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# Immunoecology of a Rodent Reservoir Host of the Lyme Disease Pathogen:

## The bank vole – *Borrelia afzelii* model

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# "Immunoecology of a Rodent Reservoir Host of the Lyme Disease Pathogen: The bank vole - *Borrelia afzelii* model"

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**Summary**

The Lyme disease is the most common vector-borne disease in the Northern Hemisphere. The etiological agents of Lyme disease are the spirochete bacteria of the species complex of *Borrelia burgdorferi* sensu lato. *B. burgdorferi*'s biology is closely linked to its host's immune response. The host immune response has been found to play a critical role in the recognition and control of *B. burgdorferi*. In response, *B. burgdorferi* has evolved multiple strategies to cope the response including immunesuppression and immune evasion. Natural reservoir hosts show clear differences in infection phenotype compared to laboratory models. Wild rodents are non-model organisms that are an example of this difference. The bank vole (*Myodes glareolus*) is a small rodent commonly found across Europe and one of the most important reservoir hosts of *B. burgdorferi* sl. The general aim of this dissertation was to test different hypotheses of the ecology and immunology of one relevant reservoir host on the Lyme disease pathogen. All the studies of this dissertation used the European Lyme disease system: the spirochete bacterium *B. afzelii,* the tick *Ixodes ricinus*, and the rodent host *M. glareolus*. This dissertation is composed of three chapters that study the effect of three different immune mechanisms on the ability of *B. afzelii* to establish infection in the rodent host and to transmit from the rodent host to the tick vector. In the first chapter, we investigated the effect of innate immune response on the pathogen by testing whether genetic variation (TLR2 gene) at an important innate immune receptor in mammals confers resistance to the pathogen in bank voles. The susceptibility to infection with *B. afzelii* following an infected tick bite was very high (95%) and did not differ between TLR2 genotypes. Therefore, we did not find that the TLR2 polymorphism in bank voles influenced variation in the susceptibility to *B. afzelii* infection under the laboratory conditions. In the second chapter, we aimed to understand the interactions between the pathogen and the acquired immune system in the rodent host by testing whether *B. afzelii* could inhibit the development of acquired anti-tick immunity in bank voles. The anti-tick immunity had negative effects on tick fitness traits. Infection with *B. afzelii* did not suppress the development of acquired immunity against *I. ricinus* ticks. The development of anti-tick immunity was strongly correlated with a dramatic temporal decline in both the bacterial abundance in the host ear tissues and the host-tick transmission success of *B. afzelii*. Our study suggests that the development of anti-tick immunity in bank voles has important consequences for the density of infected ticks and the risk of Lyme borreliosis. Finally, in the third chapter, we aimed to investigate the effect of passive immunity transmitted by mothers to their offspring on the pathogen. Our study showed that maternal antibodies provide strain-specific protection against *B. afzelii*, which could mean important consequences for the epidemiology of multiple-strain pathogens in nature.

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# General Introduction

## Immune Response

The immune system is a remarkably adaptive host defense system that has evolved different structures and processes to protect an organism against invading pathogens. In vertebrate animals, the immune system can be broadly divided in two categories known as the 'innate system' and the 'acquired system' (Owen, Punt, & Stranford, 2013). The innate immune system provides the first response against any pathogen, it is fast but non-specific. The acquired immune system takes more time to develop an immune response against the pathogen, but this response is highly specific and the development of long-lived memory cells allows a much faster response upon re-exposure to the same pathogen (Owen, Punt, & Stranford, 2013). The effects of the immune response on the epidemiology of pathogens and infectious diseases are included in the term 'immuno-epidemiology'. The immune response can act on the pathogens by different mechanisms such as resistance to infection, clearance or reduction of the pathogen's fitness (Schmid Hempel, 2011). Therefore, the two types of responses can have different effects on the ecology and epidemiology of the pathogen (Schmid Hempel, 2011). The complex responses from the immune system arise from pathogens being a phylogenetically and antigenically diverse group of organisms that interact at various levels with the host. For this reason, predicting the evolutionary potential of natural populations in response to pathogens requires the understanding of pathogenesis and immunity (Acevedo-Whitehouse & Cunningham, 2006).

## Lyme Disease and its etiological agent *Borrelia burgforferi* sensu lato

Lyme disease or Lyme borreliosis was first recognized in 1976 by the medical doctor Allen Steere and his colleagues (Steere et al., 1977). He linked the symptoms of previously described tick-transmitted disease with a cluster of children suffering from rheumatoid arthritis in Lyme, Connecticut State (Steere, Coburn, & Glickstein, 2004). The etiological agent of Lyme disease was identified in 1981 and this spirochete bacterium was named *Borrelia burgdorferi* after its co-discoverer, the medical doctor Willy Burgdorfer (Burgdorfer et al., 1982).

In humans, the first clinical manifestations of Lyme disease appear after an incubation period that ranges from a few days to one month (Steere & Sikand, 2003). A pathagnomonic skin rash called erythema migrans is formed at the site of the tick (shown in Figure 1). This manifestation appears in 70-80% of Lyme disease patients (Steere et al., 2004), and is often accompanied by flu-like symptoms like malaise, fatigue, and fever (Steere et al., 1983). The infection subsequently disseminates from the skin to other organs including the heart, brain and joints. In disseminated Lyme disease, the patient develops more complicated pathologies such as arthritis, neurological manifestations and skin manifestations (Duray & Steere, 1988). If left untreated, the pathogen establishes a chronicnfection and can persist in the human body for years (Steere et al., 2004).

Lyme disease is the most common vector-borne disease in the Northern Hemisphere (Tsao, 2009). In Europe, about 65,500 cases are reported annually (Rizzoli et al., 2011), with large differences in the case load between countries (Lindgren, Jaenson, Menne, & Organization, 2006). In the United States, about 30,000 cases are reported annually, but this

caseload is probably an underestimate because many cases are undiagnosed (Schwartz, 2017). The Center for Disease Control (CDC) recently suggested that the actual annual case load in the United States may exceed 300,000 (Kuehn, 2013). In North China, about 30,000 cases are reported annually (Y. Wang, Li, Wang, Hu, & Lu, 2011). Lyme disease is an emerging tick-borne disease with a continuous increase in geographic distribution and disease incidence in human populations (Mead, 2015; Sykes & Makiello, 2016).

The etiological agents of Lyme disease (LD) are the spirochete bacteria of the species complex of *Borrelia burgdorferi* sensu lato (s.l.). To date, the complex of bacteria includes 20 species, which are not all pathogenic to humans (G Stanek & Reiter, 2011). In Europe, at least five species can cause LD: *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto (s.s.), *B. bavariensis* and *B. spielmanii* (Rizzoli et al., 2011). In North America, *B. burgdorferi* s.s. is the only species known to cause LD. The species also differ in the disease they cause in humans. *B. burgdorferi* s.s. is associated with arthritis, *B. garinii* with neurological problems, and *B. afzelii* with skin problems (Gerold Stanek & Strle, 2008).

The spirochete bacteria from the genus *Borrelia* have one of the most complex genomes of all known bacteria (Fraser et al., 1997). The genome shows the largest number of plasmids known for any bacteria (S. R. Casjens et al., 2012; Singh & Girschick, 2004). It consists of a linear chromosome that is ~910 kb and a high number (twenty-one) of circular and linear plasmids that range in size from 9 to 62 kb (S. Casjens et al., 2000; S. R. Casjens et al., 2012; Fraser et al., 1997). The chromosome has most of the housekeeping genes (S. Casjens, Eggers, & Schwartz, 2010), whereas the plasmids carry the critical virulence genes that encode numerous lipoproteins that are essential for infecting the vertebrate host and the tick vector (Brooks, Vuppala, Jett, & Akins, 2006). Some of the plasmids are not essential for the life cycle and the number of plasmids can differ dramatically between species (S. Casjens et al., 2010).
During its enzootic life cycle, *B. burgdorferi* cycles between a tick vector and a vertebrate reservoir host (Figure 1). To establish infection and persist in these two very different environments, the pathogen expresses a different set of genes depending on whether it is in the tick vector or the vertebrate host (Hovius, van Dam, & Fikrig, 2007; Singh & Girschick, 2004). One of the best-known examples of differential gene expression between the tick vector and the vertebrate host are the genes encoding for a family of outer surface lipoproteins (Osps) (Tilly, Rosa, & Stewart, 2008). In the midgut of unfed nymphal and adult ticks, the spirochetes express outer surface protein A (OspA) (Barbour, Tessier, & Todd, 1983). OspA plays a critical role in the colonization of the spirochetes to the tick midgut and their persistence (Pal et al., 2004; Yang, Pal, Alani, Fikrig, & Norgard, 2004). Once the tick attaches to a host and starts blood feeding, the spirochetes multiply, down-regulate OspA, up-regulate the outer surface lipoprotein C (OspC), and migrate from the midgut to the salivary glands (De Silva & Fikrig, 1995; Schwan, Piesman, Golde, Dolan, & Rosa, 1995). OspC plays several critical roles during the invasion of the spirochetes to the tick salivary glands, the establishment of the infection and its dissemination (Grimm et al., 2004; Stewart et al., 2006; Tilly, Bestor, Jewett, & Rosa, 2007; Tilly et al., 2006). During the early stages of the infection in the vertebrate host, the spirochetes express OspC and not OspA (De Silva & Fikrig, 1997; Leuba-Garcia, Martinet, & Gern, 1998). The spirochetes reduce their expression of the OspC antigen during later stages of the infection in the vertebrate host (Liang, Yan, et al., 2004).

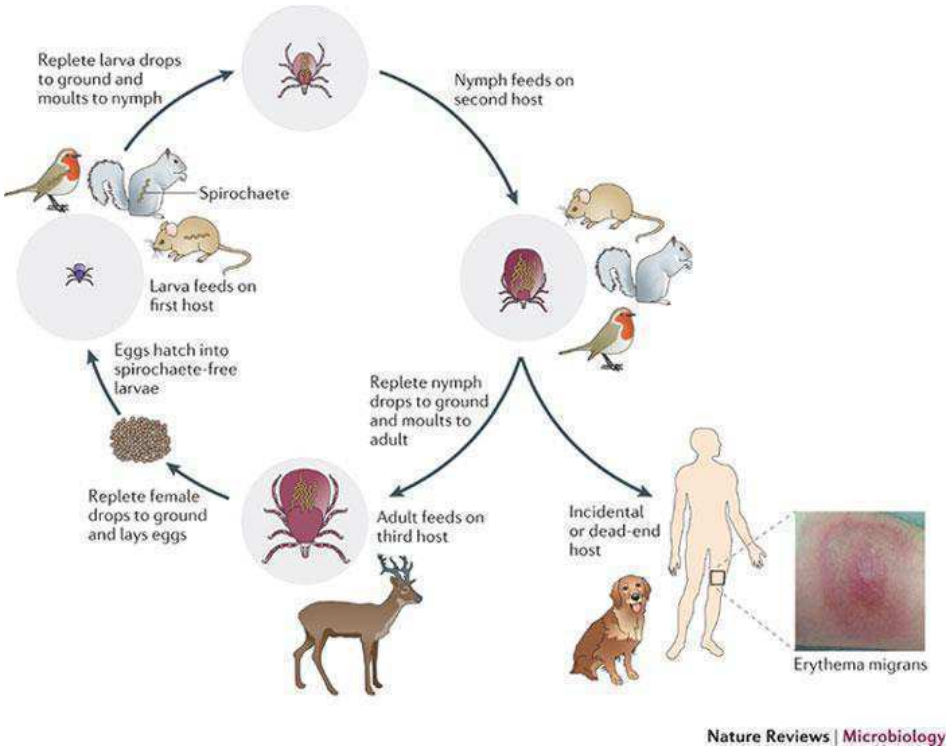


Figure 1. Enzootic life cycle of *B. burgdorferi* s.l.. Reviewed in: Radolf, Caimano, Stevenson, and Hu (2012).

*Borrelia burgdorferi* has evolved multiple strategies to cope with the host immune response. Numerous studies have characterized the detailed strategies that *B. burgdorferi* shows in the course of the infection, i.e. virulence factors, immune suppression and immune evasion (reviewed in: Singh & Girschick, 2004). I will briefly summarize some of the most important adaptations that allow *B. burgdorferi* to evade the innate and adaptive arms of the host immune system during the different stages of the infectious cycle in the vertebrate host.

During the tick-to-host transmission step, the spirochete encounters the proteins of the complement system of the vertebrate host. The complement system is part of the innate immune system of the vertebrate host which plays a key role in the clearance of pathogens. It consists of a group of proteins dissolved in the blood of the vertebrate host including serum proteins and cell membrane receptors (Kindt, Goldsby, Osborne, & Kuby, 2007). The activated complement system stimulates phagocytes to clear unknown or damaged cells. Complement regulatory proteins are present on the self-cells membrane to avoid being target by the complement system (Kindt et al., 2007). *Borrelia burgdorferi* expresses a variety of outer surface proteins called complement regulator-acquiring surface proteins (CRASPs) that allow the pathogen to evade the complement system (Kraiczy et al., 2004). These outer surface proteins bind to complement regulatory proteins and inhibit lysis by the membrane attack complex (Alitalo et al., 2001; Stevenson, El-Hage, Hines, Miller, & Babb, 2002).

Infection with *B. burgdorferi* starts after the tick deposits the spirochete into the skin of the vertebrate host (Hanson et al., 1998). At early and late stages of the infection, *B. burgdorferi*

is associated with the host's connective tissue. It uses surface adhesins (i.e. decorin-binding proteins) to adhere to and colonize the components of the extra-cellular matrix of the connective tissue (Guo, Norris, Rosenberg, & Höök, 1995). Spirochetes have been detected in bradythropic tissues like ligaments and collagen tissue (Häupl et al., 1993; Pachner, Basta, Delaney, & Hulinska, 1995). Tissue localization is a strategy to escape the host immune system and avoid immune clearance in sites that have less immune surveillance (Singh & Girschick, 2004).

In the weeks following infection by *B. burgdorferi*, the adaptive branch of the immune system of the vertebrate host develops a strong antibody response. To establish a chronic infection in the vertebrate host, the pathogen must avoid being cleared by this highly specific antibody response. Avoiding antibody-mediated clearance is probably why *B. burgdorferi* decreases expression of OspC in the vertebrate host (Liang, Jacobs, Bowers, & Philipp, 2002; Liang, Yan, et al., 2004). During chronic infections, most of the host immune response is directed at a surface lipoprotein known as VlsE (variable major protein-like sequence expressed). The pathogen uses antigenic variation at VlsE to avoid antibody-mediated clearance and to persist in the vertebrate host (Zhang, Hardham, Barbour, & Norris, 1997). VlsE is expressed at the *vlsE* gene that is flanked by a cassette of 15 *vls*-like pseudo-genes (Zhang et al., 1997). During the course of infection, recombination of the pseudo-genes in the cassettes produces new antigenic variants in the *vlsE* expression site. Recombination allows the spirochetes to escape the antibody-mediated recognition of previously encountered VlsE proteins (Graves, Ros, Stevenson, Sniegowski, & Brisson, 2013; Singh & Girschick, 2004).

Like other vector-borne pathogens, *B. burgdorferi* consist of multiple strains that are often found in mixed infections in both the vertebrate host and the tick vector (Andersson, Scherman, & Råberg, 2013; Durand, Herrmann, et al., 2017; Strandh & Råberg, 2015). Different genetic markers have been used to classify isolates of *B. burgdorferi* into species and strains (G. Wang et al., 2014). Strains from pathogens are commonly distinguished by their immunodominant antigens (Singh & Girschick, 2004). Our laboratory and others have used the *ospC* gene to type strains of *B. burgdorferi* (Andersson et al., 2013; Brisson & Dykhuizen, 2004; Durand, Jacquet, Paillard, Rais, Gern, & Voordouwa, 2015; Strandh & Råberg, 2015; N. Wang et al., 1999). The *ospC* gene is a single-copy and highly polymorphic gene that codes for the OspC protein (Grimm et al., 2004). This gene is at least one order of magnitude more diverse than other genes of *B. burgdorferi* (Brisson & Dykhuizen, 2004). OspC is an immunodominant antigen that induces a strong antibody response in the vertebrate host, which results in selection on the pathogen (Baranton, Seinost, Theodore, Postic, & Dykhuizen, 2001; Barbour & Travinsky, 2010; Brisson & Dykhuizen, 2004; Theisen et al., 1995; Wilske et al., 1993). *ospC* gene sequences can be clustered into *ospC* major group (oMG) alleles. An oMG is defined having more than 8% genetic divergence from other oMGs, and less than 2% genetic divergence from alleles belonging to the same oMG (Durand, Jacquet, Paillard, Rais, Gern, & Voordouwa, 2015; N. Wang et al., 1999). Studies have found up to 24 oMGs belonging to the three pathogenic species, only *B.afzelii* has approximately 20 oMGs and *B. burgdorferi s.s.* has around 22 oMGs (Andersson et al., 2013; Baranton et al., 2001; Barbour & Travinsky, 2010; Durand, Jacquet, Paillard, Rais, Gern, & Voordouw, 2015; Lagal, Postic, Ruzic-Sabljic, & Baranton, 2003). At a small spatial scale (~1 ha), the local population of *B. burgdorferi* sl can contain up to a dozen oMGs (Brisson & Dykhuizen, 2004; Durand, Jacquet, Paillard, Rais,

Gern, & Voordouwa, 2015; Durand, Jacquet, Rais, Gern, & Voordouw, 2017; W.-G. Qiu, Dykhuizen, Acosta, & Luft, 2002; Råberg et al., 2017; N. Wang et al., 1999).

The frequencies of the different oMG alleles suggest that this locus is under balancing selection (W.-G. Qiu et al., 2002; W. G. Qiu et al., 1997; N. Wang et al., 1999). Two alternative hypotheses for the observed pattern of balancing selection are negative frequency-dependent (NFDS) and multiple niche polymorphism (MNP) (Brisson, Drecktrah, Eggers, & Samuels, 2012; Vuong et al., 2014; N. Wang et al., 1999). Under NFDS, the adaptive branch of the host immune system is believed to play a critical role in maintaining the *ospC* polymorphism in nature (Barbour & Travinsky, 2010; N. Wang et al., 1999). Under NFDS, the most common oMG strain is recognized and targeted more efficiently by the host immune system. In contrast, the host immune system is less capable at recognizing the rare oMG strains and these strains are therefore more likely to establish an infection (Brisson et al., 2012; N. Wang et al., 1999). The MNP hypothesis suggests that the *ospC* strains are adapted to different species of vertebrate host, where each host act as a different niche (Brisson & Dykhuizen, 2004). Under the MNP hypothesis, the frequencies of the oMG strains depend on the abundances of the different vertebrate hosts. Under MNP there is no oMG strain with the highest fitness overall.

## Tick Vector

Ticks are obligate ectoparasites that vector the greatest diversity of pathogens including viruses, parasites, and bacteria (De la Fuente, Estrada-Pena, Venzal, Kocan, & Sonenshine, 2008; Rizzoli et al., 2014). They are the most important vectors for pathogens of veterinary concern and the second most important vector of human diseases after mosquitoes (Dantas-Torres, Chomel, & Otranto, 2012). Ticks are remarkably successful as disease vectors because of their longevity, high reproductive potential and large host spectrum for some species.

The spirochete bacteria of the *B. burgdorferi* sl genospecies complex are transmitted by the hard ticks from the genus *Ixodes* (Klaus Kurtenbach et al., 2006). Different tick species are involved in different geographical areas. The principal vectors of LD include *I. pacificus*, *I. scapularis*, *I. ricinus* and *I. persulcatus* in Western North America, Eastern North America, Europe, and Asia, respectively. *I. ricinus* and *I. scapularis* are generalist ticks that have been found to feed on hundreds of vertebrate species including reptiles, mammals, birds and humans (Gern et al., 1998; Mccoy, Léger, & Dietrich, 2013). Because of their generalist nature, these tick species can transmit LD to humans. In contrast, many other *Ixodes* species are host specialists, such as *I. cookei*, *I. dentatus*, *I. frontalis*, *I. arboricola*, which exclude them from transmitting *B. burgdorferi* sl to humans (Heylen et al., 2014; Mccoy et al., 2013).

*Ixodes* ticks have four life stages: egg, larva, nymph and adult. Each stage, except eggs and adult males, feed on their vertebrate host for several days and then molt into the next developmental stage (adult females produce eggs). The life cycle of *Ixodes* ticks usually corresponds to one year per stage, but it can range between 2 to 6 years depending on biotic and abiotic factors (Gerold Stanek, Wormser, Gray, & Strle, 2012). Larvae and nymphs feed on the same set of small vertebrate hosts such as rodents, insectivores and birds, which are competent reservoir hosts for *B. burgdorferi* sl pathogens. Adult females usually feed on large vertebrate hosts such as deer, which are not competent reservoir hosts for *B. burgdorferi* sl pathogens (Jaenson & Tälleklint, 1992; Matuschka et al., 1993; Telford III, Mather, Moore, Wilson, & Spielman, 1988). Thus adult females do not play a direct role in the infective cycle of *B. burgdorferi* sl (Gern & Humair, 2002). The females lay eggs in the summer that hatch into larvae after few weeks. As transovarial transmission of *B. burgdorferi* sl is virtually non-existent (Richter, Debski, Hubalek, & Matuschka, 2012), larval ticks are uninfected when they hatch and are not important vectors of the pathogen (with the exception of the relapsing fever spirochete *Borrelia miyamotoi*). The larval ticks acquire the spirochetes after feeding on an infected host (Figure1). The engorged larvae molt into flat nymphs and the infection is maintained by trans-stadial transmission. The infected nymphs transmit the infection to the next generation of susceptible hosts to complete the infective cycle.

Larval ticks can acquire *B. burgdorferi* sl pathogens by two modes of transmission: systemic transmission versus co-feeding transmission (Belli, Sarr, Rais, Rego, & Voordouw, 2017; Randolph, Gern, & Nuttall, 1996; Richter, Allgöwer, & Matuschka, 2002; Voordouw, 2015). In systemic transmission, the pathogen has established a systemic infection in the vertebrate host and the larval tick can acquire the infection from feeding anywhere on the body of the vertebrate host. In co-feeding transmission, larvae acquire the pathogen when they feed in close proximity to infected nymphs on the same host (Belli et al., 2017; Randolph et al.,

1996; Richter et al., 2002; Voordouw, 2015). Co-feeding transmission is usually but not inevitably followed by the development of a systemic infection (Voordouw, 2015).

Hard ticks are unusual compared to other arthropod vectors because of their long feeding time on the vertebrate host. Larvae and nymphs feed for about 3 to 5 days and adult females feed for about 7 days (G Stanek & Reiter, 2011). The long duration of the blood meal allows the vertebrate host to develop immune responses against ticks (Šimo, Kazimirova, Richardson, & Bonnet, 2017). These immune responses can interfere with the efficacy of blood feeding and reduce tick fitness traits (Dizij & Kurtenbach, 1995; Randolph, 1994). Ticks are therefore under strong selection to evolve strategies that evade the host immune response (Wikel, 1996, 1999). Tick saliva is the critical component that facilitates the blood meal and evades host defenses (Šimo et al., 2017).

Tick saliva is secreted by the tick salivary glands, and has anticoagulant, vasolidator, cytolytic, anti-inflammatory, and immunomodulatory activity (Ribeiro et al., 2006). It is a powerful pharmacopoeia of more than 500 proteins and peptides belonging to at least 25 different protein families (Ribeiro et al., 2006). Tick saliva also plays an important role in the transmission of tick-borne pathogens (Nuttall & Labuda, 2004). Tick saliva provides the matrix in which tick-borne pathogens are inoculated, and it modifies the local environment at the tick bite site in a way that benefits the pathogen (Šimo et al., 2017). Tick-borne pathogens can benefit from tick salivary proteins to avoid the host's immune system and colonize the host (Ramamoorthi et al., 2005). One well-studied example of how tick salivary proteins facilitate the transmission of *B. burgdorferi* ss is Salp15. Salp15 is a tick salivary gland protein of *I. scapularis* that suppresses the host immune response (Anguita et al., 2002). *B. burgdorferi* ss binds to Salp15 during tick-to-host transmission to evade detection by the host immune system (Ramamoorthi et al., 2005).The enhancement of pathogen transmission by tick saliva is called saliva-assisted transmission (SAT). SAT has been shown for several tick-borne pathogens including *B. burgdorferi* (Gern, Schaible, & Simon, 1993; Machackova, Oborník, & Kopecký, 2006; Pechova, Stepanova, Kovar, & Kopecky, 2002). This phenomenon has been supported by other studies (Machackova et al., 2006; Pechova et al., 2002). Machackova et al. (2006) and Pechova et al. (2002) showed that culturing *B. burgdorferi* in the presence of tick salivary glands extract (SGE) from *I. ricinus* ticks caused the spirochetes to grow faster in a test tube.

## Reservoir Hosts

### Host population dynamics

*B. burgdorferi* must remain in the zoonotic life cycle, because neither the tick nor the host can vertically transmit the pathogen (Bunikis et al., 2004; Hofmeister, Ellis, Glass, & Childs, 1999; Richter et al., 2012). In the ecology of Lyme disease it is important to distinguish between competent reservoir hosts for *B. burgdorferi* and incompetent reservoir hosts (Gern et al., 1998). In a competent reservoir host, *B. burgdorferi* establishes a chronic infection and achieves high host-to-tick transmission over the lifetime of the infection. Competent reservoir hosts for *B. burgdorferi* pathogens include rodents, insectivores, ground-dwelling birds, and lizards (Gern et al., 1998). Incompetent and accidental hosts include deer and humans, respectively (P.-F. Humair & Gern, 2000; Jaenson & Tälleklint, 1992). The deer feeds the adult females and its immune system kills the spirochetes (Jaenson & Tälleklint, 1992). Accidental hosts such as humans represent a dead-end for the pathogen because host-to-tick transmission never occurs. A number of *B. burgdorferi* sl genospecies such as *B. burgdorferi* ss, *B. afzelii*, *B. bavariensis*, and *B. spielmanii* are transmitted by rodent reservoir hosts. In Europe, the principal rodents species involved in the transmission of *B. burgdorferi* are the bank vole (*Myodes glareolus*) and *Apodemus* mice (*Apodemus flavicolis* and *Apodemus sylvaticus*) (Hanincova et al., 2003), whereas in North America, the principal rodent species is the white-footed mouse *Peromycus leucopus* (Bunikis et al., 2004; Donahue, Piesman, & Spielman, 1987; Hofmeister et al., 1999).

For vector-borne pathogens such as *B. burgdorferi* sl, an important biological parameter is the timescale of host-to-tick transmission relative to the lifespan of the infection in the host (Hartemink, Randolph, Davis, & Heesterbeek, 2008; Jacquet, Durand, Rais, & Voordouw, 2016; Klaus Kurtenbach et al., 2006; Randolph et al., 1996). After the tick bite, the spirochetes disseminate rapidly to different organs including the bladder, heart, joints and skin (Barthold, Persing, Armstrong, & Peeples, 1991; Shih, Pollack, Telford III, & Spielman, 1992). This multi-organ infection is often referred to as a systemic infection, which persists for the lifetime of its rodent host (Gern et al., 1994; P. Humair, Rais, & Gern, 1999; Richter, Klug, Spielman, & Matuschka, 2004). For example, naturally infected *Apodemus* mice remained infective to ticks for 40 months in captivity without decrease in infectivity (Gern et al., 1994). Chronic infections for vector-borne pathogens such as *B. burgdorferi* are adaptive because they increase the lifetime transmission success of the infection.

The long evolutionary history between the reservoir hosts and *B. burgdorferi* pathogens has resulted in the evolution of immune mechanisms that reduce pathogen-induced disease during persistent infection (Tracy & Baumgarth, 2017). To date, there is no evidence that infection with *B. burgdorferi* sl reduces the fitness of its reservoir hosts (Chambert et al., 2012; Hofmeister et al., 1999; Moody, Terwilliger, Hansen, & Barthold, 1994; A. C. Norte et al., 2018; Schwanz, Voordouw, Brisson, & Ostfeld, 2011; Voordouw, Lachish, & Dolan, 2015). Capture-mark-recapture studies have found no effect of infection with *B. burgdorferi* sl on the survival of adult mice or sea birds (Chambert et al., 2012; Hofmeister et al., 1999; Voordouw et al., 2015). We recently found that there was no effect of *B. afzelii* infection on the reproductive success of female bank voles under laboratory conditions (Heinrich, Gomez

Chamorro, Sarr, & Voordouw, 2018; in preparation). Experimental infection of the white-footed mouse (*P. leucopus*) with *B. burgdorferi* ss found that juveniles but not adults suffered carditis and arthritis (Moody et al., 1994). This study is the only demonstration that *B. burgdorferi* sl can induce pathology in a natural reservoir host, but there was no evidence that this pathology actually influenced subsequent fitness (Moody et al., 1994).

**Host immune response**

The host immune system plays a critical role in the recognition and control of *B. burgdorferi* (Radolf & Samuels, 2010). The two branches of the vertebrate immune system, innate and acquired immunity, are both important for the recognition and control of the pathogen (Radolf & Samuels, 2010). The innate immune system provides the first response against any pathogen, and is fast but non-specific. The acquired immune system takes more time to develop an immune response against the pathogen, but this response is highly specific and the development of long-lived memory cells allow a much faster response upon re-exposure to the same pathogen. The biology of *B. burgdorferi* is closely linked to the host's immune response (Klaus Kurtenbach et al., 2006).

In vertebrate hosts, the initial recognition of pathogens is mediated by pathogen-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) (Janeway Jr & Medzhitov, 1998). One of the most important families of PRRs is the Toll-like receptor (TLR) family, with 15 described receptors that function in pathogen recognition and induction of the acquired immune response (Kumar, Kawai, & Akira, 2009). Several studies have found that polymorphisms at TLR loci are associated with susceptibility to different diseases (Misch & Hawn, 2008). Toll-like receptor 2 (TLR2) is a PRR that recognizes common bacterial motifs including peptidoglycan, lipoproteins, and lipotechoic acid (Texereau et al., 2005). TLR2 has been shown to play a key role in the recognition and control of *B. burgdorferi* s.l. (Hirschfeld et al., 1999; Wooten et al., 2002). Genetically engineered mice lacking the TLR2 gene had much higher loads of *B. burgdorferi* than control mice (Hirschfeld et al., 1999; Wooten et al., 2002). A study on wild bank voles in Sweden found they contained a polymorphism at the TLR2 locus (Tschirren, Andersson, Scherman, Westerdahl, & Råberg, 2012). A follow-up study found that bank voles with different TLR2 genotypes differed in the prevalence of *B. afzelii* infection in the field (Tschirren et al., 2013).

The innate immune response has also shaped the host association of the genospecies of the *B. burgdorferi* s.l. complex. The genospecies *B. afzelii* and *B. garinii* are associated with rodents and birds, respectively (Hanincova et al., 2003; Klaus Kurtenbach, Peacey, et al., 1998; A. Norte, Ramos, Gern, Núncio, & Lopes de Carvalho, 2013; Olsén, Jaenson, & Bergström, 1995). This host specificity is mediated by the vertebrate complement system (Klaus Kurtenbach, Simona De Michelis, et al., 2002; K Kurtenbach, Schaefer, De Michelis, Etti, & Sewell, 2002; Klaus Kurtenbach, Sewell, Ogden, Randolph, & Nuttall, 1998). One proposed mechanism consists of complement-resistant spirochetes using outer surface proteins (such as CRASPs) to bind to host-derived complement inhibitors, whereas complement-sensitive spirochetes are lysed in the midgut of the feeding tick (Klaus Kurtenbach, Stefanie M Schäfer, et al., 2002). Different *B. burgdorferi* genospecies have different ability to bind the complement

regulatory proteins. For example, *B. afzelii* has a high binding affinity against the mice complement regularory proteins, but *B. garinii* does not show this affinity (Bhide et al., 2005).

The adaptive branch of the vertebrate immune system develops an antibody response that shapes other important aspects of the biology of *B. burgdorferi* (Klaus Kurtenbach et al., 1994; K Kurtenbach et al., 2002; Probert, Crawford, Cadiz, & LeFebvre, 1997). The acquired immune system creates highly specific antibodies that are an important factor for controlling the spirochete abundance in the host tissues, even if they are unable to completely clear the infection (Connolly & Benach, 2005; LaRocca & Benach, 2008). The importance of antibodies in controlling the abundance of spirochetes in the host tissues has been shown by comparing immunocompetent mice and mice with severe combined immunodeficiency (SCID) (Hodzic, Feng, Freet, & Barthold, 2003). SCID mice spirochete loads in their tissues that are 100 times higher than immunocompetent mice (Hodzic et al., 2003; Liang, Brown, Wang, Iozzo, & Fikrig, 2004; Liang, Yan, et al., 2004). It has been also shown that the spirochete load in SCID mice can be reduced by injecting antibodies from infected immunocompetent mice (Liang, Brown, et al., 2004; Liang, Yan, et al., 2004). The persistent infection in the presence of a strong antibody response poses a long-standing paradox in Lyme disease research (Klaus Kurtenbach et al., 2006). Counter-intuitively, although the antibody response cannot completely clear the primary infection, it can be very effective at preventing infection by a secondary strain that is genetically similar to the primary strain.

Immunization studies with the OspC antigen have shown that the antibody response is highly strain-specific, and that it prevents re-infection with the same strain (Jacquet, Durand, Rais, & Voordouw, 2015; Probert et al., 1997). Several antigens of *B. burgdorferi* sl have been characterized, but only a few have shown protection against the infection via tick bite (Plotkin, 2011; Schuijt, Hovius, van der Poll, van Dam, & Fikrig, 2011). To date, OspC is the only antigen that provides protective immunity against infection via tick-bite under the natural conditions of the infectious cycle (Gilmore, Kappel, Dolan, Burkot, & Johnson, 1996; Jacquet et al., 2015; Mbow, Gilmore, & Titus, 1999). In contrast, immunization with OspA also provides protective immunity against tick bite, but the OspA antigen never encounters the host immune system in nature because it is only expressed in the tick vector. OspC is an immunodominant antigen that is highly polymorphic (Brisson & Dykhuizen, 2004). The antibody response against the OspC antigen protects the mouse from re-infection with strains carrying the same oMG allele, but it does not prevent strains carrying other oMGs to establish an infection (Bhatia et al., 2018; Derdáková et al., 2004; Jacquet et al., 2015; Probert et al., 1997; Rogovskyy & Bankhead, 2014; Rynkiewicz et al., 2017). This protective antibody response against infection with strains expressing the same OspC antigen can have important consequences for strain diversity in natural populations of *B. burgdorferi* sl.

**Study species: The bank vole (*Myodes glareolus*)**

This PhD thesis used the European Lyme disease system: the spirochete bacterium *B. afzelii,* the tick *I. ricinus*, and the rodent host *M. glareolus*. The bank vole (*M. glareolus*) is a small rodent commonly found across Europe. It lives in deciduous and mixed forests where the population density depends on the site and the time of the year. It shows seasonal fluctuations with mast-driven outbreaks (Tersago et al., 2008). The breeding season is from the spring until early autumn with litter sizes that range from one to ten offspring, but the average number is three to four. It was chosen as model species because of its importance as a reservoir host of *B. afzelii* in Europe (Talleklint & Jaenson, 1997). This rodent species has been proposed to have a high competence for *B. afzelii* (P. Humair et al., 1999; Klaus Kurtenbach et al., 1994). Similar to laboratory mice, it has a short life cycle and is relatively easy to handle. This PhD thesis was motivated by the recent discovery that a TLR2 polymorphism in a wild population of bank voles in Sweden was associated with variation in prevalence of *B. afzelii* infection (Tschirren et al., 2013). Another interesting feature of bank voles is that they can develop acquired immunity against the tick vector but they are more efficient at host-to-tick transmission of *B. afzelii* than other rodent species (Dizij & Kurtenbach, 1995; P. Humair et al., 1999; Klaus Kurtenbach et al., 1994; Randolph, 1994). There are important differences in infection phenotype between the bank vole and the laboratory mouse that are discussed here (Jackson, 2015). Studies investigating the evolutionary ecology of host-parasite interactions should always try to use the relevant combinations of hosts and pathogens. Therefore, this dissertation used the bank vole because it is a highly relevant reservoir host for Lyme disease.

## Aims

The general aim of this dissertation was to test different hypotheses of the ecology and immunology of one relevant reservoir host on the Lyme disease pathogen. This dissertation is composed of three chapters that investigated different aspects of the immune response of the bank vole against *B. afzelii*. The first chapter aims to investigate the effect of innate immune response on the pathogen by testing whether genetic variation at an important innate immune receptor in mammals confers resistance to the pathogen in bank voles. The second chapter aims to investigate interactions between the pathogen and the acquired immune system in the rodent host by testing whether *B. afzelii* could inhibit the development of acquired anti-tick immunity in bank voles. In the third and last chapter we aim to investigate the effect of passive immunity transmitted by mothers to their offspring on the pathogen.

## Chapter one

Previous studies found that bank voles with different TLR2 genotypes differed in their prevalence of infection with *B. afzelii* (Tschirren et al., 2013). The TLR2 alleles fell into two different clusters: the C1 susceptibility cluster and the C2 resistance cluster. This result was supported by another study that found a strong positive correlation between the frequency of the C2 resistance allele and the case load of human LB across 19 countries in Europe (Tschirren, 2015). To date, all the work on the TLR2 polymorphism and *B. afzelii* infection in bank voles has been correlative in nature. In the absence of experimental infections, we cannot establish causality between the TLR2 polymorphism in this species and resistance/susceptibility to infection with *B. afzelii*. The purpose of this chapter was to test whether genetic variation at the TLR2 locus in wild bank voles affects susceptibility to *B. afzelii*. According to the study of Tschirren et al. (2013), the C2C2 genotype and the C1C1 genotype were expected to be resistant and susceptible, respectively, to developing a systemic infection following infestation with *B. afzelii*-infected nymphs. We tested this hypothesis by experimentally challenging bank voles of known TLR2 genotype with *B. afzelii*-infected ticks.

**Chapter two**

Several studies have shown that bank voles can develop anti-tick immunity (Dizij & Kurtenbach, 1995; Randolph, 1994). Anti-tick immunity reduces tick fitness, and will also impair the transmission of tick-borne pathogens (Wikel, Ramachandra, Bergman, Burkot, & Piesman, 1997). Thus supression of the development of anti-tick immunity in the vertebrate host would benefit both the tick and the pathogen. Chapter 2 was motivated by a recent study showing that *B. burgdorferi* ss could suppress the development of acquired immunity in laboratory mice (Elsner, Hastey, Olsen, & Baumgarth, 2015). The aim of chapter 2 was to test whether *B. afzelii* could suppress the development of acquired anti-tick immunity in bank voles and thereby increase tick fitness. For this purpose, we repeatedly infested *B. afzelii*-infected bank voles and uninfected control bank voles with *I. ricinus* larval ticks to develop acquired anti-tick immunity. We measured different tick life history traits including larval weight, nymphal weight, molting time and molting success. We expected that *B. afzelii* would suppress the development of acquired anti-tick immunity and increase tick fitness in the *B. afzelii*-infected bank voles compared to the uninfected controls.

## Chapter three

The ability for mothers to transmit induced resistance against a specific pathogen to their offspring may be an essential maternal effect that influences the fitness of offspring. Maternal antibodies can play an important role in the epidemiology of infectious diseases in wild populations (Kallio et al., 2010). Importantly, they could also influence pathogen populations that consist of multiple strains. The role of maternal antibodies in the epidemiology of *B. burgdorferi* ss has not received much attention. The aim of this chapter was to test whether maternal antibodies can protect the offspring against *B. afzelii* and whether this protection is specific for the strain of *B. afzelii* in a natural reservoir host. We created *B. afzelii*-infected and uninfected female bank voles and then tested whether their offspring were protected against infection with *B. afzelii*-infected ticks. We expected that maternal antibodies would protect the offspring against the bite of *B. afzelii*-infected ticks and that this protection would be strain-specific.

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# Chapter One

*Susceptibility to infection with*
*Borrelia afzelii and TLR2 polymorphism*
*in a wild reservoir host*

**Susceptibility to infection with *Borrelia afzelii* and TLR2 polymorphism in a wild reservoir host**

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## Abstract

The study of polymorphic immune genes in host populations is critical for understanding genetic variation in susceptibility to pathogens. Controlled infection experiments are necessary to separate variation in the probability of exposure from genetic variation in susceptibility to infection, but such experiments are rare for wild vertebrate reservoir hosts and their zoonotic pathogens. The bank vole (*Myodes glareolus*) is an important reservoir host of *Borrelia afzelii*, a tick-borne spirochete that causes Lyme disease. Bank vole populations are polymorphic for Toll-like receptor 2 (TLR2), an innate immune receptor that recognizes bacterial lipoproteins. To test whether the TLR2 polymorphism influences variation in the susceptibility to infection with *B. afzelii*, we challenged pathogen-free, lab-born individuals of known TLR2 genotype with *B. afzelii*-infected ticks. We measured the spirochete load in tissues of the bank voles. The susceptibility to infection with *B. afzelii* following an infected tick bite was very high (95%) and did not differ between TLR2 genotypes. The TLR2 polymorphism also had no effect on the spirochete abundance in the tissues of the bank voles. Under the laboratory conditions of our study, we did not find that the TLR2 polymorphism in bank voles influenced variation in the susceptibility to *B. afzelii* infection.

## Introduction

The Toll-like receptors (TLRs) are an important family of pathogen recognition receptors (PRRs) that are found in all vertebrates [1]. These receptors of the innate immune system play a critical role in the recognition of pathogen-associated molecular patterns (PAMPs). PAMPs are highly conserved molecules that are essential for the pathogen's existence such as the cell wall components of bacteria. To date, 12 to 15 different TLR genes have been discovered in humans and mice, and each TLR receptor recognizes a distinct set of PAMPs [2]. For example, TLR4 recognizes lipopolysaccharides (LPS), whereas TLR5 recognizes the bacterial flagellin protein [2,3]. The recognition of pathogens by the TLR receptors is a critical step in the activation of the adaptive immune system in the vertebrate host [4]. Recent reviews have described numerous polymorphisms in the different TLRs and their associations with susceptibility or resistance to different infectious diseases [4,5].

The toll-like receptor 2 (TLR2) recognizes a wide variety of PAMPs including peptidoglycan, zymosan, and lipoproteins [3]. TLR2 can also dimerize with TLR1 and TLR6 to recognize additional PAMPs [6]. TLR2 plays an important role in recognizing the lipoproteins of the spirochete bacteria of the *Borrelia burgdorferi* sensu lato (sl) genospecies complex, which includes the causative agents of Lyme borreliosis in humans [7,8]. Functional studies have shown that TLR2-knockout mice have much higher loads of *B. burgdorferi* sensu stricto (ss) in their tissues than immunocompetent control mice [7,9]. Furthermore, population genetic studies have found associations between genetic polymorphisms at the TLR2 locus and resistance to *B. burgdorferi* sl pathogens in humans and wild rodents [10-14].

Lyme borreliosis is the most common vector-borne disease in the northern hemisphere [15]. In Europe, *Borrelia afzelii* is the most important etiological agent of Lyme borreliosis and is transmitted by the hard tick *Ixodes ricinus* [16,17]. The main reservoir hosts of *B. afzelii* include small mammals such as mice (*Apodemus* species) and the bank vole (*Myodes glareolus*). Experimental infections have shown that *B. afzelii* establishes chronic infections in its rodent reservoir hosts that can last for months or even years [18-20]. Larval ticks acquire spirochetes after feeding on an infected reservoir host, as there is no vertical transmission [21,22]. Blood-engorged larvae subsequently moult into nymphs that infect the reservoir hosts the following year [15]. In areas with high tick densities, wild rodents are repeatedly bitten by nymphs and exposed to *B. burgdorferi* sl pathogens [23,24]. Long-term field studies in Sweden have shown that a quarter of all wild rodents were infected with *B. afzelii* [25]. Thus, *B. afzelii* is an important pathogen in wild rodent populations that could exert strong selection on the host immune system [13,14,26].

The bank vole (*M. glareolus*) is an important wild reservoir host for *B. afzelii* and for immature *Ixodes* ticks [17,23,27]. Genetic studies on the TLR2 locus in this species found three different clusters of alleles: C1, C2, and C3 [13,28]. A field study in Sweden found that individuals with the C1C1, C1C2, and C2C2 genotype had the highest (48.6%), intermediate (30.6%), and lowest (17.9%) prevalence of *B. afzelii* [13]. Thus, the C1 and C2 alleles appear to confer susceptibility and resistance, respectively, to *B. afzelii* in bank voles. The C3 allele was rare in this population and its phenotype with respect to *B. afzelii* infection was not investigated. A recent study of the TLR2 polymorphism in bank vole populations across 19 countries in Europe found a strong positive correlation between the frequency of the C2 resistance allele and the case load of human Lyme borreliosis [26]. This study suggests that *B. afzelii* is driving the evolution of the C2 resistance allele in European bank vole populations [26]. Nevertheless, it remains to be conclusively demonstrated that the TLR2 polymorphism in bank voles causes variation in susceptibility to *B. afzelii* in this important reservoir host.

To date, all the work on the TLR2 polymorphism and *B. afzelii* infection in bank voles has been correlative in nature [13,14,26]. Experimental infections are needed to further confirm whether the TLR2 polymorphism in this species influences variation in susceptibility to infection with *B. afzelii*. The two aims of this study were to determine whether the TLR2

polymorphism in wild bank voles influences variation in the probability of infection with *B. afzelii* (hereafter referred to as host susceptibility) and variation in *B. afzelii* spirochete abundance in host tissues (hereafter referred to as host spirochete load). To test this hypothesis, we challenged *Borrelia*-free, lab-born bank voles of known TLR2 genotype with *B. afzelii* via tick bite. The TLR2 gene could influence the prevalence of *B. afzelii* by different mechanisms such as preventing systemic infection and/or spirochete clearance following infection. We expected that compared to the C1C1 genotype, the C2C2 genotype would be less likely to develop a systemic infection following infestation with *B. afzelii*-infected nymphs. We also expected that the C2C2 genotype would have a lower spirochete load in the tissues than the C1C1 genotype. The importance of the C3 allele was assessed for the first time in this study.

## Materials and methods

### General experimental design

The present study consists of two separate infection experiments: the first experiment was conducted in the summer of 2015 using bank voles from a Swiss colony, whereas the second experiment was conducted in the summer of 2016 using bank voles from a Finnish colony. In these infection experiments, the Swiss and Finnish bank voles of known TLR2 genotype were challenged via tick bite with different isolates of *B. afzelii*, respectively. The Swiss bank vole colony contained the C1 susceptible allele and the C3 allele, for which the *B. afzelii* infection phenotype was unknown. The Finnish bank vole colony contained the C1 susceptible allele, the C2 resistance allele, and the C3 allele.

### Ethics statement and animal experimentation permits

All the experiments were performed following the Swiss and Finnish legislation on animal experimentation. For the infection experiment conducted in Switzerland, the commission that is part of the 'Service de la Consommation et des Affaires Vétérinaires (SCAV)' of the canton of Vaud, Switzerland evaluated and approved the ethics of this part of the study. The SCAV of the canton of Neuchâtel, Switzerland issued the animal experimentation permit (NE2/2014). The infection experiment conducted in Finland was carried out under the permits of the Finnish Animal Experiment Board (ESAVI/3834/04.10.03/2011, ESAVI/7256/04.10.07/2014 and ESAVI/3457/04.10.07/2015).

### Breeding of the bank voles in the laboratory

The Swiss and Finnish colonies of bank voles were established by trapping bank voles near Neuchâtel, Switzerland and Jyväskylä, Finland, respectively (**section 1 in the electronic supplementary material (ESM)**). TLR2 genotyping found that the Swiss bank voles carried the C1 and C3 alleles (n = 36 animals; frequencies were 42.2% and and 57.8%, respectively), but not the C2 putative resistance allele (**section 2 in the ESM**). For the Swiss infection experiment, male and female bank voles of known TLR2 genotype were crossed to maximize the number of homozygous C1C1 and C3C3 offspring. A sample of the Finnish colony (n = 48 animals) revealed that the frequencies of the TLR2 allele were as follows: 88.5% C1, 4.2% C2, and 7.3% C3. For the Finnish infection experiment, bank voles were bred to maximize the number of homozygous C1C1, C2C2 and C3C3 offspring. All lab-born offspring were genotyped with respect to their TLR2 locus. TLR2 genotyping of the bank voles is described in **section 2 in the ESM**.

### Isolates of *B. afzelii*

Our original intention was to challenge the Swiss and Finnish bank voles with ticks carrying a single Swiss isolate (NE4049) and a single Finnish isolate (Fin-Jyv-A3) of *B. afzelii*, respectively. We chose these two strains because our previous work had shown that they are competent at establishing infection in laboratory mice [29-32]. Due to a combination of experimental error and time constraints, we used nymphs that were co-infected with two strains of *B. afzelii*. The Swiss bank voles were challenged with nymphs co-infected with a local Swiss strain (NE4049) and a foreign Austrian strain (E61). The Finnish bank voles were challenged with nymphs that were co-infected with a local Finnish strain (Fin-Jyv-A3) and a foreign Swiss strain (NE4049). The origin and genetics of these *B. afzelii* strains is described in **section 3 in the ESM**. We used a strain-specific qPCR to confirm that the local isolate of *B. afzelii* had established infection in the bank voles (**section 3 in the ESM**).

**Infectious challenge of bank voles with *B. afzelii*-infected nymphal ticks**

All animals were *Borrelia*-free, lab-born individuals and were in the adult stage (older than 2 months) at the time of the challenge with *B. afzelii*-infected nymphs. For the Swiss infection experiment, 50 animals were challenged with *B. afzelii*-infected nymphs and the TLR2 genotypes were as follows: 17 C1C1 (12 females, 5 males), 15 C1C3 (7 females, 8 males), and 18 C3C3 (8 females, 10 males). For the Finnish infection experiment, 50 animals were challenged in one single trial. The TLR2 genotypes were as follows: 11 C1C1 (4 females, 7 males), 8 C1C2 (4 females, 4 males), 12 C2C2 (4 females, 8 males), 1 C2C3 (1 male), 8 C1C3 (4 females, 4 males), and 10 C3C3 (6 females, 4 males).

In the field, rodents are rarely infested with more than one *I. ricinus* nymph at a time [13,23,24,33]. To simulate the natural conditions while also ensuring a good probability of infectious challenge, each animal was infested with 3 randomly selected nymphs. The creation of the experimentally infected nymphs is described in **section 3 in the ESM**. The percentage of *B. afzelii*-infected nymphs was >90% for both the Swiss and Finnish infection experiments. The nymphs were placed in a neoprene capsule that had been glued to the shaved backs of the voles. During this procedure, animals were anesthetized with a mixture of xylazine, ketamine and PBS (1:2:9; 5 µl per gram of vole). The purpose of the capsule is to prevent the bank vole from grooming off and killing the attached nymphs. The animals were fitted with an Elizabethan collar to prevent them from removing the capsules. The capsules were checked on a daily basis over a period of 7 days, and detached engorged nymphs were collected from the capsule and frozen at –20°C.

At 49 days post-infection, the animals were sacrificed using $CO_2$ asphyxiation. Our previous work had shown that this is enough time for *B. afzelii* to disseminate to all of the organs [20,29,30]. For each animal, a tissue biopsy was taken from the ear and the dorsal skin from the site of the tick bite using a 2 mm forceps-type punch. The bladder and ankle joints were aseptically dissected [29]. These internal tissues are typically used to demonstrate that *B. burgdorferi* sl has established a systemic infection [9,34-38]. To determine the infection status of the bank voles, these four tissue samples were tested for *B. afzelii* using qPCR (see below).

***Borrelia afzelii* infection status of the engorged nymphs and the bank voles**

We extracted the DNA from engorged nymphs and bank vole tissue samples and used qPCR targeting the *flagellin* gene to estimate the spirochete load (for details, see **section 3 in the ESM**). For the bank vole tissue samples, we used the DNA concentration of the DNA extraction to standardize the spirochete load as the number of spirochetes per mg of host DNA (for details, see **section 3 in the ESM**).

**Statistical analyses**

**General approach**

The three response variables included the number of engorged nymphs collected from each bank vole, the infection status of the bank vole (infected, uninfected) and the spirochete load of *B. afzelii* in the bank vole tissues. These three response variables were analysed using generalized linear models (GLMs) with binomial errors, GLMs with binomial errors, and linear mixed effects models (LMEMs) with normal errors, respectively. The fixed effects included experiment (2 levels: Swiss, Finnish), sex (2 levels: female, male), organ (4 levels: bladder, ear, joint, dorsal skin), and TLR2 genotype. TLR2 genotype was coded in eight different ways that corresponded to different assumptions about the effects of the TLR2 alleles (**Table 1**).

Experiment and the eight different ways to code TLR2 could not be in the same model because of partial or complete redundancy between these factors. We used model selection based on the Akaike Information Criterion (AIC) to determine the best model. The advantage of this approach is that it allows us to compare non-nested models. We used the model weights to calculate the % support for each of the fixed effects. We used model averaging to obtain robust parameter estimates and 95% confidence intervals (95% CI) for the fixed effects. A fixed effect is significant when its model-averaged 95% CI does not overlap 0. We used R version 3.4.3 to analyse the data. The GLMs and LMEMs were run using the glm() function in the base package and the lmer() function in the lme4 package, respectively. Model selection and calculation of model-averaged parameter estimates were run using the model.sel() and the model.avg() functions in the MuMIn package.

