abundance level, and a few ticks were collected in September (Figure 1). In the Luzulo-Fagetum milietosum, densities were similar in

Table 2 Number of *Ixodes ricinus* and infection rates with *B. burgdorferi* s.l. at three plant communities in the Siebengebirge in 2007 and 2008

Plant community	Tick stage*	2007							2008						
		Total no. of ticks	No. of infected ticks/ no. of examined ticks					Total infection rate (%)	Total no. of ticks	No. of infected ticks/ No. of examined ticks					Total infection rate (%)
			Month#							Month#					
			M	JN	JL	A	S			M	JN	JL	A	S	
Fraxino-Aceretum pseudoplatani	A	6	0/3	0/1	0/0	0/2	0/0	0	23	4/13	0/2	1/7	0/1	0/0	21.7
	N	100	12/46	1/13	0/1	0/5	7/35	20.0	340	5/47	11/58	8/46	4/32	0/0	15.3
Luzulo-Fagetum milietosum	A	18	1/4	0/12	0/2	0/0	0/0	5.6	15	0/8	0/0	0/6	1/1	0/0	6.7
	N	288	14/91	33/88	2/35	2/12	0/10	21.6	482	5/52	27/61	4/54	18/55	4/8	25.2
Galio-Fagetum typicum	A	26	0/9	1/6	1/6	0/2	2/3	15.4	23	1/8	2/9	0/1	1/5	0/0	17.4
	N	608	14/91	18/94	12/86	3/16	14/29	19.3	708	6/53	4/52	3/59	6/55	1/9	8.8
Total	A	50			5/50			10.0	61			10/61			16.4
	N	996			132/652			20.2	1530			106/641			16.5
	All	1046			137/702			19.5	1591			116/702			16.5

* The total number of collected adult (A) and nymphal (N) of *I. ricinus* at the three study sites are separately presented.
# Ticks were collected in May (M), June (JN), July (JL), August (A) and September (S) of 2007 and 2008 at the three study sites of the Siebengebirge.

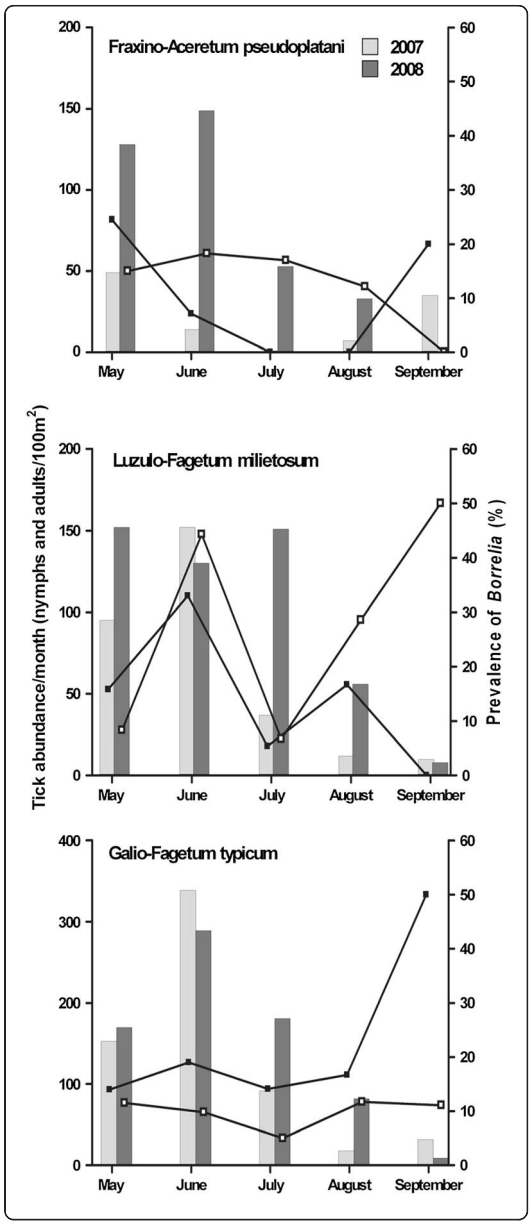

Figure 1 Monthly abundances of *I. ricinus* and *Borrelia burgdorferi* s.l. prevalences at three study sites in the Siebengebirge in 2007 and 2008. From May to September 2007 and 2008, host-seeking *I. ricinus* were collected by blanket dragging at three plant communities (Galio-Fagetum typicum, Fraxino-Aceretum pseudoplatani and Luzulo-Fagetum milietosum) in the nature reserve Siebengebirge, and the ticks were examined for *Borrelia* infection. The monthly abundances of ticks (columns) and *Borrelia burgdorferi* s.l. prevalences (line diagram) in 2007 (black squares) and 2008 (white squares) at the three study sites are presented in the figure.

May, June and July and decreased until September (Figure 1).

Summarizing the numbers of ticks of all sites per year, population densities increased significantly from 2007 to 2008 (χ^2 = 179.9, df = 4, p < 0.0001). Highest densities of 339 and 289 ticks/100m^2 were found in the Galio-Fagetum typicum in June 2007 and 2008, respectively (Figure 1), but densities did not differ statistically significantly in direct comparison of the numbers in the Galio-Fagetum typicum and the Luzulo-Fagetum milietosum in 2007 (χ^2 = 7.409, df = 4, p > 0.1). Excluding the lower numbers of collected ticks at all sites in September, densities increased from 2007 to 2008 up to 4.5-fold. This was caused by the enormous increase in the numbers of nymphal ticks since numbers of collected adults changed only slightly between the two years (Table 2).

B. *burgdorferi* s.l. infection rates in 2007 and 2008

In 2007, 137 of 702 examined ticks (19.5%; 5 adults and 132 nymphs of 50 adults and 652 nymphs) were infected with *B. burgdorferi* s.l. (Table 2). In 2008, 116 of 702 ticks (10 adults and 106 nymphs of 61 adults and 641 nymphs) were infected, resulting in a prevalence of 16.5%. In 2007, at all collection sites, infection rates of nymphal ticks were significantly higher than that of adults (χ^2 = 13.62, df = 2, p < 0.01, Table 2). However, in 2008 significantly more adults than nymphs were infected in the Galio-Fagetum typicum and Fraxino-Aceretum pseudoplatani sites (χ^2 = 13.44, df = 2, p < 0.01), but the overall *Borrelia* prevalence in 2008 did not differ between nymphs and adults (Table 2).

In the Galio-Fagetum typicum, overall *Borrelia* prevalences were lower in 2007 than in 2008 (Mann–Whitney U test, p=0.0286) (Figure 1). At the other two sites, the infection rates did not differ statistically significantly between both years. In addition, *Borrelia* prevalences and tick densities were not correlated for all sites and both years (Spearman's rank correlation, r = −0.3163, p>0.05). In 2007 and 2008, 0–33 and 0–27 infected ticks/100m^2 per month, respectively, were found in all plant communities. Excluding months representing prevalences based on <20 ticks, no strong differences in the prevalence between the different months were evident in the Fraxino-

Aceretum pseudoplatani and Galio-Fagetum typicum for both years; only the latter showed an increase of the infection rate up to 50% in September 2007. In the Luzulo-Fagetum milietosum, in both years prevalences of *Borrelia* in June were about two and six times higher than in May and July.

Distribution of *B. burgdorferi* s.l. genospecies in 2007 and 2008

The ticks of the Siebengebirge were infected with five genospecies, *B. garinii, B. afzelii, B. valaisiana, B. burgdorferi* s.s. and *B. lusitaniae*. *B. spielmanii, B. bissettii* and *B. bavariensis* could not be detected at any site in both years. In 2007, the two most prominent genospecies in single and multiple *Borrelia* infections were *B. garinii* (58.6%) and *B. afzelii* (56.4%). Overall infection rates of the other genospecies were: 9.0% of *B. valaisiana,* 5.3% of *B. burgdorferi* s.s. and 11.3% of *B. lusitaniae*. The highest rates of *B. garinii*, 78.4%, occurred in the Luzulo-Fagetum milietosum and of *B. afzelii*, 87.1%, in the Galio-Fagetum typicum (Figure 2). *B. lusitaniae* appeared only at two sites and only in spring (apart from one infected tick in the Fraxino-Aceretum pseudoplatani in September) with a prevalence of 27.5% in ticks from the Luzulo-Fagetum milietosum, and *B. burgdorferi* s.s. only occurred in the Galio-Fagetum typicum (Figure 2).

In 2008, the genospecies composition differed significantly from that of 2007 (χ^2 = 41.16, df = 4, p < 0.0001), but *Borrelia* infected *I. ricinus* also mainly contained *B. garinii* (overall infection prevalence at all sites including multiple infections: 53.4%). As in 2007, the highest prevalence with *B. garinii* occurred in the Luzulo-Fagetum milietosum (Figure 2). However, the overall infection prevalences of the other genospecies differed, with 26.7% for *B. afzelii,* 19.8% for *B. valaisiana* and 30.2% for *B. burgdorferi* s.s. At two sites *B. afzelii* prevalences were about 50% lower than in 2007, whereas *B. valaisiana* prevalences were higher in two plant communities (Figure 2). The strongest changes were evident for *B. burgdorferi* s.s. and *B. lusitaniae*. *B. burgdorferi* s.s. rates increased remarkably at two sites and this genospecies was detected for the first time in 2008 at all three sites. *B. lusitaniae* was not identified in 2008 in any plant community (Figure 2).

A comparison of the distribution of the different genospecies in nymphs and adults cannot be performed, since infections of only 15 adult ticks were identified; nine ticks with single infections covered one of the five genospecies (Table 3). Summarizing data of nymphs and adults for a comparison of single and multiple infections revealed the predominance of single *Borrelia* infections with *B. afzelii* or *B. garinii* in both years (Table 3). In 2007, both genospecies also predominated in six combinations of double infections (Table 3). The frequency of

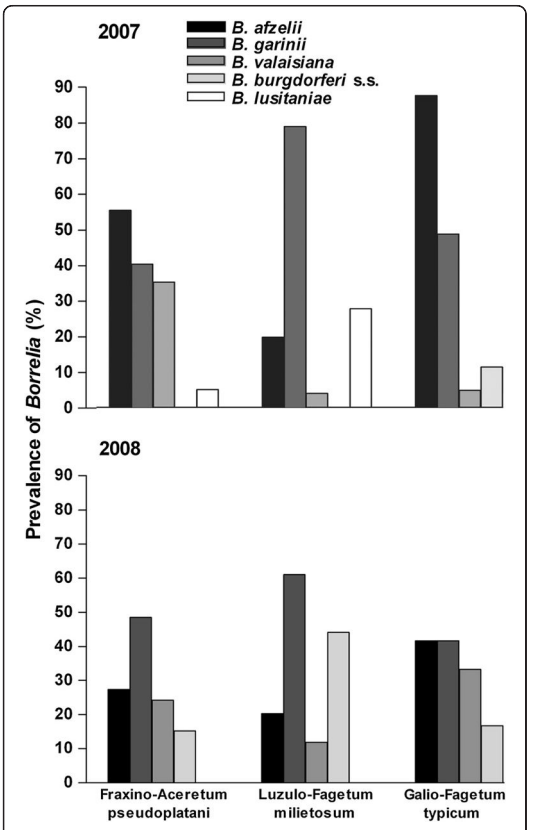

Figure 2 Prevalence of different *Borrelia burgdorferi* s.l. genospecies in *I. ricinus* at the three plant communities in the Siebengebirge in 2007 and 2008. Prevalences were calculated from the total number of infected ticks in the respective year and from the respective site used for the genospecies identification.

a specific *Borrelia* genospecies detected either as a single or a co-infection with other species did not differ significantly (χ^2 = 0.05, df = 3, p > 0.05), e.g. 35 ticks were singly infected with *B. afzelii* and 40 ticks with this genospecies as well as at least one other *Borrelia* species in 2007. A high rate of multiple infections of 32.3% is only evident for the *B. afzelii/B. garinii* double infection in the Galio-Fagetum typicum. In this biotope, also the only type of a triple infection with *B. afzelii, B. garinii* and *B. burgdorferi* s.s. occurred. Additionally, *B. burgdorferi* s.s. was only detected in double and these triple infections of *I. ricinus*, not as a single infection in 2007 (Table 3).

In 2008, significantly more single than multiple infections with *B. garinii* predominated at three study sites (χ^2 = 9.8, df = 3, p < 0.05) (Table 3). For *B. garinii* single infections ranged between 25.0% and 39.4% and for the

Table 3 Total number (No.) and percentage (%) of single and multiple infections of ticks with *Borrelia afzelii* (AF), *B. garinii* (GA), *B. valaisiana* (VA), *B. burgdorferi* s.s. (SS) and *B. lusitaniae* (LU) at the three collection sites

Plant community	Fraxino-Aceretum pseudoplatani				Luzulo-Fagetum milieotosum				Galio-Fagetum typicum			
Geno-	2007		2008		2007		2008		2007		2008	
species	No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]
AF	6	30.0	5[1]	15.2	3	5.9	2	3.4	26	41.9	4	16.7
GA	4	20.0	13[3]	39.4	25	49.0	23	39.0	6	9.7	6[1]	25.0
VA	3	15.0	6	18.2	1	2.0	4	6.8	1	1.6	5[2]	20.8
SS	0	0.0	4	12.1	0	0.0	12[1]	20.3	0	0.0	2	8.3
LU	0	0.0	0	0.0	7[1]	13.7	0	0.0	0	0.0	0	0.0
AF/GA	3	15.0	2[1]	6.1	7	13.7	1	1.7	20[3]	32.3	2	8.3
AF/VA	2	10.0	1	3.0	0	0.0	1	1.7	1	1.6	2	8.3
AF/SS	0	0.0	1	3.0	0	0.0	4	6.8	4[1]	6.5	1	4.2
GA/VA	1	5.0	1	3.0	1	2.0	2	3.4	1	1.6	0	0.0
GA/SS	0	0.0	0	0.0	0	0.0	6	10.2	0	0.0	1[1]	4.2
GA/LU	0	0.0	0	0.0	7	13.7	0	0.0	0	0.0	0	0.0
VA/LU	1	5.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
AF/GA/VA	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	4.2
AF/GA/SS	0	0.0	0	0.0	0	0.0	4	6.8	3	4.8	0	0.0
Total	20	100	33	100	51	100	59	100	62	100	24	100

[a] superscripts indicate the corresponding numbers of adult ticks with the respective infection.
[b] calculated from the total number of infected ticks used for genospecies identification in the respective year and from the respective site.

other genospecies between 3.4% and 20.8% in the different plant communities. Double infections were present in only up to 10.2% of infected ticks, mainly including *B. garinii, B. afzelii* and *B. valaisiana.* At all sites, *B. burgdorferi* s.s. was detected as a single infection. Comparing double infections of both years *B. afzelii* and *B. garinii* appeared always in combination with three of the other four genospecies. Four ticks with triple infections in the Luzulo-Fagetum milietosum contained the same genospecies combination as the ticks in 2007 from the Galio-Fagetum typicum. In the latter biotope, a new combination with *B. afzelii, B. garinii* and *B. valaisiana* occurred in one tick.

Climate conditions in the region of Bonn between 1987 and 2008

Climate records of the region of Bonn, near the Siebengebirge, revealed no striking differences in the mean monthly air temperatures between any year of 1987 until 2008 (One Way ANOVA, $p > 0.05$, Table 4). Furthermore, no statistically significant differences in the average monthly winter air temperatures from November to February before the collection periods in 1987, 1989, 2003, 2007 and 2008 were evident (Kruskal-Wallis test, $p > 0.05$, Table 4).

Precipitation levels in the region of Bonn during the tick collection months differed significantly between

Table 4 Mean monthly air temperatures [°C] 2m above the soil near the Siebengebirge[a] in different years

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Year	Winter months [b]
1986	2.6	−4.1	4.9	6.9	14.9	17.7	18.3	17.1	11.5	11.6	7.6	4.3	9.4	-
1987	−3.5	1.8	2.3	12.6	10.3	14.8	18.0	16.8	15.8	10.8	6.1	3.9	9.1	2.6
1988	5.8	4.0	4.9	9.4	15.9	20.6	17.4	17.7	14.1	11.1	5.2	6.5	11.1	-
1989	4.1	4.4	8.8	11.4	15.6	16.0	19.4	18.3	15.5	12.1	5.0	4.7	11.3	5.1
2002	3.2	7.0	7.3	9.7	14.3	18.0	17.9	18.8	13.9	10.0	8.3	3.9	11.0	-
2003	1.7	1.4	8.1	10.3	14.6	19.7	19.8	20.8	14.9	7.6	8.6	3.7	10.9	3.8
2006	0.2	1.8	3.8	9.1	14.5	17.8	23.3	16.2	18.4	14.0	8.9	5.8	11.2	-
2007	6.5	6.2	7.9	13.8	15.3	18.0	17.8	17.0	13.5	9.8	5.7	3.3	11.2	6.9
2008	5.9	5.1	5.8	8.8	16.5	17.3	18.2	18.2	13.0	10.2	6.5	2.0	10.6	5.0

[a] Data were obtained from the weather station Cologne/Bonn (no. 10513) of the Deutscher Wetterdienst.
[b] The mean temperature of the winter months were determined by calculating the mean monthly temperature from November of the previous year to February of the following year stated in the beginning of each line. For example, for 2007 the mean average temperature of the winter months was calculated from November 2006 until February 2007.

the collection years 1978, 1989, 2003, 2007 and 2008 (Kruskal-Wallis test, $p<0.05$) with high precipitation levels from May to August in 1987 and 2007 (Table 5). Significantly higher precipitation levels were also recorded in 2007 compared to 2003 (Mann–Whitney U test, $p<0.05$).

