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Growth kinetics and transmission dynamics of *Borrelia afzelii* in *Ixodes ricinus*

Bachelor thesis

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Annotation

Laboratory model for Lyme disease was used for a revision of *Borrelia afzelii* / *Ixodes ricinus* transmission cycle. The behaviour of *B. afzelii* in different phases of the transmission cycle was analyzed. The role of the tick vector *I. ricinus* in the transmission of *B. afzelii* was investigated.

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Tereza Pospíšilová

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1. Introduction

1.1. Ticks

Ticks are bloodsucking ectoparasites, transmitting a wide range of pathogens, affecting broad range of animals as well as humans. Beside the tick pathogenesis, a massive tick infestation results in a significant blood loss and represents a major economic threat to the world livestock industry (Graf et al. 2004).

These small arachnids belong to the order Parasitiformes, subclass Acari. So far, three families of ticks have been established: the Argasidae, Ixodidae, and Nuttalliellidae, represented by a single south African species *Nuttalliella namaqua,* which is considered to be the evolutionary missing link between the hard (Ixodidae) and soft (Argasidae) ticks (Nava et al. 2009; Mans et al. 2011).

1.1.1. Argasidae

Argasidae, also known as soft ticks, represent approximately 200 usually nidicolous species including *Argas*, *Nothaspis*, *Ornithodoros*, *Carios* and *Otobius* (Horak et al. 2002). Their life strategy and morphology is significantly different to hard ticks. Their oval-shaped body is covered with a leathery cuticle. They lack a hard plate on their back, called scutum, present in ixodid ticks. For the host tissues penetration and attachment, a sophisticated harpoon-like structure on the capitulum called hypostome, is required. In Argasidae, the capitulum with hypostome is located beneath the body and so not visible (Sonenshine 1991). The lifecycle of soft ticks range from months to years, and involve one larval, several nymphal and one adult stage. Each instar requires a blood meal, the engorgement is rapid and the tick is usually fully fed within a few minutes. Both, an adult male and female suck blood and mate off the host. After mating, female may lay small bunches of eggs (200-300) repeatedly during her lifetime (Sonenshine & Roe 2013b). Any excess fluid is excreted via coxal glands during feeding, which is also unique feature to argasid ticks, in contrast to ixodid ticks, that use salivary glands to eliminate surplus water during the blood intake (Sonenshine & Roe 2013a).

1.1.2. Ixodidae

The Ixodidae family (683 species) (Jongejan & Uilenberg 2004), contains *Amblyomma, Dermacentor, Haemaphysalis, Hyalomma, Ixodes, Margaporus* and *Rhipicephalus* genera (Horak et al. 2002). They are recognized as hard ticks for their sclerotized dorsal scutum, which covers the whole male dorsum, and about 1/3 of the nymphal and the female body. Hard ticks are classified as one-, two- or three host parasites (Sonenshine 1991). When feeding, they remain attached to the host for up to eight days, in case of larvae and nymphs, whereas adult female may suck blood for 12 days or even longer (Anderson & Magnarelli 2008).

1.1.2.1. *Ixodes ricinus* lifecycle

In nature, the overall lifecycle of *Ixodes ricinus* takes about two to three years to complete (Fig. 1). An adult female feeds usually on big mammals like a deer. During engorgement she mates with a male, which is important for her full repletion (a hundred times increased body mass is feasible) (Weiss & Kaufman 2004). An adult male does not require blood meal at all. His only task is to fertilize the feeding female. After mating, a fully fed female drops of the host, to lay thousands of eggs in the grass, and dies. In spring, six-legged larvae hatch from fertilized eggs. Larvae usually feed on rodents or birds. After a blood intake, engorged larvae shelter in the grass and in four to six weeks molt into nymphs. To seek for a prey, nymphs crawl on the top of grass, quest for their second host to come. When questing, they hold the first pair of legs (with a complex sensory Haller's organ) outstretched, to detect the animal's breath (CO_2), scent, humidity, body heat, and vibrations (Foelix & Axtell 1972), to be ready to attach to the passing animal. After nymphal feeding, in four to six weeks, nymphs molt to adults. Both, the larval as well as the nymphal feeding lasts approximately three days, feeding of an adult female is rather longer.

When studying the transmission of the Lyme disease, the most crucial stage is the nymphal stage (the most abundant stage a human can found attached on his body), because the transovarial transmission is rather not likely. The infection is acquired by larval or nymphal tick feeding on an infected host (usually a rodent or a bird). However, transovarial transmission of relapsing fever spirochaete *B. miyamotoi* by *I. persulcatus* was described (Rollend et al. 2013). Moreover, recent data indicate, that larval *I. ricinus* might transmit *B. afzelii* as well as a relapsing fever spirochaete *B. miyamotoi* (van Duijvendijk et al. 2016).

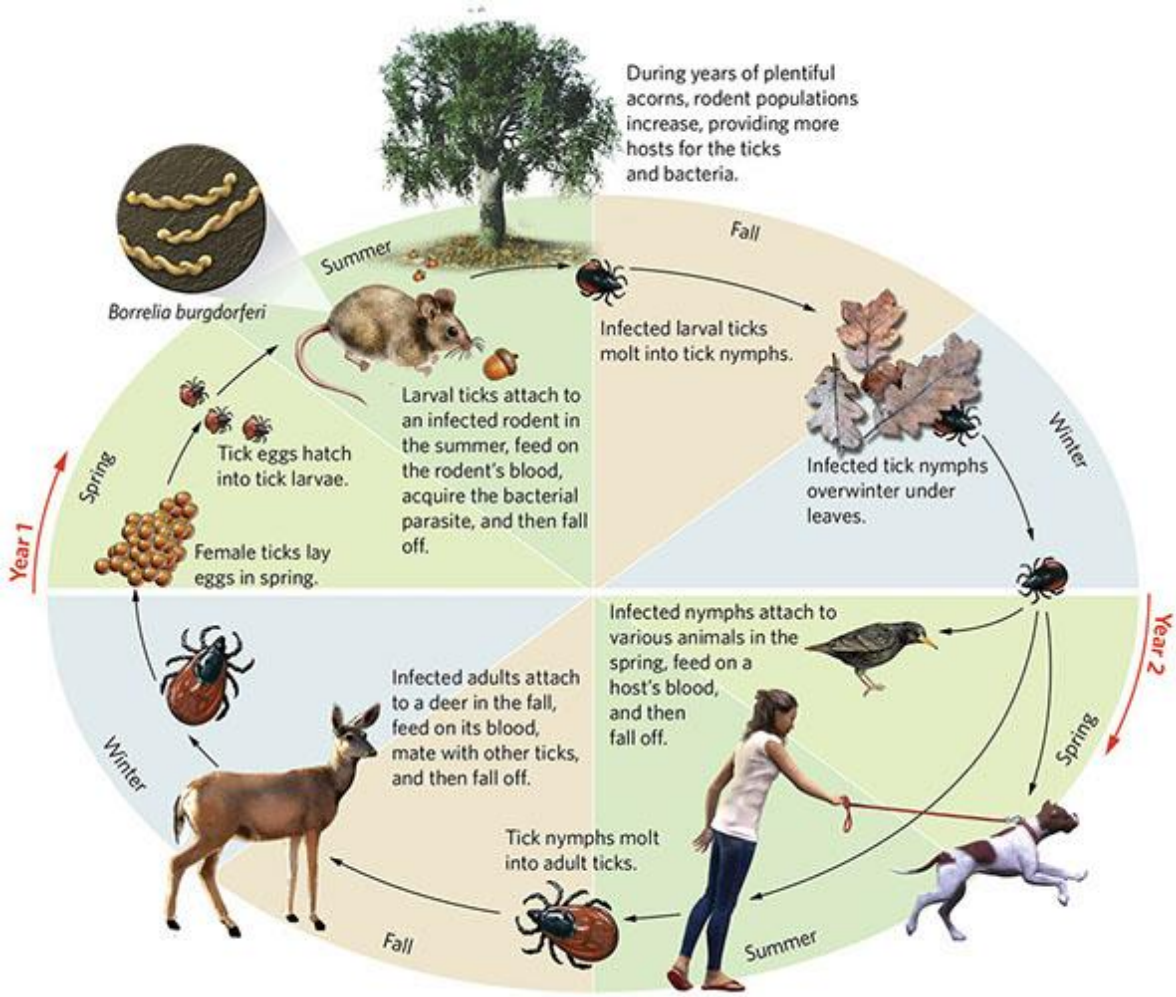

Figure 1: The three host lifecycle of *I. ricinus*, and the Lyme disease transmission. Larval ticks acquire infection via feeding on infected hosts (usually a small mammal or a bird), transovarial transmission is not likely (Rollend et al. 2013). After repletion, and dropping of the host, larvae seek for a shelter in the grass, in 4-6 weeks molt into nymphs, and overwinter. Afterwards, infected nymphs spread infection further, when feeding on another host, which might be a human as well. Both, larval and nymphal feeding lasts about 3 days to complete. Subsequently, after nymphal feeding and molting to an adult (also in 4-6 weeks), adult females attach to the third host (usually a big mammal) and start feeding. For a total repletion, mating with a male is essential. Mating may occur off the host before feeding or during feeding (Weiss & Kaufman 2004). An adult male does not require any blood meal, his only part is to fertilize the female, afterwards passes away. Scheme was designed by Nicolle R. Fuller, Sayo-Art LLC, and reproduced with her expressed written consent.

1.1.3. Tick transmitted pathogens

Together with mosquitoes, ticks are the most important vectors of diseases worldwide (Sonenshine 1991). A huge spectrum of tick-borne pathogens involves protozoan infections like theileriosis transmitted by African hard tick *Rhipicephalus appendiculatus*, malaria-like babesiosis, as well as viruses like encephalitis or hemorrhagic fever. Bacterial diseases represent rickettsiosis (spotted fever), anaplasmosis , and tularemia (Jongejan & Uilenberg 2004). In case of *Borrelia* spirochetes, both tick families (Argasidae and Ixodidae) are capable of transmitting the pathogen. *B. hermsii*, also known as a relapsing fever *Borrelia* is transmitted by the soft tick *Ornithodoros*, whereas *Borrelia burgdorferi* sensu lato (s.l.), causing Lyme borreliosis, is spread by *Ixodes* ticks (Jongejan & Uilenberg 2004).

1.1.4. Tick saliva

Tick saliva plays an important role in a process of the tick feeding. A complex mixture of biochemical compounds, injected via hypostome to the tick feeding site include anticoagulants, cytokine inhibitors, histamine binding factors, and complement inhibitors, suppress or mediate the host sensibility to the tick bite (Hajdusek et al. 2013). This results in a painless bite and facilitated tick feeding (Nuttall & Labuda 2008). Almost 70% of the water ingested during the feeding is returned to the host via salivary glands (Bowman & Sauer 2004).

Borrelia exploits tick salivary proteins in favour of its transmission to the host. Importantly, a ratio of many genes expressed in the salivary glands, and other organs of infected and uninfected ticks fluctuates a lot (Ribeiro et al. 2006; Hajdusek et al. 2013).

This includes the tick histamine release factor (tHRF) that neutralizes the inflammatory effect of histamine, secreted by host immune cells at the tick feeding site, and therefore is a critical factor for tick repletion. Silencing of this gene using RNAi or tHRF blocking by antibodies severely hampers tick feeding, and also decreases spirochetal numbers in mice (Jianfeng Dai et al. 2010).

Another salivary gland protein upregulated in *B. burgdorferi* infected ticks is Salp15, which protects the pathogen from antibody mediated killing in the host (Ramamoorthi et al. 2005; Hovius et al. 2008). Silencing of Salp15 significantly reduces spirochetal capability of infecting mice. Furthermore, antibodies raised against tick Salp15 significantly protect mice from the infection (Dai et al. 2009).

Reduced complement killing also secure tick complement inhibitors like TSLPI (tick salivary lectin pathway inhibitor) or Salp20 (Tyson et al. 2007), which protect *Borrelia* from

being destroyed by the host immune system via phagocytosis or bacterial lysis. Reviewed by (Schuijt et al. 2011; Hajdusek et al. 2013).

1.1.5. Tick gut

The tick gut serves as a storage organ of the ingested blood, before its digestion within cells. Intracellular blood digestion (neutral pH) prevents microbes from the contact with digestive proteases (usually secreted to the lumen). An engorged tick gut is full of nutrients present in the blood and therefore provides *Borrelia* and other microorganisms a friendly environment for its colonization and proliferation. Thus, ticks possess effective defense mechanisms to maintain the intestinal microflora at tolerable level (Hajdusek et al. 2013).

These mechanisms include antimicrobial defensins (Kopácek et al. 1999), lysozymes (Nakajima et al. 2002) and large antimicrobial peptides hemocidins, derived from the Hemoglobin digestion (Sonenshine et al. 2005). Tick differential innate immunity responses are demonstrated on *Ixodes scapularis* immunotolerance, and *Dermacentor variabilis* immunocompetence (both Acari: Ixodidae) for *B. burgdorferi* transmission (R Johns et al. 2001). *Dermacentor variabilis* possess defensin named varisin, which shows activity against *B. burgdorferi* s. l. (R. Johns et al. 2001).

Whether the tick serves as a competent vector for *Borrelia* transmission, rely on the tick diverse commensal microflora as well. Evidence for that might be an artificial infection of the soft tick, *Ornithodoros moubata*, with *Chryseobacterium indologenes*, a G^- bacteria which the tick hardly encounters in nature (Buresová et al. 2006). This artificial infection resulted in a prompt tick death within 3 days, whereas the increased mortality rate of similarly infected hard tick *I. ricinus* ticks was insignificant (Buresová et al. 2006). Nevertheless, scientists still lack the general understanding of the mutual interplay between the ingested pathogen, the commensal microbiome, and the tick itself (Hajdusek et al. 2013; Narasimhan & Fikrig 2015).

1.2. Genus *Borrelia*

The genus *Borrelia* belongs to the phylum Spirochaetes. *Borrelia* is a helical-shaped bacteria with a double-membrane envelope, with an average length of 20 μm and a diameter of 0.2 μm. The cell contains 7-14 periplasmic flagella running lengthwise between the peptidoglycan layer and the outer cell membrane. Periplasmic flagella are anchored at both ends of spirochete to the cytoplasmic membrane, and twist around the protoplasmic cell cylinder (Rosa et al. 2005), (Fig. 2). This extraordinary cell architecture determines the specific cell shape, and enables *Borrelia* to move relatively quickly through viscous fluids based on chemotaxis, and successfully evade host tissues (Barbour & Hayes 1986; Charon et al. 2012). In other bacteria, peptidoglycan layer governs the cell shape. However, *Borrelia* might be weakly stained as gram negative bacteria, it is neither considered G^+ nor G^-, since its unique cellular structure, and the lack of lipopolysaccharides in its cell walls (Takayama et al. 1987). Instead, the cell membranes contain glycolipids (Ben-Menachem et al. 2003).