**Number of engorged nymphs per bank vole**

Each bank vole was infested with 3 nymphs, and the number of engorged nymphs collected from each bank vole was therefore a binomial variable that ranged from 0/3 to 3/3. To test whether the 100 bank voles were exposed to the same infectious challenge, we analysed two response variables: the number of engorged nymphs that were collected per bank vole and the number of engorged *B. afzelii*-infected nymphs that were collected per bank vole. These two response variables were modelled using GLMs with binomial errors. The fixed factors were experiment, sex, and the eight different ways to code TLR2 genotype. Each vole occurred only once in the analysis so it was not necessary to include bank vole ID as a random effect.

**TLR2 genotype and infection status of the bank voles**

Bank vole infection status is a binomial variable (uninfected, infected) that was modelled using GLMs with binomial errors. The fixed effects were number of *B. afzelii*-infected nymphs (0, 1, 2, 3), experiment, sex, and the eight different ways to code TLR2 genotype. As there was no variation in infection status among the four organs, we did not include this factor in the analysis. Each vole occurred only once in the analysis so it was not necessary to include bank vole ID as a random effect. The analysis was performed for all the bank voles (n = 100) and for the subset of bank voles that had been successfully challenged with *B. afzelii*-infected nymphs (n = 88).

***B. afzelii* spirochete load of the bank vole tissue samples**

The statistical analysis of the *B. afzelii* spirochete load in the tissue samples was restricted to the subset of infected bank voles (n = 84). For each tissue sample, the geometric mean spirochete load (in 3 µl of DNA template) was calculated for the three replicate runs. The tissue spirochete loads were standardized to mg of DNA (by dividing by the DNA concentration). This variable was log10-transformed to normalize the residuals. The log10-transformed tissue spirochete loads were modelled using LMEMs with normal errors. The fixed factors were experiment, sex, organ, and the eight different ways to code TLR2 genotype.

Organs sampled from the same bank vole are not independent and bank vole identity was therefore modelled as a random factor.

## Results

### The C1, C2, and C3 allele clusters encode different TLR2 proteins

The TLR2 gene sequences of the animals in the experimental infection formed three clearly separated clusters that coded for three different protein variants: cluster 1 (C1), cluster 2 (C2) and cluster 3 (C3); these three clusters were the same as the ones described by Tschirren et al. [13]. The C1 and C2 clusters, C1 and C3 clusters, and C2 and C3 clusters were separated by a genetic distance of 12, 15, and 15 nucleotide differences, respectively, which corresponded to a protein distance of 7, 7, and 4 amino acids, respectively (**Figure S1**). A protein model of TLR2 had previously shown that the variants encoded by the C1 and C2 alleles would differ in at least one amino acid in the binding site that is believed to be critical for ligand binding [13]. We therefore expected that these different TLR2 proteins would differ in their ability to recognize the lipoproteins of *B. afzelii* and therefore to protect the bank vole against infection via tick bite.

### Definition of a successful infectious challenge

We collected at least 1 engorged *B. afzelii*-infected nymph from 83 of the 100 bank voles (**Tables S01 and S02 in the ESM**). There were 17 bank voles for which we did not recover any engorged *B. afzelii*-infected nymphs (**Tables S01 and S02 in the ESM**). Five of these 17 individuals became infected with *B. afzelii* proving that they had been exposed to an infected nymph (which we failed to recover). Hence, we have proof that 88 of the 100 bank voles were successfully challenged in this study (83 + 5 = 88). The 12 remaining individuals for which there was no proof of an infectious challenge (no infected engorged nymphs, bank voles are uninfected) all came from the Swiss experiment.

### Number of engorged nymphs per bank vole

For the 50 Swiss bank voles, we collected 84 engorged nymphs (mean = 2.00, range = 0–3 engorged nymphs), of which 52 were infected with *B. afzelii* (mean = 1.24, range = 0–3 engorged infected nymphs). For the 50 Finnish bank voles, we collected 131 engorged nymphs (mean = 2.72, range = 2–3, units = engorged nymphs per bank vole), of which 96 were infected with *B. afzelii* (mean = 1.92, range = 0–3 engorged infected nymphs).

For the model selection analysis of the number of engorged nymphs per bank vole, the top 3 models had 99.999% of the support; the remaining 26 models had <0.001% of the support (**Table 2 and Table S07 in the ESM**). The support for individual factors was as follows: experiment (99.999%), sex (32.4%), and TLR2 genotype (<0.001% for 24 models). The model-averaged parameter estimates found that significantly more engorged nymphs were collected per bank vole in the Finnish experiment (**Table S08 in the ESM**) compared to the Swiss experiment.

The results were similar for the number of engorged *B. afzelii*-infected nymphs and when the analyses were done on the subset of 88 bank voles that had been successfully challenged. These analyses show that the infectious challenge was the same for all TLR2 genotypes.

### Infection status of the bank voles

A bank vole was defined as being infected if it tested positive for *B. afzelii* for at least one of four organs: (1) bladder, (2) ear, (3) joint, and (4) dorsal skin. There was no ambiguity about the infection status of the 100 bank voles: 84 tested positive for all four organs and 16

tested negative for all four organs. The susceptibility of a host to a pathogen is the probability of acquiring an infection following exposure. In our study, the susceptibility of bank voles to infection with *B. afzelii* was 95.5% (84 infected/ 88 total; 95% CI = 88.1–98.5%) and was therefore very high.

Infection was higher in the Finnish experiment (100.0% = 50/50) than the Swiss experiment (68.0% = 34/50). When the analysis was restricted to the subset of 88 bank voles that had been successfully challenged, infection in the Finnish experiment (100.0% = 50/50) and Swiss experiment (89.5% = 34/38) were similar.

Bank vole infection status was initially modelled using GLMs with binomial errors (**Table S09 in the ESM**). However, due to the lack of variation in infection status (most voles were infected), the standard errors (SE) estimated by the glm() function had very low precision (i.e., SE was ~1000 times bigger than the parameter estimate; **Table S10 in the ESM**). To avoid this problem with parameter estimation, we re-analysed the data using linear models with normal errors.

For the model selection analysis of the linear models of infection status of 100 bank voles, the top 6 models had 99.9% of the support; the remaining 111 models had 0.1% of the support (**Table 3 and Table S11 in the ESM**). The support for individual factors was as follows: number of infected nymphs (>99.9%), experiment (>99.9%), sex (55.6%), and TLR2 genotype (<0.001% for 98 models). The model-averaged parameter estimates found that infection increased significantly with the number of engorged infected nymphs per bank vole and that infection was significantly higher in the Finnish experiment compared to the Swiss experiment (**Table S12 in the ESM**).

We do not present the model selection analysis for the subset of 88 bank voles that had been successfully challenged because there was no variation in infection status (84 infected/ 88 total).

We compared the susceptibility of bank voles to infection with *B. afzelii* between our study and the study by Tschirren et al. [13] (for details, see **section 4 in the ESM**). For the study by Tschirren et al. [13], the probability of encountering an infected tick in the field was not known, but by setting this probability to a maximum (100%) or a minimum (48.7%), we were able to estimate the susceptibility of each TLR2 genotype. This comparison found that the susceptibility was significantly different between the two studies (**Figure 1**).

### *B. afzelii* spirochete load of the bank vole tissue samples

For each organ, the repeatability of the log10-transformed tissue spirochete loads was high (range = 71–75%; **section 5 in the ESM**). For the Swiss bank voles, the mean spirochete load per mg of DNA was lowest in the bladder (540 spirochetes per mg of DNA; Table 5), 4.2 times higher in the ear, 5.1 times higher in the dorsal skin, and 8.4 times higher in the ankle joint (**Figure 2; Table 4**). For the Finnish bank voles, the mean spirochete load per mg of DNA was lowest in the bladder (1339 spirochetes per mg of DNA; Table 5), 9.4 times higher in the dorsal skin, 10.9 times higher in the ankle joint, and 15.2 times higher in the ear (**Figure 2; Table 4**). Averaged across the four organs, the tissue spirochete loads in the Finnish bank voles were 4.2 times higher than the Swiss bank voles (**Figure 2; Table 4**).

For the model selection analysis of tissue spirochete load per mg of DNA, the top 5 models had 99.4% of the support; the remaining 112 models had 0.6% of the support (**Table 5 and Table S13 in the ESM**). The support for individual factors was as follows: experiment (99.99%), sex (11.4%), organ (100.0%), and TLR2 genotype (<0.001% for 98 models). The model-averaged parameter estimates found that the ear, joints, and ventral skin had significantly higher spirochete loads than the bladder (**Figure 2; Table S14 in the ESM**). The Finnish bank voles had significantly higher spirochete loads in their tissues than the Swiss bank voles (**Figure 2; Table S14 in the ESM**). Finally, the interaction between experiment and organ was

significant because the difference in ear tissue spirochete load between Finnish and Swiss bank voles was higher than the difference for the other tissues (**Figure 2; Table S14 in the ESM**).

**Strain-specific qPCR to determine infection success of the local isolate**

For the sample of infected Swiss bank voles, 88.2% (30/34) were infected with the local isolate of *B. afzelii* (isolate NE4049). For the sample of infected Finnish bank voles, 98.0 % (49/50) were infected with the local isolate of *B. afzelii* (isolate Fin-Jyv-A3). Thus, 94.0% (79/84) of the bank voles became infected with their local (and intended) isolate of *B. afzelii* following the infectious challenge.

## Discussion

In this study, we standardized exposure to infected ticks and tested whether a genetic polymorphism at the TLR2 locus in the bank vole explained variation in susceptibility to *B. afzelii,* a common and important tick-borne pathogen. Most studies on the role of TLR2 polymorphisms in the susceptibility to *B. burgdorferi* sl pathogens have been association studies in humans and wild rodents [4,13,14,26,39,40]. To our knowledge, there are no experimental infection studies showing that a TLR2 polymorphism influences variation in susceptibility to infection with *B. burgdorferi* sl. Under the laboratory conditions of our study, ~100.0% of all bank voles become infected following exposure to *B. afzelii*-infected ticks. Thus, our study found no evidence that bank voles with different TLR2 genotypes (C1C1, C2C2, C3C3) differed in their susceptibility to infection with *B. afzelii*. One limitation of this study was the small sample size for the C2C2 genotypes (n = 12), which limits our power to detect differences in susceptibility to *B. afzelii* infection between the susceptible (C1C1) and resistant (C2C2) genotypes.

The prevalence of *B. afzelii* infection in our study (95.5%) was 2.5 times higher than the field study by Tschirren et al. (37.5%) [13] as shown in **Figure 1**. This discrepancy is due to differences in the risk of exposure between the two studies. In our study, the risk of exposure was 100.0% for the subset of 88 bank voles that had been successfully challenged (by definition). In the field study by Tschirren et al. [13], the risk of exposure was unknown, but was assumed to be identical for the three TLR2 genotypes. Under this assumption and after minimizing the risk of exposure (subject to the assumption that bank voles cannot clear their infection), the susceptibility of the resistant C2C2 TLR2 genotype remained significantly lower in the study by Tschirren et al. [13] compared to our study (**Figure 1**). Why did we not find the expected differences in susceptibility to infection with *B. afzelii* between the TLR2 genotypes?

One explanation for the different infection prevalences in the field study by Tschirren et al. [13] is that the three TLR2 genotypes differed in their risk of exposure to *B. afzelii*-infected nymphs. Fine-scale spatial studies have shown that the density of infected nymphs (DIN) and the risk of exposure can vary dramatically over very small distances [41]. If by chance, families of bank voles carrying the C2 allele live in areas of the sampling grid that have a low DIN compared to families of bank voles carrying the C1 allele, then observed differences in infection prevalence would be caused by these spatial differences in the risk of exposure and not by differences in susceptibility between the TLR2 genotypes. Differences in exposure can also explain why the prevalence of *B. afzelii* infection was 2.5 times higher in our lab study compared to the field study by Tschirren et al. [13].

A second explanation involves grooming, which is an important behaviour that protects vertebrate hosts against ectoparasites such as ticks [42]. Previous studies have shown that the probability of nymph-to-host transmission of *B. burgdorferi* sl pathogens increases with the duration of attachment of the nymphal tick to the vertebrate host [43,44]. In nature, the normal grooming behaviour of the vertebrate host is a significant cause of tick mortality that would

reduce the risk of pathogen transmission following nymphal attachment [42,45]. In the present study, we prevented the bank voles from grooming off the nymphs by fitting them with a collar and by placing the nymphs in a protective capsule. If C2C2 individuals have a more effective grooming response than C1C1 individuals, they would be less likely to become infected with *B. afzelii* following the attachment of an infected nymph. The link between the immune system and grooming behaviour is not so far-fetched because the former has itch-generation mechanisms (e.g. release of histamine by mast cells) that would stimulate the latter [46]. The absence of the grooming defence mechanism can also explain why the prevalence of *B. afzelii* infection was 2.5 times higher in our lab study compared to the field study by Tschirren et al. [13].

A third explanation involves acquired immunity, which can influence susceptibility to infection with *B. afzelii*. We have recently shown that infected female bank voles can transmit maternal antibodies to their offspring that protect against infection with *B. afzelii* [47-49]. Bank voles can also develop acquired immunity against *I. ricinus* ticks [50], and this anti-tick immunity protects against infection with *B. burgdorferi* sl [51-53]. Thus variation in maternal antibodies and/or anti-tick immunity in wild bank vole populations will influence variation in susceptibility to infection with *B. burgdorferi* sl. In our study, the bank voles did not have any antibodies to either *B. afzelii* or to *I. scapularis* ticks at the time of the infectious challenge (i.e. they were completey naive). In contrast, field studies can not control for this variation in acquired immunity [13,14,26]. The absence of maternal antibodies or anti-tick immunity can also explain why the prevalence of *B. afzelii* infection was 2.5 times higher in our lab study compared to the field study by Tschirren et al. [13]

A fourth explanation involves the ability of bank voles to clear *B. afzelii*. The consensus is that rodent reservoir hosts cannot clear *B. burgdorferi* sl [18-20,23,27,54]. In our study, there was no evidence of clearance at 49 days post-infection, as the four tissues in the 84 infected bank voles were all positive for *B. afzelii*. However, we recently found clearance of spirochetes from ear tissues in bank voles that had been experimentally infected with *B. afzelii* strain NE4049 [48]. Bank voles that had cleared the infection from their ear tissues still had very high spirochete loads in other tissues (bladder, heart, dorsal skin, ventral skin) [48]. This study suggests that ear tissue biopsies might underestimate the infection status of bank voles in the field [48]. In most field studies, the infection status of the bank voles is determined by taking ear tissue biopsies [13,14,26]. Thus, another explanation for the difference in prevalence of *B. afzelii* infection between the two studies, is that we determined infection status by testing for spirochetes in multiple internal organs.

A fifth explanation is that our laboratory environment was so stressful that it overwhelmed the variation in susceptibility to infection between the TLR2 genotypes. Physiological stress can suppress the host immune system and increase susceptibility to infection with pathogens [55-58]. Although we cannot rule out the stress-immunosuppression explanation, we believe that it is unlikely for the following reasons. First, there is a long history in Lyme disease research of performing experimental infections with wild animals in the lab, and this work has successfully determined the susceptibility of many vertebrate hosts to various *B. burgdorferi* sl pathogens [18,19,59-65]. Second, as mentioned previously, using the same experimental conditions described in the present study, female bank voles infected with *B. afzelii* transmitted antibodies to their offspring, which protected the offspring against infected ticks [47-49]. The creation of antibodies in the mothers and their ability to block infection in the offspring requires the coordinated action of both the adaptive and innate immune system. This study provides some evidence that the immune system of our bank voles is functioning as it should under our laboratory conditions [47-49]. Third, although the laboratory environment may be stressful, other stressors found in nature, such as predators, food shortage, competitive interactions, and other parasites, were absent in our lab environment. Fourth, lab mice (*Mus*

*musculus*) are presumably not stressed by our lab conditions, but they are also 100% susceptible to *B. afzelii* [29]. The most parsimonious explanation is that competent rodent reservoir hosts are highly susceptible to infection with *B. burgdorferi* sl if *Ixodes* nymphs feed to completion.

A sixth explanation for the difference in susceptibility to *B. afzelii* infection between our laboratory study and the field study by Tschirren et al. [13] is the presence of other parasites. In natural systems, hosts are often co-infected with multiple parasites including viruses, bacteria, protozoans, helminth parasites, and ectoparasites [66,67]. Field studies have shown that the susceptibility of infection to any particular parasite depends on the presence of other parasites [68]. For example, in a wild population of the field vole (*Microtus agrestis*), infection with tick-borne *Babesia microti* increased and decreased the risk of acquiring infections with tick-borne *Anaplasma phagocytophilum* and flea-borne *Bartonella* spp., respectively [68]. A recent experimental infection study using lab mice found no evidence that infection with the nematode *Heligmosomoides polygyrus* influenced the susceptibility to tick-borne *B. afzelii* [69]. In our laboratory system, co-infection with other parasites cannot increase the susceptibility of naive bank voles to tick-borne *B. afzelii* because this susceptibility is already very high (95.5%). However, we cannot exclude that co-infection with other parasites in nature decreases the susceptibility to tick-borne *B. afzelii*, but to our knowledge, no such parasite has been identified.

A unique aspect of this study was our effort to expose the bank voles to a realistic infectious challenge. Field studies have shown that rodents are rarely infested with more than one *I. ricinus* nymph at a time [13,23,24]. In the present study, we collected between 1 and 2 engorged *B. afzelii*-infected nymphs from each bank vole indicating that they had exposed to an ecologically relevant infectious tick bite challenge. We collected more engorged *B. afzelii*-infected nymphs per bank vole in the Finnish infection experiment (1.24) than the Swiss infection experiment (2.02). The reason for this difference was improved methodology; the use of collars prevented the bank voles from removing the capsules and from grooming off and killing the ticks. The improvement in the infestation methodology explains why the infection success was higher in the Finnish experiment than the Swiss experiment. However, when the infection success was restricted to the subset of 88 bank voles that had been successfully challenged, the infection success was similar between the two experiments. Important for the purpose of this study was our demonstration that within each infection experiment, the TLR2 genotypes were exposed to the same infectious tick bite challenge.

Estimates of the probability of nymph-to-host transmission of *B. burgdorferi* sl pathogens for wild reservoir hosts are rare in the scientific literature [29,31]. Under the conditions of our study, we found that bank voles were highly susceptible to acquiring *B. afzelii* infection following an infected tick bite. Of the 88 voles that had been successfully challenged, 95.5% developed a systemic infection (95% CI = 88.1–98.5%). We recently showed for *B. afzelii* strain NE4049 that 96.4% of laboratory mice developed a systemic infection following an infected tick bite [29]. Taken together, these studies suggest that tick-to-host transmission of *B. afzelii* is ~100% when a single infected nymph feeds to completion on a competent rodent host.

The TLR2 receptor plays an important role in controlling the spirochete load in the tissues of laboratory mice (*Mus musculus*). Genetically modified mice that do not have TLR2 have spirochete loads that are 100-fold higher than wild-type mice [9]. We therefore expected resistant C2C2 genotypes to have lower spirochete loads than susceptible C1C1 genotypes. However, our study found no evidence that the TLR2 polymorphism influences variation in the tissue spirochete load of *B. afzelii*. The higher tissue spirochete loads in the Finnish versus the Swiss experiment may be due to differences between the two bank vole populations or between the strains of *B. afzelii*. Our recent finding that strain Fin-Jyv-A3 establishes a higher tissue spirochete load than strain NE4049 in inbred lab mice [70] suggests that strain is part of the explanation.

We also found significant differences in spirochete load between organs (**Figure 2**). In both infection experiments, the spirochete loads were significantly higher in the ear and the dorsal skin compared to the bladder. These results are similar to a study on *B. afzelii* strain NE4049 in lab mice, which found that the spirochete loads were 1.4 to 2.1 times higher in the skin compared to the bladder [29]. Others and we have shown that the probability of host-to-tick transmission of *B. afzelii* increases with the spirochete load in the skin [30,71]. *B. burgdorferi* sl must establish a persistent infection in the skin to achieve effective host-to-tick transmission [71-74]. *B. burgdorferi* sl also invades joint tissues, which causes swelling and arthritis in the ankle joints of lab mice [35,36]. We found no evidence that infection with *B. afzelii* caused swelling of the ankle joints in bank voles (**see section 7 in the ESM**).

We chose to work with *B. afzelii* strains NE4049 and Fin-Jyv-A3 because these strains occur in the Swiss and Finnish bank vole populations and because our previous work had shown that they are competent at establishing infection in laboratory mice [29-32]. While the original study by Tschirren et al. (2013) did not suggest that the TLR2-associated susceptibility to *B. afzelii* in bank voles would be strain-dependent, we acknowledge that repeating our study with different strains of *B. afzelii* might reveal host variation in susceptibility. In each of the two infection experiments, the bank voles were challenged with nymphs that were co-infected with two different strains of *B. afzelii*. Co-infections are the norm in nature; 80% of wild *I. ricinus* nymphs are infected with multiple strains [75-77]. Likewise, small mammal hosts (including bank voles) are often infected with multiple strains of *B. afzelii* [25,78-81]. Thus, co-infected nymphs frequently bite bank voles in nature. In our study, the bank voles were simultaneously challenged with one local strain and one foreign strain. We showed that the local co-adapted strain was present in at least 94.0% (79/84) of the infected bank voles. One potential criticism of our study is that the presence of the foreign strain could have enhanced the ability of the local strain to establish infection. We believe that this explanation is unlikely for two reasons. First, we recently conducted two other studies where Swiss bank voles were experimentally infested with nymphs that were singly infected with either strain NE4049 (n = 22) or strain Fin-Jyv-A3 (n = 21) and found that 95.3% (41/43) of the bank voles became infected [48]. These studies suggest that the susceptibility of bank voles to strains NE4049 and Fin-Jyv-A3 is ~100% and does not depend on whether the nymph is infected with one strain or two strains. Second, we recently found that when lab mice are simultaneously infected with two strains (NE4049 and Fin-Jyv-A3), there can be interference but not facilitation [32]. In summary, we have consistently found that our bank voles and lab mice are ~100% susceptible to infection with *B. afzelii* when we successfully challenge them with at least 1 infected nymph.

In conclusion, after controlling for exposure to infected ticks, we did not find that bank voles with different TLR2 genotypes differed in their susceptibility to infection with *B. afzelii* under the laboratory conditions of our study. Bank voles were highly susceptible to acquiring *B. afzelii* infection when nymphs were allowed to feed to repletion. Future studies should investigate whether different strains of *B. afzelii* or different experimental conditions (e.g. semi-natural enclosures that reduce stress) could reveal the expected variation in susceptibility to *B. afzelii* infection. Our study emphasizes the importance of using controlled experimental infections to separate variation in exposure from variation in susceptibility in the study of candidate immune genes for resistance to pathogens.

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reconstruct the TLR2 haplotypes. Thanks to two anonymous reviewers whose comments improved this manuscript.

**Author contributions**

A.G.-C. and M.J.V designed the study. N.B. gave advice on the experimental design. A.G.-C. and F.B. captured bank voles at the field sites in Switzerland, bred the bank voles, performed the experimental infections and the molecular work. C.C., E.K., and T.M. helped with the experimental infections of the Finnish bank voles. D.G. and A.S. created the *B. afzelii*-infected nymphs. A.G.-C. analysed the data. A.G.-C. and M.J.V wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests: The authors declare no competing interests.

## Figures and Tables

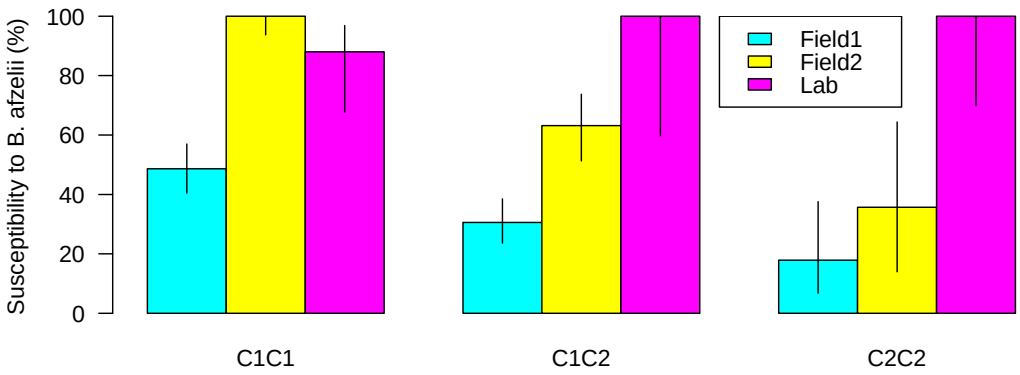


**Figure 1.** The susceptibility of the three bank vole TLR2 genotypes (C1C1, C1C2, and C2C2) to infection with *B. afzelii* is compared between our lab study (Lab) and the field study by Tschirren et al. [13] (Field1, Field2). The field study by Tschirren et al. [13] assumed that the exposure rates were identical for the three TLR2 genotypes and that bank voles do not clear the infection. Using these assumptions, the prevalence of infection can be separated into the probability of exposure and the susceptibility to infection following an infectious challenge. The Field1 estimates of susceptibility are based on the unrealistic assumption that 100% of the bank voles were exposed to an infected tick. The Field2 estimates of susceptibility are based on the assumption that 48.6% of the bank voles were exposed to an infected tick, which maximizes the susceptibility of the C1C1 genotype to 100.0%.

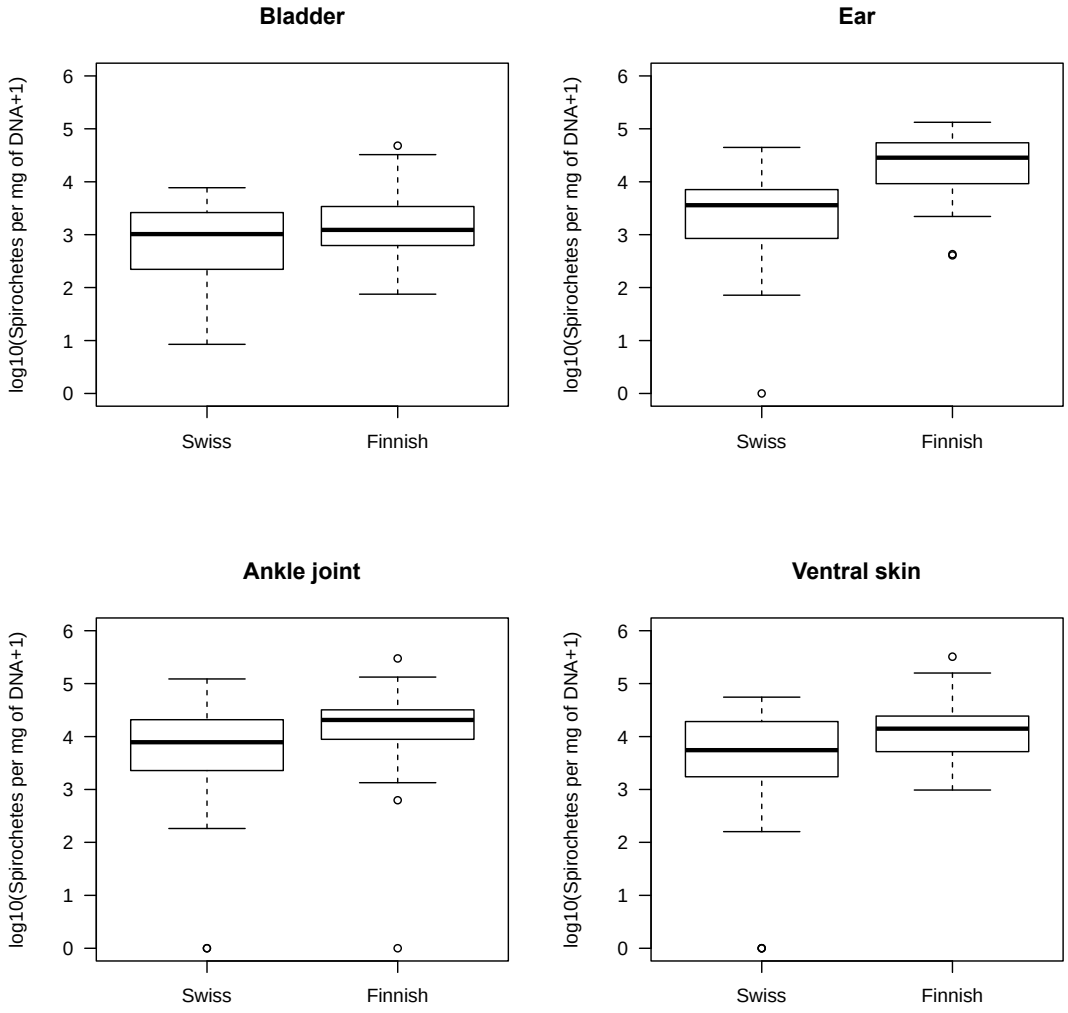


**Figure 2.** Organ and experiment had a significant effect on the tissue spirochete load of *B. afzelii* in the bank voles. The spirochete loads were standardized per mg of DNA and then log10-transformed. For the Finnish bank voles, the tissue spirochete loads were higher compared to the Swiss bank voles. The rank order of the organ spirochete loads (from lowest to highest) depended on the experiment: bladder, ear, ventral skin, ankle joint for the Swiss bank voles and bladder, ventral skin, ankle joint, ear for the Finnish bank voles. Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (circles).

**Table 1.** The eight different ways in which TLR2 genotype was modelled are shown. TLR2 genotype was either modelled as a categorical factor where each TLR2 genotype was a different category or as a covariate that counted the number of TLR2 alleles. We reduced the number of genotypes or alleles by setting pairs of alleles or genotypes as equivalent. For example, for Geno2, we assume that the C1 and C3 alleles are equivalent so that there are only 3 distinct genotypes: C2C2, C2Cx, CxCx, where Cx = C1 = C3. Combining similar genotypes (or alleles) increases the sample size for the remaining genotype categories and thereby increases the power of the statistical test.

| Name | Type of variable | # of genotypes | Identity of genotypes | # of alleles | Identity of alleles |
|---|---|---|---|---|---|
| Geno1 | Categorical | 6 | C1C1, C2C2, C3C3, C1C2, C1C3, C2C3 | NA | NA |
| Geno2 | Categorical | 3 | C2C2, C2Cx, CxCx; (Cx = C1 = C3) | NA | NA |
| Geno3 | Categorical | 3 | C1C1, C1Cy, CyCy; (Cy = C2 = C3) | NA | NA |
| Geno4 | Categorical | 3 | C3C3, C2Cz, CzCz; (Cz = C1 = C2) | NA | NA |
| Geno5 | 1 Covariate | NA | NA | 2 | # of C2 alleles; (C1 = C3) |
| Geno6 | 1 Covariate | NA | NA | 2 | # of C1 alleles; (C2 = C3) |
| Geno7 | 1 Covariate | NA | NA | 2 | # of C3 alleles; (C1 = C2) |
| Geno8 | 3 Covariates | NA | NA | 3 | # of C1, C2, and C3 alleles |

**Table 2.** Model selection table is shown for the generalized linear models (with binomial errors) of the number of engorged nymphs per bank vole (nymphs.engorged). The analysis was done on the entire data set of 100 bank voles. Of the 29 models, the top 5 models are shown; the top 3 models have 99.999% of the support. **Table S09 in the ESM** shows all 29 models. The fixed factors are experiment (E), sex (S), and TLR2 genotype. TLR2 genotype was modelled in 8 different ways (geno1, geno2, geno3, geno4, geno5, geno6, geno7, and geno8). For each model, the model structure, degrees of freedom (df), log likelihood, corrected AIC value (AICc), difference in AICc from the top model (Delta), and the support (Weight) are shown.

| Model | Model structure | df | logLik | AICc | Delta | Weight (%) |
|---|---|---|---|---|---|---|
| model003 | nymphs.engorged~E | 2 | -109.353 | 222.831 | 0.000 | 67.575 |
| model002 | nymphs.engorged~E+S | 3 | -109.316 | 224.881 | 2.051 | 24.235 |
| model001 | nymphs.engorged~E+S+E:S | 4 | -109.315 | 227.051 | 4.221 | 8.189 |
| model010 | nymphs.engorged~geno2 | 3 | -121.250 | 248.751 | 25.920 | 0.000 |
| model022 | nymphs.engorged~geno5 | 2 | -122.638 | 249.399 | 26.569 | 0.000 |

**Table 3.** Model selection table is shown for the linear models of bank vole infection status (infection). The analysis was done on the entire data set of 100 bank voles. Of the 117 models, the top 10 models are shown; the top 6 models have 99.9% of the support. **Table S11 in the ESM** shows all 117 models. The fixed factors are number of engorged *B. afzelii*-infected nymphs (N), experiment (E), sex (S), and TLR2 genotype. TLR2 genotype is modelled in 7 different ways (geno1, geno2, geno3, geno4, geno5, geno6, and geno7). For each model, the model structure, degrees of freedom (df), log likelihood, corrected AIC value (AICc), difference in AICc from the top model (Delta), and the support (Weight) are shown.

| Model | Model structure | df | logLik | AICc | Delta | Weight (%) |
|---|---|---|---|---|---|---|
| model01 | | | -35.212 | | | |
| 1 | infection ~E+N+E:N | 5 | | 12.287 | 0.000 | 44.374 |
| model00 | | | -36.507 | | | |
| 7 | infection ~E+S+N+E:N | 6 | | 11.802 | 1.295 | 23.226 |
| model00 | | | -37.546 | | | |
| 5 | infection ~E+S+N+E:N+S:N | 7 | | 11.164 | 2.334 | 13.817 |
| model00 | | | -37.851 | | | |
| 3 | infection ~E+S+N+E:S+E:N | 7 | | 11.317 | 2.639 | 11.862 |
| model00 | | | -39.646 | | | |
| 2 | infection ~E+S+N+E:S+E:N+S:N | 8 | | 11.032 | 4.434 | 4.835 |
| model00 | infection | | -41.651 | | | |
| 1 | ~E+S+N+E:S+E:N+S:N+E:S:N | 9 | | 10.826 | 6.439 | 1.774 |
| model01 | | | -51.271 | | 16.065 | |
| 4 | infection ~E+N | 4 | | 21.428 | | 0.014 |
| model60 | | | -52.463 | | 17.257 | |
| 6 | infection ~geno6+S+N+geno6:S | 6 | | 19.780 | | 0.008 |
| model00 | | | -52.517 | | 17.309 | |
| 9 | infection ~E+X+N | 5 | | 20.939 | | 0.008 |
| model00 | | | -52.645 | | 17.435 | |
| 8 | infection ~E+X+N+S:N | 6 | | 19.871 | | 0.007 |

**Table 4.** Mean tissue spirochete loads in the bank voles are shown for each organ in both the Swiss and Finnish infection experiments. Bank vole organs were dissected at 49 days post-infection. The tissue spirochete load was standardized per mg of DNA (units are spirochetes per mg of DNA). Shown are the means, and the lower limits (LL) and upper limits (UL) of the 95% confidence interval.

| Experiment | Organ | Mean | LL | UL |
|---|---|---|---|---|
| Switzerland | Bladder | 540 | 286 | 1017 |
| Switzerland | Ear | 2283 | 1211 | 4303 |
| Switzerland | Joint | 4531 | 2404 | 8541 |
| Switzerland | Skin | 2768 | 1469 | 5218 |
| Finland | Bladder | 1339 | 794 | 2259 |
| Finland | Ear | 20300 | 12036 | 34240 |
| Finland | Joint | 14533 | 8616 | 24512 |
| Finland | Skin | 12587 | 7463 | 21230 |

**Table 5.** Model selection table is shown for the linear mixed effects models of the *B. afzelii* spirochete load in bank vole tissues. Spirochete load is standardized per mg of DNA and was log10-transformed to normalize the residuals (log10(spiro.per.mg.DNA)). The analysis was done on the subset of 84 bank voles infected with *B. afzelii*. Of the 117 models, the top 10 models are shown; the top 5 models have 99.4% of the support. **Table S12 in the ESM** shows all 117 models. The fixed factors are experiment (E), organ (O), sex (S), and TLR2 genotype. TLR2 genotype is modelled in 7 different ways (geno1, geno2, geno3, geno4, geno5, geno6, and geno7). For each model, the model structure, degrees of freedom (df), log likelihood, corrected AIC value (AICc), difference in AICc from the top model (Delta), and the support (Weight) are shown.

| Model | Model structure | df | logLik | AICc | Delta | Weight (%) |
|---|---|---|---|---|---|---|
| lm.model1014 | log10(spiro.per.mg.DNA)~E+O | 7 | -408.954 | 832.250 | 0.000 | 76.959 |
| lm.model1011 | log10(spiro.per.mg.DNA)~E+O+E:O | 10 | -407.676 | 836.029 | 3.779 | 11.632 |
| lm.model1009 | log10(spiro.per.mg.DNA)~E+S+O | 8 | -410.178 | 836.796 | 4.547 | 7.925 |
| lm.model1006 | log10(spiro.per.mg.DNA)~E+S+O+E:S | 9 | -410.639 | 839.830 | 7.580 | 1.739 |
| lm.model1007 | log10(spiro.per.mg.DNA)~E+S+O+E:O | 11 | -408.900 | 840.614 | 8.364 | 1.175 |
| lm.model1003 | log10(spiro.per.mg.DNA)~E+S+O+E:S+E:O | 12 | -409.361 | 843.688 | 11.438 | 0.253 |
| lm.model1008 | log10(spiro.per.mg.DNA)~E+S+O+S:O | 11 | -410.562 | 843.939 | 11.689 | 0.223 |
| lm.model1004 | log10(spiro.per.mg.DNA)~E+S+O+E:S+S:O | 12 | -411.023 | 847.012 | 14.762 | 0.048 |
| lm.model1005 | log10(spiro.per.mg.DNA)~E+S+O+E:O+S:O | 14 | -409.259 | 847.826 | 15.576 | 0.032 |
| lm.model1002 | log10(spiro.per.mg.DNA)~E+S+O+E:S+E:O+S:O | 15 | -409.720 | 850.939 | 18.690 | 0.007 |

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**Electronic supplementary material (ESM)**

**Section 1 – Field site for trapping wild bank voles in Switzerland**

Two trapping sessions were performed at two different sites in Switzerland. The first trapping session was performed in the summer of 2014 at a site above the city of Neuchâtel (47°00'21.9"N 6°56'33.9"E). The second trapping session was performed in the summer of 2015 at a site above the city of Zurich in Zürichbergwald (47°23'41.2"N 8°33'43.0"E). The Neuchatel site was chosen for its high abundance of rodents and *B. afzelii*-infected nymphs [1]. The Zurich site was chosen because a previous study had shown that the frequency of the C2 resistance allele was high in the local bank vole population [2]. A total of 110 traps were placed along transects at each field site. The traps were baited with apple and oatmeal and were checked three times per day. All captured bank voles were transported to the animal facility of the University of Neuchâtel.

**Section 1 – Field trapping and lab rearing wild bank voles in Finland**

The bank vole breeding and rearing conditions in laboratory are detailed in [3]. The lab colony of the University of Jyväskylä is composed of descendants of wild-caught individuals, mainly first and second generation. Wild bank voles are caught within the framework of a long-term population-monitoring program; sampling occurs four times per year (in May, July, August, November) in the Konnevesi area in Central Finland. In each of 20 monitored forests, four Ugglan multiple-capture traps are placed at the corners of a 15-meter square, baited with sunflower seeds for two consecutive days and checked daily. Trapped bank voles are taken to the laboratory facility at the University of Jyväskylä where they measured and kept in standard mouse cages (43 cm*26 cm*15 cm) with wood shavings and under 16 h light:8 h dark photoperiod and temperature of ~19 °C. Animals have *ad libitum* access to food and water [4].

Initial matings took place during the spring of 2016 and the founders (F0 voles) were born between the end of January and March 2016. Offspring were separated from their mothers after weaning (21 days) and were kept in sibling groups according to their sex. Each individual was identified with a microchip inserted subcutaneously. After weaning, all individuals were measured, sampled for TLR2 genotyping and housed individually. Similarly, two directional mating sessions, based on TLR2 genotype, which aimed at maximising the number of individuals with C1C1, C2C2 and C3C3 genotypes, took place during the spring of 2016 and created the F1 and F2 generations. Bank voles from the F0, F1 and F2 generations were used in the experimental challenge.

**Section 2 – TLR2 genotyping of the bank voles**

The TLR2 genotype of each individual bank vole was determined as follows. An ear biopsy was taken from each bank vole with a punch type forceps (2 mm in diameter). Total DNA was extracted from the bank vole ear biopsies using the QIAGEN DNeasy® Blood and Tissue Kit and following the manufacturer's instructions. The DNA was eluted in a final volume of 150 µl of AE buffer and stored at –20°C. The DNA concentration of each sample was measured using a Nanodrop® 2000. The PCR protocol targeted a 1200 base pair fragment of the TLR2 gene and was described previously [5]. PCR reactions were performed in a total volume of 25 µL including 25 ng of total genomic DNA, 1x Green GoTaq® Reaction Buffer (Promega AG), 0.125 mM dNTPs, 1 mM of each primer (forward and reverse) and 2.5 U of Go TAQ polymerase (Promega AG). The thermocycler conditions were as follows: initial denaturation step at 94°C for 2 min, 37 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, and extension at 72°C for 90 sec, followed by a final extension step at 72°C for 10 min. PCR products were visualized by gel electrophoresis (0.8% agarose gel dyed with Midori Green) and then sent to Microsynth AG for forward and reverse sequencing.

**Section 2 – Assignment of TLR2 sequences to haplotypes and clusters**

The TLR2 sequences were assigned to haplotypes and to the three clusters: C1, C2, and C3. Only high quality sequences were used for haplotype reconstruction. Forward and reverse TLR2 sequences were processed, assembled, and aligned using Geneious version 6.1 (http://www.geneious.com). Consensus sequences were created and polymorphisms were examined visually. Database searches and sequence comparisons were performed with the BLAST tool provided by the National Center for Biotechnology. TLR2 haplotypes were reconstructed using the default settings of the PHASE v2.1 software [6]. Input files were created using the SeqPHASE web tool [7]. The alignment of all the individuals was processed in TCS v1.21 to construct the TLR2 haplotype network [8].

**Section 2 – Frequency of the TLR2 alleles in the wild bank vole populations**

For the Swiss bank voles, a total of 36 and 59 wild bank voles were captured at the field sites of Neuchâtel and Zurich, respectively. In Neuchatel, the frequencies of the C1 and C3 clusters were 42.2% and 57.8%, respectively, whereas in Zurich, the frequencies of the C1 and C3 clusters were 65.2% and 34.7%, respectively. For the Neuchatel and Zurich samples, 8.6% (3/35) and 18.6% (11/59) of the animals were infected with *B. afzelii* at the time of capture. In Neuchatel, the 3 bank voles infected with *B. afzelii* had the following TLR2 genotypes: 1 C1C1, 1 C1C3, and 1 C3C3. In Zurich, the 11 bank voles infected with *B. afzelii* had the following TLR2 genotypes: 4 C1C1, 6 C1C3, and 1 C3C3. Therefore, there was no association between *B. afzelii* infection and the TLR2 genotype in the Neuchatel and Zurich populations.

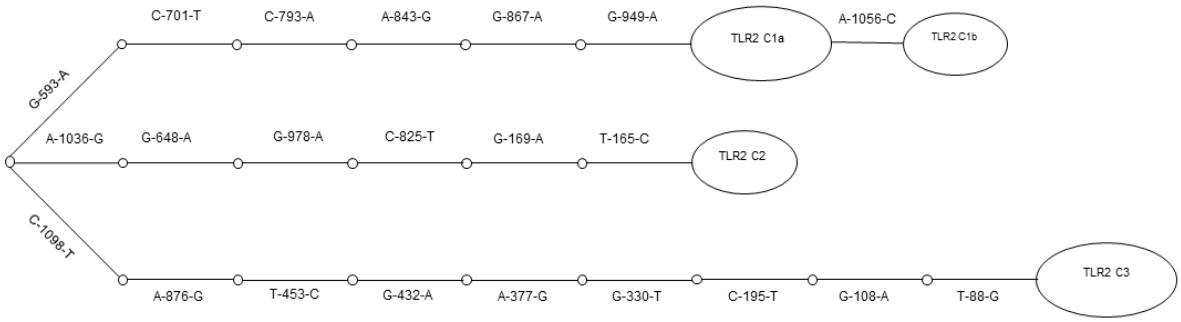


**Figure S1.** TLR2 haplotype network is shown for the 50 Swiss and 50 Finish bank voles that were included in both infection studies. The C1 and C2 clusters, C1 and C3 clusters, and C2 and C3 clusters were separated by a genetic distance of 12, 15, and 15 nucleotides, respectively, which corresponds to a protein distance of 7, 7, and 4 amino acids, respectively. The lines connecting the nodes indicate the number and types of nucleotide changes that separate these four TLR2 haplotypes from each other. For example, TLR2 C1a and TLR2 C1b are separated by a single substitution; the label "A-1056-C" indicates that at site 1056, TLR2 C1a has an adenine whereas TLR2 C1b has a cytosine.

**Section 3 – Isolates of *B. afzelii***

Our original intention was to challenge the Swiss and Finnish bank voles with ticks carrying a single Swiss isolate (NE4049) and a single Finnish isolate (Fin-Jyv-A3) of *B. afzelii*, respectively. We chose these two strains because our previous work had shown that they are highly infectious to laboratory mice [9-12]. Due to a combination of experimental error and time constraints, we used nymphs that were co-infected with two strains of *B. afzelii*. The Swiss bank voles were challenged with nymphs co-infected with a local Swiss strain (NE4049) and a foreign Austrian strain (E61). The Finnish bank voles were challenged with a local Finnish strain (Fin-Jyv-A3) and a foreign Swiss strain (NE4049).

Isolates NE4049, Fin-Jyv-A3, and E61 were originally obtained from an *I. ricinus* tick in Neuchâtel, Switzerland, a bank vole in Jyväskylä, Finland, and a human patient in Austria, respectively. We have characterized these isolates genetically [3,10,13]. Fin-Jyv-A3 has *ospC* major group (oMG) A3, multi-locus sequence type (MLST) 676, and strain ID number 1961 in the *Borrelia* MLST database. Isolate NE4049 has oMG A10, MLST 679, and strain ID number 1887 in the *Borrelia* MLST database. Isolate E61 has oMG A3, MLST ST75, and strain ID number 1888 in the *Borrelia* MLST database.

**Section 3 – Creation of nymphs infected with *B. afzelii***

The *B. afzelii*-infected nymphs used to challenge the bank voles were created as follows. Female, *Borrelia*-free *Mus musculus* BALB/c mice were infected with isolates of *B. afzelii* via tick bite. Four weeks after infection, each *B. afzelii*-infected mouse was infested with ~100 *Borrelia*-free, larval ticks from our laboratory colony of *I. ricinus* at the University of Neuchâtel. Blood-engorged larvae were placed in individual tubes and were allowed to moult into *B. afzelii*-infected nymphs. For the Swiss and Finnish infection experiments, the prevalence from a random sample of *B. afzelii*-infected nymphs was 91.3% (42 infected nymphs/46 total nymphs) and 96.8% (91 infected nymphs/94 total nymphs), respectively.

**Section 3 – Strain-specific qPCR to determine infection success of the local isolate**

The Swiss bank voles were challenged with a local Swiss isolate (NE4049) and a foreign Austrian isolate (E61). The Finnish bank voles were challenged with a local Finnish isolate (Fin-Jyv-A3) and a foreign Swiss isolate (NE4049). For the purpose of the experiment, it was important to show that the bank voles had become infected with their local isolates. We had previously developed a qPCR protocol that allows us to differentiate between *B. afzelii ospC* major group (oMG) alleles A10 and A3 [12,14]. This strain-specific qPCR uses general primers to amplify the *ospC* gene but uses different probes to detect the two oMG alleles [12,14]. This qPCR can be used to differentiate between isolates NE4049 and E61, which carry oMG alleles A10 and A3, respectively, and between isolates Fin-Jyv-A3 and NE4049, which carry oMG alleles A3 and A10, respectively. We performed this qPCR protocol to determine the infection success of the local isolate.

**Section 3 - *Borrelia afzelii* infection status of the engorged nymphs and the bank voles**

DNA extractions from engorged nymphs and bank vole tissue samples were performed using the 96-well plate QIAGEN DNeasy® Blood and Tissue Kit and following the manufacturer's instructions. Engorged nymphs were crushed using a sterile steel bead in a Tissue Lyser (Peqlab, Erlangen, Germany). For each bank vole organ, ~20 to 25 mg of tissue was used for DNA extraction and the elution volume was 200 µl. Each of the 6 DNA extraction plates included 12 tissue samples of uninfected lab mice as negative controls (total of 72 negative controls). We measured the DNA concentration of each DNA extraction using a Nanodrop machine. The DNA concentration is an estimate of the amount of host DNA and we used it to standardize our estimates of spirochete *B. afzelii* load per mg of host DNA (see below).

The *B. afzelii* infection status and spirochete load of the engorged nymphs and the tissue samples of the bank vole organs were estimated using a qPCR assay that amplified a 132 bp fragment of the *flagellin* gene [15]. The qPCR reaction was performed following the protocol of Jacquet et al. [10]; 3 µl of DNA template were used per sample. Each qPCR plate contained 81 samples from individual bank voles (and the tissue samples from the lab mice that served as negative DNA extraction controls), and triplicates of standards (10, 100, 1000, 10000 *flagellin* gene copies) and 3 negative controls for the qPCR (template was 3 µl of distilled water). Each bank vole tissue sample was replicated in three independent qPCRs. The reactions were run in the LightCycler® 96 (Roche Applied Science, Switzerland). The number of spirochetes present in each engorged nymph and bank vole tissue sample was calculated using the standard curves and the LightCycler® 96 software (Roche Applied Science, Switzerland). All of the 72 negative controls for the DNA extraction (tissue samples from lab mice) tested negative for *B. afzelii*.

**Table S01.** Infection status is shown for the 50 Swiss bank voles in the Swiss infection experiment. Bank voles were considered as having been successfully challenged if we collected at least 1 *B. afzelii*-infected engorged nymph from the capsule (Engorged nymphs = Positive) or if the individual developed a systemic infection (Vole infection = Positive). Individuals for which there was no proof that they had been exposed to at least one *B. afzelii*-infected engorged nymph (Engorged nymphs = Negative; Vole infection = Negative) were excluded from the experiment (12 animals in the top row in Table S1).

| Engorged nymphs | Vole infection | Challenged | C1C1 | C1C3 | C3C3 | Total |
|---|---|---|---|---|---|---|
| Negative | Negative | No | 3 | 6 | 3 | 12 |
| Negative | Positive | Yes | 2 | 0 | 2 | 4 |
| Positive | Negative | Yes | 3 | 0 | 1 | 4 |
| Positive | Positive | Yes | 9 | 9 | 12 | 30 |
| | | | 17 | 15 | 18 | 50 |

**Table S02.** Infection status is shown for the 50 Finnish bank voles in the Finnish infection experiment. Bank voles were considered as having been successfully challenged if we collected at least 1 *B. afzelii*-infected engorged nymph from the capsule (Engorged nymphs = Positive) or if the individual developed a systemic infection (Vole infection = Positive). Individuals for which there was no proof that they had been exposed to at least one *B. afzelii*-infected engorged nymph (Engorged nymphs = Negative; Vole infection = Negative) were excluded from the experiment (0 animals in the top row in Table S2).

| Engorged nymphs | Vole infection | Challenged | C1C1 | C1C3 | C3C3 | C1C2 | C2C2 | C2C3 | Total |
|---|---|---|---|---|---|---|---|---|---|
| Negative | Negative | No | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Negative | Positive | Yes | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Positive | Negative | Yes | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Positive | Positive | Yes | 11 | 8 | 9 | 8 | 12 | 1 | 49 |
| | | | 11 | 8 | 10 | 8 | 12 | 1 | 50 |

**Section 4 – Correspondence between field and lab estimates of susceptibility to infection:**

The probability that a bank vole is infected with *B. afzelii* in nature ($P_I$) is the probability of exposure to an infectious challenge ($P_E$) times the probability of acquiring an infection following an infectious challenge, hereafter referred to as the susceptibility ($P_S$) [16]. In the field, $P_I = P_E * P_S$. In our study, we controlled the probability of exposure (i.e. $P_E$ was set to 1.000) and we were therefore able to estimate the susceptibility to infection ($P_S$). After combining the data from our two infection experiments, the susceptibility to infection for TLR2 genotypes C1C1, C1C2, and C2C2 was 88.0% (22/25), 100.0% (8/8), and 100.0% (12/12), respectively.