Discussion

Comparison of tick densities and climate conditions between the different study years

Long-term investigations on the distribution of the tick *I. ricinus* and on *Borrelia* infection rates in these ticks are rare, and in Germany these investigations have been only performed since 1987 in the nature reserve Siebengebirge, a very popular recreation area of the Bonn-Cologne region. The nature reserve possesses a very species-rich vegetation with approximately 100 different plant communities [51] which support the development of *I. ricinus* differently [8]. According to a study monitoring tick densities including Geographic Information Systems (GIS) in 2003, 57% of the total area of the nature reserve possesses very high (≥51 ticks/100m^2) to medium tick densities (11–40 ticks/100m^2) [8]. Comparing exactly the same sites examined since 1987, tick densities changed considerably between 1987/89 and 2008 (Figure 3). In the Fraxino-Aceretum pseudoplatani (covering 0.3% of the nature reserve) the number of ticks/year/100m^2 decreased from 1987 until 2003 to a minimum of 9 ticks/100m^2 in 2003 and increased about 8-fold until 2008. In the Luzulo-Fagetum milietosum which covers 3% of the total area of the nature reserve (24% including all Luzulo-Fagetum sub-associations), the tick population density enormously decreased between 1987 and 2003, but then increased continuously to a 3-fold higher density compared to 2003. In the Galio-Fagetum typicum, the abundance increased continuously from 13 ticks/100m^2 in 1987 to 146 ticks/100m^2 in 2008. An assessment of the tick numbers for the entire Siebengebirge according to the GIS evaluation

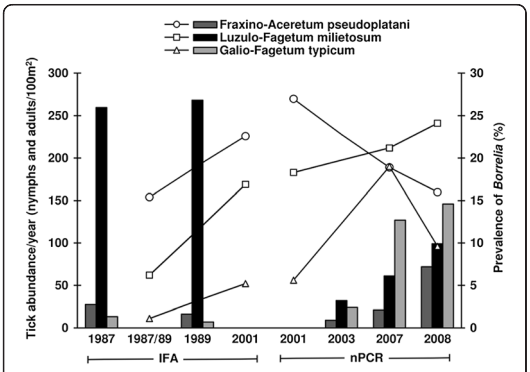

Figure 3 Abundances of ticks at three plant communities in the Siebengebirge and *Borrelia burgdorferi* s.l. prevalences from 1987 to 2008. Abundances are presented as columns: Fraxino-Aceretum pseudoplatani (dark grey), the Luzulo-Fagetum milietosum (black) and the Galio-Fagetum typicum (light grey). Monthly tick abundances per 100m^2 of study site from May to September of each study year were calculated; in 2001 tick densities were not determined [15]. *Borrelia* prevalences for the Fraxino-Aceretum pseudoplatani, the Luzulo-Fagetum milietosum and the Galio-Fagetum typicum, are presented as circles, squares and triangles, respectively. Tick samples from 1987/89 and 2001 were examined for *Borrelia* infection by the immunofluorescence assay (IFA) using the same experimental protocol [15,38]. The same ticks used for IFA in 2001 were also analyzed for *Borrelia* infection by nested PCR (nPCR) [15]. Additionally, the same nPCR approach from 2001 was employed to analyze *I. ricinus* in 2007 and 2008. Accordingly, *Borrelia* prevalences from 1987/89 were compared with results from 2001, and 2001 prevalence data were compared with the data from 2007 and 2008. *Borrelia* infection rates were not determined in 2003 [8].

of *I. ricinus* in the Siebengebirge by Schwarz *et al.* [8] suggested that the increase in tick numbers may have a huge impact on the total number of ticks in the entire Siebengebirge because the Galio-Fagetum typicum is the third largest plant community, covering 10% of the total area of the nature reserve [52]. However, for example, differences in the host cenosis at the same plant communities in different areas of the Siebengebirge can change the distribution of tick populations.

Table 5 Monthly precipitation heights [mm] near the Siebengebirge[a] in different years

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Year	Summer months[b]
1987	59.6	68.9	104.0	39.0	70.6	123.0	126.8	83.3	50.2	38.2	80.6	42.3	73.9	100.9
1988	72.3	68.1	160.0	31.0	19.2	49.9	134.8	27.1	52.2	58.2	85.9	112.1	72.6	57.8
1989	27.6	57.1	65.8	103.1	26.3	61.1	49.9	67.9	37.8	62.6	38.4	95.1	57.7	51.3
2002	56.3	122.0	58.9	75.1	44.8	43.9	89.4	97.2	29.4	89.9	93.5	90.5	74.2	68.8
2003	77.1	29.2	43.5	43.4	55.2	72.7	53.4	55.3	50.8	95.1	45.6	54.0	56.3	59.2
2006	25.3	64.6	79.3	68.4	99.3	37.5	17.0	96.5	32.8	38.0	85.0	55.9	58.3	62.6
2007	82.4	59.8	53.5	1.6	129.6	105.8	127.8	193.0	59.9	33.2	70.8	52.2	80.8	139.1
2008	35.6	38.5	71.0	64.0	36.6	89.8	131.7	62.9	52.1	80.4	45.1	55.9	63.6	80.3

[a] Data were obtained from the weather station Cologne/Bonn (no. 10513) of the Deutscher Wetterdienst.
[b] The mean precipitation heights of the summer months were obtained by calculating the monthly precipitation from May to August of the same year.

Temperature is one of the most important abiotic factors for tick development [5,53,54], and global warming during the last two decades of the 20th century is suggested to be one of the reasons for increasing tick abundances in Sweden or Great Britain [4,55]. Ticks were collected at similar air temperature conditions from May to September in all years [8,15,38] with a minimum recorded temperature of 14.7°C in August 2007 in the Luzulo-Fagetum milietosum (data not shown). Additionally, air temperatures of the region of Bonn did not significantly differ between the years. However, in comparison to other tick collection seasons relatively high air temperatures from November 2006 until April 2007 (8.9°C-13.9°C) were recorded that may have caused a higher survival rate of ticks in 2006 as well as a stronger increase of tick population densities in spring 2007 and thus an overall increase in abundances during the entire tick season in 2007.

In addition to temperature, humidity affects the development of ticks. The relative humidity at the three sites and during the tick collections differed between the years [8,15,38]. In 1987, 1989 and 2001, ticks were collected at a minimum of 80% relative humidity at the sites, and in the years 2003, 2007 and 2008 minimum relative humidities of 57% [8], 45% and 52% (Table 1), respectively, were recorded at the biotopes. During all 15 tick collections in 2003 less than 70% relative humidity occurred at all field sites [52], and in 2007 only in 7 out of 15 collections more than 70% relative humidity was recorded (data not shown). In 2008, a minimum of 70% relative humidity was reached during 6 out of 15 tick collections. Although from 2003 to 2008 in 62% of all collections drier climatic conditions occurred compared to 1987 and 1989, higher tick abundances were determined in some of the biotopes between 2003 and 2008 (Figure 3). The saturation deficit that depends on the air temperature and the relative humidity influences the questing behavior of ticks [5,56]. Therefore, numbers of questing ticks increased until a certain limit of the saturation deficit [57]. This may explain the increase in questing ticks at the different study sites from 2003 to 2008. However, the high air temperatures in summer 2003 most likely corresponded with the lower yearly tick densities in the Fraxino-Aceretum pseudoplatani and Luzulo-Fagetum milietosum (Figure 3), because the saturation deficit was very high at these sites, and a high saturation deficit can rapidly decrease the numbers of questing ticks [56].

In a correlation of abiotic factors and tick abundances, the number of host seeking ticks rose significantly with rising soil water content in 2003 [8] and that may also explain the increase in tick numbers in 2007. Comparing all three different plant communities, the Fraxino-Aceretum pseudoplatani soil had the lowest water content and this correlated with low numbers of collected ticks [8]. The soil water content is affected by the precipitation. Overall higher precipitation levels in the region of Bonn in 2007 compared to 2003 combined with the higher air temperatures during the winter and spring may explain the higher tick numbers in 2007 in comparison to 2003. In addition, the soil water content of the different sites was 1–2 times higher in May and June 2007 (data not shown) than in the dry summer 2003. Furthermore, the optimal abiotic conditions in summer 2007 most likely caused a strong increase in the density of the tick population which was not strongly reduced by the mild winter 2007/2008 (Table 4) and resulted in higher numbers of questing ticks in 2008, respectively. These data indicate an effect of global warming on the number of ticks, but a continuous monitoring of tick densities covering several years and a determination of soil water contents are necessary for a better conclusion.

Comparison of *Borrelia* prevalences between the study years

Borrelia infection rates were sometimes positively correlated with tick densities [58,59]. However in the Siebengebirge, the prevalences of *Borrelia* decreased in the Fraxino-Aceretum pseudoplatani and the Galio-Fagetum typicum from 2007 to 2008, whereas the total number of ticks increased in these biotopes (Figure 3). Only the Luzulo-Fagetum milietosum showed consistently increasing infection rates of ticks with *Borrelia* from 1987 to 2001 (IFA data comparison) and from 2001 to 2008 (nested PCR data) as well as increasing tick abundances since 1987 (apart from the exceptional high abundances in that biotope in 1987 and 1989) [8,15]. Since climate factors cannot explain this phenomenon, biotic factors should be considered. Tick populations strongly depend on their hosts and infections with *Borrelia* can only be obtained from hosts [60-62]. Rabbits, foxes, roe and red deer, wild boar, mice and voles are abundant in the Siebengebirge and hosts of *I. ricinus*. Of these, *Apodemus sylvaticus*, *A. flavicollis* and *Clethrionomys glareolus* have been confirmed as reservoir hosts of *Borrelia* in the nature reserve [37]. Reservoir hosts of *Borrelia* differ considerably in their competence to acquire the infection and to enable a multiplication of the spirochetes for a successful transmission [61]. The reservoir potential of *Apodemus* spp. and *C. glareolus* differed even between biotopes in the Siebengebirge [37] which may be caused by differing host immune responses, for example by tick density-dependent resistance of the host against tick feeding. Another reservoir host for *B. burgdorferi* s.l. is the wild boar [63-66]. In the Siebengebirge, the numbers increased enormously during the last 50 years [67], but hunted boars were not strongly infested by ticks [68,69]. Much

stronger infestation was seen in roe deer, which are abundant in the Siebengebirge, thus supporting tick populations [70]. However, roe deer are not a competent host for *B. burgdorferi* s.l. [71,72], most likely resulting in a so called dilution effect of *Borrelia* [73].

Such investigations are also required to explain the increased number of multiple infections with *Borrelia* in *I. ricinus* from the Siebengebirge. In 2007, significantly more ticks possessed multiple infections than in 2001 (χ^2 = 7.7, df = 2, p < 0.05) [15]. In 2001, only seven double infections with *Borrelia* were detected [15], whereas 7-fold and 4-fold more multiple infections were recorded in 2007 and 2008, respectively. Increased numbers of ticks within the last few years may have given rise to the probability of more ticks co-feeding on the same host and this may have led to an increased exchange of different genospecies between host and ticks resulting in a higher burden of different *Borrelia* genotypes per tick and host [74] However, the lower percentages of double infections in the Siebengebirge in 2008 in comparison to 2007 indicate the existence of specific factors and no general trend. For the first time in the Siebengebirge, triple infections were detected; one type of infection in 2007 and two different types of *Borrelia* combination in 2008. In 1987/89 no discrimination between single and multiple *Borrelia* infections were carried out. Initially, more single than double infections of ticks with *Borrelia* were reported for different sites in Europe [18,75,76]. However, not only in the Siebengebirge, but also in Ireland and Denmark the percentages of mixed infections increased, and even quadruple infections occurred [77,78]. Multiple infections can increase the risk of infections by Lyme disease since the chance of infections of a competent vector is increased.

Comparison of *Borrelia* genospecies between 2001, 2007 and 2008

Estimations of the infection risk require not only a determination of the numbers of ticks and the infection rates with *B. burgdorferi* s.l., but also determinations of the genospecies. *B. afzelii, B. garinii* and *B. valaisiana* are the three most abundant species in Europe [29]. This is also the case in the Siebengebirge, but the prevalence of *B. burgdorferi* genospecies has changed during the few last years. In 2001, at all three sites the most prominent genospecies was *B. valaisiana* (infection rate of 43.1%) [15], whereas in 2007 *B. garinii* and *B. afzelii* were detected in every second tick, and in 2008 *B. garinii* was the dominant species. Also changes of low-abundant genospecies occurred within the two collection years of the present study: For example, in 2007 *B. burgdorferi* s.s. was only detected in 7 out of 137 infected ticks, but one year later this species was found in 35 out of 116 ticks. Also *B. valaisiana* was rarely found in 2007 but in 19.8% of *Borrelia* infections in 2008.

Differences in the genospecies composition were also evident between the three study sites. In the Fraxino-Aceretum pseudoplatani, *B. valaisiana* as dominant species in 2001 did not re-appear to this extent in the present study [15]. Only the Luzulo-Fagetum milietosum showed a stable dominance of *B. garinii* with similar infection rates in all years. In the Galio-Fagetum typicum, in 2001 *B. garinii* and *B. afzelii* predominated, in 2007 only *B. afzelii,* and in 2008 similar numbers of ticks were infected with *B. garinii*, *B. valaisana* and *B. afzelii*. Such differences in the distribution of genospecies seem to be caused by differences in the host cenosis [29] and be based on different competences of vertebrate hosts for the respective genospecies. *B. afzelii* is mainly found in rodents such as *Apodemus* sp.,and *B. garinii* and *B. valaisiana* are associated with birds [31]. The complement system of rodents completely lyses different genotypes of *B. garinii* and *B. valaisiana* but not *B. afzelii* [79]. Vice versa, in birds the complement system lyses *B. afzelii,* but not *B. garinii* and *B. valaisiana* [79]. Comparing the reservoir capacity of different birds, pheasants (*Phasianus colchicus*) and passerines such as the European blackbird (*Turdus merula*) and the American robin (*T. migratorius)* were positively associated with *Borrelia* infections [80-82]. Both, rodents and birds are competent reservoir hosts of *Borrelia burgdorferi* s.s. [32]. In the Siebengebirge in all study years, the two bird genospecies, *B. garinii* and *B. valaisiana,* together predominated. Thus, birds in the Siebengebirge seem to be the most successful reservoir host for *Borrelia,* a phenomenon that was also suggested for 2001 [15]. Passerine birds are widely distributed in the Siebengebirge, and thus they have a high impact on the density of ticks.

B. lusitaniae was detected for the first time in the Siebengebirge in 2007. We cannot exclude it for 2001 since DNA probes for *B. lusitaniae*, *B. spielmanii* and *B. bissettii* were not used in that year. However, only 4 out of 65 *Borrelia*-positive tick samples in 2001 reacted only with the complex specific *B. burgdorferi* s.l. probe [15]. Initially, this genospecies was classified as non-pathogenic for humans because this species was not detected in humans but only found in animal hosts [83,84]. However, serious symptoms of Lyme borreliosis were induced in mice infected by *B. lusitaniae* [85], and recently the first isolate of this genospecies was found in a woman suffering from chronic skin lesions in Portugal [28]. Thus, *B. lusitaniae* represents a new *Borrelia* genospecies with a new risk for visitors of the Siebengebirge to be infected with Lyme disease. *B. lusitaniae* was frequently present in ticks from Mediterranean countries such as Portugal (first record), Tunisia and Morocco [84,86,87]. It was also found in the Czech Republic,

Poland, Slovakia, Moldavia, Ukraine, Spain, France, Switzerland and South Germany [36,75,83,88-91]. The recent identifications in Denmark and Sweden demonstrated the ability of this genospecies to establish even in northern Europe [77,92]. In the Siebengebirge, *B. lusitaniae* was found in 15 ticks (13 nymphs and one adult from the Luzulo-Fagetum milietosum and one from the Fraxino-Aceretum pseudoplatani). The latter site was near the Luzulo-Fagetum milietosum. Half of these ticks were co-infected with *B. garinii* and one tick with *B. valaisiana* indicating that birds may have introduced this species to the Siebengebirge; a similar observation was made in Switzerland [93]. Birds are considered as main reservoir hosts for *B. lusitaniae* [35], but sand lizards (*Lacerta agilis*) and common wall lizards (*Podarcis muralis*) were also infected with *B. lusitaniae* in Germany [36]. These two lizard species exist in the Siebengebirge, but they are rare and thus presumably less important for the distribution of *B. lusitaniae* in the Siebengebirge. Since almost all *B. lusitaniae* were detected in May and June of 2007 (apart from one infected tick in the Fraxino-Aceretum pseudoplatani in September) but not again in 2008, and since the distribution of *Borrelia* is linked to the migration of birds [31] future investigations in *Borrelia* transmission in the Siebengebirge should consider migratory birds as potential hosts of *Borrelia*. The maintenance of *B. lusitaniae* in the local bird fauna of the Siebengebirge is rather unlikely because in that case *B. lusitaniae* should have been detected frequently alongside with *B. garinii* and *B. valaisiana* in ticks. However, although recently Norte *et al.* [94] confirmed *B. lusitaniae* in questing *I. ricinus* they could never detect this *Borrelia* species in the local bird fauna in Portugal nor in migratory birds. Instead, two *Borrelia* isolates were identical to *B. lusitaniae* detected in mice skin in Portugal [95] and in ticks feeding on lizards from central Europe, Madeira and Portugal [36,96,97]. Thus, mice and lizards may maintain *B. lusitaniae*, and birds only play a minor, temporary role in the *B. lusitaniae* distribution. However, mice commonly occur in the Luzulo-Fagetum milietosum and Fraxino-Aceretum pseudoplatani and nevertheless *B. lusitaniae* was only detected for a short time in 2007. Furthermore, Amore *et al.* [98] found *B. lusitaniae* only in ticks feeding on lizards, but not in ticks feeding on mice and birds. Thus, the distribution and maintenance of *B. lusitaniae* remains unclear and further investigations are needed including the analysis of ticks feeding on mice, lizards and birds in the Siebengebirge.

Conclusions

Over the last two centuries tick densities have increased in the Siebengebirge, a dense forested nature reserve providing excellent abiotic and biotic conditions for ticks, without changes of the biotopes by human activities. These increases were most likely favored by climatic conditions. Although *Borrelia* infection prevalences did not increase simultaneously with increasing tick densities in all biotopes, significantly higher multiple infections of ticks with *Borrelia* occurred in 2007 than in 2001; for the first time triple infections with *Borrelia* were detected in 2007 and 2008 in the Siebengebirge. Furthermore, a new *Borrelia* species, *B. lusitaniae,* has been introduced to the Siebengebirge. Thus, the risk for visitors, woodmen, hunters, farmers and animals of the nature reserve Siebengebirge of being exposed to tick bites increased strongly since 1987, however, the risk of being infected by Lyme disease did not increase consequently simultaneously. Nevertheless, the increase of multiple *Borrelia* infections in ticks may represent a new potential risk factor.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AS, GAS and LG drafted the manuscript. AS and GAS designed the study and AA collected the ticks in the Siebengebirge, Germany. AS and CB performed the tick examinations of the samples in 2007, and VH and ZV carried out all further experiments of samples from 2008 and all genotyping experiments. All authors read and approved the final manuscript.