1.2.1. The *Borrelia* genome

The *Borrelia* genome represents a linear chromosome of 911 kbp, along with at least 16 linear or circular plasmids of a minimum size of 533 kbp, which undertake frequent horizontal gene transfer (Fraser et al. 1997; Casjens et al. 2012).

In contrast to the complex *B. burgdorferi* genome, its spirochetal cousin *Treponema pallidum*, the causative agent of syphilis, contains only one circular chromosome, without any extrachromosomal elements (Chamberlain et al. 1989).

Evidence, that *B. burgdorferi* differentially expressed antigens are roughly plasmid encoded (Ojaimi et al. 2003) indicates the plasmid essentiality for both, *Borrelia* infectivity as well as the maintenance of the complex enzootic cycle, which involves a cold-blooded tick and a warm-blooded vertebrate. Though a high level of *Borrelia* physiological adaptation is obligatory (Purser & Norris 2000).

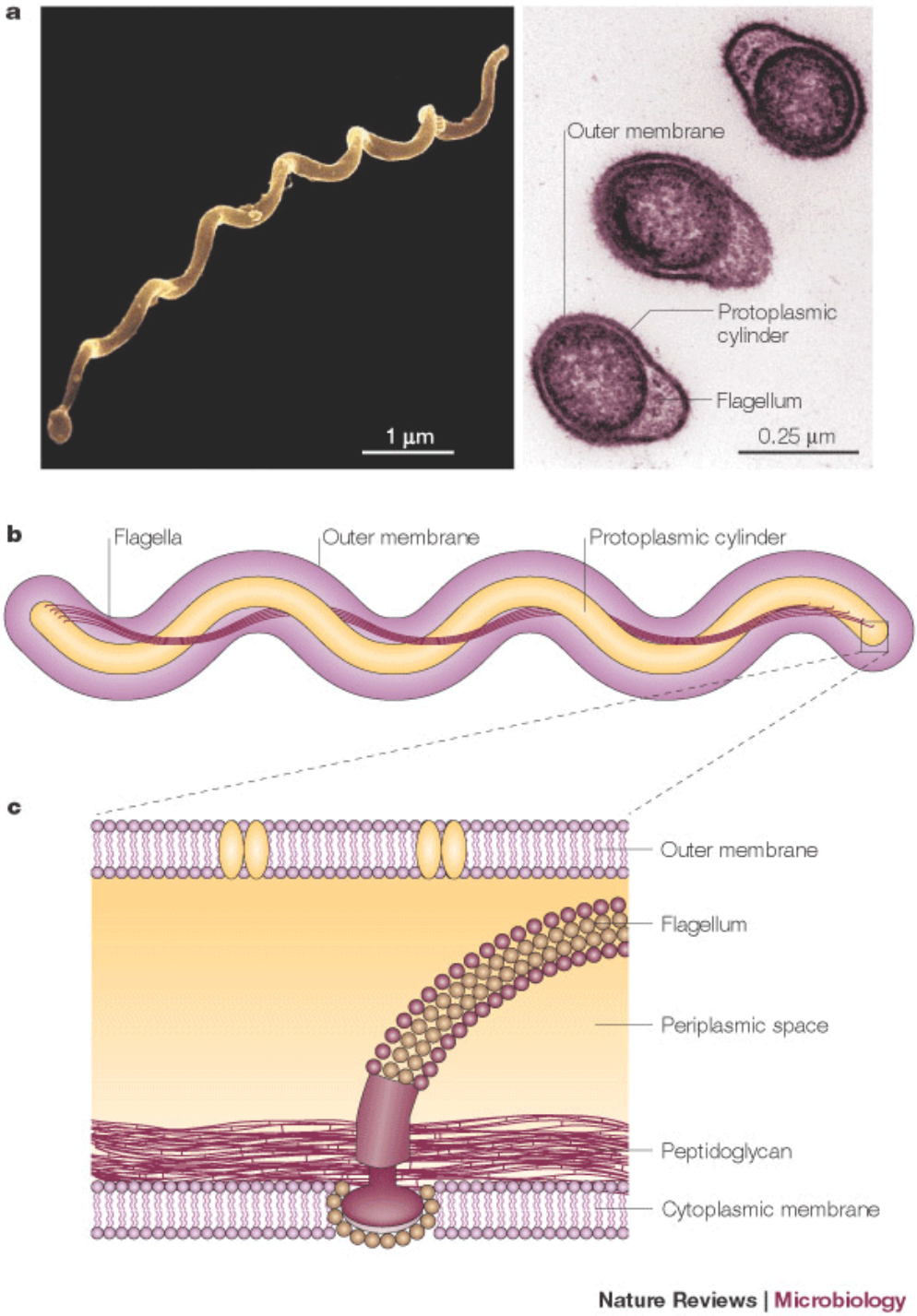

Figure 2: a Scanning (left) and transmission (right) electron micrographs of *B. burgdorferi*. The helical shape of the cell is determined by the periplasmic flagella, which cross-sectional view is displayed on the transmission electron micrograph. **b** Scheme of the spirochete. Flagellar clusters twist around the elastic, rod-shaped protoplasmic cell cylinder **c** Flagella bundles are incorporated into the cytoplasmic membrane at both ends of the cell. The outer cell membrane limits flagella within the periplasmic space. Adapted from (Rosa et al. 2005).

1.2.2. *Borrelia* surface lipoproteins

Differential expression of surface antigens allows *Borrelia* to adapt specifically to the tick or the host environment as required, and therefore plays a significant role in its pathogenesis (Liang et al. 2002). Moreover, Cox and colleagues detected OspA/B, and OspC outer surface proteins shuttle from to the periplasm of the cell during infection, which also helps to prevent the host immune response (Cox et al. 1996). The idea of antibodies shedding, and so changeable cell coating, is utilized also by protozoan pathogens *Trypanosoma brucei*, the causing agent of sleeping sickness, and *Plasmodium falciparum*, which causes malaria (Taylor & Rudenko 2006; Dzikowski et al. 2006). To come to the point, besides the differential gene expression, also the modulation of the surface lipoproteins generates the spirochetal antigenic diversity (Hefty et al. 2002).

1.2.3. Rrp2- RpoN/RpoS alternative sigma factor cascade

RNA polymerase, sigma S (RpoS) serves as a master regulator controlling expression of more than hundred *B. burgdorferi* genes including those, which play key roles in *Borrelia* transmission and pathogenicity (Caimano et al. 2007). For the RpoS (σ^S) synthesis, previous activation of RpoN (σ^{54}), via binding a putative enhancer protein Rrp2, is essential (Ouyang et al. 2014). BosR (*Borrelia* oxidative stress response regulator) is another transcription factor directly controlling the expression of the *rpoS* gene (Boylan et al. 2003).

The stimulation of *rp2- rpoN/rpoS* is initiated by nymphal blood feeding, hence spirochetes in flat nymphs as well as engorged larvae do not express RpoS (Caimano et al., 2007). An increased temperature, downregulation of *ospA/B,* and a reciprocal *rrp2-rpoN/rpoS* activation during the nymphal feeding, form a positive feedback loop, that activates *ospC, dbpA, bbk32* and other genes that play a part in *Borrelia* transmission (Schwan et al. 1995; Hübner et al. 2001; He et al. 2008; Caimano et al. 2007).

1.2.4. How the tick acquires *B. burgdorferi* infection?

It has been found, that *Borrelia* spirochetes in the tissues of the infected hosts are capable of recognizing, and subsequently acting in response to vertebrate host neuroendocrine stress hormones, epinephrine and norepinephrine, that are expected to be released at the tick feeding site (Scheckelhoff et al. 2007). In response to the host catecholamines, *B. burgdorferi* may regulate its protein expression, particularly enhance the production of the outer surface protein A (OspA), which is essential for the uninfected tick gut recolonization (Scheckelhoff et al. 2007). Thus, the host catecholamines may contribute

to the capability of the pathogen to recolonize uninfected ticks feeding on an infected mammal, which is crucial for the maintenance of *B. burgdorferi* in nature (Scheckelhoff et al. 2007).

1.2.5. Borrelia *"sleeping"* within the tick gut

OspA and OspB homologues are abundant outer surface lipoproteins typically expressed by spirochetes in the midgut of unfed tick (Yang et al. 2004). Spirochetal adherence to tick gut walls is enabled via binding to the tick receptor for OspA called TROSPA (U Pal et al. 2004). Evidence, that OspA/B variants probably originated by a gene duplication, supports the fact ,that the expression of both, OspA/B is regulated by the same operon, located on the linear plasmid (lp54) (Fraser et al. 1997).

The expression of OspA/B decreases as the tick feeds on a warm blooded host, which goes along with activation of the Rrp2-RpoN-RpoS pathway (He et al. 2008). Spirochetes in the host tissue transcribe *ospA* only during the first day of the tick feeding, but not afterwards (Hodzic et al. 2002).

Even though *ospA/B* deficient spirochetes can be transmitted from infected mice to the arthropod vector, these strains are altogether incapable of colonizing, and persisting within the tick midgut (Yang et al. 2004; Battisti et al. 2008).

1.2.6. *Borrelia* "wakes up" during the tick feeding

OspC is an alternative surface lipoprotein to OspA/B. The tick blood feeding, elevated temperature (32-37°C), reduced pH, and RpoS binding to the *ospC* promoter, enforce its synthesis (Eggers et al. 2004; Caimano et al. 2007). Complementary, OspC levels are almost undetectable in the midgut of unfed ticks at lower temperatures (24°C), where OspA/B is omnipresent (Schwan et al. 1995). Up till now, OspC is generally known for its reciprocal production to OspA/B, that mediates spirochetal transmission from the vector to the host (Radolf & Caimano 2008).

Alteration of surface antigens, particularly *ospC* rapid upregulation, seems to be crucial in early phases of *Borrelia* infectivity, and spreading (Liang 2002). These findings support the fact, that the conversion between OspA/OspC is initiated within the tick midgut during the blood intake (36-48h), and whereas guts from partially engorged ticks are infectious to mammals, infected guts from unfed ticks are not (Shih & Spielman 1993). Furthermore, *ospC* deletion mutants are not infectious for mice (Grimm et al. 2004; Tilly et al. 2006).

According to Pal et al., OspC plays a fundamental role in the *B. burgdorferi* invasion of tick salivary glands, where it binds to the tick salivary protein Salp15, mediating spirochetal transmission from the tick to the host (Pal et al. 2004). Salp15 might work as a protecting shield against the host immune system recognition, and subsequent antibody-mediated killing (Ramamoorthi et al. 2005).

Moreover, besides the ability to use a secreted tick protein as a protective shield, to avoid specific humoral response in the host, *Borrelia* sheds OspC, which is essential in the initial phase of spirochetal transmission, and then upregulates the expression of highly variable surface antigens including DbpA, Bbk32, and VlsE, required for the permanent infection in the host (Liang 2002; Crother et al. 2004).

1.2.7. Evading the host immune response *"stealth pathogen"*

To be able to persist in the host, and to avoid recognition by the host immune system, the surface- exposed lipoprotein VlsE undergoes intriguing genetic recombination, which results in enormous antigenic variability (Zhang & Norris 1998a). *vlsE* locus was originally discovered on the linear plasmid (lp28-1) of *B. burgdorferi*, and contains the *vlsE* expression site and 15 silent cassettes, which are recombined into the central *vlsE* cassette region during infection (Zhang et al. 1997). Additionally, VlsE resembles variable major protein (Vmp) of the relapsing fever *Borrelia hermsii* (Saint Girons & Barbour 1991; Zhang et al. 1997). During infection, regions of the expressed *vlsE* cassette are replaced with regions of the silent cassettes, as early as 4 days post infection, and a selection of *vlsE* variants depends on the host immune response (Zhang & Norris 1998b). In the study conducted by Coutte et al., researchers encountered a complete change of *B. burgdorferi vlsE* parental locus in mice 28 days post infection (Coutte et al. 2009).

1.2.8. Pathogen adhesion to the host tissues

Decorin binding proteins DbpA and DbpB are essential for the host cell adhesion as they bind to collagen-associated proteoglycan decorin (Guo et al. 1998). Proteoglycan decorin interacts with collagen fibers, and therefore acts as a component of the connective tissue. In relation to distinct variant of decorin binding proteins, *Borrelia* strains prefer colonization and settling in different tissues, and thus triggering diverse Lyme disease symptoms like arthritis or carditis (Lin et al. 2014). DbpA/B expression is enhanced by a temperature shift from 23 to 37 °C , and reduced pH, which evoke a significant role for these proteins in the host organism (Carroll et al. 2000; Revel et al. 2002; Ojaimi et al. 2003).

Even though, neither DbpA, nor DbpB are essential for infecting the host, both proteins play a notable part in later stages of disease**,** for instance during dissemination and establishing a chronic infection in decorin-rich tissues. Reviewed by (Kenedy et al. 2012).

Bbk32 is another spirochetal surface lipoprotein upregulated via Rrp2- RpoN/RpoS pathway during the tick feeding, and in the host tissues (Probert & Johnson 1998; He et al. 2007). It has been shown, that Bbk32 binds to the fibronectin, a host glycoprotein, that exists either as a serum protein or as a component of the extracellular matrix, glycosaminoglycans, heparin, and also dermatan sulfate (Fischer et al. 2006). However Bbk32 adhesion function contributes to the *Borrelia* pathogenesis, Bbk32 lacking spirochetes remain infectious for the mammalian host (Li et al. 2006).

Moreover, recent data show, that Bbk32 inhibits the classical pathway of human complement by binding with high affinity to the initiating C1 complement complex, a previously unknown target of bacterial anti-complement molecules. Therefore, Bbk32 plays an important part in *B. burgdorferi* protection from complement mediated killing. Eventually, such a discovery significantly advances our understanding of how disease-causing bacteria survive in immune competent hosts (Garcia et al. 2016).