In the study by Tschirren et al. [17], the prevalence of *B. afzelii* infection of adult bank voles in the field for TLR2 genotypes C1C1, C1C2, and C2C2 was 48.6% (72/148), 30.6% (48/157), and 17.9% (5/28), respectively. An important assumption in the study by Tschirren et al. [17] was that the probability of exposure ($P_E$) was the same for all three genotypes. If we assume that the probability of exposure is 100% (i.e. as in our lab experiment), then the susceptibility of infection is equal to the observed prevalence of infection and is 48.6%, 30.6%, and 17.9% for the TLR2 genotypes C1C1, C1C2, and, respectively. However, this assumption is not realistic. We can set a lower limit for the probability of exposure, by assuming that the C1C1 genotype is 100% susceptible. In this case, the probability of exposure is 48.6% and the susceptibility of infection is 100.0%, 62.8%, and 36.7% for TLR2 genotypes C1C1, C1C2, and C2C2 (**Table S03**).

We now ask, what is the probability that the susceptibilities in the study by Tschirren et al. [17] could have generated the observed susceptibilities in our laboratory study? We can use the binomial probability functions in R to calculate the probabilities that the susceptibilities in the field could have generated the observed susceptibilities in the lab as follows:

| | | |
|---|---|---|
| C1C1: | 1-pbinom(21, 25, 1.000000) | = 1.000 |
| C1C2: | dbinom(8, 8, 0.6284501) | = 0.02433136 |
| C2C2: | dbinom(12, 12, 0.3670634) | = 0.000005982642 |

**Table S03**. The probability that a bank vole is infected with *B. afzelii* ($P_I$) is the product of the probability of exposure to an infectious challenge ($P_E$) and the probability of acquiring an infection following an infectious challenge (Probability of susceptibility = $P_S$). Two opposite scenarios are shown where the probability of exposure is either maximal (100.0%) or minimal (48.6%). Under the minimal probability of exposure, the C1C1 genotype is maximally susceptible (100.0%).

| TLR2 | $P_E$ | $P_S$ | $P_I$ |
|---|---|---|---|
| C1C1 | 100.0% | 48.6% | 48.6% |
| C1C2 | 100.0% | 30.6% | 30.6% |
| C2C2 | 100.0% | 17.9% | 17.9% |
| C1C1 | 48.6%, | 100.0% | 48.6% |
| C1C2 | 48.6%, | 62.8% | 30.6% |
| C2C2 | 48.6%, | 36.7% | 17.9% |

Under the assumption of independence, the probability that the field susceptibilities estimates could generate the laboratory data is the product of the above probabilities: 2*1.000*0.02433136*0.000005982642 = 0.0000002911316. In the preceding equation, we doubled this probability because we had no 'a priori' expectation of whether the lab susceptibilities would be more or less extreme than the field susceptibilities (i.e. were are doing a two-tailed probability test). This exercise shows that it is very improbable (less than 1 in a million) for the field susceptibility estimates to generate our observed experimental data. For this calculation, we made the field and lab susceptibility estimates as close as possible to each other by assuming that the probability of exposure was as low as possible (48.6%).

**Section 5 – Repeatability of the *B. afzelii* spirochete loads in the tissues of the bank voles**

The spirochete load in the tissues of the bank voles was estimated using a qPCR assay that targeted the *flagellin* gene of *B. afzelii*. For each tissue sample, the *flagellin* gene copy number is an estimate of the spirochete load. The repeatability is a commonly used metric to assess the reliability of scoring a particular phenotype. We estimated the repeatability for two phenotypes associated with the spirochete load in the tissues of the bank voles: the quantification cycle (Cq) and the spirochete load (SL; first divided by the DNA concentration of the DNA extraction of the tissue sample, then log10-transformed). We used a generalized linear mixed effects model to estimate the 'among' and 'within' variance components of the Cq and of the SL. These variance components were used to calculate the repeatability of the Cq and the SL (**Table S04**).

**Table S04.** Repeatability of the quantification cycle (Cq) and the repeatability of the spirochete load (SL) are shown for each organ.

| Organ | N | Repeatability of Cq | Repeatability of SL |
|---|---|---|---|
| Heart | 54 | 82% | 59% |
| Joint | 96 | 96% | 71% |
| Skin | 93 | 89% | 53% |
| Ear | 99 | 95% | 59% |
| Total | 342 | 97% | 75% |

**Section 6 – Re-analysis of the *B. afzelii* infection prevalence data by Tschirren et al. [17]**

The bank voles in the field study by Tschirren et al. [17] were captured during the months of May, June, August, September, and October. Tick questing activity is highly seasonal and it is well established that the risk of exposure to infected ticks varies dramatically over the course of the transmission season [18]. The prevalence of infection in the bank voles in each month of the study is an index of the risk of infection. In the original statistical analysis in Tschirren et al. [17], infection prevalence was modelled as a generalized linear mixed effects model and month was modelled as a random effect. While this approach controls for the variance in infection risk among months, it does not examine how infection prevalence (and risk of infection) changes over time. We therefore re-analysed the data from the study by Tschirren et al. [17] to determine how infection prevalence (and risk of infection) varied over the months of the study. The infection prevalence was higher in the months of June, August, and September compared to the months of May and October (**Figure S2**) and these differences were statistically significant ($\Delta$ df = 4, $\Delta \chi^2 = 19.912$, $p < 0.001$).

We used a generalized linear model with binomial errors to model infection prevalence as a function of TLR2 genotype, sex, and their interaction separately for each month. TLR2 genotype was modelled as the number of C2 alleles. We expect the slope of the relationship between infection prevalence and the number of C2 alleles to be negative. The sex:TLR2 genotype interaction was not significant for any of the months and we therefore re-ran the models without this interaction term. The parameter estimates for TLR2 genotype and sex are shown for each month in **Table S05**. The expected negative effect of the number of C2 alleles was statistically significant for three of the five months (June, August, and September). This analysis shows that the differences in infection prevalence between the three TLR2 genotypes are not biased by differences in the risk of infection between months.

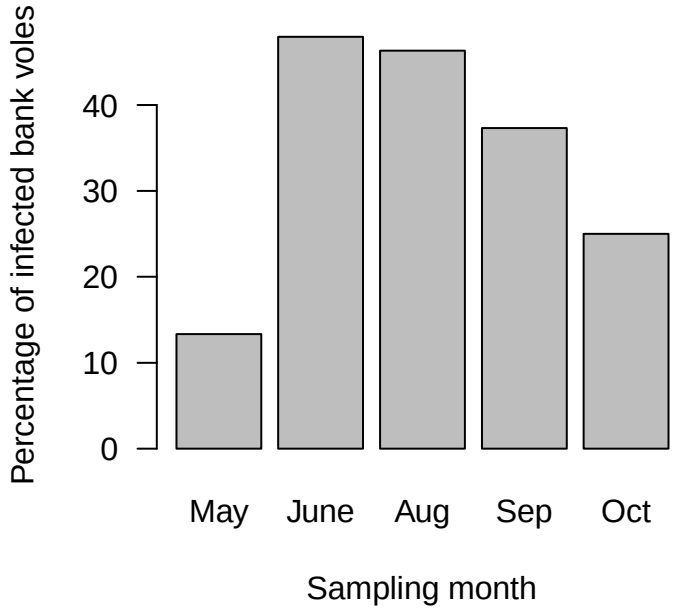


**Figure S2.** The prevalence of bank voles infected with *B. afzelii* is shown for each month for the field study by Tschirren et al. [17].

**Table S05.** Analysis of the prevalence of *B. afzelii* infection in the bank voles of the field study by Tschirren et al. [17]. For each month of the field study, infection prevalence was modelled as a function of TLR2 genotype and sex using a GLM with binomial errors. TLR2 genotype was modelled as the number of C2 alleles and sex is modelled as a contrast between males and females. Shown are the parameters, the parameter estimates, the standard errors, the z-values and associated p-values.

| Month | Parameter | Estimate | Std. Error | z-value | p |
|---|---|---|---|---|---|
| May | Intercept | -3.951 | 1.454 | -2.717 | 0.007 |
| May | C2.alleles | 1.644 | 1.125 | 1.462 | 0.144 |
| May | sexM | 1.409 | 1.264 | 1.115 | 0.265 |
| June | Intercept | 0.317 | 0.490 | 0.647 | 0.518 |
| **June** | **C2.alleles** | **-1.486** | **0.480** | **-3.099** | **0.002** |
| June | sexM | 1.020 | 0.532 | 1.919 | 0.055 |
| August | Intercept | 0.120 | 0.336 | 0.358 | 0.720 |
| **August** | **C2.alleles** | **-0.895** | **0.351** | **-2.549** | **0.011** |
| August | sexM | 0.755 | 0.452 | 1.669 | 0.095 |
| September | Intercept | 0.133 | 0.424 | 0.314 | 0.754 |
| **September** | **C2.alleles** | **-1.050** | **0.456** | **-2.304** | **0.021** |
| September | sexM | -0.089 | 0.531 | -0.167 | 0.867 |
| October | Intercept | -0.900 | 0.434 | -2.077 | 0.038 |
| October | TLR2 | -0.122 | 0.450 | -0.271 | 0.786 |
| October | sexM | -0.314 | 0.584 | -0.537 | 0.591 |

**Section 7 – Materials and methods and statistical analysis of the morphometric data**

Infection with *B. burgdorferi* sensu stricto in laboratory mice is known to cause inflammation and/or swelling in organs, such as the heart and ankle joints (Wooten et al., 2002). To test whether *B. afzelii* induces inflammation in a natural reservoir host like the bank vole, we measured a number of morphological variables in the Swiss bank voles. Ankle diameter (mm) was measured for each bank vole prior to sacrifice. The heart weight (mg) and bladder weight (mg) were measured after the bank voles had been sacrificed and frozen. LME models with normal errors were used to test whether infection with *B. afzelii* and TLR2 genotype influenced these morphological variables. The TLR2 genotype (three levels: C1C1, C1C3, C3C3), sex (two levels: males and females), infection status (two levels: infected and uninfected), and the covariate body mass were modelled as fixed factors, whereas the trial number was modelled as a random factor.

**Section 7 – Results of the morphometric data**

For the sample of Swiss bank voles, LME models with normal errors were used to test whether infection with *B. afzelii* and TLR2 genotype influenced the following three phenotypes: heart weight, bladder weight, and ankle diameter. There was no effect of *B. afzelii* infection and no effect of TLR2 genotype on heart weight, bladder weight, or ankle diameter (**Table S06**).

**Table S06.** Morphological measurements of 5 different phenotypes are compared between the uninfected and the *Borrelia*-infected bank voles (mean ± standard deviation) in the Swiss population.

| Organ | Uninfected | Infected |
|---|---|---|
| Ankle Left (mm) | 2.38 ± 0.15 | 2.35 ± 0.17 |
| Ankle Right (mm) | 2.31 ± 0.13 | 2.30 ± 0.12 |
| Heart (mg) | 132.98 ± 22.92 | 148.68 ± 34.89 |
| Bladder (mg) | 27.42 ± 8.94 | 33.21 ± 15.24 |
| Weight (g) | 23.92 ± 4.33 | 25.04 ± 5.44 |

**Table S07.** Model selection table is shown for the generalized linear models (with binomial errors) of the number of engorged nymphs per bank vole (nymphs.engorged). The analysis was done on the entire data set of 100 bank voles. Of the 29 models, the top 3 models have 99.999% of the support. The fixed factors are experiment (E), sex (S), and TLR2 genotype. TLR2 genotype was modelled in 8 different ways (geno1, geno2, geno3, geno4, geno5, geno6, geno7, and geno8). For each model, the model structure, degrees of freedom (df), log likelihood, corrected AIC value (AICc), difference in AICc from the top model (Delta), and the support (Weight) are shown.

| Model | Model Structure | df | logLik | AICc | delta | weight |
|---|---|---|---|---|---|---|
| model003 | nymphs.engorged~E | 2 | -109.353 | 222.831 | 0.000 | 67.575 |
| model002 | nymphs.engorged~E+S | 3 | -109.316 | 224.881 | 2.051 | 24.235 |
| model001 | nymphs.engorged~E+S+E:S | 4 | -109.315 | 227.051 | 4.221 | 8.189 |
| model010 | nymphs.engorged~geno2 | 3 | -121.250 | 248.751 | 25.920 | 0.000 |
| model022 | nymphs.engorged~geno5 | 2 | -122.638 | 249.399 | 26.569 | 0.000 |
| model019 | nymphs.engorged~C1+C2+C3 | 3 | -122.122 | 250.494 | 27.663 | 0.000 |
| model009 | nymphs.engorged~geno2+S | 4 | -121.250 | 250.922 | 28.091 | 0.000 |
| model021 | nymphs.engorged~geno5+S | 3 | -122.636 | 251.522 | 28.691 | 0.000 |
| model007 | nymphs.engorged~geno1 | 6 | -119.814 | 252.530 | 29.700 | 0.000 |
| model018 | nymphs.engorged~C1+C2+C3+S | 4 | -122.122 | 252.665 | 29.834 | 0.000 |
| model020 | nymphs.engorged~geno5+S+geno5:S | 4 | -122.602 | 253.625 | 30.794 | 0.000 |
| model016 | nymphs.engorged~geno4 | 3 | -124.254 | 254.758 | 31.928 | 0.000 |
| model006 | nymphs.engorged~geno1+S | 7 | -119.811 | 254.839 | 32.009 | 0.000 |
| model028 | nymphs.engorged~geno7 | 2 | -125.477 | 255.077 | 32.247 | 0.000 |
| model008 | nymphs.engorged~geno2+S+geno2:S | 6 | -121.171 | 255.246 | 32.415 | 0.000 |
| model017 | nymphs.engorged~C1+C2+C3+S+C1:S+C2:S+C3:S | 6 | -121.690 | 256.283 | 33.452 | 0.000 |
| model015 | nymphs.engorged~geno4+S | 4 | -124.165 | 256.751 | 33.920 | 0.000 |
| model027 | nymphs.engorged~geno7+S | 3 | -125.422 | 257.094 | 34.263 | 0.000 |
| model026 | nymphs.engorged~geno7+S+geno7:S | 4 | -124.629 | 257.680 | 34.850 | 0.000 |
| model029 | nymphs.engorged~1 | 1 | -128.286 | 258.613 | 35.782 | 0.000 |
| model014 | nymphs.engorged~geno4+S+geno4:S | 6 | -123.368 | 259.639 | 36.809 | 0.000 |
| model004 | nymphs.engorged~S | 2 | -128.226 | 260.576 | 37.746 | 0.000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| model025 | nymphs.engorged~geno6 | 2 | -128.273 | 260.669 | 37.838 | 0.000 |
| model013 | nymphs.engorged~geno3 | 3 | -128.015 | 262.281 | 39.450 | 0.000 |
| model024 | nymphs.engorged~geno6+S | 3 | -128.218 | 262.686 | 39.856 | 0.000 |
| model005 | nymphs.engorged~geno1+S+geno1:S | 11 | -119.121 | 263.241 | 40.411 | 0.000 |
| model012 | nymphs.engorged~geno3+S | 4 | -127.956 | 264.333 | 41.502 | 0.000 |
| model023 | nymphs.engorged~geno6+S+geno6:S | 4 | -128.130 | 264.682 | 41.851 | 0.000 |
| model011 | nymphs.engorged~geno3+S+geno3:S | 6 | -127.459 | 267.821 | 44.991 | 0.000 |

**Table S08.** Model-averaged parameter estimates are shown for the generalized linear models (with binomial errors) of the number of engorged nymphs per bank vole. The model selection table contains 29 models. The fixed factors are experiment (expt), sex (sex), and TLR2 genotype. TLR2 genotype was modeled in 8 different ways (geno1, geno2, geno3, geno4, geno5, geno6, geno7, and geno8). For each of the 38 parameter estimates, mean1, mean2, the lower limit (LL) and upper limit (UL) of the 95% confidence interval (CI) for mean2, and the statistical significance ($p < 0.05$) are shown. Mean1 is averaged over all the models that contain that parameter in the model selection table. Mean2 is averaged over a subset of models that contain that parameter.

| Parameter | Mean1 | Mean2 | 95% LL | 95% UL | Signif |
|---|---|---|---|---|---|
| Intercept | 0.252 | 0.252 | -0.109 | 0.613 | NS |
| **exptFin** | **1.693** | **1.693** | **1.077** | **2.309** | **P<0.05** |
| sexM | -0.024 | -0.074 | -0.649 | 0.501 | NS |
| exptFin:sexM | -0.002 | -0.019 | -1.203 | 1.165 | NS |
| geno2C2Cx | 0.000 | 0.704 | -1.070 | 2.477 | NS |
| **geno2CxCx** | **0.000** | **-1.106** | **-2.126** | **-0.086** | **P<0.05** |
| **geno5** | **0.000** | **-0.741** | **-1.281** | **-0.202** | **P<0.05** |
| C1 | 0.000 | 0.161 | -0.174 | 0.496 | NS |
| **C2** | **0.000** | **0.808** | **0.272** | **1.344** | **P<0.05** |
| C3 | 0.000 | NA | NA | NA | NA |
| geno1C1C2 | 0.000 | 1.423 | -0.125 | 2.971 | NS |
| geno1C1C3 | 0.000 | -0.533 | -1.225 | 0.159 | NS |
| geno1C2C2 | 0.000 | 0.848 | -0.230 | 1.926 | NS |

| | | | | | |
|---|---|---|---|---|---|
| geno1C2C3 | 0.000 | 15.180 | -2229.585 | 2259.946 | NS |
| geno1C3C3 | 0.000 | -0.282 | -0.952 | 0.389 | NS |
| geno5:sexM | 0.000 | -0.137 | -1.177 | 0.902 | NS |
| geno4C3Cz | 0.000 | -0.187 | -0.877 | 0.503 | NS |
| geno4CzCz | 0.000 | 0.621 | -0.030 | 1.272 | NS |
| geno7 | 0.000 | 0.323 | -0.025 | 0.670 | NS |
| geno2C2Cx:sexM | 0.000 | -0.095 | -3.616 | 3.425 | NS |
| geno2CxCx:sexM | 0.000 | -0.373 | -2.415 | 1.668 | NS |
| C1:sexM | 0.000 | 0.294 | -0.363 | 0.951 | NS |
| C2:sexM | 0.000 | 0.248 | -0.815 | 1.311 | NS |
| C3:sexM | 0.000 | NA | NA | NA | NA |
| geno7:sexM | 0.000 | 0.377 | -0.219 | 0.974 | NS |
| geno4C3Cz:sexM | 0.000 | 0.125 | -1.213 | 1.463 | NS |
| geno4CzCz:sexM | 0.000 | 0.725 | -0.513 | 1.964 | NS |
| geno6 | 0.000 | 0.029 | -0.295 | 0.354 | NS |
| geno3C1Cy | 0.000 | -0.173 | -0.854 | 0.509 | NS |
| geno3CyCy | 0.000 | 0.031 | -0.616 | 0.678 | NS |
| geno1C1C2:sexM | 0.000 | -0.464 | -3.568 | 2.639 | NS |
| geno1C1C3:sexM | 0.000 | -0.687 | -2.097 | 0.722 | NS |
| geno1C2C2:sexM | 0.000 | -0.128 | -2.339 | 2.084 | NS |
| geno1C2C3:sexM | 0.000 | NA | NA | NA | NA |
| geno1C3C3:sexM | 0.000 | -0.679 | -2.045 | 0.687 | NS |
| geno6:sexM | 0.000 | -0.132 | -0.757 | 0.493 | NS |
| geno3C1Cy:sexM | 0.000 | -0.672 | -2.018 | 0.674 | NS |
| geno3CyCy:sexM | 0.000 | -0.378 | -1.673 | 0.916 | NS |

**Table S09.** Model selection table is shown for the generalized linear models with binomial errors of bank vole infection status (infection). The analysis was done on the entire data set of 100 bank voles. Of the 117 models, the top 41 models have 95.0% of the support. The fixed factors are number of engorged *B. afzelii*-infected nymphs, experiment (E), sex (S), and TLR2 genotype. TLR2 genotype is modelled in 7 different ways (geno1, geno2, geno3, geno4, geno5, geno6, and geno7). For each model, the model structure, degrees of freedom (df), log likelihood, corrected AIC value (AICc), difference in AICc from the top model (Delta), and the support (Weight) are shown.

| Model ID | Model Structure | df | logLik | AICc | delta | weight |
|---|---|---|---|---|---|---|
| model014 | infection~E+N | 3 | -21.400 | 49.051 | 0.000 | 17.929 |
| model009 | infection~E+S+N | 4 | -20.592 | 49.605 | 0.554 | 13.591 |
| model011 | infection~E+N+E:N | 4 | -21.400 | 51.222 | 2.171 | 6.055 |
| model307 | infection~geno3+S+N+geno3:N | 7 | -18.038 | 51.292 | 2.242 | 5.845 |
| model008 | infection~E+S+N+S:N | 5 | -20.549 | 51.736 | 2.685 | 4.683 |
| model007 | infection~E+S+N+E:N | 5 | -20.592 | 51.822 | 2.771 | 4.485 |
| model006 | infection~E+S+N+E:S | 5 | -20.592 | 51.822 | 2.771 | 4.485 |
| model302 | infection~geno3+S+N+geno3:S+geno3:N+S:N | 10 | -14.771 | 52.014 | 2.964 | 4.074 |
| model311 | infection~geno3+N+geno3:N | 6 | -19.744 | 52.391 | 3.340 | 3.375 |
| model601 | infection~geno6+S+N+geno6:S+geno6:N+S:N+geno6:S:N | 8 | -17.475 | 52.532 | 3.481 | 3.145 |
| model303 | infection~geno3+S+N+geno3:S+geno3:N | 9 | -16.361 | 52.721 | 3.670 | 2.861 |
| model402 | infection~geno4+S+N+geno4:S+geno4:N+S:N | 10 | -15.469 | 53.410 | 4.359 | 2.028 |
| model305 | infection~geno3+S+N+geno3:N+S:N | 8 | -17.934 | 53.450 | 4.400 | 1.987 |
| model407 | infection~geno4+S+N+geno4:N | 7 | -19.328 | 53.873 | 4.823 | 1.608 |
| model701 | infection~geno7+S+N+geno7:S+geno7:N+S:N+geno7:S:N | 8 | -18.148 | 53.878 | 4.827 | 1.605 |
| model005 | infection~E+S+N+E:N+S:N | 6 | -20.549 | 54.001 | 4.950 | 1.509 |
| model004 | infection~E+S+N+E:S+S:N | 6 | -20.549 | 54.001 | 4.950 | 1.509 |
| model003 | infection~E+S+N+E:S+E:N | 6 | -20.592 | 54.087 | 5.036 | 1.445 |
| model403 | infection~geno4+S+N+geno4:S+geno4:N | 9 | -17.311 | 54.621 | 5.571 | 1.106 |
| model015 | infection~S+N | 3 | -24.246 | 54.742 | 5.692 | 1.042 |
| model607 | infection~geno6+S+N+geno6:N | 5 | -22.248 | 55.134 | 6.084 | 0.856 |
| model018 | infection~N | 2 | -25.668 | 55.459 | 6.408 | 0.728 |

| | | | | | | |
|---|---|---|---|---|---|---|
| model509 | infection~geno5+S+N | 4 | -23.532 | 55.484 | 6.434 | 0.719 |
| model514 | infection~geno5+N | 3 | -24.645 | 55.540 | 6.490 | 0.699 |
| model606 | infection~geno6+S+N+geno6:S | 5 | -22.455 | 55.549 | 6.498 | 0.696 |
| model411 | infection~geno4+N+geno4:N | 6 | -21.337 | 55.577 | 6.527 | 0.686 |
| model706 | infection~geno7+S+N+geno7:S | 5 | -22.623 | 55.885 | 6.834 | 0.588 |
| model614 | infection~geno6+N | 3 | -24.830 | 55.910 | 6.860 | 0.581 |
| model609 | infection~geno6+S+N | 4 | -23.766 | 55.953 | 6.902 | 0.569 |
| model405 | infection~geno4+S+N+geno4:N+S:N | 8 | -19.222 | 56.026 | 6.975 | 0.548 |
| model002 | infection~E+S+N+E:S+E:N+S:N | 7 | -20.549 | 56.315 | 7.264 | 0.474 |
| model611 | infection~geno6+N+geno6:N | 4 | -24.008 | 56.438 | 7.387 | 0.446 |
| model603 | infection~geno6+S+N+geno6:S+geno6:N | 6 | -21.776 | 56.456 | 7.405 | 0.442 |
| model709 | infection~geno7+S+N | 4 | -24.068 | 56.557 | 7.507 | 0.420 |
| model012 | infection~S+N+S:N | 4 | -24.207 | 56.835 | 7.784 | 0.366 |
| model707 | infection~geno7+S+N+geno7:N | 5 | -23.143 | 56.924 | 7.873 | 0.350 |
| model406 | infection~geno4+S+N+geno4:S | 7 | -20.896 | 57.010 | 7.959 | 0.335 |
| model714 | infection~geno7+N | 3 | -25.380 | 57.010 | 7.959 | 0.335 |
| model301 | infection~geno3+S+N+geno3:S+geno3:N+S:N+geno3:S:N | 12 | -14.771 | 57.129 | 8.078 | 0.316 |
| model605 | infection~geno6+S+N+geno6:N+S:N | 6 | -22.246 | 57.396 | 8.345 | 0.276 |
| model306 | infection~geno3+S+N+geno3:S | 7 | -21.199 | 57.615 | 8.564 | 0.248 |
| model409 | infection~geno4+S+N | 5 | -23.515 | 57.668 | 8.618 | 0.241 |
| model508 | infection~geno5+S+N+S:N | 5 | -23.521 | 57.679 | 8.629 | 0.240 |
| model506 | infection~geno5+S+N+geno5:S | 5 | -23.532 | 57.702 | 8.651 | 0.237 |
| model209 | infection~geno2+S+N | 5 | -23.532 | 57.702 | 8.651 | 0.237 |
| model507 | infection~geno5+S+N+geno5:N | 5 | -23.532 | 57.702 | 8.651 | 0.237 |
| model214 | infection~geno2+N | 4 | -24.645 | 57.711 | 8.661 | 0.236 |
| model511 | infection~geno5+N+geno5:N | 4 | -24.645 | 57.711 | 8.661 | 0.236 |
| model703 | infection~geno7+S+N+geno7:S+geno7:N | 6 | -22.424 | 57.752 | 8.701 | 0.231 |
| model604 | infection~geno6+S+N+geno6:S+S:N | 6 | -22.429 | 57.761 | 8.710 | 0.230 |
| model309 | infection~geno3+S+N | 5 | -23.600 | 57.839 | 8.788 | 0.221 |
| model314 | infection~geno3+N | 4 | -24.731 | 57.884 | 8.833 | 0.217 |

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|---|---|---|---|---|---|---|
| model608 | infection~geno6+S+N+S:N | 5 | -23.623 | 57.884 | 8.833 | 0.216 |
| model704 | infection~geno7+S+N+geno7:S+S:N | 6 | -22.622 | 58.148 | 9.097 | 0.190 |
| model414 | infection~geno4+N | 4 | -24.906 | 58.233 | 9.182 | 0.182 |
| model401 | infection~geno4+S+N+geno4:S+geno4:N+S:N+geno4:S:N | 12 | -15.469 | 58.524 | 9.473 | 0.157 |
| model708 | infection~geno7+S+N+S:N | 5 | -23.952 | 58.542 | 9.491 | 0.156 |
| model711 | infection~geno7+N+geno7:N | 4 | -25.116 | 58.653 | 9.602 | 0.147 |
| model001 | infection~E+S+N+E:S+E:N+S:N+E:S:N | 8 | -20.549 | 58.680 | 9.629 | 0.145 |
| model602 | infection~geno6+S+N+geno6:S+geno6:N+S:N | 7 | -21.761 | 58.739 | 9.688 | 0.141 |
| model705 | infection~geno7+S+N+geno7:N+S:N | 6 | -23.138 | 59.179 | 10.128 | 0.113 |
| model404 | infection~geno4+S+N+geno4:S+S:N | 8 | -20.894 | 59.370 | 10.320 | 0.103 |
| model408 | infection~geno4+S+N+S:N | 6 | -23.377 | 59.658 | 10.607 | 0.089 |
| model308 | infection~geno3+S+N+S:N | 6 | -23.428 | 59.760 | 10.709 | 0.085 |
| model304 | infection~geno3+S+N+geno3:S+S:N | 8 | -21.107 | 59.797 | 10.746 | 0.083 |
| model505 | infection~geno5+S+N+geno5:N+S:N | 6 | -23.521 | 59.944 | 10.893 | 0.077 |
| model504 | infection~geno5+S+N+geno5:S+S:N | 6 | -23.521 | 59.944 | 10.893 | 0.077 |
| model208 | infection~geno2+S+N+S:N | 6 | -23.521 | 59.944 | 10.893 | 0.077 |
| model503 | infection~geno5+S+N+geno5:S+geno5:N | 6 | -23.532 | 59.967 | 10.916 | 0.076 |
| model702 | infection~geno7+S+N+geno7:S+geno7:N+S:N | 7 | -22.391 | 60.000 | 10.949 | 0.075 |
| model211 | infection~geno2+N+geno2:N | 6 | -24.645 | 62.194 | 13.143 | 0.025 |
| model502 | infection~geno5+S+N+geno5:S+geno5:N+S:N | 7 | -23.521 | 62.258 | 13.208 | 0.024 |
| model207 | infection~geno2+S+N+geno2:N | 7 | -23.532 | 62.281 | 13.230 | 0.024 |
| model206 | infection~geno2+S+N+geno2:S | 7 | -23.532 | 62.281 | 13.230 | 0.024 |
| model114 | infection~geno1+N | 7 | -23.718 | 62.652 | 13.602 | 0.020 |
| model109 | infection~geno1+S+N | 8 | -22.733 | 63.048 | 13.997 | 0.016 |
| model107 | infection~geno1+S+N+geno1:N | 12 | -17.987 | 63.561 | 14.510 | 0.013 |
| model111 | infection~geno1+N+geno1:N | 11 | -19.552 | 64.103 | 15.052 | 0.010 |
| model205 | infection~geno2+S+N+geno2:N+S:N | 8 | -23.521 | 64.623 | 15.573 | 0.007 |
| model204 | infection~geno2+S+N+geno2:S+S:N | 8 | -23.521 | 64.623 | 15.573 | 0.007 |
| model501 | infection~geno5+S+N+geno5:S+geno5:N+S:N+geno5:S:N | 8 | -23.521 | 64.623 | 15.573 | 0.007 |
| model108 | infection~geno1+S+N+S:N | 9 | -22.619 | 65.237 | 16.187 | 0.005 |

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|---|---|---|---|---|---|---|
| model105 | infection~geno1+S+N+geno1:N+S:N | 13 | -17.883 | 65.999 | 16.948 | 0.004 |
| model016 | infection~E | 2 | -31.343 | 66.811 | 17.760 | 0.002 |
| model203 | infection~geno2+S+N+geno2:S+geno2:N | 9 | -23.532 | 67.063 | 18.012 | 0.002 |
| model106 | infection~geno1+S+N+geno1:S | 12 | -20.253 | 68.092 | 19.041 | 0.001 |
| model013 | infection~E+S | 3 | -30.998 | 68.247 | 19.196 | 0.001 |
| model202 | infection~geno2+S+N+geno2:S+geno2:N+S:N | 10 | -23.521 | 69.513 | 20.462 | 0.001 |
| model102 | infection~geno1+S+N+geno1:S+geno1:N+S:N | 17 | -14.289 | 70.041 | 20.990 | 0.000 |
| model010 | infection~E+S+E:S | 4 | -30.998 | 70.418 | 21.367 | 0.000 |
| model104 | infection~geno1+S+N+geno1:S+S:N | 13 | -20.233 | 70.698 | 21.647 | 0.000 |
| model103 | infection~geno1+S+N+geno1:S+geno1:N | 16 | -16.131 | 70.816 | 21.765 | 0.000 |
| model201 | infection~geno2+S+N+geno2:S+geno2:N+S:N+geno2:S:N | 12 | -23.521 | 74.627 | 25.576 | 0.000 |
| model101 | infection~geno1+S+N+geno1:S+geno1:N+S:N+geno1:S:N | 21 | -14.289 | 82.424 | 33.373 | 0.000 |
| model516 | infection~geno5 | 2 | -39.807 | 83.739 | 34.688 | 0.000 |
| model513 | infection~geno5+S | 3 | -39.351 | 84.952 | 35.902 | 0.000 |
| model216 | infection~geno2 | 3 | -39.807 | 85.865 | 36.814 | 0.000 |
| model510 | infection~geno5+S+geno5:S | 4 | -39.351 | 87.123 | 38.073 | 0.000 |
| model213 | infection~geno2+S | 4 | -39.351 | 87.123 | 38.073 | 0.000 |
| model610 | infection~geno6+S+geno6:S | 4 | -40.023 | 88.467 | 39.416 | 0.000 |
| model710 | infection~geno7+S+geno7:S | 4 | -40.063 | 88.548 | 39.497 | 0.000 |
| model410 | infection~geno4+S+geno4:S | 6 | -37.841 | 88.585 | 39.534 | 0.000 |
| model019 | infection~1 | 1 | -43.967 | 89.975 | 40.924 | 0.000 |
| model310 | infection~geno3+S+geno3:S | 6 | -38.580 | 90.064 | 41.013 | 0.000 |
| model616 | infection~geno6 | 2 | -43.054 | 90.232 | 41.181 | 0.000 |
| model017 | infection~S | 2 | -43.267 | 90.658 | 41.607 | 0.000 |
| model613 | infection~geno6+S | 3 | -42.501 | 91.253 | 42.202 | 0.000 |
| model116 | infection~geno1 | 6 | -39.233 | 91.369 | 42.318 | 0.000 |
| model210 | infection~geno2+S+geno2:S | 6 | -39.351 | 91.606 | 42.555 | 0.000 |
| model716 | infection~geno7 | 2 | -43.873 | 91.870 | 42.819 | 0.000 |
| model316 | infection~geno3 | 3 | -42.887 | 92.023 | 42.973 | 0.000 |
| model416 | infection~geno4 | 3 | -43.064 | 92.379 | 43.328 | 0.000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| model713 | infection~geno7+S | 3 | -43.176 | 92.601 | 43.550 | 0.000 |
| model113 | infection~geno1+S | 7 | -38.763 | 92.744 | 43.693 | 0.000 |
| model413 | infection~geno4+S | 4 | -42.288 | 92.997 | 43.946 | 0.000 |
| model313 | infection~geno3+S | 4 | -42.320 | 93.062 | 44.011 | 0.000 |
| model110 | infection~geno1+S+geno1:S | 11 | -34.655 | 94.310 | 45.259 | 0.000 |

**Table S10.** Model-averaged parameter estimates are shown for the generalized linear models (with binomial errors) of bank vole infection status. The model selection table contains 117 models. The fixed factors are experiment (expt), sex (sex), number of engorged infected nymphs (N), and TLR2 genotype. TLR2 genotype was modeled in 7 different ways (geno1, geno2, geno3, geno4, geno5, geno6, and geno7). For each of the 64 parameter estimates, mean1, mean2, the lower limit (LL) and upper limit (UL) of the 95% confidence interval (CI) for mean2, and the statistical significance ($p < 0.05$) are shown. Mean1 is averaged over all the models that contain that parameter in the model selection table. Mean2 is averaged over a subset of models that contain that parameter. Due to problems with parameter estimation, the standard errors are very large, and none of the 95% confidence intervals overlap zero.

| Parameter | Mean1 | Mean2 | 95% LL | 95% UL | Signif |
|---|---|---|---|---|---|
| Intercept | -0.969 | -0.969 | -5240 | 5238 | NS |
| exptFin | 10.759 | 19.105 | -7862 | 7900 | NS |
| N | 3.982 | 3.983 | -4580 | 4588 | NS |
| sexM | 7.395 | 10.860 | -11761 | 11782 | NS |
| exptFin:N | -0.318 | -2.252 | -6392 | 6388 | NS |
| geno3C1Cy | -5.046 | -26.127 | -16236 | 16183 | NS |
| geno3CyCy | -0.792 | -4.099 | -4964 | 4956 | NS |
| geno3C1Cy:N | 8.488 | 45.986 | -13727 | 13819 | NS |
| geno3CyCy:N | 1.885 | 10.213 | -6550 | 6571 | NS |
| N:sexM | -2.234 | -8.994 | -12553 | 12535 | NS |
| exptFin:sexM | -0.045 | -0.553 | -9689 | 9688 | NS |
| geno3C1Cy:sexM | -4.298 | -56.690 | -32833 | 32719 | NS |
| geno3CyCy:sexM | -4.962 | -65.439 | -29271 | 29140 | NS |
| geno6 | -0.562 | -7.393 | -6606 | 6591 | NS |

| | | | | | |
|---|---|---|---|---|---|
| geno6:sexM | 0.559 | 12.000 | -8419 | 8443 | NS |
| geno6:N | 1.170 | 22.038 | -9459 | 9504 | NS |
| geno6:N:sexM | -1.713 | -54.475 | -15453 | 15344 | NS |
| geno4C3Cz | -1.539 | -21.728 | -19685 | 19641 | NS |
| geno4CzCz | 0.368 | 5.199 | -5255 | 5266 | NS |
| geno4C3Cz:sexM | 0.332 | 8.891 | -24161 | 24179 | NS |
| geno4CzCz:sexM | 2.470 | 66.232 | -25151 | 25283 | NS |
| geno4C3Cz:N | 2.188 | 35.664 | -14122 | 14194 | NS |
| geno4CzCz:N | -0.843 | -13.742 | -7501 | 7474 | NS |
| geno7 | 0.290 | 6.888 | -7769 | 7783 | NS |
| geno7:sexM | -0.284 | -10.571 | -9741 | 9720 | NS |
| geno7:N | -0.590 | -23.405 | -12067 | 12020 | NS |
| geno7:N:sexM | 0.860 | 53.600 | -16311 | 16418 | NS |
| geno5 | -0.484 | -18.416 | -11887 | 11850 | NS |
| geno3C1Cy:N:sexM | 0.008 | 2.682 | -50554 | 50560 | NS |
| geno3CyCy:N:sexM | -0.118 | -37.222 | -43830 | 43755 | NS |
| geno5:sexM | 0.006 | 1.487 | -10574 | 10577 | NS |
| geno2C2Cx | 0.006 | 1.013 | -12416 | 12418 | NS |
| geno2CxCx | -0.103 | -16.070 | -8620 | 8588 | NS |
| geno5:N | 0.039 | 5.855 | -10462 | 10474 | NS |
| geno4C3Cz:N:sexM | 0.060 | 38.469 | -29354 | 29431 | NS |
| geno4CzCz:N:sexM | 0.057 | 36.182 | -24189 | 24262 | NS |
| exptFin:N:sexM | -0.001 | -0.424 | -13160 | 13159 | NS |
| geno2C2Cx:N | 0.000 | 0.020 | -15153 | 15153 | NS |
| geno2CxCx:N | 0.001 | 2.517 | -9628 | 9633 | NS |
| geno2C2Cx:sexM | 0.000 | -0.633 | -18042 | 18041 | NS |
| geno2CxCx:sexM | 0.000 | -0.072 | -12308 | 12308 | NS |
| geno1C1C2 | 0.013 | 18.511 | -4574995 | 4575032 | NS |
| geno1C1C3 | -0.006 | -8.034 | -6425 | 6409 | NS |
| geno1C2C2 | 0.012 | 17.450 | -39530 | 39565 | NS |

| | | | | | |
|---|---|---|---|---|---|
| geno1C2C3 | 0.010 | 13.584 | -40431 | 40458 | NS |
| geno1C3C3 | 0.000 | 0.251 | -818 | 819 | NS |
| geno1C1C2:N | 0.000 | -0.498 | -7399411 | 7399410 | NS |
| geno1C1C3:N | 0.010 | 38.559 | -11909 | 11986 | NS |
| geno1C2C2:N | 0.000 | -0.489 | -28215 | 28214 | NS |
| geno1C2C3:N | 0.000 | NA | NA | NA | NA |
| geno1C3C3:N | 0.001 | 2.570 | -1780 | 1785 | NS |
| geno5:N:sexM | 0.000 | -1.893 | -17771 | 17767 | NS |
| geno1C1C2:sexM | -0.001 | -29.594 | -24246896 | 24246837 | NS |
| geno1C1C3:sexM | -0.001 | -33.719 | -21436 | 21368 | NS |
| geno1C2C2:sexM | -0.001 | -25.682 | -35670 | 35619 | NS |
| geno1C2C3:sexM | 0.000 | NA | NA | NA | NA |
| geno1C3C3:sexM | -0.001 | -37.748 | -18280 | 18204 | NS |
| geno2C2Cx:N:sexM | 0.000 | 0.000 | -38136 | 38136 | NS |
| geno2CxCx:N:sexM | 0.000 | 0.211 | -26508 | 26508 | NS |
| geno1C1C2:N:sexM | 0.000 | 1.278 | -97527 | 97530 | NS |
| geno1C1C3:N:sexM | 0.000 | 2.341 | -32387 | 32391 | NS |
| geno1C2C2:N:sexM | 0.000 | 1.278 | -75446 | 75448 | NS |
| geno1C2C3:N:sexM | 0.000 | NA | NA | NA | NA |
| geno1C3C3:N:sexM | 0.000 | -36.605 | -29219 | 29145 | NS |

**Table S11.** Model selection table is shown for the linear models with normal errors of bank vole infection status (infection). The analysis was done on the entire data set of 100 bank voles. Of the 117 models, the top 6 models have 99.9% of the support. The fixed factors are number of engorged *B. afzelii*-infected nymphs (N), experiment (E), sex (S), and TLR2 genotype. TLR2 genotype is modelled in 7 different ways (geno1, geno2, geno3, geno4, geno5, geno6, and geno7). For each model, the model structure, degrees of freedom (df), log likelihood, corrected AIC value (AICc), difference in AICc from the top model (Delta), and the support (Weight) are shown.

| Model ID | Model Structure | df | logLik | AICc | delta | weight |
|---|---|---|---|---|---|---|
| model011 | infection~E+N+E:N | 5 | -12.287 | 35.212 | 0.000 | 44.374 |
| model007 | infection~E+S+N+E:N | 6 | -11.802 | 36.507 | 1.295 | 23.226 |
| model005 | infection~E+S+N+E:N+S:N | 7 | -11.164 | 37.546 | 2.334 | 13.817 |
| model003 | infection~E+S+N+E:S+E:N | 7 | -11.317 | 37.851 | 2.639 | 11.862 |
| model002 | infection~E+S+N+E:S+E:N+S:N | 8 | -11.032 | 39.646 | 4.434 | 4.835 |
| model001 | infection~E+S+N+E:S+E:N+S:N+E:S:N | 9 | -10.826 | 41.651 | 6.439 | 1.774 |
| model014 | infection~E+N | 4 | -21.428 | 51.277 | 16.065 | 0.014 |
| model606 | infection~geno6+S+N+geno6:S | 6 | -19.780 | 52.463 | 17.251 | 0.008 |
| model009 | infection~E+S+N | 5 | -20.939 | 52.517 | 17.305 | 0.008 |
| model008 | infection~E+S+N+S:N | 6 | -19.871 | 52.645 | 17.433 | 0.007 |
| model402 | infection~geno4+S+N+geno4:S+geno4:N+S:N | 11 | -13.990 | 52.981 | 17.769 | 0.006 |
| model604 | infection~geno6+S+N+geno6:S+S:N | 7 | -18.949 | 53.115 | 17.903 | 0.006 |
| model704 | infection~geno7+S+N+geno7:S+S:N | 7 | -19.177 | 53.572 | 18.359 | 0.005 |
| model303 | infection~geno3+S+N+geno3:S+geno3:N | 10 | -15.571 | 53.614 | 18.402 | 0.004 |
| model006 | infection~E+S+N+E:S | 6 | -20.602 | 54.107 | 18.895 | 0.004 |
| model603 | infection~geno6+S+N+geno6:S+geno6:N | 7 | -19.512 | 54.241 | 19.029 | 0.003 |
| model511 | infection~geno5+N+geno5:N | 5 | -21.875 | 54.388 | 19.176 | 0.003 |
| model403 | infection~geno4+S+N+geno4:S+geno4:N | 10 | -16.020 | 54.513 | 19.300 | 0.003 |
| model004 | infection~E+S+N+E:S+S:N | 7 | -19.858 | 54.934 | 19.722 | 0.002 |
| model706 | infection~geno7+S+N+geno7:S | 6 | -21.044 | 54.991 | 19.779 | 0.002 |
| model401 | infection~geno4+S+N+geno4:S+geno4:N+S:N+geno4:S:N | 13 | -12.445 | 55.122 | 19.910 | 0.002 |
| model302 | infection~geno3+S+N+geno3:S+geno3:N+S:N | 11 | -15.082 | 55.164 | 19.951 | 0.002 |

| | | | | | | |
|---|---|---|---|---|---|---|
| model702 | infection~geno7+S+N+geno7:S+geno7:N+S:N | 8 | -18.794 | 55.171 | 19.959 | 0.002 |
| model602 | infection~geno6+S+N+geno6:S+geno6:N+S:N | 8 | -18.910 | 55.402 | 20.189 | 0.002 |
| model404 | infection~geno4+S+N+geno4:S+S:N | 9 | -17.722 | 55.445 | 20.233 | 0.002 |
| model507 | infection~geno5+S+N+geno5:N | 6 | -21.278 | 55.460 | 20.248 | 0.002 |
| model018 | infection~N | 3 | -24.743 | 55.736 | 20.524 | 0.002 |
| model012 | infection~S+N+S:N | 5 | -22.764 | 56.166 | 20.953 | 0.001 |
| model505 | infection~geno5+S+N+geno5:N+S:N | 7 | -20.530 | 56.277 | 21.065 | 0.001 |
| model411 | infection~geno4+N+geno4:N | 7 | -20.621 | 56.460 | 21.248 | 0.001 |
| model015 | infection~S+N | 4 | -24.026 | 56.473 | 21.261 | 0.001 |
| model405 | infection~geno4+S+N+geno4:N+S:N | 9 | -18.304 | 56.608 | 21.396 | 0.001 |
| model701 | infection~geno7+S+N+geno7:S+geno7:N+S:N+geno7:S:N | 9 | -18.346 | 56.693 | 21.481 | 0.001 |
| model306 | infection~geno3+S+N+geno3:S | 8 | -19.602 | 56.787 | 21.575 | 0.001 |
| model407 | infection~geno4+S+N+geno4:N | 8 | -19.628 | 56.838 | 21.626 | 0.001 |
| model406 | infection~geno4+S+N+geno4:S | 8 | -19.683 | 56.949 | 21.737 | 0.001 |
| model703 | infection~geno7+S+N+geno7:S+geno7:N | 7 | -20.967 | 57.151 | 21.939 | 0.001 |
| model614 | infection~geno6+N | 4 | -24.404 | 57.228 | 22.016 | 0.001 |
| model304 | infection~geno3+S+N+geno3:S+S:N | 9 | -18.663 | 57.327 | 22.115 | 0.001 |
| model311 | infection~geno3+N+geno3:N | 7 | -21.091 | 57.399 | 22.187 | 0.001 |
| model514 | infection~geno5+N | 4 | -24.512 | 57.445 | 22.233 | 0.001 |
| model503 | infection~geno5+S+N+geno5:S+geno5:N | 7 | -21.144 | 57.506 | 22.294 | 0.001 |
| model601 | infection~geno6+S+N+geno6:S+geno6:N+S:N+geno6:S:N | 9 | -18.791 | 57.582 | 22.370 | 0.001 |
| model307 | infection~geno3+S+N+geno3:N | 8 | -20.038 | 57.659 | 22.447 | 0.001 |
| model714 | infection~geno7+N | 4 | -24.710 | 57.841 | 22.629 | 0.001 |
| model608 | infection~geno6+S+N+S:N | 6 | -22.532 | 57.968 | 22.756 | 0.001 |
| model414 | infection~geno4+N | 5 | -23.682 | 58.002 | 22.790 | 0.000 |
| model301 | infection~geno3+S+N+geno3:S+geno3:N+S:N+geno3:S:N | 13 | -13.904 | 58.040 | 22.828 | 0.000 |
| model508 | infection~geno5+S+N+S:N | 6 | -22.591 | 58.085 | 22.873 | 0.000 |
| model609 | infection~geno6+S+N | 5 | -23.775 | 58.187 | 22.975 | 0.000 |
| model408 | infection~geno4+S+N+S:N | 7 | -21.515 | 58.248 | 23.036 | 0.000 |
| model211 | infection~geno2+N+geno2:N | 7 | -21.539 | 58.296 | 23.084 | 0.000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| model708 | infection~geno7+S+N+S:N | 6 | -22.745 | 58.393 | 23.181 | 0.000 |
| model509 | infection~geno5+S+N | 5 | -23.885 | 58.407 | 23.195 | 0.000 |
| model214 | infection~geno2+N | 5 | -23.887 | 58.413 | 23.201 | 0.000 |
| model611 | infection~geno6+N+geno6:N | 5 | -23.893 | 58.423 | 23.211 | 0.000 |
| model502 | infection~geno5+S+N+geno5:S+geno5:N+S:N | 8 | -20.517 | 58.616 | 23.404 | 0.000 |
| model709 | infection~geno7+S+N | 5 | -23.991 | 58.621 | 23.409 | 0.000 |
| model305 | infection~geno3+S+N+geno3:N+S:N | 9 | -19.331 | 58.662 | 23.449 | 0.000 |
| model409 | infection~geno4+S+N | 6 | -22.885 | 58.673 | 23.461 | 0.000 |
| model208 | infection~geno2+S+N+S:N | 7 | -22.019 | 59.255 | 24.043 | 0.000 |
| model314 | infection~geno3+N | 5 | -24.376 | 59.391 | 24.179 | 0.000 |
| model209 | infection~geno2+S+N | 6 | -23.244 | 59.392 | 24.180 | 0.000 |
| model607 | infection~geno6+S+N+geno6:N | 6 | -23.273 | 59.450 | 24.238 | 0.000 |
| model207 | infection~geno2+S+N+geno2:N | 8 | -20.974 | 59.531 | 24.319 | 0.000 |
| model711 | infection~geno7+N+geno7:N | 5 | -24.695 | 60.028 | 24.816 | 0.000 |
| model308 | infection~geno3+S+N+S:N | 7 | -22.429 | 60.075 | 24.863 | 0.000 |
| model605 | infection~geno6+S+N+geno6:N+S:N | 7 | -22.432 | 60.082 | 24.870 | 0.000 |
| model705 | infection~geno7+S+N+geno7:N+S:N | 7 | -22.552 | 60.322 | 25.110 | 0.000 |
| model309 | infection~geno3+S+N | 6 | -23.741 | 60.385 | 25.173 | 0.000 |
| model504 | infection~geno5+S+N+geno5:S+S:N | 7 | -22.590 | 60.398 | 25.186 | 0.000 |
| model506 | infection~geno5+S+N+geno5:S | 6 | -23.757 | 60.418 | 25.206 | 0.000 |
| model205 | infection~geno2+S+N+geno2:N+S:N | 9 | -20.211 | 60.422 | 25.210 | 0.000 |
| model501 | infection~geno5+S+N+geno5:S+geno5:N+S:N+geno5:S:N | 9 | -20.408 | 60.816 | 25.604 | 0.000 |
| model707 | infection~geno7+S+N+geno7:N | 6 | -23.960 | 60.823 | 25.611 | 0.000 |
| model111 | infection~geno1+N+geno1:N | 12 | -17.483 | 62.551 | 27.339 | 0.000 |
| model114 | infection~geno1+N | 8 | -22.502 | 62.586 | 27.374 | 0.000 |
| model109 | infection~geno1+S+N | 9 | -21.716 | 63.432 | 28.220 | 0.000 |
| model107 | infection~geno1+S+N+geno1:N | 13 | -16.653 | 63.539 | 28.326 | 0.000 |
| model108 | infection~geno1+S+N+S:N | 10 | -20.619 | 63.711 | 28.499 | 0.000 |
| model206 | infection~geno2+S+N+geno2:S | 8 | -23.184 | 63.950 | 28.738 | 0.000 |
| model204 | infection~geno2+S+N+geno2:S+S:N | 9 | -22.003 | 64.006 | 28.794 | 0.000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| model203 | infection~geno2+S+N+geno2:S+geno2:N | 10 | -20.835 | 64.141 | 28.929 | 0.000 |
| model103 | infection~geno1+S+N+geno1:S+geno1:N | 17 | -11.410 | 64.283 | 29.071 | 0.000 |
| model106 | infection~geno1+S+N+geno1:S | 13 | -17.278 | 64.789 | 29.577 | 0.000 |
| model105 | infection~geno1+S+N+geno1:N+S:N | 14 | -16.093 | 65.127 | 29.915 | 0.000 |
| model104 | infection~geno1+S+N+geno1:S+S:N | 14 | -16.099 | 65.139 | 29.927 | 0.000 |
| model202 | infection~geno2+S+N+geno2:S+geno2:N+S:N | 11 | -20.181 | 65.362 | 30.150 | 0.000 |
| model102 | infection~geno1+S+N+geno1:S+geno1:N+S:N | 18 | -10.810 | 66.064 | 30.851 | 0.000 |
| model016 | infection~E | 3 | -30.982 | 68.213 | 33.001 | 0.000 |
| model013 | infection~E+S | 4 | -30.637 | 69.695 | 34.483 | 0.000 |
| model201 | infection~geno2+S+N+geno2:S+geno2:N+S:N+geno2:S:N | 13 | -20.136 | 70.505 | 35.293 | 0.000 |
| model010 | infection~E+S+E:S | 5 | -30.293 | 71.223 | 36.011 | 0.000 |
| model101 | infection~geno1+S+N+geno1:S+geno1:N+S:N+geno1:S:N | 22 | -9.519 | 76.181 | 40.969 | 0.000 |
| model516 | infection~geno5 | 3 | -39.221 | 84.692 | 49.480 | 0.000 |
| model610 | infection~geno6+S+geno6:S | 5 | -37.123 | 84.884 | 49.671 | 0.000 |
| model513 | infection~geno5+S | 4 | -38.794 | 86.009 | 50.797 | 0.000 |
| model216 | infection~geno2 | 4 | -38.949 | 86.319 | 51.107 | 0.000 |
| model710 | infection~geno7+S+geno7:S | 5 | -37.947 | 86.533 | 51.321 | 0.000 |
| model019 | infection~1 | 2 | -41.547 | 87.218 | 52.006 | 0.000 |
| model616 | infection~geno6 | 3 | -40.618 | 87.487 | 52.275 | 0.000 |
| model213 | infection~geno2+S | 5 | -38.489 | 87.616 | 52.404 | 0.000 |
| model510 | infection~geno5+S+geno5:S | 5 | -38.645 | 87.929 | 52.717 | 0.000 |
| model017 | infection~S | 3 | -40.848 | 87.945 | 52.733 | 0.000 |
| model310 | infection~geno3+S+geno3:S | 7 | -36.463 | 88.144 | 52.932 | 0.000 |
| model613 | infection~geno6+S | 4 | -40.064 | 88.550 | 53.338 | 0.000 |
| model716 | infection~geno7 | 3 | -41.452 | 89.154 | 53.942 | 0.000 |
| model410 | infection~geno4+S+geno4:S | 7 | -37.014 | 89.246 | 54.034 | 0.000 |
| model316 | infection~geno3 | 4 | -40.505 | 89.431 | 54.219 | 0.000 |
| model416 | infection~geno4 | 4 | -40.565 | 89.551 | 54.339 | 0.000 |
| model713 | infection~geno7+S | 4 | -40.755 | 89.931 | 54.719 | 0.000 |
| model413 | infection~geno4+S | 5 | -39.789 | 90.217 | 55.005 | 0.000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| model313 | infection~geno3+S | 5 | -39.939 | 90.517 | 55.305 | 0.000 |
| model116 | infection~geno1 | 7 | -38.231 | 91.680 | 56.468 | 0.000 |
| model210 | infection~geno2+S+geno2:S | 7 | -38.374 | 91.966 | 56.754 | 0.000 |
| model113 | infection~geno1+S | 8 | -37.755 | 93.092 | 57.879 | 0.000 |
| model110 | infection~geno1+S+geno1:S | 12 | -33.611 | 94.809 | 59.597 | 0.000 |