Acknowledgements
The authors thank Carina Schleiden for her assistance in the tick collections and Kathrin Hartelt and Rainer Oehme for providing *Borrelia* strains as positive control samples for the experiments. A.S. was funded by the Alexander von Humboldt Foundation (Feodor Lynen Research Fellowship). We also acknowledge the financial support of the Grant Agency of the Czech Republic (GACR grant no. 206/09/H026), the Ministry of Education, Youth and Sports of the Czech Republic (MSMT grant no. LC 06009 and MSM 6007665801), the European Union (FP7 HEALTH, ANTIGONE project 278976) and the Academy of Sciences of the Czech Republic (grant no. Z60220518). We are also very grateful for the financial support of parts of this study by WEPA APOTHEKENBEDARF GmbH & Co KG, Hillscheid, Germany.

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Received: 13 May 2012 Accepted: 4 November 2012
Published: 21 November 2012

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doi:10.1186/1756-3305-5-268
Cite this article as: Schwarz *et al.*: Abundance of *Ixodes ricinus* and prevalence of *Borrelia burgdorferi* s.l. in the nature reserve Siebengebirge, Germany, in comparison to three former studies from 1978 onwards. *Parasites & Vectors* 2012 **5**:268.

4.3 Paper III

Prevalence of *Borrelia miyamotoi* in *Ixodes* Ticks in Europe and the United States

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Emerging Infectious Diseases 20(10): 1678-1682

Prevalence of *Borrelia miyamotoi* in *Ixodes* Ticks in Europe and the United States

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Borrelia miyamotoi, a relapsing fever-related spirochete transmitted by *Ixodes* ticks, has been recently shown to be a human pathogen. To characterize the prevalence of this organism in questing *Ixodes* ticks, we tested 2,754 ticks for a variety of tickborne pathogens by PCR and electrospray-ionization mass spectrometry. Ticks were collected from California, New York, Connecticut, Pennsylvania, and Indiana in the United States and from Germany and the Czech Republic in Europe from 2008 through 2012. In addition, an isolate from Japan was characterized. We found 3 distinct genotypes, 1 for North America, 1 for Europe, and 1 for Japan. We found *B. miyamotoi* infection in ticks in 16 of the 26 sites surveyed, with infection prevalence as high as 15.4%. These results show the widespread distribution of the pathogen, indicating an exposure risk to humans in areas where *Ixodes* ticks reside.

Ixodes ticks can transmit a variety of pathogens, including viruses, bacteria, and protozoa (*1*). *Borrelia* spirochetes are one of the genera of bacteria transmitted by *Ixodes* ticks. Most *Borrelia* that infect ticks belong to the *Borrelia burgdorferi* senso lato group and include *B. burgdorferi* senso stricto, *B. garinii*, and *B. afzelii*, all of which cause Lyme disease in humans (*1*). *Borrelia miyamotoi* has been found in a variety of *Ixodes* ticks and is more closely related to the relapsing fever spirochetes that infect soft ticks than to the bacteria that cause Lyme disease (*2*).

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DOI: http://dx.doi.org/10.3201/eid2010.131583

B. miyamotoi found in Europe and the United States also cause disease in humans (*3–5*). A study in Russia has shown that the spirochete *B. miyamotoi* has the ability to infect humans; infections with *B. miyamotoi* cause symptoms similar to those seen with relapsing fever, as well as erythema migrans-like skin lesions on rare occasions (*6*). *B. miyamotoi* has been found in ticks of the following species: *Ixodes scapularis* and *I. pacificus* in the United States, *I. persulcatus* in Japan, and *I. ricinus* and *I. persulcatus* in Europe and Asia (*2*,*7–11*). In North America, *B. miyamotoi* has been found as far north as the Canadian provinces of Ontario and Nova Scotia (*12*). In the United States, the geographic range of *B. miyamotoi* is from the Northeast to California and has been reported as far south as Tennessee (*7*,*8*,*13–15*). Previous studies have shown that *B. miyamotoi* can be placed into different genetic groups based upon its geographic location and has some variation within the genographic groups (*6*,*9*).

To examine the prevalence distribution and diversity of *B. miyamotoi* in *Ixodes* ticks, we screened individual ticks by PCR and electrospray ionization mass spectrometry (PCR/ESI-MS) to detect tickborne pathogens, including *B. miyamotoi* (*16*). This approach has been used to characterize tickborne microorganisms, including *Ehrlichia* and *Borrelia*, from clinical specimens, heartworms in canine blood, and naturally occurring tick endosymbionts (*16–19*). Ticks that tested positive for *B. miyamotoi* were further characterized by using a *Borrelia* genotyping assay to assess genetic diversity (*20*).

Materials and Methods

B. miyamotoi Culture Isolate

The *B. miyamotoi* strain Fr74B was obtained by the Centers for Disease Control and Prevention (Fort Collins, CO, USA), as a culture isolate. This strain was originally isolated from an infected *Apodemus argenteus* field mouse

from Japan. The DNA from this strain was isolated by diluting the culture 1:10 with phosphate-buffered saline and heating to 95°C for 10 min. The raw lysate was then used in the *Borrelia* PCR/ESI-MS genotyping assay (Abbott Laboratories, Des Plaines, IL, USA) at 1 mL per PCR well (*20*).

Ixodes Tick Collection and Extractions

Ticks were obtained from most locations by flagging during 2008–2012. In Germany, a subset of ticks were also obtained after they were removed from persons. The species of *Ixodes* tick was determined by an entomologist and confirmed by the detection of the species-specific endosymbionts (*19*). The numbers and locations of the collection sites are described in Table 1.

Nucleic acids were extracted from ticks according to a published protocol by using bead-beating homogenization followed by isolation of RNA and DNA with DNeasy Blood and Tissue Kit columns (QIAGEN, Valencia, CA, USA) instead of the published QiaAmp Virus Elute Kits (*21*). A negative control consisting of a lysis buffer without a tick was with each set of extractions. Ticks from the United States were processed at Ibis Biosciences (Carlsbad, CA, USA). Ticks collected from the European countries were isolated at their respective sources. Nucleic acid samples from Germany and the Czech Republic were shipped to Ibis at ambient temperatures; those from Czech Republic were shipped after being stabilized by RNAstable (Biomatrica, San Diego, CA, USA) per the manufacturer's instructions.

Molecular Detection and Genotyping of *B. miyamotoi* from Nucleic Acid Extracts

B. miyamotoi was detected and identified by using a previously described broad-range PCR/ESI-MS assay designed to detect tickborne pathogens (*16*). For each set of samples analyzed with the assay, an extraction negative control sample as well as a PCR plate negative-control sample of water was included. A PCR-positive control was already built into the plate for each well in the form of a calibrant (*20*). Amplicons were analyzed by using a research use only PLEX-ID system (Abbott Laboratories). Samples positive for *B. miyamotoi* were further characterized by using a *Borrelia* PCR/ESI-MS genotyping assay as described that is designed to differentiate between *Borrelia* species and genotypes (*20*). PCR/

Table 1. Prevalence of *Borrelia miyamotoi* in *Ixodes* ticks, Europe and the United States, 2008–2012*

Region/subregion	Species	Total no. ticks tested (nymphs; adults)	No. ticks positive for *B. miyamotoi* (% of total)
Czech Republic			
Zavadilka	*I. ricinus*	153 (153; 0)	4 (2.6)
Blatna	*I. ricinus*	100 (100; 0)	2 (2.0)
Dacice	*I. ricinus*	93 (93; 0)	3 (3.2)
Netolice	*I. ricinus*	89 (89; 0)	0 (0)
Germany			
Constance	*I. ricinus*	226 (0; 48)*	4 (1.8)
United States			
Connecticut			
Fairfield County	*I. scapularis*	322 (309; 13)	16 (5.0)
Litchfield County	*I. scapularis*	18 (18; 0)	0
New London County	*I. scapularis*	29 (29; 0)	0
New York			
Dutchess County	*I. scapularis*	357 (357; 0)	2 (0.56)
Suffolk County	*I. scapularis*	180 (24; 156)	2 (1.1)
Westchester County	*I. scapularis*	44 (0; 44)	3 (6.8)
Pennsylvania			
Chester County	*I. scapularis*	80 (79; 1)	2 (2.5)
Indiana			
Pulaski County	*I. scapularis*	81 (0; 81)	10 (12.3)
California			
Alameda County	*I. pacificus*	22 (0; 22)	1 (4.5)
Del Norte County	*I. pacificus*	33 (0; 33)	0
Glenn County	*I. pacificus*	44 (0; 44)	0
Humbolt County	*I. pacificus*	74 (0; 74)	0
Lake County	*I. pacificus*	129 (0; 129)	0
Marin County	*I. pacificus*	85 (0; 85)	1 (1.2)
Mendocino County	*I. pacificus*	57 (0; 57)	2 (3.5)
Napa County	*I. pacificus*	65 (0; 65)	10 (15.4)
Orange County	*I. pacificus*	15 (0; 15)	0
Placer County	*I. pacificus*	250 (0; 250)	4 (1.6)
San Bernardino County	*I. pacificus*	18 (0; 18)	0
Santa Cruz County	*I. pacificus*	64 (0; 64)	0
Sonoma County	*I. pacificus*	126 (126; 0)	2 (1.6)

*A total of 119 ticks were removed from humans, and the life stage of 178 of the 226 ticks tested was not recorded.

ESI-MS assay provides genetic information about the PCR amplicon in the form of A, G, C, and T basecounts, and *B. miyamotoi* detection was defined as positive when one or more primer pairs produced an amplicon basecount signature that was unique to *B. miyamotoi*. Although most researchers agree that the nymphal stage of *Ixodes* ticks is the most epidemiologically essential life stage for transmission of *B. burgdorferi* sensu lato, because little is known about the transmission of *B. miyamotoi* from *Ixodes* ticks to humans, the data for both nymphs and adults were combined.

Sequence Confirmation of *B. miyamotoi* Detections

Representative samples positive for *B. miyamotoi* were selected for 16S Sanger sequencing. Primers were designed to amplify a 676-bp region of the 16S rRNA gene for *Borrelia*. A M13 tag was added to each primer for sequencing. The M13 forward sequence tag was 5′-CCC AGT CAC GAC GTT GTA AAA CG-3′, and the reverse tag was 5′-AGC GGA TAA CAA TTT CAC ACA GG-3′. The forward primer used was 5′-M13-CGC TGG CAG TGC GTC TTA AG-3′, and the reverse primer was 5′-M13-GCG TCA GTC TTG ACC CAG AAG TTC-3′. The amplification of the 16S rRNA genes was performed in a 50 mL reaction containing 1 mL nucleic acid extract, 1 unit of Platinum Taq High Fidelity polymerase (Invitrogen, Carlsbad, CA, USA) or Immolase Taq (Bioline, Randolph, MA, USA), the manufacturer's PCR buffer, 2.0 mmol/L $MgSO_4$, 200 μmol/L dATP, 200 μmol/L dCTP, 200 μmol/L dTTP, 200 μmol/L dGTP (Bioline), and 250 nmol/L of each primer. The following PCR cycling conditions were used on an MJ Dyad 96-well thermocycler (Bio-Rad Inc., Hercules, CA, USA): 95°C for 2 min, followed by 8 cycles of 95°C for 15 s, 50°C for 45 s, and 68°C for 90 s, with the 50°C annealing temperature increasing 0.6°C for each cycle. PCR was continued for 37 additional cycles of 95°C for 15 s, 60°C for 15 s, and 68°C for 60 s. The PCR cycle ended with a final extension of 4 min at 72°C. Reactions were visualized by electrophoresis on 1% agarose gels to ensure the presence of appropriately-sized products before being sent to SeqWright (Houston, TX, USA) for purification and sequencing with M13 primers. Resulting sequences were trimmed of primer sequences and a consensus created. The consensus sequence was analyzed with NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nucleotide database to determine the species.

Results

Multilocus PCR/ESI-MS Genotyping of *B. miyamotoi*

The multilocus *Borrelia* PCR/ESI-MS genotyping assay differentiates strains and species of *Borrelia* by their unique combination of basecount signatures. To characterize the prevalence of *B. miyamotoi* in *Ixodes* ticks we examined the basecount signatures from ticks that were positive for *B. miyamotoi*. Positive specimens from each of the 3 regions (United States, Europe, and Japan) typically produced basecount signatures at 5 of the 8 loci evaluated in the *Borrelia* genotyping assay. Based upon these 5 signatures, *B. miyamotoi* from the United States, Europe, and Japan are distinct genotypes (Table 2). All the specimens from North America had the same basecount signatures for the 5 detecting primer pairs. A separate signature combination was found for all of the European isolates detected in ticks from Germany and the Czech Republic. A third signature was observed from the CDC culture isolate from the Japanese strain. Although all 3 genotypes shared the same basecount for the locus BCT3515, the European genotype did not have any other basecount signatures in common with the other 2 genotypes. The North American and Japanese genotypes had the same signatures for 2 of the 4 remaining loci, BCT 3519 and BCT3511. We detected *B. miyamotoi* with 3 or more primers in the *Borrelia* genotyping assay in all but 4 of the 68 positive specimens. Several factors may explain why all 5 primers did not detect the bacteria, including nucleic acid quality and quantity or differences in primer sensitivities.

Prevalence of *B. miyamotoi* in Europe and the United States

I. ricinus ticks from the Czech Republic and Germany in Europe and *I. scapularis* and *I. pacificus* ticks from 5 states in the United States were screened for *B. miyamotoi* by PCR/ESI-MS. *B. miyamotoi* was found in all regions examined in varying degrees (Table 1) and in all 3 *Ixodes* species examined. Germany had a low incidence rate; only 4 of the 226 ticks tested were infected (1.8%). Incidence of *B. miyamotoi* infection of ticks from the Czech Republic varied by region and ranged from 0% to 3.2% with an average infection rate of 2%. In North America, the infection rates of ticks varied from 0% to 15.4%. All negative controls were negative and all positive controls were positive.

Table 2. *Borrelia miyamotoi* PCR/ESI-MS basecount signatures*

Region	Genotype	BCT3515 (*rplB*)	BCT3517 (*flaB*)	BCT3519 (*hbb*)	BCT3520 (*hbb*)	BCT3511 (*gyrB*)
Europe	1	A13G22C15T18	A41G30C23T27	A41G29C19T46	A52G29C13T47	A36G32C13T35
North America	2	A13G22C15T18	A43G28C23T27	A40G30C18T47	A52G30C13T46	A37G31C13T35
Japan	3	A13G22C15T18	A41G29C23T28	A40G30C18T47	A53G29C13T46	A37G31C13T35

*PCR/ESI-MS, PCR and electrospray ionization mass spectrometry.

Sequence Confirmation of *B. miyamotoi* detections

Representative samples were selected for 16S rRNA sequencing: 1 sample from Pennsylvania in the United States, 1 from Germany, and 1 from the Czech Republic. The samples from Germany and the Czech Republic were identical (KF740842 and KF740841, respectively) and matched 99.11% (669 bp out of 675 bp) of the *B. miyamotoi* LB-2001 sequence, a North American isolate from the East Coast (GenBank accession no. NC_022079). The sample from Pennsylvania (KF740843) was identical (675 bp of 675 bp) to the *B. miyamotoi* LB-2001 sequence.

Discussion

In this study, we identified 3 distinct *B. miyamotoi* genotypes in the United States, Europe, and Japan. Results show that *B. miyamotoi* is widely distributed across North America and Europe.We observed no genotypic differences using this PCR/ESI-MS assay between the *B. miyamotoi* detected in *I. scapularis* from the eastern US states and the midwest or between these bacteria and the *B. miyamotoi* detected in *I. pacificus* from California. In a study by Mun et al., a 766-bp region of the flagellin gene sequence were shown to have and a 0.9% difference between *B. miyamotoi* found in *I. pacificus* and those found in *I. scapularis* in the United States (*8*). However, our flagellin primers targeted a region of the flagellin gene that does not contain the differences identified by Mun et al., thus explaining why we found a single North American genotype. Previous studies that examined the sequence of the 16S rRNA gene from multiple *B. miyamotoi* strains indicated that strains from the United States and Europe were located in their own clusters (*6*). The Japanese strain FR64b grouped with isolates found in infected humans and *I. persulcatus* ticks in Russia, whereas the *B. miyamotoi* found in *I. ricinus* ticks from Russia grouped with those found in Europe (*6*). In our genetic analysis, the Japanese strain also differed from that found in *I. ricinus* in Europe.

Our study demonstrates that the presence of *B. miyamotoi* in *Ixodes* ticks is widespread across the regions examined and was observed in all 3 species of field-collected *Ixodes* ticks. In Europe we observed *B. miyamotoi* in ≈2.0% of *I. ricinus* ticks tested, consistent with the detection rates in other studies examining *I. ricinus* prevalence at other locations in Europe (*9*,*10*). Our detection rates were also similar to those seen in an earlier study on ticks from Mendocino County, California (*8*). *I. scapularis* ticks from the East Coast region (New York, Connecticut, and Pennsylvania) were found to have infection rates ranging from 0% to 6.8% for ticks. In Indiana, however, a much higher percentage, ≈12%, of *I. scapularis* ticks examined were infected with *B. miyamotoi*. Other studies have also shown that local site-to-site prevalence of *B. miyamotoi* can vary greatly from the overall regional mean (*13*).

Our study indicates that *B. miyamotoi* is likely present in any region where *Ixodes* ticks reside but that infection rates can vary greatly by region. Since the original description of *B. miyamotoi* as a human pathogen, studies have shown clinical infection in both healthy and immunocompromised patients in both Europe and the United States (*3*–*6*,*22*). If physicians know the regional infection rate in ticks, they will be alert for possible exposure risks for their patients. Standard Lyme borreliosis serologic tests offered by commercial laboratories cannot be relied on to detect *B. miyamotoi* infection in patients. *B. miyamotoi* has been shown to have transovarial transmission, suggesting that larval ticks may also pose a risk (*7*). Little is yet known about the transmission rates to humans, and further studies are required to better gauge the risk to humans in these *B. miyamotoi*-endemic regions.

Acknowledgments

We thank our many collaborators who collected ticks for this study. We also thank Wakoli Wekesa, Robert Cummings, Paul Binding, David James, Ronald Keith, Angella Falco, Ann Donohue, Jamesina Scott, Stacy Berden, and Jack Cavier for collection of the California *Ixodes* ticks; Jianmin Zhong for ticks from Humbolt County; Scott Campbell and Keith Clay for ticks from New York and Indiana, respectively; John Bruno and Dan Qui for ticks from New York and Connecticut; and Kirby Stafford for ticks from Connecticut.

