University of South Bohemia in České Budějovice
Faculty of science

Growth kinetics of the Lyme disease spirochetes in vector ticks *Ixodes ricinus* and *Ixodes scapularis*

Master thesis

Bc. Hana Velanová

Supervisor: RNDr. Radek Šíma, Ph.D.

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Annotation

Growth kinetics of *Borrelia afzelii* CB43 in *Ixodes ricinus* and *Ixodes scapularis* were obtained. Based on this data, *I. scapularis* was marked as capable of acquiring *B. afzelii* infection. Growth kinetics of *B. burgdorferi* N40 in *I. ricinus* was obtained as well with the same outcome, *I. ricinus* was able to acquire *B. burgdorferi* infection. Following transmission experiments showed that *I. ricinus* as well as *I. scapularis* are able to acquire the infection by *B. burgdorferi* and *B. afzelii*. Moreover, we proved that both ticks are able to transmit the infection back to naïve mice.

Declaration [in Czech]

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České Budějovice, 20. 5. 2020 ……..

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Obsah

1 Introduction

1.1 Ticks

Ticks are hematophagous ectoparasites of terrestrial vertebrates, including mammals, birds, reptiles, and even amphibians. They are widespread in tropical and temperate regions of the world. Ticks can cause harm to their hosts either directly through paralysis, allergic reaction, and tick-caused toxicoses, or indirectly. Owing to their lifecycle, ticks can transmit a great variety of pathogens, including viruses, bacteria, protozoa, and fungi, which can cause numerous severe diseases of both humans and animals (Sonenshine and Roe, 2014a). For many of these, there is not an effective treatment or vaccine developed, which leads to high economic losses, mainly in the livestock industry (Betancur Hurtado and Giraldo-Ríos, 2019).

The order Ixodida with 896 species (Guglielmone et al., 2002) belongs to the phylum Arthropoda, class Arachnida, subclass Acari, and Superorder Parasitiformes. There are three known families among Ixodida: Ixodidae, Argasidae, and Nuttalliellidae (Nava et al., 2009). The latter comprises a single African species, *Nuttalliella namaqua*. Phylogenetic analysis indicates that *N. namaqua* is a basal group to Ixodidae and Argasidae and is the closest existing lineage to the last common ancestral tick lineage (Mans et al., 2011). The family Argasidae comprises 193 species. However, there is widespread disagreement concerning the genera in this family (Guglielmone et al., 2002; Sonenshine and Roe, 2014a). The Ixodidae consists of 702 species in 14 genera (Guglielmone et al., 2002).

1.1.1 Argasidae

Ticks from the family Argasidae, also called soft ticks, differ from Ixodidae (hard ticks) in both morphology and life cycle. One feature, for which they also earned their second name: soft ticks, is a missing scutum (a sclerotized hard plate) on their dorsum. Instead, they possess a leathery, folded cuticle, which enables limited expansion when feeding (see Fig. 1). This is one of the reasons why there is a distinct difference in the lifecycle as well. The folded cuticle allows rapid engorgement usually within minutes and mostly up to 1 to 2 hours. For the attachment to the host skin, ticks use the hypostome with rows of recurved teeth. This organ is located beneath the body in Argasidae, therefore it is not visible (see Fig. 1). Although the cuticle does not allow to take up as much blood as the growing cuticle of hard ticks, the soft ticks can feed multiple times within one life stage. There are four stages in tick development:

egg, larva, nymph, and adult. Argasid ticks may have two or more nymphal instars, and each of them has to find a host, feed, detach and molt again until the final molt to the adult stage. The adult females can feed multiple times, and after each blood meal, they lay a small clutch of eggs (a few hundred). The lifecycles of soft ticks are usually very long, up to 20 years, due to the presence of many nymphal instars, which require separate blood meals. This longevity gives them a unique adaption for long periods of starvation (Sonenshine and Roe, 2014a).

The majority of Argasids are nidicolous, meaning that they live in or nearby shelters of their vertebrate hosts. Mostly they can be found in nests, burrows, caves, crevices, and in few cases in human dwellings. Soft ticks have a wide geographical distribution, and they can be found mostly in arid or semiarid regions (Sonenshine and Roe, 2014b).

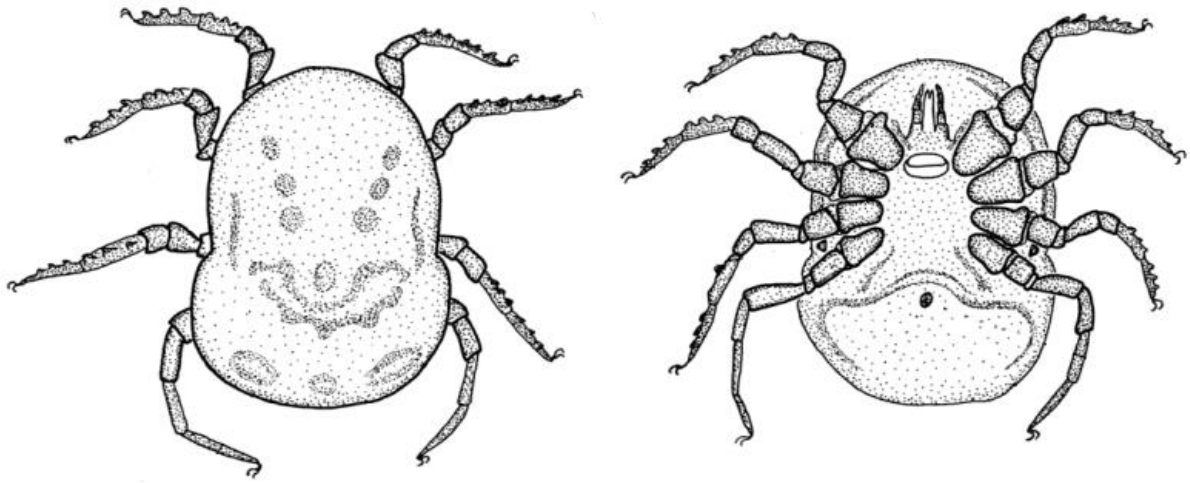


Fig. 1: Morphological features of soft ticks. Adult female of genus *Ornithodoros*, dorsal view on the left, ventral view on the right. Adapted from (Barker and Walker, 2014).

1.1.2 Ixodidae

Ixodidae are also called hard ticks due to the occurrence of a hard, sclerotized plate on the dorsal body surface called the scutum. The scutum covers the entire dorsum in adult males however, in adult females, larvae, and nymphs, it only covers the anterior half (see Fig. 2). In males, the large scutum allows only a small blood intake, whereas in females and other life stages, the remainder of the body cuticle can expand greatly. The cuticle expansion is achieved rather by a synthesis of the new cuticle, than by simple unfolding and stretching of the original one. For the attachment, Ixodid ticks use the hypostome with recurved teeth, connected to the capitulum. Both body parts are, unlike in soft ticks, visible from the dorsal view (see Fig. 2).

After attachment, the ticks secrete cement from their salivary glands, which attaches the tick firmly to the host skin. This enables ticks to feed on the host for a long period of time, which is ranging from a few days to several weeks. During slow feeding, the ticks are synthesizing new cuticle in order to accommodate the immense volume of blood meal (Sonenshine and Roe, 2014a).

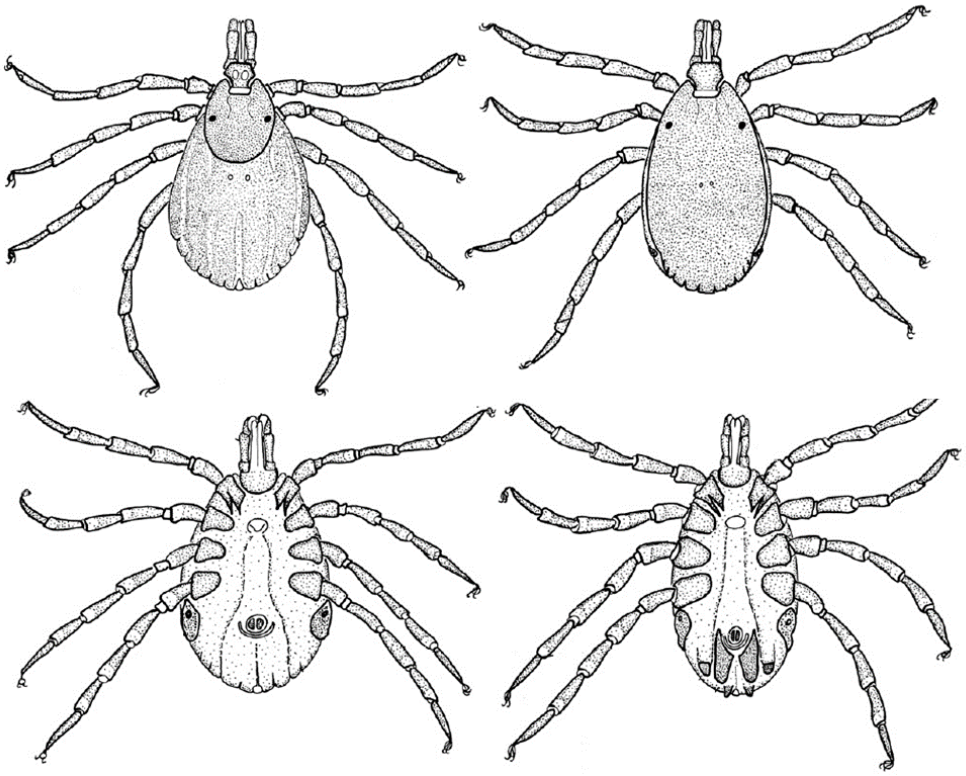


Fig. 2: Morphological features of hard ticks. Adult female on the left and adult male on the right of genus *Hyalomma*, dorsal view on the top, ventral view on the bottom. Adapted from (Barker and Walker, 2014).

The life cycle of all Ixodid ticks consists of the egg, larva, nymph, and adult, and each active stage (the latter three) feed only once in its life. The majority of hard ticks feature a three-host life cycle (see Fig. 3). The hatched larvae attach to the first host, and after full engorgement, they drop off and molt into nymphs. Unfed nymphs are questing for the second host, which can be the same or different from the previous one. They feed, drop off, and subsequently molt into adults. The adults find a host, they mate, and the adult females feed, drop off, lay eggs, and die. The size of the clutch depends on the tick species and the degree of engorgement, but the average number is a few thousand (Sonenshine and Roe, 2014a).

Some males require a blood meal before mating (Olivier, 1982). However, the majority of males do not feed at all.

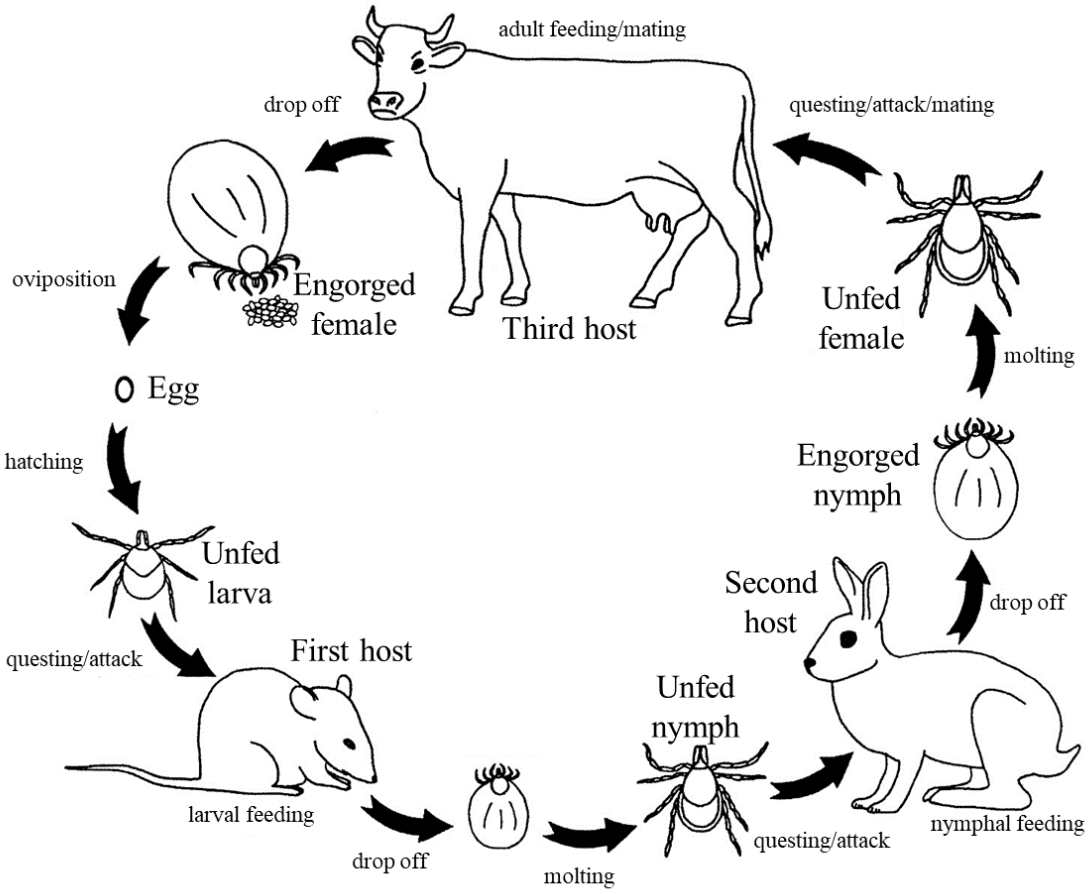


Fig. 3: Three-host life cycle of hard ticks. Adapted from (Sonenshine and Roe, 2014a).

The three-host life cycle is not the only one occurring among Ixodid ticks. Some ticks exhibit a two- or one-host life cycle. In the case of ticks exhibiting the two-host life cycle, larvae and nymphs feed on one host, because engorged larvae molt directly on the host. After that, fully engorged nymphs drop off and molt into adults. Whereas the full engorged nymphs with one-host life cycle remain on the host and molt into adults there (Sonenshine and Roe, 2014a).

The majority of hard ticks are non-nidicolous, which means that they live in an open environment such as forests, brush-lands, savannahs, and meadows, where they crawl up the emergent vegetation and wait for a passing host. To keep themselves from dehydration, they occasionally descend to seek shelter in a more humid climate, for example, in leaf litter. There

are nidicolous ticks within the Ixodidae family as well, for example, the genus *Ixodes* is mostly nidicolous. However, the best known species of this genus (*I. ricinus, I. scapularis*), which are responsible for the transmission of a wide spectrum of pathogens, are strictly non-nidicolous (Sonenshine and Roe, 2014b).