**Table S12.** Model-averaged parameter estimates are shown for the linear models (with normal errors) of bank vole infection status. The model selection table contains 117 models. The fixed factors are experiment (expt), sex (sex), number of engorged infected nymphs (N), and TLR2 genotype. TLR2 genotype was modeled in 7 different ways (geno1, geno2, geno3, geno4, geno5, geno6, and geno7). For each of the 64 parameter estimates, mean1, mean2, the lower limit (LL) and upper limit (UL) of the 95% confidence interval (CI) for mean2, and the statistical significance ($p < 0.05$) are shown. Mean1 is averaged over all the models that contain that parameter in the model selection table. Mean2 is averaged over a subset of models that contain that parameter.

| Parameter | Mean1 | Mean2 | 95% LL | 95% UL | Signif |
|---|---|---|---|---|---|
| **Intercept** | **0.350** | **0.350** | **0.203** | **0.497** | **P<0.05** |
| **exptFin** | **0.625** | **0.626** | **0.374** | **0.878** | **P<0.05** |
| **N** | **0.298** | **0.298** | **0.201** | **0.395** | **P<0.05** |
| **exptFin:N** | **-0.290** | **-0.290** | **-0.425** | **-0.156** | **P<0.05** |
| sexM | 0.057 | 0.103 | -0.085 | 0.292 | NS |
| N:sexM | -0.013 | -0.063 | -0.193 | 0.066 | NS |
| exptFin:sexM | -0.019 | -0.105 | -0.383 | 0.174 | NS |
| exptFin:N:sexM | 0.002 | 0.085 | -0.189 | 0.358 | NS |
| geno6 | 0.000 | 0.125 | -0.012 | 0.263 | NS |
| **geno6:sexM** | **0.000** | **-0.204** | **-0.360** | **-0.049** | **P<0.05** |
| geno4C3Cz | 0.000 | -0.375 | -0.754 | 0.004 | NS |
| geno4CzCz | 0.000 | -0.141 | -0.478 | 0.195 | NS |
| geno4C3Cz:sexM | 0.000 | 0.207 | -0.169 | 0.584 | NS |
| **geno4CzCz:sexM** | **0.000** | **0.436** | **0.031** | **0.841** | **P<0.05** |

| | | | | | |
|---|---|---|---|---|---|
| geno4C3Cz:N | 0.000 | 0.156 | -0.028 | 0.341 | NS |
| geno4CzCz:N | 0.000 | -0.020 | -0.208 | 0.167 | NS |
| geno7 | 0.000 | -0.078 | -0.220 | 0.063 | NS |
| **geno7:sexM** | **0.000** | **0.196** | **0.023** | **0.368** | **P<0.05** |
| geno3C1Cy | 0.000 | -0.106 | -0.468 | 0.256 | NS |
| geno3CyCy | 0.000 | 0.252 | -0.073 | 0.576 | NS |
| geno3C1Cy:sexM | 0.000 | -0.252 | -0.621 | 0.117 | NS |
| **geno3CyCy:sexM** | **0.000** | **-0.442** | **-0.791** | **-0.093** | **P<0.05** |
| geno3C1Cy:N | 0.000 | 0.172 | -0.010 | 0.353 | NS |
| geno3CyCy:N | 0.000 | -0.023 | -0.194 | 0.148 | NS |
| geno6:N | 0.000 | -0.025 | -0.112 | 0.062 | NS |
| geno5 | 0.000 | -0.246 | -0.576 | 0.085 | NS |
| **geno5:N** | **0.000** | **0.130** | **0.007** | **0.253** | **P<0.05** |
| geno4C3Cz:N:sexM | 0.000 | 0.018 | -0.321 | 0.357 | NS |
| geno4CzCz:N:sexM | 0.000 | -0.207 | -0.525 | 0.112 | NS |
| geno7:N | 0.000 | -0.017 | -0.114 | 0.080 | NS |
| geno7:N:sexM | 0.000 | -0.073 | -0.231 | 0.086 | NS |
| geno5:sexM | 0.000 | 0.045 | -0.242 | 0.331 | NS |
| geno6:N:sexM | 0.000 | 0.040 | -0.129 | 0.208 | NS |
| geno3C1Cy:N:sexM | 0.000 | 0.239 | -0.121 | 0.599 | NS |
| geno3CyCy:N:sexM | 0.000 | 0.217 | -0.126 | 0.559 | NS |
| geno2C2Cx | 0.000 | 0.057 | -0.544 | 0.658 | NS |
| geno2CxCx | 0.000 | -0.247 | -0.875 | 0.381 | NS |
| geno2C2Cx:N | 0.000 | 0.000 | -0.403 | 0.403 | NS |
| geno2CxCx:N | 0.000 | 0.219 | -0.052 | 0.489 | NS |
| geno5:N:sexM | 0.000 | -0.072 | -0.393 | 0.249 | NS |
| geno1C1C2 | 0.000 | 0.320 | -0.308 | 0.949 | NS |
| geno1C1C3 | 0.000 | -0.139 | -0.489 | 0.210 | NS |
| geno1C2C2 | 0.000 | 0.251 | -0.372 | 0.875 | NS |
| geno1C2C3 | 0.000 | -0.143 | -0.804 | 0.518 | NS |

| | | | | | |
|---|---|---|---|---|---|
| geno1C3C3 | 0.000 | 0.126 | -0.166 | 0.418 | NS |
| geno1C1C2:N | 0.000 | -0.161 | -0.625 | 0.303 | NS |
| geno1C1C3:N | 0.000 | 0.176 | -0.005 | 0.357 | NS |
| geno1C2C2:N | 0.000 | -0.168 | -0.457 | 0.120 | NS |
| geno1C2C3:N | 0.000 | NA | NA | NA | NA |
| geno1C3C3:N | 0.000 | -0.015 | -0.191 | 0.160 | NS |
| geno2C2Cx:sexM | 0.000 | -0.018 | -0.635 | 0.600 | NS |
| geno2CxCx:sexM | 0.000 | 0.033 | -0.430 | 0.496 | NS |
| geno1C1C2:sexM | 0.000 | -0.276 | -0.802 | 0.251 | NS |
| geno1C1C3:sexM | 0.000 | -0.252 | -0.594 | 0.090 | NS |
| geno1C2C2:sexM | 0.000 | -0.291 | -0.741 | 0.160 | NS |
| geno1C2C3:sexM | 0.000 | NA | NA | NA | NA |
| **geno1C3C3:sexM** | **0.000** | **-0.474** | **-0.798** | **-0.151** | **P<0.05** |
| geno2C2Cx:N:sexM | 0.000 | 0.000 | -1.119 | 1.119 | NS |
| geno2CxCx:N:sexM | 0.000 | -0.082 | -0.873 | 0.710 | NS |
| geno1C1C2:N:sexM | 0.000 | 0.233 | -0.777 | 1.244 | NS |
| geno1C1C3:N:sexM | 0.000 | 0.256 | -0.114 | 0.626 | NS |
| geno1C2C2:N:sexM | 0.000 | 0.233 | -0.555 | 1.021 | NS |
| geno1C2C3:N:sexM | 0.000 | NA | NA | NA | NA |
| geno1C3C3:N:sexM | 0.000 | 0.190 | -0.185 | 0.565 | NS |

**Table S13.** Model selection table is shown for the linear mixed effects models of the *B. afzelii* spirochete load in bank vole tissues. Spirochete load is standardized per mg of DNA and was log10-transformed to normalize the residuals (log10(spiro.per.mg.DNA)). The analysis was done on the subset of 84 bank voles infected with *B. afzelii*. Of the 117 models, the top 5 models have 99.4% of the support. The fixed factors are experiment (E), organ (O), sex (S), and TLR2 genotype. TLR2 genotype is modelled in 7 different ways (geno1, geno2, geno3, geno4, geno5, geno6, and geno7). For each model, the model structure, degrees of freedom (df), log likelihood, corrected AIC value (AICc), difference in AICc from the top model (Delta), and the support (Weight) are shown.

| Model | Model Structure | df | logLik | AICc | delta | weight |
|---|---|---|---|---|---|---|
| model014 | log10(spiro.per.mg.DNA)~E+O | 7 | -408.954 | 832.250 | 0.000 | 76.959 |
| model011 | log10(spiro.per.mg.DNA)~E+O+E:O | 10 | -407.676 | 836.029 | 3.779 | 11.632 |
| model009 | log10(spiro.per.mg.DNA)~E+S+O | 8 | -410.178 | 836.796 | 4.547 | 7.925 |
| model006 | log10(spiro.per.mg.DNA)~E+S+O+E:S | 9 | -410.639 | 839.830 | 7.580 | 1.739 |
| model007 | log10(spiro.per.mg.DNA)~E+S+O+E:O | 11 | -408.900 | 840.614 | 8.364 | 1.175 |
| model003 | log10(spiro.per.mg.DNA)~E+S+O+E:S+E:O | 12 | -409.361 | 843.688 | 11.438 | 0.253 |
| model008 | log10(spiro.per.mg.DNA)~E+S+O+S:O | 11 | -410.562 | 843.939 | 11.689 | 0.223 |
| model004 | log10(spiro.per.mg.DNA)~E+S+O+E:S+S:O | 12 | -411.023 | 847.012 | 14.762 | 0.048 |
| model005 | log10(spiro.per.mg.DNA)~E+S+O+E:O+S:O | 14 | -409.259 | 847.826 | 15.576 | 0.032 |
| model002 | log10(spiro.per.mg.DNA)~E+S+O+E:S+E:O+S:O | 15 | -409.720 | 850.939 | 18.690 | 0.007 |
| model018 | log10(spiro.per.mg.DNA)~O | 6 | -419.902 | 852.059 | 19.809 | 0.004 |
| model514 | log10(spiro.per.mg.DNA)~geno5+O | 7 | -420.093 | 854.527 | 22.277 | 0.001 |
| model001 | log10(spiro.per.mg.DNA)~E+S+O+E:S+E:O+S:O+E:S:O | 18 | -408.398 | 854.955 | 22.705 | 0.001 |
| model015 | log10(spiro.per.mg.DNA)~S+O | 7 | -420.954 | 856.250 | 24.001 | 0.000 |
| model214 | log10(spiro.per.mg.DNA)~geno2+O | 8 | -419.992 | 856.424 | 24.174 | 0.000 |
| model714 | log10(spiro.per.mg.DNA)~geno7+O | 7 | -421.123 | 856.588 | 24.338 | 0.000 |
| model614 | log10(spiro.per.mg.DNA)~geno6+O | 7 | -421.364 | 857.069 | 24.819 | 0.000 |
| model414 | log10(spiro.per.mg.DNA)~geno4+O | 8 | -420.500 | 857.441 | 25.191 | 0.000 |
| model314 | log10(spiro.per.mg.DNA)~geno3+O | 8 | -421.045 | 858.530 | 26.280 | 0.000 |
| model509 | log10(spiro.per.mg.DNA)~geno5+S+O | 8 | -421.178 | 858.797 | 26.547 | 0.000 |
| model411 | log10(spiro.per.mg.DNA)~geno4+O+geno4:O | 14 | -415.171 | 859.651 | 27.402 | 0.000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| model209 | log10(spiro.per.mg.DNA)~geno2+S+O | 9 | -421.070 | 860.692 | 28.442 | 0.000 |
| model709 | log10(spiro.per.mg.DNA)~geno7+S+O | 8 | -422.189 | 860.818 | 28.569 | 0.000 |
| model609 | log10(spiro.per.mg.DNA)~geno6+S+O | 8 | -422.412 | 861.264 | 29.014 | 0.000 |
| model311 | log10(spiro.per.mg.DNA)~geno3+O+geno3:O | 14 | -416.101 | 861.510 | 29.260 | 0.000 |
| model409 | log10(spiro.per.mg.DNA)~geno4+S+O | 9 | -421.559 | 861.670 | 29.420 | 0.000 |
| model506 | log10(spiro.per.mg.DNA)~geno5+S+O+geno5:S | 9 | -421.879 | 862.310 | 30.060 | 0.000 |
| model309 | log10(spiro.per.mg.DNA)~geno3+S+O | 9 | -422.099 | 862.751 | 30.501 | 0.000 |
| model012 | log10(spiro.per.mg.DNA)~S+O+S:O | 10 | -421.339 | 863.354 | 31.104 | 0.000 |
| model114 | log10(spiro.per.mg.DNA)~geno1+O | 11 | -420.349 | 863.513 | 31.263 | 0.000 |
| model611 | log10(spiro.per.mg.DNA)~geno6+O+geno6:O | 10 | -421.544 | 863.764 | 31.514 | 0.000 |
| model706 | log10(spiro.per.mg.DNA)~geno7+S+O+geno7:S | 9 | -422.635 | 863.823 | 31.573 | 0.000 |
| model407 | log10(spiro.per.mg.DNA)~geno4+S+O+geno4:O | 15 | -416.230 | 863.960 | 31.711 | 0.000 |
| model606 | log10(spiro.per.mg.DNA)~geno6+S+O+geno6:S | 9 | -422.844 | 864.239 | 31.990 | 0.000 |
| model206 | log10(spiro.per.mg.DNA)~geno2+S+O+geno2:S | 11 | -420.902 | 864.618 | 32.369 | 0.000 |
| model711 | log10(spiro.per.mg.DNA)~geno7+O+geno7:O | 10 | -422.354 | 865.386 | 33.136 | 0.000 |
| model307 | log10(spiro.per.mg.DNA)~geno3+S+O+geno3:O | 15 | -417.156 | 865.811 | 33.561 | 0.000 |
| model406 | log10(spiro.per.mg.DNA)~geno4+S+O+geno4:S | 11 | -421.500 | 865.816 | 33.566 | 0.000 |
| model508 | log10(spiro.per.mg.DNA)~geno5+S+O+S:O | 11 | -421.562 | 865.939 | 33.690 | 0.000 |
| model306 | log10(spiro.per.mg.DNA)~geno3+S+O+geno3:S | 11 | -421.645 | 866.104 | 33.855 | 0.000 |
| model511 | log10(spiro.per.mg.DNA)~geno5+O+geno5:O | 10 | -422.779 | 866.235 | 33.985 | 0.000 |
| model109 | log10(spiro.per.mg.DNA)~geno1+S+O | 12 | -421.412 | 867.789 | 35.540 | 0.000 |
| model208 | log10(spiro.per.mg.DNA)~geno2+S+O+S:O | 12 | -421.454 | 867.874 | 35.624 | 0.000 |
| model708 | log10(spiro.per.mg.DNA)~geno7+S+O+S:O | 11 | -422.573 | 867.961 | 35.711 | 0.000 |
| model607 | log10(spiro.per.mg.DNA)~geno6+S+O+geno6:O | 11 | -422.592 | 867.998 | 35.749 | 0.000 |
| model403 | log10(spiro.per.mg.DNA)~geno4+S+O+geno4:S+geno4:O | 17 | -416.172 | 868.268 | 36.018 | 0.000 |
| model608 | log10(spiro.per.mg.DNA)~geno6+S+O+S:O | 11 | -422.796 | 868.407 | 36.157 | 0.000 |
| model408 | log10(spiro.per.mg.DNA)~geno4+S+O+S:O | 12 | -421.943 | 868.852 | 36.602 | 0.000 |
| model303 | log10(spiro.per.mg.DNA)~geno3+S+O+geno3:S+geno3:O | 17 | -416.701 | 869.326 | 37.077 | 0.000 |
| model504 | log10(spiro.per.mg.DNA)~geno5+S+O+geno5:S+S:O | 12 | -422.263 | 869.492 | 37.242 | 0.000 |
| model707 | log10(spiro.per.mg.DNA)~geno7+S+O+geno7:O | 11 | -423.420 | 869.655 | 37.405 | 0.000 |

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|---|---|---|---|---|---|---|
| model308 | log10(spiro.per.mg.DNA)~geno3+S+O+S:O | 12 | -422.483 | 869.933 | 37.683 | 0.000 |
| model211 | log10(spiro.per.mg.DNA)~geno2+O+geno2:O | 14 | -420.581 | 870.470 | 38.220 | 0.000 |
| model507 | log10(spiro.per.mg.DNA)~geno5+S+O+geno5:O | 11 | -423.865 | 870.544 | 38.294 | 0.000 |
| model704 | log10(spiro.per.mg.DNA)~geno7+S+O+geno7:S+S:O | 12 | -423.020 | 871.005 | 38.755 | 0.000 |
| model405 | log10(spiro.per.mg.DNA)~geno4+S+O+geno4:O+S:O | 18 | -416.426 | 871.010 | 38.760 | 0.000 |
| model603 | log10(spiro.per.mg.DNA)~geno6+S+O+geno6:S+geno6:O | 12 | -423.024 | 871.013 | 38.763 | 0.000 |
| model604 | log10(spiro.per.mg.DNA)~geno6+S+O+geno6:S+S:O | 12 | -423.228 | 871.421 | 39.172 | 0.000 |
| model204 | log10(spiro.per.mg.DNA)~geno2+S+O+geno2:S+S:O | 14 | -421.286 | 871.880 | 39.630 | 0.000 |
| model703 | log10(spiro.per.mg.DNA)~geno7+S+O+geno7:S+geno7:O | 12 | -423.867 | 872.699 | 40.450 | 0.000 |
| model404 | log10(spiro.per.mg.DNA)~geno4+S+O+geno4:S+S:O | 14 | -421.884 | 873.077 | 40.827 | 0.000 |
| model304 | log10(spiro.per.mg.DNA)~geno3+S+O+geno3:S+S:O | 14 | -422.029 | 873.366 | 41.116 | 0.000 |
| model305 | log10(spiro.per.mg.DNA)~geno3+S+O+geno3:O+S:O | 18 | -417.623 | 873.405 | 41.155 | 0.000 |
| model503 | log10(spiro.per.mg.DNA)~geno5+S+O+geno5:S+geno5:O | 12 | -424.565 | 874.096 | 41.847 | 0.000 |
| model106 | log10(spiro.per.mg.DNA)~geno1+S+O+geno1:S | 16 | -420.390 | 874.485 | 42.235 | 0.000 |
| model207 | log10(spiro.per.mg.DNA)~geno2+S+O+geno2:O | 15 | -421.659 | 874.818 | 42.568 | 0.000 |
| model108 | log10(spiro.per.mg.DNA)~geno1+S+O+S:O | 15 | -421.796 | 875.092 | 42.842 | 0.000 |
| model605 | log10(spiro.per.mg.DNA)~geno6+S+O+geno6:O+S:O | 14 | -422.973 | 875.254 | 43.004 | 0.000 |
| model402 | log10(spiro.per.mg.DNA)~geno4+S+O+geno4:S+geno4:O+S:O | 20 | -416.368 | 875.402 | 43.152 | 0.000 |
| model705 | log10(spiro.per.mg.DNA)~geno7+S+O+geno7:O+S:O | 14 | -423.785 | 876.879 | 44.629 | 0.000 |
| model302 | log10(spiro.per.mg.DNA)~geno3+S+O+geno3:S+geno3:O+S:O | 20 | -417.169 | 877.004 | 44.754 | 0.000 |
| model111 | log10(spiro.per.mg.DNA)~geno1+O+geno1:O | 26 | -410.264 | 877.071 | 44.821 | 0.000 |
| model505 | log10(spiro.per.mg.DNA)~geno5+S+O+geno5:O+S:O | 14 | -424.355 | 878.018 | 45.768 | 0.000 |
| model602 | log10(spiro.per.mg.DNA)~geno6+S+O+geno6:S+geno6:O+S:O | 15 | -423.404 | 878.309 | 46.059 | 0.000 |
| model203 | log10(spiro.per.mg.DNA)~geno2+S+O+geno2:S+geno2:O | 17 | -421.491 | 878.906 | 46.656 | 0.000 |
| model702 | log10(spiro.per.mg.DNA)~geno7+S+O+geno7:S+geno7:O+S:O | 15 | -424.232 | 879.963 | 47.714 | 0.000 |
| model107 | log10(spiro.per.mg.DNA)~geno1+S+O+geno1:O | 27 | -411.326 | 881.562 | 49.312 | 0.000 |
| model502 | log10(spiro.per.mg.DNA)~geno5+S+O+geno5:S+geno5:O+S:O | 15 | -425.056 | 881.611 | 49.361 | 0.000 |
| model301 | log10(spiro.per.mg.DNA)~geno3+S+O+geno3:S+geno3:O+S:O+geno3:S:O | 26 | -412.656 | 881.856 | 49.607 | 0.000 |
| model104 | log10(spiro.per.mg.DNA)~geno1+S+O+geno1:S+S:O | 19 | -420.774 | 881.953 | 49.703 | 0.000 |
| model205 | log10(spiro.per.mg.DNA)~geno2+S+O+geno2:O+S:O | 18 | -422.147 | 882.452 | 50.202 | 0.000 |

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|---|---|---|---|---|---|---|
| model401 | log10(spiro.per.mg.DNA)~geno4+S+O+geno4:S+geno4:O+S:O+geno4:S:O | 26 | -413.093 | 882.731 | 50.481 | 0.000 |
| model601 | log10(spiro.per.mg.DNA)~geno6+S+O+geno6:S+geno6:O+S:O+geno6:S:O | 18 | -423.670 | 885.498 | 53.248 | 0.000 |
| model202 | log10(spiro.per.mg.DNA)~geno2+S+O+geno2:S+geno2:O+S:O | 20 | -421.979 | 886.624 | 54.374 | 0.000 |
| model501 | log10(spiro.per.mg.DNA)~geno5+S+O+geno5:S+geno5:O+S:O+geno5:S:O | 18 | -424.965 | 888.088 | 55.839 | 0.000 |
| model701 | log10(spiro.per.mg.DNA)~geno7+S+O+geno7:S+geno7:O+S:O+geno7:S:O | 18 | -425.387 | 888.932 | 56.682 | 0.000 |
| model103 | log10(spiro.per.mg.DNA)~geno1+S+O+geno1:S+geno1:O | 31 | -410.304 | 889.135 | 56.885 | 0.000 |
| model105 | log10(spiro.per.mg.DNA)~geno1+S+O+geno1:O+S:O | 30 | -411.840 | 889.778 | 57.529 | 0.000 |
| model201 | log10(spiro.per.mg.DNA)~geno2+S+O+geno2:S+geno2:O+S:O+geno2:S:O | 26 | -417.129 | 890.801 | 58.551 | 0.000 |
| model102 | log10(spiro.per.mg.DNA)~geno1+S+O+geno1:S+geno1:O+S:O | 34 | -410.818 | 897.543 | 65.293 | 0.000 |
| model016 | log10(spiro.per.mg.DNA)~E | 4 | -448.869 | 905.860 | 73.610 | 0.000 |
| model101 | log10(spiro.per.mg.DNA)~geno1+S+O+geno1:S+geno1:O+S:O+geno1:S:O | 46 | -400.595 | 908.151 | 75.902 | 0.000 |
| model013 | log10(spiro.per.mg.DNA)~E+S | 5 | -450.093 | 910.368 | 78.119 | 0.000 |
| model010 | log10(spiro.per.mg.DNA)~E+S+E:S | 6 | -450.554 | 913.364 | 81.114 | 0.000 |
| model019 | log10(spiro.per.mg.DNA)~1 | 3 | -459.817 | 925.707 | 93.457 | 0.000 |
| model516 | log10(spiro.per.mg.DNA)~geno5 | 4 | -460.008 | 928.137 | 95.887 | 0.000 |
| model017 | log10(spiro.per.mg.DNA)~S | 4 | -460.870 | 929.860 | 97.611 | 0.000 |
| model216 | log10(spiro.per.mg.DNA)~geno2 | 5 | -459.907 | 929.996 | 97.746 | 0.000 |
| model716 | log10(spiro.per.mg.DNA)~geno7 | 4 | -461.039 | 930.198 | 97.948 | 0.000 |
| model616 | log10(spiro.per.mg.DNA)~geno6 | 4 | -461.279 | 930.679 | 98.429 | 0.000 |
| model416 | log10(spiro.per.mg.DNA)~geno4 | 5 | -460.415 | 931.013 | 98.763 | 0.000 |
| model316 | log10(spiro.per.mg.DNA)~geno3 | 5 | -460.960 | 932.102 | 99.852 | 0.000 |
| model513 | log10(spiro.per.mg.DNA)~geno5+S | 5 | -461.093 | 932.369 | 100.119 | 0.000 |
| model213 | log10(spiro.per.mg.DNA)~geno2+S | 6 | -460.985 | 934.226 | 101.976 | 0.000 |
| model713 | log10(spiro.per.mg.DNA)~geno7+S | 5 | -462.104 | 934.390 | 102.141 | 0.000 |
| model613 | log10(spiro.per.mg.DNA)~geno6+S | 5 | -462.327 | 934.836 | 102.586 | 0.000 |
| model413 | log10(spiro.per.mg.DNA)~geno4+S | 6 | -461.474 | 935.204 | 102.954 | 0.000 |
| model510 | log10(spiro.per.mg.DNA)~geno5+S+geno5:S | 6 | -461.794 | 935.843 | 103.594 | 0.000 |
| model313 | log10(spiro.per.mg.DNA)~geno3+S | 6 | -462.015 | 936.285 | 104.035 | 0.000 |
| model116 | log10(spiro.per.mg.DNA)~geno1 | 8 | -460.264 | 936.969 | 104.719 | 0.000 |
| model710 | log10(spiro.per.mg.DNA)~geno7+S+geno7:S | 6 | -462.551 | 937.357 | 105.107 | 0.000 |

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|---|---|---|---|---|---|---|
| model610 | log10(spiro.per.mg.DNA)~geno6+S+geno6:S | 6 | -462.759 | 937.773 | 105.523 | 0.000 |
| model210 | log10(spiro.per.mg.DNA)~geno2+S+geno2:S | 8 | -460.817 | 938.074 | 105.825 | 0.000 |
| model410 | log10(spiro.per.mg.DNA)~geno4+S+geno4:S | 8 | -461.416 | 939.272 | 107.022 | 0.000 |
| model310 | log10(spiro.per.mg.DNA)~geno3+S+geno3:S | 8 | -461.560 | 939.560 | 107.311 | 0.000 |
| model113 | log10(spiro.per.mg.DNA)~geno1+S | 9 | -461.327 | 941.206 | 108.956 | 0.000 |
| model110 | log10(spiro.per.mg.DNA)~geno1+S+geno1:S | 13 | -460.305 | 947.740 | 115.491 | 0.000 |

**Table S14.** Model-averaged parameter estimates are shown for the linear mixed effects models (with normal errors) of spirochete loads in the bank vole tissues. The spirochete loads were standardized per mg of DNA before being log10-transformed. The model selection table contains 117 models. The fixed factors are experiment (expt), sex (sex), organ (org), and TLR2 genotype. TLR2 genotype was modelled in 7 different ways (geno1, geno2, geno3, geno4, geno5, geno6, and geno7). For each of the 124 parameter estimates, mean1, mean2, the lower limit (LL) and upper limit (UL) of the 95% confidence interval (CI) for mean2, and the statistical significance ($p < 0.05$) are shown. Mean1 is averaged over all the models that contain that parameter in the model selection table. Mean2 is averaged over a subset of models that contain that parameter.

| Parameter | Mean1 | Mean2 | 95% LL | 95% UL | Signif |
|---|---|---|---|---|---|
| **Intercept** | **2.613** | **2.613** | **2.358** | **2.868** | **P<0.05** |
| **exptFin** | **0.596** | **0.596** | **0.297** | **0.895** | **P<0.05** |
| **orgEar** | **0.913** | **0.913** | **0.586** | **1.240** | **P<0.05** |
| **orgJoint** | **0.981** | **0.981** | **0.733** | **1.229** | **P<0.05** |
| **orgSkin** | **0.846** | **0.846** | **0.581** | **1.110** | **P<0.05** |
| **exptFin:orgEar** | **0.073** | **0.554** | **0.104** | **1.005** | **P<0.05** |
| exptFin:orgJoint | 0.015 | 0.111 | -0.339 | 0.562 | NS |
| exptFin:orgSkin | 0.034 | 0.263 | -0.188 | 0.713 | NS |
| sexM | -0.002 | -0.015 | -0.288 | 0.258 | NS |
| exptFin:sexM | 0.001 | 0.073 | -0.401 | 0.547 | NS |
| orgEar:sexM | 0.000 | 0.118 | -0.331 | 0.566 | NS |
| orgJoint:sexM | 0.001 | 0.389 | -0.059 | 0.838 | NS |
| orgSkin:sexM | 0.001 | 0.251 | -0.198 | 0.699 | NS |

| | | | | | |
|---|---|---|---|---|---|
| geno5 | 0.000 | -0.145 | -0.325 | 0.035 | NS |
| exptFin:orgEar:sexM | 0.000 | 0.361 | -0.543 | 1.265 | NS |
| exptFin:orgJoint:sexM | 0.000 | 0.665 | -0.239 | 1.569 | NS |
| exptFin:orgSkin:sexM | 0.000 | 0.576 | -0.328 | 1.480 | NS |
| geno2C2Cx | 0.000 | -0.148 | -0.687 | 0.391 | NS |
| geno2CxCx | 0.000 | -0.293 | -0.679 | 0.093 | NS |
| geno7 | 0.000 | 0.068 | -0.091 | 0.226 | NS |
| geno6 | 0.000 | 0.043 | -0.126 | 0.212 | NS |
| geno4C3Cz | 0.000 | -0.175 | -0.601 | 0.251 | NS |
| geno4CzCz | 0.000 | 0.073 | -0.292 | 0.439 | NS |
| geno3C1Cy | 0.000 | -0.111 | -0.517 | 0.295 | NS |
| geno3CyCy | 0.000 | 0.065 | -0.299 | 0.429 | NS |
| geno4C3Cz:orgEar | 0.000 | 0.322 | -0.301 | 0.944 | NS |
| geno4CzCz:orgEar | 0.000 | 0.151 | -0.360 | 0.662 | NS |
| **geno4C3Cz:orgJoint** | **0.000** | **-0.630** | **-1.252** | **-0.007** | **P<0.05** |
| geno4CzCz:orgJoint | 0.000 | -0.136 | -0.647 | 0.375 | NS |
| geno4C3Cz:orgSkin | 0.000 | 0.521 | -0.102 | 1.143 | NS |
| geno4CzCz:orgSkin | 0.000 | 0.501 | -0.010 | 1.012 | NS |
| geno3C1Cy:orgEar | 0.000 | 0.250 | -0.334 | 0.835 | NS |
| geno3CyCy:orgEar | 0.000 | 0.102 | -0.436 | 0.640 | NS |
| geno3C1Cy:orgJoint | 0.000 | -0.446 | -1.030 | 0.138 | NS |
| geno3CyCy:orgJoint | 0.000 | 0.186 | -0.352 | 0.724 | NS |
| geno3C1Cy:orgSkin | 0.000 | -0.278 | -0.862 | 0.306 | NS |
| geno3CyCy:orgSkin | 0.000 | -0.432 | -0.970 | 0.106 | NS |
| geno5:sexM | 0.000 | 0.056 | -0.319 | 0.430 | NS |
| geno1C1C2 | 0.000 | 0.048 | -0.456 | 0.553 | NS |
| geno1C1C3 | 0.000 | -0.223 | -0.619 | 0.172 | NS |
| geno1C2C2 | 0.000 | 0.219 | -0.221 | 0.658 | NS |
| geno1C2C3 | 0.000 | 0.254 | -0.992 | 1.500 | NS |
| geno1C3C3 | 0.000 | -0.035 | -0.396 | 0.326 | NS |

| | | | | | |
|---|---|---|---|---|---|
| geno6:orgEar | 0.000 | 0.035 | -0.235 | 0.305 | NS |
| geno6:orgJoint | 0.000 | 0.136 | -0.134 | 0.406 | NS |
| geno6:orgSkin | 0.000 | -0.211 | -0.481 | 0.059 | NS |
| geno7:sexM | 0.000 | 0.155 | -0.154 | 0.464 | NS |
| geno6:sexM | 0.000 | -0.157 | -0.483 | 0.170 | NS |
| geno2C2Cx:sexM | 0.000 | 0.061 | -1.053 | 1.176 | NS |
| geno2CxCx:sexM | 0.000 | 0.113 | -0.699 | 0.926 | NS |
| geno7:orgEar | 0.000 | 0.066 | -0.190 | 0.323 | NS |
| geno7:orgJoint | 0.000 | -0.020 | -0.277 | 0.237 | NS |
| geno7:orgSkin | 0.000 | 0.241 | -0.016 | 0.497 | NS |
| geno4C3Cz:sexM | 0.000 | -0.021 | -0.786 | 0.744 | NS |
| geno4CzCz:sexM | 0.000 | 0.255 | -0.374 | 0.883 | NS |
| geno3C1Cy:sexM | 0.000 | -0.474 | -1.190 | 0.241 | NS |
| geno3CyCy:sexM | 0.000 | -0.358 | -1.018 | 0.302 | NS |
| geno5:orgEar | 0.000 | -0.133 | -0.434 | 0.169 | NS |
| geno5:orgJoint | 0.000 | -0.138 | -0.439 | 0.163 | NS |
| geno5:orgSkin | 0.000 | -0.069 | -0.371 | 0.232 | NS |
| geno2C2Cx:orgEar | 0.000 | -0.101 | -1.009 | 0.807 | NS |
| geno2CxCx:orgEar | 0.000 | -0.254 | -0.902 | 0.395 | NS |
| geno2C2Cx:orgJoint | 0.000 | -0.324 | -1.231 | 0.584 | NS |
| geno2CxCx:orgJoint | 0.000 | -0.297 | -0.945 | 0.351 | NS |
| geno2C2Cx:orgSkin | 0.000 | -0.392 | -1.300 | 0.516 | NS |
| geno2CxCx:orgSkin | 0.000 | -0.193 | -0.841 | 0.455 | NS |
| geno1C1C2:sexM | 0.000 | -0.400 | -1.425 | 0.625 | NS |
| geno1C1C3:sexM | 0.000 | -0.500 | -1.303 | 0.303 | NS |
| geno1C2C2:sexM | 0.000 | -0.413 | -1.339 | 0.514 | NS |
| geno1C3C3:sexM | 0.000 | -0.423 | -1.158 | 0.312 | NS |
| geno1C1C2:orgEar | 0.000 | 0.181 | -0.648 | 1.011 | NS |
| geno1C1C3:orgEar | 0.000 | 0.283 | -0.366 | 0.931 | NS |
| geno1C2C2:orgEar | 0.000 | 0.320 | -0.400 | 1.041 | NS |

| | | | | | |
|---|---|---|---|---|---|
| geno1C2C3:orgEar | 0.000 | 0.525 | -1.529 | 2.578 | NS |
| geno1C3C3:orgEar | 0.000 | -0.025 | -0.618 | 0.567 | NS |
| geno1C1C2:orgJoint | 0.000 | -0.226 | -1.055 | 0.604 | NS |
| geno1C1C3:orgJoint | 0.000 | -0.550 | -1.199 | 0.099 | NS |
| geno1C2C2:orgJoint | 0.000 | 0.206 | -0.514 | 0.927 | NS |
| geno1C2C3:orgJoint | 0.000 | 0.750 | -1.304 | 2.803 | NS |
| geno1C3C3:orgJoint | 0.000 | 0.152 | -0.441 | 0.745 | NS |
| geno1C1C2:orgSkin | 0.000 | -0.575 | -1.404 | 0.254 | NS |
| geno1C1C3:orgSkin | 0.000 | -0.138 | -0.787 | 0.510 | NS |
| geno1C2C2:orgSkin | 0.000 | -0.086 | -0.807 | 0.635 | NS |
| geno1C2C3:orgSkin | 0.000 | 0.296 | -1.758 | 2.349 | NS |
| **geno1C3C3:orgSkin** | **0.000** | **-0.635** | **-1.228** | **-0.042** | **P<0.05** |
| geno3C1Cy:orgEar:sexM | 0.000 | -0.458 | -1.626 | 0.710 | NS |
| geno3CyCy:orgEar:sexM | 0.000 | -0.239 | -1.316 | 0.838 | NS |
| **geno3C1Cy:orgJoint:sexM** | **0.000** | **-1.445** | **-2.613** | **-0.278** | **P<0.05** |
| geno3CyCy:orgJoint:sexM | 0.000 | -0.821 | -1.898 | 0.256 | NS |
| geno3C1Cy:orgSkin:sexM | 0.000 | -0.661 | -1.829 | 0.507 | NS |
| geno3CyCy:orgSkin:sexM | 0.000 | 0.022 | -1.055 | 1.098 | NS |
| geno4C3Cz:orgEar:sexM | 0.000 | -0.564 | -1.818 | 0.689 | NS |
| geno4CzCz:orgEar:sexM | 0.000 | 0.315 | -0.715 | 1.345 | NS |
| geno4C3Cz:orgJoint:sexM | 0.000 | -0.659 | -1.913 | 0.594 | NS |
| geno4CzCz:orgJoint:sexM | 0.000 | 0.234 | -0.796 | 1.264 | NS |
| geno4C3Cz:orgSkin:sexM | 0.000 | -0.845 | -2.099 | 0.409 | NS |
| geno4CzCz:orgSkin:sexM | 0.000 | 0.271 | -0.759 | 1.301 | NS |
| geno6:orgEar:sexM | 0.000 | -0.091 | -0.633 | 0.451 | NS |
| geno6:orgJoint:sexM | 0.000 | -0.332 | -0.874 | 0.210 | NS |
| geno6:orgSkin:sexM | 0.000 | 0.064 | -0.478 | 0.606 | NS |
| geno5:orgEar:sexM | 0.000 | -0.103 | -0.737 | 0.531 | NS |
| geno5:orgJoint:sexM | 0.000 | 0.161 | -0.474 | 0.795 | NS |
| geno5:orgSkin:sexM | 0.000 | -0.300 | -0.935 | 0.334 | NS |

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|---|---|---|---|---|---|
| geno7:orgEar:sexM | 0.000 | 0.196 | -0.323 | 0.714 | NS |
| geno7:orgJoint:sexM | 0.000 | 0.217 | -0.302 | 0.735 | NS |
| geno7:orgSkin:sexM | 0.000 | 0.189 | -0.329 | 0.708 | NS |
| geno2C2Cx:orgEar:sexM | 0.000 | 0.849 | -1.028 | 2.727 | NS |
| geno2CxCx:orgEar:sexM | 0.000 | 0.023 | -1.346 | 1.392 | NS |
| geno2C2Cx:orgJoint:sexM | 0.000 | 0.056 | -1.822 | 1.934 | NS |
| geno2CxCx:orgJoint:sexM | 0.000 | 0.352 | -1.017 | 1.721 | NS |
| geno2C2Cx:orgSkin:sexM | 0.000 | 0.239 | -1.638 | 2.117 | NS |
| geno2CxCx:orgSkin:sexM | 0.000 | -0.427 | -1.796 | 0.942 | NS |
| geno1C1C2:orgEar:sexM | 0.000 | 0.493 | -1.169 | 2.156 | NS |
| geno1C1C3:orgEar:sexM | 0.000 | -0.911 | -2.213 | 0.392 | NS |
| geno1C2C2:orgEar:sexM | 0.000 | -0.374 | -1.877 | 1.129 | NS |
| geno1C3C3:orgEar:sexM | 0.000 | -0.297 | -1.488 | 0.895 | NS |
| geno1C1C2:orgJoint:sexM | 0.000 | -1.219 | -2.882 | 0.443 | NS |
| **geno1C1C3:orgJoint:sexM** | **0.000** | **-1.540** | **-2.843** | **-0.237** | **P<0.05** |
| geno1C2C2:orgJoint:sexM | 0.000 | -1.076 | -2.579 | 0.427 | NS |
| geno1C3C3:orgJoint:sexM | 0.000 | -0.733 | -1.925 | 0.458 | NS |
| geno1C1C2:orgSkin:sexM | 0.000 | 0.237 | -1.426 | 1.899 | NS |
| geno1C1C3:orgSkin:sexM | 0.000 | -1.102 | -2.404 | 0.201 | NS |
| geno1C2C2:orgSkin:sexM | 0.000 | 0.104 | -1.399 | 1.607 | NS |
| geno1C3C3:orgSkin:sexM | 0.000 | -0.182 | -1.374 | 1.009 | NS |

**Section 8 – Materials and methods of the *Borrelia*-specific IgG antibody response**

At 35 days post-infection, a blood sample was collected from each animal (from the saphenous vein or the retro orbital sinus) to determine its infection status using ELISA. The SERION® ELISA classic *Borrelia burgdorferi* IgG/IgM immunoassay plates (Ruwag, Germany) were used to detect *Borrelia*-specific IgG antibodies following the manufacturer's instructions. The wells were incubated with serum samples diluted to 1:100 in PBS for 45 min at room temperature. Each well was rinsed three times with 0.1% PBS-Tween before and after adding the secondary antibody for 45 min at room temperature. The secondary antibody was goat anti-*Mus musculus* IgG conjugated to horseradish peroxidase (Promega, Switzerland) and diluted 1:5000 in PBS. A volume of 100 µl of TMB product was added to each well. The absorbance was measured at 652 nm every 2 minutes for a total duration of 60 minutes. The strength of the *Borrelia*-IgG antibody response was calculated as the integral of the absorbance versus time curve over the total duration of 60 minutes of absorbance measurements. Positive controls were serum samples from *Mus musculus* mice that had been experimentally infected with *B. afzelii* isolate NE4049 via tick bite. Negative controls were serum samples from *M. musculus* mice that had never been exposed to ticks.

**Section 8 – Statistical analysis of the *Borrelia*-specific IgG antibody response**

Infection with *B. afzelii* induces a strong *Borrelia*-specific antibody response [19]. To test whether our ELISA assay could discriminate between uninfected bank voles and *B. afzelii*-infected bank voles, the *Borrelia*-specific IgG antibody response was compared between these two groups with a t-test. This analysis was only done for the Swiss infection experiment, which had 16 uninfected individuals and 34 infected individuals. This analysis was not done for the Finnish infection experiment, because all 50 individuals were infected with *B. afzelii*.

To test whether the TLR2 genotype and sex influenced the *Borrelia*-specific IgG antibody response, we analysed the subset of 84 bank voles that were infected with *B. afzelii*. We applied the same model selection analysis approach that we had applied for the other response variables. The antibody response against *B. afzelii* was log10-transformed to normalize the residuals. This response variable was modelled using linear models with normal errors. The fixed factors were experiment, sex, and the eight different ways to code TLR2 genotype. Each vole occurred only once in the analysis so it was not necessary to include bank vole ID as a random effect.

**Section 8 – Results of the *Borrelia*-specific IgG antibody response**

As shown in previous studies [20], the goat anti-*Mus musculus* IgG secondary antibody was effective at binding the IgG antibodies of *M. glareolus*. The bank voles infected with *B. afzelii* had a *Borrelia*-specific IgG antibody response that was 5.2 times higher than that of the uninfected bank voles and this difference was highly significant ($t = -11.978$, $df = 33.593$, p-value $< 0.001$). This result shows that the ELISA was highly effective for distinguishing between *B. afzelii*-infected and uninfected bank voles.

For the model selection analysis of the number of engorged nymphs per bank vole, the top 3 models had 99.997% of the support; the remaining 26 models had 0.003% of the support (**Table S15**). The support for individual factors was as follows: experiment (99.997%), sex (37.5%), and TLR2 genotype (<0.003% for 24 models). The model-averaged parameter estimates found that the bank voles in the Finnish experiment had a significantly higher antibody titre than the bank voles in the Swiss experiment (**Figure S3**; **Table S16**).

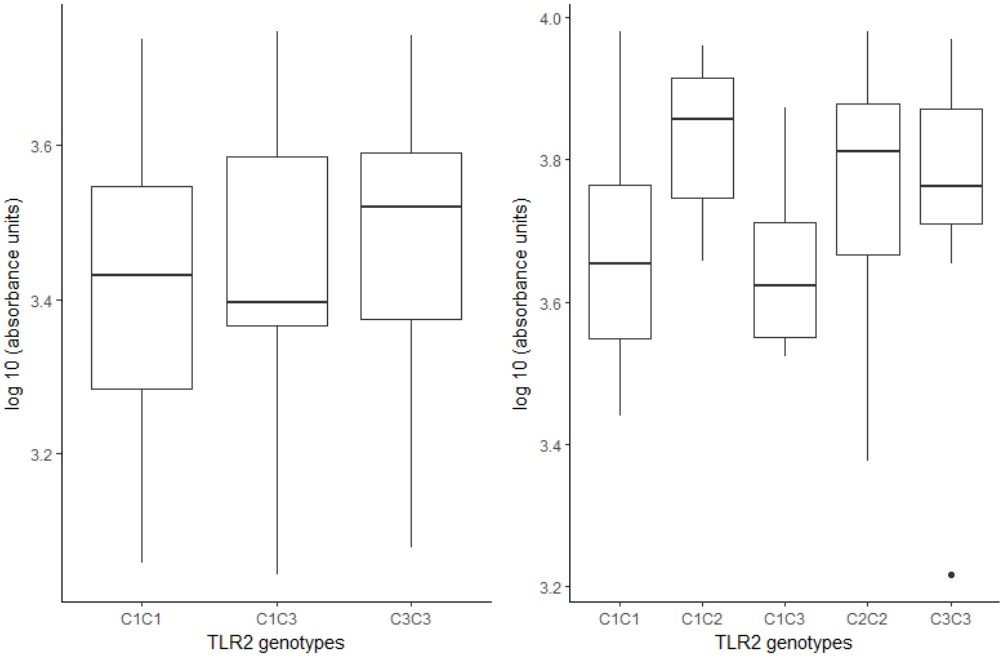


**Figure S3.** The TLR2 genotype of the bank vole has no effect on the *Borrelia*-specific IgG antibody response. The left and right panels refer to the Swiss infection experiment (n = 34 bank voles) and the Finnish infection experiment (n = 50), respectively. The *Borrelia*-specific IgG antibody response was measured using commercially available Lyme borreliosis ELISA plates. Absorbance values were integrated over the 60 minutes of the ELISA assay and log10-transformed to improve their fit to the normal distribution.