This study was supported by National Institutes of Health grant no. 2R44AI077156.

M.W.E., C.D.C., H.E.M., M.A.R., D.J.E. are employees of Ibis Biosciences Inc., an Abbott Company that developed the PCR/ESI-MS assays and instrumentation used in these studies. Assays described are for research use only. H.H., B.M., and O.N. are employees of the laboratory of Dr Brunner, which was contracted to test ticks.

Dr Crowder is a researcher at Ibis Biosciences working on vectorborne disease diagnostics. His research interests include tick-transmitted diseases in both the vector and in clinical patients.

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4.4 Paper IV

Ticks and Tick-Borne Pathogens in South Bohemia (Czech Republic) - Spatial Variability in *Ixodes ricinus* Abundance, *Borrelia burgdorferi* and Tick-borne Encephalitis Virus Prevalence

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Ticks Tick-Borne Diseases, 2015, 6(5): 559-567
Supplementary data available in Appendix I

Ticks and Tick-borne Diseases 6 (2015) 559–567

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Ticks and tick-borne pathogens in South Bohemia (Czech Republic) – Spatial variability in *Ixodes ricinus* abundance, *Borrelia burgdorferi* and tick-borne encephalitis virus prevalence

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ARTICLE INFO

Article history:
Received 21 October 2014
Received in revised form 19 April 2015
Accepted 20 April 2015
Available online 27 April 2015

Keywords:
Tick
Ixodes ricinus
Lyme borreliosis
Tick-borne encephalitis
Environmental factors
GIS

ABSTRACT

Spatial distribution of *Ixodes ricinus* tick host-seeking activity, as well as prevalence of *Borrelia burgdorferi* sensu lato and tick-borne encephalitis virus (TBEV) were studied in the TBE endemic area of South Bohemia (Czech Republic). High variability in tick abundance detected in a network of 30 study sites was most closely associated with characteristics of vegetation cover. Of 11,182 tested tick samples, 12% carried DNA of spirochete from *B. burgdorferi* s.l. complex. *B. afzelii* and *B. garinii* prevailed among spirochete species. The presence of *B. spielmanii* in the region was confirmed. The median number of borrelial genome copies in positive samples reached 6.6×10^3 by real-time PCR. The total prevalence of TBEV in pooled samples reached 0.32% (20,057 samples tested), at least one TBEV positive tick was present in 21 out of 30 sampling sites.

Introduction

Concerning the distribution of human tick-borne diseases, the hard tick *Ixodes ricinus* is the most important recognized vector of tick borne pathogens in (Central) Europe. This tick species inhabits an extensive area reaching from Ireland to western parts of Russia and from Scandinavia to North Africa. It is the major vector of the causative agents of Lyme borreliosis (spirochetes of the *Borrelia burgdorferi* sensu lato complex), tick-borne encephalitis (tick-borne encephalitis virus), human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), babesiosis (*Babesia divergens*, *B. microti*), some other less frequent human diseases and pathogens of veterinary importance (Charrel et al., 2004; Parola and Raoult, 2001). With an annual number of over 85 thousand cases of Lyme borreliosis and almost 2900 cases of tick-borne encephalitis (TBE) (ECDC Meeting report, 2012) these two diseases are the vector-borne diseases with largest impact on human health in Europe.

In the last decades ticks and tick-borne pathogens have received increasing attention, both from experts (medical, scientific) and general public. Concerning eco-epidemiological studies the research interest has focused mainly on estimation of tick activity, prevalence of tick-borne pathogens (including meta-analysis) and subsequently, efforts to identify the key factors, that determine the above mentioned parameters and might be used for disease risk prediction (e.g. Hubalek et al., 2003; Medlock et al., 2013; Schwarz et al., 2009, 2012; Swei et al., 2011). In Europe, such studies focus mainly on variability in time, following few local tick populations for several years. Apart from EDEN and EDENext projects, which cover almost whole Europe, studies that take into consideration multiple sampling sites and the spatial variability in tick and tick-borne pathogen abundance are scarce in Central Europe (Altobelli et al., 2008; James et al., 2013; Nazzi et al., 2010; Rizzoli et al., 2002).

To fill the gaps in this field we collected the data in a typical central European tick-borne disease endemic area – the region of South Bohemia in the Czech Republic. An annual average of 23.3 TBE disease cases per 100,000 inhabitants is registered in this region

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http://dx.doi.org/10.1016/j.ttbdis.2015.04.010
1877-959X/

compared to 6.5 cases per 100,000 inh. for the whole country (Kriz and Benes, 2011). The first part of our project consisted mainly of extensive collection of data on variability in *I. ricinus* activity, and on distribution of the two major wide-spread tick-borne pathogens: *B. burgdorferi* s.l. and tick borne-encephalitis virus (TBEV). The goal of the second part of our project was accumulation of a large quantity of environmental data, analysis and elucidation of the relationship between environmental variables and spatial distribution of ticks and tick-transmitted pathogens.

Methods

Area under survey

The geographic area of interest is located in the southern part of the Czech Republic – region of South Bohemia. It is well known for a high occurrence of tick-borne diseases. The presence of natural and cultural attractions and a developed tourism industry makes it also an attractive site for high number of visiting tourists. This combination increases the epidemiological impact of the area on public health of local human population as well as visitors. An average of 154 human cases of Lyme borreliosis and 146 human cases of TBE is registered per year in South Bohemia (data provided by the National Institute of Public Health, Prague).

Geographically, climatically and biologically, the area is considerably heterogeneous ranging from 350 m above the sea level (a.s.l.) of the Orlik dam to the highest sections of the Sumava forest mountain range (1378 m a.s.l.). The total area under survey comprised 10,056 km^2. More than 38% of the area is covered by forests. Lower parts are covered mostly by deciduous and mixed forests, whereas in upper parts coniferous forests predominate.

Tick sampling

I. ricinus ticks were collected and their host-seeking activity was estimated by flagging. Based on a thorough GIS analysis of the vegetation cover, altitude, TBE incidence and tourist activity, the areas principally suitable for tick survival (and having epidemiological importance at the same time) were identified (Svec et al., 2009). Subsequently, semi-random selection procedure was run to select 35 potential sites of which later 30 definite study sites were selected in field survey (Fig. 1). GPS coordinates, altitude (ArcPad, ESRI) and basic phytocenological characteristics were recorded in field.

Ticks were collected in three sampling events: April, June/July and September 2008. In each campaign all 30 localities were sampled for a maximum time span of 10 days, each locality for three flaghours (one flaghour defined as one hour of continual flagging of one worker). The numbers of nymphal, male and female adult ticks were recorded. Different development stages, female and male ticks were stored in separate tubes. Tick density (number of individuals per 100 m^2 area) has a close and linear relationship to relative abundance (number of ticks per hour). According to experience obtained in Central European types of habitat 1 flag-hour corresponds approximately to 200 m^2 area (see Danielova et al., 2010). Tick relative abundance was recalculated for tick density (number of ticks per 100 m^2) ensuring comparability with other studies.

Tick samples were transported within hours after flagging in a cooled box to the laboratory where they were stored at −74 °C until isolation of nucleic acids. DNA was isolated from individual ticks, RNA from pooled samples. Using stainless steel beads the ticks were homogenized individually in 200 μl of sterile PBS (phosphate buffered saline, pH 7.4) for 2 min at 30 shakes/s (Tissue Lyser II, Qiagen). After brief centrifugation, 15 μl of each sample was transferred to a pooled sample (maximum of 10 original samples to one pool). The original samples were subjected to DNA isolation, the pooled samples to RNA isolation. DNA and RNA in parallel were extracted from all adult ticks and maximum of 125 nymphal ticks per sampling event and locality. The remaining nymphal samples were directly homogenized as pools of 10 individuals in 400 μl of sterile PBS and only RNA was isolated. Altogether 20,057 ticks were subjected to TBEV RNA detection (in pools) and 11,182 individual ticks were tested for the presence of *B. burgdorferi* DNA.

Detection of tick-borne pathogens

DNA was extracted using a modified Chelex protocol (Rauter et al., 2002; Walsh et al., 1991). Briefly: 350 μl of 10% Chelex® 100 Resin solution in TE (Tris–EDTA) buffer was added to each sample and incubated overnight at 56 °C with shaking. After vortexing the samples were incubated for 10 min at 95 °C and immediately cooled on ice. The samples were centrifuged, the supernatant was collected and either directly used for PCR or stored at −20 °C. RNA was isolated using QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's instructions. RNA was used immediately for TBEV detection or stored at −74 °C.

The presence of borrelial DNA was detected by PCR targeting a fragment of o*spA* gene. PCR reactions of 25 μl total volume contained: 10.5 μl of sample and 12.5 μl of Combi PPP Mastermix (Top-Bio) (final concentrations: 75 mM Tris–HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dTTP, 200 μM dGTP, 2.5 U Taq purple DNA polymerase) and 1 μl of each of 10 mM primers SL1 (5′-AATAGGTCTAATAATAGCCTTAATAGC-3′), SL2 (5′-CTAGTGTTTTGCCATCTTCTTTGAAAA-3′) (Demaerschalck et al., 1995). After initial denaturation at 95 °C, the template was amplified in 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s with final elongation step at 72 °C for 3 min. Positive and negative controls were included in each run. PCR products were analyzed by gel electrophoresis. Positive samples were subjected to genospecies identification by reverse line blotting (RLB). *Borrelia* genospecies were identified according to the protocol of Gern et al. (2010). Briefly: a fragment of borrelial 23S–5S intergenic spacer region was amplified by PCR using a biotin-labelled primer and hybridized with multiple species-specific probes (Gern et al., 2010) immobilized on a membrane. After washing, the hybridized products were visualized by chemiluminiscence.

TBEV was detected by two-step RT-PCR amplification of a fragment of gene encoding viral E protein. First, 2 μl of 10 mM reverse primer (E(R): 5′-CCGTTGGAAGGTGTTCCACT-3′) (Ruzek et al., 2007) and 4 μl of template RNA were incubated at 70 °C for 10 min. Subsequently 1.25 μl of 10 mM dNTPs, 20 U of SUPERase-In RNA inhibitor (Ambion), 200 U of M-MLV reverse transcriptase (Promega) and DEPC-treated water to the final reaction volume of 25 μl were added. The cDNA synthesis was conducted at 37 °C for 60 min. Subsequently, 4 μl of cDNA were used for PCR amplification with primers E(F) (5′-GGGGACYACGAGGGTYACCT-3′) and E(R): (5′-CCGTTGGAAGGTGTTCCACT-3′) (Ruzek et al., 2007). The same PCR protocol as for amplification of spirochete DNA was used. Annealing temperature of the primers was set to 50 °C. Positive and negative controls were included for reverse transcription and PCR. The PCR products were analyzed by agarose gel electrophoresis.

From the *Borrelia* positive samples, 267 were semi-randomly selected (with respect to representation of different genospecies) for quantification of copies of borrelial genome by real-time PCR (targeting a portion of the *flagellin* gene of *B. burgdorferi*). The samples were analyzed in triplicates in 25 μl reactions consisting of 12.5 μl Probe qPCR Master Mix ($(NH_4)_2SO_4$, MgCl2, KCl, dNTP mix, Hot Start Taq DNA polymerase) (Fermentas), primer FlaF1 (5′-AGCAAATTTAGGTGCTTTCCAA-3′) (300 nM final concentration), primer FlaR1: (5′-GCAATCATTGCGATTGCAGA-3′) (900 nM final concentration), TaqMan probe FlaProbe1

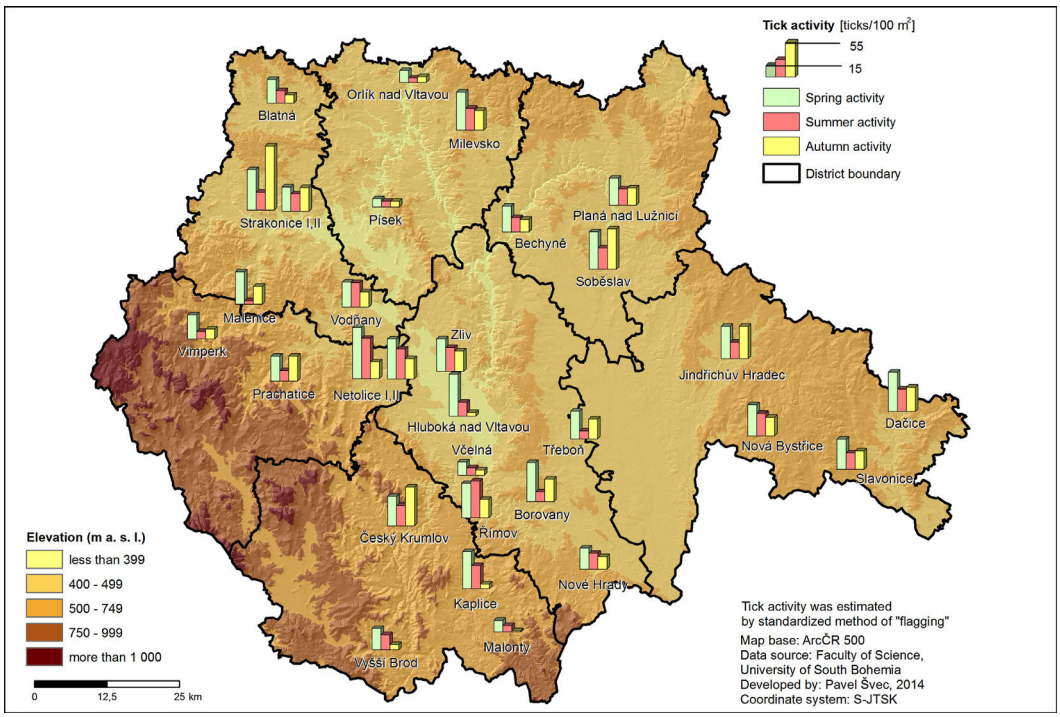

Fig. 1. Host seeking activity of *Ixodes ricinus* ticks. Tick activity was estimated as the number of ticks collected by flagging by one person per hour. In order to allow the comparison to other studies, the number of ticks was recalculated per 100 m^2 – one flag-hour corresponds to 200 m^2 (own experience and Danielova et al., 2010).

(5′-BHQ-TGCTACAACCTCATCTGTCATTGTAGCATCTTTTATTTG-FAM-3′ (200 nM final concentration) (nucleotide sequence from Schwaiger et al., 2001), DEPC-treated H_2O, and 4 μl of sample DNA. The amplification was carried out in a Rotor Gene 3000 cycler (Corbett Research) using a profile consisting of initial denaturation 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Standard calibration curve was created using serially diluted borrelial DNA corresponding to $2 \times 10^7 - 20$ cells/ml. The initial concentration of *Borrelia* cells was determined in the original culture using Petroff-Hausser counting chamber and dark-field microscopy.

Environmental data

One of the main aims of the study was to identify environmental factors, which influence the geographical distribution of ticks and tick-borne pathogens. The data on environmental variables were obtained from various sources. Selected climatic data were kindly supplied by the Czech Hydrometeorological Institute (CHMI). More specifically, data interpolated in 10×10 km grid from regular measurements of meteorological stations were available for the whole surveyed area. Daily, monthly, seasonal (May–September) and annual averages, minimum and maximum values for the amount of precipitation and air temperatures were estimated for each of the sampling sites. Data on vegetation cover contained: classified remote sensing data (CORINE Landcover, 2006, 100 m resolution) (EEA, 2006) and Ellenberg indicator values. Ellenberg indicator values were calculated for each of the sampling sites from the phytocenological releves collected by our own field survey. Indicator values calculated for herb (E1) and herb/shrub (E1/E2) stratum contained: light, temperature, continentality, moisture, soil reaction, nutrients and Shannon-Wiener index of biological diversity. The following parameters were derived via GIS: exposition and slope from ArcCR 500 (ArcDATA Prague, CR), land surface temperature (LST), normalized difference vegetation index (NDVI) from MODIS database (NASA, 2010). The closest reading to the date of sampling was assigned to each sampling/locality. For NDVI the Vegetation Indices 16-Day L3 Global 250 m product was downloaded and processed, for LST Land Surface Temperature & Emissivity 8-Day L3 Global 1 km was used. Forest types surrounding sampling site were calculated as portion of deciduous, mixed and coniferous forest in the collection site pixel and surrounding 8 cells in raster data (EEA, 2006). Furthermore, demographic data on numbers of inhabitants (Czech Statistical Office) and tourist activity were available (Vystoupil et al., 2006).

Prevalence of TBEV was calculated using EpiTool utility (http://epitools.ausvet.com.au; AusVet Animal Health Services) for perfect test and fixed pool size (Sergeant, 2014). GIS analysis and computations were done in ArcGIS 9.3 (ESRI), statistical analysis were performed using STATISTICA ver. 9 (StatSoft), phytocenological data were processed using TURBOVEG (Hennekens and Schaminee, 2001) and JUICE 9.0 (Tichy, 2002).

Results

Ixodes ricinus *tick abundance*

Altogether, 20,057 host-seeking *I. ricinus* ticks were collected (18,829 nymphs, 578 females, 650 males) at 30 study sites spread over the region of South Bohemia (Czech Republic) (Fig. 1) in three sampling events in 2008. The tick activity was analyzed in more detail. No significant differences were found between the density of

Table 1
Mean host-seeking activity of ticks [ticks/100 m^2] and standard deviation. Tick activity was estimated as the number of ticks collected by flagging by one person per hour. In order to allow the comparison to other studies, the number of ticks was recalculated per 100 m^2 – one flag-hour corresponds to 200 m^2 (own field experience and Danielova et al., 2010). Pairs of results with significant differences between the sampling events are indicated by symbols *#.

Sampling event	Mean density of host-seeking *I. ricinus* (S.D.)				
	Nymphs	Females	Males	Adults	Total
Spring	*#45.97 (16.30)	2.30 (1.23)	2.37 (1.23)	*#4.67 (2.38)	*#50.64 (17.43)
Summer	*27.82 (15.03)	0.55 (0.57)	0.86 (0.57)	*1.41 (1.20)	*30.03 (15.54)
Autumn	#30.81 (22.40)	0.36 (22.4)	0.38 (0.33)	#0.74 (0.59)	#31.56 (22.57)
Average	34.87	1.07	1.20	2.27	37.41

host-seeking female and male ticks, while density of nymphal ticks significantly exceeded the adult tick density (Wilcoxon pair test, $p < 0.01$). Concerning the seasonal pattern of occurrence in general, the nymphal tick densities decreased from spring sampling to summer and again increased in autumn. Whereas adult tick densities showed higher activity in spring lower in summer and even lower in autumn sampling event. The differences in the total tick density (and density of nymphs and adults separately) between spring and summer as well as spring and autumn sampling events were significant (ANOVA, Tukey HSD, $p < 0.01$). Neither the decrease in host-seeking activity of adult ticks in autumn compared to summer nor the increase in case of nymphs were found significant. More detailed results on host-seeking tick density are summarized in Table 1.