1.1.2.1. *Ixodes ricinus*

Ixodes ricinus is the most widespread European tick species, and it is also the principal vector of pathogens in Europe (Salman et al., 2013). *I. ricinus* occurs through whole Europe, west to east from Ireland to the Urals, and north to south from northern Sweden to North Africa (Estrada-Peña et al., 2013). The distribution of this tick species has broadened in the past few decades, so it can also be found in more northern areas and higher altitudes (Medlock et al., 2013). *Ixodes ricinus* is sensitive to climatic conditions requiring the minimal air humidity of 80 % (Milne, 1949). Natural habitats typically include both deciduous and coniferous woodlands, heathland, moorland, rough pasture forests, and urban parks (Medlock et al., 2013). *Ixodes ricinus* ticks are generalists, so they are able to feed on a great variety of both cold- and warm-blooded animals. The immature stages prefer to feed on smaller mammals and birds, whereas the adults feed particularly on larger hosts like cattle and deer. The time of feeding ranges from 3-5 days in larvae, 4-7 days in nymphs, and 7-11 days in females, whereas the period between feeding can last for several months or even years (Rizzoli et al., 2014). *Ixodes ricinus* transmits a great variety of pathogens, including several strains of *Borrelia*, *Anaplasma phagocytophilum*, two species of *Babesia*, Tick-borne encephalitis virus, and few others (Rizzoli et al., 2014).

1.1.2.2. *Ixodes scapularis*

Ixodes scapularis is a tick occurring mostly in woodland habitats. It has a three-host life cycle, which usually lasts for two up to four years (Yuval and Spielman, 1990). The ticks in their immature life stages (larvae and nymphs) have a broad spectrum of hosts, including rodents, insectivores, birds, lagomorphs, and ungulates (LoGiudice et al., 2003). In their adult stage, they mainly feed on medium- and large-sized mammals, mostly white-tailed deer (Piesman et al., 1979). The distribution of *I. scapularis* is concentrated in North America occupying the eastern part of the USA and south-eastern part of Canada, and the geographic range within the USA is gradually expanding (Eisen and Eisen, 2018). *Ixodes scapularis* is a

significant vector of several diseases in the USA. Until the year 2017, seven *I. scapularis*-borne human pathogens were described: *Borrelia burgdorferi*, *B. miyamotoi,* and *B. mayonii* causing the Lyme disease and relapsing fever; *Babesia microti* causing Babesiosis; *Anaplasma phagocytophilum* causing Human granulocytic anaplasmosis; *Ehrlichia muris* causing ehrlichiosis and Powassan virus (Eisen and Eisen, 2018)

1.2 Borreliae

Borrelia is a genus of bacteria belonging to the spirochete phylum. The structural characteristics shared with other spirochetes are as followed: cells are helically shaped and motile with three modes of movement; an outer cell membrane surrounds the protoplasmic cylinder complex, which consists of the cytoplasm, the inner cell membrane, and the peptidoglycan; and the flagella are located in the periplasmic space rather than on the surface (see Fig 4).

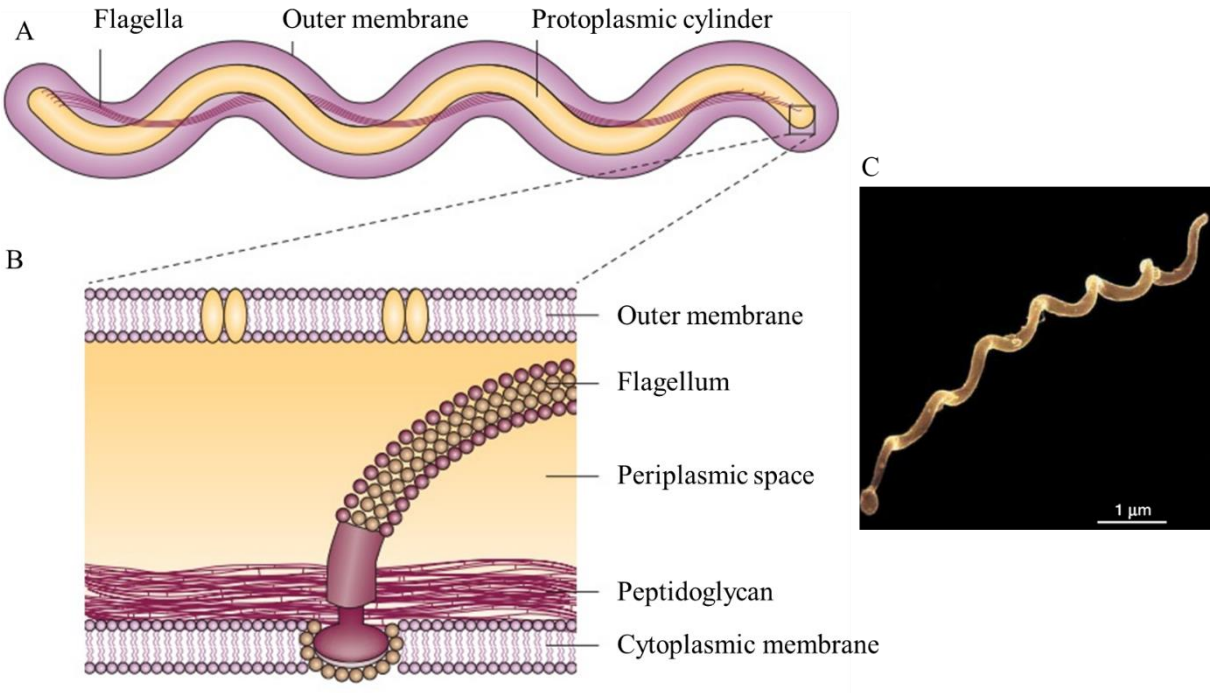


Fig. 4: Structure and morphology of *B. burgdorferi*. A: Diagram of the spirochete. Bundles of flagella wind around the protoplasmic cylinder. B: Diagram of flagella. Each flagellum is inserted in the cytoplasmic membrane and extends to the periplasm. The motility of *Borrelia* results from the coordinated motion of flagella, which form a complex structure. C: Picture of *B. burgdorferi* from scanning electron microscopy. Adapted from (Rosa et al., 2005).

Genus *Borrelia* can be well distinguished from other spirochetes in their characteristics of being transmitted to vertebrates by hematophagous arthropods (Barbour and Hayes, 1986). Members of this genus are of an obligate parasitic lifestyle and well recognized as agents of Lyme disease and relapsing fever in humans. According to the disease they cause, *Borrelia* can be divided into two clades. One associated with ixodid ticks and causing the Lyme disease, and the other being transmitted mainly by argasid ticks, and causing the relapsing fever (Margos et al., 2018). Based on molecular phylogeny, some authors propose that these two groups should be divided into two genera (Adeolu and Gupta, 2014). Others claim that they belong to the same genus (Margos et al., 2018). Both groups are dependent on their vertebrate and arthropod hosts for most of their nutritional requirements and share a unique genomic structure. Their genome comprises one linear chromosome, which is highly conserved and numeral extrachromosomal structures, which involve both linear and circular plasmids highly variable between strains (Casjens et al., 2012; Miller et al., 2013). By the year 2014, there were 21 species of the relapsing fever causing *Borrelia* described (Sonenshine and Roe, 2014b). By now, there are 22 species causing the Lyme disease known and well established around the world, but descriptions of new species and variants continue to be recognized, so the current number of described species is probably not final.

Considering the human sensitivity to *B. burgdorferi* sensu lato, the complex of 22 *Borrelia* species can be divided into two groups:

- Twelve species that have not been detected in or isolated from humans yet: *B. americana* (Rudenko et al., 2009b), *B. andersonii* (Marconi et al., 1995), *B. californiensis* (Postic et al., 2007), *B. carolinensis* (Rudenko et al., 2009a), *B. chilensis* (Ivanova et al., 2014), *B. finlandensis* (Casjens et al., 2011), *B. japonica* (Kawabata et al., 1993), *B. lanei* (Margos et al., 2017), *B. tanukii* (Fukunaga et al., 1996), *B. turdi* (Fukunaga et al., 1996), *B. sinica* (Masuzawa et al., 2001), and *B. yangtzensis* (Chu et al., 2008).

- Ten species with pathogenic potential, that were detected or isolated from humans: *B. afzelii* (Canica et al., 1993), *B. bavariensis* (Margos et al., 2009), *B. bissettii* (Postic et al., 1998), *B. burgdorferi* sensu stricto. (Baranton et al., 1992), *B. garinii* (Baranton et al., 1992), *B. kurtenbachii* (Margos et al., 2010), *B. lusitaniae* (Fleche et al., 1997), *B. mayonii* (Pritt et al., 2016), *B. spielmanii* (Richter et al., 2006), and *B. valaisiana* (Wang et al., 1997).

1.2.1 Geographic distribution of *Borrelia burgdorferi* sensu lato

The Lyme disease causing agent is also referred to as *B. burgdorferi* sensu lato, or *B. burgdorferi* complex. The species complex consists of 22 species, which are distributed all around the Holarctic region, and it is also the most prevalent vector-borne disease there (Margos et al., 2011). Due to the obligate parasitic lifestyle, the biology of *Borreliae* is tightly linked to that of their hosts, which of course, define their ecological niches and geographical distribution (Kurtenbach et al., 2002). The compatibility of spirochetes with tick vectors has not been studied very much. However, some studies show that although many ticks of genus *Ixodes* can transmit several species of *Borrelia*, some *Borrelia*-vector associations are not compatible or less efficient (Dolan et al., 1998). Host specialization and vector compatibility most likely influence the global distribution of *Borrelia* species. In Europe, there are eight *Borrelia* species, of which three (*B. garinii*, *B. afzelii*, and *B. bavariensis*) can also be found in Asia. *B. garinii* has the biggest areal since it can be found in Eurasia and also on the east coast of Canada. In America, there are seven *Borrelia* species (Margos et al., 2011). All the species with their distribution can be found in Fig. 5.

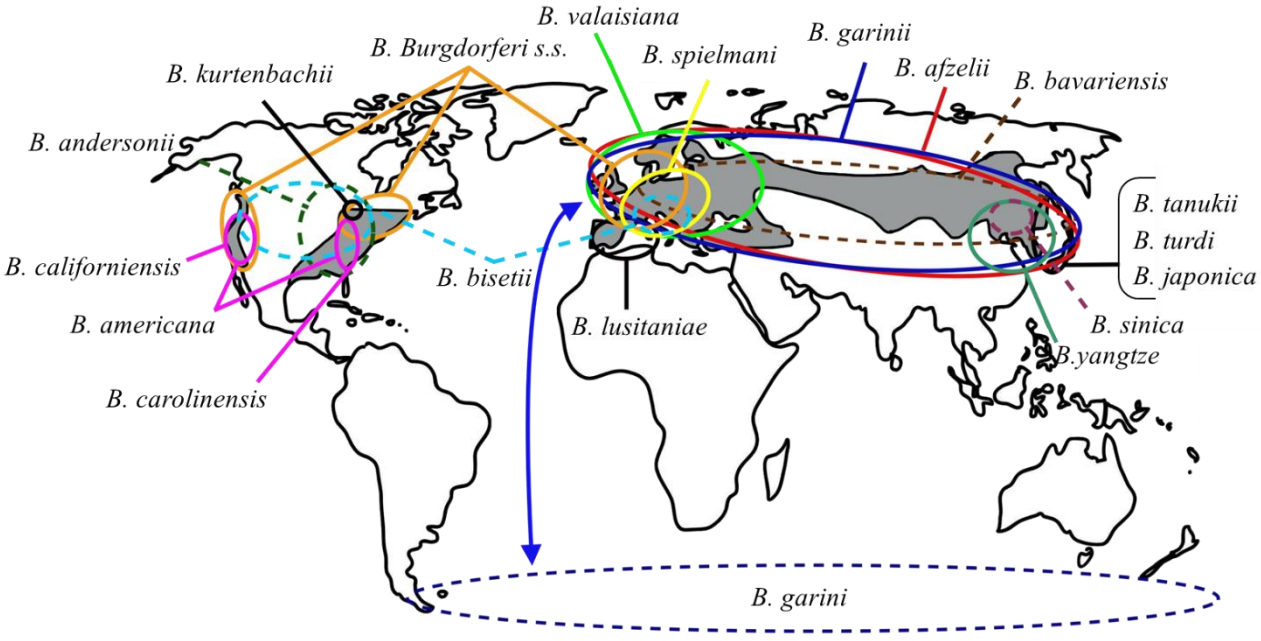


Fig. 5: A map of the global geographical distribution of *B. burgdorferi* complex. The grey areas represent the distribution of vectors. The circles and ellipses represent an approximate distribution of *Borrelia* species. Adapted from (Margos et al., 2011).

1.2.2 Life cycle of *Borreliae*

Borreliae are maintained in nature in an enzootic cycle that involves the transmission from tick vector to a vertebrate host, and acquisition from the vertebrate host back to a tick vector. The transmission cycles are maintained by the ticks from the *I. ricinus-I. persulcatus* complex, which is represented by *I. ricinus* in Western and Central Europe, *I. persulcatus* in Eastern Europe and Asia, *I. scapularis* in central and eastern North America, and *I. pacificus* in western North America (Lane et al., 1991; Sonenshine and Roe, 2014b). Ticks from this complex have three developmental stages, and each stage requires a blood meal from a vertebrate host, with the exception of adult males. *Borrelia* spirochetes are mainly preserved in nature by horizontal transmission between ticks and vertebrate hosts (Tsao, 2009). The typical transmission cycle involves infection of rodents, birds, or other medium-sized mammals by infected nymphs. Nymphs are also principal developmental stages to infect humans (Matuschka et al., 1992). Larvae can become infected either by feeding on infected vertebrate hosts or less likely by co-feeding on naïve host in close vicinity of an infected tick (Voordouw, 2015). Engorged larvae later molt into infected nymphs, which are further questing for hosts to complete their lifecycle, and continue to spread the *Borrelia* spirochetes (see Fig. 6). Adult ticks usually do not participate in the transmission cycle because they mostly feed on incompetent hosts such as deer or ungulates (Gern, 2008).

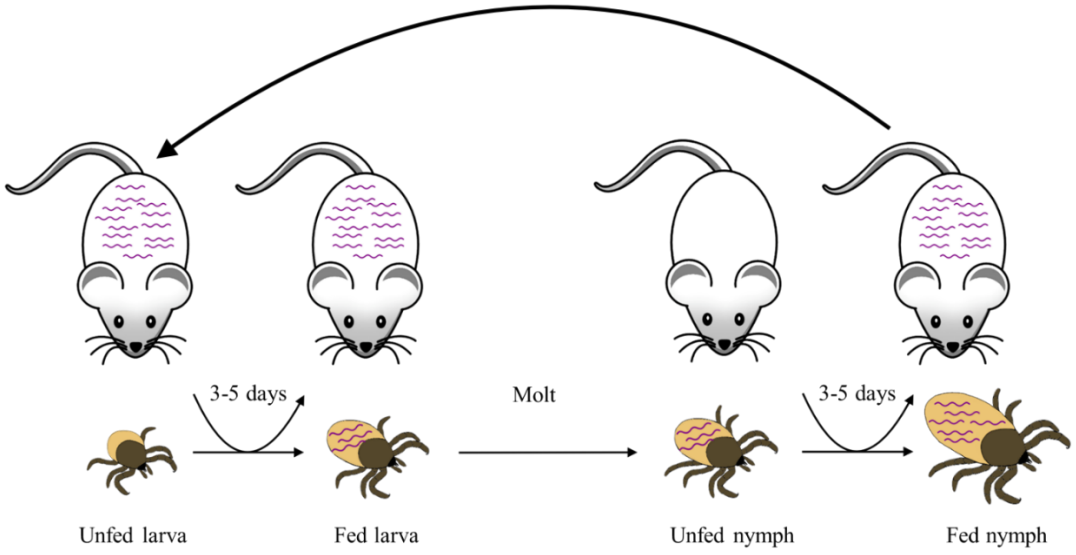


Fig. 6: Infectious cycle of *B. burgdorferi* sensu lato Naïve larvae acquire infection from infected hosts, mainly from rodents. Engorged larvae subsequently molt into infected nymphs, which further feed on other, possibly naïve hosts and establish *Borrelia* infection in them.