1.3. Lyme disease

The causing agent of Lyme disease, *B. burgdorferi*, was first identified by Willy Burgdorfer as a Treponema-like spirochete. The disease is named after the town of Lyme in Connecticut, USA, where children, suffering from an atypical arthritis, were treated (Burgdorfer et al. 1982). Lyme disease is the most common vector-borne infection in the northern hemisphere transmitted by *Ixodes* ticks. As a multisystemic disease, it causes huge spectrum of problems, ranging from arthritis to neuromuscular disorders. In early stages, flu-like symptoms and tiredness are common. Prior to dissemination and establishing a systemic infection, pathogens migrate through the skin. Consequently, characteristic skin lesion Erythema migrans (also called the bulls eye rash for its typical shape), might or might not develop. In chronic stages, *Borrelia* spirochetes usually colonize various tissues, including heart, joints, central nervous system and brain, where they hide away from the host immunity. Reviewed by (Cook 2015).

In the USA, Lyme disease is caused mostly by *B. burgdorferi* sensu stricto (s. s.), spread by *I. scapularis* (in the east), and I*xodes pacificus* (in the west). Whereas in Europe, *B. afzelii*, and *B. garinii*, transmitted by *I. ricinus* ticks, are widespread (Sonenshine & Roe 2013b). Moreover, a recent review data indicate, that *B. burgdorferi* s.l. enhances the fitness of *I. ricinus* (Herrmann & Gern 2015).

The model of selective transmission, that different *Borrelia* genospecies prefer different hosts, is based on the host complement system ability to clear out the particular genospecies both from the infested host, and feeding tick as well. *B. afzelii* spirochete is a rodent specialist, and *B. garinii* an avian specialist, however, *B. burgdorferi* s. s. persists in both, rodents and birds (Kurtenbach et al. 2002). Big ungulates like deer are incompetent hosts for *B. burgdorferi* (Jaenson & Tälleklint 1992). In humans, *B. afzelii* usually causes bluish-red lesions (Acrodermatitis chronica atrophicans), *B. burgdorferi* sensu stricto is likely to cause Lyme arthritis, whereas *B. garinii* infection ends in neuroborreliosis (Wang et al. 1999).

1.3.1. Transmission of Lyme disease by *Ixodes* ticks

It is generally believed, that the risk of acquiring the Lyme disease increases with length of the tick attachment. However, the minimum tick attachment time for the spirochetal transmission has never been established. Both the spirochetal transmission times and virulence differ in reliance on the tick and *Borrelia* species. Reviewed by (Cook 2015).

Previous studies state, that *I. scapularis* ticks do not transmit the *B. burgdorferi* s. s. spirochetes during the first 2 days of attachment (Piesman et al. 1987; Falco et al. 1996; Piesman et al. 2001).

However, spirochetes appear in the host dermis even the first day of the tick blood meal (Ohnishi et al. 2001; Hodzic et al. 2002; Crippa et al. 2002). Culture grown spirochetes as well as the spirochetal population within the tick gut, is rather homogenous, producing mainly OspA on its surface. Antigenic variability, essential for establishing of a permanent infection, is generated during the tick feeding (Ohnishi et al. 2001). Spirochetes transmitted to the host during the first day of the tick feeding express only OspA but not OspC on its surface (Hodzic et al. 2002). However, spirochetes in the host tissue express OspA only during the first day of the tick attachment, but not afterwards (Hodzic et al. 2002), when a permanent infection is more likely to be established. *ospA/B* deficient spirochetes can be transmitted from infected mice to the tick, but are unable to persist within the tick gut (Yang et al. 2004), whereas *ospC* deletion mutants are not infectious for mice (Grimm et al. 2004; Tilly et al. 2006)**.**

ospC rapid upregulation seems to be crucial in early phases of *Borrelia* infectivity, and spreading (Liang 2002). OspC might be important for the development of a protective immunity, when facing *B. burgdorferi* infection (Hughes et al. 1993). Conversion between OspA/OspC via stimulation of the Rrp2- RpoN/RpoS pathway (Caimano et al., 2007) is initiated within the tick midgut during the blood intake (36-48 hours), which goes along with the fact, that guts from partially engorged ticks are infectious to mammals, whereas infected guts from unfed ticks are not (Piesman 1993; Crippa et al. 2002). Partially fed nymphal ticks transmit spirochetal infection more rapidly than do ticks that have never been attached to a host (Shih & Spielman 1993). Nevertheless, an elevated temperature alone, seems to be an insufficient factor for the *Borrelia* pathogenity, based on the fact, that homogenates prepared from infected unfed ticks incubated at 37°C, did not establish a permanent infection, when injected to mice (Piesman 1993).

Correspondingly, all mice exposed to the bite of *B. burgdorferi* s.s. infected ticks up to 48 hours remained uninfected. In contrast, one of seven (14%) and four of eight (50%) mice exposed for 24 and 48 hours to *B. afzelii* infected ticks became infected. Thus, when comparing *B. afzelii* and *B. burgdorferi* transmission by *I.ricinus*, *B. afzelii* is transmitted faster and seems to be more infectious than *B. burgdorferi* (Crippa et al. 2002) .

1.3.2. Salivary route of *Borrelia* transmission or regurgitation

In 1995, using confocal fluorescent microscopy, De Silva and Fikrig described *B. burgdorferi* proliferation within the tick midgut during the nymphal *I. scapularis* feeding (De Silva & Fikrig 1995). Whereas in unfed nymphs, spirochetes (a mean of 496) were restricted only to the midgut, after 48 hours of the tick blood feeding spirochetal dissemination to the salivary glands seemed widespread. A highest number of spirochetes within the tick (166 575 spirochetes/nymph), was observed 72 hours post attachment. Furthermore, 96 hours post attachment, after the tick repletion and dropping of the host, the pathogen appeared to be restricted strictly to the midgut again, and the number of spirochetes decreased (95 410 spirochetes/nymph). Based on these data, scientists established a critical timepoint of spirochetal dissemination and infection of the salivary glands to 36-48 hours. These data has been taken as s solid evidence of the previously suggested hypothesis of the salivary route of *B. burgdorferi* transmission (Ribeiro et al. 1987).

In contrast, the homogenates of the tick salivary glands, derived from infected ticks, did not produce infection in mice unless gathered from ticks feeding for ≥60 hours (Piesman 1995).

In actual fact, the hypothesis of the salivary route of *B. burgdorferi* transmission is nowadays generally accepted, regardless these two totally opposing statements.

On the other hand, when the pathogen was discovered, even Willy Burgdorfer alone purposed a potential way of *Borrelia* transmission via regurgitation, which is a direct migration of spirochetes from the tick gut to the host. Actually, using radiolabeling, regurgitation of gut content was demonstrated on the ticks *Ornithodoros moubata* as well as *Amblyomma americanum*, which unique midgut antigens were observed in the host (Brown 1988; Connat 1991). Such a data may support the pathogen transmission via regurgitation, originally suggested by Willy Burgdorfer (Burgdorfer et al. 1984).

1.3.3. Vaccination

A lot of effort has been put into the investigation and developing of a possible vaccine based on the OspA recombinant (Steere et al. 1998), and other surface antigens thwarting *Borrelia* transmission. Unfortunately, until these days, all vaccination attempts failed due to the *Borrelia* antigens shedding abbility, wide polymorphism, and a great strain diversity of *Borrelia* species (Bunikis et al. 2004; Wilske et al.; Kenedy et al. 2012). Moreover, negative side-effects of vaccination including arthritis and facial paralysis emerged (Lathrop et al. 2002).

Nowadays, scientists focus rather on a developing of a possible anti-tick vaccine, thwarting the tick blood feeding, thus consequently preventing the transmission of the tick-borne pathogens in general.

Several tick antigens with potential to protect against tick-transmitted diseases have been published. Labuda et al. tested an anti-tick vaccine derived from a tick cement protein 64TRP. This vaccine had a dual effect in immunized animals and resulted in impaired blood feeding and death of the ticks and protected against tick borne encephalitis virus (TBEV) (Labuda et al. 2006). Subsequently, Dai et al. characterized a tick histamine release factor (tHRF) derived from *I. scapularis*. Blocking of the tick histamine release factor tHRF by RNAi or by immunization significantly impaired tick feeding and decreased *B. burgdorferi* burden in mice (Dai et al. 2010). To conclude, in future, it might be feasible to use tick antigens to prevent tick-borne diseases.

2. Objectives

- Quantification of *B. afzelii* spirochetes at timepoints during *I. ricinus* development and blood feeding
- Transmission of *B. afzelii* by nymphal *I. ricinus*- the critical length of nymphal blood feeding to establish *B. afzelii* infection
- The effect of tick molecules on *B. afzelii* survival in mice
- *B. afzelii* gene expression

3. Materials and methods

3.1. Laboratory animals

I. ricinus larvae and nymphs were obtained from the breeding facility of the Institute of Parasitology, Biology Centre, CAS. All *I. ricinus* developmental stages (eggs, larvae, nymphs and adults) were maintained in wet chambers in glass boxes with air humidity around 95% at constant temperature of 24 °C and with photo-period light: dark – 11h: 13h. To prepare both, infected and uninfected *I. ricinus* nymphs, the larvae were fed on either infected, or uninfected mice, allowed to molt to nymphs, and after 4–6weeks were used for further experiments. Inbred, pathogen free C3H/HeN mice (Jackson Laboratory, Germany), were used for the pathogen transmission experiments. All experimental animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 137/2008.

3.2. Infection of mice and ticks

Low passage *Borrelia* strains of *B. afzelii* CB43 (Stěpánová-Tresová et al. 2000), (we are grateful to Prof. Kopecký for generously sharing *B. afzelii* CB43 strain with us), and *B. burgdorferi* s.s. SLV-2 (collection site 46°3′17″N,14°30′21″E) (Golovchenko et al. 2014) were grown in BSK-H medium (Sigma-Aldrich, USA) at 33°C for 5–7 days. Six weeks old female C3H/HeN mice were infected by subcutaneous injections of 10^5 spirochetes in 100 µl of BSK-H medium per mouse (Fig. 3). Presence of spirochetes in ear biopsies was determined 3 weeks post injection by standard PCR.

Four weeks after inoculation, uninfected larvae were allowed to feed on infected mice (100 larvae per mouse) and left to molt (Fig. 3). Generally, nymphs were considered to be infected if >80% of them were PCR positive. However, the acquisition of *B. afzelii* CB43 by feeding ticks was almost 100%.

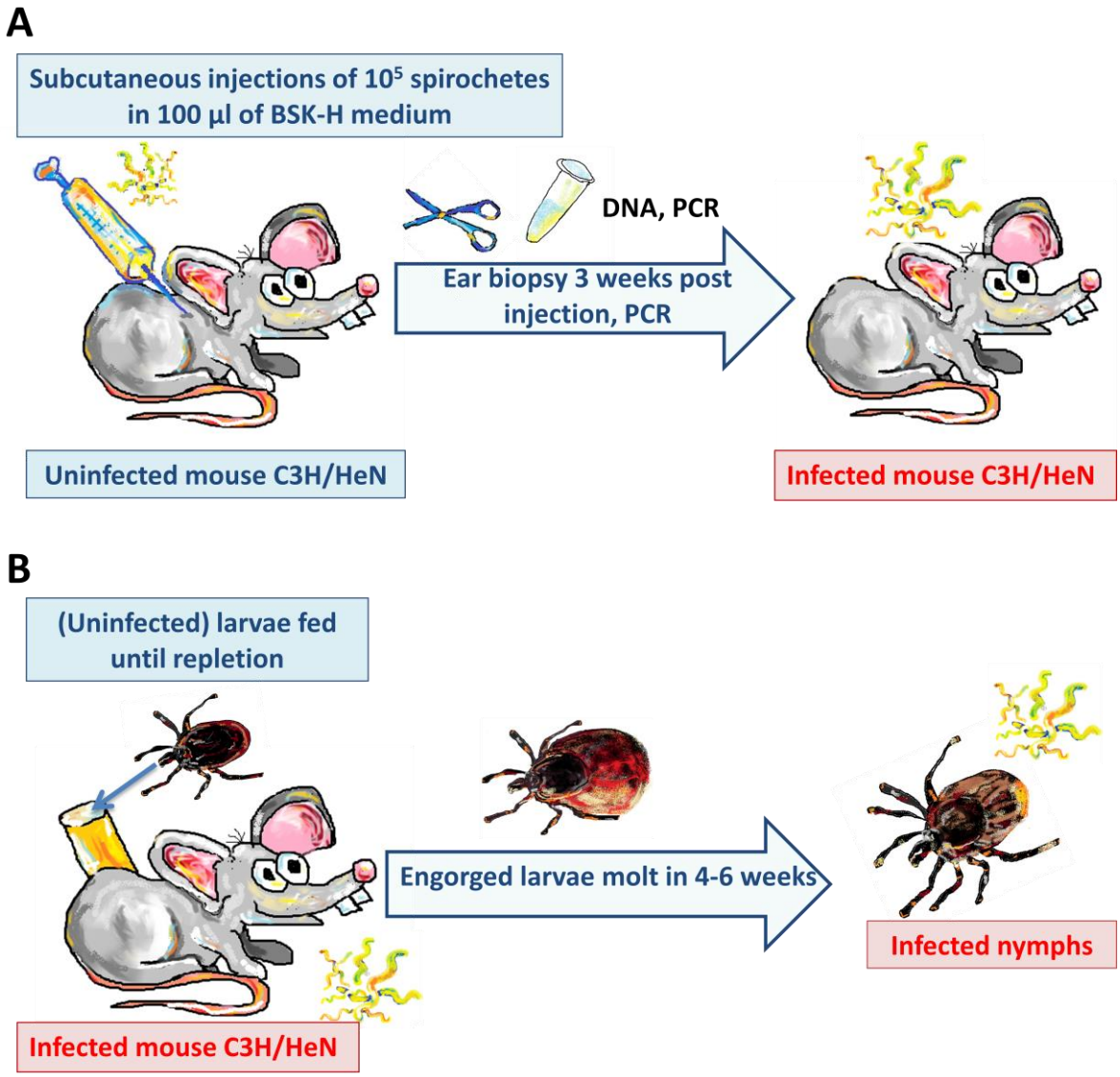

Figure 3: Infection of mice and ticks. **A** Six weeks old female C3H/HeN mice were infected by subcutaneous injections of 10^5 spirochetes in 100 µl of BSK-H medium per mouse. Presence of spirochetes in ear biopsies was determined 3 weeks post injection by standard PCR. **B** To prepare infected nymphs, four weeks after inoculation, uninfected larvae were allowed to feed on infected mice (100 larvae per mouse) and left to molt. Infected nymphs were used for further experiments.