The variability among the localities was largest in autumn for nymphs and in spring for adults. Although significant seasonal differences were found in individual localities, the ranking of the localities correlated among spring, summer and autumn (Spearman $\rho = 0.38–0.75$, $p < 0.01$,), indicating the tick density being locality specific.

The association of *I. ricinus* with specific types of habitats was tested. Significant differences in total tick host-seeking density were found among the CORINE land cover categories (EEA, 2006) (ANOVA, $p < 0.05$; confirmed by Kruskal–Wallis nonparametric test, $p < 0.05$). Significantly lower tick counts were recorded in coniferous and broad-leaved than mixed type forests (Fisher LSD, $p < 0.05$). The seasonal dynamics remained similar for different land cover classes.

These results were further supported by the regression analysis. Tick density correlated positively with portion of the surrounding area covered by mixed forests (calculated from the pixel of the collection site localization and eight surrounding pixels) (Pearson $R^2 = 0.11$, $p < 0.05$), whereas the correlation was negative for coniferous and deciduous forests. Negative impact of broad-leaved forests on tick activity was also expressed by observed negative correlation with normalized difference vegetation index (NDVI) for July (Spearman $\rho = -0.45$, $p < 0.05$; Pearson $R^2 = 0.16$, $p < 0.05$) and August (Spearman $\rho = -0.46$, $p < 0.05$; Pearson $R^2 = 0.15$, $p < 0.05$).

As the association of tick activity with specific habitat type was confirmed, the relationship was surveyed in more detail. Climatic factors, physical–geographical, and phytocenologic characteristics were analyzed at the beginning independently and subsequently as co-existing and interfering factors. Spearman ranking correlation analysis was performed to identify the best representation for each type of environmental factors and to exclude cross-correlated variables. Tick densities were highly correlated among tick stages and sampling events (Spearman $\rho = 0.38–0.99$, $p < 0.05$). High level of correlation was observed also for most climatic characteristics (Spearman $\rho = 0.37–0.99$, $p < 0.05$) except of land surface temperature (LST) – August and September means (MODIS derived remote-sensing data) and annual mean air temperature (derived from CHMI data). Ellenberg indicator values and NDVI remained mostly uncorrelated. From groups of cross-correlated variables only one was selected for further analysis.

Multiple step-wise linear regression analysis was performed using total tick counts as dependent variable and LST July, LST August, Moisture E1, Shannon-Wiener diversity index E2E1, Light E2E1, Moisture E2E1, Nutrients E2E1, portion of deciduous forest in neighboring pixels, portion of coniferous forest in neighboring pixels, portion of mixed forest in neighboring pixels, portion of northwards facing neighboring pixels. Forward step-wise regression analysis resulted in a final equation ($R^2 = 0.31$, $p < 0.01$):

$$\text{total tick activity} = 1.77(\text{portion of mixed forests}) - 2.50(\text{portion of northwards facing pixels}) + 12.42(\text{S-W diversity index E2E1})$$

Borrelia burgdorferi *sensu lato prevalence*

Tick samples were tested for presence of *B. burgdorferi* s.l. and TBEV. Altogether 11,182 ticks were tested for the presence of borrelial DNA. A total of 1356 positive tick samples resulted in a total prevalence rate of 12.1%. The 12.6% prevalence rate of *Borrelia* in nymphs exceeded the 8.5% prevalence in adults (χ^2 test, $p < 0.01$). More detailed results are presented in Table 2 and Supplementary data.

No statistically significant differences in the prevalence rate of *B. burgdorferi* s.l. were found among the collection events. The prevalence rate of *Borrelia* correlated significantly with tick activity only for adult ticks (Spearman $\rho = 0.31$, $p < 0.05$), but not for nymphs.

Concerning the environmental variables, prevalence of LB spirochetes in ticks correlated positively with precipitation (Spearman $\rho = 0.35$, $p < 0.05$) and negatively with seasonal (most significantly July) temperatures (Spearman $\rho = -0.42$, $p < 0.05$).

No significant correlation was found for number of human LB cases and prevalence of *B. burgdorferi* s.l. in ticks or activity of *Borrelia* infected ticks.

Borrelia *genospecies identification*

Randomly selected group of 1001 *Borrelia* positive samples was submitted for genospecies identification by reverse line blotting. The identification was possible in 628 tick samples. Some of the ticks were co-infected with more than one genospecies resulting

Table 2
Prevalence rate of *B. burgdorferi* s.l. and mean density of *Borrelia* infected ticks. Tick activity was estimated as the number of ticks collected by flagging by one person per hour. In order to allow the comparison to other studies, the number of ticks was recalculated per 100 m^2 – one flag-hour corresponds to 200 m^2 (own field experience and Danielova et al., 2010).

	B. burgdorferi s.l. prevalence (positive/tested)	Mean density of borrelia-infected [infected ticks/100 m^2] (±SD)
Nymphs	12.6% (1239/9809)	4.4 (0.3–9.0)
Adults	8.5% (117/1373)	0.2 (0–0.6)
Total	12.1% (1356/11,182)	4.5 (0.3–9.0)

Table 3
Representation of *B. burgdorferi* s.l. genospecies in *I. ricinus* ticks.

	N (% of all identifications)	*N* (% of all nymphal identifications)	*N* (% of all adult identifications)
B. afzelii	447 (61.7)	433 (62.6)	14 (42.4)
B. garinii	187 (25.8)	178 (25.7)	9 (27.3)
B. burgdorferi s. s.	59 (8.2)	51 (7.4)	8 (24.2)
B. valaisiana	17 (2.4)	17 (2.5)	0 (0)
B. lusitaniae	9 (1.2)	8 (1.2)	1 (3)
B. spielmanii	5 (0.7)	4 (0.6)	1 (3)

in a total of 724 genospecies identifications. The most prevalent genospecies was *B. afzelii* followed by *B. garinii* and *B. burgdorferi* sensu stricto (Table 3). *B. bavariensis* was not identified as separate species and thus is included among *B. garinii*. Occurrence of *B. spielmanii* in South Bohemia was confirmed ($N = 5$).

Differences in the genospecies prevalence were identified between the stages of *I. ricinus*. *B. burgdorferi* s. s. was detected remarkably more frequently in adults than in nymphs, whereas *B. afzelii* displayed lower frequency of occurrence in adult compared to nymphal ticks (Pearson Chi-square; $p < 0.01$). *B. valaisiana* was found exclusively in nymphal ticks ($N = 17$).

Infection by a single genospecies was recorded in 53.3% of the samples subjected for species identification ($N = 534$). The presence of two *Borrelia* species in one tick was identified in 9.2% ($N = 92$), while triple infections were found in 0.2% ($N = 2$) of the samples. *Borrelia* species in the remaining 373 (37.3%) samples could not be identified. The most common co-infection was *B. afzelii–B. garinii* ($N = 58$), followed by *B. afzelii–B. burgdorferi* s.s. ($N = 14$), and *B. garinii–B. valaisiana* ($N = 10$). All other combinations of genospecies were found rarely (*B. garinii–B. lusitaniae* ($N = 3$), *B. afzelii–B. lusitaniae* ($N = 2$), *B. afzelii–B. spielmanii* ($N = 2$), *B. afzelii–B. valaisiana* ($N = 1$), *B. burgdorferi* s.s.–*B. lusitaniae* ($N = 1$), *B. burgdorferi* s.s.–*B. garinii* ($N = 1$)). Both triple infections consisted exclusively of *B. burgdorferi* s.s.–*B. afzelii–B. garinii*. Interestingly, one of them was found in nymphal tick. No statistically significant differences in the frequency of occurrence of multiple infections were observed between nymphal and adult tick stages.

Estimation of Borrelia *genome copy number*

The number of borrelial genome copies in 267 tick samples (253 nymphal, 14 adult) was estimated by real-time PCR. The median number of borrelial genomes per tick reached 6.6×10^3 (range 115–1.13×10^5). Higher median numbers of *Borrelia* were observed in nymphs (6.6×10^3) compared to adult ticks (4.7×10^3), nevertheless the difference was not statistically significant. Significant differences in the numbers of borrelial genome copies per tick were observed between ticks infected by *B. afzelii* (median 4324 copies/tick) and *B. garinii* (13,769 copies/tick) (ANOVA, $p < 0.01$). No significant differences were observed in the comparison of single and mixed infections.

Detection of tick-borne encephalitis virus

A total of 20,057 ticks (18,829 nymphs, 1228 adults) was tested for the presence of TBEV. The prevalence rate of pooled samples was calculated using Epitools application (http://epitools.ausvet.com.au; AusVet Animal Health Services) for perfect test and fixed pool size (Sergeant, 2014). The total prevalence reached 0.32%. TBEV prevalence in adult ticks (0.81%) significantly exceeded the prevalence in nymphs (0.32%) (χ^2 test, $p < 0.01$). Detailed data on prevalence of TBEV and activity of infected ticks are summarized in Table 4.

From the total number of 30 localities at least one TBEV positive sample was found on 21 (70%) of them. TBEV positive localities displayed significantly higher tick activities than the TBEV negative ones (t-test; $p < 0.02$).

No statistically significant differences in the prevalence rate of TBEV were found among the collection periods. The total prevalence rate correlated significantly with total and nymphal tick activity (Spearman $\rho = 0.39$, $p < 0.05$), but not with activity of adult ticks. TBEV prevalence in ticks as well as activity of TBEV infected ticks remained uncorrelated with almost all of the environmental variables tested. TBEV prevalence and activity of TBEV infected ticks were found correlated with absolute number of TBE cases in 2008 (Spearman $\rho = 0.43, 0.42$, $p < 0.05$) but not for the total sum of disease cases 2001–2008.

Discussion

Risk of tick-borne infection is given by the probability of human encountering an infected tick. The probability of infected tick occurrence is influenced by pathogen prevalence and tick abundance (Randolph, 2001). Both factors may vary in time and space depending on numerous abiotic and biotic environmental factors. Spatial variability in tick activity and pathogen prevalence was explored in the region of South Bohemia – an area of known permanent occurrence of Lyme borreliosis disease cases and a TBE high risk area.

Tick abundance

Tick abundance was found highly variable in space (among sampling sites) and time (sampling event). Overall, highest adult tick abundance was observed in spring sampling event and it decreased in summer and autumn, whereas nymphs displayed highest level of activity in spring, lower abundance in summer and an than an increase in activity in autumn again. Some localities displayed a different pattern (data from three samplings per locality). Concerning the overall seasonal dynamics of tick abundance based on multiple samplings, mixed uni- and bimodal patterns were observed throughout Europe (Perret et al., 2004).

Because tick activities were apparently correlated among the sampling sites for different sampling periods, we assume a globally acting factor ruling the seasonal fluctuations. Jensen (2000) has shown that observed seasonal differences in tick abundance result rather from differences in tick host-seeking activity

Table 4
Pooled prevalence rate estimates of TBEV and mean activity of TBEV infected ticks. Tick activity was estimated as the number of ticks collected by flagging by one person per hour. In order to allow the comparison to other studies, the number of ticks was recalculated per 100 m^2 – one flag-hour corresponds to 200 m^2 (own field experience and Danielova et al., 2010).

	Mean TBEV prevalence (positive/tested)	Mean activity of TBEV infected [infected ticks/100 m^2] (min.–max.)
Nymphs	0.29% (54/18,829)	0.10 (0–0.33)
Adults	0.73% (10/1228)	0.18 (0–0.63)
Total	0.32% (64/20,057)	0.12 (0–0.61)

(particularly from differences in duration of questing period) than from population fluctuations.

Tick activity is influenced by numerous interacting environmental factors with global or local effect in time and space. The complexity of effects of the environment may be expressed by surrogate variables like vegetation cover (Halos et al., 2010; Rizzoli et al., 2002), NDVI or other indices (Bisanzio et al., 2008; Eisen et al., 2010, 2006). Such complex characteristics of particular habitats incorporate climatic, physical–geographical and most importantly also biotic conditions that may have impact on tick populations (e.g. habitat suitability for tick hosts competent or incompetent for pathogen transmission) (James et al., 2013; Prusinski et al., 2006). In our study, the highest total tick counts were found in mixed forests, somewhat lower tick abundance was found in agricultural areas with significant portion of natural vegetation and coniferous forests. Interestingly, deciduous forests, generally considered as more suitable tick habitats (Estrada-Peña, 2001; James et al., 2013; Lindström and Jaenson, 2003), presented with significantly lower tick counts. On the other hand, it was shown for areas with higher humidity, that coniferous forests are able to support high tick densities as well (Estrada-Peña, 2001; Walker et al., 2001).

Concerning the particular factors controlling tick activity, the most important are generally considered temperature in spring and autumn, and combinations of temperature and humidity (saturation deficit) in summer (Eisen et al., 2010; Knap et al., 2009; Perret et al., 2004; Schwarz et al., 2012). We were able to construct a simple model predicting nymphal host-seeking activity using portion of mixed forests in the vicinity of the collection site, portion of north oriented slopes and Shannon-Wiener biological diversity index. Similar approach was applied by Eisen et al. (2006). Mixed forests are known to provide a more stable environment in terms of humidity and temperatures and thus may serve as a surrogate of saturation deficit (Knap et al., 2009). We have used climatic data interpolated from the measurements of meteorological stations to get geographically as close as possible to the location of the sampling sites, but still we were working with a considerably large resolution of 10×10 km grid. Obviously it is rather the microclimatic than macroclimatic conditions, which influence the activity of ticks. Nevertheless it was shown previously, that the microclimatic and macroclimatic conditions are to a high degree correlated at least in the case of temperature and precipitation (Daniel et al., 2006). Thus macroclimatic data (combined with other types of data) may be useful in prediction of tick activity.

Also we hypothesize that the mixed forests offer a more complex and stable source of food for vertebrate animals (the potential hosts of ticks) due to their larger plant species diversity (illustrated by Shannon-Wiener diversity index). Host availability may influence the total tick population size and hence also actual tick activities (James et al., 2013; Jensen, 2000). Northward orientation of the locality may result in decreased access to light, delay in temperature increase in spring and thus delayed start of the spring tick activity. Greenfield (2011) has previously shown an association of tick presence/absence with soil humidity, temperature and light levels.

B. burgdorferi *s.l. in ticks*

Two most important tick-borne pathogens–Lyme borreliosis spirochetes and tick-borne encephalitis virus were detected in the tick samples. The total prevalence rate of *B. burgdorferi* as well as prevalence of individual *Borrelia* genospecies was not substantially different from other studies (Hubalek and Halouzka, 1998, 1997; Rauter and Hartung, 2005). The prevalence of the spirochetes in adult ticks reached surprisingly low level. However, it displayed also high variability among collection sites and reached expected values of 20–30% in some of the localities but also extremely low values in others. The variability among the localities may be partly caused by insufficient numbers of adult tick samples from some of the sampling sites. Furthermore, different developmental stages of *I. ricinus* are known to have different hosts (Mejlon and Jaenson, 1997; Randolph and Storey, 1999). Some hosts (e.g. large ungulates), that are important hosts for nymphal ticks, are known to be incompetent in *Borrelia* transmission (Jaenson and Tälleklint, 1992; Gern, 2008; Gern et al., 1998). Significant involvement of such hosts as food source for nymphal ticks may be a reason of lower spirochete prevalence in adults. At that case our conclusions correlate with similar explanation, proposed by Tälleklint and Jaenson (1996). Similar or even higher prevalence in nymphs than in adults was reported also in other studies (Nazzi et al., 2010; Skarphedinsson et al., 2007; Wielinga et al., 2006).

Precipitation and temperature seemed to be associated with *B. burgdorferi* prevalence. It was shown that *Borrelia* infected ticks may be more capable of survival in challenging conditions like high saturation deficit (Herrmann and Gern, 2010). In our case, the association was completely the opposite – the prevalence of infected ticks increased with rising precipitation values and decreased with rising temperatures. We assume that if there is a causal dependence, it is mostly indirect – mediated via *Borrelia* competent hosts, which prefer conditions with higher humidity and lower temperature (Eisen et al., 2010).

Concerning the relationship of tick density and *B. burgdorferi* prevalence, correlation was found only for adult ticks, but not for nymphal and total tick density. No correlation was found also in studies from Germany (Schwarz et al., 2009), Italy (Nazzi et al., 2010) and Scotland (James et al., 2013). Some authors indicate there might be positive correlation for lower tick densities and negative correlation for higher tick activities (Tälleklint and Jaenson, 1996). No such pattern was observed in our study.

Borrelia *genospecies identification*

Although the predominance of *B. afzelii* (62%) and *B. garinii* (26%) is concordant with the data from the reviews on the occurrence of *B. burgdorferi* s.l. genospecies in Europe: 37.1% and 39.7% (Hubalek and Halouzka, 1997), and 38% and 33% (Rauter and Hartung, 2005) for *B. afzelii* and *B. garinii* respectively, in our study the prevalence of *B. afzelii* was almost twice as high as in the cited reviews. Nevertheless regionally such high proportion of *B. afzelii* is not exceptional (e.g. Geller et al., 2013, Herrmann et al., 2013). Higher prevalence of *B. afzelii* found in our study may be caused by increased involvement of rodents as *B. afzelii* specific hosts (Hanincova et al., 2003). *B. burgdorferi* s.s. is less frequent in Central and Eastern Europe: 15.9% (Hubalek and Halouzka, 1997) and 18% (Rauter and Hartung, 2005). Previous report of presence of *B. spielmanii* (Derdakova et al., 2003) in the region of South Bohemia was confirmed in our study. Interesting differences in the prevalence of *Borrelia* genospecies were found between nymphal and adult ticks. *B. afzelii* was more frequently detected in nymphal ticks whereas *B. burgdorferi* s.s. in adults. High prevalence of *B. afzelii* in nymphal ticks was also found by Herrmann and Gern (2010) and Wilhelmsson et al. (2013). In the latter study the prevalence of *B. garinii* and *B. valaisiana* in adult ticks exceeded significantly the prevalence of these genospecies in nymphal ticks. The differences may be again caused by host preference of the tick developmental stages in combination with the host specificity of *Borrelia* genospecies (Margos et al., 2009). Particularly *B. afzelii* is known to be associated mainly with rodents (Hanincova et al., 2003), which are frequent hosts of larval ticks and thus may serve as a source of *Borrelia* (*afzelii*) for future nymphs. Presence of multiple *Borrelia* genospecies within single tick samples were recorded. Interestingly, one nymphal tick was co-infected with three genospecies, indicating either

co-infection of a single host by all of these genospecies or sequential acquisition of *Borrelia* from different hosts due to interrupted feeding. The above mentioned genospecies host specificity makes the first option less likely. On the other hand transmission of *B. burgdorferi* among co-feeding ticks may occur directly bypassing the necessity of systemic infection (Gern and Rais, 1996) and thus such transmission may be possible even on an incompetent host. For co-infection of a nymphal tick by three *Borrelia* genospecies probably a combination of above mentioned mechanisms would be necessary. Since in the time of tick sample analysis, there was no rapid and financially efficient method for distinguishing *B. garinii* and *B.bavariensis*, we may also hypothesize, that some of the frequent *B.* afzelii–*B. garinii* co-infections are in fact *B.* afzelii–*B. bavariensis*. Such co-infection would be more likely, because these two genospecies share rodents as the main host species (Huegli et al., 2002; Margos et al., 2009). The presence of hidden *B. bavariensis* in the group of *B. garinii* should not have a large impact since prevalence of this genospecies seems to be generally low in *I. ricinus* ticks (Geller et al., 2013; Glatz et al., 2014; Herrmann et al., 2013).