1.2.3. Interaction of *Borrelia* spirochetes with their tick vectors

Ticks of the genus *Ixodes* acquire *Borrelia* spirochetes when feeding on their infected reservoir hosts. Spirochetes remain within the tick's midgut as larvae molt into nymphs. When nymphs start feeding, the spirochetes sense a signal to migrate to the vertebrate host. There are three routes of spirochete transmission proposed. The first two hypotheses assumed *Borrelia* transmission via regurgitation, which is a direct migration of spirochetes from the tick gut to the host, or infection via contaminated feces (Benach et al., 1987). However, the latter was rejected (Cook, 2014). The most widely accepted today is the salivary route, which will be described further (Spielman et al., 1987).

For the successful colonization of the host, and for the survival in different environmental conditions, *Borrelia* spirochetes show differential gene expression at specific locations. After ingestion by ticks, spirochetes express their outer surface proteins (Osp), which were first described by Schwan et al. (1995). *Borrelia* spirochetes synthesize OspA when entering the ticks, which has three known functions. It protects the spirochetes from the tick's acquired immunity, it facilitates the adhesion of spirochetes to midgut cells via TROSPA receptor, and it enables interaction and adherence among spirochetes (Battisti et al., 2008; Pal et al., 2004). Spirochetes stay attached to the midgut until the next blood meal, when they sense certain stimuli (temperature, pH, cell density) that initiate their rapid replication. During nymphal feeding, OspA is downregulated, whereas another outer surface protein (OspC) is upregulated (Schwan et al., 1995). Together with the downregulation of TROSPA by ticks, it helps the spirochetes to detach from the tick's midgut and start migrating through the hemocoel into the salivary glands. Spirochetes can bind to the salivary glands of their vector, and they can be transmitted to the vertebrate host together with the saliva (Pal et al., 2004).

1.2.3.1 Infection of the vertebrate hosts

During feeding of infected nymph on a vertebrate host, a change in the expression of outer surface protein occurs, which helps the *Borrelia* spirochetes to migrate into the new host. The expression of OspC facilitates not only the migration to the host but also the establishment of early infection in the host (Grimm et al., 2004). During the initial phase, the success of infection does not depend on exploiting the host tissues, instead of the strategy of evading the host's immune system is applied. The rate of survival of *Borrelia* spirochetes is likely enhanced by tick saliva, which contains a variety of immunomodulatory substances that

protect the tick against the host immune system (Nuttall et al., 2000). During the early infection spirochetes disseminate locally in the dermis and later enter the bloodstream.

To continue evading the immune system, spirochetes eventually downregulate OspC and introduce a new mechanism. The adaptive immunity of the host is deceived by the antigenic variation of an outer membrane lipoprotein of the *vls* system. This system comprises of *vlsE* protein with highly variable cassettes combined with invariable regions, which allows random recombination and though many possible variants of this protein (Zhang and Norris, 1998). After the host adaptive immune system becomes active, spirochetes exit the bloodstream and invade various tissues, including skin, joints, heart, and bladder, where the persistent infection can be developed (Barthold et al., 1991). Not to be detected by the immune system, the spirochetes often migrate to the extracellular matrix, which may provide immunoprivileged sites for them, or perhaps survive intracellularly (Cabello et al., 2007).

The process of migration of spirochetes from the vertebrate host into the feeding tick is poorly understood. Spirochetes may be driven to the feeding site by chemotaxis since salivary gland extracts attract *Borrelia* spirochetes in vitro (Tsao, 2009).

1.2.4 Genome of *Boreliae*

The genomes of *Borreliae* are from the most complex of any bacteria (Casjens et al., 2012). It consists of approximately 950 kb linear chromosome and various circular and linear plasmids with a size of 9 to 62 kb. Their genome has a low GC content with app. 28% (Brisson et al., 2012). Most housekeeping genes can be found on the chromosome. However, the majority of genes encoding outer lipoproteins, which mediate transition through the *Borrelia* life cycle, are on the plasmids. The importance of such lipoproteins can be demonstrated by their abundance since they form slightly less than 8% of all open reading frames in *B. burgdorferi* (Samuels, 2011). Additionally, 6% of the genes on the chromosome are genes encoding products, which ensure the motility and chemotaxis of spirochetes. This demonstrates another key attribute to navigate within the enzootic cycle (Charon et al., 2012).

Borreliae lack the capacity to synthesize amino acids, nucleotides, fatty acids, and enzyme cofactors since they were probably lost during coevolution with their vectors and hosts (Fraser et al., 1997). Instead of synthesizing these, more than 50 genes encoding transporters or binding proteins of such molecules can be found in the genome of *B. burgdorferi* (Saier and Paulsen, 2000). It also lacks genes encoding enzymes for the tricarboxylic acid cycle and

oxidative phosphorylation. Therefore *Borreliae* are completely dependent on nutrients from the host (Von Lackum and Stevenson, 2005).

1.2.5 Evolution of different *Borrelia* species

Many species in Europe seem to be adapted on either mammals or birds, whereas the major species in America, *B. burgdorferi* sensu stricto, is rather a generalist (Hanincova et al., 2003; Kurtenbach et al., 2002). Not every host species can be infected by each *Borrelia* species. It is due to the different sensitivity of the spirochetes to the host serum (Kurtenbach et al., 1998b). The key point occurs immediately after nymphs start feeding on a host when spirochetes residing in the tick midgut are challenged with the serum of the vertebrate host. The spirochetes are attacked by non-specific alternative complement pathway ingested in host blood. If the *Borrelia* species is susceptible to the alternative pathway complement of the host on which it is feeding, the spirochetes are killed by the membrane attack complexes coupled with the complement before they can migrate to the host (Tsao, 2009). On the other hand, if the *Borrelia* species is compatible with a given vertebrate host, it can express several complement regulator-acquiring surface proteins (CRASPs), which can bind to the various proteins of the alternative complement pathway to avoid recognition and elimination by the complement. These bacterial surface proteins, which include virulence factors from OspE/F family and Csp proteins, recruit human factor H, factor H-like protein, and other H-related proteins, which serve as negative regulators of the alternative complement pathway (Garcia et al., 2016; Kurtenbach et al., 2002; McDowell et al., 2003). This is the fundamental mechanism of specialization of different *Borrelia* species (Kurtenbach et al., 2006).

It was found that not only *Borrelia* can inhibit the alternative pathway of complement, but there are other molecules, which can interfere with the classical pathway of complement as well. It was reported that borrelial lipoprotein BBK32 inhibits the classical pathway by binding to C1 component of the complement pathway. This is an example of a spirochetal protein that acts as a direct inhibitor of the classical pathway (Garcia et al., 2016).

The strains of *B. burgdorferi* sensu lato can be separated into three ecological groups based on the specialization on either mammals, birds, or both. The least specialized is *B. burgdorferi* sensu stricto, which can be transmitted to ticks via both rodents and birds (Donahue et al., 1987; Rand et al., 1998), and therefore must be relatively resistant to rodent and avian complement (Kurtenbach et al., 1998b). *B. afzelii* is closely associated with rodents,

however, it is eliminated from nymphs feeding on birds (Hanincova et al., 2003). On the other hand, another European species, *B. garinii* and *B. valaisiana*, are associated with birds and would be eliminated from a nymph feeding on a small mammal (Kurtenbach et al., 1998a; Olsen et al., 1995).

1.3 Lyme disease

The Lyme disease was first described almost forty years ago by the American scientist Willy Burgdorfer, who also identified the causative agent as tick-borne spirochete *B. burgdorferi* (Burgdorfer et al., 1982). It is a multisystemic disease, which can affect many organs (e.g., heart, joints, CNS), and it can have many symptoms like extreme fatigue, flu-like symptoms, arthritis, peripheral neuropathy, or cognitive dysfunction. Lyme disease is the most common tick-borne disease in the northern temperate zone, with approximately 85 000 cases reported annually in Europe (Lindgren and Jaenson, 2006). More than 30 000 cases are reported annually in the US by the Centers for Disease Control and Prevention, although the actual number is estimated to be much higher (according to CDC, 2013).

Lyme disease is typically divided into three stages. During the early phase of Lyme disease (stage I), spirochetes are present in the skin, and the typical manifestations include erythema migrans, which is an inflammatory skin rash, often accompanied by influenza-like symptoms. Stage II, early disseminated Lyme disease, involves spirochetes migrating and settling in tissues including heart, joints, or nervous system, where they can cause carditis, arthritis, neuropathies, and sometimes manifests neurological symptoms, such as meningitis. If left untreated, *Borrelia* infection can progress to late-persistent disease (stage III), with chronic arthritis, neuroborreliosis, and skin disorders (Steere, 2001).

The Lyme disease is caused by several *Borrelia* species, which belong to the *B. burgdorferi* complex. Among these, three are the main cause of the disease in humans: *B. burgdorferi* sensu stricto in America, and *B. afzelii* and *B. garinii* in Europe. Different species are also coupled with different clinical manifestations of Lyme disease (Steere, 2001; van Dam et al., 1993). *B. burgdorferi* sensu stricto is often associated with arthritis, *B. afzelii* with chronic skin disorders, and *B. garinii* with neurological symptoms. However, the connection between *Borrelia* species and manifestations is not absolute (van Dam et al., 1993). Although the species listed above are the most reported agents causing Lyme disease in humans, there are also others, mainly in Europe, reported in human infections such as *B. spielmanii*,

B. bavariensis, *B. bissettii* or *B. lusitaniae* (Fingerle et al., 2008; Rudenko et al., 2009c; Stanek et al., 2011).

1.3.1 Lyme disease transmission models

It is essential to understand interactions between *Borrelia* spirochetes, their tick vectors, and vertebrate hosts. This comprehension is the key element for the development of treatment and vaccines for Lyme disease. Although there were many studies conducted, since the first discovery of spirochetes causing the Lyme disease, most of them were focused on a single species: *B. burgdorferi* sensu stricto (De Silva and Fikrig, 1995; Hodzic et al., 2002; Ohnishi et al., 2001; Piesman et al., 1990, 2001; Schwan et al., 1995; Spielman et al., 1987). Therefore the generally accepted model of *Borrelia* transmission is not always consistent with the recent findings of other *Borrelia* species (Crippa et al., 2002; Pospisilova et al., 2019; Van Duijvendijk et al., 2015). Major differences between the generally accepted American model for *B. burgdorferi* sensu stricto and *I. scapularis* and newly described European model for *B. afzelii* and *I. ricinus* will be further discussed.

1.3.1.1 American transmission model: *Borrelia burgdorferi*/*Ixodes scapularis*

Borrelia spirochetes are acquired by naïve *I. scapularis* nymphs feeding on an infected host within 24 hours after attachment. The spirochetes then reside in tick midgut through molting until the next blood meal. The kinetics of spirochetes in the midgut after repletion involves an initial period of immediate rapid multiplication, reaching the maximum before molting. When the molting process is initiated, the amount of spirochetes present in midgut starts to decrease dramatically, and during the post-molting period, it remains low (Piesman et al., 1990). The decrease in numbers of *B. burgdorferi* during molting could be explained by depletion of the fundamental component of spirochetal development, N-acetylglucosamine, which is also an important building block of integumentary chitin, that is needed for the reformation of exoskeleton during molting (Barbour, 1986; Piesman et al., 1990).

As soon as the flat nymphs start feeding on a vertebrate host, the spirochetes present in *I. scapularis* midgut undergo many dramatic changes. First of all, they start to multiply rapidly within the tick midgut resulting in overall higher spirochetes density (De Silva and Fikrig, 1995). According to Piesman et al., the number of spirochetes increased ten times, compared to unfed nymphs (Piesman et al., 2001). Together with an increased density, a

change in expression of outer surface proteins occurs when the OspA present during the dormant phase is replaced by OspC as described in the previous chapter (Schwan et al., 1995).

It was shown that also in salivary glands, the number of spirochetes during feeding is increasing. Piesman et al. observed that although only a small number of spirochetes was present during the first two days of feeding, this number started to increase rapidly from 48 to 60 hours, and after repletion, the number of spirochetes present in salivary glands started decreasing again (Piesman et al., 2001). This trend is supported by other studies as well (De Silva and Fikrig, 1995; Ohnishi et al., 2001). Therefore the generally accepted model for *B. burgdorferi* transmission is the salivary route. After getting certain stimuli, spirochetes start to penetrate into the hemolymph, and they migrate to the salivary glands. However, the mechanism remains unclear (De Silva and Fikrig, 1995).

Generally, it is known that the risk of Lyme disease increases with the length of tick attachment. The establishment of minimal time needed for the development of infection is challenging. Several studies were conducted stating that if the tick is attached for less than two days, the infection is not transmitted (Ohnishi et al., 2001; Piesman et al., 2001).

1.3.1.2 European transmission model: *Borrelia afzelii*/*Ixodes ricinus*

The research of Lyme disease in Europe lags behind the USA. This can be due to the fact that in Europe there are many *Borrelia* species acting as agents, whereas the majority of cases in the USA are caused by a single species. Up to date, there were only a few studies focused on European *Borrelia* species and *I. ricinus* (Crippa et al., 2002; Koci et al., 2006; Pospisilova et al., 2019; Van Duijvendijk et al., 2015). However, this data suggest that European transmission model distinctly differs from the American one in many ways.

The *B. afzelii*-*I. ricinus* transmission model was established in our laboratory with the following results (Pospisilova et al., 2019).

When *I. ricinus* acquires *B. afzelii* from the infected rodent, the number of spirochetes in the first few weeks after repletion remains low. However, this initial phase is followed by rapid growth. Unlike in *B. burgdorferi*/*I. scapularis* model, the growth does not stop until the second week after molting. This phase of rapid multiplication is followed by a quick decrease in the spirochetes population, and from the fourth week post-molt, the number of spirochetes remains stable.