**Table S15.** Model selection table is shown for the linear models of antibody titres against *B. afzelii* (Ab.titre). The analysis was done on the subset of 84 bank voles infected with *B. afzelii*. Of the 29 models, the top 3 models have 99.997% of the support. The fixed factors are experiment (E), sex (S), and TLR2 genotype. TLR2 genotype was modelled in 8 different ways (geno1, geno2, geno3, geno4, geno5, geno6, geno7, and geno8). For each model, the model structure, degrees of freedom (df), log likelihood, corrected AIC value (AICc), difference in AICc from the top model (Delta), and the support (Weight) are shown.

| Model ID | Model Structure | df | logLik | AICc | delta | weight |
|---|---|---|---|---|---|---|
| model003 | Ab.titre~E | 3 | 24.871 | -43.442 | 0.000 | 62.525 |
| model002 | Ab.titre~E+S | 4 | 25.032 | -41.558 | 1.883 | 24.383 |
| model001 | Ab.titre~E+S+E:S | 5 | 25.542 | -40.314 | 3.128 | 13.089 |
| model010 | Ab.titre~geno2 | 4 | 15.243 | -21.979 | 21.462 | 0.001 |
| model009 | Ab.titre~geno2+S | 5 | 15.377 | -19.985 | 23.457 | 0.001 |
| model022 | Ab.titre~geno5 | 3 | 12.888 | -19.477 | 23.965 | 0.000 |
| model021 | Ab.titre~geno5+S | 4 | 13.074 | -17.641 | 25.800 | 0.000 |
| model008 | Ab.titre~geno2+S+geno2:S | 7 | 16.530 | -17.586 | 25.856 | 0.000 |
| model019 | Ab.titre~C1+C2+C3 | 4 | 13.031 | -17.555 | 25.887 | 0.000 |
| model020 | Ab.titre~geno5+S+geno5:S | 5 | 13.713 | -16.657 | 26.784 | 0.000 |
| model007 | Ab.titre~geno1 | 7 | 15.911 | -16.349 | 27.093 | 0.000 |
| model018 | Ab.titre~C1+C2+C3+S | 5 | 13.200 | -15.631 | 27.810 | 0.000 |
| model006 | Ab.titre~geno1+S | 8 | 16.033 | -14.145 | 29.297 | 0.000 |
| model017 | Ab.titre~C1+C2+C3+S+C1:S+C2:S+C3:S | 7 | 14.321 | -13.168 | 30.274 | 0.000 |
| model025 | Ab.titre~geno6 | 3 | 7.910 | -9.519 | 33.922 | 0.000 |
| model029 | Ab.titre~1 | 2 | 6.209 | -8.271 | 35.171 | 0.000 |
| model028 | Ab.titre~geno7 | 3 | 7.047 | -7.793 | 35.649 | 0.000 |
| model013 | Ab.titre~geno3 | 4 | 8.125 | -7.744 | 35.698 | 0.000 |
| model024 | Ab.titre~geno6+S | 4 | 7.918 | -7.330 | 36.112 | 0.000 |
| model005 | Ab.titre~geno1+S+geno1:S | 12 | 17.751 | -7.108 | 36.334 | 0.000 |
| model016 | Ab.titre~geno4 | 4 | 7.422 | -6.338 | 37.104 | 0.000 |
| model004 | Ab.titre~S | 3 | 6.218 | -6.135 | 37.306 | 0.000 |
| model027 | Ab.titre~geno7+S | 4 | 7.083 | -5.660 | 37.782 | 0.000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| model023 | Ab.titre~geno6+S+geno6:S | 5 | 8.206 | -5.642 | 37.800 | 0.000 |
| model012 | Ab.titre~geno3+S | 5 | 8.132 | -5.495 | 37.947 | 0.000 |
| model015 | Ab.titre~geno4+S | 5 | 7.443 | -4.117 | 39.325 | 0.000 |
| model026 | Ab.titre~geno7+S+geno7:S | 5 | 7.232 | -3.695 | 39.746 | 0.000 |
| model011 | Ab.titre~geno3+S+geno3:S | 7 | 8.657 | -1.839 | 41.602 | 0.000 |
| model014 | Ab.titre~geno4+S+geno4:S | 7 | 8.153 | -0.832 | 42.610 | 0.000 |

**Table S16.** Model-averaged parameter estimates are shown for the linear models of antibody titres against *B. afzelii*. The model selection table contains 29 models. The fixed factors are experiment (expt), sex (sex), and TLR2 genotype. TLR2 genotype was modeled in 8 different ways (geno1, geno2, geno3, geno4, geno5, geno6, geno7, and geno8). For each of the 38 parameter estimates, mean1, mean2, the lower limit (LL) and upper limit (UL) of the 95% confidence interval (CI) for mean2, and the statistical significance ($p < 0.05$) are shown. Mean1 is averaged over all the models that contain that parameter in the model selection table. Mean2 is averaged over a subset of models that contain that parameter.

| Parameter | Mean1 | Mean2 | 95% LL | 95% UL | Signif |
|---|---|---|---|---|---|
| **Intercept** | **3.457** | **3.457** | **3.387** | **3.528** | **P<0.05** |
| **exptFin** | **0.280** | **0.280** | **0.189** | **0.371** | **P<0.05** |
| sexM | -0.002 | -0.006 | -0.113 | 0.102 | NS |
| exptFin:sexM | -0.011 | -0.081 | -0.243 | 0.082 | NS |
| geno2C2Cx | 0.000 | 0.073 | -0.136 | 0.281 | NS |
| **geno2CxCx** | **0.000** | **-0.196** | **-0.347** | **-0.046** | **P<0.05** |
| **geno5** | **0.000** | **-0.124** | **-0.201** | **-0.047** | **P<0.05** |
| geno2C2Cx:sexM | 0.000 | 0.257 | -0.116 | 0.630 | NS |
| geno2CxCx:sexM | 0.000 | 0.174 | -0.097 | 0.446 | NS |
| C1 | 0.000 | -0.018 | -0.083 | 0.047 | NS |
| **C2** | **0.000** | **0.113** | **0.039** | **0.187** | **P<0.05** |
| C3 | 0.000 | NA | NA | NA | NA |
| geno5:sexM | 0.000 | 0.072 | -0.057 | 0.202 | NS |
| **geno1C1C2** | **0.000** | **0.284** | **0.113** | **0.456** | **P<0.05** |
| geno1C1C3 | 0.000 | 0.001 | -0.133 | 0.136 | NS |
| **geno1C2C2** | **0.000** | **0.210** | **0.059** | **0.362** | **P<0.05** |
| geno1C2C3 | 0.000 | 0.371 | -0.053 | 0.795 | NS |
| geno1C3C3 | 0.000 | 0.057 | -0.066 | 0.180 | NS |
| C1:sexM | 0.000 | 0.056 | -0.067 | 0.178 | NS |
| C2:sexM | 0.000 | -0.051 | -0.191 | 0.090 | NS |
| C3:sexM | 0.000 | NA | NA | NA | NA |
| geno6 | 0.000 | 0.057 | -0.007 | 0.121 | NS |
| geno7 | 0.000 | 0.035 | -0.025 | 0.095 | NS |
| geno3C1Cy | 0.000 | 0.092 | -0.042 | 0.225 | NS |
| geno3CyCy | 0.000 | 0.116 | -0.007 | 0.240 | NS |
| geno1C1C2:sexM | 0.000 | 0.044 | -0.303 | 0.390 | NS |
| geno1C1C3:sexM | 0.000 | 0.039 | -0.233 | 0.310 | NS |
| geno1C2C2:sexM | 0.000 | -0.203 | -0.516 | 0.110 | NS |
| geno1C2C3:sexM | 0.000 | NA | NA | NA | NA |
| geno1C3C3:sexM | 0.000 | -0.091 | -0.339 | 0.158 | NS |
| geno4C3Cz | 0.000 | -0.039 | -0.187 | 0.109 | NS |
| geno4CzCz | 0.000 | 0.056 | -0.063 | 0.174 | NS |
| geno6:sexM | 0.000 | -0.045 | -0.164 | 0.075 | NS |
| geno7:sexM | 0.000 | 0.031 | -0.084 | 0.146 | NS |
| geno3C1Cy:sexM | 0.000 | 0.033 | -0.232 | 0.297 | NS |
| geno3CyCy:sexM | 0.000 | -0.078 | -0.322 | 0.166 | NS |
| geno4C3Cz:sexM | 0.000 | 0.163 | -0.121 | 0.447 | NS |
| geno4CzCz:sexM | 0.000 | 0.053 | -0.180 | 0.287 | NS |

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# Chapter Two

*Lyme disease pathogens do not suppress the development of anti-tick immunity in a rodent reservoir host*

**Lyme disease pathogens do not suppress the development of anti-tick immunity in a rodent reservoir host**

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## Abstract

Vector-borne pathogens manipulate their vertebrate hosts to enhance their transmission to arthropod vectors. The ability of vertebrate hosts to develop acquired immunity against arthropod vectors represents an existential threat for both the vector and the pathogen. The purpose of the study was to test whether the tick-borne spirochete bacterium *Borrelia afzelii* could suppress the development of acquired immunity to its tick vector *Ixodes ricinus* in the bank vole *Myodes glareolus*, which is an important host for both the tick and the pathogen. We created a group of *B. afzelii*-infected bank voles and an uninfected control group by exposing lab-reared animals to infected or uninfected ticks. At 1, 2, and 3 months post-infection, all bank voles were infested with larval *I. ricinus* ticks. The bank voles developed a strong antibody response against tick salivary gland extract proteins. This anti-tick immunity had negative effects on tick fitness traits including engorged larval weight, unfed nymphal weight, larva-to-nymph moulting time and larva-to-nymph moulting success. Infection with *B. afzelii* did not suppress the development of acquired immunity against *I. ricinus* ticks. The development of anti-tick immunity was strongly correlated with a dramatic temporal decline in both the bacterial abundance in the host ear tissues and the host-tick transmission success of *B. afzelii*. Our study suggests that the development of anti-tick immunity in bank voles has important consequences for the density of infected ticks and the risk of Lyme borreliosis.

## Introduction

Many pathogens exploit the obligate blood-feeding habits of hematophagous arthropods to achieve transmission between vertebrate hosts. These vector-borne pathogens and their arthropod vectors have co-evolved an intimate relationship that is characterized by specificity and adaptive complexity (Woolhouse et al. 2001, Lefèvre et al. 2006, Lefevre and Thomas 2008). Specificity is shown by the fact that many vector-borne pathogens are only effectively acquired and transmitted by one or a few closely related vector species (Dolan et al. 1998, Zeidner et al. 2002, Heylen et al. 2014). Examples of adaptive complexity in pathogens include increasing the vector's production of immunosuppressive saliva molecules to enhance their own transmission (Ramamoorthi et al. 2005), protecting the vector against changes in the environment (Neelakanta et al. 2010, Herrmann and Gern 2015), and manipulating the odor profile of the vertebrate host to increase host-vector encounter rates and pathogen transmission (Lacroix et al. 2005, Cornet et al. 2013, De Moraes et al. 2014). Thus, whenever it suits their interests, we expect vector-borne pathogens to enhance the fitness of their arthropod vectors.

From the perspective of the pathogen and vector, the ability of the vertebrate host to develop immunity or resistance against the vector represents a shared existential threat. Compared to other hematophagous arthropods, this threat of inducing acquired resistance is particularly important for ticks, probably because they feed on the host in large numbers and for longer periods of time (several days). Studies on a variety of host-tick systems have shown that this anti-tick immunity or acquired tick resistance can reduce tick fitness components including measures of larval engorgement, duration of attachment, percentage of recovered larvae, and larva-to-nymph molting success (Randolph 1979, 1991, Dizij and Kurtenbach 1995, Ogden et al. 2002). Acquired immunity against ticks also enhances the host defense against tick-borne pathogens (Wikel et al. 1997). Acquired immunity against ticks can impair transmission of tick-borne pathogens, establishment of the pathogen within the host, and alter the cutaneous environment at the tick attachment site (Wikel et al. 1997, Nazario et al. 1998, Narasimhan et al. 2007). In summary, the development of acquired immunity in the vertebrate host against ticks reduces the fitness of both the tick vector and the tick-borne pathogen.

Ticks and specifically tick salivary glands and tick saliva have evolved to cope with this existential threat of host resistance. Tick saliva contains nearly 500 proteins and peptides that belong to at least 25 different protein families (Ribeiro et al. 2006). These tick saliva molecules have powerful anti-haemostatic, anti-inflammatory and immunomodulatory properties (Simo et al. 2017) that seek to subvert the host immune system at every turn. This powerful pharmacopeia also enhances the transmission of pathogens, a phenomenon known as saliva-assisted transmission (Nuttall and Labuda 2004). Conversely, a number of tick-borne pathogens have developed a variety of strategies that allow them to evade or suppress the host immune system (Woldehiwet 2008). In turn, pathogen-induced immunosuppression may reduce the development of anti-tick immunity and thereby enhance tick feeding success.

To study whether vector-borne pathogens can suppress the development of acquired immunity against the arthropod vector in the vertebrate host, we used a tick-borne spirochete bacterium belonging to the *B. burgdorferi* sensu lato (sl) genospecies complex and that causes Lyme borreliosis (LB) in humans as a model system (Kurtenbach et al. 2006, Stanek and Reiter 2011). Our study was motivated by a recent demonstration that *B. burgdorferi* sensu stricto (ss) could suppress the development of acquired immunity in the lab mouse *Mus musculus* (Elsner et al. 2015). We wanted to test whether other *B. burgdorferi* sl genospecies could induce immunosuppression in their natural rodent reservoir hosts. The most common LB system in Europe includes the bacterium *B. afzelii*, the tick *Ixodes ricinus*, and rodent hosts such as the bank vole (*Myodes glareolus*) (Kurtenbach et al. 1994, Talleklint and Jaenson 1995, Humair et al. 1999, van Duijvendijk et al. 2015). The bank vole is a good host for *B. afzelii* (Kurtenbach

et al. 1994, Talleklint and Jaenson 1995, Humair et al. 1999), but it develops acquired immunity against *Ixodes* ticks (Dizij and Kurtenbach 1995, Humair et al. 1999). Previous workers have suggested that this anti-tick immunity in bank voles reduces the production of *B. afzelii*-infected ticks (Humair et al. 1999).

The purpose of this study was to test whether *B. afzelii* could inhibit the development of acquired anti-tick immunity in bank voles. We created *B. afzelii*-infected bank voles and uninfected bank voles, and repeatedly infested them with larval *I. ricinus* ticks to induce acquired anti-tick immunity. We predicted that bank voles would develop a strong antibody response against tick salivary gland proteins, which would decrease tick fitness. We expected that infection with *B. afzelii* would suppress the development of acquired anti-tick immunity in bank voles. We therefore expected tick fitness to be higher on the *B. afzelii*-infected bank voles compared to the uninfected controls.

## Materials and Methods

### Bank voles, *Ixodes ricinus* ticks and *Borrelia afzelii*:

The bank voles came from a laboratory colony at the University of Neuchâtel. This colony was descended from bank voles that had been captured at a field site near Neuchâtel, Switzerland in the summer of 2014 (Gomez-Chamorro et al. 2019). All animals used in the study were lab-born, 5 to 10 weeks old at the start of the study, and not infected with *B. afzelii*. During the experiment, bank voles were maintained in individual cages and were given food and water *ad libitum*. The *I. ricinus* ticks came from the University of Neuchâtel colony, and larval *I. ricinus* ticks were also purchased from Insect Services (Germany). Bank voles were experimentally infected via tick-bite with *B. afzelii* isolate NE4049. This isolate was originally obtained from an *I. ricinus* tick in Neuchâtel, it has multi-locus sequence type 679, *ospC* major group (oMG) A10, and strain ID number 1887 in the *Borrelia* MLST database. We used isolate NE4049 because our previous work has shown that it is highly infectious to *I. ricinus* ticks and rodents including bank voles (Tonetti et al. 2015, Jacquet et al. 2016, Belli et al. 2017, Gomez-Chamorro et al. 2019). *B. afzelii* strains carrying oMG A10 also have the highest frequency in wild *I. ricinus* populations near Neuchâtel (Durand et al. 2017b).

### Ethics statement and animal experimentation permits:

The study was performed at the University of Neuchâtel and followed the Swiss legislation on animal experimentation. The commission that is part of the 'Service de la Consommation et des Affaires Vétérinaires (SCAV)' of canton Vaud evaluated and approved the ethics of this part of the study. The SCAV of canton Neuchâtel issued the animal experimentation permits for the study (NE1/2017) and for the maintenance of the *I. ricinus* tick colony at the University of Neuchâtel (NE5/2014).

### Creation of nymphs infected with *B. afzelii*:

Larval *I. ricinus* ticks were fed on BALB/c mice (*Mus musculus*) that had been previously infected with *B. afzelii* isolate NE4049 via tick-bite. The resultant engorged larval ticks were collected, stored in individual Eppendorf tubes, and allowed to molt into nymphs. A random sample of 30 nymphs was tested for *B. afzelii* using qPCR, and the prevalence of infection was 93.3% (28 infected/ 30 total). To create uninfected control nymphs, larval *I. ricinus* ticks were fed on uninfected BALB/c mice and the resultant engorged larvae were allowed to molt into nymphs.

**Experimental design:**

Forty voles were randomly assigned to one of two experimental groups: uninfected control (n = 20) and infected with *B. afzelii* (n = 20). Each vole in the uninfected control group was infested with 5 uninfected nymphs, whereas each vole in the *B. afzelii*-infected group was infested with 5 *B. afzelii*-infected nymphs. During the nymphal challenge, voles were anesthetized with a ketamine/xylazine cocktail via intraperitoneal injection (1:2:9 xylazine: ketamine: PBS; dose of 5 µl per 10 g of vole body mass). Six of the 40 voles died under anesthesia leaving 34 animals. To prevent the voles from removing the nymphs via grooming, the nymphs were placed in a plastic capsule attached to a shaved area on the back of the vole. Each vole was fitted with a collar to prevent the animal from removing the capsule. The capsules were checked on a daily basis and the engorged nymphs were collected and tested for their *B. afzelii* infection status. The infection status of the voles was confirmed using additional diagnostic criteria (see below).

**Larval infestations:**

Voles were infested with larval ticks on three separate occasions at 27, 54, and 84 days post-infection (PI). For the first and second infestation, we used larvae from our *I. ricinus* tick colony at the University of Neuchâtel. For the third infestation, we had a shortage of larvae at our colony, and we therefore purchased them from Insect Services (Germany). For each infestation, the voles were anaesthetized and approximately 100–150 larvae were put on each vole. For the first infestation, voles were anesthetized with the ketamine/xylazine cocktail described previously. Unexpectedly, of the remaining 34 voles, another 6 died under anesthesia leaving 28 animals. For the second and third infestations, voles were anesthetized with 2% isoflurane using the Combi-vet® anesthesia system (Rothacher Medical, Switzerland) and no additional animals were lost. For each vole, an ear biopsy and a blood sample (from the saphenous vein) were collected on four occasions at -1, 26, 51, and 106 days PI. Ear tissue biopsies and serum samples were kept at –20°C for future analysis. On day 106 PI, the voles were euthanized with $CO_2$ and exsanguinated. Of the 28 voles that survived to the end of the study, there were 14 in the uninfected control group and 14 in the *B. afzelii*-infected group.

**Tick phenotypes:**

Engorged larval ticks detached from voles after 2–4 days of blood feeding. For each of the 84 combinations of infestation and vole, a maximum of 50 engorged larvae were collected and placed in individual 1.5 ml Eppendorf tubes. The tubes contained a strip of moistened paper towel to maintain a high humidity. The engorged larvae were kept in an incubator (Sanyo, Japan) with a day-night cycle of 16 hours of light and 8 hours of darkness, and a relative humidity of 85% (section 1 in the supplementary material). For each infestation and each vole, a maximum of 20 engorged larvae were randomly selected and weighed within 5 days of drop off (total of 1680 engorged larvae). Once the engorged larval ticks started molting, they were checked two or three times per week until the percentage of molted nymphs reached 80%. The median molting time was defined as the date when 50% of the engorged larvae had molted into nymphs. At two weeks after the median molting time, the ticks that had been weighed as larvae were weighed again as nymphs. At four weeks after the median molting time, 10 nymphs were randomly selected for each of the 84 combinations of vole and infestation (total of 840 nymphs), and these nymphs were frozen at –80°C to assess their *B. afzelii* infection status.

Different tick phenotypes were used to determine the effect of acquired anti-tick immunity and *B. afzelii* infection on tick fitness. The tick phenotypes included: engorged larval weight, unfed nymphal weight, moulting time and moulting success. Moulting time was defined as the number of days between the detachment of the engorged larvae and the molting to unfed nymphs. Moulting success was the percentage of engorged larvae that moulted into flat nymphs.

**ELISA and qPCR:**

The serum samples of the voles were tested for the presence of *B. afzelii*-specific IgG antibodies using a commercial ELISA assay as previously described (Gomez-Chamorro et al. 2019). The DNA was extracted from the vole tissue samples, the engorged nymphs, and the flat nymphs as previously described (Jacquet et al. 2015, Gomez-Chamorro et al. 2019). All the DNA extractions were eluted into 65 µl of water. The vole ear tissue samples and the ticks were tested for infection with *B. afzelii* using a qPCR assay that targets a 132 bp fragment of the *flagellin* gene as previously described (Gomez-Chamorro et al. 2019). For each vole tissue sample and tick, 3.0 µl of DNA template was used in the qPCR reaction.

**Culture of viable *B. afzelii* spirochetes from xenodiagnostic nymphs:**

To demonstrate that the voles transmitted a viable *B. afzelii* infection to the *I. ricinus* ticks, flat nymphs (that had fed as larvae on the voles) were cultured in BSK-H media (Bio&Sell). Six nymphs were randomly selected for each bank vole (total of 168 nymphs). Each tick was washed with 70% ethanol, cut in half, and placed in an individual 1.5 ml Eppendorf tube containing BSK-H medium. The culture tubes were kept at 34°C in an incubator and were screened for motile *B. afzelii* spirochetes over a period of 4 weeks using a dark field microscope.

**Tick salivary gland extract protein solution:** Tick salivary gland extract (SGE) proteins were obtained from the salivary glands of engorged adult female *I. ricinus* ticks from our laboratory colony. A total of 28 female and 5 male adult ticks were fed on one rabbit. After 96 hours of blood feeding, the 24 engorged female ticks were removed from the rabbit with tweezers, washed with 70% ethanol, and washed with 1x PBS. The tick salivary glands were dissected on ice using a dissecting microscope, washed with 1x PBS, pooled and homogenized in 400 µl of 1x PBS, and kept at –80 °C. The tick salivary gland cells were lysed by keeping the suspension on ice and conducting three short pulse sonication sessions (5 s pulse, 30 s pause) using a SKAN sonifier 450 (SKAN AG, Switzerland). Soluble antigens were obtained after centrifugation at 20,000 g for 15 min. The protein concentration was determined using the Bradford assay. The concentration of the tick SGE sample was 1.62 mg/ml for a total of 648 µg of tick SGE.

**Tick salivary gland extract ELISA:**

The IgG antibody response of the voles against tick SGE was measured using a homemade ELISA. 96-well tissue culture plates (Fisher Scientific, Switzerland) were coated overnight at 4°C with 1 µg of tick SGE protein per well. Wells were washed 3 times with PBS-Tween 0.1% between each change of solution. The wells were incubated with a BSA 2% blocking solution for 2 hours, followed by the bank vole serum samples (diluted 1:100 in 1x PBS) for 45 minutes, and the secondary antibody for 45 minutes (diluted 1:5000 in 1x PBS). The secondary antibody was a goat anti-*Mus musculus* IgG conjugated to horseradish peroxidase (Promega). After adding 100 µl of TMB product (Promega) to each well, the absorbance was measured at 652 nm every 2 minutes for one hour using a plate reader (Synergy HT, Multi-detection plate reader, Bio-Tek, United States). The strength of the IgG antibody response against tick SGE was determined for each serum sample by calculating the area under the curve of the optical density (OD) versus time.

**Infection status of bank voles:**

A vole was considered to be infected with *B. afzelii*, if it tested positive for one or more of four criteria: (1) optical density > 500 absorbance units indicating the presence of *B. afzelii*-specific IgG antibodies, (2) spirochete load in the ear tissue biopsy > 0, (3) spirochete load in the xenodiagnostic nymphs > 0, and (4) culture of live spirochetes from xenodiagnostic

nymphs. Thirteen of the 14 voles in the *B. afzelii*-infected group tested positive for 3 or 4 criteria, and these 13 voles were therefore considered as infected with *B. afzelii* (section 2 in the supplementary material). The vole that tested negative for all five criteria was excluded from the analysis. As expected, all of the 14 voles in the control group tested negative for the 4 infection criteria. All statistical analyses are therefore based on 13 *B. afzelii*-infected voles and 14 uninfected voles.

**Statistical Analysis**

All statistical analyses were done in R version 1.0.143 (R Development Core Team 2015-08-14). Means are reported with their 95% confidence intervals (95% CI). Normal versus binomial response variables were modelled using linear mixed effects models (LMMs) with normal errors versus generalized linear mixed effects models (GLMMs) with binomial errors, respectively. To determine the statistical significance of each fixed factor, pairs of models that differed in the fixed factor of interest were compared using log-likelihood ratio (LLR) tests. To calculate the p-value, the change in deviance between models is compared to the Chi-square distribution. Bank vole identity was included as a random factor.

**Tick life history traits**

The response variables included four tick life history traits: (1) engorged larval weight, (2) unfed nymphal weight, (3) moulting duration, and (4) moulting success. The engorged larval weight and unfed nymphal weight were log10-transformed to improve the normality of the residuals. All response variables followed a normal distribution, except moulting success, which followed a binomial distribution. Each response variable was modelled as a function of two fixed factors: infection status (two levels: uninfected control and infected with *B. afzelii*), infestation number (two levels: first and third infestation), and their interaction. For infestation number, only the first and third infestations were used in the analysis to simplify the presentation of the results (full analyses in section 4 of the supplementary material). We also modelled the tick SGE-specific IgG antibody levels in the voles (log10-transformed) using an LMM model. The fixed factors were infection status, day of blood sample (four levels: -1, 26, 51, and 106 days PI), and their interaction. The background variation in the OD values was corrected for each plate as follows: the mean OD of the BSA controls for a given plate was subtracted from the OD values of the bank vole serum samples in that plate.

**Relationships between *B. afzelii* infection phenotypes in the infected voles**

For the subset of infected voles, we had measured three *B. afzelii* infection phenotypes around the time of each larval infestation (27, 54, and 84 days PI): (1) tick SGE-specific IgG antibody levels of the vole (hereafter the anti-tick IgG response), (2) spirochete load in the vole ear biopsy (number of spirochetes in the entire ear tissue biopsy), and (3) host-to-tick transmission (percentage of flat nymphs that were infected with *B. afzelii*). The spirochete loads in the ear biopsies were log10-transformed to improve their fit to a normal distribution. The anti-tick IgG response and the log10(ear tissue spirochete load) followed a normal distribution, whereas host-to-tick transmission followed a binomial distribution. We conducted two separate analyses that were based on causal relationships between these three variables. First, we modelled the ear biopsy spirochete load as a function of the anti-tick IgG response. Second, we modelled host-to-tick transmission as a function of the anti-tick IgG response and the ear biopsy spirochete load.

## Results

### Collection of engorged nymphal ticks from the voles:

We collected 47 engorged nymphs from the 14 voles in the control group (mean = 3.36, range = 1–5 engorged nymphs per vole). As expected, none of these nymphs tested positive for *B. afzelii*. We collected 46 engorged nymphs from the 14 voles in the *B. afzelii*-infected group (mean = 3.29, range = 0–5 engorged nymphs per vole). Our qPCR assay found that 58.7% (27/46) of the engorged nymphs were infected with *B. afzelii*. For 12 of the 14 voles in the infected group, we collected at least 1 engorged *B. afzelii*-infected nymph (mean = 1.93, range = 1–4; Table S1 in the supplementary material). These data show that the voles in the infected group and the control group were infested with similar numbers of nymphs.

### Tick phenotypes

### Weight of blood-engorged larvae:

The engorged larval weight decreased from the first to the third infestation (Figure 2). The LMM analysis of engorged larval weight found a significant interaction between infection status and infestation number (LLR: $\chi^2 = 8.316$, df = 2, $p = 0.016$). Infestation number had a significant effect on the engorged larval weight (LLR: $\chi^2 = 45.996$, df = 2, $p < 0.001$), but the effect of infection status was not significant (LLR: $\chi^2 = 0.028$, df = 1, $p = 0.866$). For the first, second, and third larval infestation, the mean engorged larval weight was 483 µg (95% CI: 475–492), 452 µg (95% CI: 444–461), and 456 µg (95% CI: 448–465), respectively. Compared to the first infestation, the mean engorged larval weight in the second and third infestation was reduced by 6.4% ($p = 0.006$) and 5.6% ($p < 0.001$), respectively.

### Weight of the unfed nymphs:

The flat nymphal weight decreased from the first to the third infestation (Figure 3). The LMM analysis of flat nymphal weight found no significant interaction between infection status and infestation number (LRR: $\chi^2 = 5.299$, df = 2, $p = 0.071$). After removing the interaction, infestation number had a significant effect on the flat nymphal weight (LRR: $\chi^2 = 74.807$, df = 2, $p < 0.001$), but the effect of infection status was not significant (LRR: $\chi^2 = 0.656$, df = 1, $p = 0.418$). For the first, second, and third larval infestation, the mean nymphal weight was 188 µg (95% CI: 183–192), 166 µg (95% CI: 162–171), and 177 µg (95% CI: 172–181), respectively. Compared to the first infestation, the mean flat nymphal weight in the second and third infestation was reduced by 11.7% ($p < 0.001$) and 5.9% ($p < 0.001$), respectively.

### Moulting time of engorged larval ticks to nymphs:

The larva-to-nymph moulting time was defined as the number of days between the drop-off of the engorged larval ticks and the moult into flat nymphs. The moulting time was monitored for a total of 2611 engorged larval ticks, of which 83.80% (2188/2611) moulted into nymphs. The moulting time decreased over the three successive larval infestations (Figure 4). The LMM analysis of moulting time found no significant interaction between infection status and infestation number (LRR: $\chi^2 = 4.341$, df = 2, $p = 0.114$). After removing the interaction, infestation number had a significant effect on the moulting time (LRR: $\chi^2 = 246.7$, df = 2, $p < 0.001$), but the effect of infection status was not significant (LRR: $\chi^2 = 0.004$, df = 1, $p = 0.946$). For the first, second, and third larval infestation, the mean moulting time was 51 days (95% CI: 50–53), 47 days (95% CI: 45–49), and 35 days (95% CI: 33–37), respectively. Compared to the first infestation, the mean moulting time in the second and third infestation was reduced by 7.8% ($p < 0.001$) and 31.4% ($p < 0.001$), respectively.

**Larva-to-nymph moulting success:**

The moulting success was defined as the percentage of engorged larval ticks that moulted into flat nymphs. The moulting success decreased over the three successive larval infestations (Figure 5). The GLMM analysis of moulting success found no significant interaction between infection status and infestation number (LRR: $\chi^2 = 2.276$, df = 2, p = 0.320). After removing the interaction, infestation number had a significant effect on the moulting success (LRR: $\chi^2 = 35.121$, df = 2, $p < 0.001$), but the effect of infection status was not significant (LRR: $\chi^2 = 1.489$, df = 1, p = 0.222). For the first, second, and third larval infestation, the mean moulting success was 88% (95% CI: 86–90%), 87% (95% CI: 84–91%), and 78% (95% CI: 75–81%), respectively. Compared to the first infestation, the mean moulting success in the second and third infestation was reduced by 1.1% (p = 0.883) and 11.4% ($p < 0.001$), respectively.

**Bank voles developed a strong IgG antibody response against the salivary gland extract of *I. ricinus* ticks:**

The tick SGE-specific IgG antibody levels (hereafter the anti-tick IgG response) increased over the duration of the study (from day -1 to day 106; Figure 6) and this change was significant (LME: $\chi^2 = 166.61$, df = 1, $p < 0.001$). For day -1, day 26, day 51, and day 106, the mean anti-tick IgG response (measured in absorbance units) was 1176 (95% CI: 1040–1328), 1559 (95% CI: 1370–1774), 2391 (95% CI: 2112–2706), and 6351 (95% CI: 5635–7157), respectively. Compared to day -1, the mean anti-tick IgG response on day 106 had increased 5.4-fold.

The LMM analysis of the anti-tick IgG response found that the interaction between infection status and the day of the blood sample was significant (LRR: $\chi^2 = 12.638$, df = 3, p = 0.005). We therefore tested the effect of infection status on the anti-tick IgG response separately for each day. This analysis found a significant difference between the infected group and the control group at day -1 (t-test: df = 25, t = 6.818, $p < 0.001$), day 26 (t-test: df = 22, t = 2.218, p = 0.037), day 51 (t-test: df = 24, t = 3.1036, p = 0.004), but not at day 106 (t-test: df = 25, t = 0.117, p = 0.908). Thus, the anti-tick IgG response reached similar levels in both groups of bank voles at the end of the experiment. On day 26 and day 51, the mean anti-tick IgG response of the infected group was 17.4% and 45.6% higher than the control group, respectively (Figure 6). On day -1, the mean anti-tick IgG response of the infected group was 55.6% lower than the control group (Figure 6).

***Borrelia* transmission**

**Spirochete load in the bank vole ear biopsy:**

The spirochete loads in the vole ear biopsies (2 mm diameter) decreased over the three infestations (section 5 in the supplementary material). For the first, second, and third larval infestation, the mean spirochete load in the ear biopsy (measured in number of spirochetes) was 5883 (95% CI: 1516–22834), 202 (95% CI: 52–786), and 13 (95% CI: 3–50), respectively. The anti-tick IgG response had a significant negative effect on the spirochete load in the ear biopsy (Figure 7; LME: $\chi^2 = 17.748$, df = 1, $p < 0.001$; slope ± S.E. = -2.26 ± 0.537).

**Host-to-tick transmission of *Borrelia afzelii*:**

After combining the three infestations, host-to-tick transmission was 38.5% (144 infected nymphs/ 374 total nymphs). Host-to-tick transmission decreased over the three infestations (section 6 in the supplementary material). For the first, second, and third larval infestations, the host-to-tick transmission was 59.4% (76/128), 35.6% (42/118), and 20.3% (26/128), respectively. For 30.8% (4/13) of the voles, the host-to-tick transmission was 0% by

the third infestation. The anti-tick IgG response had a significant negative effect (GLME: $\chi^2$ = 5.407, df = 1, p = 0.020; logit slope ± S.E. = -1.49 ± 0.639), whereas the log10-transformed spirochete load in the ear biopsy had a significant positive effect on host-to-tick transmission (Figure 8; GLME: $\chi^2$ = 10.369, df = 1, p = 0.001; logit slope ± S.E. = 0.52 ± 0.162).

## Discussion

**Ability of *B. burgdorferi* sl to suppress acquired immunity in the vertebrate host:** Infection with *B. afzelii* in a natural rodent host did not prevent the development of acquired immunity against larval *I. ricinus* ticks and its negative effects on tick life history traits. Our study contrasts with a recent study showing that *B. burgdorferi* ss suppressed the development of acquired immunity in laboratory *Mus musculus* mice (Elsner et al. 2015). Specifically, this study found that infected mice immunized with the influenza vaccine did not develop protective antibodies against the influenza virus (Elsner et al. 2015). The mechanism of immunosuppression is that *B. burgdorferi* ss migrates to the mouse lymph nodes where it inhibits the development of long-lived plasma cells and memory B cells, (Elsner et al. 2015). Differences between our study and (Elsner et al. 2015) include the genospecies of *B. burgdorferi* sl (*B. burgdorferi* ss versus *B. afzelii*), the antigen (influenza vaccine versus live ticks), the rodent host (lab mice versus natural host), and mode of infection (needle inoculation versus tick bite). Other tick-borne pathogens such as *Anaplasma phagocytophilum* and *Babesia microti* have been documented to induce immunosuppression in vertebrate hosts (Randolph 1994, Woldehiwet 2008). Interestingly, the protozoan parasite *B. microti* was able to induce immunosuppression in laboratory mice, but not in the bank vole or any known natural reservoir host (Gray and Phillips 1983, Randolph 1994). These studies suggest that the ability of *B. burgdorferi* sl to suppress the adaptive immune system may depend on the identity of the vertebrate host.

From an ecological and public health perspective, pathogen-induced immunosuppression is important because it can facilitate the infection and emergence of opportunistic pathogens (Vaumourin et al. 2015). For example, the ability of HIV to suppress the immune system has led to the re-emergence of tuberculosis in human populations in the developing world (Porter and McAdam 1994). Community ecology studies often find positive associations between *B. burgdorferi* sl genospecies and other tick-borne pathogens (Vaumourin et al. 2015). For example, the emergence of *B. microti* was strongly associated with the prevalence of *B. burgdorferi* ss in the northeast United States (Diuk-Wasser et al. 2016, Walter et al. 2016). In addition, *B. burgdorferi* sl genospecies and strains within a genospecies have been found to be positively associated (Andersson et al. 2013, Herrmann et al. 2013a, Durand et al. 2017a). In summary, *B. burgdorferi* sl-induced immunosuppression in the vertebrate host can facilitate mixed infections and the emergence of other tick-borne pathogens.

**Development of acquired immunity against ticks in vertebrate hosts:** Our study found that repeated infestations with larval *I. ricinus* ticks caused the bank voles to develop a strong IgG antibody response against the SGE proteins of *I. ricinus*. The development of this anti-tick immunity was correlated with a reduction in tick fitness including the size of the engorged larval ticks and the resultant flat nymphs, the duration of the larva-to-nymph molt, and the larvae-to-nymph molting success. One complication with our study was that the *I. ricinus* larvae for the first and second infestation came from the University of Neuchâtel colony and the larvae for the third infestation came from Insect Services (Germany). This happened because the University of Neuchâtel colony failed to produce sufficient numbers of larvae for the third infestation. As a result, any differences in tick phenotype between the first and third infestation could be due to innate differences between these two tick colonies rather than acquired anti-tick immunity. However, the statistically significant decreases in engorged larval

weight and flat nymphal weight between the first and second larval infestation (which used larvae from the University of Neuchâtel colony) suggests that our bank voles developed acquired anti-tick immunity after the first larval infestation.

Our results are in agreement with previous studies showing that bank voles develop anti-tick immunity to larval ticks of *I. ricinus* or *I. trianguliceps* (Randolph 1994, Dizij and Kurtenbach 1995, Humair et al. 1999). These studies found that anti-tick immunity reduced the percentage of fully engorged larvae, larval engorgement index, duration of attachment, percentage of recovered larvae, and larva-to-nymph molting success (Randolph 1994, Dizij and Kurtenbach 1995, Humair et al. 1999). The mechanism by which anti-tick immunity reduces tick fitness is that it impairs tick blood feeding and therefore reduces the quality and quantity of the blood meal (Simo et al. 2017). With respect to body size, previous studies on *I. ricinus* have shown that larger nymphs have higher fat reserves (Herrmann et al. 2013b) and that such nymphs can quest for longer periods of time, which increases their chances of finding a host in the field (Crooks and Randolph 2006, Herrmann and Gern 2012, Herrmann and Gern 2015). Body size also influences female fecundity, with larger female ticks laying larger clutches of eggs (Gray 1981). The reduced molting success is the most direct fitness cost because for arthropods such as ticks, the inability to complete the molt is equivalent to death (Ogden et al. 2004). Population ecology models of *Ixodes* ticks have shown that the tick population growth rate is highly sensitive to molting success (Ogden et al. 2007, Dobson et al. 2011). In summary, the negative effects of acquired anti-tick immunity on tick life history traits can reduce the abundance of ticks in the field.

***B. burgdorferi* sl pathogens establish chronic infections in rodents with high lifetime host-to-tick transmission:** We showed that *B. afzelii* isolate NE4049 established a long-lived systemic infection in the bank voles. At the time of sacrifice, all of the 13 bank voles showed systemic infections where the dorsal skin, ventral skin, ear, bladder and heart were all infected with *B. afzelii*. These results are in agreement with previous studies that have shown that *B. burgdorferi* sl pathogens established chronic systemic infections in their rodent reservoir hosts (Gern et al. 1994, Humair et al. 1999, Richter et al. 2004, Jacquet et al. 2016). For vector-borne pathogens such as *B. burgdorferi* sl, chronic infections are adaptive because they increase the lifetime transmission success of the infection (Kurtenbach et al. 2006, Tsao 2009, Jacquet et al. 2016). In the present study, host-to-tick transmission of *B. afzelii* decreased three-fold over time from 60% in the first infestation (4 weeks PI) to 20% in the third infestation (12 weeks PI). Less dramatic declines have been observed in another important rodent reservoir, the wood mouse (*Apodemus sylvaticus*), where host-to-tick transmission of *B. afzelii* decreased from 100% at 3 weeks PI to ~40% at 9 weeks PI (Richter et al. 2004). A study that used the same isolate of *B. afzelii* (NE4049) and the same colony of *I. ricinus* ticks to infect *M. musculus* mice found that host-to-tick transmission decreased from 90.8% at 5 weeks PI to 68.9% at 13 weeks PI (Jacquet et al. 2016). Likewise, experimental infection studies on *B. burgdorferi* ss have shown that the pattern of host-to-tick transmission over time can vary depending on the particular combination of strain and rodent species (Derdakova et al. 2004, Hanincova et al. 2008, Rynkiewicz et al. 2017). In general, host-to-tick transmission of *B. burgdorferi* sl pathogens decreases over time in most rodent species, but the bank voles in this study showed a particularly steep decline.

**Relationship between pathogen abundance in host tissues and host-to-vector transmission:** For vector-borne pathogens, there is a direct relationship between the pathogen's abundance in the relevant tissues and host-to-vector transmission. For example, this relationship has been shown in rodent malaria where the parasite density in the blood is critical for mouse-to-mosquito transmission (de Roode et al. 2005). For *B. burgdorferi* sl pathogens, the skin rather than the blood is the critical tissue for host-to-tick transmission (Kern et al. 2015, Grillon et al. 2017). Field studies on *B. afzelii* in bank voles and other wild rodents found a positive

relationship between the spirochete load in the ear tissues and transmission to larval *I. ricinus* ticks (Raberg 2012). Similarly, an infection experiment with laboratory *M. musculus* mice and the *B. afzelii* strain used in the present study (NE4049), also found a positive relationship between the spirochete load in the ears and the host-to-tick transmission (Jacquet et al. 2015). Studies on laboratory *M. musculus* mice have shown that the spirochete load in the host tissues can change over time (Wang et al. 2001, Wooten et al. 2002, Hodzic et al. 2003), but these studies have not investigated host-to-tick transmission. The present study found that the spirochete load of *B. afzelii* in the ear biopsies decreased 450-fold over time from 5883 spirochetes per biopsy in the first infestation (4 weeks PI) to 13 spirochetes per biopsy in the third infestation (12 weeks PI). This decline in spirochete load was strongly correlated with the temporal increase of anti-tick immunity. Our study suggests that an increasingly effective host immune response (against the tick and/or the pathogen) reduced the spirochete load in the skin of the bank voles, which subsequently reduced transmission of *B. afzelii* to feeding larval ticks.

**Acquired immunity against *B. burgdorferi* sl and *Ixodes* ticks:** Rodents develop strong antibody responses against *B. burgdorferi* sl pathogens, which are believed to play an important role in controlling the infection (Barthold 1999, Connolly and Benach 2005, LaRocca and Benach 2008). For example, SCID mice that cannot produce antibodies have spirochete loads that are an order of magnitude higher than immunocompetent mice, and inoculation of SCID mice with *B. burgdorferi* sl anti-serum reduces the tissue spirochete load (Hodzic et al. 2003, Liang et al. 2004a, Liang et al. 2004b, Barthold et al. 2006). Previous studies on bank voles and *Apodemus* mice suggested that the strength of the spirochete-reactive antibody response was important for controlling host-to-tick transmission (Kurtenbach et al. 1994). The bank voles in this study developed a strong IgG antibody response against *B. afzelii* as shown by the results from the commercial ELISA. Thus, one plausible explanation is that the *B. afzelii*-targeted antibody response was able to reduce the spirochete load in the skin and thereby reduce host-to-tick transmission. A second explanation is that the anti-tick immunity developed against the sequential larval infestations reduced host-to-tick transmission success of *B. afzelii*. Previous studies have shown that the development of acquired immunity against ticks in the vertebrate host has important consequences for the acquisition and transmission of tick-borne pathogens (Wikel et al. 1997, Nazario et al. 1998). We found a highly significant negative relationship between the anti-tick IgG antibody response and host-to-tick transmission, even after controlling for the temporal decline in spirochete load in the ear tissues. This observation suggests that anti-tick immunity caused the temporal decline in host-to-tick transmission. A plausible mechanism for this phenomenon is that the anti-tick antibodies opsonize the tick salivary gland proteins and thereby transform the feeding lesion into an immunologically hostile environment where spirochete survival and spirochete migration to the mouthparts of feeding larval ticks are compromised (Shih et al. 2002, Scheckelhoff et al. 2007, Dunham-Ems et al. 2009). A third explanation for the decline in host-to-tick transmission was that anti-tick immunity shortened the feeding time of the larval ticks, which reduced the probability of acquiring *B. afzelii*. A recent manipulative study showed that there was a positive relationship between the duration of the blood meal of *I. scapularis* larvae and the probability of host-to-tick transmission of *B. burgdorferi* ss (Couret et al. 2017). In summary, anti-spirochete immunity, anti-tick immunity, and shorter feeding times of larval ticks are three alternative explanations for the observed temporal decline in the ear tissue spirochete loads and host-to-tick transmission of *B. afzelii*.

**Ecological consequences of acquired immunity in bank voles and associated reductions in tick life history traits and host-to-tick transmission:** Several studies have suggested that the bank vole is an important reservoir host of *B. afzelii* (Kurtenbach et al. 1994, Talleklint and Jaenson 1994, Dizij and Kurtenbach 1995, Humair et al. 1999, Hanincova et al. 2003, Jacquot et al. 2014, Raberg et al. 2017). Early studies on the vertebrate host community

in Sweden suggested that the bank vole was the second-most important host of *B. afzelii* and produced 17% of the infected *I. ricinus* nymphs (Talleklint and Jaenson 1994). In this experimental study, we found that the anti-tick immunity developed by bank voles had negative consequences for both the tick vector and the tick-borne pathogen. Population ecology models of *I. ricinus* have shown that the tick population size and growth rate are highly sensitive to density-dependent mortality on the host, which can be mediated by acquired immunity (Dobson et al. 2011). A recent field study in France found that the abundance of bank voles had negative effects on the abundance of *I. ricinus* nymphs the following year (Perez et al. 2016). The explanation was that acquired immunity in bank voles had a negative effect on the recruitment of feeding larval ticks into nymphs the following year. Theoretical models have shown that the reproduction number ($R_0$) of *B. burgdorferi* sl pathogens is strongly influenced by the duration of the infection and the host-to-tick transmission rate (Hartemink et al. 2008). Both of these life history traits were strongly reduced in the present study showing the importance of acquired immunity in controlling the ecology of *B. burgdorferi* sl pathogens and the epidemiology of Lyme borreliosis.

**Conclusions:**

We found no evidence that infection with *B. afzelii* suppressed the development of acquired immunity against *I. ricinus* ticks in bank voles. Future research needs to confirm whether suppression of acquired immunity is a widespread strategy in the *B. burgdorferi* sl genospecies complex or whether it is restricted to certain strains or unnatural hosts, such as laboratory mice. Repeated infestations with larval *I. ricinus* ticks induced the bank voles to develop a strong IgG antibody response against the salivary gland extract proteins of *I. ricinus*. This anti-tick antibody response reduced tick fitness and was also associated with a dramatic temporal decline in the ear spirochete load and host-to-tick transmission. Our study suggests that acquired anti-tick immunity in the vertebrate host can play an important role in controlling the abundance of *B. burgdorferi* sl-infected nymphs and hence the risk of Lyme borreliosis.

## Figures

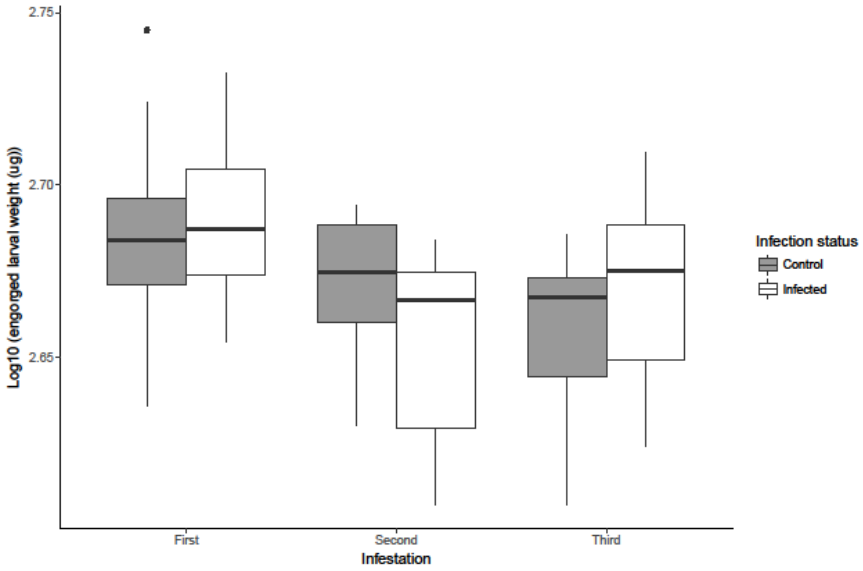


**Figure 2.** The engorged weight of *I. ricinus* larval ticks feeding on the bank voles decreased over the three successive infestations. Infection of bank voles with *B. afzelii* did not affect the engorged larval weight. The bank voles in the *B. afzelii*-infected group (n = 13) and the control group (n = 14) were infested with larval ticks at 27, 55, and 84 days PI. Each data point represents the mean engorged larval weight for an individual bank vole and is based on ~20 ticks. Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (circles).

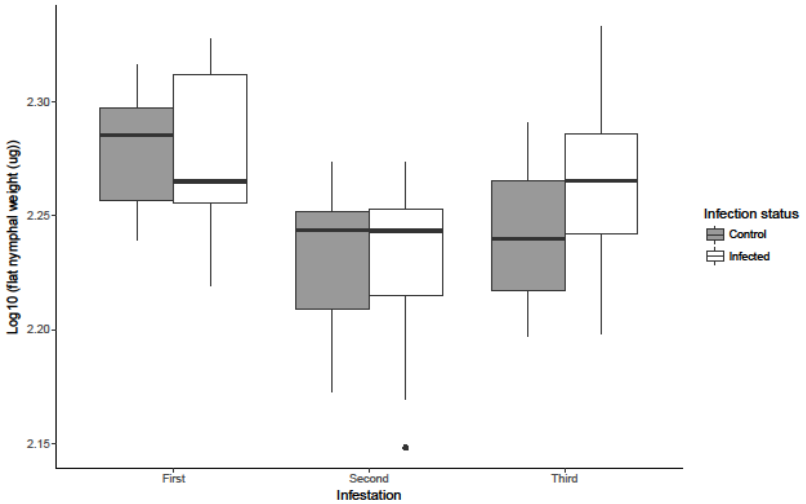


**Figure 3**. The weight of the flat *I. ricinus* nymphs decreased over the three successive infestations. The flat nymphs had fed on the bank voles during the larval stage. Infection of bank voles with *B. afzelii* did not affect the flat nymphal weight. The bank voles in the *B. afzelii*-infected group (n = 13) and the control group (n = 14) were infested with larval ticks at 27, 55, and 84 days PI. Each data point represents the mean flat nymphal weight for an individual bank vole and is based on ~20 flat nymphs. Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (circles).

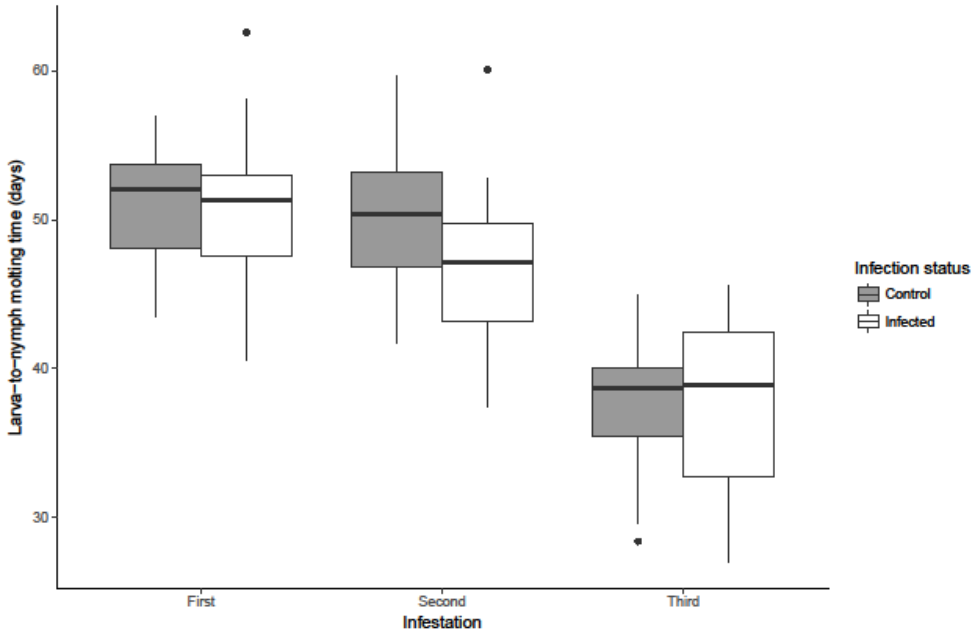


**Figure 4**. The larva-to-nymph moulting time of *I. ricinus* ticks decreased over the three successive infestations. The moulting time refers to the number of days for an engorged larval tick to moult into a nymphal tick. Infection of bank voles with *B. afzelii* did not affect the moulting time. The bank voles in the *B. afzelii*-infected group (n = 13) and the control group (n = 14) were infested with larval ticks at 27, 55, and 84 days PI. Each data point represents the mean larva-to-nymph moulting time for an individual bank vole and is based on ~50–100 ticks. Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (circles).

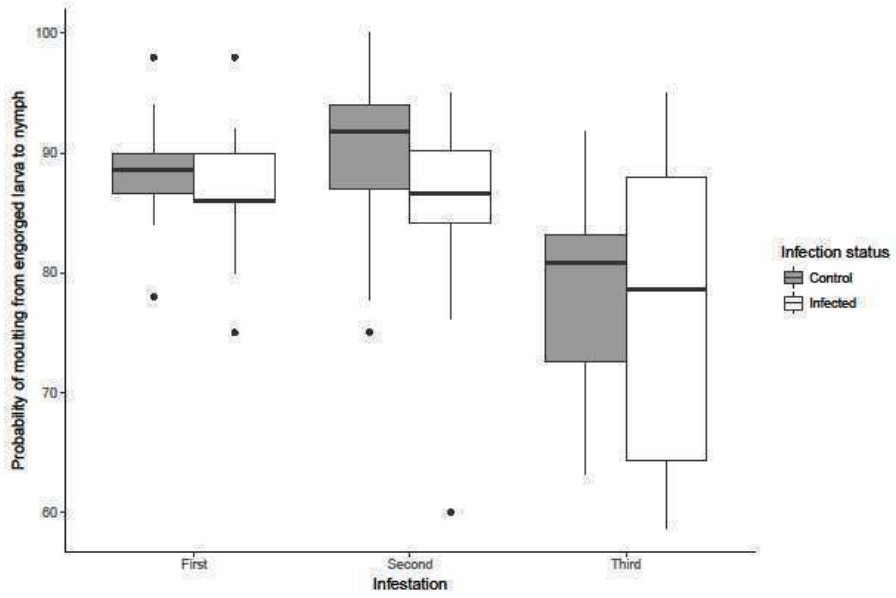


**Figure 5.** The moulting success of *I. ricinus* larval ticks decreased over the three successive infestations. Moulting success refers to the percentage of engorged larval ticks that developed into the nymphal stage. Infection of bank voles with *B. afzelii* did not affect the moulting success. The bank voles in the *B. afzelii*-infected group (n = 13) and the control group (n = 14) were infested with larval ticks at 27, 55, and 84 days PI. Each data point represents the mean larva-to-nymph moulting success for an individual bank vole and is based on ~50–100 ticks. Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (circles).