3.3. Nucleic acid isolation

3.3.1. DNA isolation

Total DNA was isolated from individual larvae, nymphs, as well as murine tissues using a NucleoSpin Tissue Kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. Quality of DNA in every sample was verified by standard PCR amplifying a 600 bp part of murine chromosome 2 (primers Mm-600F, Mm-600R), or 600 bp part of tick sorting nexin 24 (primers Ir-600F, Ir-600R). Sequences of primers are shown in Table I (Tab. I).

3.3.2. RNA isolation

Total RNA was extracted from murine tissue samples and from *I. ricinus* nymphs using NucleoSpin RNA (Macherey-Nagel) according to manufacturer's protocol. The quality of RNA was checked on the 1% agarose gel. Subsequently, RNA concentration was measured on spectrometer NanoDrop^{TM} 1000 (NanoDrop, USA).

3.3.2.1. cDNA preparation

1 μg of isolated RNA served as a template for reverse-transcription into cDNA. The procedure of cDNA sythesis was carried on following the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany) protocol, using the anchored-oligo (dT)18 primers. Obtained cDNAs were diluted 10x and served as templates for the following quantitative expression analyses by qRT-PCR.

3.4. PCR

3.4.1. Standard PCR

Detection of spirochetes in ticks, as well as in murine tissues was performed by PCR amplification of a 154 bp fragment of *flagellin*. A reaction volume of 25 µl contained 12.5 µl of FastStart PCR MasterMix (Roche), 4 µl of purified DNA, 10 pmol of each primer (FlaF1A, FlaR1) and PCR water up to 25 µl. Primer sequences are shown in Table I (Tab. I). Amplification program for detection of *Borrelia* spirochetes by PCR using Fla primers consisted of denaturation at 94°C for 10 minutes, then 40 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 40s. The program was finished by final extension at 72°C for 7 minutes.

3.4.2. Nested PCR

Small loads of *Borrelia* spirochetes (particularly spirochetes in the murine tissues from the tick feeding sites) were undetectable by standard PCR. Therefore, ten times more sensitive method, nested PCR, was applied. Another purpose of applying nested PCR was genotyping of *Borrelia* species.

Nested PCR was run similarly to standard PCR (described above) except particular annealing temperatures and the primers, which are shown in Table II (Tab. II). For a detection of small loads of spirochetes following primers encoding borrelial *23S rRNA* were applied (the 1st round: Bor 1, Bor 2; the 2nd round: Bor 3, Bor 4, 10 µM). For a discrimination of different *Borrelia* strains, following primers amplifying 16S-23S rRNA (rrs-rrlA) intergenic spacer region were used (the 1st round: IGS F, IGS R, the 2nd round: IGS Fn, IGS Rn, 10 µM).

3.5. Quantitative PCR

3.5.1. Absolute quantification (qPCR)

Total spirochete load was determined in *B. afzelii* CB43 positive samples by quantitative real-time PCR (qPCR) using a LightCycler 480 (Roche). qPCR was performed in a 25 µl reaction volume containing 12.5 µl of FastStart Universal Probe Master (Rox) (Roche), 5 µl of purified DNA, 10 pmol of each, forward and reverse primer, and 5 pmol of particular probe as required (described below). The remaining reaction volume was adjusted with sterile water. qPCR reaction was run according to the following amplification program: denaturation at 95°C for 10 minutes, followed by 50 cycles of denaturation at 95°C for 15 sec and annealing + elongation at 60°C for 1 min. Sequences of particular primers and probes are in Table I (Tab. I).

For quantification of *B. afzelii* CB 43 spirochetes in ticks, primers FlaF1A + FlaR1 and TaqMan FlaProbe1 were used. The total number of borrelial genomes per tick was determined using external *B. burgdorferi* flagellin standard curve.

To quantify spirochetes in murine tissues, primers FlaF1A+ FlaR1 and TaqMan FlaProbe1 for *B. burgdorferi flagellin* and also primers Mm actin F + Mm actin R and probe Mm actin P for *Mus musculus actin* were used. In this case, the number of spirochete burden in tissues was determined per 10^5 of *M. musculus actin* copies. Murine *actin* was absolutely quantified using external *M. musculus actin* standard curve.

All samples were analysed in triplicates. The threshold cycles were determined as the resulting arithmetic means of the two measurements in triplicates.

3.5.2. Relative quantification (qRT-PCR)

The cDNA samples prepared from ticks and mice (described above) served as templates for the following quantitative *B. afzelii* CB43 gene expression analyses by quantitative reverse transcription PCR (qRT-PCR). A reaction of a total volume of 25 µl contained 12.5 µl of FastStart Universal Sybr Green Master, Rox (Roche), 10 pmol of each primer, 5.5 µl H_2O (nuclease free) and 5 µl cDNA template. Sequences of primers are in Table III (Tab. III). All samples were analysed in triplicates using the LightCycler 480 (Roche). Reaction conditions followed protocol (95 °C/10s – denaturation, 60 °C/10s – annealing, 72 °C/10s – extension) in 50 cycles and included the melting curve analysis. The threshold cycles were determined as the resulting arithmetic means of the three measurements. Melting curve for

each sample was checked. The relative gene expression levels were calculated using the mathematical model of Pfaffl (Pfaffl 2001). The expression levels of examined *B. afzelii* CB43 genes were normalized to borrelial housekeeping gene *flaB.*

Table I: PCR and qPCR primers, and probes for detection and quantification of spirochetes in ticks and murine tissues, and primers for control of DNA presence in samples.

PCR / qPCR primers for a detection of spirochetes in ticks and murine tissues				
Target	**Name**	**Sequence (5'→3')**	**Product size**	**Source**
Borrelia flagellin	**FlaF1A**	AAGCAAATTTAGGTGCTTTCCAA	154 bp	Schwaiger et al. 2001
	FlaR1	GCAATCATTGCCATTGCAGA		
	TaqMan FlaProbe1	TGCTACAACCTCATCTGTCATTGTA GCATCTTTTATTTG		
Mus musculus B-actin	**Mm actin F**	AGAGGGAAATCGTGCGTGAC	137 bp	Dai et al. 2009
	Mm actin R	CAATAGTGATGACCTGGCCGT		
	Mm actin P	CACTGCCGCATCCTCTTCCTCCC		
PCR primers for *Ixodes ricinus* and *Mus musculus* control genes				
Target	**Name**	**Sequence (5'→3')**	**Product size**	**Source**
Ixodes ricinus	**Ir-600R**	GACCTGCACGAAAATGATTG	600 bp	Šíma R. unpublished
	Ir-600F	GAGGCATGAGGGTGTGTTTT		
Mus musculus	**Mm-600F**	GCTTCTGGAAGAACCACAGG	600 bp	Šíma R. unpublished
	Mm-600R	AAGCACTTCGAACCACTGCT		

Table II: Nested PCR primers for detection of small loads of spirochetes in murine tissues; Nested PCR primers for IGS *Borrelia* genotyping.

Nested PCR primers for detection of small loads of spirochetes in murine tissues					
Target	**Name**	**Sequence (5'→3')**	**Product size**	**Annealing**	**Source**
Borrelia 23S rRNA	**Bor1**	AGAAGTGCTGGAGTCGA	260 bp	53°C	Šíma R. unpublished
	Bor2	TAGTGCTCTACCTCTATTAA			
	Bor3	GCGAAAGCGAGTCTTAAAAGG	222 bp	58°C	
	Bor4	ACTAAAATAAGGCTGAACTTAAAT			
Nested PCR IGS primers for *Borrelia* genotyping					
Target	**Name**	**Sequence (5'→3')**	**Product size**	**Annealing**	**Source**
***Borrelia* rrs–rrlA IGS**	**IGS F**	GTATGTTTAGTGAGGGGGGTG	450 bp* 500 bp** 760 bp***	56°C	Bunikis et al. 2004
	IGS R	GGATCATAGCTCAGGTGGTTAG			
	IGS Fn	AGGGGGGTGAAGTCGTAACAAG		58°C	
	IGS Rn	GTCTGATAAACCTGAGGTCGGA			

**B. afzelii*, ***B. garinii*, ****B. burgdorferi* sensu stricto.

Table III: *Borrelia* gene expression primers (qRT-PCR).

***Borrelia* gene expression primers (qRT-PCR)**				
Target	**Name**	**Sequence (5'→3')**	**Product size**	**Source**
OspA lipoprotein	**RTospA-FN**	CGCATGGGATTCAAAAACTT	119 bp	new primers
	RTospA-RN	TGGTACCTGCGGAGTCGTAT		
OspC lipoprotein	**RTospC-F**	ATGCTTCAGAACAGTTTTTAGCC	124 bp	Koci et al. 2006
	RTospC-R	AATGGATCGTTGTTAGCAGGA		
Bbk32 lipoprotein	**RTbbk32-F**	CACGTCTTGACAACCTTGCT	117 bp	
	RTbbk32-R	CCTTGCACTCACTTGAATATAG		
DbpA lipoprotein	**RTdbpA-F**	TACGCGTCGCTGACTTAACA	129 bp	
	RTdbpA-R	CTTTTGCGGCGTTGAGTATTA		
Borrelia flagellin	**RTflaB-F**	GTTCATGTGGGAGCAAATCA	120 bp	
	RTflaB-R	ACCCTCTTGAACAGGTGCAG		

3.6. Preparation of tick tissues for confocal microscopy

Guts and salivary glands of unfed, 24 hours fed, 48 hours fed and fully fed nymphs infected with *B. afzelii* CB43 were dissected in phosphate buffer. After that, guts were pierced by tip of blade and both tissues were immersed into 4% paraformaldehyde for 4 hours at room temperature. Tissues were washed 3x20 min in PBS, permeabilized in 1% BSA (Bovine serum albumin, Sigma) in 1% PBS-Tx (Triton X 100) at 4°C, overnight. Next day, *Borrelia* spirochetes in tissues were stained with primary anti *B. burgdorferi* antibody (*Borrelia burgdorferi* Antibody, FITC conjugate, Rabbit/IgG, Thermo Fisher Scientific, USA) 1:200 in 0,1% PBS-Tx, for 2 hours at room temperature. After incubation with primary antibody, tissues were washed 3x20 min in 0,1% PBS-Tx and stained with fluorescently labeled secondary antibody (Alexa Fluor 488 Goat anti Rabbit, life technologies, USA), 1:500 in 0,1%PBS-Tx, for 1 hour at room temperature. Gut and salivary gland cells were counterstained with DAPI, mounted in DABCO and examined using a confocal Olympus FluoView FV1000 microscope (Olympus, Japan). The images were subsequently processed using the Fluoview (FV10-ASW, Version 1.7) software.

3.7. Gel electrophoresis

PCR products were visualized using gel electrophoresis. Ethidium bromide (Sigma-Aldrich) stained 2% agarose gel in TAE buffer was used for separation and size determination of PCR products. 10 µl of each sample was mixed with DNA loading dye (Top-Bio, Czech Republic) and subsequently loaded on the gel. PCR product size was determined according to the 100 bp DNA ladder (Thermo Scientific, USA).

3.8. Statistical analysis

The statistics and graphs of obtained data were processed using GraphPad Prism 6 (version 6.01 for Windows, GraphPad Software, USA). Ap value of $P<0.05$ was considered to be statistically significant. For sample groups comparisons, either unpaired t-test (two-tailed, F-test used to compare variances) or one-way ANOVA (Tukey test), was used. Data normalization was done via logarithm where required. The error bars show standard error of the mean in the graphs as independent biological replicates were used to obtain the results.

4. Results

4.1. Quantification of *Borrelia* spirochetes at timepoints during tick development and blood feeding

The vast majority of the overall knowledge of the Lyme disease transmission is based upon the research performed on American *B. burgdorferi* and its vector *I. scapularis*. So far, a little is known about the behaviour of their European relatives, *B. afzelii* and *I. ricinus*. However, it is well-known that both, the disparity of spirochetal transmission times and virulence remarkably depend on the tick and *Borrelia* species (Cook 2015).

Therefore, to build our investigation on solid foundations, in which we can trust and refer to, when conducting transmission experiments in future, it was essential to examine the specific behaviour of *B. afzelii* CB43 during lifecycle of *I. ricinus* ticks.

4.1.1. Growth kinetics of *B. afzelii* CB43 in molting *I. ricinus*

Absolute quantitative PCR was used for a determination of the growth kinetics of *B. afzelii* CB43 spirochetes in different stages of the tick development (Fig. 4). In engorged larvae, as well as during larval molting, spirochetes multiplied quite rapidly. In fully fed *I. ricinus* larvae, the mean number of spirochetes per tick was 618 ± (158=SEM) immediately after repletion. The maximum number of spirochetes, 21005 ± (4805=SEM) spirochetes per tick, was observed in unfed nymphs 2^{nd} week after larval molting. Afterwards, spirochetal proliferation decelerated, diminished or even stopped. During the 4^{th} to 20^{th} week post repletion, an average spirochetal number moved around 8900 spirochetes per tick.

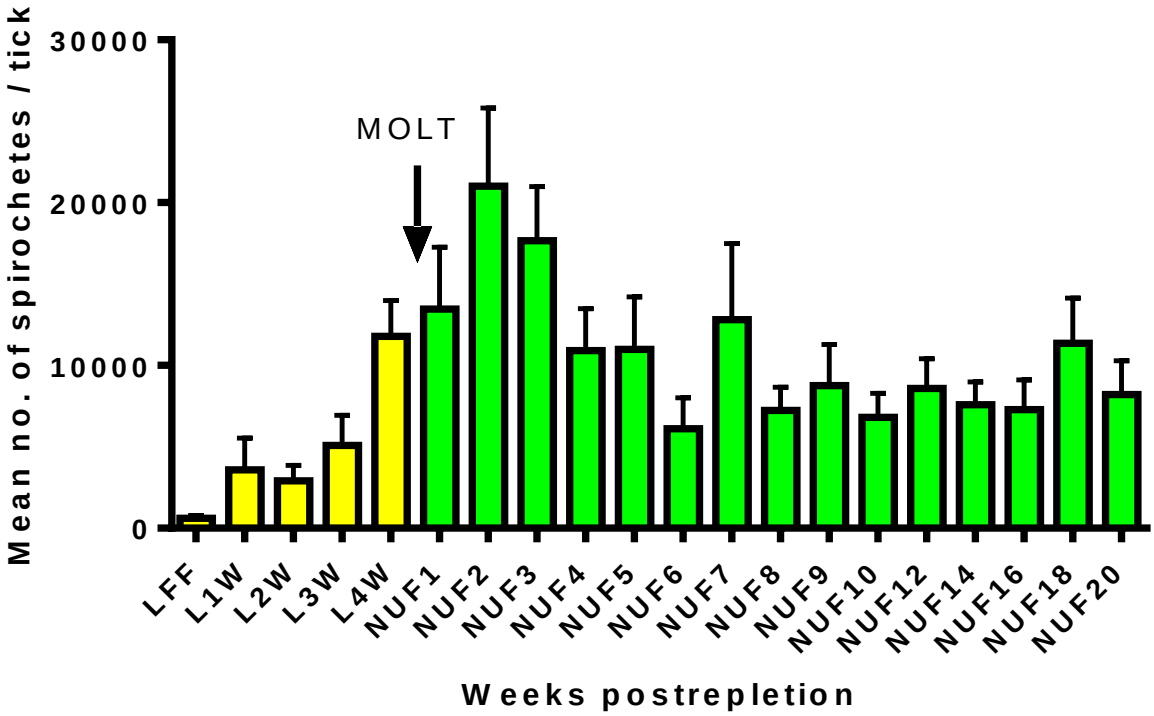

Figure 4: Growth kinetics of *B. afzelii* CB43 in molting *I. ricinus.*
Developmental stage: (LFF) fully fed larva, (L1W-L4W) larva 1st-4th week after detachment, (NUF1-NUF20) unfed nymph 1st -20th week after molting. Each column represents a mean number of spirochetes per tick. At each timepoint, DNA of 20 individual ticks was examined by qPCR separately. The error bars indicate SEM.