During feeding of *I. ricinus* nymph on the vertebrate host, an opposite trend in the number of spirochetes was observed. Instead of rapid multiplication, the number of spirochetes in the midgut was decreasing as the spirochetes were migrating to the new host. In addition, when the presence of spirochetes in salivary glands was tested, there were no spirochetes detected at any stage, and this is also contradictory to the American model. The absence of spirochetes in salivary glands may point to another transmission route than observed for *B. burgdorferi*, the direct 'gut to mouth' route proposed by Burgdorfer, where the spirochetes should be actively migrating from the midgut to the host (Burgdorfer, 1984).

The length of tick attachment required for the development of Lyme disease has proven to be shorter than for *B. burgdorferi* as the time interval of 24 up to 48 hours of exposure to *B. afzelii* infected tick is critical for the development of infection in mice. This is also in accordance with previous findings (Crippa et al., 2002).

Although these two models differ in a number of features described above, there are others that are very similar. For instance, it was found that *B. afzelii* has homologous outer surface proteins, which are expressed in the same manner as in *B. burgdorferi*. OspA is upregulated in larvae and nymphs, but as soon as the nymph starts feeding, OspA is downregulated, whereas OspC is upregulated.

2 Objectives

1. Growth kinetics of the European *B. afzelii* in American vector *I. scapularis*.
2. Growth kinetics of the American *B. burgdorferi* in European vector *I. ricinus*.
3. Comparison with known transmission models for *B. afzelii*/*I. ricinus* and *B. burgdorferi*/*I. scapularis*.
4. Transmission of *Borrelia* spirochetes by nymphal *I. ricinus* and *I. scapularis*.

3 Materials and methods

3.1 Laboratory animals

3.1.1 Ticks

For the experiments, ticks from the breeding facility of the Institute of Parasitology, Biology Centre of Academy of Sciences of the Czech Republic were used. There were two species used, *I. ricinus* and *I. scapularis*. Both species were kept under the same conditions, in glass vials placed in wet chambers with the air humidity of about 95%. The temperature was maintained constant at 24 °C, and the photoperiod was set to 12 hours of light and 12 hours of dark.

3.1.2 Mice

For the pathogen transmission experiments, inbred pathogen-free mouse strain CH3/HeN (Jackson Laboratory, Germany) was used. The mice were kept in the animal facility of the Institute of Parasitology under standard conditions. All experimental animals were treated according to the Animal Protection Law of the Czech Republic No. 246/992 Sb., ethic approval No. 137/2008.

3.2 Infection of mice and ticks

For the infection, two species of *Borrelia* were used: *B. afzelii* CB43 – European strain isolated from local *I. ricinus* ticks (Stepanova-Tresova et al., 2000); and *B. burgdorferi* N40 – North American strain obtained from Prof. Erol Fikrig, Yale School of Medicine. In order to infect the mice, *Borrelia* spirochetes were first cultivated in BSK-H medium (Sigma-Aldrich) for 5 to 7 days at 33 °C. After that, female C3H/HeN mice were infected by subcutaneous injection of 10^5 spirochetes in 100 µl BSK-H medium. The presence of *Borrelia* spirochetes was tested three weeks after injection in ear biopsies by standard PCR. When proven positive, tick larvae were enabled to feed on infected mice (approximately 100 larvae per mouse). Fully fed larvae were collected and stored in wet chambers while allowed to molt into nymphs.

In the first experiment, four mice were infected with *B. afzelii* CB43. On two of them, larvae of *I. ricinus* were placed, on the other two mice, larvae of *I. scapularis* were placed and allowed to feed. Fully fed larvae were stored in standard conditions listed above. In the course

of ten weeks, ten individuals (first larvae, later nymphs) of *I. ricinus* and ten individuals of *I. scapularis* were randomly selected every week in order to quantify the load of *Borrelia* spirochetes in each tick by qPCR.

The second experiment was carried out in a similar way. Four mice were infected with *B. burgdorferi* N40, and larvae of *I. ricinus* were used to feed on them. In order to improve the statistical results, 20 individuals of *I. ricinus* were tested for the presence of *Borrelia* spirochetes every week for a period of 10 weeks.

3.3 Transmission of *Borrelia* spirochetes

Ten weeks after taking their last blood meal on infected mice, already molted nymphs were tested whether they are able to transmit *Borrelia* spirochetes back to naïve mice. From the first experiment, 25 *I. scapularis* nymphs were randomly selected and placed on five mice (5 ticks on each mouse). The nymphs were allowed to feed, and after full engorgement, they were collected and tested for *Borrelia* presence by standard PCR. The presence of spirochetes in mice was inspected four weeks after engorgement. Samples were taken from the ear, bladder, and heart.

From the second experiment, 60 nymphs were selected and placed on six mice (ten nymphs per mouse). Mice were separated into three groups, which differed from each other by a length of tick attachment (24 h, 48 h, and until full engorgement) The presence of spirochetes in mouse tissues was tested four weeks after engorgement in ear, bladder, heart, and joint.

3.4 Growth kinetics of *Borrelia* spirochetes

The kinetics of *Borrelia* growth within the feeding tick was inspected. Therefore, mice from the second experiment were separated into three groups (two mice per group). From the first group, ticks were collected after 24 hours, from the second group, ticks were collected after 48 hours, and ticks from the third group were collected after full engorgement. DNA from the ticks from all groups was isolated, and the number of *Borrelia* spirochetes was quantified by qPCR.

3.5 Isolation of DNA

For the isolation of DNA from both ticks and murine tissues, a NucleoSpin Tissue Kit (Macherey-Nagel) was used. Prior to isolation, ticks were disrupted either by bead beats or manually cut into pieces. The DNA was isolated following the manufacturer's standard protocol for human or animal tissue. The only change to the protocol was the volume of elution buffer, which was changed to 60 µl to yield a higher DNA concentration.

3.6 PCR

Detection of *Borrelia* spirochetes in ticks and in murine tissues was performed by the standard as well as by nested PCR. Standard PCR was performed using amplification of 154 bp long fragment of *flagellin*, the gene encoding a globular protein in borrelia's flagellum. The volume for one reaction was 25 µl, and it consisted of 12,5 µl FastStart PCR MasterMix (Roche), 10 pmol of each primer FlaF1A and FlaR1 (sequence can be found in Tab. I), 4 µl of DNA and the rest was filled up with PCR water. Amplification profile for detection of *Borrelia* spirochetes consisted of initial denaturation at 94 °C for 10 minutes followed by 40 cycles of denaturation at 94 °C for 30s, annealing at 60 °C for 30s and elongation at 70° C for 40s. After 40 cycles, the program was completed with a final extension at 70 °C for 7 minutes.

Where the presence of spirochetes could not be certainly detected by standard PCR, nested PCR with ten times higher sensitivity was applied. Amplification of a 222 bp long fragment of *Borrelia* 23S rRNA was performed. The reaction mixture for nested PCR was similar to standard PCR. The only difference was in using different primers. Bor1 and Bor2 were the primers used for the first round, Bor3 and Bor4 were the primers used for the second round of PCR amplification (sequences of all the primers can be found in Tab. I). The amplification profile was similar to standard PCR. Only the annealing temperatures were different. The first set of primers required the annealing temperature of 53 °C and the other pair of 58 °C.

3.7 Gel electrophoresis

The fragments amplified by PCR were visualized by gel electrophoresis. For the separation of products, 1,5 % agarose gel in TAE buffer was used. The gel was stained with ethidium bromide (Sigma-Aldrich) for easy visualization. Before loading, 10 µl of the sample

was mixed with 2 µl of DNA loading dye (Top-Bio). For size determination, GeneRuler 100bp DNA Ladder (Thermo Fisher Scientific) was used. The gel electrophoresis was running for 30 minutes at 100 V. After separation, pictures were taken under UV light, and the sizes of fragments were determined according to the DNA ladder.

3.8 Quantitative PCR

Quantitative real-time PCR was used for the determination of total spirochetes load in tick DNA samples. For the analysis, QuantStudio 6 Flex real-time PCR system (Thermo Fisher Scientific) was used. The reaction volume of 25 µl consisted of 12,5 µl of FastStart Universal Probe Master (Rox) (Roche), 10 pmol of each primer amplifying *Borrelia flagellin* (FlaF1A, FlaR1), 5 pmol of TaqMan probe FlaProbe 1 (sequences can be found in Tab. I) and 5 µl of DNA. The remaining volume was filled in with PCR water. Amplification profile was set to denaturation at 95 °C for 10 minutes followed, by 50 cycles of denaturation at 95 °C for 15 s, and annealing and elongation at 60 °C for 1 minute. All samples were analysed in duplicates to minimize random deviation.

For determination of both *B. afzelii* CB43 and *B. burgdorferi* N40 spirochetes loads in ticks a calculation of number of spirochetes in whole tick body using external *B. burgdorferi flagellin* standard curve was done.

Tab. I: Primers and probes for PCR determination and quantification of *Borrelia* spirochetes in ticks and murine tissues.

PCR and qPCR primers for detection and quantification of spirochetes in ticks				
Target	Name	Sequence (5'→3')	Product size	Source
Borrelia flagellin	FlaF1A	AAGCAAATTTAGGTGCTTTCCAA	154 bp	Schwaiger et al., 2001
	FlaR1	GCAATCATTGCCATTGCAGA		
	TaqMan FlaProbe1	TGCTACAACCTCATCTGTCATTGTA GCATCTTTTATTTG		
Nested PCR primers for detection of spirochetes in murine tissues				
Target	Name	Sequence (5'→3')	Product size	Source
Borrelia 23S rRNA	Bor1	AGAAGTGCTGGAGTCGA	260 bp	(Boudova et al., 2005)
	Bor2	TAGTGCTCTACCTCTATTAA		
	Bor3	GCGAAAGCGAGTCTTAAAAGG	222 bp	
	Bor4	ACTAAAATAAGGCTGAACTTAAAT		

3.9 Statistical analysis

The graphs were created using GraphPad Prism 6 for Windows version 6.07. The error bars indicate standard error of the mean since the data were obtained from independent biological replicates. The statistics was calculated using the same software using the following tests. For the comparison of 2 groups, an unpaired t-test was used. For the comparison of more than 2 groups, one-way ANOVA was used. A p-value not greater than 0,05 was considered statistically significant.

4 Results

There are two well-developed models inspecting the interaction of ticks and *Borrelia*. The one drawing greater attention is an American model which investigates *B. burgdorferi* sensu stricto and *I. scapularis* (De Silva and Fikrig, 1995; Hodzic et al., 2002; Ohnishi et al., 2001; Piesman et al., 1990, 2001; Schwan et al., 1995; Spielman et al., 1987). Another model, which was established in our laboratory, is studying the relationship between a European tick, *I. ricinus*, and a European pathogen, *B. afzelii* (Pospisilova et al., 2019). It seems that many variables in both acquisition and transmission of *Borrelia* spirochetes vary between these models, and that is the reason for future investigation. In our experiments, the aim was to find out whether an American tick *I. scapularis* would be able to transmit European *Borrelia* strain and vice versa if a European tick *I. ricinus* would be able to transmit American *Borrelia* strain. Another intention was to describe the growth kinetics of *Borrelia* spirochetes in tick larvae, later nymphs, in both experimental settings.

4.1 Acquisition of *Borrelia afzelii* CB43 by *Ixodes ricinus* and *Ixodes scapularis*

To examine whether *I. scapularis* is able to acquire the European strain of *Borrelia*, mice were infected with *B. afzelii* CB43 and larvae of both *I. ricinus* and *I. scapularis* were allowed to feed on them. Both European and American ticks were used at the same time to get the most accurate comparison since all ticks, mice, and *Borrelia* spirochetes were subjected to the same conditions. After larvae finished feeding, they were collected, and a sample of 10 larvae (later nymphs) of each species was inspected for *Borrelia* presence.

4.1.1 Growth kinetics of *Borrelia afzelii* CB43 in *Ixodes ricinus*

For determination of *B. afzelii* growth kinetics in ticks of different developmental stages, absolute quantification of *Borrelia* spirochetes per tick was used. This was performed by quantitative real-time PCR. The first quantification was taken immediately after the full engorgement, and the following ones were taken every week for the time period of nine weeks. The graph presenting the growth kinetics during the monitored period is shown in Fig. 7. The load of *Borrelia* spirochetes in fully fed *I. ricinus* larvae was fairly low. However, spirochetes multiplied within the ticks during subsequent molting to nymphs. The mean number of

B. afzelii spirochetes in *I. ricinus* larvae was 390 ± 60 (SEM). The mean number of spirochetes in nymphs was 1406 ± 370 (SEM). The highest number of spirochetes was observed in the seventh week, which was the third week after molting, the mean value was 2856, but the variance was quite high (SEM = 1636).

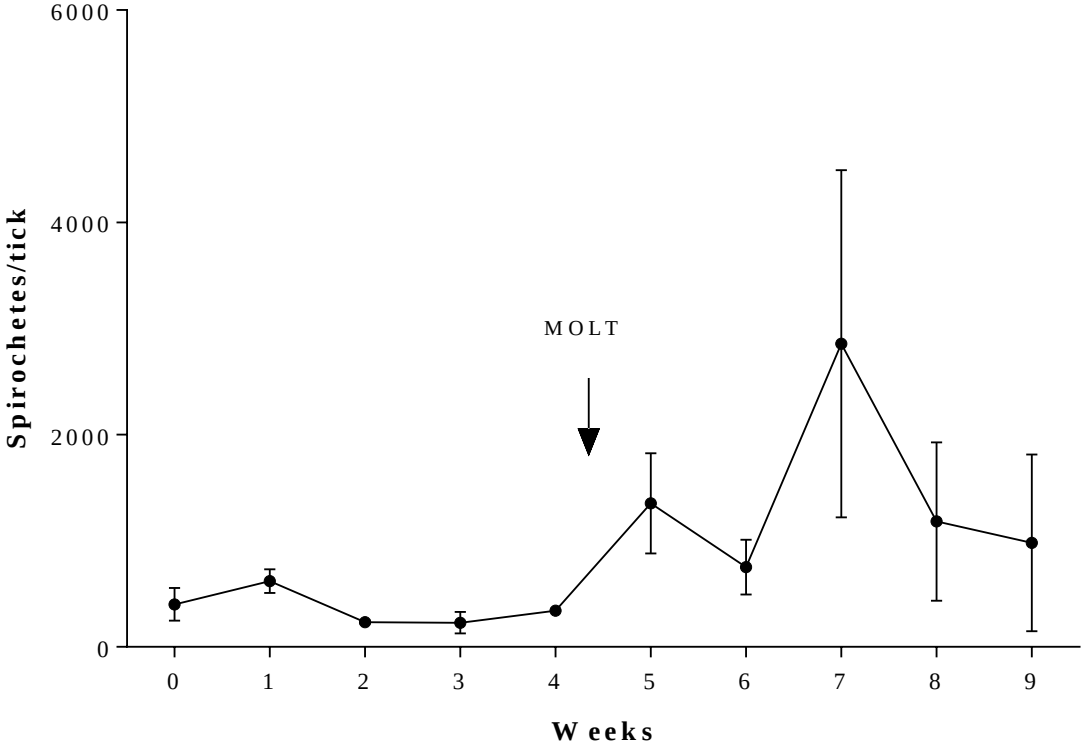


Fig. 7: Growth kinetics of *B. afzelii* in *I. ricinus*. Developmental stages: 0 - 4. week: larvae, 5. – 9. week: nymphs. Each point represents the mean number of spirochetes per tick and consists of 10 separate quantifications. The error bars indicate SEM.