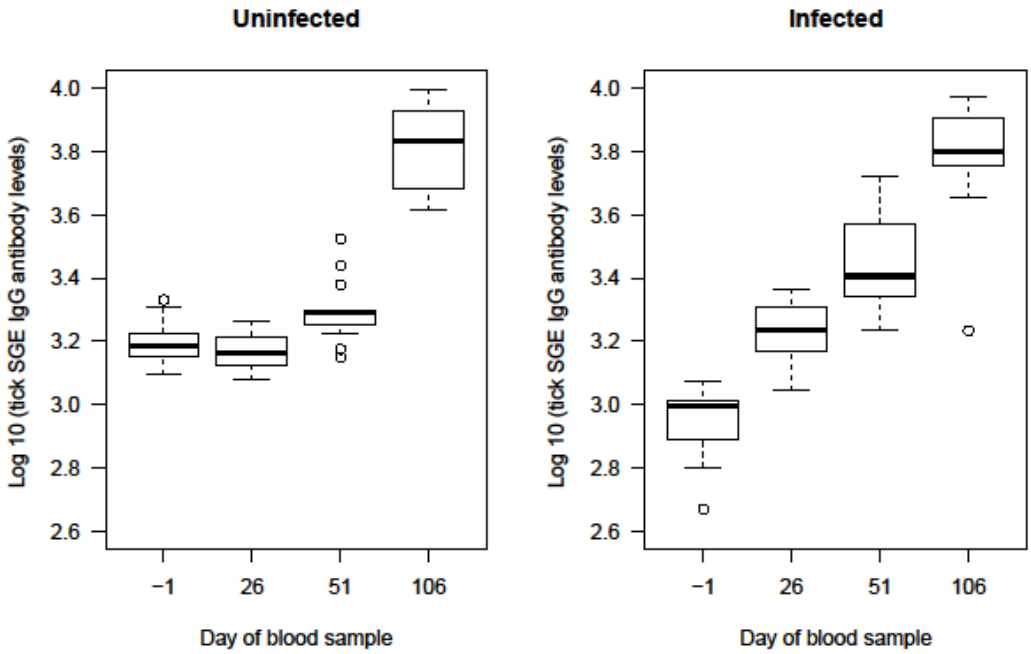


**Figure 6**. The bank voles developed a strong IgG antibody response against the salivary gland extract of *I. ricinus* ticks over the three successive larval infestations in both the control group and the *B. afzelii*-infected group. Serum samples were taken on -1, 26, 51, and 106 days PI, which corresponded to time points before the nymphal challenge, and after the first, second, and third larval infestation. The strength of the IgG antibody response against tick salivary gland extract was measured as the absorbance from an ELISA. Each data point represents the log10-transformed absorbance value for an individual bank vole. Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (circles).

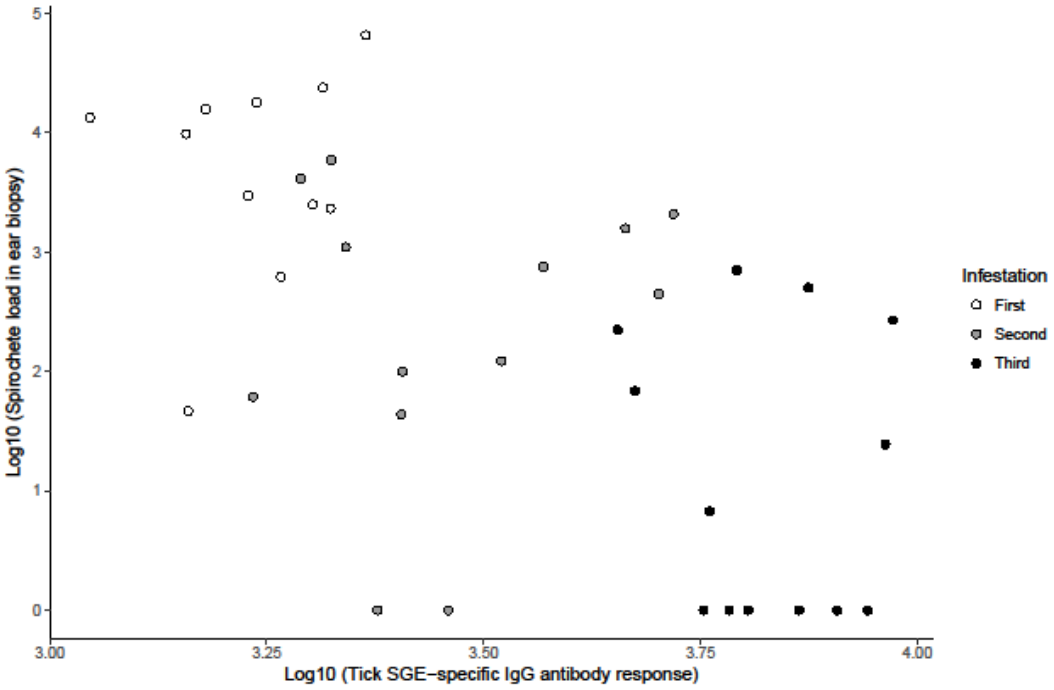


**Figure 7**. The spirochete load in the bank vole ear biopsy is negatively related to the tick SGE-specific IgG antibody response. Data are shown for the subset of *B. afzelii*-infected bank voles (n = 13 individuals) at the time of the first (open white circles), second (solid grey circles), and third infestation (solid black circles). Ear tissue biopsies were taken at 26, 51, and 106 days PI and the serum samples were taken at 26, 51, and 106 days PI. The 39 data points represent the 13 *B. afzelii*-infected bank voles at each of the three infestations. Spirochete loads refer to the number of spirochetes in the whole ear tissue biopsy (2 mm diameter).

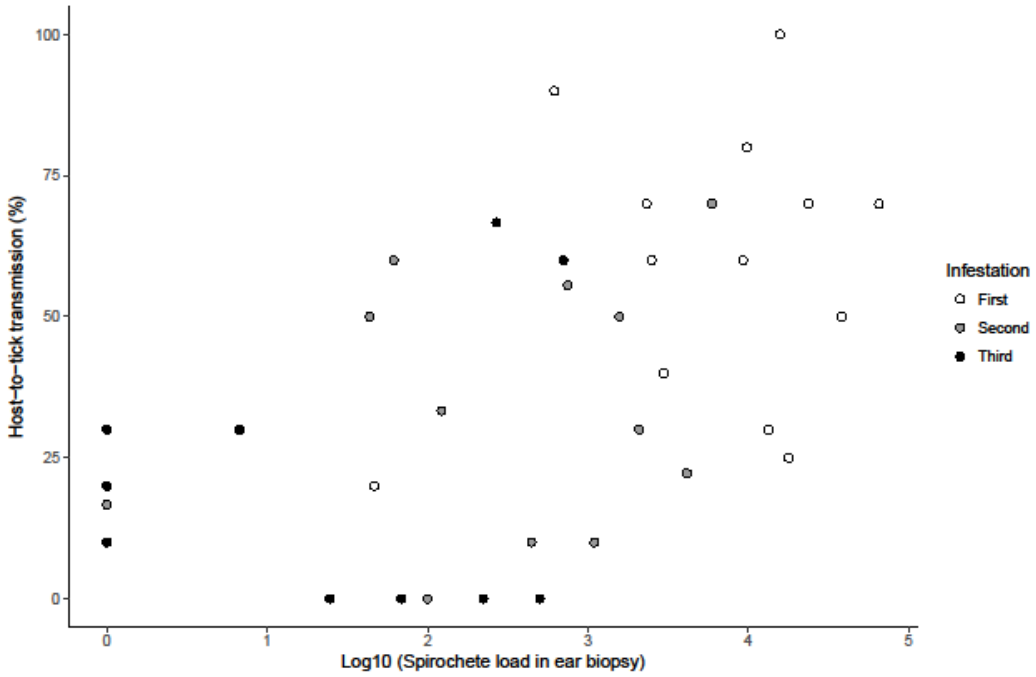


**Figure 8**. Host-to-tick transmission is positively correlated with spirochete load in the ear tissue of the bank voles. Host-to-tick transmission is the percentage of nymphs that acquired the *B. afzelii* infection during the larval blood meal. Data are shown for the subset of *B. afzelii*-infected bank voles (n = 13 individuals) at the time of the first (open white circles), second (solid grey circles), and third infestation (solid black circles). Ear tissue biopsies were taken at 26, 51, and 106 days PI and the bank voles were infested with larval ticks at 27, 55, and 84 days PI. The 39 data points represent the 13 *B. afzelii*-infected bank voles at each of the three infestations. Spirochete loads refer to the number of spirochetes in the whole ear tissue biopsy (2 mm diameter).

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**Author contributions**

A.G.-C. and M.J.V designed the study. A.G.-C., Y.L. and A.S performed the experimental infections and the molecular work. O.R. created the larvae necessary for the infestations. A.G.-C. analysed the data. A.G.-C. and M.J.V wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests: The authors declare no competing of interest.

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**Supplementary material**

**Section 1 – Phytotron conditions for development of engorged larval ticks:**

The engorged larval ticks were placed in individual tubes and these tubes were kept in boxes that were stored in a phytotron. The conditions of the phytotron were as follows. Between 5:00–19:00 (14 hours), the light intensity was 3000 lumens and the temperature was 25°C. Between 4:00–5:00 and 19:00–20:00 (2 hours), the light intensity was 1000 lumens and the temperature was 21.5 °C. Between 20:00–04:00 (8 hours), the light intensity was 0 lumen and the temperature was 18°C. The relative humidity was maintained at 85%

**Section 2 – *B. afzelii* infection status of the bank voles after challenge with uninfected *I. ricinus* nymphs and infected nymphs**

Bank voles in the uninfected control group (n = 14) were infested with uninfected *I. ricinus* nymphs whereas bank voles in the infected group (n = 14) were infested with *B. afzelii*-infected *I. ricinus* nymphs. After the nymphal infestation, all bank voles were tested with respect to four infection criteria to determine their actual *B. afzelii* infection status. The four infection criteria were as follows: (1) presence of *B. afzelii*-specific IgG antibodies (optical density of the ELISA > 500 absorbance units), (2) presence of *B. afzelii* in ear tissue biopsy (i.e., spirochete load of *B. afzelii* > 0), (3) presence of *B. afzelii* in xenodiagnostic nymphs (i.e., spirochete load of *B. afzelii* > 0 in at least 1 xenodiagnostic nymph), and (4) presence of live *B. afzelii* spirochetes in culture (i.e. at least 1 culture derived from a xenodiagnostic nymph contains live spirochetes).

The four infection criteria for the 28 bank voles are shown in Table S1. As expected, all of the 14 bank voles in the control group tested negative for the 4 infection criteria (Table S1). Thirteen of the 14 bank voles in the *B. afzelii*-infected group tested positive for 3 or 4 criteria, and these 13 bank voles were therefore considered as infected with *B. afzelii* (Table S1). The bank vole that had been challenged with infected nymphs, but that tested negative for all five criteria was excluded from the analysis. All statistical analyses are therefore based on 13 *B. afzelii*-infected bank voles and 14 uninfected bank voles.

**Table S1**. The *B. afzelii* infection status is shown for each of the 28 bank voles that belonged to either the *B. afzelii*-infected group or the uninfected control group. The infection status of each bank vole was based on four criteria: (1) *B. afzelii*-specific IgG antibodies, (2) spirochetes in the ear biopsy, (3) *B. afzelii*-infected xenodiagnostic nymphs, and (4) culture of live spirochetes from xenodiagnostic nymphs.

| Vole ID[a] | Treatment[b] | Engorged nymphs[c] | ELISA[d] | Spirochetes in ear biopsy[e] | Xenodiagnosis[f] | Culture of nymphs[g] | Criteria[h] | Infection status[i] |
|---|---|---|---|---|---|---|---|---|
| 193 | Control | 0/3 | 301 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 195 | Control | 0/4 | 342 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 196 | Control | 0/4 | 369 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 197 | Control | 0/1 | 252 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 198 | Control | 0/3 | 248 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 199 | Control | 0/4 | 239 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 206 | Control | 0/4 | 281 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 209 | Control | 0/4 | 344 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 211 | Control | 0/3 | 233 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 216 | Control | 0/2 | 239 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 224 | Control | 0/5 | 223 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 228 | Control | 0/2 | 238 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 230 | Control | 0/5 | 254 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 231 | Control | 0/3 | 403 | 0 | 0/30 | 0/0 | 0 | Uninfected |
| 192 | Infected | 3/4 (75.0%) | 1120 | 2550 | 4/30 (13.3%) | 2/6 (33.3%) | 4 | Infected |
| 200 | Infected | 1/2 (50.0%) | 5001 | 4315 | 12/27 (44.4%) | 1/6 (16.7%) | 4 | Infected |
| 202 | Infected | 2/2 (100.0%) | 1405 | 21196 | 13/29 (44.8%) | 1/6 (16.7%) | 4 | Infected |
| 204 | Infected | 0/1 (0.0%) | 3865 | 348 | 19/30 (63.3%) | 3/6 (50.0%) | 3 | Infected |
| 205 | Infected | 2/4 (50.0%) | 5475 | 519 | 8/25 (32.0%) | 1/6 (16.7%) | 4 | Infected |
| 214 | Infected | 1/5 (20.0%) | 4309 | 6412 | 2/22 (9.1%) | 0/6 (0.0%) | 3 | Infected |
| 217 | Infected | 4/4 (100.0%) | 3336 | 979 | 7/29 (24.1%) | 2/5 (40.0%) | 4 | Infected |
| 218 | Infected | 1/2 (50.0%) | 2809 | 3704 | 13/27 (44.4%) | 1/4 (25.0%) | 4 | Infected |
| 220 | Infected | 1/3 (33.0%) | 1168 | 17670 | 11/30 (36.7%) | 3/5 (60.0%) | 4 | Infected |
| 222 | Infected | 4/5 (80.0%) | 4096 | 10465 | 11/30 (36.7%) | 1/6 (16.7%) | 4 | Infected |
| 223 | Infected | 0/1 (0.0%) | 218 | 0 | 0/26 (0.0%) | 0/0 (0.0%) | 0 | Uninfected[j] |
| 225 | Infected | 2/3 (66.7%) | 1204 | 6589 | 17/29 (58.6%) | 0/6 (0.0%) | 3 | Infected |
| 226 | Infected | 3/5 (80.0%) | 1587 | 36 | 7/28 (25.0%) | 2/5 (40.0%) | 4 | Infected |
| 229 | Infected | 3/5 (60.0%) | 4011 | 3704 | 12/29 (41.4%) | 1/6 (16.7%) | 4 | Infected |

[a] Vole id is the unique identification number assigned to each bank vole in the study
[b] Treatment refers to whether the bank vole was randomly assigned to the *B. afzelii*-infected group or the uninfected control group.
[c] Engorged nymphs is the number of engorged challenge nymphs that tested positive for *B. afzelii* compared to the total number of engorged challenge nymphs that were collected. The percentage of engorged challenge nymphs that tested positive for *B. afzelii* is shown in brackets.
[d] ELISA is the strength of the *Borrelia afzelii*-specific IgG antibody response as measured by the commercial ELISA assay. The optical density (OD) was measured every 2 minutes over a period of 60 minutes. The area under the curve of OD versus time was integrated to give the values shown. Individuals with an optical density > 500 units are considered infected.
[e] Spirochetes in ear biopsy is the number of spirochetes in the ear tissue biopsy (2 mm diameter) as estimated by our qPCR assay. Individuals with a spirochete load > 1 per ear tissue biopsy are considered infected.
[f] Xenodiagnosis refers to the xenodiagnostic larvae that fed on the bank voles and that subsequently moulted into xenodiagnostic nymphs. The number of xenodiagnostic nymphs that tested positive for *B. afzelii* compared to the total number of xenodiagnostic nymphs that were tested using qPCR are shown. The percentage of xenodiagnostic nymphs that tested positive for *B. afzelii* is shown in brackets. Individuals that infect > 0% of the xenodiagnostic nymphs are considered infected.
[g] Culture is the number of xenodiagnostic nymphs that yielded a live spirochete culture compared to the total number of xenodiagnostic nymphs that were placed into BSK media. Individuals that produce at least 1 live spirochete culture are considered infected.
[h] Criteria is the number of infection status criteria that were met by each bank vole and ranges from 0 to 4.
[i] Infection status is whether a vole was considered to be infected with *B. afzelii* or not.
[j] Bank vole 223 did not become infected following exposure to *B. afzelii*-infected nymphs.

**Section 3 – Relationship between engorged larval weight and flat nymphal weight**

For a sample of 880 *I. ricinus* ticks, the engorged larval weight (μg) and the unfed nymphal weight (μg) were measured for each individual tick. A Pearson correlation test was used to test whether there was a relationship between the engorged larval weight and the flat nymphal weight. The engorged larval weight was strongly and positively correlated with the flat nymphal weight (Figure 3; Pearson correlation: $r = 0.796$, $df = 879$, $t = 38.943$, $p < 0.001$). This result shows that the weight of the engorged larva influences the weight of the resultant flat nymph.

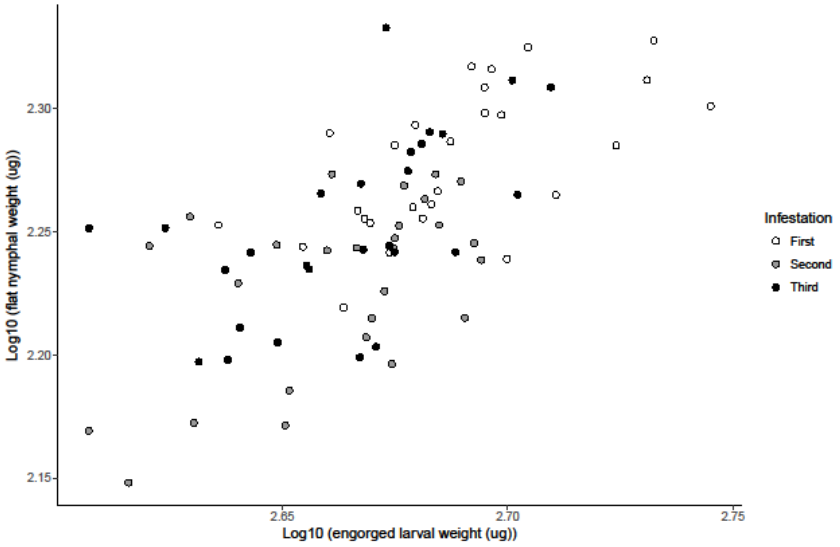


**Figure S1**. There is a strong and positive relationship between the weight of the engorged larvae (μg) and the weight of the resultant flat nymphs (μg). Larval ticks that take a larger blood meal moult into larger nymphs. The correlation between engorged larval weight and flat nymphal weight was highly significant ($r = 0.796$, $df = 879$, $t = 38.943$, $p < 0.001$).

**Section 4 – Additional analysis of the tick phenotypes**

In the main manuscript, we tested whether the bank voles developed acquired immunity against *I. ricinus* ticks over the three successive infestations and whether infection with *B. afzelii* influenced the development of this anti-tick immunity. The four tick phenotypes that were analyzed included engorged larval weight, flat nymphal weight, larva-to-nymph moulting time, and larva-to-nymph moulting success. Each of these four tick phenotypes was analyzed as a function of two fixed factors: *B. afzelii* infection status, infestation number, and their interaction using linear mixed effects models (LMMs) or generalized linear mixed effects models (GLMMs). Here we present the parameter estimates of the LMMs and the GLMMs for each of the four tick phenotypes. In Tables S2, S3, S4, S5, S6, S7, S8, and S9 we show the parameter estimates from the most parsimonious model for each tick phenotype.

**Table S2A**. LMM parameter estimates are shown for the main effects and interaction of infection status and infestation on the log10-transformed weight of the engorged *I. ricinus* larvae. The engorged larval weight was measured in µg. The intercept refers to the log10-transformed engorged weight of larval ticks that fed on uninfected bank voles during the first infestation.

| Fixed effects | Estimate | Standard error | t-value | p-value |
|---|---|---|---|---|
| (Intercept) | 2.682 | 0.005 | 489.314 | < 0.001 |
| $2^{nd}$ – $1^{st}$ infestation | -0.019 | 0.007 | -2.747 | 0.006 |
| $3^{rd}$ – $1^{st}$ infestation | -0.029 | 0.007 | -4.373 | < 0.001 |
| Infected – Control | 0.005 | 0.008 | 0.620 | 0.537 |
| $2^{nd}$ – $1^{st}$ infestation\| Infected | -0.021 | 0.010 | -2.175 | 0.030 |
| $3^{rd}$ – $1^{st}$ infestation\| Infected | 0.006 | 0.009 | 0.691 | 0.490 |

**Table S2B**. LMM parameter estimates are shown for the main effects of infection status and infestation on the log10-transformed weight of the engorged *I. ricinus* larvae. The engorged larval weight was measured in µg. The intercept refers to the log10-transformed engorged weight of larval ticks that fed on uninfected bank voles during the first infestation.

| Fixed effects | Estimate | Standard error | t-value | p-value |
|---|---|---|---|---|
| (Intercept) | 2.684 | 0.005 | 549.492 | < 0.001 |
| $2^{nd}$ – $1^{st}$ infestation | -0.029 | 0.005 | -6.044 | < 0.001 |
| $3^{rd}$ – $1^{st}$ infestation | -0.025 | 0.005 | -5.464 | < 0.001 |
| Infected – Control | 0.001 | 0.006 | 0.169 | 0.867 |

**Table S3**. The parameter estimates of the weight of the engorged *I. ricinus* larvae are shown for each of the six combinations of infection status and infestation. Engorged larval weight is measured in µg. The means are shown with their 95% confidence intervals where LL and UL refer to the lower limit and upper limit, respectively.

| Infection Status | Infestation | Larval weight | 95% LL | 95% UL |
|---|---|---|---|---|
| Control | First | 480.84 | 469.32 | 492.86 |
| Control | Second | 460.26 | 448.37 | 473.39 |
| Control | Third | 449.78 | 438.77 | 462.05 |
| Infected | First | 486.41 | 474.39 | 498.59 |
| Infected | Second | 443.61 | 432.15 | 456.08 |
| Infected | Third | 462.38 | 450.58 | 473.77 |

**Table S4**. LMM parameter estimates are shown for the main effects of infection status and infestation on the log10-transformed weight of the unfed *I. ricinus* nymphs. The unfed nymphal weight was measured in μg. The intercept refers to the log10-transformed weight of unfed nymphs that fed as larvae on uninfected bank voles during the first infestation.

| Fixed effects | Estimate | Standard error | t-value | p-value |
|---|---|---|---|---|
| (Intercept) | 2.270 | 0.007 | 348.258 | < 0.001 |
| $2^{nd}$ – $1^{st}$ infestation | -0.052 | 0.006 | -8.731 | < 0.001 |
| $3^{rd}$ – $1^{st}$ infestation | -0.026 | 0.006 | -4.361 | < 0.001 |
| Infected – Control | 0.006 | 0.008 | 0.782 | 0.441 |

**Table S5**. The parameter estimates of the weight of the unfed *I. ricinus* nymphs are shown for of the six combinations of infection status and infestation. Unfed nymphal weight is measured in μg. The means are shown with their 95% confidence intervals where LL and UL refer to the lower limit and upper limit, respectively.

| Infection Status | Infestation | Nymphal weight | 95% LL | 95% UL |
|---|---|---|---|---|
| Control | First | 187.37 | 181.50 | 193.43 |
| Control | Second | 166.82 | 160.88 | 172.99 |
| Control | Third | 171.82 | 165.75 | 178.12 |
| Infected | First | 187.77 | 181.87 | 193.87 |
| Infected | Second | 165.61 | 159.70 | 171.74 |
| Infected | Third | 181.64 | 175.24 | 188.28 |

**Table S6**. LMM parameter estimates are shown for the main effects of infection status and infestation on the larva-to-nymph moulting time of *I. ricinus*. The larva-to-nymph moulting time is measured in days. The intercept refers to the larva-to-nymph moulting time of larvae that fed on uninfected bank voles during the first infestation.

| Fixed effects | Estimate | Standard error | t-value | p-value |
|---|---|---|---|---|
| (Intercept) | 51.261 | 1.134 | 45.191 | < 0.001 |
| $2^{nd}$ – $1^{st}$ infestation | -4.046 | 1.134 | -3.568 | < 0.001 |
| $3^{rd}$ – $1^{st}$ infestation | -16.256 | 1.018 | -15.973 | < 0.001 |
| Infected – Control | -0.080 | 1.489 | -0.054 | 0.958 |

**Table S7**. The parameter estimates of the moulting time are shown for each infestation. The infestation had a significant effect on the larva-to-nymph moulting time. The moulting time refers to the number of days it takes for an engorged larval tick to molt into a nymphal tick. The means are shown with their 95% confidence intervals where LL and UL refer to the lower limit and upper limit, respectively.

| Infestation | Moulting time | 95% LL | 95% UL |
|---|---|---|---|
| First | 51.22 | 49.55 | 52.89 |
| Second | 47.18 | 44.99 | 49.37 |
| Third | 34.96 | 33.00 | 36.93 |

**Table S8**. GLMM parameter estimates are shown for the main effects of infection status and infestation on the larva-to-nymph moulting success of *I. ricinus*. Larva-to-nymph moulting success is a binomial variable (Not moulted = 0, Moulted = 1). The intercept refers to the moulting success of the larvae that fed on uninfected bank voles during the first infestation.

| Fixed effects | Estimate | Standard error | z-value | p-value |
|---|---|---|---|---|
| (Intercept) | 2.055 | 0.112 | 18.269 | < 0.001 |
| $2^{nd}$ – $1^{st}$ infestation | -0.023 | 0.156 | -0.148 | 0.883 |
| $3^{rd}$ – $1^{st}$ infestation | -0.690 | 0.123 | -5.633 | < 0.001 |
| Infected – Control | -0.158 | 0.128 | -1.241 | 0.215 |

**Table S9**. The parameter estimates of the moulting success are shown for each infestation. The infestation had a significant effect on the moulting success. The larva-to-nymph moulting success refers to the percentage of engorged larval ticks that developed into the nymphal stage. The means are shown with their 95% confidence intervals where LL and UL refer to the lower limit and upper limit, respectively.

| Infestation | Molting success (%) | 95% LL | 95% UL |
|---|---|---|---|
| First | 87.7 | 85.6 | 89.9 |
| Second | 87.4 | 84.2 | 90.5 |
| Third | 78.1 | 75.4 | 80.8 |

**Section 5 – The spirochete load of *B. afzelii* in the ear tissues of the bank voles decreased over the three infestations**

The spirochete loads in the bank vole ear biopsies (2 mm diameter) decreased over the three infestations (Figure S2) and this effect was statistically significant (LLR test: $\chi^2 = 38.231$, df = 2, $p < 0.001$). For the first, second, and third larval infestation, the mean spirochete load in the ear biopsy (measured in number of spirochetes) was 5883 (95% CI: 1516–22834), 202 (95% CI: 52–786), and 13 (95% CI: 3–50), respectively.

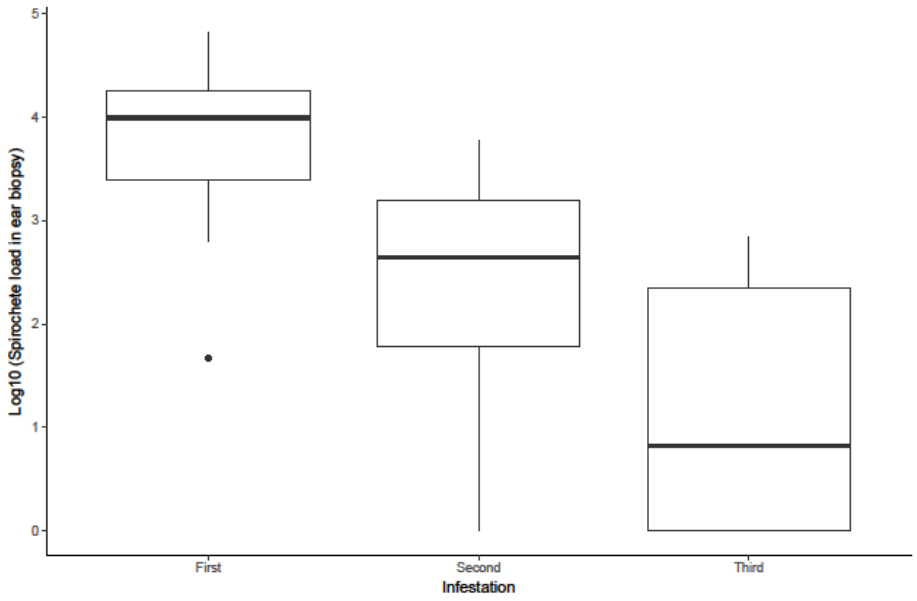


**Figure S2**. The spirochete load of *B. afzelii* in the ear biopsies of the bank voles decreased over the three successive infestations. The spirochete load is the number of *B. afzelii* spirochetes in the whole ear tissue biopsy (2 mm diameter). Each of the infected bank voles (n = 13) was biopsied at 26, 51, and 106 days PI. Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (circles).

**Section 6 – Host-to-tick transmission of *B. afzelii* from infected bank voles to *I. ricinus* ticks decreased over the three infestations**

Host-to-tick transmission decreased over the three infestations (Figure S3) and this effect was statistically significant (LLR test: $\chi^2 = 41.808$, df = 2, $p < 0.001$). For the first, second, and third larval infestations, the host-to-tick transmission was 59.4% (76/128), 35.6% (42/118), and 20.3% (26/128), respectively.

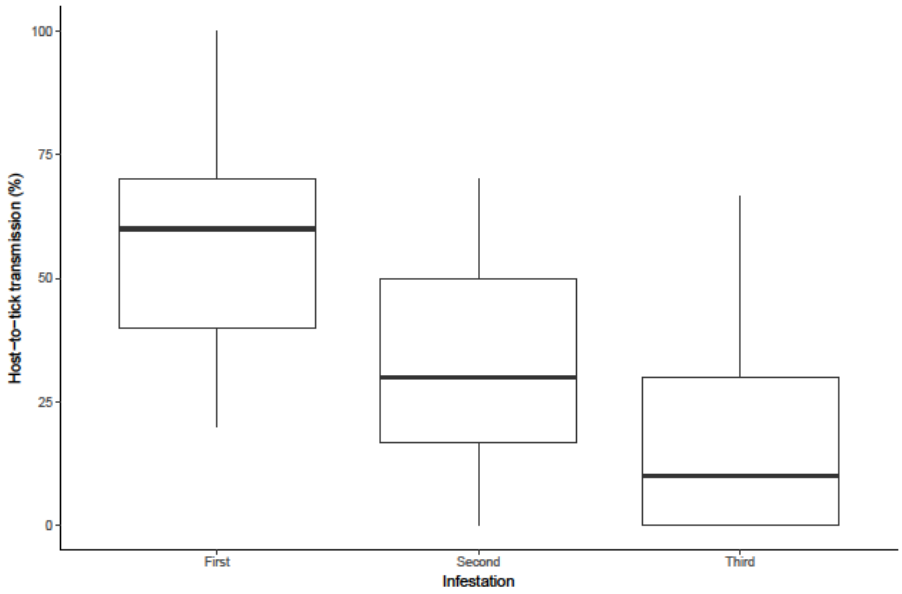


**Figure S3**. The host-to-tick transmission of *B. afzelii* from the infected bank voles to the *I. ricinus* ticks decreased over the three successive infestations. Host-to-tick transmission refers to the percentage of nymphs that acquired the *B. afzelii* infection during the larval blood meal. Each of the infected bank voles (n = 13) was infested with 50–100 larval *I. ricinus* ticks at 27, 55, and 84 days PI. The engorged larval ticks were allowed to molt into nymphs and the percentage of *B. afzelii*-infected nymphs was estimated using qPCR. Each host-to-tick transmission data point is based on 10 nymphs. Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (circles).

**Section 7 – Analysis of the spirochete load of *B. afzelii* in the *I. ricinus* nymphs that had fed as larval ticks on the infected bank voles**

We modeled the spirochete load in the flat nymphs as a function of the tick SGE-specific IgG antibody response, the spirochete load in the ear biopsy, and host-to-tick transmission. The mean spirochete load in the nymphs decreased over the three infestations and this effect was statistically significant (LMM: $\chi^2 = 7.807$, df = 2, p = 0.020). For the first, second, and third larval infestation, the mean spirochete load in the nymph was 608.9 (95% CI: 241.8–824.4), 234.1 (95% CI: 84.8–302.9), and 455.1 (95% CI: 174.8–743.9), respectively.

The tick SGE-specific IgG antibody response (LLR: $\chi^2 = 0.089$, df = 1, p = 0.766) and the ear spirochete load (LLR: $\chi^2 = 0.073$, df = 1, p = 0.787) had no effect on the spirochete load in the nymphs. Host-to-tick transmission had a significant positive effect on the mean spirochete load in the nymphs (Figure S4; LLR: $\chi^2 = 6.246$, df = 1, p = 0.012; slope = 1.12 ± 0.450).

The positive relationship between host-to-tick transmission and the nymphal spirochete load occurred because both of these two variables decreased over the three infestations. One explanation is that acquired anti-tick immunity created an increasingly hostile environment for the *B. afzelii* spirochetes in the feeding lesion of the larval ticks. This anti-tick immunity would have reduced the probability of host-to-tick transmission and spirochete growth in the subset of larval ticks that did become infected with *B. afzelii*. These results are consistent with previous studies that found a positive relationship between host-to-tick transmission and the nymphal spirochete load of *B. afzelii* (Gern et al., 1994; Jacquet, Durand, Rais, & Voordouw, 2015; Råberg, 2012). These results are also consistent with two recent studies that found that the spirochete load of *B. afzelii* was much lower in nymphs that had fed as larvae under conditions that induced a stronger anti-tick response in the rodent host (Belli, Sarr, Rais, Rego, & Voordouw, 2017; Jacquet, Durand, Rais, & Voordouw, 2016). The nymphal spirochete load may have important consequences for tick-to-host transmission (Durand et al., 2017; Rego, Bestor, Štefka, & Rosa, 2014). We recently showed that *ospC* strains of *B. afzelii* that establish a high spirochete load in field-collected *I. ricinus* nymphs have a higher frequency in these populations of *I. ricinus* (Durand et al., 2017) and we suggested that such strains have higher nymph-to-host transmission of *B. afzelii*. In contrast, a recent lab study showed that a 20-fold difference in nymphal spirochete load had no effect on the probability of nymph-to-host transmission of *B. afzelii* (Belli et al., 2017). Thus, the importance of nymphal spirochete load for nymph-to-host transmission of *B. burgdorferi* sl pathogens is currently not clear.

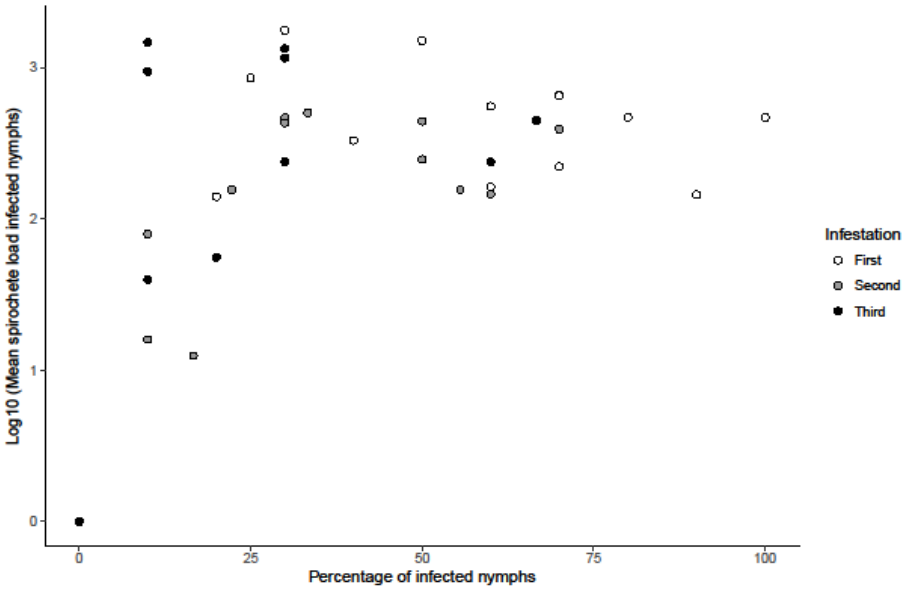


**Figure S4**. The mean spirochete load in the *B. afzelii*-infected nymphs is positively correlated with host-to-tick transmission. Data are shown for the subset of *B. afzelii*-infected bank voles (n = 13 individuals) at the time of the first (open white circles), second (solid grey circles), and third infestation (solid black circles). The bank voles were infested with larval ticks at 27, 55, and 84 days PI. The 39 data points represent the 13 *B. afzelii*-infected bank voles at each of the three infestations. Spirochete loads are calculated for the entire nymph.

## Section 8 – Analysis of the spirochete load of *B. afzelii* in the organ tissue samples of the bank voles at 106 days post-infection

### Materials and methods

The dramatic decline in the spirochete load of *B. afzelii* in the ear tissue biopsies (Figure S1) led us to suspect that the bank voles were clearing their infections. We therefore decided to test the *B. afzelii* infection status of various organs after the bank voles were euthanized. There were 28 bank voles that survived to the end of the experiment: 14 in the *B. afzelii*-infected group (of which 13 were truly infected) and 14 in the uninfected control group. All 28 bank voles were sacrificed at 106 days post-infection (PI) via asphyxiation with $CO_2$. The bank voles were dissected under sterile conditions and the following five organs were removed: ear, bladder, heart, ventral skin, and dorsal skin. The ear was sampled using an ear punch (2 mm diameter). For the bladder, heart, ventral skin, and dorsal skin, ~20 to 25 mg of tissue were obtained. The dissection equipment was sterilized with bleach in between individuals and in between organs of the same individual to avoid contamination. A total of 140 bank vole tissue samples were obtained (28 bank voles* 5 tissue samples per bank vole = 140 tissue samples).

The DNA was extracted from 140 tissue samples using the QIAGEN DNeasy® Blood and Tissue Kit and following the manufacturer's protocol. For the ear, the whole biopsy (2 mm diameter) was used for DNA extraction. For the bladder, heart, ventral skin, and dorsal skin, we obtained the wet weight of each tissue sample using a balance before DNA extraction. The DNA concentration of each DNA extraction was measured using a Nanodrop. The spirochete load of the tissue samples was estimated using a qPCR that amplifies a 132 bp fragment of the *flagellin* gene of *B. afzelii*. Each qPCR contained 84 samples from individual bank voles, triplicates of the standards (10, 100, 1000, 10000 flagellin gene copies) and negative controls ($H_20$). For each tissue sample, we standardized the spirochete load in two different ways. The number of spirochetes in the DNA extraction was divided by the amount of tissue that was extracted to calculate the number of spirochetes per mg of tissue. The numbers of spirochetes in 1 µl of DNA extract were divided by the DNA concentration of the extract to calculate the number of spirochetes per mg of DNA. The spirochete density in rodent tissues is low so that the DNA concentration of *B. burgdorferi* sl-infected rodent tissue is mostly a measure of rodent DNA (and hence rodent cell content).

### Statistical analysis

We standardized the spirochete load in the bank vole tissue samples in two different ways: relative to the mass of the tissue sample and relative to the DNA concentration of the tissue sample. For the subset of *B. afzelii*-infected bank voles (n = 13), we used a Pearson correlation test to determine whether there was a significant correlation between these two different ways to standardize the spirochete load.

To test whether there were differences in the probability of infection between the five organs, we used a GLMM with binomial errors for the subset of *B. afzelii*-infected bank voles (n = 13). In this model, the binomial response variable was whether the organ tissue sample was uninfected (0) or infected (1). Organ was modeled as a fixed factor and the bank vole ID was used as a random factor.

To test whether there were differences in tissue spirochete load between the five organs, we used an LMM with normal errors for the subset of *B. afzelii*-infected bank voles (n = 13). In this model, the response variable was the standardized spirochete load, which was transformed using log10(spirochete load + 1), to normalize the residuals. Organ was modeled as a fixed factor and bank vole ID as a random factor.

In addition, we wanted to test whether the spirochete loads in some organs, such as the skin, are more important for host-to-tick transmission than other organs. We had estimated the

spirochete load at 106 days PI in five organs: bladder, ear, heart, dorsal skin, and ventral skin. For each organ, the spirochete loads were expressed as the number of spirochetes per mg of tissue or as the number of spirochetes per mg of DNA. The spirochete loads were log10(X + 1)-transformed. For each infected bank vole, the lifetime host-to-tick transmission was calculated as the percentage of *B. afzelii*-infected nymphs summed over the three infestations. We modelled the lifetime host-to-tick transmission as a function of the spirochete loads in five organs: heart, bladder, ear, dorsal skin, and ventral skin using a generalized linear model (GLM) with binomial errors.

**Results**

The analysis of the tissue spirochete loads of the organs further confirmed the infection status of the 28 bank voles. In the control group, 0 of 14 bank voles and 0 of the 70 tissue samples tested positive for *B. afzelii* (Tables S10 and S11). In the *B. afzelii*-infected group, 12 the 14 bank voles and 39 of the 70 tissue samples tested positive for *B. afzelii* (Tables S10 and S11). The two bank voles in the *B. afzelii*-infected group that tested negative for *B. afzelii* for all 5 tissues samples were bank voles 223 and 205 (Tables S10 and S11). According to our 4 other infection criteria (Table S1), bank voles 223 and 205 were uninfected and infected, respectively.

Figure S3 shows that the two methods of standardizing the tissue spirochete load give very similar results. The correlation between the two methods of standardizing the spirochete load was highly significant (Figure S3; r = 0.989, t = 54.043, df = 63, p < 2.2e-16).

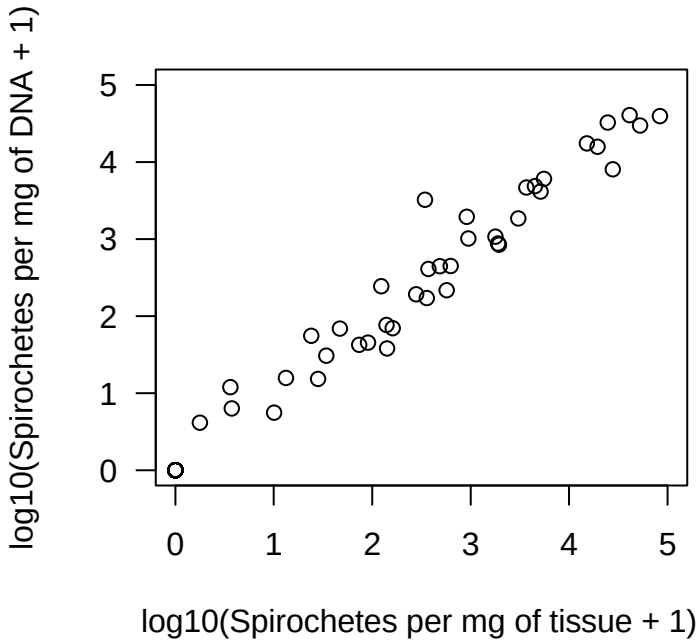


**Figure S5**. The two methods of standardizing the spirochete load in the bank vole tissues give very similar results. The tissue spirochete loads were expressed as the spirochetes per mg of tissue (horizontal axis) or the spirochetes per mg of DNA (vertical axis).

Organ had a highly significant effect on the probability of whether the tissue sample tested positive for *B. afzelii* in the qPCR ($\Delta \chi^2 = 34.93$, $\Delta$ df =4, p = 4.802e-07). The percentage of infected tissue samples was ranked as follows: 15.4% (2/13) in the heart, 38.4% (5/13) in the

ear, 61.5% (8/13) in the bladder, 92.3% (12/13) in the belly skin, and 92.3% (12/13) in the neck skin.

Organ had a highly significant effect on the spirochete load per mg of tissue ($\Delta \chi^2$ = 53.271, $\Delta$ df =4, p = 7.479e-11) and on the spirochete load per mg of DNA. ($\Delta \chi^2$ = 46.724, $\Delta$ df =4, p = 1.741e-09). The mean spirochete load was ranked from lowest to highest as follows: heart, ear, bladder, neck skin, and belly skin (Table S7). The mean spirochete load in the belly skin was ~1000 times higher compared to the heart. Interestingly, the spirochete load was highest in the skin (Table S7), which is the organ that is most important for host-to-tick transmission.

**Table S6**. Mean tissue spirochete loads of *B. afzelii* differ among the five organs in the subset of infected bank voles. The spirochete loads were standardized per mg of tissue and per mg of DNA. Shown are the means and the lower limit (LL) and upper limit (UL) of the 95% confidence interval.

| Organ | Standardization | Mean | 95% LL | 95% UL |
|---|---|---|---|---|
| Bladder | Mg of Tissue | 16.7 | 3.8 | 74.6 |
| Ear | Mg of Tissue | 10.2 | 2.3 | 45.4 |
| Heart | Mg of Tissue | 1.2 | 0.3 | 5.2 |
| Skin belly | Mg of Tissue | 1413.5 | 317.0 | 6302.0 |
| Skin neck | Mg of Tissue | 490.9 | 110.1 | 2188.5 |
| Bladder | Mg of DNA | 22.3 | 4.9 | 102.2 |
| Ear | Mg of DNA | 10.3 | 2.2 | 47.0 |
| Heart | Mg of DNA | 1.3 | 0.3 | 5.9 |
| Skin belly | Mg of DNA | 1151.3 | 251.4 | 5271.9 |
| Skin neck | Mg of DNA | 278.4 | 60.8 | 1274.7 |

The correlations in spirochete load were all positive, except the correlation between heart and ear, but none were statistically significant, except the correlation between the belly skin and the neck skin ($r = 0.728$, $df = 11$, $p = 0.005$; Table S8). The GLM analysis of lifetime host-to-tick transmission as a function of the spirochete loads in the five different organs (heart, bladder, ear, belly skin, and neck skin) was overdispersed (residual deviance = 22.630, residual degrees of freedom = 7), and the data was therefore re-analyzed using a quasibinomial distribution. For the spirochete loads that were standardized to the DNA concentration, the spirochete load in the belly skin was positively related to the lifetime host-to-tick transmission, but the slope was not significantly different from zero (Table S9; $p = 0.0738$). For the spirochete loads that were standardized to the weight of the tissue sample, the spirochete load in the belly skin was positively related to the lifetime host-to-tick transmission, but the slope was not significantly different from zero (Table S9; $p = 0.0551$).

**Table S7**. Correlation matrix of the tissue spirochete loads between the five organs. The five organs include: bladder, ear, heart, belly skin, and neck skin. Data are based on the subset of 13 bank voles that were infected with *B. afzelii*. All tissue spirochete loads were log10(X + 1)-transformed to improve their fit to the normal distribution. The Pearson correlation coefficients are shown on the right side of the diagonal and the p-values of the correlation on the left side of the diagonal.

| Organ | Bladder | Ear | Heart | Belly skin | Neck skin |
|---|---|---|---|---|---|
| Bladder | *** | 0.312 | 0.360 | 0.508 | 0.393 |
| Ear | 0.299 | *** | -0.053 | 0.428 | 0.164 |
| Heart | 0.227 | 0.862 | *** | 0.317 | 0.215 |
| Belly skin | 0.077 | 0.144 | 0.291 | *** | **0.728** |
| Neck skin | 0.185 | 0.592 | 0.481 | **0.005** | *** |

**Table S8**. The mean spirochete loads in five different organs do not influence the lifetime host-to-tick transmission. The five organs include: bladder, ear, heart, belly skin, and neck skin. The mean tissue spirochete load for each organ was standardized by either the DNA concentration of the tissue DNA extraction (units are spirochetes per mg of DNA) or the weight of the tissue sample (units are spirochetes per mg of tissue).

| Correction | Organ | Slope | S.E. | t-statistic | P |
|---|---|---|---|---|---|
| DNA | Intercept | -0.91479 | 0.51333 | -1.782 | 0.1179 |
| DNA | Log10(Bladder+1) | -0.07309 | 0.17752 | -0.412 | 0.6929 |
| DNA | Log10(Ear+1) | -0.20156 | 0.16539 | -1.219 | 0.2624 |
| DNA | Log10(Heart+1) | -0.14006 | 0.81856 | -0.171 | 0.869 |
| DNA | Log10(Belly skin+1) | 0.47081 | 0.22409 | 2.101 | 0.0738 |
| DNA | Log10(Neck skin+1) | -0.31438 | 0.26042 | -1.207 | 0.2666 |
| Tissue | Intercept | -0.9774 | 0.58363 | -1.675 | 0.1379 |
| Tissue | Log10(Bladder+1) | -0.07508 | 0.19189 | -0.391 | 0.7072 |
| Tissue | Log10(Ear+1) | -0.26381 | 0.17887 | -1.475 | 0.1837 |
| Tissue | Log10(Heart+1) | -0.43307 | 1.25817 | -0.344 | 0.7408 |
| Tissue | Log10(Belly skin+1) | 0.56235 | 0.28186 | 1.995 | 0.0862 |
| Tissue | Log10(Neck skin+1) | -0.34201 | 0.29665 | -1.153 | 0.2868 |

**Table S9**. The *B. afzelii* spirochete loads are shown for the dissected organs of the bank voles. The units of the spirochete load are the number of spirochetes per mg of DNA.

| Vole ID[a] | Sex [b] | Treatment [c] | N positive[d] | Bladder[e] | Ear[f] | Heart[g] | Skin belly[h] | Skin neck[i] | Status[j] |
|---|---|---|---|---|---|---|---|---|---|
| 193 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 195 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 196 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 197 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 198 | M | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 199 | M | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 206 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 209 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 211 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 216 | M | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 224 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 228 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 230 | M | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 231 | M | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 192 | F | Infected | 3 | 41 | 0 | 0 | 37 | 872 | Infected |
| 200 | F | Infected | 3 | 1848 | 0 | 0 | 4647 | 1070 | Infected |
| 202 | M | Infected | 3 | 68 | 0 | 0 | 17450 | 170 | Infected |
| 204 | M | Infected | 4 | 11 | 0 | 3 | 6039 | 68 | Infected |
| 205 | M | Infected | 0 | 0 | 0 | 0 | 0 | 0 | Infected |
| 214 | F | Infected | 2 | 0 | 0 | 0 | 44 | 75 | Infected |
| 217 | NA | Infected | 3 | 0 | 446 | 0 | 4845 | 190 | Infected |
| 218 | F | Infected | 4 | 1939 | 4126 | 0 | 29764 | 216 | Infected |
| 220 | M | Infected | 4 | 54 | 243 | 0 | 442 | 408 | Infected |
| 222 | F | Infected | 4 | 14 | 1015 | 0 | 15767 | 844 | Infected |
| 223 | M | Infected | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected[k] |
| 225 | M | Infected | 2 | 0 | 0 | 0 | 40599 | 39389 | Infected |
| 226 | M | Infected | 5 | 3230 | 30 | 5 | 32525 | 8011 | Infected |
| 229 | M | Infected | 2 | 0 | 0 | 0 | 5 | 15 | Infected |

[a] Vole ID is the unique identification number assigned to each bank vole in the study.
[b] Sex refers to whether the bank vole is female (F) or male (M).
[c] Treatment refers to whether the bank vole was randomly assigned to the *B. afzelii*-infected group or the uninfected control group.
[d] N positive is the number of organs that tested positive for *B. afzelii* for each bank vole.
[e] Bladder is the number of spirochetes per mg of DNA in the bladder as estimated by our qPCR assay.
[f] Ear is the number of spirochetes per mg of DNA in the ear as estimated by our qPCR assay.
[g] Heart is the number of spirochetes per mg of DNA in the heart as estimated by our qPCR assay.
[h] Skin belly is the number of spirochetes per mg of DNA in the belly skin as estimated by our qPCR assay.
[i] Skin neck is the number of spirochetes per mg of DNA in the neck skin as estimated by our qPCR assay.
[j] Infection status is whether a vole was considered to be infected with *B. afzelii* or not (from Table S1).
[k] Bank vole 223 did not become infected following exposure to *B. afzelii*-infected ticks.

**Table S10**. The *B. afzelii* spirochete loads are shown for the dissected organs of the bank voles. The units of the spirochete load are the number of spirochetes per mg of tissue.

| Vole ID[a] | Sex [b] | Treatment[c] | N positive[d] | Bladder[e] | Ear[f] | Heart[g] | Skin belly[h] | Skin neck[i] | Status[j] |
|---|---|---|---|---|---|---|---|---|---|
| 193 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 195 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 196 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 197 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 198 | M | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 199 | M | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 206 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 209 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 211 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 216 | M | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 224 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 228 | F | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 230 | M | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 231 | M | Control | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected |
| 192 | F | Infected | 3 | 73 | 0 | 0 | 140 | 1898 | Infected |
| 200 | F | Infected | 3 | 3040 | 0 | 0 | 3677 | 1780 | Infected |
| 202 | M | Infected | 3 | 46 | 0 | 0 | 15149 | 357 | Infected |
| 204 | M | Infected | 4 | 3 | 0 | 1 | 5539 | 160 | Infected |
| 205 | M | Infected | 0 | 0 | 0 | 0 | 0 | 0 | Infected |
| 214 | F | Infected | 2 | 0 | 0 | 0 | 89 | 138 | Infected |
| 217 | NA | Infected | 3 | 0 | 624 | 0 | 4498 | 278 | Infected |
| 218 | F | Infected | 4 | 912 | 5128 | 0 | 52521 | 570 | Infected |
| 220 | M | Infected | 4 | 23 | 122 | 0 | 485 | 370 | Infected |
| 222 | F | Infected | 4 | 27 | 947 | 0 | 19384 | 1941 | Infected |
| 223 | M | Infected | 0 | 0 | 0 | 0 | 0 | 0 | Uninfected[k] |
| 225 | M | Infected | 2 | 0 | 0 | 0 | 41119 | 83549 | Infected |
| 226 | M | Infected | 5 | 342 | 33 | 3 | 24691 | 27739 | Infected |
| 229 | M | Infected | 2 | 0 | 0 | 0 | 9 | 12 | Infected |

[a] Vole ID is the unique identification number assigned to each bank vole in the study.
[b] Sex refers to whether the bank vole is female (F) or male (M).
[c] Treatment refers to whether the bank vole was randomly assigned to the *B. afzelii*-infected group or the uninfected control group.
[d] N positive is the number of organs that tested positive for *B. afzelii* for each bank vole.
[e] Bladder is the number of spirochetes per mg of tissue in the bladder as estimated by our qPCR assay.
[f] Ear is the number of spirochetes per mg of tissue in the ear as estimated by our qPCR assay.
[g] Heart is the number of spirochetes per mg of tissue in the heart as estimated by our qPCR assay.
[h] Skin belly is the number of spirochetes per mg of tissue in the belly skin as estimated by our qPCR assay.
[i] Skin neck is the number of spirochetes per mg of tissue in the neck skin as estimated by our qPCR assay.
[j] Infection status is whether a vole was considered to be infected with *B. afzelii* or not (from Table S1).
[k] Bank vole 223 did not become infected following exposure to *B. afzelii*-infected ticks.