4.1.2. Kinetics of *B. afzelii* CB43 during feeding of *I. ricinus* nymph

qPCR was performed to evaluate an absolute number of *B. afzelii* CB43 spirochetes within the feeding *I. ricinus* nymph. Infected flat nymphs from the 4th week after larval molting were placed on uninfected C3H/HeN mice and removed at 24, 48 and 72 hours after attachment (Fig. 5).

During the tick blood intake, an overall number of spirochetes within the tick dropped dramatically. Importantly, the migration of spirochetes started immediately the 1st day of the tick attachment. Prior to feeding, an average number of spirochetes per tick was 10907 ± (2590 =SEM). After 24 hours of the tick feeding, the number of spirochetes was reduced to 7492 ± (3294=SEM). In the following 2nd and 3rd day of blood intake, the spirochetal population within tick decreased even more, from 2447 ± (801=SEM) to 720± (138=SEM) spirochetes per tick respectively.

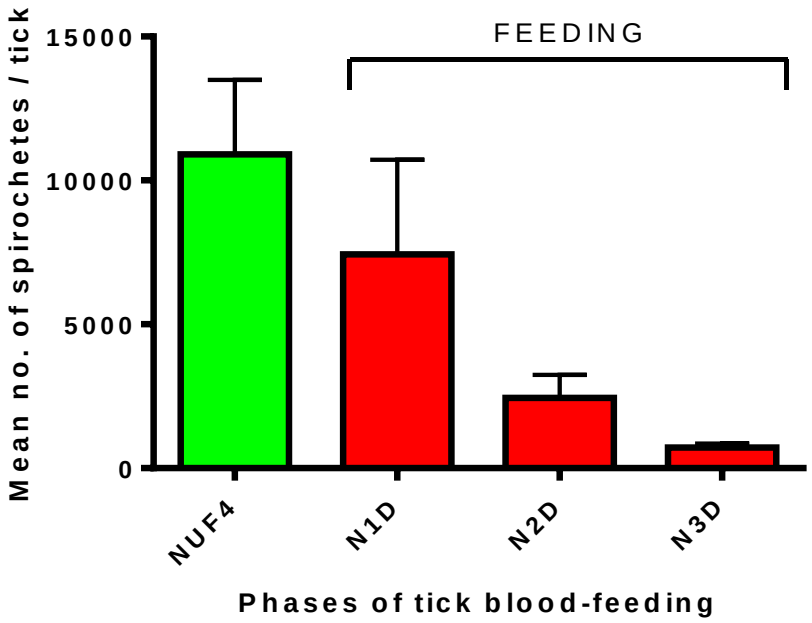

Figure 5: Kinetics of *B. afzelii* CB43 during feeding of *I. ricinus* nymph.
(NUF4) unfed nymph 4th week after molting, (N1D-N3D) nymph during 1st -3rd day of blood intake. Each column represents a mean number of spirochetes per tick. At each timepoint, qPCR was performed on DNA samples isolated from 20 individual nymphs separately. The error bars indicate SEM.

4.2. Transmission of *B. afzelii* by nymphal *I. ricinus*

It is generally believed, that the risk of acquiring the Lyme disease increases with the length of tick attachment (Cook 2015). Considering the fact, that both, spirochetal transmission times and virulence differ in reliance on the tick and *Borrelia* species, the minimum tick feeding time for the spirochetal transmission has never been established (Cook 2015).

Therefore, we focused particularly on the infectivity of *B. afzelii* CB43 transmitted via *I. ricinus.*

4.2.1. Critical length of nymphal blood feeding to establish *B. afzelii* infection

To determine a minimum length of tick blood-sucking required for an establishment of a permanent infection in mice, *B. afzelii* CB43 infected tick nymphs were allowed to feed on pathogen free C3H/HeN mice for 24, 48, or 72 hours (10 nymphs per mouse).

An ability of *B. afzelii* CB43 spirochetes to develop a chronic infection in mice increases with the length of tick blood-sucking (Tab. IV), (Fig. 6). In the first group of 9 mice, where infectious ticks were removed after 24 hours of the attachment, no mice showed a positive ear biopsy for the presence of *Borrelia* spirochetes three weeks after the tick removal. Moreover, when ticks were allowed to feed for 48 hours, 8/10 mice became infected. Eventually, the infection developed in all 9 mice, which challenged the tick bite for 72 hours (Tab. IV), (Fig. 6).

Table IV: PCR detection of *B. afzelii* CB43 in mice 3 weeks after the removal of infectious *I. ricinus* nymphs in the established timepoints of the nymphal blood-feeding.

Duration of tick feeding (hours)	**24**	**48**	**72**
no. of mice *B. afzelii* CB43 infected/ no. of mice exposed	0/9 (0%)	8/10 (80%)	9/9 (100%)

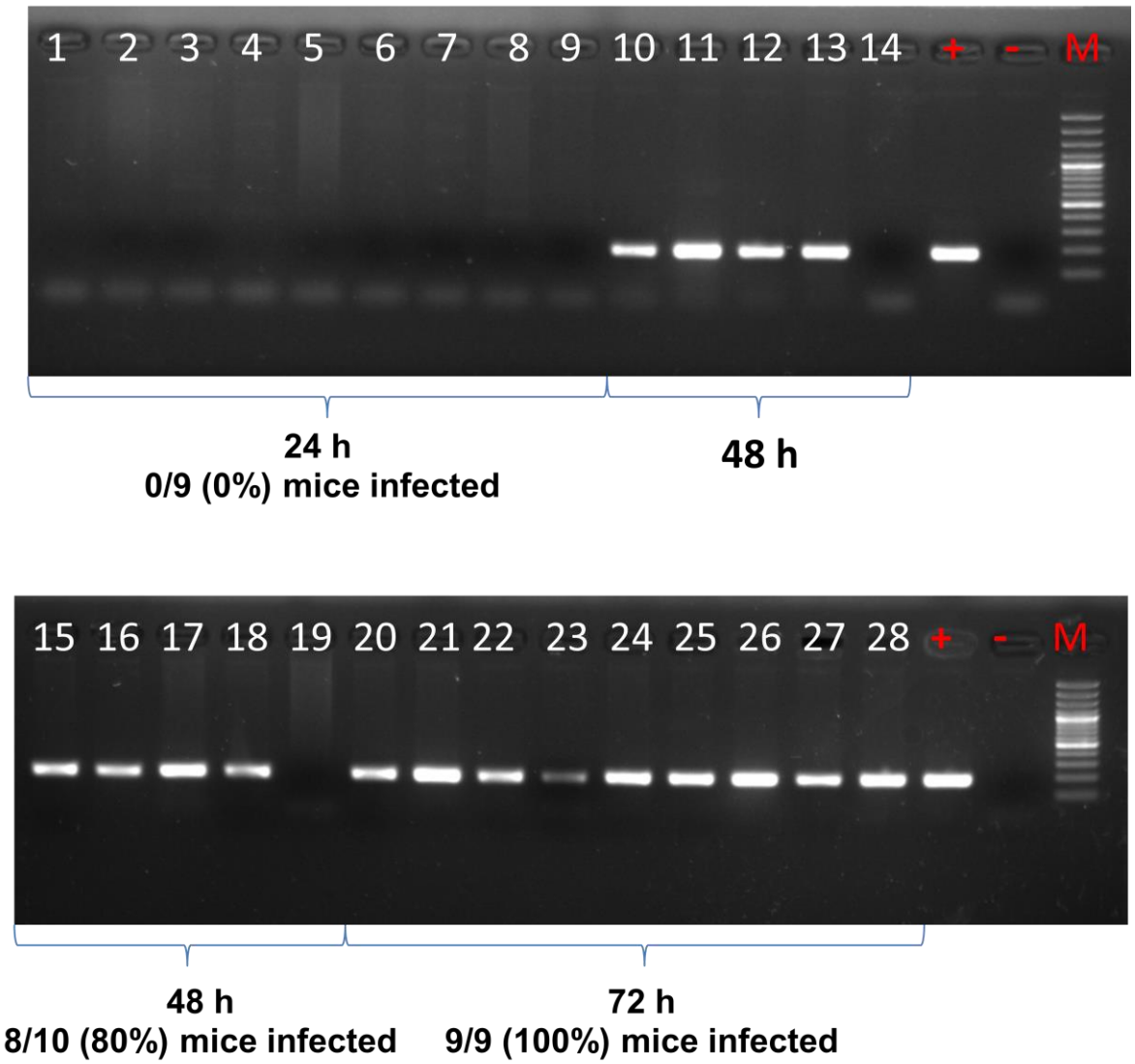

Figure 6: PCR detection of *B. afzelii* CB43 in mice 3 weeks after the removal of infectious *I. ricinus* nymphs in the established timepoints of the nymphal blood-feeding. (1-28) samples of murine ear punch biopsies, (+) positive control, (-) negative control, (M) molecular weight marker.

4.2.2. Presence of spirochetes in murine skin

Previous data on the kinetics of *B. afzelii* CB43 during the feeding of *I. ricinus* nymph suggested, that the migration of spirochetes to the host starts immediately the 1st day of the tick attachment (Fig. 5). To confirm this hypothesis, presence and quantity of *B. afzelii CB43* spirochetes was tested by nested PCR and qPCR in murine skin biopsies from the tick feeding site at time intervals of 24, 48 and 72 hours.

Surprisingly, skin biopsies from 9/10 (90%), 10/10 (100%), and 10/10 (100%) mice were PCR positive at time intervals of 24, 48 and 72 hours respectively (Tab. V), (Fig. 7).

Moreover, there were not significant differences in quantity of spirochetes in skin samples at defined time intervals and the presence of *B. afzelii* CB43 spirochetes in the murine skin at time interval of 24 hours was confirmed even by confocal microscopy (Fig. 7B). Thus, these data confirm the statement that *B. afzelii* CB43 spirochetes migrate to the host yet during the 1st day of the tick feeding.

Table V: Nested PCR analysis of the presence of *Borrelia* spirochetes in murine skin immediately after ticks were removed.

Duration of tick feeding (hours)	**24**	**48**	**72**
no. of *B. afzelii* CB43 positive mice / no. of mice exposed	9/10 (90%)	10/10 (100%)	10/10 (100%)

A **B**

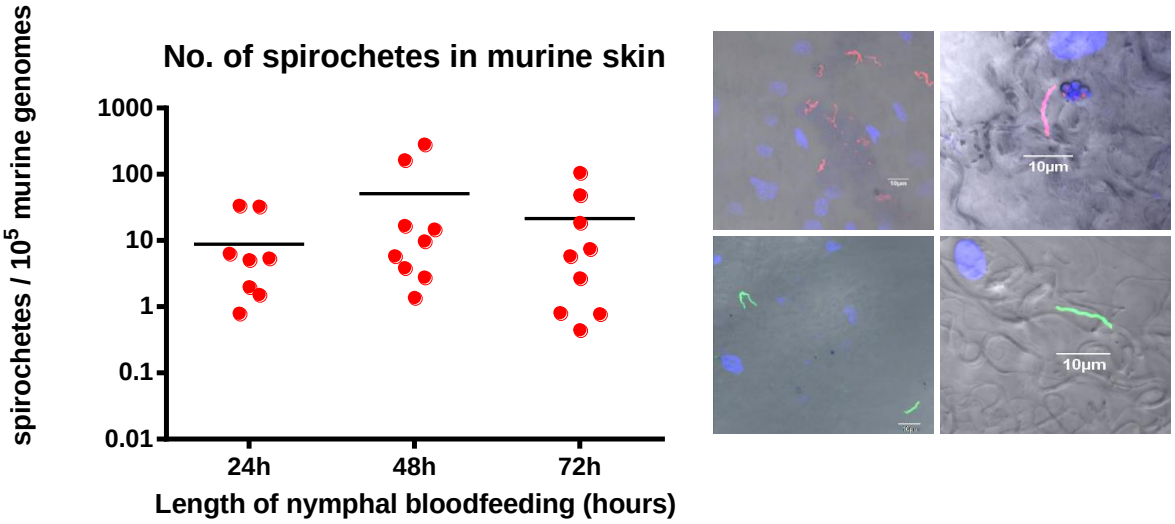

Figure 7: **A** qPCR detection of *Borrelia* spirochetes in murine skin immediately after ticks were removed. Horizontal bars indicate a mean number of spirochetes in the tick bite site at each timepoint. Differences in quantity of spirochetes in skin samples at defined time intervals are insignificant ($P<0.05$). **B** Presence of *B. afzelii* CB43 spirochetes in the murine skin at time interval of 24 hours visualized by confocal microscopy.