Borrelia *genome copy quantification*

The number of copies of *B. burgdorferi* genome per tick was estimated by real-time PCR. The median value of 7×10^3 is slightly higher than in other studies performed on host-seeking (Herrmann and Gern, 2012, 2010) and partially engorged *I. ricinus* (Wilhelmsson et al., 2013) and host-seeking *I. scapularis* ticks (Wang et al., 2003). The difference may be caused by differences in methodology of quantification and/or detection. Higher sensitivity of detection method would result in higher percentage of positive ticks with low numbers of *Borreliae* and thus in lower average load. No significant difference between the spirochetal load in nymphal and adult ticks were found in our study, which correlates with results presented by Wang et al. (2003) and Wilhelmsson et al. (2013). Significantly higher spirochete loads were found in ticks infected by *B. garinii* than in ticks infected by *B. afzelii*. Similar patterns were observed previously (Herrmann et al., 2013; Wilhelmsson et al., 2013), although the differences were not found significant.

Tick-borne encephalitis virus in ticks

The prevalence of TBEV 0–1.2% corresponds with other reports from geographically close areas (Gäumann et al., 2010; Oehme et al., 2002; Pinter et al., 2013) and also directly from the area of South Bohemia (Danielova et al., 2002). No particular association with environmental variables was found for TBEV. TBEV positive sites presented with significantly higher tick density than TBEV negative ones. Since four of the sampling sites out of 30 did not reach 400 samples, one could speculate that the difference is caused rather by low sample size than by real absence of TBEV. All of these four studied sites were TBEV negative. On the other hand similar findings were reported earlier based on studies with higher sample numbers (Hudson et al., 2001). High tick densities may support the natural virus circulation due to viremic transmission as well as frequency of co-feeding transmission. TBEV foci are known to be far more scattered than foci of Lyme borreliosis (Zeman and Januska, 1999). The reason might be in local variation in tick activity and high dependence of the natural virus circulation on sufficient tick abundance (Norman et al., 1999) and/or synchronization of activity dynamics of different tick developmental stages supporting their co-feeding (Burri et al., 2011; Randolph et al., 2000) and/or variations in population density of TBEV competent/incompetent hosts (Jaenson et al., 2012).

Conclusion

Host-seeking activity of ticks as well as tick-borne pathogen prevalence was found to be highly variable in space. Vegetation cover seems to be a crucial factor influencing the rate of *I. ricinus* tick occurrence. The region of South Bohemia was confirmed to be a TBE high-risk area. LB spirochetes were found at all the sampled localities and displayed high variability in the representation of different genospecies.

Acknowledgements

The research was co-financed by the European Development Fund of the European Union (project title: "Ticks and tick borne infectious diseases in the conditions of South Bohemia and Bavaria"), by the Region of South Bohemia and by the following grants: SP/2010184, GAČR 206/09/H026 and Z60220518). This publication was also supported by the project Postdok_BIOGLOBE (CZ.1.07/2.3.00/30.0032) co-financed by the European Social Fund and the State Budget of the Czech Republic. The authors would like to thank the Czech Hydrometeorological Institute, Faculty of Economics and Administration, MU Brno, National Institute of Public Health, Prague for making the data available. We are also grateful for the advice and help of Dr. Danielova and Dr. Daniel in the beginning of the project. Special thanks belong to Radka Bunesova for all the help with the administration.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.04.010

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4.5 Paper V

Estimation of Acarological Risk of Exposure to Lyme Borreliosis or Tick-Borne Encephalitis Infected Ticks in the Border Area of the Czech Republic (South Bohemia) and Germany (Lower Bavaria and Upper Palatinate) and its Presentation in a Form of Map Portal

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Manuscript submitted to Parasites and Vectors

Estimation of acarological risk of exposure to Lyme borreliosis or tick-borne encephalitis infected ticks in the border area of the Czech Republic (South Bohemia) and Germany (Lower Bavaria and Upper Palatinate) and its presentation in a form of map portal

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Keywords: tick, Lyme borreliosis, tick-borne encephalitis, risk modeling, GIS

Abstract:

Background

In Europe Lyme borreliosis (LB) and tick-borne encephalitis (TBE) are the two vector-borne diseases with largest impact on human health. In order to apply appropriate preventive measures to protect the public health it is important to estimate the risk of infection. Based on data on tick activity and pathogen prevalence and using a variety of environmental data, we have attempted to create a model of acarological risk.

Methods

The study areas comprised of the region of South Bohemia (Czech Republic) and regions of Lower Bavaria and Upper Palatinate (Bavaria, Germany). The data on tick activity were acquired by flagging 50 sampling sites 3 times in a single season. Prevalence of the causative agents of LB and TBEV was estimated using PCR and real-time PCR. Data on environmental variables (altitude, slope, exposition, vegetation cover, NDVI, land surface temperature) were obtained from different sources and processed by GIS. Generalized linear models were used to obtain prediction of tick activity, probability of tick infection and activity of infected ticks prediction for the whole area.

Results

Apart from the model outputs which were also made available to the public using an online map portal (http://gis.vsb.cz/klistata/), statistically significant differences were found in the incidence of TBE in South Bohemia and Bavarian regions under survey. Significantly higher activity of tick populations was found in South Bohemia whereas the prevalence of pathogens was in general not found significant.

Conclusions

A spatial infection risk model based on estimation of infected tick activity was created. Based on the data acquired in the study, the differences in the incidence of TBE may be caused not only by differences in human behavior but also by different level of tick activity.

4.6 Paper VI

Ixodes ricinus Tick Host Species Spectrum and Its Association with Genospecies of *Borrelia burgdorferi* Sensu Lato Complex

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Manuscript based on unpublished data

***Ixodes ricinus* tick host species spectrum and its association with genospecies of *Borrelia burgdorferi* sensu lato complex**

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Keywords: tick, *Ixodes ricinus*, host identification, blood-meal, Lyme borreliosis, reverse-line blotting

Abstract:

Background

Ixodes ricinus tick is the main vector of bacteria of the *Borrelia burgdorferi* sensu lato complex, which cause Lyme borreliosis. The pathogen is transmitted by the ticks among its vertebrate hosts. *I. ricinus* is a host generalist known to be able to feed on more than 300 vertebrate host species. Nevertheless, there is still little information on the overall species composition of the tick host fauna.

Methods

Questing *I. ricinus* ticks were sampled by flagging in 5 study sites in South Bohemia (Czech Republic). The ticks were analyzed for their blood-meal source using reverse line blotting. The same technique was used for detection and identification of *B. burgdorferi* s.l. genospecies.

Results

From a total of 669 ticks (642 nymphs and 27 adults), the host identification was successful in 66 % (444). The most prevalent hosts were species of rodents and artiodactyls, followed by birds. Also DNA of carnivorous and insectivorous species was recorded. The prevalence of *B.*

burgdorferi reached 17.8 %. *B. afzelii* was the most frequently detected genospecies followed by *B. garinii* and *B. burgdorferi* s.s. *B. burgdorferi* s.l. genospecies associations with specific host species were confirmed, although from our data they seem to be less strict than reported previously. Multiple host species were more frequently detected in adult than in nymphal tick samples. The majority of co-infections by two *B. burgdorferi* genospecies occurred in ticks with two hosts identified.

Conclusions

The study has brought important information on *I. ricinus* host fauna composition. The results imply that the importance of large mammals as hosts of ticks and source of borrelia may be underestimated and that *B. burgdorferi* associations with specific host species may be more loose.

5 Summary and conclusions

The six research papers and manuscripts included in the thesis contribute to the knowledge on tick and tick-borne pathogen spatial distribution in the region of Central Europe. The mechanisms determining the spatial distribution and recent changes were outlined and discussed. The host species spectrum of *I. ricinus* was analyzed in more detail, including *B. burgdorferi* genospecies host associations.

Relatively recently reported presence of species of relapsing fever *Borrelia* was confirmed in European and North American *Ixodes* tick species. The wide spread distribution of *B. miyamotoi* in tick populations was reported.

5.1 Vertical distribution of *I. ricinus*, tick-borne encephalitis virus and *B. burgdorferi* s.l.

Paper nr. I brings an insight in the current situation in distribution of *I. ricinus* tick population in higher altitudes. Since there were substantial changes recorded in the altitudinal distribution of the ticks and pathogens that they transmit in several locations in Central Europe, one of the aims of the presented study was to find out whether such pattern is geographically universal.

Ticks were sampled in elevation up to 1300 m a.s.l. Occurrence of all developmental stages, including larvae in altitudes above 1000 m and considerably high tick activity in general imply that the ticks are members of stable autochthonous populations rather than accidentally occurring individuals randomly brought up by their hosts. Ticks were not found higher than 1350 m a.s.l., which ix approximately the timber line level in the studied area.

None of the tick samples was positive for TBEV, which might be a result of insufficient size of sample group rather than true absence, as TBEV prevalence is generally considerably lower. Presence of TBEV was previously confirmed in the neighboring Krkonose mountains where the minimum infection rate reached 0.4 %.

B. burgdorferi was found in adult (prevalence rate 6.3 %), nymphal (5.4 %) and larval (0.7 %) *I. ricinus.* Although the prevalence rate is lower than usually observed (10-30 %), LB spirochetes seem to be naturally circulating via the tick population

among the local natural hosts. All three major pathogenic genospecies, *B. afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto, were identified among the positive samples. Large proportion of *B. afzelii* indicates high importance of rodents as tick hosts and borrelia source in such type of habitat. *B. garinii* associated typically with avian hosts contributed also significantly, whereas *B. burgdorferi* s.s. was less frequent and *B. valaisiana* was detected rarely.

Analyses of climatic data have shown a substantial rise in annual mean temperatures (1979-2007). Furthermore, the increase in temperature is more pronounced in mountain areas. Most importantly, it was the spring and summer warming, crucial parts of the tick season, which accounted for the major part of the total difference. Rise in temperatures in particularly these parts of the season may promote the total duration of the period of tick activity, level of tick activity, as well as developmental rate.

The shift of tick populations as well as tick transmitted pathogens in higher altitudes continues and seems to be universal in the region of the Czech Republic. The mechanism is probably associated with the increase of mean annual temperatures. The detection of *B. burgdorferi* in larval questing ticks again raises the question of transovarial transmission of the spirochetes or possible alternative ways of infection including interrupted feeding and repeated questing.

5.2 Temporal changes in distribution of *I. ricinus* and *B. burgdorferi* s.l.

Temporal differences in the activity of tick populations as well as prevalence of *B. burgdorferi* s.l. were investigated in paper nr. II. Data on tick and borrelia occurrence from years 1987, 1989, 2001, 2007 and 2008 in 3 specific sampling sites (different plant communities) were compared.

High variability in the tick activity, *B. burgdorferi* s.l. prevalence and prevalence of the individual genospecies was revealed among the different sampling years as well as among the different plant communities. The long-term overall trend showed increase in tick activity, although not for all types of habitats, and also rise of overall *B. burgdorferi* prevalence. No apparent correlation was found with changes in air temperatures or precipitations. Only indirect evidence shows possible influence of saturation deficit on questing tick abundance and its annual fluctuations.

Interestingly, the prevalence of the individual *B. burgdorferi* s.l. genospecies changed considerably in time as well. Previously prevailing *B. valaisiana*, genospecies of limited pathogenity to human, was substituted by the common combination of *B. garinii* and *B. afzelii* accounting for the majority of infected samples. and *B. burgdorferi* s.s. and *B. valaisiana* were less frequently detected. This finding has an important impact on the LB infection risk for human entering this area. The occurrence of *B. lusitaniae* seems to be spatially restricted and probably closely associated with its competent host species, since a relatively high prevalence was recorded only in a single year on a single locality and never before or after.

Tick activity and *B. burgdorferi* s.l. prevalence undergo significant annual variations. Although major tendencies may be identified, discrepancies among different sampling sites are observed and may be attributed to differences in specific habitat conditions.

5.3 Spatial distribution of *I. ricinus*, tick-borne encephalitis virus and *B. burgdorferi* s.l.

Whereas in paper nr. II the temporal differences were the main focus of interest, papers nr. IV and nr. V were focused on spatial variability concerning a larger set of sampling sites including a variety of different tick habitats.

Significant variability in tick activity was observed. Nevertheless concerning the data from one season only, tick activity seemed to be locality specific. The tick activity was best correlated with vegetation cover characteristics (portion of mixed forests, NDVI), land surface temperature and orientation of the sampling site.

Paper nr. V contains comparison among sampling sites placed in South Bohemia, Czech Repblic and in selected regions of Bavaria, Germany. Significant differences in tick activity were observed between those two areas. The difference in tick activity may contribute to the significant differences in TBE disease case incidence between the two areas.

The total prevalence rates of *B. burgdorferi* and TBEV did not differ significantly among the South Bohemian sampling sites or in comparison of South Bohemian and Bavarian localities. While *B. burgdorferi* was found in all sampling sites, the

distribution of TBEV was much more scattered. Localities with at least one TBEV positive tick presented with significantly higher tick activity than TBEV negative sites.

Presence of *B. spielmanii* was confirmed in the area of South Bohemia. Other genospecies of *B. burgdorferi* s.l. were present in expected proportions: *B. afzelii* and *B. garinii* prevailed, *B. burgdorferi* s.s., *B. valaisiana* followed, *B. lusitanie* and *B. spielmanii* were found rarely.

Concerning the abundance of *B. burgdorferi* (genome copies) in ticks, *B. afzelii* reached significantly lower median numbers than *B. garinii* indicating possible differences in the intake, amplification or survival of different genospecies in ticks.

In the paper nr. V a model predicting the activity of ticks infected either by *B. burgdorferi* or TBEV is presented. The map outputs of the model and other data acquired in the study have been made available to the public through an online map portal: http://gis.vsb.cz/klistata/.

5.4 The host species of *Ixodes ricinus* tick estimated by molecular analysis of blood-meal source

Apart from environmental factors, discussed above, the intensity of occurrence of ticks and tick-borne pathogens is also influenced by interactions of ticks with other animal species. Obviously the most close and important interactions are parasite-host association.

In an unpublished study summarized in paper VI, molecular method of blood-meal analysis was used for identification of the host in questing *I. ricinus* ticks. It was possible to identify the host in 66 % of the (mostly nymphal) tick samples. A surprisingly high portion of ticks was fed on artiodactyls (including wild boar), also rodent species have contributed significantly. Birds were recorded as an important group of tick hosts only on one of the sampling sites situated in a forest park.

Genospecies of *B. burgdorferi* were detected and identified in the same set of samples. Although *B. afzelii* prevailed in the borrelia positive ticks fed on rodents, also *B. garinii* and *B. valaisiana*, previously shown to be associated with birds, were detected. Similarly, the proportion of *B. garinii* and *B. valaisiana* among bird-fed ticks was relatively low. Co-infection of one tick by multiple genospecies of *B. burgdorferi*

s.l. were frequently associated with ticks with two host identified by blood meal analysis. This finding supports the importance of interrupted feeding and re-attachment of a tick to another host in nature, as proposed in other studies.

Identification of the tick host from blood remnants in the questing ticks brings valuable information on the overall composition of the tick host fauna and may also significantly contribute to the identification of the role of vertebrate hosts in the circulation of tick-borne pathogens.

5.5 Occurrence of *B. miyamotoi* in *Ixodes ticks* in Europe and in the United States

Relapsing fever borrelia, *Borrelia miyamotoi* was detected in *I. ricinus*, *I. scapularis* and *I. pacificus* ticks. Positive samples were found in 4 of 5 European sampling sites and in 12 of 21 US sampling sites. The prevalence ranged from 0 to 3.2 % for the European tick populations sampled, from 0 to 12.3 % for *I. scapularis* and from 0 to 15.4 % for *I. pacificus*. A novel PCR – electrospray-ionization mass spectrometry approach was used for the detection of *B. miyamotoi*.

5.6 Summary remarks

Comparisons of the level of tick activity in time show an increase in the occurrence of ticks in different tick habitats (paper I and II). These changes seem are to a certain extent associated with global climatic changes.

Spatial distribution of ticks and tick-borne diseases is influenced by numerous interacting environmental factors. The crucial ones are represented by climatic factors and habitat type. Changes in environmental factors may have different impact in different types of habitats. Environmental factors may be successfully used for prediction of tick and pathogen activity in space. Obviously these factors do not influence only tick populations but also other components of the ecosystems shaping a particular habitat into a more/less tick and tick-borne disease prone environment.

The composition of the host fauna of *I. ricinus* tick may have a significant influence on tick abundance, as well as on pathogen prevalence. The overall contribution of particular host species may be larger than previously assumed, as shown for

artiodactyl species. Also, the host association of *B. burgdorferi* genospecies is probably less strict than previously suggested.

The prevalence of *B. burgdorferi* s.l. genospecies may change in time and be affected by significant annual variations. Since different genospecies present with different level of pathogenicity, the proportions of individual genospecies influence the overall risk of human LB infection.

Occurrence of relapsing fever spirochete in *I. ricinus* tick in Central Europe (and other *Ixodes* tick species in the US) was confirmed. Since recently, this spirochete has been shown to be associated with human disease cases, it represents a novel tick associated risk to public health. It is obvious that even in consistently studied vector species like *I. ricinus* new pathogens may emerge.