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# Chapter Three

*Maternal antibodies provide strain-specific protection against infection with the Lyme disease pathogen in bank voles*

**Maternal antibodies provide strain-specific protection against infection with the Lyme disease pathogen in bank voles**

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**Abstract**

Multiple-strain microbial pathogens often induce strain-specific antibody responses in their vertebrate hosts. Mothers can transmit antibodies to their offspring, which can provide short-term, strain-specific protection against infection. Few experimental studies have investigated this phenomenon for multiple-strain zoonotic pathogens occurring in wildlife reservoir hosts. The tick-borne bacterium *Borrelia afzelii* causes Lyme disease in Europe and consists of multiple strains that cycle between the tick vector (*Ixodes ricinus*) and vertebrate hosts such as the bank vole (*Myodes glareolus*). We used a controlled experiment to show that female bank voles infected with *B. afzelii* via tick bite transmit protective antibodies to their offspring. To test the specificity of protection, the offspring were challenged using a natural tick bite challenge with either the maternal strain to which the mothers had been exposed or a different strain. The maternal antibodies protected the offspring against a homologous infectious challenge, but not against a heterologous infectious challenge. The offspring from the uninfected control mothers were equally susceptible to both strains. *Borrelia* outer surface protein C (OspC) is an antigen that is known to induce strain-specific immunity. Maternal antibodies in the offspring reacted more strongly with homologous than heterologous recombinant OspC, but other antigens may also mediate strain-specific immunity. Our study shows that maternal antibodies provide strain-specific protection against *B. afzelii* in an ecologically important rodent reservoir host. The transmission of maternal antibodies may have important consequences for the epidemiology of multiple-strain pathogens in nature.

**Key words**
*Borrelia afzelii*, Lyme disease, Ecology of infectious disease, Maternal antibodies, Maternal effects, Outer surface protein C, Strain-specific immunity, Tick-borne disease

**Importance**

Many microbial pathogen populations consist of multiple strains that induce strain-specific antibody responses in their vertebrate hosts. Females can transmit these antibodies to their offspring thereby providing them with short-term strain-specific protection against microbial pathogens. We investigated this phenomenon using the multiple-strain tick-borne microbial pathogen, *Borrelia afzelii*, and its natural rodent reservoir host, the bank vole, as a model system. We found that female bank voles infected with *B. afzelii* transmitted maternal antibodies to their offspring that provided highly efficient but strain-specific protection against a natural tick bite challenge. Trans-generational transfer of antibodies could be a mechanism that maintains high strain diversity of this tick-borne pathogen in nature.

## Introduction

The maternal environment and maternal phenotype can have important consequences for offspring phenotype and offspring fitness (1). In vertebrate hosts, an important maternal effect is the transmission of antibodies from mothers to offspring (2, 3). Young vertebrates are susceptible to infectious diseases while their immune systems are developing (3). Maternally transmitted antibodies protect the offspring against pathogens until they can produce their own antibodies (3). Theoretical models have shown that the evolution of maternal transfer of immunity depends on a number of factors including host lifespan, rate of loss of maternal protection, pathogen virulence, and host recovery rate (4). The transgenerational transmission of acquired immunity can have important consequences for the epidemiology of the pathogen (2, 5). Despite its potential importance in nature, the transgenerational transfer of acquired immunity has not received much attention in the ecology of zoonotic diseases (2, 5).

Maternal antibodies may be particularly important for the epidemiology of pathogens that consist of multiple genetically distinct strains that circulate in the host population at the same time. These strains can be distinguished by the acquired immune system of the vertebrate host resulting in the development of strain-specific antibody responses. Theoretical models of acquired immunity (without maternal transmission) have shown that strain-specific antibodies play a critical role in shaping the epidemiology and population structure of pathogen strains (6-8). Theoretical models of host populations infected with multiple pathogen strains have shown that the evolution of maternal transfer of immunity depends on a number of factors including the force of infection, the level of cross-immunity between strains, and the probability that the offspring will encounter the same strain as their mother (4). In such systems, a pathogen strain that is common in the maternal generation would be at a selective disadvantage in the offspring generation due to the maternal transmission of strain-specific antibodies against this common strain (4).

Tick-borne spirochete bacteria belonging to the *Borrelia burgdorferi* sensu lato (sl) genospecies complex are the etiological agents of Lyme borreliosis (9, 10). *B. burgdorferi* sl is a good model system for studying whether maternally transmitted antibodies can influence strain-specific infection success. The populations of *B. burgdorferi* sl consist of multiple strains that circulate between *Ixodes* ticks and vertebrate hosts such as rodents and birds (11-15). Immature *Ixodes* ticks search for a blood meal from spring until early autumn, and the transmission of *B. burgdorferi* sl therefore coincides with the reproduction and population expansion of their vertebrate hosts (10, 16). There is no vertical transmission of *B. burgdorferi* sl in either the tick (17) or the vertebrate host (18-20). In nature, vertebrate hosts develop a strong antibody response against *B. burgdorferi* sl (18, 19), and infection studies in rodents have shown that this antibody response is strain-specific (21-24). This antibody response is not effective at clearing the pathogen, which is why rodent hosts remain infected for months or even years (25-28). However, this antibody response is effective at preventing re-infection with the same strain (29, 30) and the transfer of anti-sera from infected donors to naïve recipients (i.e. passive immunization) prevents infection in the latter (31-33). Studies in various vertebrate species showed that infected neonates develop much more disease than infected adults (34, 35), suggesting that it is important for mothers to protect their young offspring. Previous field studies on sea birds (36, 37) and one dog (38) found that infected mothers transmit antibodies to their offspring (36, 37). However, to date no one has used an experimental approach to test whether maternal antibodies protect offspring against infection with *B. burgdorferi* sl and whether this protection is strain-specific.

In this study, we used *Borrelia afzelii*, which is the most common cause of Lyme borreliosis in Europe (39), its tick vector *I. ricinus*, and the bank vole (*Myodes glareoulus*), which is an important reservoir host for both *B. afzelii* and *I. ricinus* (39). The purpose of this

study was to test (1) whether female bank voles that were experimentally infected with *B. afzelii* transmit maternal antibodies to their offspring, (2) whether maternal antibodies can protect bank vole offspring against *B. afzelii*-infected *I. ricinus* ticks, and (3) whether this maternal antibody protection is specific for the strain of *B. afzelii*.

## Results

### Maternal infection status and maternal antibody transmission

The maternal infection status was unambiguous: control mothers tested negative for 4 of 4 infection criteria, whereas infected mothers tested positive for at least 3 of 4 infection criteria (Table S1). The mean *B. afzelii*-specific IgG antibody response of the infected mothers (mean = 3811 absorbance units, 95% CI = 2692–5395) was 7.4 times higher than the uninfected mothers (mean = 512 absorbance units, 95% CI = 371–706), and this was significant (Figure S1; $t = -9.335$, $df = 11$, $p < 0.001$). This result shows that infected mothers developed a strong IgG antibody response against the *B. afzelii* infection.

For the offspring blood sample that was taken the day before the infectious challenge (at 34 days PB), the mean level of *B. afzelii*-specific IgG antibodies was 1.6 times higher for the MatAb+ offspring (mean = 815 absorbance units, 95% CI = 731–906) compared to the MatAb- offspring (mean = 511 absorbance units, 95% CI = 459–566) and this was significant (Figure 1; $t = -5.589$, $df = 39$, $p < 0.001$). This result shows that *B. afzelii*-infected mothers transmitted maternal antibodies to the MatAb+ offspring, whereas uninfected mothers did not transmit such antibodies to the MatAb- offspring.

The OspC A10 specificity ratio of the maternally transmitted IgG antibodies was 3.07 times higher in the MatAb+ offspring compared to the MatAb- offspring, and this difference was significant (Figure S3; $t = -10.015$, $df = 39$, $p < 0.001$). This result shows that the maternal IgG antibodies in the MatAb+ offspring reacted more strongly with rOspC A10 than rOspC A3 compared to the MatAb- offspring.

### Offspring infection status following the infectious challenge

As expected, there was no mother-offspring transmission of the pathogen; the ear tissue biopsies of all offspring before the infectious challenge (34 days PB) tested negative for *B. afzelii*. The infectious challenge was successful: we collected at least one *B. afzelii*-infected nymph from 38 of the 40 offspring that were challenged with infected ticks. The 2 offspring for which no infected ticks were collected were excluded from the analysis (Tables S2 and S3). The final sample sizes were therefore 8, 11, 9, and 10 offspring for groups MatAb-/NE4049, MatAb-/Fin-Jyv-A3, MatAb+/NE4049, and MatAb+/Fin-Jyv-A3, respectively. The infection status of the offspring was unambiguous: uninfected offspring tested negative for at least 5 of the 6 infection criteria, whereas infected offspring tested positive for at least 4 of the 6 infection criteria (Tables S2 and S3).

### Maternal antibody protection and strain specificity

The analysis of offspring infection status found a highly significant interaction between maternal antibody status and challenge strain (GLM: $\Delta\ df = 1$, $\Delta\ \chi^2 = 71.659$, $p < 0.001$). All of the MatAb- offspring became infected regardless of whether they were challenged with strain NE4049 (100.0% = 8 infected /8 total) or strain Fin-Jyv-A3 (100.0% = 11 infected /11 total). This result shows that both strains were highly infectious to naive offspring. The MatAb+ offspring were perfectly protected against strain NE4049 (0.0% = 0 infected /9 total), but almost completely susceptible to strain Fin-Jyv-A3 (90.0% = 9 infected /10 total), and this difference was significant ($\chi^2 = 11.992$, $df = 1$, $p < 0.001$). This result shows that maternal antibodies

developed against the maternal strain (strain NE4049) protected offspring against this strain but not against a different strain (Fin-Jyv-A3).

**The *B. afzelii*-specific IgG antibody response in the post-infection offspring**

In the offspring, the *B. afzelii*-specific IgG antibody response at 35 days PI followed the expectation for strain-specific protection (Figure 3). The interaction between maternal antibody status and challenge strain was significant ($F_{1, 33} = 54.664$, $p < 0.001$). After splitting the analysis by maternal antibody status, the challenge strain had a significant effect on the *B. afzelii*-specific IgG antibody response at 35 days PI of the MatAb+ offspring ($F_{1, 17} = 75.174$, $p < 0.001$), but not the MatAb- offspring ($F_{1, 16} = 0.088$; $p = 0.771$). As expected, the two negative control offspring showed no sign of infection.

**The *B. afzelii* spirochete load in the ear tissue biopsy of the post-infection offspring**

In the offspring, the *B. afzelii* spirochete load in the ear tissue biopsies at 35 days PI followed the expectation for strain-specific protection (Figure 4). The interaction between maternal antibody status and challenge strain was significant ($F_{1, 34} = 16.101$, $p < 0.001$). After splitting the analysis by maternal antibody status, the challenge strain had a significant effect on the ear tissue spirochete load at 35 days PI in the MatAb+ offspring ($F_{1, 17} = 44.082$, $p < 0.001$), but not the MatAb- offspring ($F_{1, 17} = 0.609$; $p = 0.446$). As expected, two negative control offspring showed no sign of infection.

**Discussion**

**Maternal antibodies are protective and strain-specific:** Our study provides experimental evidence that maternally transmitted antibodies protect offspring against infection with *B. burgdorferi* sl in an important reservoir host. The maternal antibodies were highly protective for the MatAb offspring, despite the fact that the ELISA absorbance values in the MatAb+ offspring (815 absorbance units) were not much higher compared to the MatAb- offspring (511 absorbance units) and were much lower compared to the mothers (3811 absorbance units). One explanation is that not many antibodies are needed for protection because during their blood meal, nymphs inoculate only a few hundred spirochetes into the vertebrate host (40). Earlier studies on a marine Lyme borreliosis system that consists of *B. garinii* and sea birds had shown a correlation in antibody concentrations between mothers and their chicks, but there was no proof of protection (36). Our results are similar to a study on the relapsing fever spirochete (*Borrelia duttonii*) in laboratory mice (*Mus musculus* strain ddY), which found that maternal antibodies protected offspring against infection (41). A strength of our study is that we used a natural rodent reservoir host rather than lab mice, and that we used ticks rather than needles to deliver the infectious challenge. The mode of delivery (needle versus tick bite) influences the antibody response and its protective efficacy (42-44).

Our study also suggests that the protection afforded by the maternal antibodies is strain-specific. Offspring from mothers infected with *B. afzelii* strain NE4049 were 100% protected against this strain, but they were highly susceptible to *B. afzelii* strain Fin-Jyv-A3 to which their mothers had not been exposed. Our results are consistent with numerous studies that have shown that strains of *B. burgdorferi* sl (or their OspC antigens) induce strain-specific antibody responses in their rodent hosts (21, 22, 28, 45, 46). Again, it is critical to use the natural route of infection (i.e. ticks), as needle inoculation of *B. burgdorferi* sl can produce strong patterns of cross-immunity between strains (47). Field studies at small spatial scales have shown that local populations of *B. burgdorferi* sl contain a community of a dozen strains that circulate in the same reservoir host and tick populations (11-15, 48-52). Theoretical models have shown that strain-specific antibody responses are important for structuring pathogen populations into

communities of antigenically distinct strains (6-8). Numerous Lyme disease researchers have suggested that the host immune response against the immunodominant OspC antigen could drive the population structure of *B. burgdorferi* sl pathogens (10, 11, 13, 14, 53, 54). The results from our study suggest that the trans-generational transfer of antibodies in vertebrate reservoir hosts could play a critical role in structuring the local community of pathogen strains.

**Duration of protection of maternal antibodies:** In rodents, maternally transmitted antibodies can protect the offspring for 6 to 10 weeks (3, 55). In the present study, maternally transmitted antibodies protected bank vole offspring at 5 weeks post-birth. The study on *B. duttonii* in ddY mice also found that the maternally transmitted antibodies protected offspring at 5 weeks post-birth (41). A study on Mongolian gerbils (*Meriones unguiculatus*), where the mothers were vaccinated against *B. burgdorferi*, found that antibody levels remained stable between 8 to19 days of age (when offspring fed on milk), but then decreased rapidly and reached undetectable levels at 40 days of age (56). A study on bank voles found that maternally transmitted antibodies against Puumala hantavirus can protect offspring for a period of two and a half months post-birth (55). Our study suggests that maternal antibodies may protect sub-adult rodents against *B. burgdorferi* sl and help to reduce the prevalence of infection in this age group (18, 19, 57).

**Maternally transmitted antibodies and offspring fitness:** Our study did not show whether protected and uninfected offspring had higher fitness than the unprotected and infected offspring. Lyme disease causes morbidity in human patients (58), but whether infection reduces the fitness of reservoir hosts is less clear (10, 53). Long-term mark-recapture studies of the white-footed mouse (*P. leucopus*) and the black-legged kittiwake (*Rissa tridactyla*) have found no effect of *B. burgdorferi* sl infection on host survival (18, 57, 59). An enclosure study on bank voles found subtle effects of *B. afzelii* infection on host reproduction, but not on host survival (60). Numerous studies have shown that infection with *B. burgdorferi* induces pathology (e.g. arthritis and carditis) in laboratory mice (*Mus musculus*) (61-63), but not in wild rodents (20, 35, 64, 65). Interestingly, young individuals are more likely to develop disease than old individuals (34, 35), suggesting that maternally transmitted antibodies increase offspring fitness by delaying pathogen acquisition to an older and more disease-resistant stage.

**Importance of maternal antibodies for the ecology of Lyme borreliosis:** Previous studies on wild bank vole populations in Finland have shown that maternally transmitted antibodies are important for the epidemiology of the Puumala Hantavirus (5). The ecology of Lyme borreliosis suggests that maternally transmitted antibodies could be important for controlling the epidemiology of *B. burgdorferi* sl pathogens in nature. The search for a blood meal by the tick vector, the resultant transmission of *B. burgdorferi* sl, and the reproduction of the rodent host all occur at the same time of the year (10, 16). *Ixodes* nymphs, which transmit *B. burgdorferi* sl, search for reservoir hosts from the spring to the autumn (66). Field studies have shown that wild rodent populations can build up high levels of acquired immunity to *B. burgdorferi* sl (18, 19). For example, >90% of white-footed mice (*Peromyscus leucopus*) in Connecticut were seropositive for *B. burgdorferi* by the end of August (19). This study suggests that the majority of female rodents are transferring protective antibodies to their offspring during the summer. In summary, the Lyme disease system is characterized by strong seasonal interactions between pathogen transmission, offspring production, and maternal transmission of highly protective antibodies to offspring (67).

**OspC and strain-specific immunity:** Our study also showed that the protection of the maternal antibodies was strain-specific. The *ospC* is the most polymorphic gene in the genome of *B. burgdorferi* sl (11-13) and encodes for outer surface protein C (OspC). Studies have shown that OspC induces a strain-specific antibody response that protects rodents from tick bite (21-23, 30). The two *ospC* alleles used in this study (A3 and A10) have a genetic distance of 23.19% and an amino acid distance of 62.57%. We had previously shown in a vaccination trial that

rOspC proteins A3 and A10 induce strain-specific protection against strains of *B. afzelii* carrying the corresponding *ospC* alleles and that cross-immunity was low (21). In the present study, we showed that maternal antibodies against *B. afzelii* strain NE4049, which carries *ospC* allele A10, reacted much more strongly with rOspC A10 than rOspC A3. Numerous studies have shown that infection with a particular *ospC* strain induces a much stronger antibody response against the homologous OspC antigen compared to the heterologous OspC antigen (21, 24, 45, 68). In summary, our study is consistent with the idea that OspC plays a role in inducing strain-specific immunity, but other antigens could also be important.

**Importance of maternal antibodies for population structure of *ospC* type strains:** *B. burgdorferi* sl pathogens often contain a high *ospC* diversity at small spatial scales (12-15, 48, 52, 69, 70). An important goal of Lyme disease ecology is to understand the factors that allow this diversity of *ospC* strains co-exist in nature (10, 12, 14, 15, 53, 54). Long-term field studies on *B. afzelii* in tick and rodent populations have shown that the community of strains carrying different *ospC* major groups (oMGs) was stable over more than a decade, with some strains an order of magnitude more common than others (14, 15). Strains that were common in the field had higher rates of host-to-tick transmission in laboratory studies (14, 71). An important question is why these high transmission strains do not eliminate the low transmission strains. The *ospC* polymorphism is maintained by balancing selection and two alternative hypotheses are multiple niche polymorphism (MNP) and negative frequency-dependent selection (NFDS) (11, 12, 54). Under MNP, the different oMG strains are adapted to different host species and the frequency of each oMG strain depends on the abundance of its host species (12, 54). Under NFDS, the immune system of the vertebrate host is more efficient at controlling the common oMG strains, and the rare oMG strains therefore have a selective advantage (11, 13). Our study suggests that balancing selection could result from the maternal transfer of OspC-specific antibodies. At the start of the transmission season, the naïve mothers will become infected with the common oMG strains. Later on in the transmission season the MatAb+ offspring will be protected against the common oMG strains, which gives the selective advantage to the rare oMG strains. In summary, the seasonal trans-generational transmission of strain-specific antibodies could play a role in maintaining the high local diversity of *ospC* strains in nature.

## Conclusions

We used experimental infections with a common Lyme disease pathogen (*B. afzelii*) and its natural reservoir host (the bank vole) to show that females transmit maternal antibodies to their offspring. These maternal antibodies were completely protective against the strain that the mother had encountered but provided no protection against a different strain. The binding affinity for homologous and heterologous rOspC antigen was consistent with the expected pattern, suggesting that this immunodominant antigen was involved in the maternal transmission of strain-specific immunity. The inter-generational transfer of protective strain-specific antibodies could have important implications for the epidemiology of multiple strain pathogen populations in the field.

Future studies should investigate whether maternally transmitted antibodies are important for protecting other important reservoir host species against *B. burgdorferi* sl. They should investigate the duration of protection, whether the mechanism of antibody transfer involves the placenta, milk, or both (41, 56), and whether females infected with multiple strains transmit antibody responses that are protective against each of those strains. Studies are needed to determine which *B. burgdorferi* sl antigens are responsible for strain-specific immunity. Finally, theoretical models should investigate how the maternal transfer of antibodies in the host population would influence the epidemiology of this multi-strain tick-borne pathogen.

## Materials and Methods

### Bank voles, *Ixodes ricinus* ticks and *Borrelia afzelii*

In 2014, we used field-captured bank voles to establish a breeding colony at the University of Neuchâtel (72). The bank voles used in this study were from the third and fourth lab-born generation and are therefore free from tick-borne pathogens. The *I. ricinus* ticks came from a laboratory colony established in 1978 at the University of Neuchâtel. During the study, the bank voles were maintained in individual cages and were given food and water *ad libitum*. Bank voles were experimentally infected via tick bite with one of two isolates of *B. afzelii*: NE4049 and Fin-Jyv-A3. NE4049 was isolated from an *I. ricinus* tick in Switzerland, has multi-locus sequence type (MLST) 679, and strain ID number 1887 in the *Borrelia* MLST database. Fin-Jyv-A3 was isolated from a bank vole in Finland, has MSLT 676, and strain ID number 1961 in the *Borrelia* MLST database. These two isolates (hereafter strains) are highly infectious to both rodents and *I. ricinus* ticks (21, 73). Furthermore, these two strains carry two different *ospC* alleles, A10 and A3, which code for two different variants of outer surface protein C (OspC). Immunization with recombinant OspC (rOspC) A10 and rOspC A3 induces strain-specific protective antibody responses in laboratory mice (21).

### Creation of *I. ricinus* nymphs infected with *B. afzelii*

Nymphs infected with *B. afzelii* strain NE4049 or Fin-Jyv-A3 were created experimentally (see section 1 in the supporting information for details). The percentage of infected nymphs was 77.9% and 91.8% for strain NE4049 and strain Fin-Jyv-A3, respectively.

### Infectious challenge of the bank vole mothers

Five-week-old female bank voles were randomly assigned to one of two experimental groups: control (n = 9) and infected with *B. afzelii* strain NE4049 (n = 11). Each female in the control group was infested with 4 uninfected nymphs; each female in the infected group was infested with 4 nymphs infected with strain NE4049. At 5 weeks PI, a blood sample and an ear tissue biopsy were taken from each female to confirm their infection status. Females were coupled with different males at 2 and at 6 weeks PI, and offspring from the first successful coupling was used in the present study. Seven control mothers and 6 *B. afzelii*-infected mothers produced a total of 22 offspring and 20 offspring, respectively (Table S1). At 18 weeks PI, the mothers were sacrificed using $CO_2$ asphyxiation and the following organs were aseptically dissected: bladder, left ear, right ear, left rear tibiotarsal joint, and right rear tibiotarsal joint. The tissue samples were stored at –80°C until further analysis.

### Rearing the bank vole offspring

At 21 days post-birth (PB), offspring were separated from their mothers and moved to individual cages. At 34 days PB, a blood sample and an ear tissue biopsy were taken from each of the 42 offspring. The blood samples at 34 days PB were tested for maternal IgG antibodies against *B. afzelii* using a *Borrelia*-specific ELISA (see below). To confirm that there was no mother-to-offspring transmission of *B. afzelii*, the ear tissue biopsies at 34 days PB were tested for the presence of *B. afzelii* using qPCR (see below). As the offspring from the uninfected mothers and the infected mothers are expected to test negative and positive for maternal antibodies (MatAb), they will hereafter be referred to as the MatAb- and MatAb+ offspring, respectively. At 35 days PB, the offspring were challenged with *I. ricinus* nymphs that were infected with either strain NE4049 or strain Fin-Jyv-A3 (see below).

**Infectious challenge of the bank vole offspring**

To test whether maternal antibodies provide strain-specific protection, the MatAb- offspring (n = 22) and the MatAb+ offspring (n = 20) were challenged via tick bite with strain NE4049 or strain Fin-Jyv-A3 at 35 days PB. Offspring were assigned to balance sample sizes and family effects among the four combinations of MatAb status and challenge strain, which were as follows: MatAb-/NE4049 (n = 9), MatAb-/Fin-Jyv-A3 (n = 11), MatAb+/NE4049 (n = 10), and MatAb+/Fin-Jyv-A3 (n = 10). The remaining 2 MatAb-offspring were challenged with uninfected nymphs as controls. The infectious tick bite challenge for the offspring was the same as for the mothers. At 35 days PB, offspring were challenged with 4 nymphs infected with either strain NE4049 or strain Fin-Jyv-A3. The engorged nymphs were collected and tested for *B. afzelii* to confirm that each offspring had been infested with at least one infected nymph (Tables S3 and S4). At 35 days PI (70 days PB), a second blood sample and a second ear tissue biopsy were taken from each of the 42 offspring to confirm their infection status. At 70 days PI (105 days PB), the offspring were sacrificed using $CO_2$ asphyxiation and the following organs were aseptically dissected: bladder, left ear, right ear, left rear tibiotarsal joint, right rear tibiotarsal joint, ventral skin, and dorsal skin. Tissue samples (20–25 mg) from the bladder, left ear, and left rear tibiotarsal joint were tested for the presence of *B. afzelii* using qPCR (see below). Tissue samples from the right ear, right rear tibiotarsal joint, ventral skin, and dorsal skin were cultured in BSK-II medium (see below).

**Infection status of the bank vole offspring**

A bank vole offspring was considered as having been successfully challenged with *B. afzelii* if at least one engorged *B. afzelii*-infected nymph was collected and/or if it developed a systemic infection following the infectious tick challenge. A bank vole was defined as having a systemic infection with *B. afzelii* if it tested positive for more than one of six criteria: (1) presence of *B. afzelii*-specific IgG antibodies at 35 days PI, (2) *B. afzelii* in ear biopsy at 35 days PI, (3) *B. afzelii* in bladder at 70 days PI, (4) *B. afzelii* in left ear at 70 days PI, (5) *B. afzelii* in left rear joint at 70 days PI, and (6) culture of live spirochetes from dissected organs at 70 days PI. Note that 35 days PI and 70 days PI correspond to 70 days post-birth (PB) and 105 days PB, respectively.

***Borrelia*-specific qPCR and *ospC*-specific qPCR**

The *B. afzelii* infection status of the engorged nymphs and the bank vole tissue samples was tested using qPCR. The DNA was extracted from the engorged nymphs and the bank vole tissue samples as previously described (21, 72). The qPCR assay targets a 132-bp fragment of the *flagellin* gene of *B. burgdorferi* sl and was performed as previously described (21, 72). The strain identity of the engorged nymphs and the offspring ear biopsies was confirmed using a strain-specific qPCR (73). This qPCR targets a 143-bp fragment of the *ospC* gene and uses two different probes that detect either *ospC* allele A3 or *ospC* allele A10 and was performed as previously described (73).

***Borrelia*-specific ELISA and OspC-specific ELISA**

The serum samples of the bank voles were tested for the presence of *B. afzelii*-specific IgG antibodies with an ELISA assay using SERION® ELISA classic *Borrelia burgdorferi* IgG/IgM immunoassay plates (Ruwag, Germany), as previously described (21, 72). These ELISA plates use the conserved fragments of three recombinant antigens of *B. burgdorferi* sl: OspC, Flagellin, and VlsE. The maternally transmitted OspC-specific IgG antibody level in the offspring before the infectious challenge (34 days PB) was measured using a homemade ELISA with recombinant OspC (rOspC) proteins A3 and A10 (21) (see section 4 in the supporting information for details).

**Culture of *B. afzelii* spirochetes from bank vole tissues**

To demonstrate that the bank vole offspring were infected with live *B. afzelii*, tissue biopsies were cultured in BSK-II media. Tissue biopsies from the skin (ventral skin and/or dorsal skin), right ear, and right rear tibiotarsal joint were placed in individual tubes for each of the 42 offspring. The culture tubes were kept in an incubator at 34°C and were screened for live spirochetes over a period of 4 weeks using a dark field microscope.

**Statistical analysis**

All statistical analyses were done in R version 1.0.143 (R Development Core Team 2015-08-14). In what follows, we use the word 'response' to refer to the amount of IgG antibodies that developed in response to the *B. afzelii* infection in infected individuals (mothers or offspring). In contrast, we use the word 'level' to refer to the amount of maternally transmitted IgG antibodies that were measured in the offspring the day before their infectious challenge. The IgG antibody response or level is measured in absorbance units and was log10-transformed to improve the normality of the residuals. All means are reported with their 95% confidence intervals (95% CI).

**Maternal infection status and maternal antibody transmission**

To test whether the mother bank voles developed an IgG antibody response against *B. afzelii* at 35 days PI, we compared this variable (log10-transformed) between infected mothers and uninfected mothers using an independent two samples t-test.

To test whether there was maternal transmission of *B. afzelii*-specific IgG antibodies from mothers to their offspring, we compared the level of this variable (log10-transformed) in the pre-infection blood sample (at 34 days PB) of the offspring between the MatAb- offspring and the MatAb+ offspring using an independent two samples t-test.

The specificity of the maternal IgG antibodies in the pre-infection blood sample (at 34 days PB) of the offspring was measured as their relative ability to bind the two strain-specific OspC antigens A3 and A10. We calculated an OspC A10 specificity ratio for each offspring by dividing the level of IgG antibodies that bound to rOspC A10 by the level of IgG antibodies that bound to rOspC A3. We compared the log10-transformed OspC A10 specificity ratio between the MatAb+ offspring and the MatAb- offspring using an independent two samples t-test.

**Maternal antibody protection and strain specificity**

We tested whether the maternal antibodies protected offspring against infection with *B. afzelii* and whether this protection was strain-specific. Offspring were classified as being uninfected (0) or infected (1) depending on the 7 infection criteria. Offspring infection status was modeled using generalized linear models (GLMs) with binomial errors. The two explanatory factors included offspring maternal antibody status (2 levels: MatAb+ and MatAb-) and offspring challenge strain (2 levels: NE4049, Fin-Jyv-A3), and their interaction. Statistical significance of explanatory factors was determined using log-likelihood ratio tests that compared the change in deviance between nested models to a Chi-square distribution.

**The *B. afzelii*-specific IgG antibody response and the *B. afzelii* ear tissue spirochete load in the post-infection offspring**

In the previous analysis, the comprehensive infection status of the offspring was based on 6 infection criteria. In our experience, scientists differ with respect to their preference for these infection criteria (e.g. some prefer antibody data, whereas others prefer direct detection of microbes via qPCR or culture). We therefore present 2 of the 6 infection criteria for the offspring to show that they have the same pattern as the comprehensive infection status. To

avoid redundancy, the other 4 infection criteria are not shown, but they all show the same pattern.

The two infection criteria analyzed here are: (1) the *B. afzelii*-specific IgG antibody response in the post-infection offspring and (2) the *B. afzelii* ear tissue spirochete load in the post-infection offspring. Both response variables were log10-transformed to normalize the residuals. We used linear models with normal errors to model both response variables as a function of two explanatory factors: offspring maternal antibody status (2 levels: MatAb+ and MatAb-), offspring challenge strain (2 levels: NE4049, Fin-Jyv-A3), and their interaction. The statistical significance of each explanatory factor was based on the type II sums of squares, which was calculated using the Anova() function in the R package 'car'.

**Data Accessibility**

The raw data for this study will be stored on DRYAD in an Excel file titled "**Raw data for maternal antibodies ms_v03.xlsx**".

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**Author contributions**

A.G.-C. and M.J.V designed the study. A.G.-C., A.S. and D.G. created the *B. afzelii*-infected nymphs. A.G.-C., V.H, and A.S. performed the experimental infections. A.G.-C., A.S, C.B. and O.R. performed the molecular work. M.J. created the recombinant proteins. A.G.-C. analysed the data. A.G.-C. and M.J.V wrote the manuscript. All authors read and approved the final version of the manuscript.

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## Figures

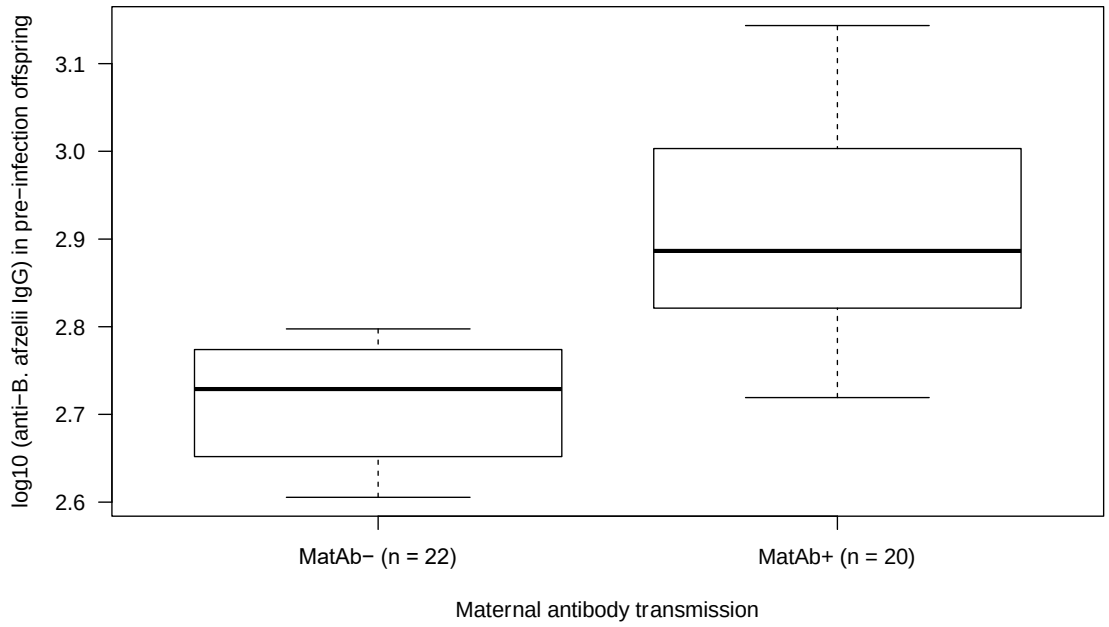


**Figure 9**. The level of maternally transmitted *B. afzelii*-specific IgG antibodies was significantly higher in the MatAb+ offspring (n = 20) compared to the MatAb- offspring (n = 22) at 34 days post-birth (PB). The MatAb- and the MatAb+ are the offspring of 7 uninfected control mothers and 6 *B. afzelii*-infected mothers, respectively. The level of the maternally transmitted *B. afzelii*-specific IgG antibody response was measured in the blood of the offspring at 34 days PB using a commercial Lyme borreliosis ELISA. Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (circles).

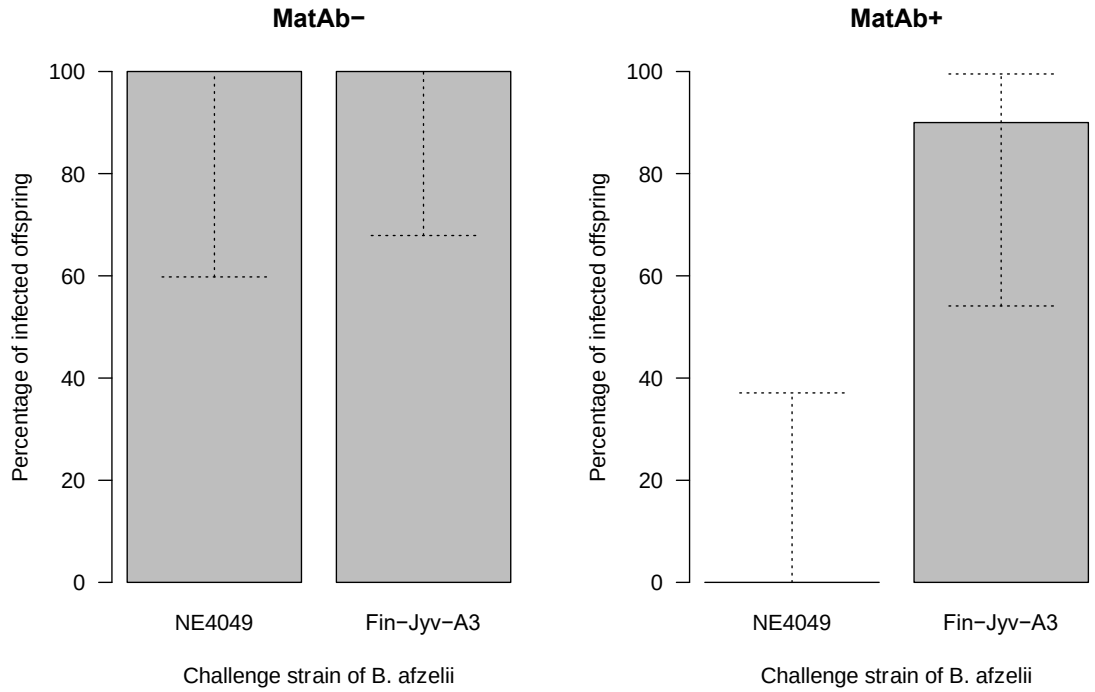


**Figure 10**. The percentage of infected offspring depends on the maternal antibody status of the offspring and on the strain with which they were challenged. The MatAb- (left panel) and MatAb+ (right panel) refer to the offspring from the uninfected control mothers and the mothers infected with *B. afzelii* strain NE4049, respectively. The offspring were challenged via tick bite with either *B. afzelii* strain NE4049 or *B. afzelii* strain Fin-Jyv-A3 at 35 days post-birth (PB). The infection status of the offspring was determined using 7 different infection phenotypes at 35 days post-infection (PI) and at 70 days PI, which correspond to 70 days PB and 105 days PB, respectively. The MatAb- offspring were equally susceptible to both strains. The MatAb+ offspring were protected against the maternal strain (NE4049) but not the new strain (Fin-Jyv-A3). The grey solid bars show the means and the stippled bars show the 95% confidence intervals.

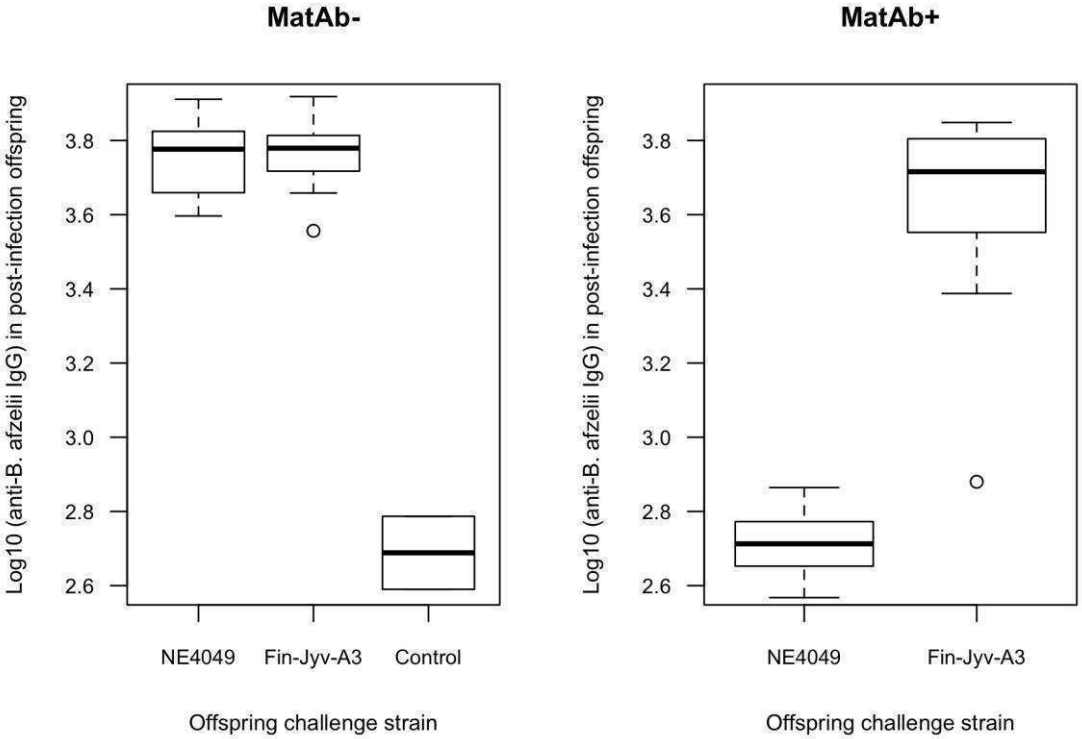


**Figure 3**. The *B. afzelii*-specific IgG antibody response of the offspring at 35 days post-infection (PI) depends on the maternal antibody status and the challenge strain. The MatAb- (left panel) and MatAb+ (right panel) refer to the offspring from the uninfected control mothers and the mothers infected with *B. afzelii* strain NE4049, respectively. The offspring were challenged via tick bite with either strain NE4049 or strain Fin-Jyv-A3 at 35 days post-birth (PB). The MatAb- offspring were equally susceptible to both strains. The MatAb+ offspring were protected against the maternal strain (NE4049) but not the new strain (Fin-Jyv-A3). The control group refers to 2 MatAb- offspring that were each infested with 4 uninfected I. *ricinus* nymphs. These two individuals show the baseline *B. afzelii*-specific IgG antibody response for bank vole offspring that were bitten by ticks, but not infected with *B. afzelii*.

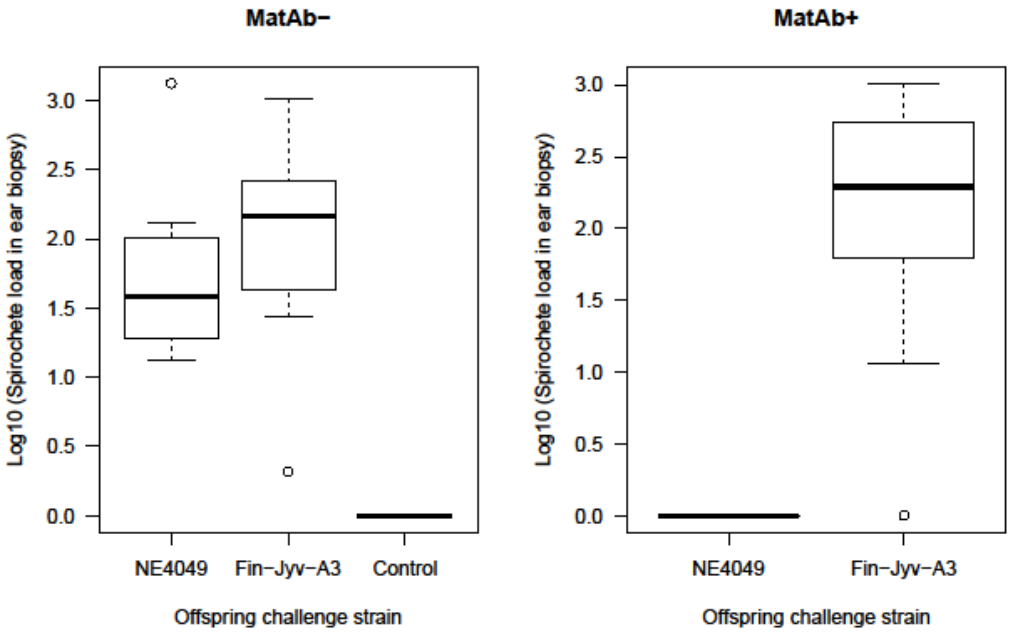


**Figure 4**. The *B. afzelii* spirochete load in the ear biopsies of the bank vole offspring at 35 days post-infection (PI) depends on the maternal antibody status and the challenge strain. The MatAb- (left panel) and MatAb+ (right panel) refer to the offspring from the uninfected control mothers and the mothers infected with *B. afzelii* strain NE4049, respectively. The offspring were challenged via tick bite with either strain NE4049 or strain Fin-Jyv-A3 at 35 days post-birth (PB). The MatAb- offspring were equally susceptible to both strains. The MatAb+ offspring were protected against the maternal strain (NE4049) but not the new strain (Fin-Jyv-A3). The control group refers to 2 MatAb- offspring that were each infested with 4 uninfected I. *ricinus* nymphs. These two individuals show the baseline *B. afzelii* spirochete load in the ear biopsies for bank vole offspring that were bitten by ticks, but not infected with *B. afzelii*.

**Supplementary material**

**Table S1.** *B. afzelii* infection status of the mothers. The *B. afzelii* infection status is shown for each of the 20 females in the study, of which 13 became mothers and produced offspring. There were 7 uninfected control females that produced 22 MatAb- offspring and 6 *B. afzelii*-infected females that produced 20 MatAb+ offspring. All mothers in the uninfected control group tested positive for 0 of the 4 criteria whereas all mothers in the infected group tested positive for 3 or 4 criteria.

| ID[a] | Treat[b] | Male[c] | Offspring[d] | Nymphs[e] | ELISA[f] | Ear[g] | Bladder[h] | Joint[i] | Criteria[j] | Infected[k] |
|---|---|---|---|---|---|---|---|---|---|---|
| V634 | Control | V162 | 3 | 0/0 | 480 | - | - | - | 0 | No |
| V635 | Control | V523 | 3 | 0/3 | 481 | - | - | - | 0 | No |
| V639 | Control | VD4 | 4 | 0/4 | 569 | - | - | - | 0 | No |
| V643 | Control | V174 | 3 | 0/2 | 503 | - | - | - | 0 | No |
| Z533 | Control | Z57 | 3 | 0/1 | 535 | - | - | - | 0 | No |
| Z536 | Control | Z59 | 4 | 0/1 | 506 | - | - | - | 0 | No |
| Z540 | Control | Z109 | 2 | 0/3 | 513 | - | - | - | 0 | No |
| V637 | Control | V184 | 0 | 0/0 | 1104 | - | - | - | 0 | No |
| Z539 | Control | Z107 | 0 | 0/1 | 1099 | - | - | - | 0 | No |
| V631 | Infected | V141 | 5 | 3/3 | 6611 | + | + | + | 4 | Yes |
| V662 | Infected | V185 | 2 | 1/1 | 6162 | - | + | + | 3 | Yes |
| V665 | Infected | V242 | 3 | 2/2 | 3800 | + | + | + | 4 | Yes |
| V666 | Infected | V151 | 2 | 0/1 | 4135 | + | + | + | 4 | Yes |
| V667 | Infected | V146 | 4 | 3/4 | 1352 | + | + | + | 4 | Yes |
| Z538 | Infected | Z101 | 4 | 1/2 | 3530 | - | + | + | 4 | Yes |
| V632 | Infected | V162 | 0 | 1/2 | 6409 | + | + | + | 4 | Yes |
| V636 | Infected | V153 | 0 | 1/1 | 2616 | + | + | + | 4 | Yes |
| V638 | Infected | V208 | 0 | 1/1 | 7630 | - | + | + | 3 | Yes |
| V641 | Infected | V154 | 0 | 2/2 | 10913 | + | + | + | 4 | Yes |
| Z534 | Infected | Z108 | 0 | 1/2 | 8150 | + | + | + | 4 | Yes |

[a] ID is the unique identification number of each female bank vole that was mated to produce offspring.
[b] Treatment has two levels: Control and Infected. Control females were infested with uninfected nymphs whereas infected females were infested with nymphs infected with *B. afzelii* strain NE4049.
[c] Male refers to the identity of the male bank vole that sired the offspring.
[d] Offspring is the number of offspring that were produced by each female bank vole.
[e] Nymphs gives a fraction where the numerator and denominator refer to the number of engorged *B. afzelii*-infected nymphs and the number of engorged nymphs, respectively, collected for each female bank vole.
[f] ELISA absorbance values indicate the strength of the IgG antibody response against *B. afzelii*. Individuals with absorbance values > 2000 are infected with *B. afzelii* strain NE4049. Absorbance values were obtained from a commercial Lyme disease ELISA.
[g] Ear indicates whether ear tissue tested positive or negative for *B. afzelii* spirochetes using the qPCR assay.
[h] Bladder indicates whether bladder tissue tested positive or negative for *B. afzelii* spirochetes using the qPCR assay.
[i] Joint indicates whether joint tissue tested positive or negative for *B. afzelii* spirochetes using the qPCR assay.
[j] Criteria is the number of the four infection criteria for which each female tested positive for *B. afzelii*.
[k] Infected refers to whether the female is considered to be infected with *B. afzelii* (Yes) or not (No).

**Table S2.** *B. afzelii* infection status of the MatAb- offspring. Infection status of the MatAb- offspring is shown following the infectious challenge via tick bite with *B. afzelii* strain NE4049 or *B. afzelii* strain Fin-Jyv-A3. The 22 MatAb- offspring were descended from the 7 mothers that were uninfected. The 6 infection criteria for the offspring are ELISA2, Biop2, Ear, Joint, Bladder, and Culture. Offspring that tested positive for 0 or 1 of the 6 infection criteria were considered as uninfected. Offspring that tested positive for 4 or more of the 6 infection criteria were considered as infected with *B. afzelii*.

| ID1[a] | ID2[b] | Strain[c] | Nymphs[d] | ELISA1[e] | Biop1[f] | ELISA2[g] | Biop2[h] | Ear[i] | Joint[j] | Bladder[k] | Culture[l] | Criteria[m] | Infected[n] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| V643 | V725 | NE4049 | 2/3 | 621 | 0 | 4549 | 18 | - | + | + | + | 5/6 | Yes |
| V643 | V728 | NE4049 | 4/4 | 556 | 0 | 8152 | 32 | + | + | + | + | 6/6 | Yes |
| V634 | V735 | NE4049 | 1/2 | 627 | 0 | 5979 | 46 | + | + | + | + | 6/6 | Yes |
| V635 | V740 | NE4049 | 4/4 | 479 | 0 | 6820 | 20 | + | + | + | + | 6/6 | Yes |
| V639 | V752 | NE4049 | 2/2 | 413 | 0 | 3950 | 129 | + | - | + | + | 5/6 | Yes |
| V639 | V753 | NE4049 | 0/2 | 579 | 0 | 847 | 0 | NA | NA | NA | - | 0/3[o] | No |
| Z536 | Z554 | NE4049 | 3/3 | 474 | 0 | 6543 | 1326 | + | - | + | - | 4/6 | Yes |
| Z533 | Z558 | NE4049 | 1/2 | NA | 0 | NA | 78 | NA | NA | NA | NA | 1/1[p] | Yes |
| Z540 | Z562 | NE4049 | 2/2 | 413 | 0 | 4579 | 13 | + | + | + | + | 6/6 | Yes |
| V643 | V727 | Fin-Jyv-A3 | 2/2 | 575 | 0 | 5518 | 139 | + | + | + | + | 6/6 | Yes |
| V634 | V736 | Fin-Jyv-A3 | 1/1 | 627 | 0 | 6016 | 163 | + | + | + | + | 6/6 | Yes |
| V634 | V737 | Fin-Jyv-A3 | 2/4 | 603 | 0 | 8288 | 377 | + | + | + | + | 6/6 | Yes |
| V635 | V741 | Fin-Jyv-A3 | 3/3 | 543 | 0 | 3602 | 1022 | + | + | + | - | 5/6 | Yes |
| V635 | V742 | Fin-Jyv-A3 | 1/2 | 594 | 0 | 6451 | 367 | + | + | + | + | 6/6 | Yes |
| V639 | V754 | Fin-Jyv-A3 | 3/3 | 403 | 0 | 7993 | 27 | + | + | + | + | 6/6 | Yes |
| V639 | V755 | Fin-Jyv-A3 | 4/4 | 449 | 0 | 5043 | 45 | + | + | + | + | 6/6 | Yes |
| Z536 | Z556 | Fin-Jyv-A3 | 1/3 | 529 | 0 | 6390 | 41 | + | + | + | + | 6/6 | Yes |
| Z536 | Z557 | Fin-Jyv-A3 | 3/3 | 420 | 0 | 6575 | 190 | + | + | + | + | 6/6 | Yes |
| Z533 | Z560 | Fin-Jyv-A3 | 2/2 | 488 | 0 | 5397 | 146 | + | + | + | - | 5/6 | Yes |
| Z540 | Z563 | Fin-Jyv-A3 | 4/4 | 594 | 0 | 4555 | 2 | + | + | + | - | 5/6 | Yes |
| Z536 | Z555 | Control | 0/3 | 351 | 0 | 389 | 0 | - | - | - | - | 0/6 | No |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Z533 | Z561 | Control | 0/4 | 529 | 0 | 612 | 0 | - | - | - | - | 0/6 | No |

[a] ID1 is the unique identification number of each mother bank vole that give birth to the offspring.
[b] ID2 is the unique identification number of each offspring bank vole.
[c] Strain refers to the strain of *B. afzelii* with which the offspring were challenged via tick bite at 35 days post-birth (PB): strain NE4049 or strain Fin-Jyv-A3.
[d] Nymphs gives a fraction where the numerator and denominator refer to the number of engorged *B. afzelii*-infected nymphs and the number of engorged nymphs, respectively, collected for each offspring bank vole.
[e] ELISA1 absorbance values indicate the level of maternally transmitted *B. afzelii*-specific IgG antibodies in the pre-infected offspring at 34 days PB. All individuals have absorbance values < 2000 indicating that they are not infected with *B. afzelii*. Absorbance values were obtained from a commercial Lyme disease ELISA.
[f] Biop1 indicates the spirochete load in the ear biopsy of the pre-infected offspring at 34 days PB. Samples were considered positive and negative if the *flagellin* gene copy number in the DNA template were > 0 and = 0, respectively.
[g] ELISA2 absorbance values indicate the strength of the IgG antibody response against *B. afzelii* in the post-infected offspring at 35 days post-infection (PI, which is 70 days post-birth). Individuals with absorbance values > 2000 are infected with *B. afzelii* strain NE4049 or Fin-Jyv-A3. Absorbance values were obtained from a commercial Lyme disease ELISA.
[h] Biop2 indicates the spirochete load in the ear biopsy of the post-infected offspring at 35 days PI (70 days PB). Samples were considered positive and negative if the *flagellin* gene copy number in the DNA template were > 0 and = 0, respectively.
[i] Ear indicates whether ear tissue tested positive or negative for *B. afzelii* at 70 days PI (105 days PB) using qPCR. Samples were considered positive and negative if the Cq value was < 38.5 cycles and > 38.5 cycles, respectively.
[j] Joint indicates whether joint tissue tested positive or negative for *B. afzelii* at 70 days PI (105 days PB) using qPCR. Samples were considered positive and negative if the Cq value was < 38.5 cycles and > 38.5 cycles, respectively.
[k] Bladder indicates whether bladder tissue tested positive or negative for *B. afzelii* at 70 days PI (105 days PB) using qPCR. Samples were considered positive and negative if the Cq value was < 38.5 cycles and > 38.5 cycles, respectively.
[l] Culture indicates whether live spirochetes were detected in at least one of the organ tissue cultures at 70 days PI (105 days PB).
[m] Criteria is the number of the six infection criteria for which each female tested positive for *B. afzelii*. These 6 criteria include: ELISA2, Biop2, ear, joint, bladder, and culture.
[n] Infected refers to whether the individuals is considered to be infected with *B. afzelii* (Yes) or not (No).
[o] Offspring V753 had 3 missing criteria (NA). This individual is uninfected because it tested positive for 0 of 3 criteria.
[p] Offspring Z558 had 5 missing criteria (NA). This individual is infected because it tested positive for 1 of 1 criterion.