4.3. Infectivity of *B. afzelii* CB43 in different phases of tick engorgement

To reveal infectivity of *B. afzelii* CB43 during different phases of nymphal tick feeding, guts containing *B. afzelii* CB43 spirochetes were dissected from unfed, 24 hours fed, 48 hours fed and 72 hours fed *I. ricinus* nymphs and subsequently injected into C3H/HeN mice. *B. afzelii* CB43 spirochetes from guts of unfed nymphs were not infectious to mice, however, spirochetes derived from guts of infectious nymphs attached for 24 h, infected 3/5 of inoculated mice. Furthermore, all mice become infected after an injection of spirochetes derived from guts of 48 hours fed nymphs. In contrast, mice inoculated with spirochetes from guts of 72h fed nymphs established *B. afzelii* infection only in 1/5 mice (Tab VI), (Fig. 8). Nevertheless, this finding correlates with the statement, that during the tick blood intake almost all spirochetes migrate to the host (Fig. 5). Thus, after the tick engorgement, the tick gut remains relatively uninfectious.

Table VI: PCR analysis of infectivity of *B. afzelii* CB43 in different phases of tick engorgement.

Duration of tick feeding (hours)	**0**	**24**	**48**	**72**
no. of mice *B. afzelii* CB43 infected / no. of mice inoculated	0/5 (0%)	3/5 (60%)	5/5 (100%)	1/5 (20%)

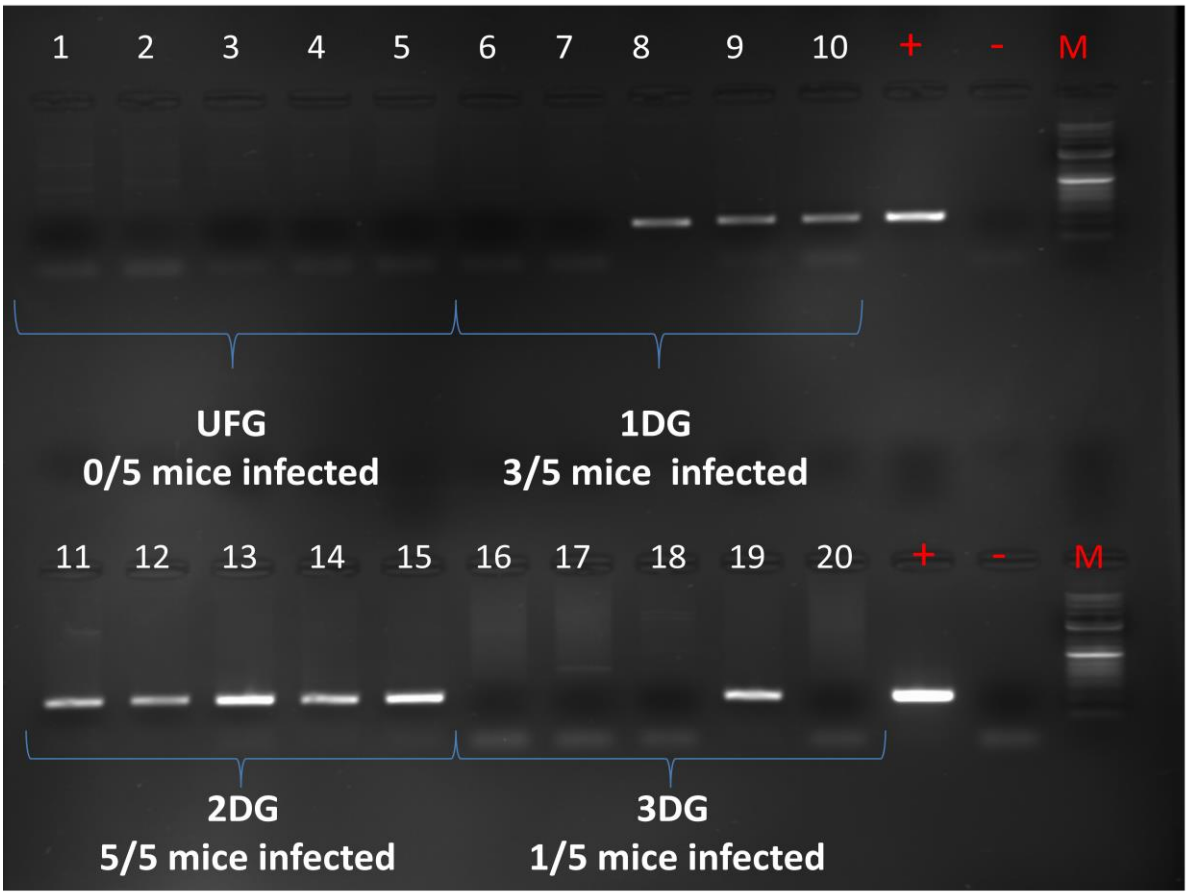

Figure 8: PCR analysis of infectivity of *B. afzelii* CB43 in different phases of tick engorgement. (1-20) samples, (+) positive control, (-) negative control, (M) marker, (UFG) mice inoculated with *B. afzelii* CB43 guts dissected from unfed *I. ricinus* nymphs, (1DG-3DG) mice inoculated with *B. afzelii* CB43 guts dissected from *I. ricinus* nymphs at the 1st - 3rd day of blood intake.

4.4. The effect of tick saliva on the *Borrelia* survival in mice

Previous results show that *B. afzelii* CB43 spirochetes enter vertebrate host in early stages of tick feeding but are not able to develop a permanent infection when the tick is not present. These findings indicate an important role of the tick for a successful dissemination and survival of spirochetes within the host body. To confirm the crucial role of the tick and its molecules for *B. afzelii* CB43 survival in mice, following experiments were performed.

4.4.1. Preparation of labeled tick nymphs

When investigating the effect of tick saliva on the survival of *Borrelia* spirochetes in mice, to be capable of distinguishing among uninfected, *B. afzelii* CB43 and *B. burgdorferi* sensu stricto SLV-2 infected ticks, the nymphs were marked with different colours (edding 780, edding, Japan), prior to infestation of mice. It was crucial to ensure the label covers just the dorsal scutum, and do not sticks the whole nymphal body or/and essential organs like the hypostome which would rather prevent the nymph from successful blood feeding (Fig. 9).

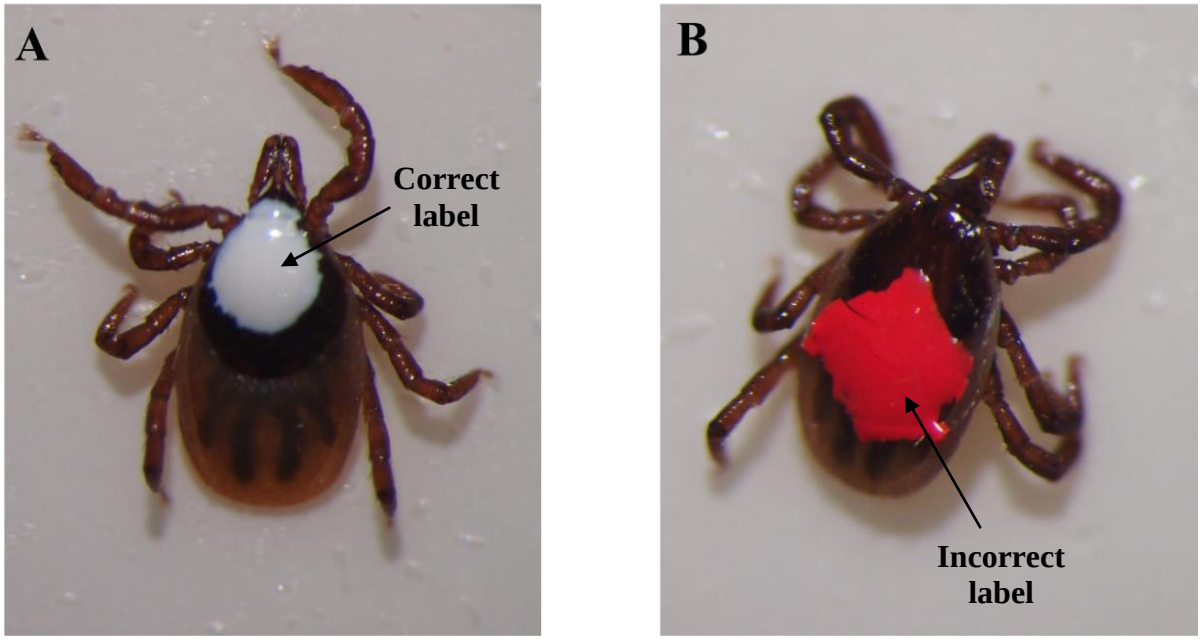

Figure 9: Different-colour labeling of tick nymphs. **A** a correct label covering only the dorsal scutum. **B** an incorrect mark besides the scutum, covers the dorsum, and so may prevent the enlargement of the nymphal body during blood engorgement (or else, the mark might be lost during blood engorgement). Such a nymph was excluded from the experiment prior to infestation of mice.

4.4.2. The effect of the saliva of uninfected ticks

To determine whether the saliva of uninfected tick nymphs has an effect on the *B. afzelii* CB43 survival in the host, different-colour labeled (described above) uninfected and *B. afzelii* CB43 infected nymphs cofed at the same feeding site for 24 hours. To assure, that the tick bite site is modulated by the tick saliva, supportive, uninfected ticks were attached to the host one day prior to infestation of *B. afzelii* CB43 infected ticks. Subsequently, after 24 hours of cofeeding, *B. afzelii* CB43 nymphs were removed and supportive uninfected nymphs were allowed to feed until repletion, providing *B. afzelii* CB43 in the murine skin a source of saliva.

As mentioned before, 24 hours is sufficient time for *B. afzelii* CB43 spirochetes to be transmitted to the host, but not for establishing a permanent infection (Tab. VI-V), (Fig. 5-7). Four weeks after the tick removal, ear, heart, and urinary bladder biopsies were performed. However, no infection was detected in mice (Tab. VII), (Fig 10).

In the control group, labeled infectious *B. afzelii* CB43 ticks were fed for 24 hours without any support of cofeeding uninfected ticks. As well as in the control group, no mice showed a positive biopsy four weeks after the tick detachment (Tab. VII), which corresponds with the previous data (Tab. IV), (Fig. 6).

4.4.3. The effect of the saliva of infected ticks

Considering the fact, that the gene expression of infected and uninfected ticks differs (Ribeiro et al. 2006; Hajdusek et al. 2013), in the next experiment, as an alternative to uninfected supportive ticks, we used *B. burgdorferi* sensu stricto SLV-2 infected nymphs instead. After 4 weeks, all mice in the experimental group were *B. burgdorferi* positive. Nonetheless, any mice were positive for the examined *B. afzelii* CB43 spirochetes (Tab. VII), (Fig 10).

In this experiment, the control group of mice was infested with both, *B. burgdorferi*, and *B. afzelii* CB43 infected ticks. All ticks were fed until repletion, which resulted in coinfection with both spirochetal strains in 2/5 mice. 3 mice were infected only with *B. afzelii* CB34 strain (Tab. VII).

To conclude, the interpretation of results of the influence of the tick saliva is in both cases quite similar. The presence of neither uninfected, nor infected *B. burgdorferi* supportive ticks is sufficient for *B. afzelii* CB43 spirochetes (transmitted to the host the 1st day of the tick feeding) to survive and develop chronic infection in mice (Tab VII), (Fig 10).

Table VII: Neither the uninfected ticks, nor the *B. burgdorferi* sensu stricto SLV-2 ticks support *B. afzelii* CB43 spirochetes (transmitted to the host the 1st day of the tick feeding) to develop a permanent infection in mice.

Experimental group	**No. of mice exposed/ no. of mice infected**		
	Ba^+	**Bb^+**	**Ba^+Bb^+**
Ba-1D/clean-FF	0/3*	-	-
Ba-1D	0/4**	-	-
Ba-1D/Bb-FF	0/5	5/5	0/5
Ba-FF/Bb-FF	3/5	2/5	2/5

Ba-1D *B. afzelii* CB43 infected nymphs fed for 1 day, **clean-FF** uninfected nymphs fed until repletion, **Bb-FF** *B. burgdorferi* sensu stricto SLV-2 infected nymphs fed until repletion, **Ba-FF** *B. afzelii* CB43 infected nymphs fed until repletion, **Ba^+** *B. afzelii* CB43 infection, **Bb^+** *B. burgdorferi* sensu stricto SLV-2 infection.

* 2 mice excluded from analysis due to the unremoved *B. afzelii* CB43 nymph.

**1 mouse died during the experiment.

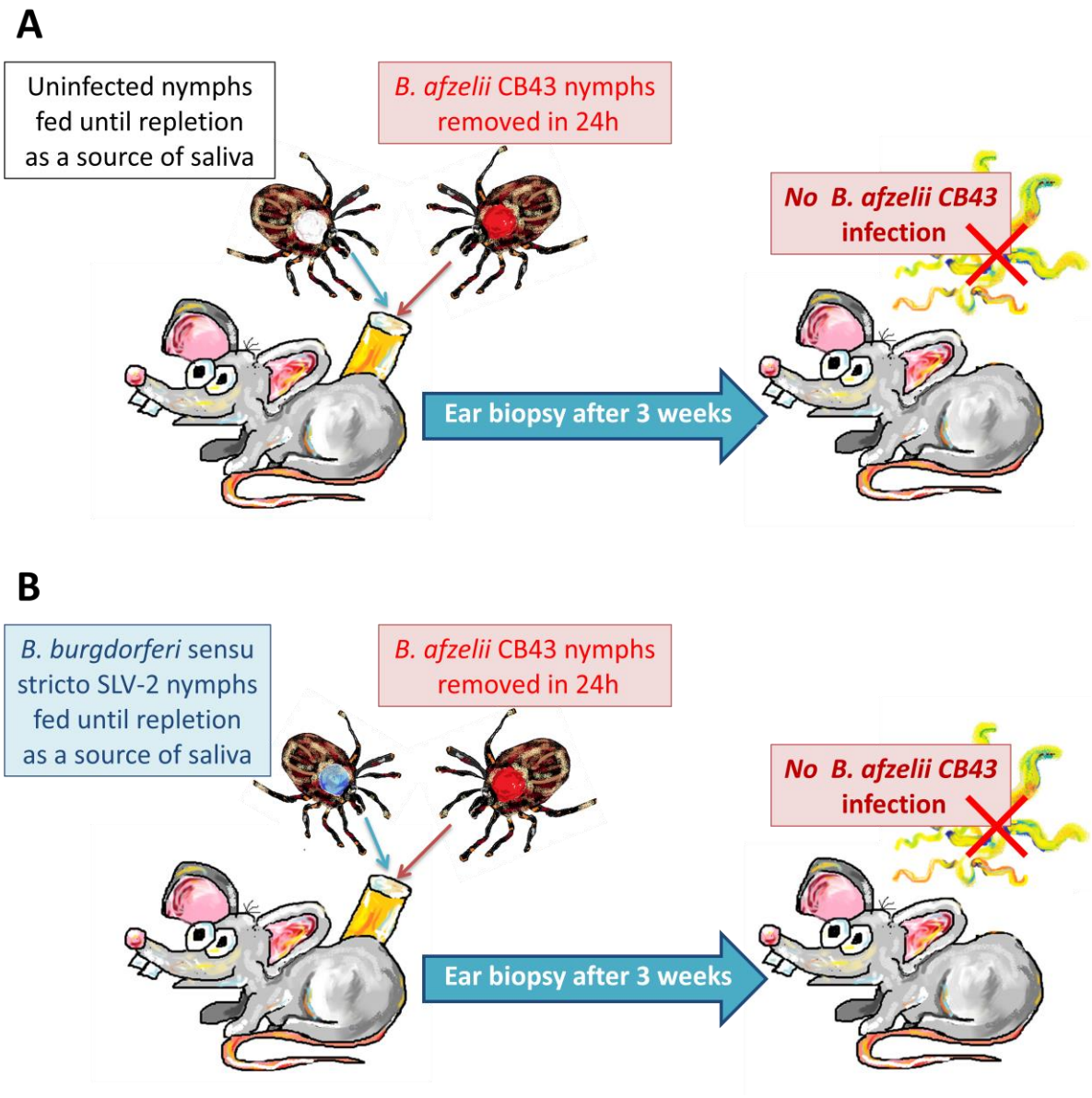

Figure 10: Neither the uninfected ticks **A**, nor the *B. burgdorferi* sensu stricto SLV-2 ticks **B** reinforce *B. afzelii* CB43 spirochetes (transmitted to the host the 1st day of the tick feeding) to develop a permanent infection in mouse.