4.1.2 Growth kinetics of *Borrelia afzelii* CB43 in *Ixodes scapularis*

For the determination of *B. afzelii* growth kinetics in *I. scapularis,* the same approach simultaneously with the previous experiment, was applied. The graph showing the growth kinetics over nine weeks is shown in Fig. 8. The number of spirochetes in *I. scapularis* larvae during the first four weeks was very low. However, a week before molting, the number of spirochetes increased very rapidly and stayed around the same level even after the larvae molted to nymphs. The average load of *B. afzelii* spirochetes in larvae during the first four weeks (week 0 to 3) was 190 ± 24 (SEM). In the fourth week, the week before molting, the number of spirochetes has increased significantly, which shifted the mean number of

spirochetes in *I. scapularis* larvae to 719 ± 247 (SEM). The mean value of spirochetes in nymphs was 2370 ± 527 (SEM). The highest number of spirochetes was observed in the last week when the infection reached 3899 ± 125 (SEM) spirochetes/tick.

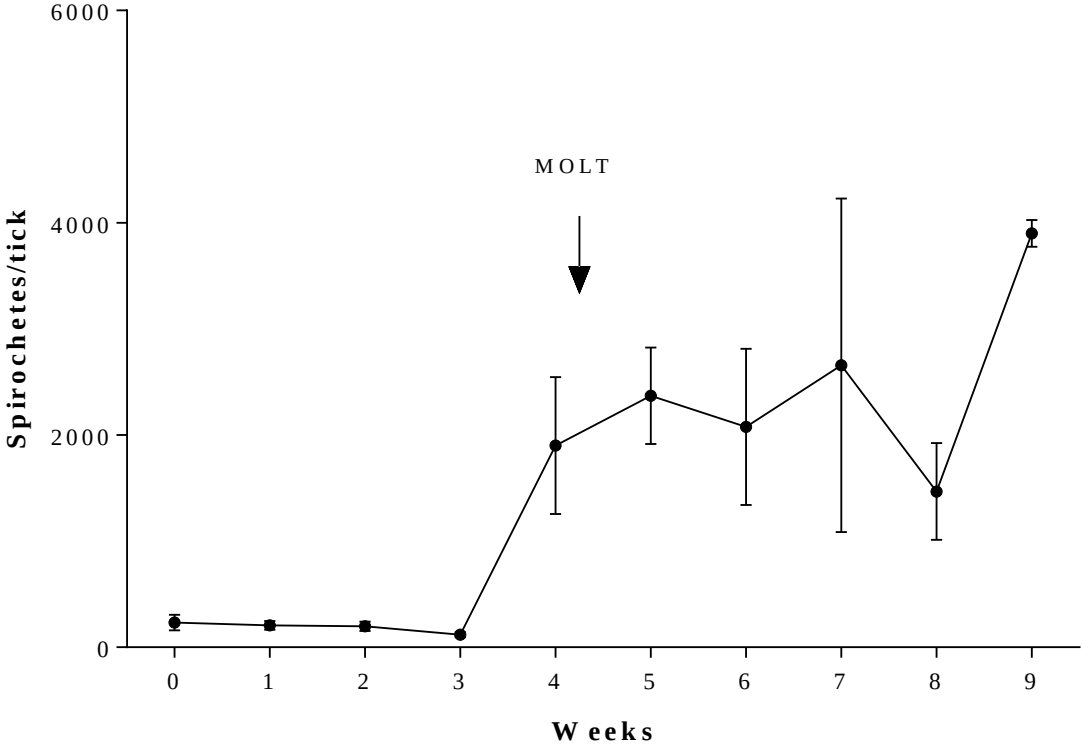


Fig. 8: Growth kinetics of *B. afzelii* in *I. scapularis*. Developmental stages: 0 - 4. week: larvae, 5. – 9. week: nymphs. Each point represents the mean number of spirochetes per tick and consists of 10 separate quantifications. The error bars indicate SEM.

4.1.3 Comparison of the load of *Borrelia afzelii* spirochetes in *Ixodes ricinus* and *Ixodes scapularis* over time

The comparison of the average number of spirochetes in larvae and nymphs of both species of ticks is shown in Fig. 9. In both *I. ricinus* and *I. scapularis*, the number of spirochetes was significantly lower in larvae than in nymphs, whereas the difference of both larvae and nymphs between the two tick species was not significantly different.

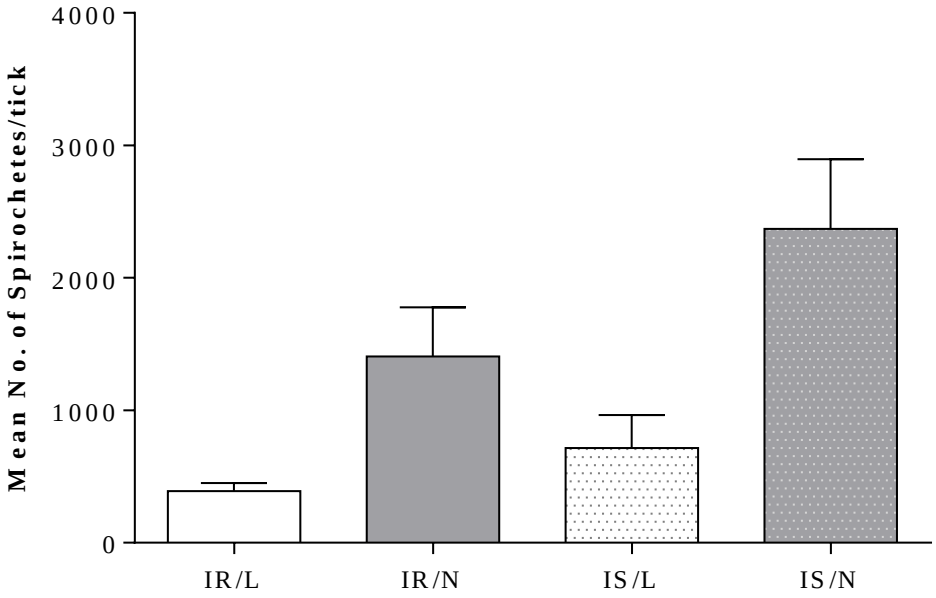
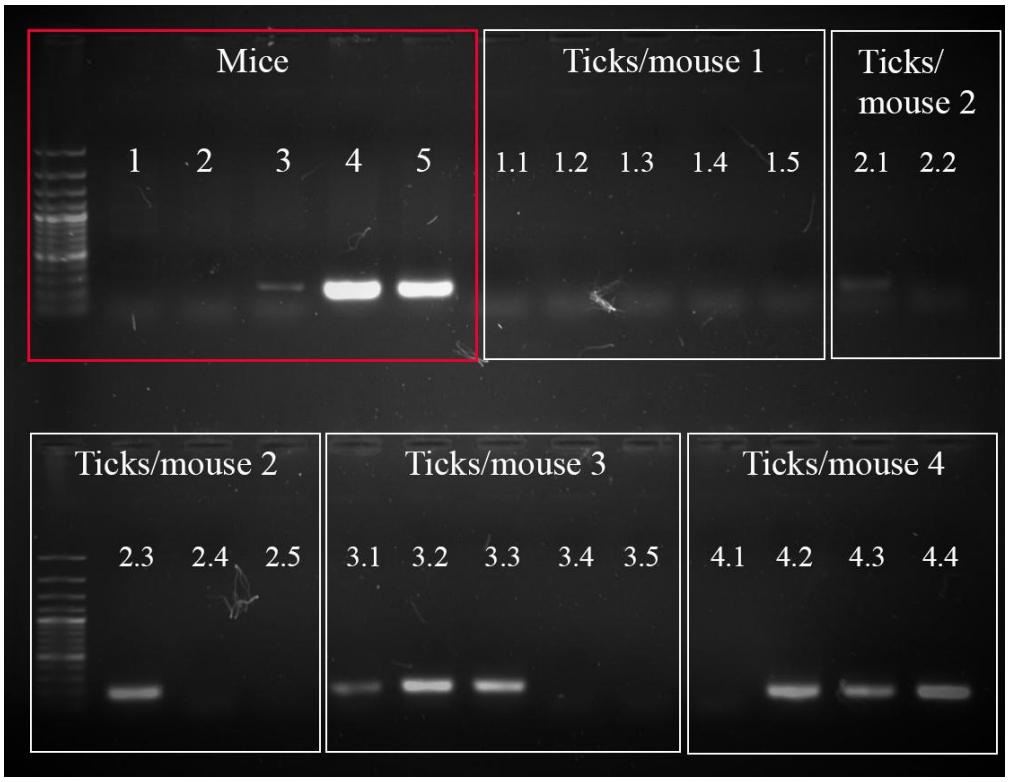


Fig. 9: Mean number of *B. afzelii* spirochetes in larvae and nymphs of both *I. ricinus* and *I. scapularis*. IR/L and IR/N denote larvae and nymphs of *I. ricinus*, respectively, IS/L and IS/N denote larvae and nymphs of *I. scapularis*. The error bars indicate SEM.

4.2 Transmission of *Borrelia afzelii* CB43 by *Ixodes scapularis*

The *I. scapularis* nymphs infected with *B. afzelii* from the previous experiment were used to find out whether the American tick species is able to transmit the European strain of *Borrelia*. In this experiment, the infected nymphs were allowed to feed on naïve mice. Fully fed nymphs were collected and tested for *Borrelia* spirochetes presence, and after four weeks, also the infection in mice was tested with standard PCR. The pictures of the gels can be found in Fig. 10. The presence of spirochetes was confirmed in 3 out of 5 mice. On the first two mice, where there were no spirochetes present, 0 and 2 ticks out of 5 were found positive, respectively. On the other 3 mice, which were infected, 3, 4, and 5 ticks out of 5 were positive (see Tab. II). Therefore it can be inferred that *I. scapularis* is able to transmit the *B. afzelii* CB43, and for the development of infection, at least three infected ticks are needed.



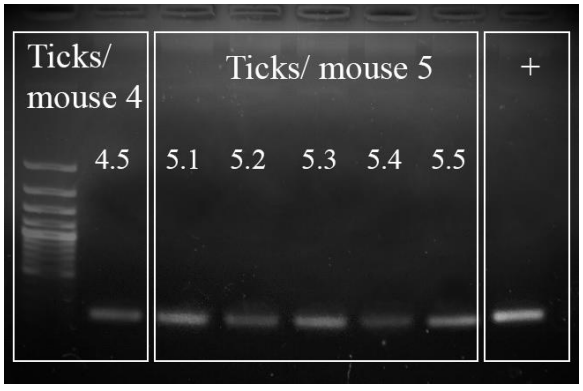


Fig. 10: Detection of *B. afzelii* CB43 in mice four weeks after *I. scapularis* nymphs infestation and in repleted nymphs. Tested mice are in the red frame (3/5 positive). In the white frames are the tested nymphs. Each white box represents ticks from one of the mice, and are assigned with numbers, respectively. The white frame assigned with + denotes positive control.

Tab. II: Summary of the transmission experiment with *I. scapularis* nymphs and *B. afzelii* CB43

Mouse	Infected	Infected ticks
1	NO	0/5
2	NO	2/5
3	YES	3/5
4	YES	4/5
5	YES	5/5

4.3 Acquisition of *Borrelia burgdorferi* N40 by *Ixodes ricinus*

To inspect whether European tick is able to acquire American strain of *Borrelia*, mice were infected with *B. burgdorferi* N40, and larvae of *I. ricinus* were allowed to feed on them. After larvae finished feeding, they were collected, and a sample of 20 larvae (later nymphs) was inspected for *Borrelia* presence.

4.3.1 Growth kinetics of *Borrelia burgdorferi* N40 in *Ixodes ricinus*

To describe the growth kinetics of *B. burgdorferi* in *I. ricinus*, 20 ticks were used for absolute quantification over the period of ten weeks (20 ticks per week). The graph presenting the growth kinetics is shown in Fig. 11. It can be inferred from the graph that there is no such trend visible as in the previous experiments. The amount of spirochetes is very low during the first two weeks, then the number starts to grow and then oscillates around the same value. Also, the molting did not occur in the same way as last time. It occurred later, and the larvae did not molt at the same time. The molting started after the sixth week and was going on until the ninth week.

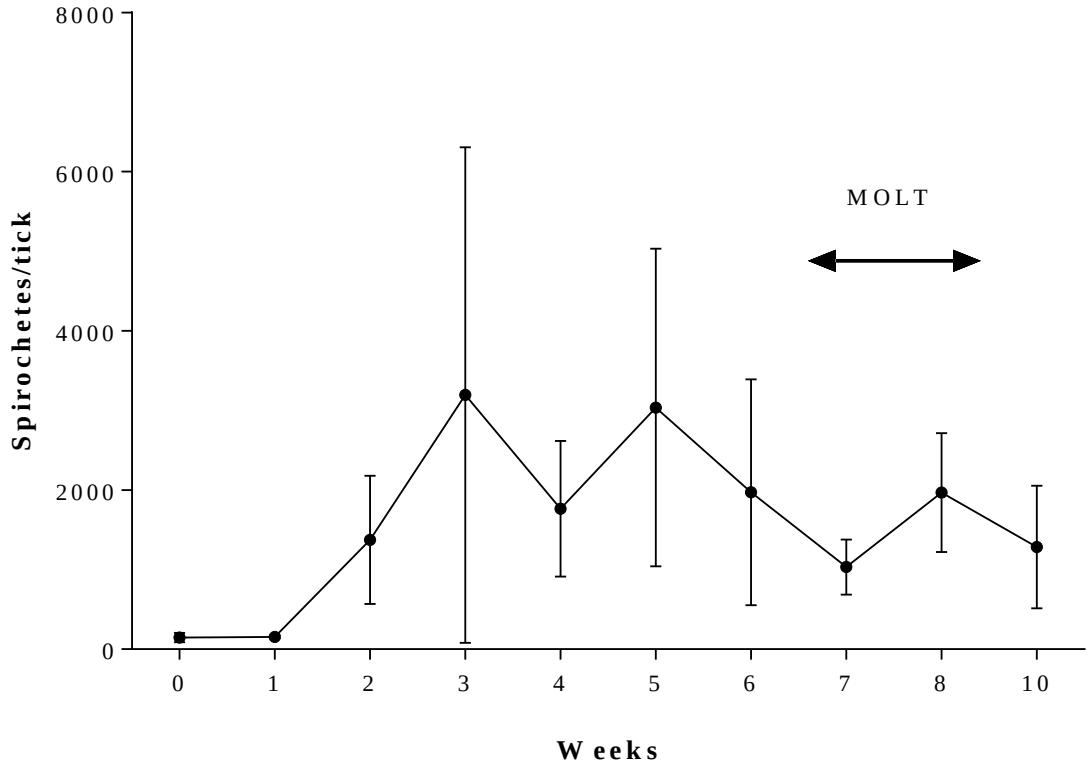


Fig. 11: Growth kinetics of *B. burgdorferi* in *I. ricinus*. Developmental stages: 0 - 6. week: larvae, 7. – 8. week: larvae and nymphs, 10. week: nymphs. Each point represents the mean number of spirochetes per tick and consists of 20 separate quantifications. The error bars indicate SEM.