6 Open questions, future direction of research

Tick-borne pathogen systems undergo permanent development associated with environmental and socio-economic changes, as it was confirmed and described in presented thesis. Simple but regular monitoring of pathogen and vector populations brings us valuable data concerning protection of public health. From the ecological point of view, it may also show us a way and reason to support naturally stable ecosystems.

Apart from that, as shown in the case of *B. miyamotoi* or *Candidatus* Neoehrlichia mikurensis, new pathogens emerge, known pathogens may change hosts or vectors and/or distribution range.

Moreover, attention should be focused on real natural vector and host species as it was shown that various interactions are strictly species (genospecies, strain) specific and generalization may be misleading.

Also the intraspecies (intragenospecies) variability and its global impact on tick-borne pathogen circulation should be addressed in more detail. Particularly recognition of host associated races together with pronounced host preference of *B. burgdorferi* genospecies may have a potential of discovery of mechanisms in the borrelial transmission cycles.

Epidemiological and surveillance studies have brought a considerable amount of geographically specific data on incidence of disease cases and pathogen distribution. Laboratory transmission experiments and models bring detailed data on transmission competence, mechanisms or efficiency, often for a very specific laboratory model host or vector species. Ecological studies accumulate data on tick-host–pathogen interactions with environment on a more general and less accurate level but in real natural conditions. In general, there is a substantial amount of information available, but scattered in different disciplines, geographic locations and other contexts, which makes it difficult to draw some more general but accurate conclusions. It seems a more multidisciplinary and integrative approach is needed. Definitely, there are technical possibilities available. GIS offer a wide range of tools of spatial data gain, visualization and analysis, mathematical and statistical approaches allow creation and testing of very specific and complex models. Studies integrating ecological,

epidemiological and biological findings need to be preformed in different scales and spatial resolutions from the level of single natural focus of pathogen circulation to up the intercontinental level concerning global climatic changes and evolutionary tendencies.

Appendix I – supplementary data to paper nr. IV

Prevalence of *B. burgdorferi* s.l. among different localities.

	Tick samples total		Nymphal ticks		Adult ticks	
Locality	**Tested/ positive**	**Prevalence**	**Tested/ positive**	**Prevalence**	**Tested/ positive**	**Prevalence**
Netolice I.	434/35	8.06%	375/34	9.07%	59/1	1.69%
Netolice II.	455/36	7.91%	375/33	8.80%	80/3	3.75%
Prachatice	384/65	16.93%	344/59	17.15%	40/6	15.00%
Vimperk	330/59	17.88%	292/56	19.18%	38/3	7.89%
Malenice	345/70	20.29%	292/58	19.86%	53/12	22.64%
Strakonice I.	413/36	8.72%	375/35	9.33%	38/1	2.63%
Strakonice II.	417/39	9.35%	375/38	10.13%	42/1	2.38%
Blatna	372/25	6.72%	314/22	7.01%	58/3	5.17%
Pisek	194/19	9.79%	177/17	9.60%	17/2	11.76%
Vcelna	256/41	16.02%	212/37	17.45%	44/4	9.09%
Rimov	406/19	4.68%	375/19	5.07%	31/0	0.00%
Borovany	370/39	10.54%	344/38	11.05%	26/1	3.85%
Nove Hrady	397/42	10.58%	374/39	10.43%	23/3	13.04%
Malonty	183/32	17.49%	181/30	16.57%	2/2	100.00%
Kaplice	306/54	17.65%	298/52	17.45%	8/2	25.00%
Vyssi Brod	337/59	17.51%	292/54	18.49%	45/5	11.11%
Cesky Krumlov	428/66	15.42%	374/61	16.31%	54/5	9.26%
Hluboka n. Vlt.	311/45	14.47%	281/43	15.30%	30/2	6.67%
Zliv	447/86	19.24%	375/76	20.27%	72/10	13.89%
Vodnany	422/49	11.61%	375/48	12.80%	47/1	2.13%
Bechyne	391/49	12.53%	250/28	11.20%	141/21	14.89%
Milevsko	408/55	13.48%	375/53	14.13%	33/2	6.06%
Orlik n. Vlt.	230/5	2.17%	181/4	2.21%	49/1	2.04%
Plana n. Luz.	410/35	8.54%	375/32	8.53%	35/3	8.57%
Sobeslav	425/67	15.76%	375/62	16.53%	50/5	10.00%
Trebon	397/30	7.56%	375/28	7.47%	22/2	9.09%
J. Hradec	414/42	10.14%	357/37	10.36%	57/5	8.77%
Nova Bystrice	423/51	12.06%	375/45	12.00%	48/6	12.50%
Slavonice	434/62	14.29%	371/59	15.90%	63/3	4.76%
Dacice	443/44	9.93%	375/42	11.20%	68/2	2.94%
Total, average	**11,182/1,356**	**12.13%**	**9,809/1239**	**12.63%**	**1,373/117**	**8.52%**

Appendix II – examples of map outputs

Fig. A1 Interpolated map of TBE disease case occurrence in South Bohemia

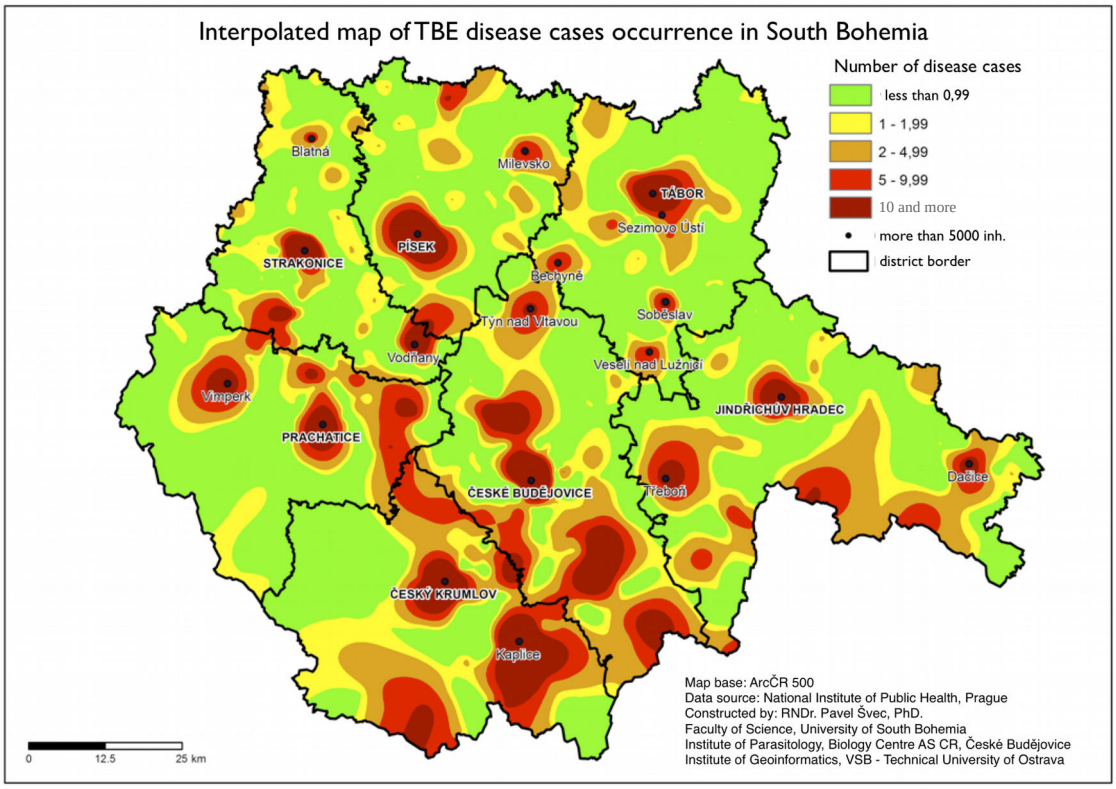


Fig. A2 Tick activity at selected study sites in South Bohemia (2008)

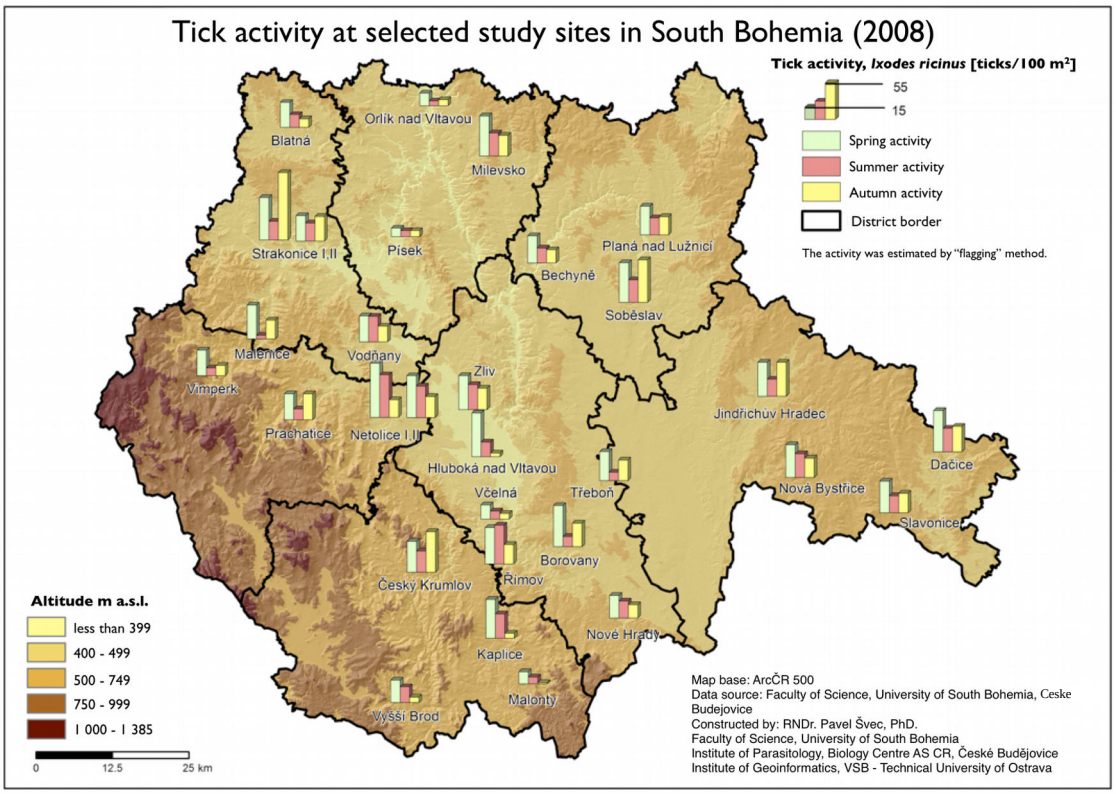


Fig. A3 Prediction of tick activity – spring

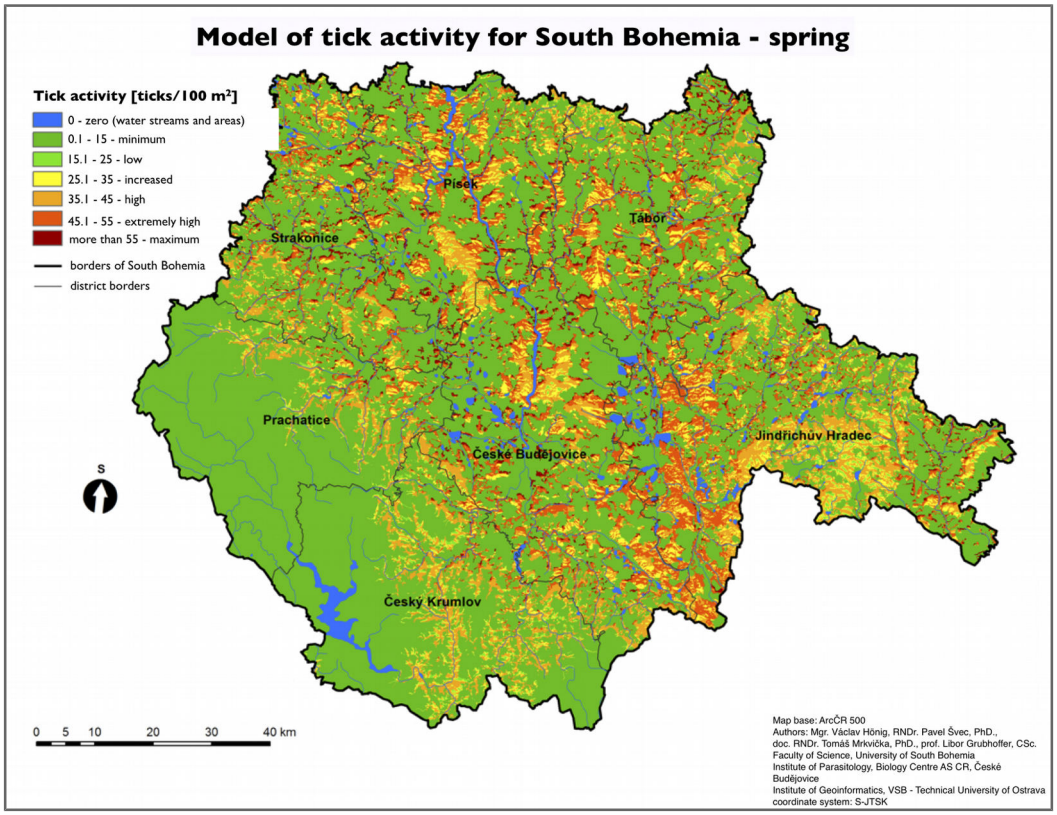


Fig. A4 Prediction of tick activity – summer

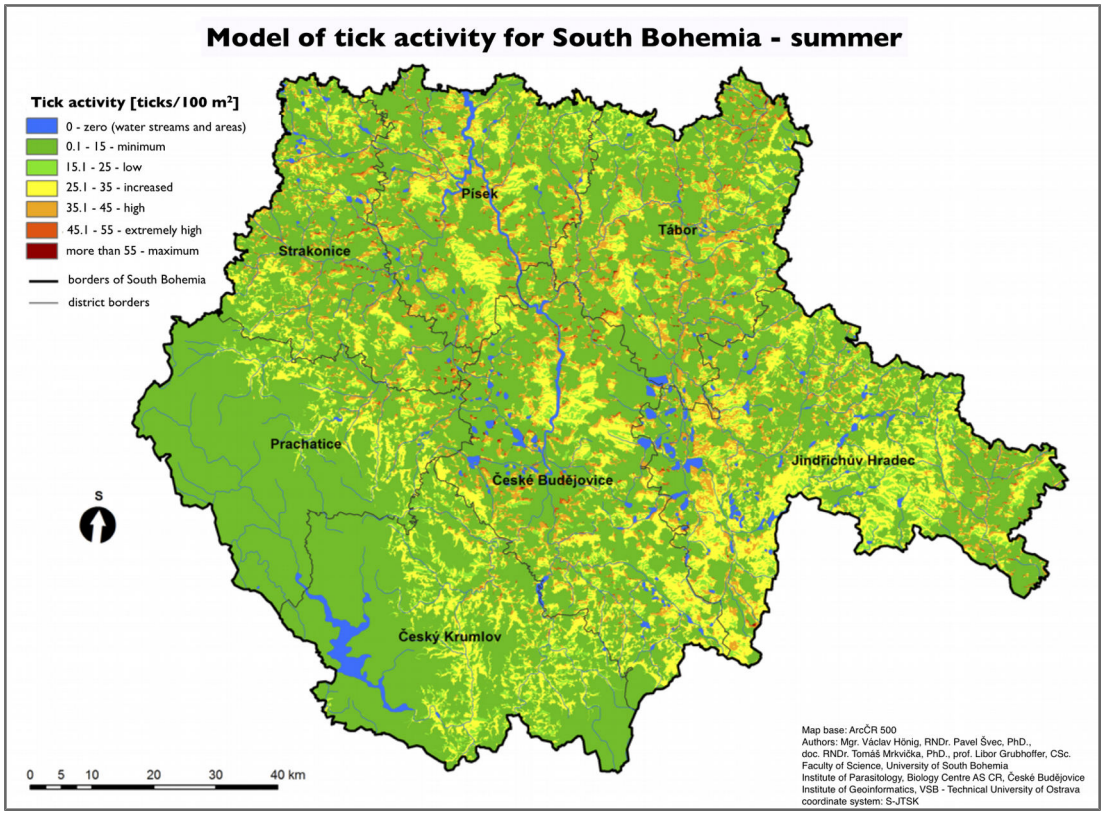


Fig. A5 Prediction of tick activity - autumn

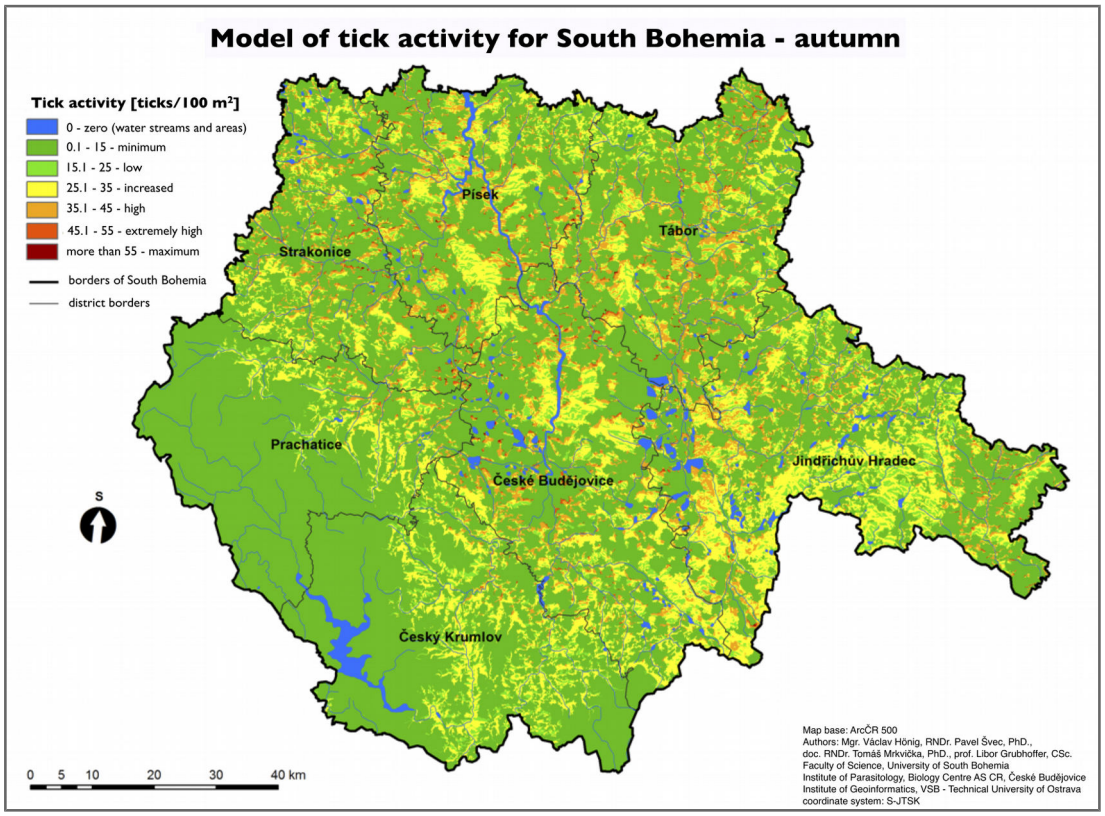


Fig. A6 Probability of tick infection by *B. burgdorferi* s.l.