4.5. *Borrelia* gene expression

Previous investigation did not confirm a crucial role of the tick and its molecules for *B. afzelii* CB43 spirochetes, transmitted to vertebrate host in early stages of tick feeding, to establish a permanent infection in mice (Tab VII), (Fig. 9). Nevertheless, differential infectivity of *B. afzelii* CB43 in different phases of tick engorgement (Tab VI), (Fig. 8) sheds a light on *Borrelia* itself.

Differential expression and modulation of surface antigens allows *Borrelia* to adapt specifically either to the tick or the host environment as required (Liang et al. 2002; Hefty et al. 2002). Therefore, to examine the connection between *B. afzelii* CB43 differential gene expression and its pathogenicity, following experiments were performed.

4.5.1. *ospA* expression

Gene encoding OspA, one of the major proteins in the borrelial outer membrane, was abundantly expressed in unfed ticks, downregulated during the tick blood-feeding, and hardly detectable in the warm-blooded mammalian host. Moreover, the expression of *ospA* might be regulated simply by temperature shift. With increasing temperature (resembling environmental conditions of the mammalian host) the expression of *ospA* in *B. afzelii* CB43 culture decreases (Fig. 11).

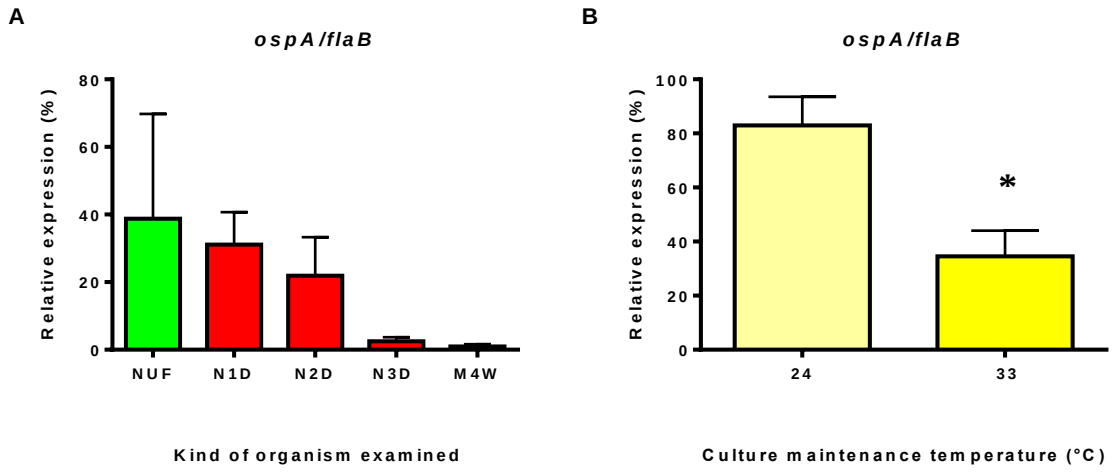

Figure 11: A *ospA/flaB* relative expression in different phases of the tick feeding, and during the chronic infection in mouse. (NUF) unfed *B. afzelii* CB43 infected nymph, (N1D-N3D) infected nymph detached from mouse at 1st-3rd day of the blood intake, (M4W) mouse with chronic *B. afzelii* CB43 infection, examined 4 weeks after detachment of infected ticks. Error bars indicate SEM. **B** Expression of *ospA* in *B. afzelii* CB43 culture maintained in different temperatures. *($P < 0.05$). Error bars indicate SEM.

4.5.2. *ospC* expression

OspC lipoprotein is supposed to be abundantly expressed on the bacterium's outer surface in vertebrate host. Neither in ticks, nor in *B. afzelii* CB43 culture, any *ospC* mRNA was detected. In contrast, we observed high levels of *ospC* mRNA in mice with permanent *B. afzelii* CB43 infection (Fig. 12).

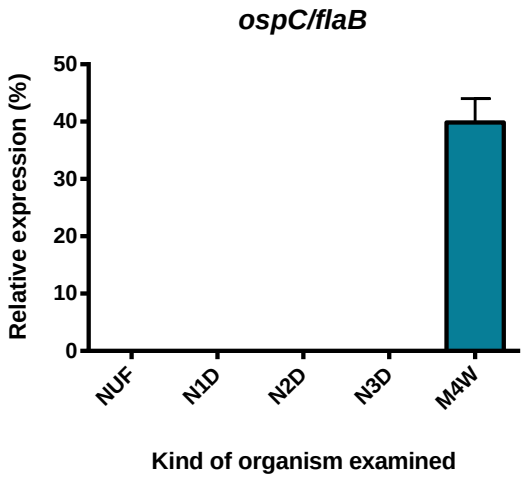

Figure 12: *ospC/flaB* relative expression in different phases of tick feeding, and during the chronic infection in mouse. (NUF) unfed infected *B. afzelii* CB43 nymph, (N1D-N3D) infected nymph detached from mouse at 1st-3rd day of the blood intake, (M4W) mouse with chronic *B. afzelii* CB43 infection, examined 4 weeks after detachment of infected ticks. Error bars indicate SEM.

4.5.3. *bbk32* expression

Besides binding the host extracellular ligand fibronectin, Bbk32 ability to inhibit the Classical Complement Pathway, contributes significantly to the pathogenesis of *B. burgdorferi* (Garcia et al. 2016). Even though, upregulation of *bbk*32 in *B. afzelii* CB43 is slightly induced yet during the tick feeding, afterwards, during the chronic mammalian infection stage, the expression is enhanced considerably more. Similarly to *ospA*, even *bbk32* gene expression might be modulated merely by treating the spirochetal culture in different temperatures. In contrast to *ospA*, the expression of *bbk*32 in *B. afzelii* CB43 culture is upregulated with increasing temperature (Fig. 13).

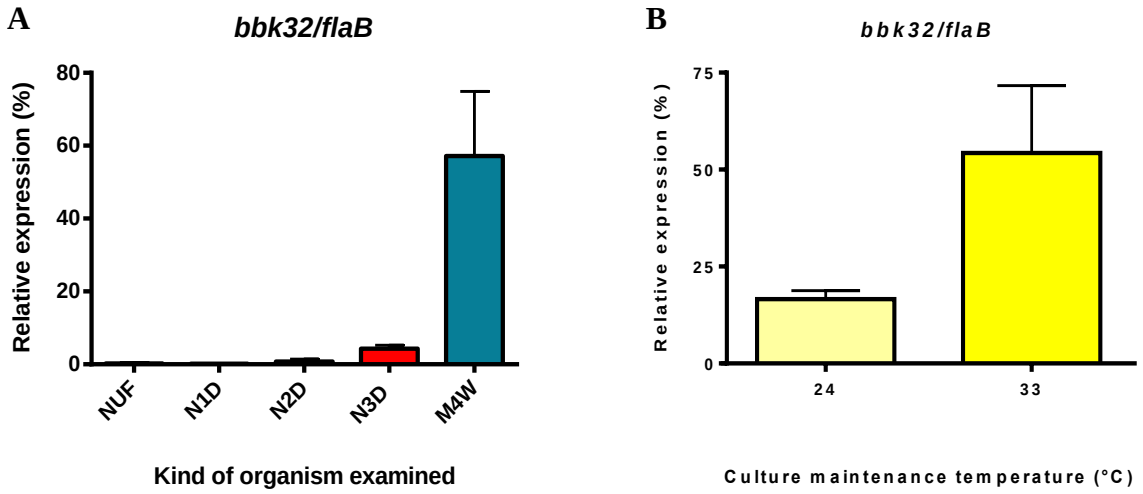

Figure 13: A *bbk*32*/flaB* relative expression in different phases of tick feeding, and during the chronic infection in mouse. (NUF) unfed infected *B. afzelii* CB43 nymph, (N1D-N3D) infected nymph detached from mouse at 1st-3rd day of the blood intake, (M4W) mouse with chronic *B. afzelii* CB43 infection, examined 4 weeks after detachment of infected ticks. Error bars indicate SEM. **B** Expression of *bbk32* in *B. afzelii* CB43 culture maintained in different temperatures. Error bars indicate SEM.

4.5.4. *dbpA* expression

Decorin binding protein A is an adhesin binding to decorin, which is a proteoglycan on the surface of human cells, expressed during the mammalian phase of infection. *B. afzelii* CB43 *dbpA* expression seems to be independent on temperature. Considerably higher *dbpA* expression rate was observed in the mammalian host, during the chronic infection stage, compared to the expression in ticks. However, *dbpA* expression levels do not show any significant trend neither in ticks, during different phases of the blood intake, nor in cultures treated in different temperatures (Fig. 14).

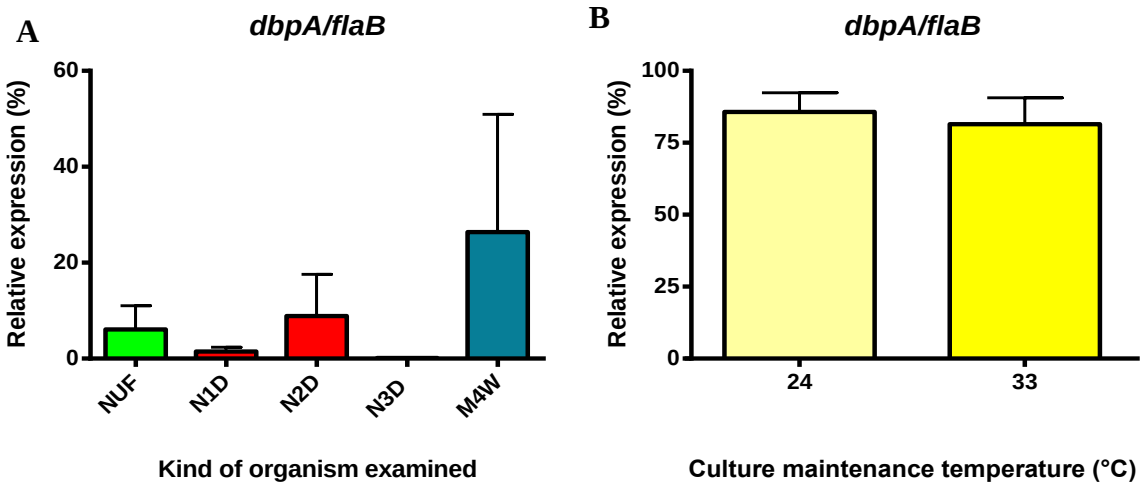

Figure 14: A *dbpA/flaB* relative expression in different phases of the tick feeding, and during the chronic infection in mouse. (NUF) unfed infected *B. afzelii* CB43 nymph, (N1D-N3D) infected nymph detached from mouse at 1st-3rd day of the blood intake, (M4W) mouse with chronic *B. afzelii* CB43 infection, examined 4 weeks after detachment of infected ticks. Error bars indicate SEM. **B** Expression of *dbpA* in *B. afzelii* CB43 culture maintained in different temperatures. Error bars indicate SEM.

5. Discussion

A mouse model for the Lyme disease together with modern tools of molecular biology enables us to study the transmission of *B. afzelii* CB43 via its vector *I. ricinus* in vivo, with high precision. Undoubtedly, the risk of acquiring the Lyme disease increases with the length of the tick attachment. However, it is hardly possible to establish the minimum tick attachment time for the *Borrelia* transmission, because the particular spirochetal transmission times and virulence depend on the tick and *Borrelia* species (Cook 2015). Nevertheless, one might expect life strategies of close related species would be comparable at least in general.

However, our data on the growth kinetics and transmission dynamics reveal an unforeseen behaviour of European *B. afzelii* CB43 in its vector *I. ricinus* and show rather too divergent trends, comparing to their long-term studied American cousins *B. burgdorferi* sensu stricto and *I. scapularis*. Furthermore, *Borrelia* differential gene expression during different phases of the tick feeding plays a crucial role in its pathogenesis. Actually, the synthesis of these findings sheds a light on the current inconsistencies and unanswered questions, relating to the transmission of the Lyme disease, from a different point of view. Eventually, regardless an indisputable fact that *Borrelia* spirochetes exploit the tick as its vector, our results query even an extent of the spirochetal dependence on the tick itself.

B. burgdorferi is maintained in nature through an enzootic cycle involving small vertebrates, primarily rodents and birds, and ticks of the genus *Ixodes* (Lane et al. 1991). As the transovarial transmission of *Borrelia* in ticks is rather not likely, the infection is acquired by larval or nymphal ticks feeding on an infected host. Absolute quantification of *B. afzelii* CB43 spirochetes at timepoints during the tick development shows, that in engorged, as well as molting larvae, spirochetes multiply within the *I. ricinus* gut quite rapidly, presumably until they have enough nutrients from host blood to live on. Afterwards, in molted nymph, the blood supplies within the tick gut dwindle, thus spirochetal proliferation decelerates, or even stops. Presumably, spirochetes enter a quiescent form “fall asleep”, just dwelling in the tick gut, saving energy and waiting for the opportunity to be transmitted further, when the next blood meal comes. However, our results do not correlate much with the data on the growth kinetics of *B. burgdorferi* in *I. scapularis* (formerly named as *I. dammini*), where numbers of spirochetes dropped yet during larval molting (Piesman et al. 1990).