From the obtained data, the mean number of spirochetes per larvae and nymph was calculated. Larvae had, on average 1309 ± 353 (SEM) spirochetes, nymphs had the average number of 2022 ± 663 (SEM) spirochetes. The difference in the number of spirochetes per larvae and nymphs was not significant. The comparison is shown in Fig. 12.

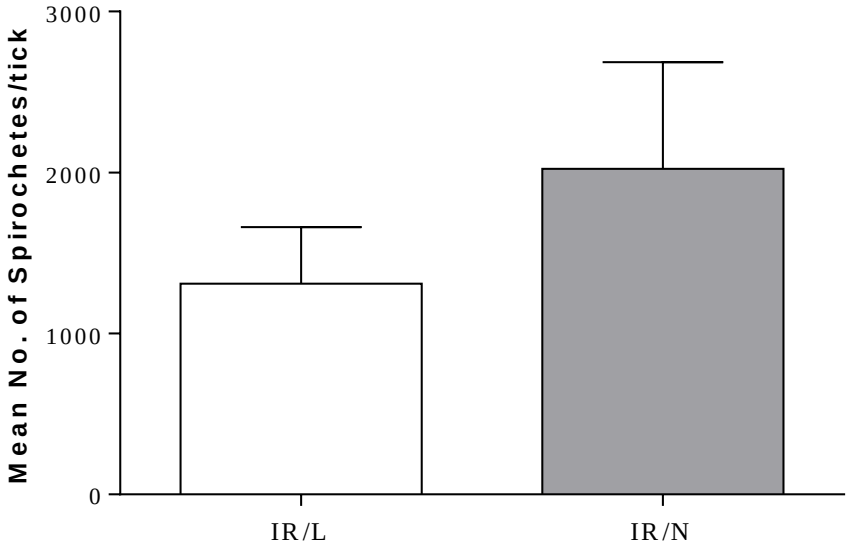


Fig. 12: Mean number of *B. burgdorferi* spirochetes in larvae and nymphs of *I. ricinus*. IR/L and IR/N denote larvae and nymphs of *I. ricinus,* respectively. The error bars indicate SEM.

4.4 Transmission of *Borrelia burgdorferi* N40 by *Ixodes ricinus*

The *I. ricinus* nymphs infected with *B. burgdorferi* from the previous experiment were used to find out whether the European tick species is able to transmit the American strain of *Borrelia*. In this experiment, the infected nymphs were allowed to feed on naïve mice. There were three experimental groups. The first group of ticks was allowed to feed on mice for 24 hours, the second group for 48 hours, and the last group of ticks was left to feed on mice until fully fed. The ability to transmit the spirochetes was inspected as well as the number of spirochetes in ticks after detachment.

4.4.1 Kinetics of *Borrelia burgdorferi* during feeding of *Ixodes scapularis* nymph

After detachment, the *Borrelia* load in ticks was determined using quantitative PCR, and the results are shown in Fig. 13. The number of spirochetes in ticks that were feeding on mice for 24 hours was very low, with an average value of 676 ± 488 (SEM). The number of spirochetes in the second group (48H) was higher, reaching an average of 14417 ± 8453 (SEM). The number of spirochetes in the third group (FF) was the highest with a mean of 197722 ± 152622 (SEM). However, the variance in all three groups was so high that they were

not proven to be significantly different from each other. Nevertheless, it can be inferred that the number of *B. burgdorferi* spirochetes in *I. ricinus* during feeding is increasing.

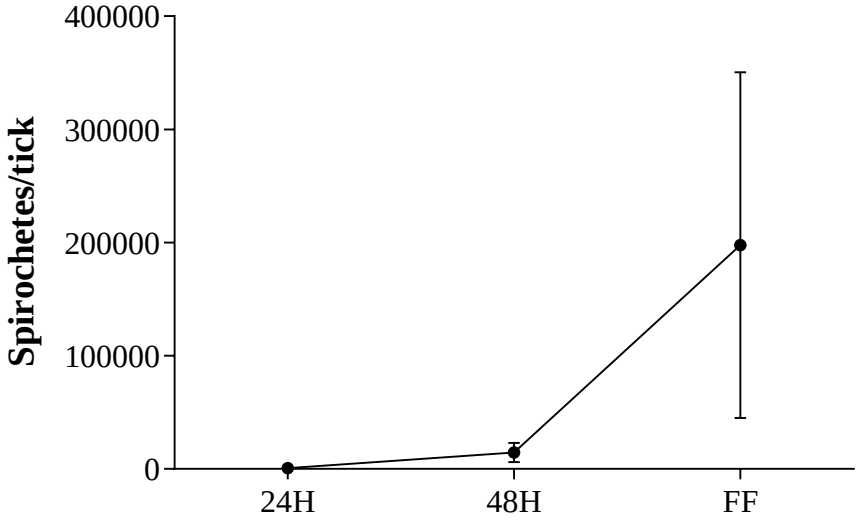


Fig. 13: Mean number of *B. burgdorferi* spirochetes in nymphs of *I. ricinus* during feeding. 24H and 48H denote the first two groups, which were allowed to feed on mice for 24 and 48 hours, respectively. FF denotes the third group, which was allowed to feed on mice until fully fed.

4.4.2 Presence of *Borrelia* spirochetes in murine tissues

Mice were tested for *Borrelia* presence four weeks after tick detachment in ear biopsies. Standard PCR was applied; however, only one mouse was proven positive. Later, three other tissues were dissected from each mouse to confirm the infection, namely bladder, heart, and joint. DNA isolated from these tissues was amplified by standard PCR, but it showed unclear results. For more precise results, nested PCR was used, the result is shown in Fig. 14. *Borrelia* spirochetes were found in joints of mice 1, 2, 3, and 4, which are the mice that were exposed to ticks for 48hours or to full engorgement. Therefore it can be inferred that *I. ricinus* is able to transmit *B. burgdorferi*, and the minimal time of attachment to cause the infection is 48 hours.

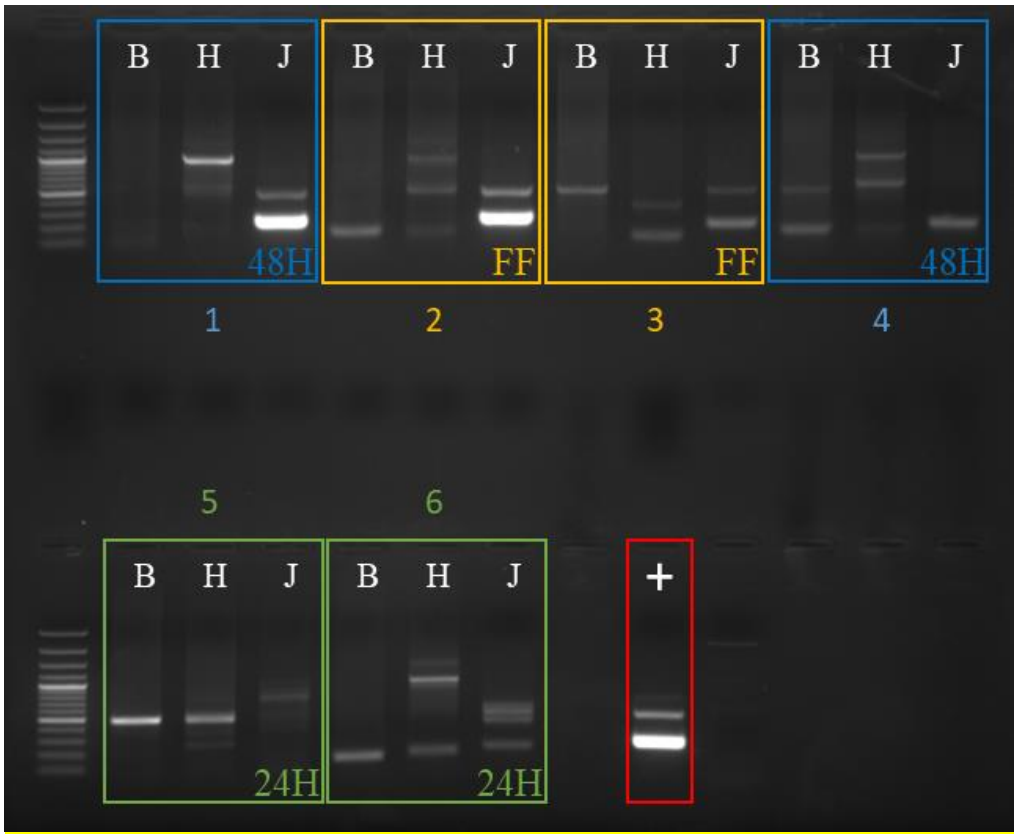


Fig. 14: Detection of *B. burgdorferi* N40 in murine tissues four weeks after the removal of *I. ricinus* nymphs. DNA was isolated from three tissues, namely bladder (B), heart (H), and joint (J). The green frame (mouse 5 and 6) denotes the first group: 24 hours long attachment. The blue frame (mouse 2 and 3) denotes the second group: 48 hours long attachment. The yellow frame (mouse 1 and 4) denotes the third group: attached until fully fed. The red frame denotes positive control.

5 Discussion

Since Lyme disease is the most common tick-borne disease in the temperate zone, perfect comprehension of relationships between the pathogen, its host, and vector is required to establish novel strategies for the prevention. Transmission models are playing a crucial role in such development. However, for a long time, attention has been drawn to a single species of *Borrelia* (*B. burgdorferi* sensu stricto), assuming the trends would be similar among other species as well (De Silva and Fikrig, 1995; Hodzic et al., 2002; Ohnishi et al., 2001; Piesman et al., 1990, 2001; Schwan et al., 1995; Spielman et al., 1987). Yet recent findings, focused on another *Borrelia* species (*B. afzelii*), have shown lots of discrepancies, and even contradictory results in few cases (Crippa et al., 2002; Eisen, 2020; Genné et al., 2019; Pospisilova et al., 2019). The latter transmission model could be described as the European model since it studies the relationship of the European tick *I. ricinus* and *B. afzelii*, the *Borrelia* species occurring in Eurasia. The other, widely accepted, transmission model could be described as the American model since it concerns the American tick *I. scapularis* and *B. burgdorferi* sensu stricto, the *Borrelia* species occurring mainly in North America, but to a lesser extent in Europe as well. Owing to the various differences between results observed for both models, the idea was to try out a different approach to assess the strategies of both *Borrelia* species. The experiments were settled to find out whether the European tick would be able to acquire and transmit the American strain of *Borrelia* and vice versa if the American tick would be able to acquire and transmit the European *Borrelia* species. All the steps in both acquisition and transmission have been well studied and will be further compared below.

5.1 Acquisition of *Borrelia*

First, *Ixodes scapularis* and *I. ricinus* were simultaneously tested on the ability to acquire the *B. afzelii* CB43 infection. The simultaneity of these experiments was introduced to secure the same conditions for further comparison. Weekly testing of larvae, which were collected off the infected mice, for *Borrelia* presence provided growth kinetics diagrams. Growth kinetics of *B. afzelii* in *I. ricinus* showed a similar trend with the data reported by Pospisilova et al. (2019). The number of spirochetes in larvae after full engorgement was low, then increased right before molting in the case of Pospisilova et al. (2019), or with molting in this experiment, and later dropped again.

Growth kinetics of *B. afzelii* in *I. scapularis* showed a similar trend as in *I. ricinus*, which confirms that *I. scapularis* is able to acquire *B. afzelii*. The number of *Borrelia* spirochetes remains low after full engorgement, and a week before molting, the spirochetes start to multiply rapidly. This is in accordance with the data by Pospisilova et al. (2019). After molting, no decrease in the number of spirochetes per nymph was observed, and the number of spirochetes stayed stable. No decrease could be explained by not depleted nutrients in the tick gut, or a short period of observation.

The absolute numbers of spirochetes in larvae and nymphs were calculated for each tick species. In both *I. ricinus* and *I. scapularis*, the number of spirochetes was significantly lower in larvae than in nymphs, whereas the difference of both larvae and nymphs between the two tick species was not significantly different. This is in accordance with the data observed by Pospisilova et al. (2019) for the same time period. Therefore it can be said that the American tick is able to acquire *B. afzelii* spirochetes with no difficulty, and the development over time is similar.

The following experiment was testing whether *I. ricinus* would be able to acquire *B. burgdorferi* N40 infection. Simultaneous experiment with *I. scapularis* was not possible because *I. scapularis* larvae were not available in our laboratory at that time. This experiment is planned to be carried out this spring, though. Growth kinetics diagrams of *B. burgdorferi* in *I. ricinus* were obtained monitoring the period of 10 weeks after full engorgement on infected mice. The results show that there is no such trend as in the previous experiments with *B. afzelii*. In the first two time points, the level of infection stays very low. During the two following weeks, the spirochetes multiply rapidly, and then the number of them oscillates around the same value. Growth kinetic reported earlier for *I. scapularis* and *B. burgdorferi* sensu stricto shows a different trend. The number of spirochetes in larvae grows rapidly after full engorgement reaching the maximum around the second week post repletion. Between the second and third week, the number of *Borrelia* decreases rapidly and continues decreasing after molting, which occurs after the third week (Piesman et al., 1990). This indicates that there would be way more spirochetes present in the larvae than in nymphs, which is not the case in this experiment. The values for the mean number of spirochetes per larva and nymph came out not significantly different from each other since the variance was too high, if anything there was slightly fewer spirochetes in larvae than in nymphs, which is an exactly opposite outcome than in the study by Piesman et al. (1990), and it rather resembles our previous results.

It is not only the growth kinetics that is very different from the one of *B. afzelii*. Also, the molting occurred in an altered manner. In the previous experiments, the larvae managed to molt in one week, specifically between a fourth and fifth week (fourth week all larvae, fifth week all nymphs). This time the molting period was longer and occurred later. First larvae were molting between a sixth and seventh week, and the last larvae were molting up to the tenth week. The reason for such a long molting period could be the timing of the experiment. While the experiment with *B. afzelii* was carried out in the spring, which is the period when ticks feed and molt naturally, the experiment with *B. burgdorferi* was carried out late in the fall, which is the period when the ticks are not active anymore in nature. Although the ticks were kept in the stable laboratory conditions, it results from the experience in our laboratory that some ticks "sense" the time of the year anyway, and they are facing difficulties with feeding, molting, or mating.

To confirm how the acquisition of *B. burgdorferi* by *I. ricinus* differs from the acquisition of *B. burgdorferi* by *I. scapularis*, further experiment with the latter settings would be needed. However, there is no doubt that *I. ricinus* is able to acquire the *B. burgdorferi* infection.