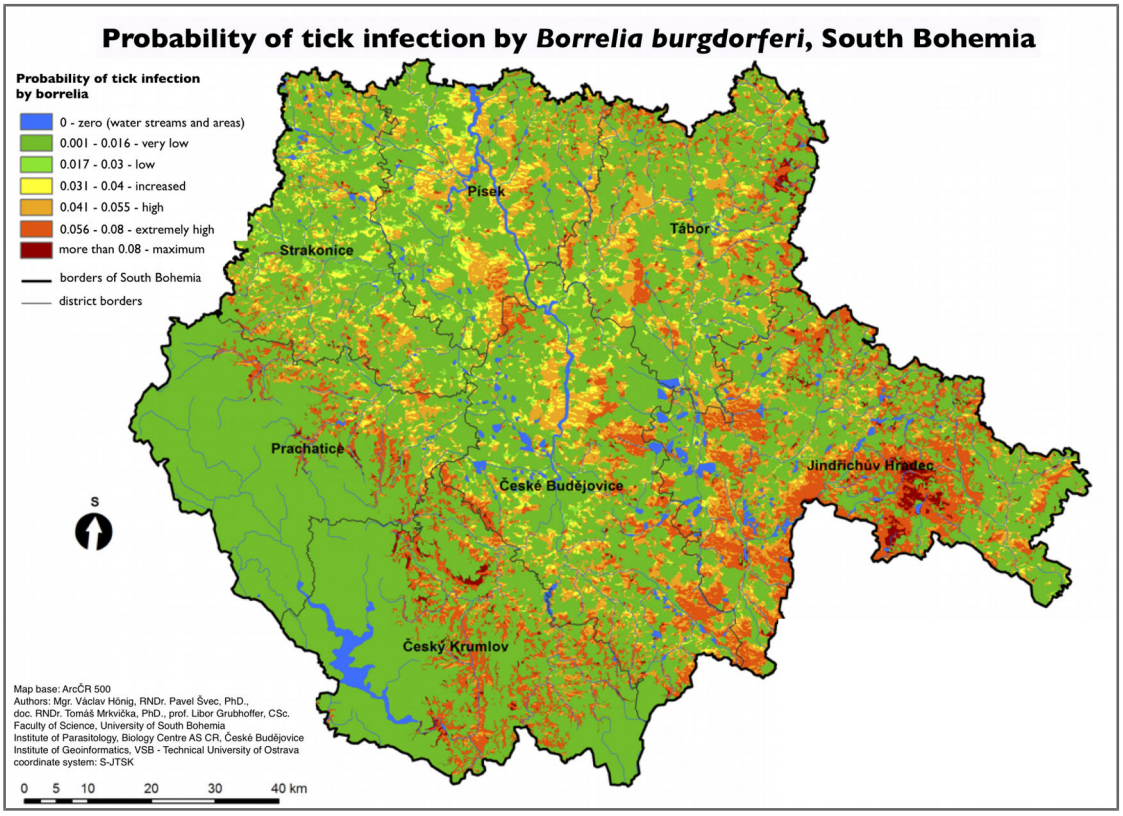
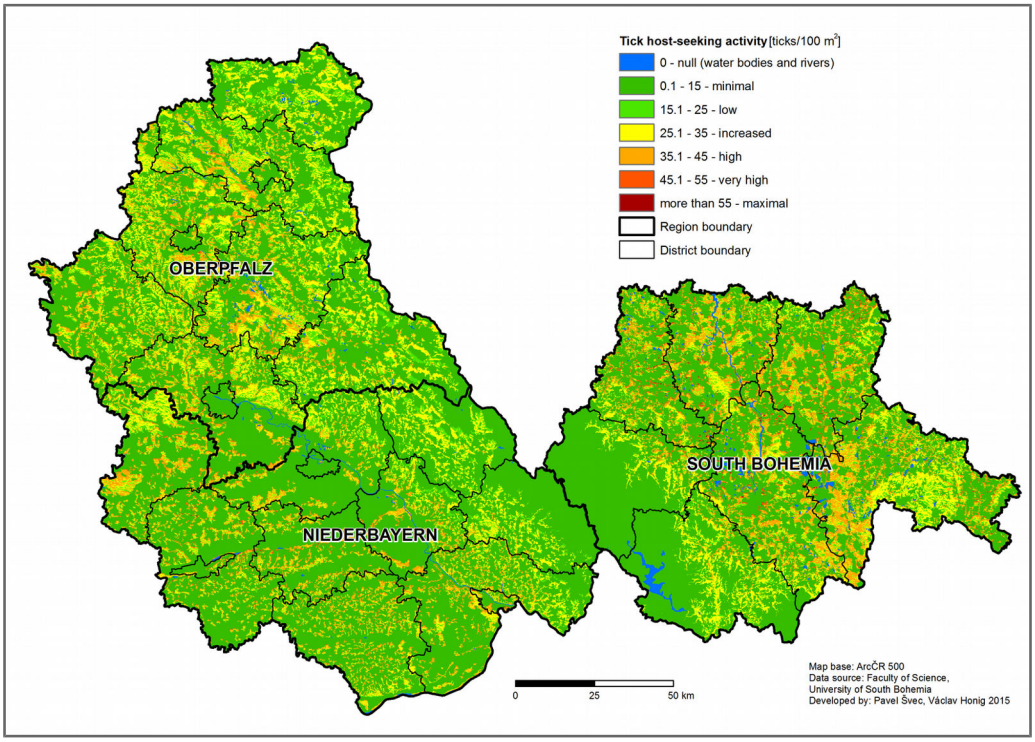


Fig. A7 Prediction of tick activity general (without seasonal effect)



Appendix III - Curriculum vitae

Václav Hönig

Born: November 23rd 1982, Písek, Czech Republic
email: honigva@gmail.com

Education

2007 - present (interrupted 2012 - 2014)	**Ph.D. studies in Parasitology**; Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic
2005 - 2007	**M.Sc. in General Biology – Microbiology**; Masaryk University, Brno Czech Republic; thesis: Genomospecies of *Borrelia burgdorferi* s.l. in the common tick in southern Moravia
2002 - 2005	**BSc. in General Biology and Medical Laboratory Techniques**; Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic; thesis: Variability of Lyme disease spirochetes analyzed by molecular biology methods.

Work experience

2011 - present	**GEN-TREND, s. r. o.** laboratory manager, research and development, QMS maintenance, marketing
2008 - 2012	**Institute of Parasitology, Biology Centre of the Academy of Sciences** of the Czech Republic, České Budějovice, Czech Republic
2009 - 2011	**Faculty of Science, University of South Bohemia,** České Budějovice, Czech Republic

Internships, stays abroad, Courses

2009	Laboratory of Eco-Epidemiology of Parasites, Institute of Biology, University of Neuchâtel, Switzerland
2009	Institute of Applied Statistic, Johannes Kepler University, Linz, Austria supported by AKTION scholarship

2009, 2010 several shorter visits, Institute of Comparative Tropical Medicin and Parasitology, Ludwig-Maximilian University, Munich

2010, 2011 PREFEKT – Systemic complex education of R&D scientists in R&D management and administration (Systematické komplexní vzdělávání pracovníků VaV v oblasti širší problematiky řízení VaV jako nezbytný PRvek EFEKTivity výzkumu). Brno, Czech Republic

Teaching activities

Teaching assistant in practical courses: Biochemistry, Medical virology and workshops POSTICK Spring school 2011 (Tick sampling, Detection of tick-borne pathogens).

Consultant of bachelor (3 students) and master theses (3 students).

Grant activities

Contributed to project proposals:
Grant agency of the Czech Republic, Grant agency of the Ministry of Health of the Czech Republics, International Co-Operation Central Europe, Technology Agency of The Czech Republic, The Enterprises and Innovations Operational Programme - Innovations

Participated as member of the research team:
"Ticks and tick borne infectious diseases in the conditions of South Bohemia and Bavaria"; European Development Fund of the European Union; University of South Bohemia, Faculty of Science

AKTION 53p19 "Spatial mapping of ticks and tick-borne infectious diseases of the region of South Bohemia and Upper Austria"; Ministry of Education, Youth, and Sports of the Czech Republic; University of South Bohemia, Faculty of Science)

Innovation of a diagnostic product – enhancement of the method of detection of periodontal pathogens; The Enterprises and Innovations Operational Programme – Innovations; GEN-TREND s. r. o.

Expansion of the services of the molecular biology laboratory to the field of veterinary medicine and innovation of the process "international lab report"; The Enterprises and Innovations Operational Programme – Innovations; GEN-TREND s. r. o.

Organizational skills

Participated on organization of following events:

- **POSTICK Spring school 2011**
- co-ordination of the project: **Ticks and tick borne infectious diseases in the conditions of South Bohemia and Bavaria**, organization of project workshops
- conference **Ticks and Tick-borne Diseases in South Bohemia and Bavari**a, 2011
- editing the project proceedings: Hönig, V., Zubriková, D., Vögerl, M., Švec, P., Pfister, K., Grubhoffer, L., 2011: Klíšťata a jimi přenášená onemocnění v Jihočeském kraji a Bavorsku / Zecken und zecken-übertragene Krankheiten in Südböhmen und Bayern. Jihočeská univerzita, České Budějovice, pp. 80, ISBN: 878-80-7394-309-7.

Publication in journal with impact factor

Schwarz, A., Honig, V., Vavruskova, Z., Grubhoffer, L., Balczun, C., Albring, A., Schaub, G.A., 2012. Abundance of *Ixodes ricinus* and prevalence of *Borrelia burgdorferi* s.l. in the nature reserve Siebengebirge, Germany, in comparison to three former studies from 1978 onwards. Parasit. Vectors 5:268. doi:10.1186/1756-3305-5-268.

Rudenko, N., Golovchenko, M., Hönig, V., Mallátová, N., Krbková, L., Mikulásek, P., Fedorova, N., Belfiore, N.M., Grubhoffer, L., Lane, R.S., Oliver, J.H. Jr. 2013. Detection of *Borrelia burgdorferi* sensu stricto ospC alleles associated with human lyme borreliosis worldwide in non-human-biting tick *Ixodes affinis* and rodent hosts in Southeastern United States. Appl Environ Microbiol. 79(5):1444-1453. doi: 10.1128/AEM.02749-12.

Crowder, C.D., Carolan, H.E., Rounds, M.A., Honig, V., Mothes, B., Haag, H., Nolte, O., Luft, B.J., Grubhoffer, L., Ecker, D.J., Schutzer, S.E., Eshoo, M.W., 2014. Prevalence of *Borrelia miyamotoi* in *Ixodes* Ticks in Europe and the United States. Emerg. Infect. Dis. 20(10):1678-1682. doi:10.3201/eid2010.131583

Honig, V., Svec, P., Halas, P., Vavruskova, Z., Tykalova, H., Kilian, P., Vetiskova, V., Dornakova, V., Sterbova, J., Simonova, Z., Erhart, J., Sterba, J., Golovchenko, M.,

Rudenko, N., Grubhoffer, L., 2015. Ticks and tick-borne pathogens in South Bohemia (Czech Republic) - Spatial variability in *Ixodes ricinus* abundance, *Borrelia burgdorferi* and tick-borne encephalitis virus prevalence. Ticks Tick-Borne Dis. 6(5):559-567. doi:10.1016/j.ttbdis.2015.04.010

Submitted manuscripts

Honig, V., Svec, P., Mrkvicka, T., Zubrikova, D., Vögerl-Wittmann, M., Masar, O., Szturcova, D., Pfister, K., Grubhoffer, L., Estimation of acarological risk of exposure to Lyme borreliosis or tick-borne encephalitis infected ticks in the border area of the Czech Republic (South Bohemia) and Germany (Lower Bavaria and Upper Palatinate) and its presentation in a form of map portal.
The manuscript submitted to Parasites and Vectors.

Publications in peer-reviewed journals and proceedings

Daniel, M., Materna, J., Honig, V., Metelka, L., Danielova, V., Harcarik, J., Kliegrova, S., Grubhoffer, L., 2009. Vertical distribution of the tick Ixodes ricinus and tick-borne pathogens in the northern Moravian mountains correlated with climate warming (Jeseniky Mts., Czech Republic). Cent. Eur. J. Public Health 17, 139–145.

Švec, P., Hönig, V., Daniel, M., Danielová, V. Grubhoffer, L. (2009): Use of GIS for mapping of ticks and tick-borne pathogens in South Bohemia In: Geografie-sborník České geografické společnosti. 114: 157-168. [article in czech]

Hönig, V., Stehlík, M., Danielová, V., Daniel, M., Švec, P., Grubhoffer, L. (2010): Tick host-seeking activity and tick-borne encephalitis incidence: regression and homogeneity. JAMSI, 6 (1): 83-88.

Hönig V., Švec P., Masař O., Grubhoffer L. (2011): Tick-borne diseases risk model for South Bohemia (Czech Republic). In: Ruzicka J., Peskova K.Symposium GIS Ostrava 2011 - Proceeding, VSB - Technical University of Ostrava, ISBN 978-80-248-2366-9.

Monograph – project proceedings

Hönig, V., Zubriková, D., Vögerl, M., Švec, P., Pfister, K., Grubhoffer, L., 2011: Klíšťata a jimi přenášená onemocnění v Jihočeském kraji a Bavorsku / Zecken und zecken-übertragene Krankheiten in Südböhmen und Bayern. Jihočeská univerzita, České Budějovice, pp. 80, ISBN: 878-80-7394-309-7. [in czech and german]

Popularization articles

Švec, P., Hönig, V., Daniel, M., Danielová, V., Grubhoffer, L., 2009: Mapping of ticks and tick-borne pathogens in the Region of South Bohemia with the use of GIS. ArcRevue 2/2009. p. 13-17. [article in czech]

Švec, P., Hönig, V., Daniel, M., Danielová, V., Grubhoffer, L., 2009: Ticks and GIS. Vesmír 88, č. 2009/6. p 409. [article in czech]

Hönig V. 2011: Vampires of South Bohemian forests. Krasec, 17: 22-23. [article in czech]

Conference contributions

Hönig V., Burri C., Materna J., Danielová V., Daniel M., Harčarik J., Gern L., Grubhoffer L. (2009): Identification of the hosts of *Ixodes ricinus* ticks collected in the Jeseníky mountain area (Czech Republic). Labudove dni conference, Bratislava, Slovak Republic, poster presentation

Hönig V., Švec P., Danielová V., Daniel M., Grubhoffer L. (2009): Complex approach in mapping andirsk prediction of tick-borne diseases. Tomáškovy dny 2009 XVIII.. conference of young microbiologists, Brno, Czech Republic, talk in czech

Hönig V., Švec P., Danielová V., Daniel M., Grubhoffer L. (2010): Ticks and tick-borne diseases in South Bohemia (Czech Republic). EDEN 2010 International Conference proceedings. Montpellier, talk in english

Hönig V., Švec P., Danielova V., Daniel M., Grubhoffer L. (2010): Ticks and Tick-borne Diseases in South Bohemia (Czech Republic). 12th international conference on Lyme Borreliosis, Ljubljana, Slovenia, poster presentation

Švec, P., Hönig, V., Masař., O., V., Grubhoffer, L. (2010): Model of prediction of tick-borne diseases – an example of South Bohemia. Ostrava, Czech Republic, talk in czech

Hönig V., Švec P., Masař O., Kilian P., Dorňáková V., Dupejová J., Palus M., Vavrušková Z., Grubhoffer L. (2011): Tick and tick-borne disease research in South

Bohemia with the use of GIS. XI. International Jena Symposium on Tick-Borne Diseases 2011, Weimar, Germany, poster presentation

Vögerl M., Čerňanská D., Švec P., Hönig V. Pfister K. (2011): The seasonal activity of questing *Ixodes ricinus* in two regions of Bavaria (Southern Germany), Oberpfalz and Niederbayern. XI. International Jena symposium on tick-borne diseases, Weimar, Germany, poster presentation

Honig V., Crowder C., Rounds M., Matthews H., Vavruskova Z., Grubhoffer L., Ecker D., Luft B., Eshoo M. (2011): *Ixodes ricinus* transmitted pathogens and potential host associations. TTP 7 Conference, Zaragoza, poster presentation

Hönig V., Švec P., Vavrušková Z., Halas P., Crowder C., Rounds M., Mathews H., Ecker, D. Eshoo, M., Luft, B., Grubhoffer L. (2011): Tick – Host – Pathogen: romantic triangle in the environment of South Bohemia. II. Labudove dni conference, Bratislava, Slovak Republic, invited lecture

Honig V. (2014): Ticks and tick borne infectious diseases in the conditions of South Bohemia and Bavaria, 16. meeting of the monitoring committee - Cross-border cooperation programme Czech Republic – Free State Of Bavaria

Applied research outputs

Specialized maps:

Number of Lyme borreliosis disease cases in the years 2001-2008 in relation with the activity of tick populations and prevalence of the causative agent in the ticks.

Number of tick-borne encephalitis disease cases in the years 2001-2008 in relation with the activity of tick populations and prevalence of the causative agent in the ticks.

The acarological risk of tick attack.

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Spatial Distribution of Tick-Borne Pathogens as a Consequence of Vector-Host-Pathogen Interactions with Environment
Ph.D. Thesis Series, 2015, No. 6

Printed in the Czech Republic by Typodesign
Edition of 20 copies

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