As the tick nymph starts feeding and the warm host blood enters the tick gut, the pathogen senses it, “wakes up”, and migrates to the host. Importantly, according to our data, migration of *B. afzelii* CB43 spirochetes to the host starts immediately the 1st day of the

nymphal *I. ricinus* feeding. In fact, during nymphal *I. ricinus* blood feeding, *B. afzelii* CB43 spirochetes do not proliferate within the tick. Rationally, there is no reason for proliferation within the tick gut at all. The ultimate goal of *Borrelia* is to be spread further. For the spirochetes, the tick might practically serve as a "bus" to get on, be transmitted further, and get off. Actually, during the nymphal blood intake, almost all spirochetes migrate to the host.

However, in 1995, using confocal microscopy, De Silva and Fikrig described *B. burgdorferi* enormous proliferation within the tick during *I. scapularis* feeding and established a critical timepoint of spirochetal dissemination and infection of the salivary glands to 36-48 hours (De Silva & Fikrig 1995). In fact, up till now, these particular results are taken as a solid evidence in favour of the salivary route of *B. burgdorferi* transmission, the hypothesis previously suggested by (Ribeiro et al. 1987).

Indisputably, the risk of acquiring the Lyme disease increases with the length of the tick attachment. All mice exposed to the bite of *B. afzelii* CB43 infected *I. ricinus* nymphs for 24 hours remained uninfected, whereas 80% of mice exposed for 48 hours and 100% of mice exposed for 72 hours became infected These results show that 48 hours exposure to the *B. afzelii* CB43 infected tick is critical for the development of systemic infection in mice.

However, previous data on the kinetics of *B. afzelii* CB43 during the feeding of *I. ricinus* nymph revealed, that the migration of spirochetes to the host starts immediately the 1st day of the tick attachment. Likewise, murine skin biopsies taken from the tick bite sites immediately after the tick removal were PCR positive in 90%, 100%, and 100% of mice at time intervals of 24, 48 and 72 hours respectively. Moreover, there were not significant differences in quantity of spirochetes in skin samples at defined time intervals. Additionally, results were confirmed by confocal microscopy, spirochetes were clearly visible in all three time intervals.

Taken together, even though, *B. afzelii* CB43 spirochetes are transmitted to the host during the first day of the tick attachment, a removal of infected *I. ricinus* nymphs within 24 hours prevents from the infection of the host organism. Our investigation is indeed in concordance with the previous detection of noninfectious *B. afzelii*, and even *B. burgdorferi* spirochetes, transmitted by *I. ricinus* and *I. scapularis* respectively, in the host dermis in the early period (24 hours) of the tick blood feeding (Ohnishi et al. 2001; Hodzic et al. 2002; Crippa et al. 2002).

These findings might indicate an important role of the tick for a successful dissemination and survival of spirochetes within the host body. Tick saliva contains many biochemical substances (anticoagulants, cytokine inhibitors, histamine binding factors, and

complement inhibitors), which suppress or mediate the host sensibility to the tick bite (Nuttall & Labuda 2008; Hajdusek et al. 2013). It was also supposed, that *Borrelia* exploits tick salivary proteins in favour of its transmission to the host, since a ratio of many genes (tHRF, Salp15) expressed in the salivary glands and other organs of infected and uninfected ticks, differs a lot (Ribeiro et al. 2006; Hajdusek et al. 2013). Silencing of these genes using RNAi or their blocking by antibodies severely hamper tick feeding, and significantly reduces the spirochetal capability to infect mice (Dai et al. 2009; J Dai et al. 2010; Hajdusek et al. 2013). In summary, the substances present in the tick saliva facilitate a painless bite, hence plays an important role in the tick feeding, and therefore may be even a factor involved in the pathogen transmission as well.

To determine whether the tick saliva has an effect on the *B. afzelii* CB43 survival in the host, supportive either uninfected, or *B. burgdorferi* sensu stricto SLV-2 infected ticks, were cofed with *B. afzelii* CB43 infected nymphs at the same feeding site for 24 hours. Nevertheless, the presence of neither uninfected, nor *B. burgdorferi* sensu stricto SLV-2 infected supportive ticks is sufficient for *B. afzelii* CB43 spirochetes (transmitted to the host the 1st day of the tick feeding) to survive and develop chronic infection in mouse. Hence, the crucial role of the tick and its (salivary) molecules for the survival of *B. afzelii* CB43 spirochetes transmitted to vertebrate host in early stages of tick feeding was not supported.

To get to the point and puzzle out the obscure feature, why are the spirochetes transmitted to the host during the first day of the tick attachment noninfectious, we need to picture the scene from the *Borrelia* point of view. We have to focus on the specific tools the pathogen possesses in its toolkit to fight against its enemy, the host immunity.

The differential expression of surface antigens, allowing *Borrelia* to adapt specifically to the tick or the host environment as required, might represent the pathogen's arsenal that stands behind, and generates the pathogen's insidious strategy full of twists and turns. It is controlled by the Rrp2- RpoN/RpoS alternative sigma factor cascade (Liang et al. 2002; Caimano et al. 2007) which is activated by nymphal blood feeding. As the tick nymph starts feeding and the warm blood enters the tick gut, spirochetes downregulate *ospA*, (essential for spirochetal survival within the tick). Downregulation of *ospA* results in a reciprocal activation of the Rrp2-RpoN-RpoS pathway (He et al. 2008), that subsequently activates transcription of *ospC, dbpA, bbk32* and other genes that play a part in *Borrelia* transmission to the host (Schwan et al. 1995; Hübner et al. 2001; Caimano et al. 2007; He et al. 2008). Alteration of surface antigens, particularly rapid *ospC* upregulation, seems to be crucial in early phases of *Borrelia* infectivity, and spreading (Liang 2002). *ospC* deletion mutants are

not infectious for mice (Grimm et al. 2004; Tilly et al. 2006). The conversion between OspA/OspC occurs during the blood intake (36-48 hours) within the tick midgut (Caimano et al. 2007). Correspondingly, *ospA* transcription in the host tissue was detected transiently only during the first day of the tick feeding, not afterwards, in contrast to *ospC* RNA, which was detected after 3 days but not earlier (Hodzic et al. 2002). The guts from partially engorged ticks are infectious to mammals, whereas infected guts from unfed ticks are not (Piesman 1993; Crippa et al. 2002). Likewise, *B. afzelii* CB43 infected guts dissected from *I. ricinus* nymphs in different phases of the blood engorgement (0, 24, 48 and 72 hours) developed infection in 0%, 60%, 100%, and 25% of inoculated mice respectively.

Thus, differential pathogenesis of *B. afzelii* CB43 in different phases of the tick engorgement is caused by *B. afzelii* CB43 gene expression shift in the midgut of feeding *I. ricinus* nymph. The evidence, that during the first 24 hours of the *I. ricinus* feeding, *B. afzelii* CB43 is spread to the host in its harmless form and even dissected guts from unfed, infected nymphs, are not infectious when injected to mice, evokes a potential natural way of acquiring protective immunity, at least against the particular strain. However, these assumptions need further investigation, because spirochetal antigenic variability, different transmission times, and virulence, depending on the tick and *Borrelia* species, must be considered (Cook 2015). The permanent infection is enabled later on. In case of *B. afzelii* CB43, it takes about 24-48 hours of the tick feeding to shed its surface antigens to become invisible for the host immune system. Additionally, the fact that mice inoculated with infected guts dissected from fully engorged nymphs established *B. afzelii* infection only in 1/5 mice correlates with the statement, that during the tick blood intake almost all spirochetes migrate to the host. Thus, after the tick engorgement, the tick gut remains relatively noninfectious.

To sum up, at the particular moment, when the tick starts feeding on the warm host blood, the pathogen within the tick gut senses it, "wakes up" and migrates from the tick gut to the host. However, to be able to establish a chronic infection, it is essential to travel incognito. At the early period of transmission (24 hours), spirochetes are unable to develop a chronic infection in the host, since they do not put on "an invisible coat" working as a protective shield against the host immunity yet. Hence, we assume the central core of the pathogen strategy is to spread insidiously. Simply put, it is crucial to remove an infected tick soon enough before *Borrelia* wakes up and become infectious and invisible, so the host immune system is able to recognize it, destroy it, and perhaps, even remember it, to be protected when facing another *Borrelia* challenge in future.

Furthermore, there are studies confirming, that the activation of the Rrp2-RpoN/RpoS regulatory system and related gene expression might be stimulated principally by treating spirochetal cultures under the conditions, which mimic either the tick or the mammalian host environment (Schwan et al. 1995; Carroll et al. 2000; Hübner et al. 2001; Caimano et al. 2007; Garcia et al. 2016).

Correspondingly, cultures of *B. afzelii* CB43 spirochetes maintained at 33°C (imitating the conditions in the mammalian host), upregulate the expression of *bbk32*, acting as an specific inhibitor of the classical pathway of human complement (Garcia et al. 2016), and downregulate the expression of *ospA*, which is essential for the *Borrelia* survival within the tick gut. In contrast, treating *B. afzelii* CB43 culture at 24°C (resembling the environment within the tick vector), causes upregulation of *ospA*, and downregulation of *bbk32*. Expression rates of *dbpA* seem to be independent on temperature. Surprisingly, expression of *ospC* was not detected in *B. afzelii* CB43 culture at all, which might indicate that spirochetes require a specific stimulus, perhaps an immunological factor present in the host blood.

Eventually, the reality, that *Borrelia*, unlike its close relative *Treponema pallidum* causing syphilis (Porcella & Schwan 2001), may be cultured easily in a medium in vitro and afterwards develop infection, when inoculated to mice, provides another evidence on the spirochetal independence on the tick saliva, if not virtually the whole tick itself.

Apparently, our data on *B. afzelii* CB43 and *I. ricinus* transmission dynamics diverge greatly from the previous findings, and do not favour the salivary route of *B. burgdorferi* transmission, originally suggested in 1987 (Ribeiro et al. 1987) and supported by confocal fluorescent microscopy analysis in 1995 (De Silva & Fikrig 1995), when modern tools of molecular biology were unavailable. Obviously, our results do not fit into nowadays generally accepted hypothesis of the salivary route of *B. burgdorferi* transmission. Our findings rather indicate a possibility of regurgitation or direct migration of spirochetes from the tick gut to the host as was originally suggested by Willy Burgdorfer (Burgdorfer). Regurgitation of the gut content was indeed demonstrated on the ticks *Amblyomma americanum* and *Ornithodoros moubata* (Brown 1988; Connat 1991).

In actual fact, mainly in engorged ticks, the gut can be really easily penetrated during tick dissection, and so simple contamination of salivary glands with leaked gut content might cause the false detection of spirochetes in the salivary glands. Likewise homogenates derived from *B. burgdorferi* infectious salivary glands of *I. scapularis* ticks did not develop infection in mice, unless gathered from ticks feeding for ≥60 hours (Piesman 1995). Therefore, we made no effort to conduct any transmission experiments with salivary glands, because we

would have no trust in such a data anyway. To reveal the truth, whether such a big disparity in the behaviour of the close related species is likely, further research using modern tools of molecular biology is required.

Our research on the tick - pathogen interface raised many unanswered questions that are of great importance and need further investigation. To analyze whether *B. afzelii* CB43 spirochetes transmitted to the host in the first 24 hours of tick feeding provide protective immunity, at least against the particular spirochetal strain, following experiments will be performed.

Firstly, to find out whether mice develop antibody response against spirochetes present in the skin during first 24 hours of tick feeding, *B. afzelii* CB43 infected *I. ricinus* nymphs will be fed on naive mice and removed at time interval of 24 hours. Blood serum will be collected for subsequent ELISA detection of antibodies against *B. afzelii* CB43. If mice develop antibody response, this might be one of the explanations of false positive ELISA results in patients who never contracted Lyme disease (but most likely had several tick bites during their life).

Secondly, naive mice will be either inoculated with dissected guts from unfed *B. afzelii* CB43 infected nymphs or "immunized" by feeding of *B. afzelii* CB43 infected *I. ricinus* nymphs for 24 hours. Subsequently *B. afzelii* CB43 infected *I. ricinus* nymphs will be fed on such as immunized mice until repletion. Eventually, *B. afzelii* CB43 infection will be examined.

Intended research should contribute to defining new strategies for tick and tick-borne pathogens control.

6. Conclusion

Investigation on the growth kinetics of *B. afzelii* CB43 in molting and feeding *I. ricinus* revealed surprising data. Primarily, *B. afzelii* CB43 spirochetes do not proliferate within the tick gut during the nymphal *I. ricinus* blood feeding. Our results show not only divergent, but sometimes even totally opposite trends compared to their long-term studied American cousins *B. burgdorferi* sensu stricto and *I. scapularis*. Thus, our data does not favor the renowned and nowadays generally accepted hypothesis of the salivary route of *B. burgdorferi* transmission. In fact, our findings coincide with an underestimated alternative way of *Borrelia* transmission, called regurgitation (a direct migration of spirochetes from the tick gut to the host).

Consequently, the revision of *B. afzelii* CB43 transmission cycle confirmed, that the risk of acquiring the Lyme disease increases with the length of the tick attachment. 48 hours exposure to the *B. afzelii* CB43 infected tick is critical for the development of systemic infection in mice. Even though spirochetes are transmitted to the host during the first day of tick attachment, the removal of infected *I. ricinus* nymphs within 24 hours prevents from the infection of the host organism. These findings suggest a potential natural way of acquiring protective immunity, at least against the particular spirochetal strain. Hence, these finding will be investigated further.

Additionally, the crucial role of the tick and its (salivary) molecules for the survival of *B. afzelii* CB43 spirochetes transmitted to vertebrate host in early stages of tick feeding was not supported. Moreover, it was found, that he differential expression of *B. afzelii* CB43 surface antigens, which causes the differential pathogenesis of *B. afzelii* CB43 in different phases of the tick engorgement, can be actually stimulated even in vitro, by treating spirochetal cultures at different temperatures. Eventually, this finding supports the evidence of the spirochetal independence on the tick saliva, possibly on the whole tick at all, unless in nature, where the tick mediates the pathogen transport to another host.

7. References

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