5.2 Transmission of *Borrelia*

After confirmation that *I. scapularis* can acquire *B. afzelii* CB43 infection, naturally followed the experiment investigating whether the infected nymph can transmit the infection back to a naïve mouse. Five mice were used for the experiment, and out of these three were proven positive. Testing of the nymphs collected from the mice revealed that there was a correlation between the number of positive ticks per mouse and a developed infection in mice. From the results, it can be inferred that probably at least three infected ticks are needed to cause infection in mice. This can mean that *I. scapularis* is a less effective vector of *B. afzelii*. However, there is no doubt that it can transmit the infection, and the probability of transmitting is higher with the higher number of infected ticks.

The transmission experiment was carried out with *I. ricinus* and *B. burgdorferi* N40 as well. This time focused on the time needed for the establishment of the infection. Three experimental groups involved feeding of infected nymphs for 24 hours, 48 hours, and until full engorgement. Three types of tissues were tested, bladder, heart, and joints. After 24 hours, no mouse in any tissue was proven positive. After 48 hours, the infection was confirmed in

joints but not in any other tissue, and after full engorgement, the result was the same. The fact that infection was detected in joints is not surprising since *B. burgdorferi* sensu stricto is predominantly known to infest joints and cause arthritis (van Dam et al., 1993). The time needed for the successful transmission of *Borrelia* to its host has never been well established, but it is thought that if the tick is attached for less than 48 hours, the infection is not transmitted (Ohnishi et al., 2001; Piesman et al., 2001). During this experiment, it was observed that although no mouse was infected after 24 hours of exposure to infected nymphs, all the mice were infected after 48 hours which leads to the thought that the time interval between 24 and 48 hours is actually crucial for the development of infection, and removing the tick in two days would probably not prevent the infection. The load of spirochetes in mice was most likely fairly low since the standard PCR was unable to detect them reliably, and a more sensitive method, nested PCR, had to be applied. However, it was proven that *I. ricinus* can transmit *B. burgdorferi* N40, and the infection is established between the first and second day after tick attachment.

5.3 Kinetics of *Borrelia* during feeding

The time points, in the previous experiment discussed above, were not only introduced to assess the time needed for the establishment of infection in mice but also to describe the kinetics of *B. burgdorferi* N40 in *I. ricinus* ticks. It was shown that *B. afzelii* and *B. burgdorferi* sensu stricto display an opposite strategy. While the number of *B. afzelii* spirochetes in *I. ricinus* nymph dramatically drops during feeding as they migrate to the host (Pospisilova et al., 2019), the *B. burgdorferi* sensu stricto spirochetes in *I. scapularis* nymphs are multiplying rapidly during feeding before starting to migrate to the host (De Silva and Fikrig, 1995; Piesman et al., 2001). The outcome of the experiment with *B. burgdorferi* N40 and *I. ricinus* is in accordance with the data of the latter authors. The mean number of spirochetes present in nymphs increased more than ten times between 24 and 48 hours, and again ten times between 48 hours and full engorgement. However, the variation in the data was so high that the groups were not proven significantly different from each other. For statistically better outcome, a bigger sample of nymphs in each group would be necessary.

5.4 Geographic distribution of *Borrelia* species

Both studied *Borrelia* species (*B. afzelii* CB43 and *B. burgdorferi* N40) were proven to be acquired and transmitted by both studied tick species (*I. ricinus* and *I. scapularis*), which raises the question whether the *Borrelia* species could be introduced to new areas where they could be a threat. *Borrelia burgdorferi* sensu stricto is a generalist regarding its hosts (Donahue et al., 1987; Rand et al., 1998), and apart from the North America, where it is the main carrier of Lyme disease, it occurs to a lesser extent in Europe as well. Therefore it is not a great surprise that *I. ricinus*, the European tick, is able to acquire and transmit the infection by predominantly American *Borrelia* species. *Borrelia afzelii*, on the other hand, which was also proven to be acquired and transmitted by *I. scapularis*, has a broad areal in Eurasia, but it was never observed in North America. Therefore the potential expansion to the west could be of importance. With today's very high frequencies of all kinds of transportation between these two continents, the introduction of *B. afzelii* to North America could seem inevitable. However, an important variable was not taken to an account, and this is the laboratory environment. All experiments have been carried out using CH3/HeN mice, so the compatibility with a vector was confirmed, but the compatibility with the host was not assessed at all. Whether *B. afzelii* could be introduced to North America depends on the availability of competent hosts in the area.

6 Conclusion

Growth kinetics of the European *B. afzelii* in American vector *I. scapularis* was obtained and compared with the data available for the European vector *I. ricinus*. The transmission experiment showed that the probability of transmitting *B. afzelii* infection raises with the number of infected nymphs feeding on the host. Growth kinetics showed a similar trend as the described transmission model. Therefore *I. scapularis* was marked as capable of acquiring and transmitting *B. afzelii*.

Growth kinetics of the American *B. burgdorferi* in European vector *I. ricinus* was obtained and compared with the existing data of the American vector *I. scapularis*. The transmission experiment revealed the crucial time for the development of *B. burgdorferi* infection and confirmed the earlier observed data on the kinetics of *B. burgdorferi* during nymphal feeding. Although growth kinetics had a different trend from the established model, *I. ricinus* was determined as capable of acquiring and transmitting *B. burgdorferi*.

7 References

Adeolu, M., and Gupta, R. S. (2014). A phylogenomic and molecular marker based proposal for the division of the genus Borrelia into two genera: The emended genus Borrelia containing only the members of the relapsing fever Borrelia, and the genus Borreliella gen. nov. containing the members of the Lyme disease Borrelia (Borrelia burgdorferi sensu lato complex). *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* 105, 1049–1072.

Baranton, G., Postic, D., Saint Girons, I., Boerlin, P., Piffaretti, J. C., Assous, M., et al. (1992). Delineation of Borrelia burgdorferi sensu stricto, Borrelia garinii sp. nov., and group VS461 associated with Lyme borreliosis. *Int. J. Syst. Bacteriol.* 42, 378–383.

Barbour, A. G. (1986). Cultivation of Borrelia: A historical overview. *Zentralblatt fur Bakteriol. Mikrobiol. und Hyg. - Abt. 1 Orig. A* 263, 11–14.

Barbour, A. G., and Hayes, S. F. (1986). Biology of Borrelia Species. *Microbiol. Rev.* 50, 381–400.

Barker, S. C., and Walker, A. R. (2014). Ticks of Australia. The species that infest domestic animals and humans. *Zootaxa* 3816, 1–144.

Barthold, S. W., Persing, D. H., Armstrong, A. L., and Peeples, R. A. (1991). Kinetics of Borrelia burgdorferi dissemination and evolution of disease after intradermal inoculation of mice. *Am. J. Pathol.* 139, 263–273.

Battisti, J. M., Bono, J. L., Rosa, P. A., Schrumpf, M. E., Schwan, T. G., and Policastro, P. F. (2008). Outer surface protein A protects lyme disease spirochetes from acquired host immunity in the tick vector. *Infect. Immun.* 76, 5228–5237.

Benach, J. L., Coleman, J. L., Skinner, R. A., and Bosler, E. M. (1987). Adult Ixodes dammini on rabbits: a hypothesis for the development and transmission of Borrelia burgdorferi. *J. Infect. Dis.* 155, 1300–6.

Betancur Hurtado, O. J., and Giraldo-Ríos, C. (2019). "Economic and Health Impact of the Ticks in Production Animals," in *Ticks and Tick-Borne Pathogens* (IntechOpen).

Boudova, L., Kazakov, D. V., Sima, R., Vanecek, T., Torlakovic, E., Lamovec, J., et al. (2005). Cutaneous lymphoid hyperplasia and other lymphoid infiltrates of the breast nipple: A retrospective clinicopathologic study of fifty-six patients. *Am. J. Dermatopathol.* 27, 375–386.

Brisson, D., Drecktrah, D., Eggers, C. H., and Samuels, D. S. (2012). Genetics of Borrelia burgdorferi. *Annu. Rev. ofGenetics* 46, 515–36.

Burgdorfer, W. (1984). Discovery of the Lyme disease spirochete and its relation to tick vectors. *Yale J. Biol. Med.* 57, 515–520.

Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E., and Davis, J. P. (1982). Lyme disease - A tick-borne spirochetosis? *Science.* 216, 1317–1319.

Cabello, F. C., Godfrey, H. P., and Newman, S. A. (2007). Hidden in plain sight: Borrelia burgdorferi and the extracellular matrix. *Trends Microbiol.* 15, 350–354.

Canica, M. M., Nato, F., Merle, L. du, Mazie, J. C., Baranton, G., and Postic, D. (1993). Monoclonal antibodies for identification of borrelia afzelii sp. Nov. Associated with late cutaneous manifestations of lyme borreliosis. *Scand. J. Infect. Dis.* 25, 441–448.

Casjens, S. R., Fraser-Liggett, C. M., Mongodin, E. F., Qiu, W. G., Dunn, J. J., Luft, B. J., et al. (2011). Whole genome sequence of an unusual Borrelia burgdorferi sensu lato isolate. *J. Bacteriol.* 193, 1489–1490.

Casjens, S. R., Mongodin, E. F., Qiu, W. G., Luft, B. J., Schutzer, S. E., Gilcrease, E. B., et al. (2012). Genome stability of lyme disease spirochetes: Comparative genomics of borrelia burgdorferi plasmids. *PLoS One* 7, e33280.

Charon, N. W., Cockburn, A., Li, C., Liu, J., Miller, K. A., Miller, M. R., et al. (2012). The Unique Paradigm of Spirochete Motility and Chemotaxis. *Annu. Rev. Microbiol.* 66, 349–370.

Chu, C. Y., Liu, W., Jiang, B. G., Wang, D. M., Jiang, W. J., Zhao, Q. M., et al. (2008). Novel genospecies of Borrelia burgdorferi sensu lato from rodents and ticks in Southwestern China. *J. Clin. Microbiol.* 46, 3130–3133.

Cook, M. J. (2014). Lyme borreliosis: A review of data on transmission time after tick attachment. *Int. J. Gen. Med.* 8, 1–8.

Crippa, M., Rais, O., and Gern, L. (2002). Investigations on the mode and dynamics of transmission and infectivity of Borrelia burgdorferi sensu stricto and Borrelia afzelii in Ixodes ricinus ticks. *Vector Borne Zoonotic Dis.* 2, 3–9.

De Silva, A. M., and Fikrig, E. (1995). Growth and migration of Borrelia burgdorferi in Ixodes ticks during blood feeding. *Am. J. Trop. Med. Hyg.* 53, 397–404.

Dolan, M. C., Piesman, J., Mbow, M. L., Maupin, G. O., Péter, O., Brossard, M., et al. (1998). Vector competence of Ixodes scapularis and Ixodes ricinus (Acari: Ixodidae) for three genospecies of Borrelia burgdorferi. *J. Med. Entomol.* 35, 465–70.

Donahue, J. G., Piesman, J., and Spielman, A. (1987). Reservoir competence of white-footed mice for Lyme disease spirochetes. *Am. J. Trop. Med. Hyg.* 36, 92–96.

Eisen, L. (2020). Vector competence studies with hard ticks and Borrelia burgdorferi sensu lato spirochetes: A review. *Ticks Tick. Borne. Dis.* 11.

Eisen, R. J., and Eisen, L. (2018). The Blacklegged Tick, Ixodes scapularis: An Increasing Public Health Concern. *Trends Parasitol.* 34, 295–309.

Estrada-Peña, A., Farkas, R., Jaenson, T. G. T., Koenen, F., Madder, M., Pascucci, I., et al. (2013). Association of environmental traits with the geographic ranges of ticks (Acari: Ixodidae) of medical and veterinary importance in the western Palearctic. A digital data set. *Exp. Appl. Acarol.* 59, 351–366.

Fingerle, V., Schulte-Spechtel, U. C., Ruzic-Sabljic, E., Leonhard, S., Hofmann, H., Weber, K., et al. (2008). Epidemiological aspects and molecular characterization of Borrelia burgdorferi s.l. from southern Germany with special respect to the new species Borrelia spielmanii sp. nov. *Int. J. Med. Microbiol.* 298, 279–290.

Fleche, A. L., Postic, D., Girardet, K., Peter, O., and Baranton, G. (1997). Characterization of Borrelia lusitaniae sp. nov. by 16S Ribosomal DNA Sequence Analysis. *Int. J. Syst. Bacteriol.* 47, 921–925.

Fraser, C. M., Casjens, S., Huang, W. M., Sutton, G. G., Clayton, R., Lathigra, R., et al. (1997). Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi. *Nature* 390, 580–586.

Fukunaga, M., Hamase, A., Okada, K., and Nakao, M. (1996). Borrelia tanukii sp. nov. and Borrelia turdae sp. nov. found from ixodid ticks in Japan: Rapid species identification by 16S rRNA gene-targeted PCR analysis. *Microbiol. Immunol.* 40, 877–881.

Garcia, B. L., Zhi, H., Wager, B., Höök, M., and Skare, J. T. (2016). Borrelia burgdorferi BBK32 Inhibits the Classical Pathway by Blocking Activation of the C1 Complement Complex. *PLoS Pathog.* 12, e1005404.

Genné, D., Sarr, A., Rais, O., and Voordouw, M. J. (2019). Competition Between Strains of Borrelia afzelii in Immature Ixodes ricinus Ticks Is Not Affected by Season. *Front. Cell. Infect. Microbiol.* 9.

Gern, L. (2008). Borrelia burgdorferi sensu lato, the agent of Lyme borreliosis: Life in the wilds. *Parasite* 15, 244–247.

Grimm, D., Tilly, K., Byram, R., Stewart, P. E., Krum, J. G., Bueschel, D. M., et al. (2004). Outer-surface protein C of the Lyme disease spirochete: A protein induced in ticks for infection of mammals. *Proc. Natl. Acad. Sci. U. S. A.* 101, 3142–3147.

Guglielmone, A. A., Robbins, R. G., Apanaskevich, D. A., Petney, T. N., Estrada-Peña, A., Horak, I. G., et al. (2002). The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. *Exp Appl Acarol.* 28, 27–54.

Hanincova, K., Schäfer, S. M., Etti, S., Sewell, H. S., Taragelová, V., Ziak, D., et al. (2003). Association of Borrelia afzelii with rodents in Europe. *Parasitology* 126, 11–20.

Hodzic, E., Feng, S., Freet, K. J., Borjesson, D. L., and Barthold, S. W. (2002). Borrelia burgdorferi population kinetics and selected gene expression at the host-vector interface. *Infect. Immun.* 70, 3382–3388.

Ivanova, L. B., Tomova, A., González-Acuña, D., Murúa, R., Moreno, C. X., Hernández, C., et al. (2014). Borrelia chilensis, a new member of the Borrelia burgdorferi sensu lato complex that extends the range of this genospecies in the Southern Hemisphere. *Environ. Microbiol.* 16, 1069–1080.

Kawabata, H., Masuzawa, T., and Yanagihara, Y. (1993). Genomic Analysis of Borrelia japonica Sp. Nov. Isolated from Ixodes ovatus in Japan. *Microbiol. Immunol.* 37, 843–848.

Koci, J., Derdakova, M., Peterkova, K., Kazimirova, M., Selyemova, D., and Labuda, M. (2006). Borrelia afzelli gene expression in ixodes ricinus (acari: Ixodidae) ticks. *Vector-Borne Zoonotic Dis.* 6, 296–304.

Kurtenbach, K., De Michelis, S., Etti, S., Schäfer, S. M., Sewell, H. S., Brade, V., et al. (2002). Host association of Borrelia burgdorferi sensu lato - The key role of host complement. *Trends Microbiol.* 10, 74–79.

Kurtenbach, K., Hanincová, K., Tsao, J. I., Margos, G., Fish, D., and Ogden, N. H. (2006). Fundamental processes in the evolutionary ecology of Lyme borreliosis. *Nat. Rev. Microbiol.* 4, 660–669.

Kurtenbach, K., Peacey, M., Rijpkema, S. G. T., Hoodless, A. N., Nuttall, P. A., and Randolph, S. E. (1998a). Differential transmission of the genospecies of Borrelia burgdorferi sensu lato by game birds and small rodents in England. *Appl. Environ. Microbiol.* 64, 1169–1174.

Kurtenbach, K., Sewell, H. S., Ogden, N. H., Randolph, S. E., and Nuttall, P. A. (1998b). Serum complement sensitivity as a key factor in Lyme disease ecology. *Infect. Immun.* 66, 1248–1251.

Lane, R. S., Piesman, J., and Burgdorfer, W. (1991). Lyme Borreliosis: Relation of Its Causative Agent to Its Vectors and Hosts in North America and Europe. *Annu. Rev. Entomol.* 36, 587–609.

Lindgren, E., and Jaenson, T. G. T. (2006). Lyme borreliosis in Europe: influences of climate and climate change, epidemiology, ecology and adaptation measures. Available at: http://www.euro.who.int/pubrequest [Accessed March 18, 2020].

LoGiudice, K., Ostfeld, R. S., Schmidt, K. A., and Keesing, F. (2003). The ecology of infectious disease: Effects of host diversity and community composition on lyme disease risk. *Proc. Natl. Acad. Sci. U. S. A.* 100, 567–571.

Mans, B. J., De Klerk, D., Pienaar, R., and Latif, A. A. (2011). Nuttalliella namaqua: A Living Fossil and Closest Relative to the Ancestral Tick Lineage: Implications for the Evolution of Blood-Feeding in Ticks. *PLoS ONE*. 6(8):e23675.

Marconi, R. T., Liveris, D., and Schwartz, I. (1995). Identification of novel insertion elements, restriction fragment length polymorphism patterns, and discontinuous 23S rRNA in Lyme disease spirochetes: Phylogenetic analyses of rRNA genes and their intergenic spacers in Borrelia japonica sp. nov. and genomic group 21038 (Borrelia andersonii sp. nov.) isolates. *J. Clin. Microbiol.* 33, 2427–2434.

Margos, G., Fedorova, N., Kleinjan, J. E., Hartberger, C., Schwan, T. G., Sing, A., et al. (2017). Borrelia lanei sp. Nov. extends the diversity of borrelia species in California. *Int. J. Syst. Evol. Microbiol.* 67, 3872–3876.

Margos, G., Gofton, A., Wibberg, D., Dangel, A., Marosevic, D., Loh, S. M., et al. (2018). The genus Borrelia reloaded. *PLoS One* 13, : e0208432.

Margos, G., Hojgaard, A., Lane, R. S., Cornet, M., Fingerle, V., Rudenko, N., et al. (2010). Multilocus sequence analysis of Borrelia bissettii strains from North America reveals a new Borrelia species, Borrelia kurtenbachii. *Ticks Tick. Borne. Dis.* 1, 151–158.

Margos, G., Vollmer, S. A., Cornet, M., Garnier, M., Fingerle, V., Wilske, B., et al. (2009). A new Borrelia species defined by multilocus sequence analysis of housekeeping genes. *Appl. Environ. Microbiol.* 75, 5410–5416.

Margos, G., Vollmer, S. A., Ogden, N. H., and Fish, D. (2011). Population genetics, taxonomy, phylogeny and evolution of Borrelia burgdorferi sensu lato. *Infect. Genet. Evol.* 11, 1545–1563.

Masuzawa, T., Takada, N., Kudeken, M., Fukui, T., Yano, Y., Ishiguro, F., et al. (2001). Borrelia sinica sp. nov., a Lyme disease-related Borrelia species isolated in China. *Int. J. Syst. Evol. Microbiol.* 51, 1817–1824.

Matuschka, F. R., Fischer, P., Heiler, M., Blümcke, S., and Spielman, A. (1992). Stage-associated risk of transmission of the lyme disease spirochete by European Ixodes ticks. *Parasitol. Res.* 78, 695–698.

McDowell, J. V, Wolfgang, J., Tran, E., Metts, M. S., Hamilton, D., and Marconi, R. T. (2003). Comprehensive analysis of the factor h binding capabilities of borrelia species associated with lyme disease: delineation of two distinct classes of factor h binding proteins. *Infect. Immun.* 71, 3597–602.

Medlock, J. M., Hansford, K. M., Bormane, A., Derdakova, M., Estrada-Peña, A., George, J. C., et al. (2013). Driving forces for changes in geographical distribution of Ixodes ricinus ticks in Europe. *Parasites and Vectors* 6, 1.

Miller, S. C., Porcella, S. F., Raffel, S. J., Schwan, T. G., and Barboura, A. G. (2013). Large linear plasmids of borrelia species that cause relapsing fever. *J. Bacteriol.* 195, 3629–3639.

Milne, A. (1949). The ecology of the sheep tick, Ixodes ricinus L.; host relationships of the tick, review of previous work in Britain. *Parasitology* 39, 167–72.

Nava, S., Guglielmone, A. A., and Mangold, A. J. (2009). An overview of systematics and evolution of ticks. *Front. Biosci.* 14, 2857–2877.

Nuttall, P. A., Paesen, G. C., Lawrie, C. H., and Wang, H. (2000). Vector-host interactions in disease transmission. *J. Mol. Microbiol. Biotechnol.* 2, 381–386.

Ohnishi, J., Piesman, J., and De Silva, A. M. (2001). Antigenic and genetic heterogeneity of Borrelia burgdorferi populations transmitted by ticks. *Proc. Natl. Acad. Sci. U. S. A.* 98, 670–675.

Olivier, J. H. (1982). "Tick Reproduction: Sperm Development and Cytogenetics," in *Physiology of Ticks* (Elsevier), 245–275.

Olsen, B., Jaenson, T. G. T., and Bergstrom, S. (1995). Prevalence of Borrelia burgdorferi sensu lato-infected ticks on migrating birds. *Appl. Environ. Microbiol.* 61, 3082–3087.

Pal, U., Yang, X., Chen, M., Bockenstedt, L. K., Anderson, J. F., Flavell, R. A., et al. (2004). OspC facilitates Borrelia burgdorferi invasion of Ixodes scapularis salivary glands. *J. Clin. Invest.* 113, 220–230.

Piesman, J., Oliver, J. R., and Sinsky, R. J. (1990). Growth kinetics of the lyme disease spirochete (Borrelia burgdorferi) in vector ticks (Ixodes dammini). *Am. J. Trop. Med. Hyg.* 42, 352–357.

Piesman, J., Schneider, B. S., and Zeidner, N. S. (2001). Use of quantitative PCR to measure density of Borrelia burgdorferi in the midgut and salivary glands of feeding tick vectors. *J. Clin. Microbiol.* 39, 4145–4148.

Piesman, J., Spielman, A., Etkind, P., Ruebush, T. K., and Juranek, D. D. (1979). Role of deer in the epizootiology of Babesia microti in Massachusetts, USA. *J. Med. Entomol.* 15, 537–40.

Pospisilova, T., Urbanova, V., Hes, O., Kopacek, P., Hajdusek, O., and Sima, R. (2019). Tracking of Borrelia afzelii Transmission from Infected Ixodes ricinus Nymphs to Mice. *Infect. Immun.* 87:e00896-18.

Postic, D., Garnier, M., and Baranton, G. (2007). Multilocus sequence analysis of atypical Borrelia burgdorferi sensu lato isolates - Description of Borrelia californiensis sp. nov., and genomospecies 1 and 2. *Int. J. Med. Microbiol.* 297, 263–271.

Postic, D., Marti Ras, N., Lane, R. S., Hendson, M., and Baranton, G. (1998). Expanded diversity among Californian Borrelia isolates and description of Borrelia bissettii sp. nov. (formerly Borrelia group DN127). *J. Clin. Microbiol.* 36, 3497–3504.

Pritt, B. S., Respicio-Kingry, L. B., Sloan, L. M., Schriefer, M. E., Replogle, A. J., Bjork, J., et al. (2016). Borrelia mayonii sp. nov., a member of the Borrelia burgdorferi sensu lato complex, detected in patients and ticks in the upper midwestern United States. *Int. J. Syst. Evol. Microbiol.* 66, 4878–4880.

Rand, P. W., Lacombe, E. H., Smith, R. P., and Ficker, J. (1998). Participation of birds (Aves) in the emergence of Lyme disease in southern Maine. *J. Med. Entomol.* 35, 270–6.

Richter, D., Postic, D., Sertour, N., Livey, I., Matuschka, F. R., and Baranton, G. (2006). Delineation of Borrelia burgdorferi sensu lato species by multilocus sequence analysis and confirmation of the delineation of Borrelia spielmanii sp. nov. *Int. J. Syst. Evol. Microbiol.* 56, 873–881.

Rizzoli, A., Silaghi, C., Obiegala, A., Rudolf, I., Hubálek, Z., Földvári, G., et al. (2014). Ixodes ricinus and its transmitted pathogens in urban and peri-urban areas in Europe: New hazards and relevance for public health. *Front. Public Heal.* 2.

Rosa, P. A., Tilly, K., and Stewart, P. E. (2005). The burgeoning molecular genetics of the Lyme disease spirochaete. *Nat. Rev. Microbiol.* 3, 129–143.

Rudenko, N., Golovchenko, M., Grubhoffer, L., and Oliver, J. H. (2009a). Borrelia carolinensis sp. nov., a new (14th) member of the borrelia burgdorferi Sensu lato complex from the southeastern region of the United States. *J. Clin. Microbiol.* 47, 134–141.

Rudenko, N., Golovchenko, M., Lin, T., Gao, L., Grubhoffer, L., and Oliver, J. H. (2009b). Delineation of a new species of the Borrelia burgdorferi sensu lato complex, Borrelia americana sp. nov. *J. Clin. Microbiol.* 47, 3875–3880.

Rudenko, N., Golovchenko, M., Růžek, D., Piskunova, N., Mallátová, N., and Grubhoffer, L. (2009c). Molecular detection of Borrelia bissettii DNA in serum samples from patients in the Czech Republic with suspected borreliosis. *FEMS Microbiol. Lett.* 292, 274–281.

Saier, M. H., and Paulsen, I. T. (2000). Whole genome analyses of transporters in spirochetes: Borrelia burgdorferi and Treponema pallidum. in *Journal of molecular microbiology and biotechnology*. 393–9.

Salman, M. D., Tarrés-Call, J., Estrada-Peña, A., Farkas, R., Jaenson, T. G. T., Koenen, F., et al. (2013). *Ticks and tick-borne diseases : geographical distribution and control strategies in the Euro-Asia region*. CABI Publishing.

Samuels, D. S. (2011). Gene Regulation in Borrelia burgdorferi . *Annu. Rev. Microbiol.* 65, 479–499.

Schwan, T. G., Piesmant, J., Goldet, W. T., Dolant, M. C., and Rosa, P. A. (1995). Induction of an outer surface protein on Borrelia burgdorferi during tick feeding. *Proc. Natl. Acad. Sci. USA* 92, 2909–2913.

Sonenshine, D. E., and Roe, R. M. (2014a). *Biology of ticks. Volume 1*. Oxford University Press.

Sonenshine, D. E., and Roe, R. M. (2014b). *Biology of ticks. Volume 2*. Oxford University Press.

Spielman, A., Ribeiro, J. M. C., Mather, T. N., and Piesman, J. (1987). Dissemination and Salivary Delivery of Lyme Disease Spirochetes in Vector Ticks (Acari: Ixodidae). *J. Med. Entomol.* 24, 201–205.

Stanek, G., Fingerle, V., Hunfeld, K. P., Jaulhac, B., Kaiser, R., Krause, A., et al. (2011). Lyme borreliosis: Clinical case definitions for diagnosis and management in Europe. *Clin. Microbiol. Infect.* 17, 69–79.

Steere, A. C. (2001). Lyme disease. *N. Engl. J. Med.* 345, 115–125.

Stepanova-Tresova, G., Kopecký, J., and Kuthejlová, M. (2000). Identification of Borrelia burgdorferi sensu stricto, Borrelia garinii and Borrelia afzelii in Ixodes ricinus ticks from southern Bohemia using monoclonal antibodies. *Zentralblatt fur Bakteriol.* 289, 797–806.

Tsao, J. I. (2009). Reviewing molecular adaptations of Lyme borreliosis spirochetes in the context of reproductive fitness in natural transmission cycles. *Vet. Res.* 40, 36.

van Dam, A. P., Kuiper, H., Vos, K., Widjojokusumo, A., de Jongh, B. M., Spanjaard, L., et al. (1993). Different genospecies of Borrelia burgdorferi are associated with distinct clinical manifestations of Lyme borreliosis. *Clin. Infect. Dis.* 17, 708–17.

Van Duijvendijk, G., Sprong, H., and Takken, W. (2015). Multi-trophic interactions driving the transmission cycle of Borrelia afzelii between Ixodes ricinus and rodents: a review. *Parasit. Vectors.* 8:643.

Von Lackum, K., and Stevenson, B. (2005). Carbohydrate utilization by the Lyme borreliosis spirochete, Borrelia burgdorferi. *FEMS Microbiol. Lett.* 243, 173–179.

Voordouw, M. J. (2015). Co-feeding transmission in Lyme disease pathogens. *Parasitology* 142, 290–302.

Wang, G., Van Dam, A. P., Le Fleche, A., Postic, D., Peter, O., Baranton, G., et al. (1997). Genetic and phenotypic analysis of Borrelia valaisiana sp. nov. (Borrelia, genomic groups VS116 and M19). *Int. J. Syst. Bacteriol.* 47, 926–932.

Yuval, B., and Spielman, A. (1990). Duration and Regulation of the Developmental Cycle of Ixodes dammini (Acari: Ixodidae). *J. Med. Entomol.* 27, 196–201.

Zhang, J. R., and Norris, S. J. (1998). Genetic variation of the Borrelia burgdorferi gene vlsE involves cassette-specific, segmental gene conversion. *Infect. Immun.* 66, 3698–3704.