**Table S3.** *B. afzelii* infection status of the MatAb+ offspring. The *B. afzelii* infection status of the MatAb+ offspring is shown following the infectious challenge via tick bite with *B. afzelii* strain NE4049 or *B. afzelii* strain Fin-Jyv-A3. The 20 MatAb+ offspring were descended from the 6 mothers that were infected with strain NE4049. The 6 infection criteria for the offspring are ELISA2, Biop2, Ear, Joint, Bladder, and Culture. Offspring that tested positive for 0 or 1 of the 6 infection criteria were considered as uninfected. Offspring that tested positive for 4 or more of the 6 infection criteria were considered as infected with *B. afzelii*.

| ID1[a] | ID2[b] | Strain[c] | Nymphs[d] | ELISA1[e] | Biop1[f] | ELISA2[g] | Biop2[h] | Ear[i] | Joint[j] | Bladder[k] | Culture[l] | Criteria[m] | Infected[n] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| V665 | V729 | NE4049 | 3/4 | 1237 | 0 | 592 | 0 | - | - | - | - | 0/6 | No |
| V666 | V734 | NE4049 | 2/3 | 880 | 0 | 732 | 0 | - | - | - | - | 0/6 | No |
| V667 | V745 | NE4049 | 3/3 | 644 | 0 | 522 | 0 | - | - | - | - | 0/6 | No |
| V667 | V746 | NE4049 | 2/3 | 727 | 0 | 458 | 0 | - | - | - | - | 0/6 | No |
| V631 | V747 | NE4049 | 2/3 | 755 | 0 | 652 | 0 | - | - | - | - | 0/6 | No |
| V631 | V748 | NE4049 | 2/2 | 792 | 0 | 449 | 0 | - | - | - | - | 0/6 | No |
| V631 | V749 | NE4049 | 2/3 | 713 | 0 | 369 | 0 | - | - | - | - | 0/6 | No |
| V662 | V757 | NE4049 | 3/3 | 1293 | 0 | 566 | 0 | - | - | - | - | 0/6 | No |
| Z538 | Z550 | NE4049 | 2/4 | 615 | 0 | 510 | 0 | - | - | - | - | 0/6 | No |
| Z538 | Z551 | NE4049 | 0/3 | 532 | 0 | 428 | 0 | - | - | + | - | 1/6 | No |
| V665 | V730 | Fin-Jyv-A3 | 2/3 | 1156 | 0 | 5120 | 62 | + | + | + | + | 6/6 | Yes |
| V665 | V731 | Fin-Jyv-A3 | 1/3 | 1101 | 0 | 5277 | 547 | + | + | + | - | 5/6 | Yes |
| V666 | V732 | Fin-Jyv-A3 | 3/3 | 682 | 0 | 6994 | 124 | + | + | + | + | 6/6 | Yes |
| V667 | V743 | Fin-Jyv-A3 | 1/3 | 749 | 0 | 6379 | 12 | - | + | + | NA | 4/5 | Yes |
| V667 | V744 | Fin-Jyv-A3 | 2/3 | 598 | 0 | 7055 | 305 | - | + | + | + | 5/6 | Yes |
| V631 | V750 | Fin-Jyv-A3 | 3/4 | 921 | 0 | 3567 | 333 | + | + | + | + | 6/6 | Yes |
| V631 | V751 | Fin-Jyv-A3 | 3/4 | 882 | 0 | 2441 | 614 | + | - | + | + | 5/6 | Yes |
| V662 | V756 | Fin-Jyv-A3 | 1/3 | 1392 | 0 | 758 | 0 | - | - | - | - | 0/6 | No |
| Z538 | Z552 | Fin-Jyv-A3 | 1/2 | 785 | 0 | 5556 | 84 | + | + | + | - | 5/6 | Yes |
| Z538 | Z553 | Fin-Jyv-A3 | 2/3 | 524 | 0 | 3997 | 1022 | + | + | + | + | 6/6 | Yes |

[a] ID1 is the unique identification number of each mother bank vole that give birth to the offspring.
[b] ID2 is the unique identification number of each offspring bank vole.
[c] Strain refers to the strain of *B. afzelii* with which the offspring were challenged via tick bite at 35 days post-birth (PB): strain NE4049 or strain Fin-Jyv-A3.
[d] Nymphs gives a fraction where the numerator and denominator refer to the number of engorged *B. afzelii*-infected nymphs and the number of engorged nymphs, respectively, collected for each offspring bank vole.
[e] ELISA1 absorbance values indicate the level of maternally transmitted *B. afzelii*-specific IgG antibodies in the pre-infected offspring at 34 days PB. All individuals have absorbance values < 2000 indicating that they are not infected with *B. afzelii*. Absorbance values were obtained from a commercial Lyme disease ELISA.
[f] Biop1 indicates the spirochete load in the ear biopsy of the pre-infected offspring at 34 days PB.
[g] ELISA2 absorbance values indicate the strength of the IgG antibody response against *B. afzelii* in the post-infected offspring at 35 days post-infection (PI, which is 70 days post-birth). Individuals with absorbance values > 2000 are infected with *B. afzelii* strain NE4049 or Fin-Jyv-A3. Absorbance values were obtained from a commercial Lyme disease ELISA.
[h] Biop2 indicates the spirochete load in the ear biopsy of the post-infected offspring at 35 days PI (70 days PB).
[i] Ear indicates whether ear tissue tested positive or negative for *B. afzelii* at 70 days PI (105 days PB) using qPCR.
[j] Joint indicates whether joint tissue tested positive or negative for *B. afzelii* at 70 days PI (105 days PB) using qPCR.
[k] Bladder indicates whether bladder tissue tested positive or negative for *B. afzelii* at 70 days PI (105 days PB) using qPCR.
[l] Culture indicates whether live spirochetes were detected in at least one of the organ tissue cultures at 70 days PI (105 days PB).

[m] Criteria is the number of the six infection criteria for which each female tested positive for *B. afzelii*. These 6 criteria include: ELISA2, Biop2, ear, joint, bladder, and culture.

[n] Infected refers to whether the individuals is considered to be infected with *B. afzelii* (Yes) or not (No).

## Section 1 – Creation of *I. ricinus* nymphs infected with *B. afzelii*

The nymphs used for the experimental infections were created as follows. BALB/c mice were infected with *B. afzelii* strain NE4049 or Fin-Jyv-A3 via tick bite. At 4 weeks post-infection (PI), the mice were infested with larval ticks from our *I. ricinus* colony. The engorged larval ticks were stored in individual 1.7 ml Eppendorf tubes; each tube contained a piece of moistened paper towel to ensure high humidity. The engorged larvae were kept at room temperature under ambient light conditions and were allowed to molt into nymphs. A random sample of nymphs was tested to determine the infection prevalence, which was 77.9% (67 infected nymphs/ 86 total nymphs) for NE4049 and 91.8% (67 infected nymphs/ 73 total nymphs) for Fin-Jyv-A3. Larval *I. ricinus* ticks were also fed on uninfected BALB/c mice to create uninfected control nymphs.

## Section 2 – Antibody response against *B. afzelii* in the bank vole mothers

The raw data for the *B. afzelii*-specific IgG antibody response in the mother at 35 days post-infection (PI) are shown in Table S1 in the column titled 'ELISA'.

To test whether the bank vole mothers developed an IgG antibody response against *B. afzelii* at 35 days post-infection (PI), we compared this variable (the ELISA variable in Table S1) between infected mothers (n = 6) and uninfected control mothers (n = 7) using an independent two sample t-test. The IgG antibody response against *B. afzelii* was log10-transformed to improve the normality of the residuals.

The mean *B. afzelii*-specific IgG antibody response of the infected mothers (mean = 3811, 95% CI = 2692–5395) at 35 days PI was 7.4 times higher compared to the uninfected control mothers (mean = 512, 95% CI = 371–706), and this difference was significant (Figure S1; $t = -9.335$, $df = 11$, $p < 0.001$). This result shows that infected bank vole mothers developed a strong IgG antibody response against the *B. afzelii* infection.

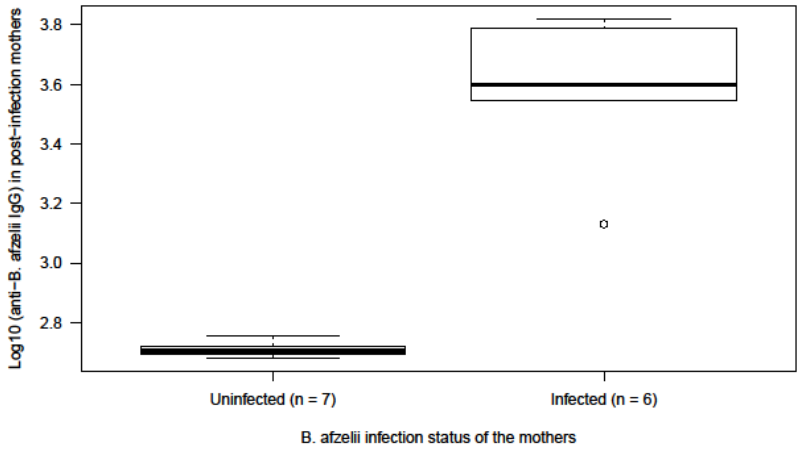


**Figure S1**. The *B. afzelii*-specific IgG antibody response of the infected bank vole mothers at 35 days post-infection (n = 6) was 7.4 times higher compared to the uninfected control bank vole mothers (n = 7). The strength of the *B. afzelii*-specific IgG antibody response was measured in absorbance units using a commercial Lyme disease ELISA. The infected mothers and the uninfected control mothers produced the MatAb+ and the MatAb- bank vole offspring, respectively.

## Section 3 – Maternal antibody transmission differs among bank vole mothers

The raw data for the level of maternally transmitted IgG antibodies in the offspring at 34 days post-birth (PB) are shown in Tables S2 and S3 in the column titled 'ELISA1'. The raw data for the *B. afzelii*-specific IgG antibody response in the mother at 35 days post-infection (PI) are shown in Table S1 in the column titled 'ELISA'.

There were significant differences among infected bank vole mothers (n = 6) in the level of maternally transmitted IgG antibodies present in their offspring at 34 days PB (Figure S2; one-way ANOVA: $F_{12, 28} = 29.25$, $p < 0.001$). However, there was no correlation between the strength of the *B. afzelii*-specific IgG antibody response in the mother at 35 days PI and the level of maternally transmitted *B. afzelii*-specific IgG antibodies in the offspring at 34 days PB (Figure S2; r = 0.361, df =18, p = 0.118).

One possible explanation for this result is as follows. The antibody levels of the mothers were measured at 35 days PI, whereas the females were coupled with males at 2 and at 6 weeks PI. Thus the window of maternal antibody transmission occurred at 5–8, or 9–12 weeks PI depending on whether the female produced her first litter with the first or second male. This variation in the time lag between maternal infection and reproduction may explain the lack of a relationship in the IgG antibodies levels between bank vole mothers and their offspring.

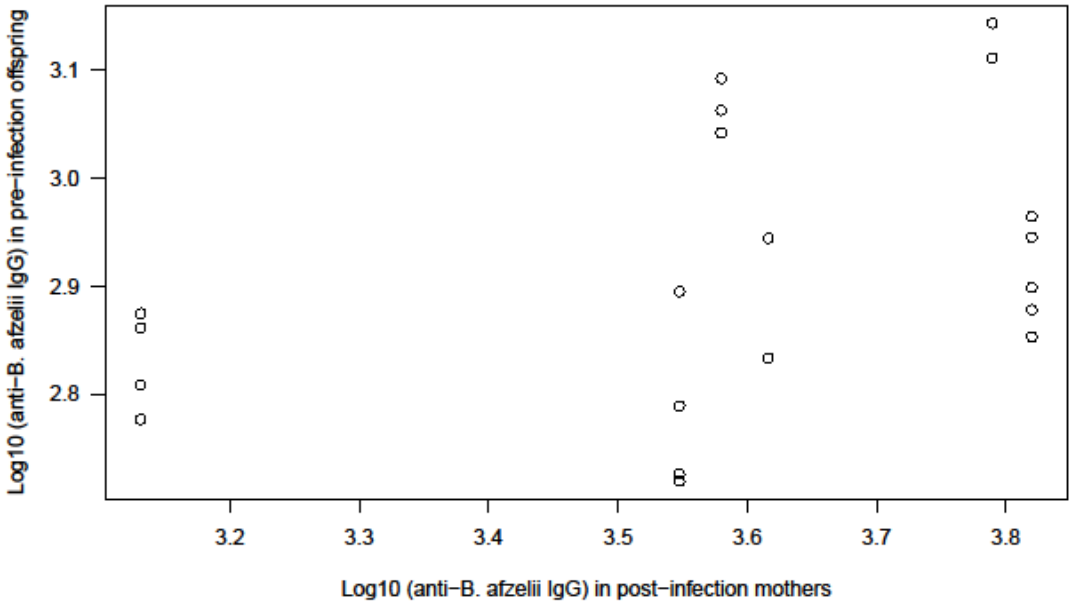


**Figure S2**. The relationship between the *B. afzelii*-specific IgG antibody response of the infected bank vole mothers (n = 6) at 35 days post-infection and the level of maternally transmitted *B. afzelii*-specific IgG antibodies of their MatAb+ offspring (n = 20) at 34 days post-birth was not significant. There were significant differences in the level of maternally transmitted *B. afzelii*-specific IgG antibodies among the 6 families of bank voles. In the graph, each of the 6 families is shown by a vertical cluster of data points.

## Section 4 – The maternally transmitted IgG antibodies are specific for the OspC antigen

The maternally transmitted OspC-specific IgG antibody level in the offspring before the infectious challenge (34 days PB) was measured using a homemade ELISA with recombinant OspC (rOspC) proteins A3 and A10 [1]. 96-well tissue culture plates were coated overnight at 4°C with rOspC proteins A3 and A10 (1 µg of protein per well). Wells were washed three times with PBS-Tween 0.1% between each step. The plate was incubated with a BSA 2% blocking solution for 2 hours, followed by the bank vole serum samples (diluted 1:100 in 1x PBS) for 45 minutes, and the secondary antibody for 45 minutes (diluted 1:5000 in 1x PBS). The secondary antibody was a goat anti-*Mus musculus* IgG conjugated to horseradish peroxidase. After adding 100 µl of TMB, we measured the absorbance at 652 nm every 2 minutes for one hour using a plate reader (Synergy HT, Multi-detection plate reader, Bio-Tek, United States). The level of IgG antibodies against each rOspC antigen in the offspring was determined by integrating the area under the absorbance versus time curve and is measured in absorbance units.

The specificity of the maternally transmitted IgG antibodies against the OspC antigen in the naive bank vole offspring (i.e., before the infectious challenge at 35 days PB) was quantified as follows. For each offspring, we measured the ability of the maternally transmitted IgG antibodies (sampled from offspring at 34 days PB) to bind recombinant outer surface protein C (rOspC) A10 and rOspC A3 using a homemade ELISA. We calculated an OspC A10 specificity ratio for each offspring by dividing the level of IgG antibodies that bound to rOspC A10 by the level of IgG antibodies that bound to rOspC A3. Thus, the OspC A10 specificity ratio measures the ability of the maternally transmitted antibodies to recognize the maternal OspC A10 antigen compared to the foreign OspC A3 antigen. The OspC A10 specificity ratio was log10-transformed to improve the normality of the residuals.

We compared the log10-transformed OspC A10 specificity ratio in the pre-infection blood sample (at 34 days PB) between the MatAb+ offspring and the MatAb- offspring using an independent two samples t-test. The OspC specificity ratio in the MatAb+ offspring was 3.07 times higher compared to the MatAb- offspring, and this difference was significant (independent two samples t-test: $t = -10.015$, $df = 39$, $p < 0.001$). This result shows that the preference of the maternally transmitted IgG antibodies for the OspC A10 antigen (compared to the OspC A3 antigen) was stronger in the MatAb+ offspring compared to the MatAb- offspring (Figure S3). For the MatAb+ offspring, the mean log10-transformed OspC A10 specificity ratio was 0.45 (95% CI = 0.40–0.50) and this was significantly greater than 0 (paired samples t-test: $t = 18.145$, $df = 18$, $p < 0.001$). This result shows that the infected mothers transmitted IgG antibodies to their offspring that were specific for OspC A10. For the MatAb- offspring, the mean log10-transformed OspC A10 specificity ratio was 0.15 (95% CI = 0.11–0.18) and this was also significantly different from 0 (paired samples t-test: $t = 7.981$, $df = 21$, $p < 0.001$). Thus, even in the absence of OspC-specific antibodies, the rOspC A10 antigen induced a stronger color reaction than the rOspC A3 antigen. This result suggests that the rOspC A10 antigen is better at binding mouse IgG or the secondary antibody compared to rOspC A3.

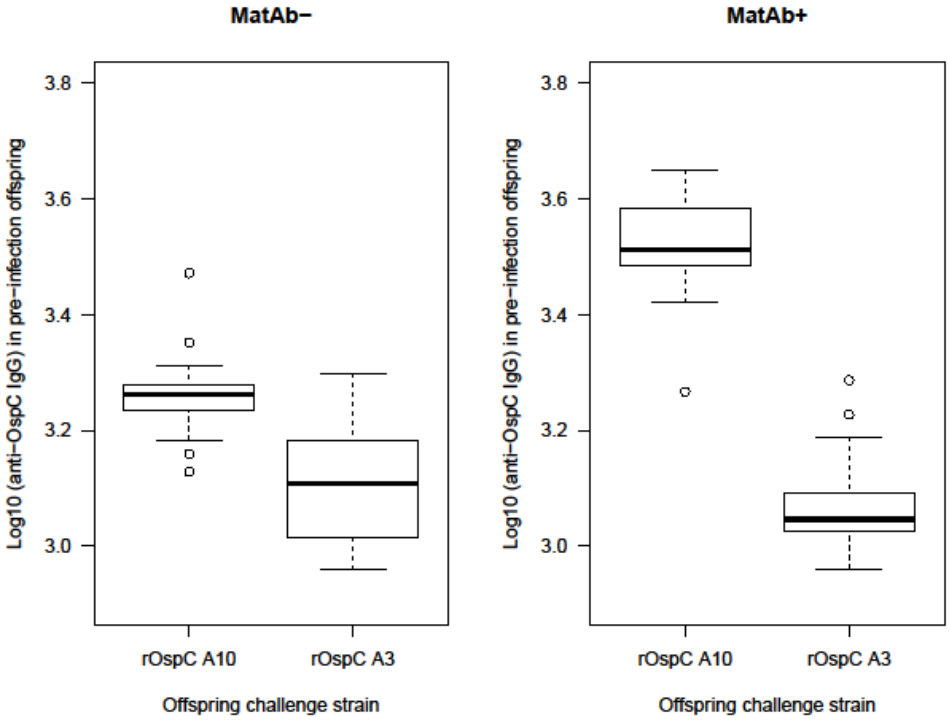


**Figure S3**. The ability of maternally transmitted IgG antibodies to bind *B. afzelii* OspC antigens A10 and A3 is shown for the MatAb- offspring (left panel) and the MatAb+ offspring (right panel). Blood samples were taken from the pre-infection offspring at 34 days post-birth. For the MatAb+ offspring, the ability of the maternally transmitted IgG antibodies to bind rOspC A10 was 2.82 times stronger compared to rOspC A3. For the MatAb- offspring, the ability of the maternally transmitted IgG antibodies to bind rOspC A10 was 1.40 times stronger compared to rOspC A3. The left panel of the graph suggests that our homemade ELISA is biased towards the rOspC A10 antigen because the serum samples from the pre-infection MatAb- offspring (which are not expected to have any *B. afzelii*-specific antibodies) bound more strongly to the rOspC A10 antigen compared to the rOspC A3 antigen. Despite this bias, this graph also shows that the serum samples of the pre-infection MatAb+ offspring bound much more strongly to the rOspC A10 antigen (right panel) compared to the MatAb- offspring (left panel). In contrast, the ability of the maternally transmitted antibodies to bind the rOspC A3 antigen was similar between the MatAb+ and MatAb- offspring.

## Section 5 – The *B. afzelii*-specific IgG antibody levels changed dramatically after offspring were exposed to infected nymphs

For the bank vole offspring, the pre-infection blood sample was taken at 34 days post-birth (PB), whereas the post-infection blood sample was taken at 35 days post-infection (PI), which corresponds to 70 days PB. The raw data for the level of *B. afzelii*-specific IgG antibodies in the pre-infection and post-infection blood samples are shown in Tables S2 and S3 in the columns titled 'ELISA1' and 'ELISA2', respectively.

The level of *B. afzelii*-specific IgG antibodies increased dramatically from the pre-infection blood sample to the post-infection blood sample for the MatAb- offspring (left column in Figure S4) and for the MatAb+ offspring that were challenged with strain Fin-Jyv-A3 (bottom-right panel in Figure S4). This result indicates that all of these offspring acquired *B. afzelii* following the infectious challenge. One MatAb+ offspring (V756) was protected from infectious challenge with strain Fin-Jyv-A3 (bottom-right panel in Figure S4). The level of *B. afzelii*-specific IgG antibody response decreased from the pre-infection blood sample to the post-infection blood sample for the MatAb+ offspring that were challenged with strain NE4049 (top-right panel in Figure S4). This result indicates that the MatAb+ offspring were protected against the infectious challenge with strain NE4049 and that the level of maternal antibodies in these offspring waned over time.

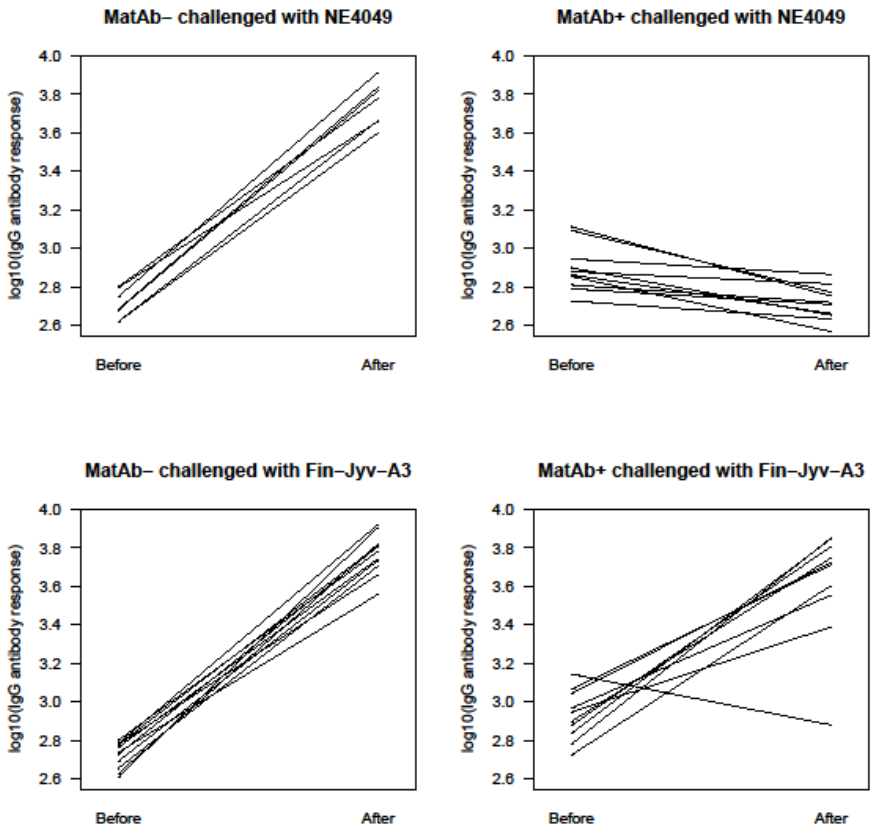


**Figure S4**. Interaction plot that shows the level of *B. afzelii*-specific IgG antibodies in the offspring before and after the infectious challenge. The pre-infection blood sample was taken at 34 days post-birth; the post-infection blood sample was taken at 35 days post-infection, which corresponds to 70 days post-birth. The MatAb- (left column) and MatAb+ (right column) refer to the offspring without and with maternal antibodies against *B. afzelii* strain NE4049, respectively. The offspring were either challenged with strain NE4049 or strain Fin-Jyv-A3. The MatAb- offspring (left column) were equally susceptible

to both strains. The MatAb+ offspring were protected against the maternal strain (NE4049; top-right panel) but not the new strain (Fin-Jyv-A3; bottom-right panel). The top-right panel also shows that the level of maternal IgG antibodies in the MatAb+ offspring waned over time.

## Section 6 – The *B. afzelii* spirochete load in the ear tissue changed dramatically after offspring were exposed to infected nymphs

For the bank vole offspring, the pre-infection ear tissue biopsy was taken at 34 days post-birth (PB), whereas the post-infection ear tissue biopsy was taken at 35 days post-infection (PI), which corresponds to 70 days PB. The raw data for the spirochete loads in the pre-infection and post-infection ear tissue biopsies are shown in Tables S2 and S3 in the columns titled 'Biop1' and 'Biop2', respectively.

The *B. afzelii* spirochete load in the ear tissue increased dramatically from the pre-infection ear biopsy to the post-infection ear biopsy for the MatAb- offspring (left column in Figure S5) and for the MatAb+ offspring that were challenged with strain Fin-Jyv-A3 (bottom-right panel in Figure S5). This result indicates that all of these offspring acquired *B. afzelii* following the infectious challenge. One MatAb+ offspring (V756) was protected from infectious challenge with strain Fin-Jyv-A3 (bottom-right panel in Figure S5). The *B. afzelii* spirochete load in the ear tissue was zero for the pre- and post-infection ear biopsies of the MatAb+ offspring that were challenged with strain NE4049 (top-right panel in Figure S7). This result indicates that the MatAb+ offspring were protected against the infectious challenge with strain NE4049. Figure S5 also shows that there is no vertical transmission of *B. afzelii* from mothers to their offspring.

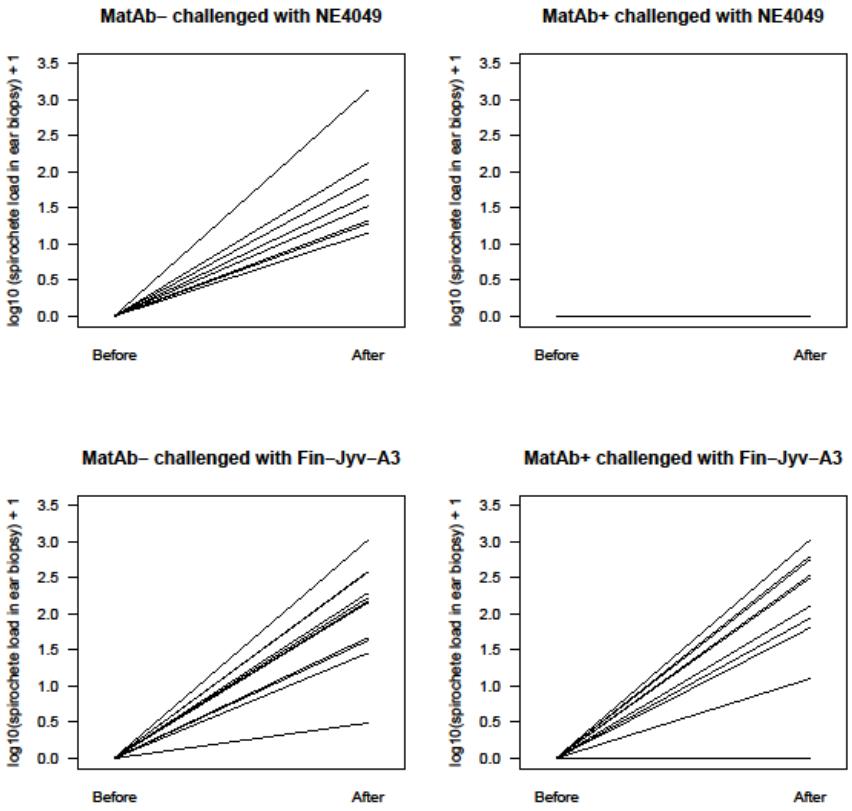


**Figure S5**. Interaction plot that shows the ear biopsy spirochete loads of the offspring before and after the infectious challenge. The pre-infection ear biopsy was taken at 34 days post-birth; the post-infection ear biopsy was taken at 35 days post-infection, which corresponds to 70 days post-birth. The MatAb-

(left column) and MatAb+ (right column) refer to the offspring without and with maternal antibodies against *B. afzelii* strain NE4049, respectively. The offspring were either challenged with strain NE4049 or strain Fin-Jyv-A3. The MatAb- offspring (left column) were equally susceptible to both strains. The MatAb+ offspring were protected against the maternal strain (NE4049; top-right panel) but not the new strain (Fin-Jyv-A3; bottom-right panel). Figure S7 shows that there is no vertical transmission of *B. afzelii* from mothers to their offspring.

## Section 7 – Culture of tissue biopsies to detect live *B. afzelii* spirochetes

The tissue biopsy culture confirmed the detection of live spirochetes in most animals (Table S3 and Table S4). Live spirochetes were found for 0% (0/9) of the MatAb+/NE4049 offspring, 66.6% (6/9) of the MatAb+/Fin-Jyv-A3 offspring, 75% (6/8) of the MatAb-/NE4049 offspring, and 72.7% (8/11) of the MatAb-/Fin-Jyv-A3 offspring. In summary, live spirochetes were recovered from tissue samples for most of the infected animals (71.4% = 20/28), but for none of the uninfected animals (0.0% = 0/9).

## Section 8 – The *ospC*-specific qPCR to confirm identity of infecting strain

Strain NE4049 carries *ospC* allele A10 and strain Fin-Jyv-A3 carries *ospC* allele A3. The *ospC*-specific qPCR confirmed that all animals were infected with the expected strain (Table S3 and Table S4). Spirochetes carrying *ospC* allele A10 were detected in 0% (0/9) of the MatAb+/NE4049 offspring (i.e. because these individuals were protected from the infectious challenge with strain NE4049), 0% (0/10) of the MatAb+/Fin-Jyv-A3 offspring, 100% (8/8) of the MatAb-/NE4049 offspring, and 0% (0/11) of the MatAb-/Fin-Jyv-A3. In contrast, spirochetes carrying *ospC* allele A3 were detected in 0% (0/9) of the MatAb+/NE4049 offspring, 100% (10/10) of the MatAb+/Fin-Jyv-A3 offspring, 0% (0/8) of the MatAb-/NE4049 offspring, and 100% (11/11) of the MatAb-/Fin-Jyv-A3 offspring. In summary, the *ospC* allele corresponding to the expected strain was always found in the tissues of the experimentally infected bank vole offspring.

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# General Discussion

Natural reservoir hosts show clear differences in infection phenotype compared to laboratory models. Wild rodents are non-model organisms that are an example of this difference with lab models (Jackson, 2015). While laboratory mice have been a useful model for human Lyme disease because they present similar symptoms (e.g. arthritis), studying wild rodents is critical for understanding the ecology of Lyme disease. In this PhD dissertation, we aimed to study different kinds of immune responses of the bank vole against *B. afzelii*, which is a natural reservoir host of the pathogen. We studied the effects of the innate and the acquired immune responses to the pathogen. In the first chapter, we studied the effect of innate immune response on the pathogen; we aimed to test whether genetic variation at an important innate immunity locus (TLR2) in bank voles confers resistance to the pathogen. In the second and third chapters, we studied the reciprocal interaction between the pathogen and the acquired immunity of the host. In chapter 2, we studied the effect the pathogen has on acquired immnunity by testing whether *B. afzelii* could inhibit the development of acquired anti-tick immunity in bank voles. In chapter 3, we studied the effect of trans-generational acquired immunity (transmited by mothers to their offspring) on the pathogen by testing whether this immunity provided protection against the pathogen and whether this protection was strain-specific. In summary, this PhD dissertation aimed to study the effect of three different immune mechanisms on the ability of *B afzelii* to establish infection in the rodent host and to transmit from the rodent host to the tick vector.

In the innate immune system of vertebrates, the most important family of recognition receptors is the Toll-like receptor (TLR) family. Several polymorphisms have been characterized in these receptors and their role on the susceptibility to diseases has been a hot topic that has received significant attention from different fields (reviewed in: Misch & Hawn, 2008; Schröder & Schumann, 2005; Takeda, Kaisho, & Akira, 2003). Many studies have screened for associations between different haplotypes or single nucleotide polymorphism and the susceptibility to a large range of diseases (Misch & Hawn, 2008; Schröder & Schumann, 2005; Takeda et al., 2003). The TLR2 has been particularly important because of its role in the recognition of the largest set of pathogens (Texereau et al., 2005). The importance of the TLR2 receptor suggested that functional polymorphism could impair host response (Wooten et al., 2002). Studies have looked for associations between TLR2 polymorphisms and susceptibility to Lyme borreliosis, tuberculosis, febrile infections, among others (Kutukculer, Yeniay, Aksu, & Berdeli, 2007; Misch & Hawn, 2008; Ogus et al., 2004; Schröder et al., 2005). In humans, genetic variation at the TLR2 locus is associated with variation in the probability of testing positive for Lyme disease (Alexopoulou et al., 2002; Schröder et al., 2005). TLR polymorphisms, unlike major histocompatibility complex, have received less attention from ecologists investigating host-parasite interactions (Tschirren et al., 2013). Few association studies between TLRs and disease susceptibility have been conducted in natural populations (Morger et al., 2014; Tschirren et al., 2013). One clear advantage of association studies compared to experimental studies is the sample sizes. However, these studies have the disadvantage to be potentially confounded by spatio-temporal differences in pathogen exposure that can lead to misleading conclusions. The aim of this chapter was to test the causal relationship between the TLR2 polymorphism and the resistance to *Borrelia* in two bank vole populations. We detected three clusters that were previously reported (Tschirren et al., 2013).

The C1 cluster “the susceptible”, the C2 “the resistant” and the C3 without any expected phenotype. The field study of Tschirren et al. (2013) found a 2.5-fold difference in prevalence between the clusters. This result was then supported (Tschirren, 2015). Our results did not support a relationship between the polymorphism in the clusters and resistance to *Borrelia* pathogens. The limitations of our study are: (a) the sample size, (b) the infection method, (c) tick-bite site, and (d) timing of infection. (a) The sample size of the experiment was restricted because of the low frequency of the C2 cluster. (b) To be able to infect the animals and then have a proof of infection (i.e. an infected engorged nymph), we glued a capsule and limited the nymphs to attach on the back. One of the criticism to this method is the stress that provokes to the animals that could induce an immunosuppressive effect. Suppression of the immune system induced by stress has been frequently reported in several organisms. I believe a potential immunosuppression caused by stress would not undermine the TLR2 effect. We have also shown in the third chapter of this dissertation that experimental infections using the same method did not immunosuppress the animals to become infected. (c) Usually ticks are found in the ear and the head, in this study the tick-bite was at the dorsal back. It is possible to find differences in the immune response depending the site, but I expect a potential protective effect from the TLR2 polymorphism to not be localized to certain tissue. (d) The animals were sacrificed at 7 weeks post-infection and dissemination of the pathogen was assessed. As part of the innate immunity, I expected the role of the TLR2 polymorphism to occur during the first weeks of infection. In summary, this study presents some limitations that are difficult to avoid in an experimental infection.

The most important difference between our study and the field study by Tschirren et al. (2013) was that we experimentally controlled the infectious challenge whereas they did not. The field study by Tschirren et al. (2013) was conducted at a single field site (Kalvs Mosse) over a period of 6 months (May to October). Seasonal and spatial differences in the density of *Borrelia*-infected nymphs (DIN) can result in dramatic spatiotemporal variation in the risk of Lyme borreliosis (Klaus Kurtenbach et al., 2006). The probability of acquiring a *Borrelia* infection can vary 10-fold over the course of the transmission season (May to September) (Voordouw et al., 2015). Moreover, the density of infected nymphs per area can vary by orders of magnitude over a spatial scale of < 1 km (Vourc'h et al., 2016). Thus, if the C1C1 individuals were captured in months or patches of the forest where the risk of Lyme disease was high, and the reverse was true for the C2C2 individuals, one could obtain the observed association between TLR2 genotype and the prevalence of *B. afzelii* infection. This study showed the importance of performing experimental infections to test whether a candidate gene is responsible for the observed variation in infectious disease prevalence in the field. Our results suggest that natural selection by *B. afzelii* on the bank vole immune system does not appear to be driving the evolution of the TLR2 polymorphism.

In contrast to the innate immune response, the specific response of the acquired immune system to the pathogen can create different dynamics in the host-pathogen interaction. In the European Lyme disease system that includes bank voles, *I. ricinus*, and *Borrelia*, one feature that has been noted is that bank voles develop acquired immunity against *Ixodes* ticks after repeated exposure. The phenomenon that bank voles develop a strong anti-tick immunity over successive infestations has been shown by several studies (Dizij & Kurtenbach, 1995; P. Humair et al., 1999). As a result, ticks that feed on bank voles that have developed acquired

resistance have reduced fitness. This phenomenon has not been found in lab mice or other reservoir hosts (Dizij & Kurtenbach, 1995; P. Humair et al., 1999). For example, *Apodemus* mice do not develop acquired immunity against ticks, but they do develop acquired immunity against *B. burgdorferi* (Dizij & Kurtenbach, 1995; P. Humair et al., 1999; Klaus Kurtenbach et al., 1994; S. Randolph, 1994). This indicates that there are different reservoir host strategies against the vector and the pathogen that result in different contributions of these reservoir hosts to the enzootic life cycle (P. Humair et al., 1999). This arms race could lead the pathogen to develop strategies to increase their own fitness.

A recent study demonstrated that infection with *B. burdorferi* ss in laboratory mice inhibits the development of long-lived plasma cells and memory B cells, and results in the temporary immunosuppression of the host (Elsner et al., 2015). The study showed that mice immunized with the influenza vaccine did not develop protective antibodies against the virus during the state of immunosuppression (Elsner et al., 2015). This study motivated us to test whether *B. afzelii* could inhibit the development of acquired anti-tick immunity in bank voles that were repeatedly infested with *I. ricinus* larval ticks. We found no evidence that *B. afzelii* suppresses the development of acquired immunity against ticks in the bank vole and thereby improve the fitness of the ticks and its own fitness. The main differences between our study and the study from Elsner et al. (2015) were the genospecies of *B. burgdorferi* sl (*B. burgdorferi* ss versus *B. afzelii*), the antigen and mode of infection (injection of influenza vaccine versus tick-bite), the rodent host (lab mice versus bank vole). For the *Borrelia* genospecies, the antigen and the mode of infection, I was expecting these factors to show a small effect in the response variable, while the rodent host to show a stronger difference in the immune response. It has been demonstrated that laboratory mice and reservoir hosts show differences in relevant phenotypes such as suppression of the immune system, development of acquired immunity against the tick vector, differences in host-to-tick transmission. For example, differences in immunosuppression between lab mice and natural host have been also reported in the tick-borne pathogen *B. microti* (S. Randolph, 1994). Laboratory mice, like humans, acquire persistent infection and develop the same manifestations (arthritis, ankle swelling and carditis) with the exception of the neuroborreliosis (Pachner & Steiner, 2007). In contrast, experimental studies in reservoir hosts have not found any pathology of the pathogen (Brown & Lane, 1994; Moody et al., 1994; Schwanz et al., 2011; Wright & Nielsen, 1990).

One interesting result we found in this study was the dramatic decrease in spirochete load in the ear tissues over time. The spirochete load has direct relationship with the host-to-tick transmission success. Field studies have shown similar results, for example, a study on field-captured bank voles and yellow-necked mice found a positive correlation between the spirochete load in the ear tissues and transmission of *B. afzelii* to larval *I. ricinus* ticks (Råberg, 2012). Likewise, an infection experiment performed in our laboratory with *Mus musculus* mice and the *B. afzelii* strain NE4049 found a positive relationship between the spirochete load in the ears tissues and the host-to-tick transmission (Jacquet et al., 2015). However, Jacquet, Margos, et al. (2016) did not find a dramatic decrease in spirochete load in the ear tissues over time. We also found that the decrease in spirochete load was strongly correlated to the developed anti-tick immunity. However, the experimental design used in this chapter did not aim to test whether acquired immunity against ticks reduces host-to-tick transmission. We strongly encourage future studies to test this hypothesis.

The adaptive immunity acts by different mechanisms that can play a role in the ecology of parasites. Another mechanism that was considered in this PhD dissertation was passive trans-generational immunity. This passive trans-generational immunity refers to antibodies created by the mother that are passed through the placenta or the milk to their offspring (Owen, Punt, & Stranford, 2013). The role of maternal antibodies in evolutionary ecology has not received much attention (Boulinier & Staszewski, 2008). Our results suggest that maternal antibodies could play an important role in maintaining strain diversity of the B. burgdorferi in nature. In this chapter, we used the ospC as marker t|o type two B. afzelii strains (namely oMG A10 and oMG A3). Numerous studies have shown this gene to show the highest gene diversity with approximately 24 oMGs (defined as within group and between groups by a 2% and 8% divergence) (Durand, Jacquet, Paillard, Rais, Gern, & Voordouw, 2015; Lagal et al., 2003). This polymorphism is maintained by balancing selection with two proposed explanatory hypotheses: the multiple niche polymorphism (MNP) and negative frequency-dependent selection (NFDS) (Barbour & Travinsky, 2010; Brisson et al., 2012b; Brisson & Dykhuizen, 2004; N. Wang et al., 1999). The results from our study support the NFDS hypothesis, but do not contradict or exclude the MNP. Recently, Andersson et al. (2013) proposed that another mechanism that could explained ospC diversity is facilitation during infection. Their study showed a stronger association between more distant ospC genotypes, which suggested that genetically diverse infections have higher infectivity. I suggest there are several mechanisms that could act together to maintain the diversity of strains at the population level. Therefore, the protective role of the maternal antibodies against a given group of strains, probably the most common ones would be in benefit of the rare strains.

There is no known fitness cost for the reservoir host produced by a *B. burgdorferi s.l* infection (Chambert et al., 2012; Voordouw et al., 2015). The only fitness cost known is the carditis and arthritis developed in 3 day old *P. leucopus* mice (Moody et al., 1994). This result is expected as infant mice take a few weeks to develop active immunity, even though the scenario where the mother transports infected nymphs to the nest seems unlikely (Grindstaff et al., 2003). Field studies on wild rodents have found that juveniles or sub-adults (less than 20 g) are less likely to be infected than adults, because sub-adults have limited exposure to the pathogen (Hofmeister et al., 1999; Tschirren et al., 2013). The difference in prevalence could also be contributed or partially contributed to the maternal protection. Even without a known cost of infection for the rodents, the transmission of maternal antibodies has shown additional benefits such as the priming of the immune system and the facilitation of growth and survival (Gustafsson, Mattsson, Holmdahl, & Mattsson, 1994; Sheldon & Verhulst, 1996).

Our study opens the field to different questions that remain to be investigated. The cost to the mother for producing the antibodies and transmitting them, the source of variation in the transfer of antibodies, the effect of age on the susceptibility of individuals, whether the maternal antibody transmission is adaptive in natural bank vole populations. I think one important question that remains open is the role of the maternal antibodies in mixed infections.

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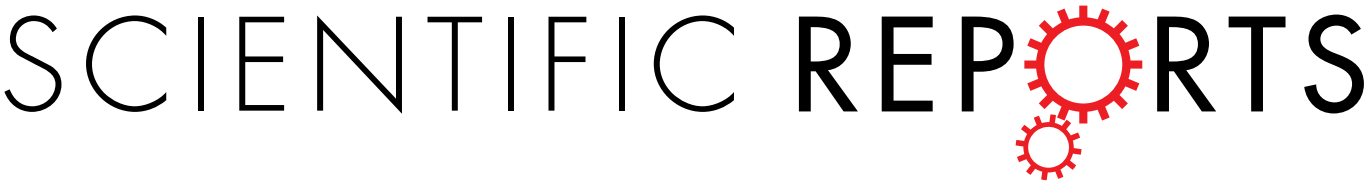
# Appendix



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# Inefficient co-feeding transmission of *Borrelia afzelii* in two common European songbirds

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**The spirochete bacterium *Borrelia afzelii* is the most common cause of Lyme borreliosis in Europe. This tick-borne pathogen can establish systemic infections in rodents but not in birds. However, several field studies have recovered larval *Ixodes ricinus* ticks infected with *B. afzelii* from songbirds suggesting successful transmission of *B. afzelii*. We reviewed the literature to determine which songbird species were the most frequent carriers of *B. afzelii*-infected *I. ricinus* larvae and nymphs. We tested experimentally whether *B. afzelii* is capable of co-feeding transmission on two common European bird species, the blackbird (*Turdus merula*) and the great tit (*Parus major*). For each bird species, four naïve individuals were infested with *B. afzelii*-infected *I. ricinus* nymphal ticks and pathogen-free larval ticks. None of the co-feeding larvae tested positive for *B. afzelii* in blackbirds, but a low percentage of infected larvae (3.33%) was observed in great tits. Transstadial transmission of *B. afzelii* DNA from the engorged nymphs to the adult ticks was observed in both bird species. However, BSK culture found that these spirochetes were not viable. Our study suggests that co-feeding transmission of *B. afzelii* is not efficient in these two songbird species.**

The tick-borne spirochete bacterium *Borrelia afzelii* is the most common etiological agent of Lyme borreliosis (LB) in Europe[1–3]. This pathogen is transmitted by *Ixodes ricinus* ticks and is adapted to infect rodent reservoir hosts[3–7]. In these hosts, *B. afzelii* establishes a long-term, systemic infection that facilitates high rates of host-to-tick transmission[6,8–11]. In contrast to bird-adapted *Borrelia* species such as *B. garinii* and *B. valaisiana*, experimental infection studies with blackbirds, pheasants, and great tits have shown that *B. afzelii* is not able to establish a systemic infection in these bird species[12–14]. The ability of *B. afzelii* to infect rodent but not avian hosts (and vice versa for the bird-adapted *Borrelia* species) appears to be mediated by the vertebrate complement system[15,16]. Thus, the general consensus is that *B. afzelii* is unable to use avian hosts to infect new ticks[1–3,17].

Recent field studies on birds have questioned this consensus of whether *B. afzelii* is strictly incompatible with avian hosts. Many species of birds are frequently exposed to *B. afzelii*-infected *I. ricinus* nymphs[18–24]. More importantly, *B. afzelii*-infected larval ticks have been recovered from a number of bird species including *Fringilla coelebs* L., *Troglodytes troglodytes* L., *Parus major* L., *Turdus merula* L., and *Turdus iliacus* L. (see Table 1). Given that vertical transmission of LB pathogens is thought to be rare in *Ixodes* ticks[25–27], these observations suggest that these larval ticks acquired *B. afzelii* spirochetes from avian hosts.

Co-feeding transmission is one strategy by which *B. afzelii* might infect larval ticks feeding on avian hosts. This mode of transmission occurs when infected and uninfected ticks feed in close spatial and temporal proximity on the same host[28–30]. A number of studies have documented co-feeding transmission of *B. afzelii* on competent rodent reservoir hosts[28,31–34]. The observation that this mode of transmission can occur in the absence of a systemic infection raised the hypothesis that co-feeding transmission could allow *Borrelia* pathogens to evade the hostile immune system of otherwise incompetent hosts[29,30,35]. For example, co-feeding transmission of *B. afzelii* and *B. garinii* has been documented on ungulates, which are believed to be refractory to systemic infection[36,37]. An experimental infection study using a Japanese strain of *B. garinii* demonstrated co-feeding transmission on

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| Bird Species | *Ixodes ricinus* larvae | | | | *Ixodes ricinus* nymphs | | | |
|---|---|---|---|---|---|---|---|---|
| | # studies reporting *B. afzelii* infections | # birds tested | # ticks tested | # infected ticks | # studies reporting *B. afzelii* infections | # birds tested | # ticks tested | # infected ticks |
| *Anthus trivialis* | | | | | 1 ([53]) | 120 | 85 | 4 |
| *Carduelis cabaret* | | | | | 1 ([22]) | ** | 5 | 1 |
| *Carduelis chloris* | | | | | 1 ([19]) | 1 | 3 | 1 |
| *Coccothraustes coccothraustes* | | | | | 1 ([53]) | 2 | 2 | 1 |
| *Erithacus rubecula* | 2 ([52,61]) | 124 | 38* | 8 | 5 ([19,22,61,73,83]) | 316 | 366 | 11 |
| *Fringilla coelebs* | 1 ([53]) | 37 | 42 | 1 | 2 ([19,53]) | 52 | 50 | 6 |
| *Locustella naevia* | | | | | 1 ([73]) | 2 | 5 | 1 |
| *Motacilla cinerea* | 1 ([73]) | 3 | 1 | 1 | 1 ([73]) | 3 | 9 | 2 |
| *Parus major* | 2 ([73,75]) | 187 | 266 | 3 | 4 ([19,20,73,75]) | 220 | 403 | 15 |
| *Phoenicurus phoenicurus* | | | | | 1 ([22]) | ** | 38 | 1 |
| *Phylloscopus trochilus* | | | | | 1 ([22]) | ** | 37 | 2 |
| *Prunella modularis* | | | | | 5 ([19,22,24,73,83]) | 87 | 430 | 27 |
| *Saxicola rubetra* | | | | | 1 ([22]) | ** | 2 | 1 |
| *Sylvia atricapilla* | | | | | 1 ([24]) | 16 | 18 | 1 |
| *Sylvia communis* | | | | | 2 ([53,73]) | 12 | 13 | 4 |
| *Sylvia curruca* | | | | | 1 ([22]) | ** | 22 | 2 |
| *Troglodytes troglodytes* | 1 ([83]) | 4 | 5 | 1 | | | | |
| *Turdus iliacus* | 1 ([61]) | 19 | 4 | 1 | 2 ([53,61]) | 28 | 60 | 5 |
| *Turdus merula* | 1 ([61]) | 11 | 2 | 1 | 7 ([19,22–24,53,61,73]) | 141 | 1009 | 35 |
| *Turdus philomelos* | | | | | 6 ([19,22,24,52,53,73]) | 131 | 436 | 11 |
| *Turdus viscivorus* | | | | | 1 ([53]) | 2 | 2 | 1 |

**Table 1. *Borrelia afzelii* infections have been found in *Ixodes ricinus* larvae and nymphs feeding on many different species of birds.** Data are from a literature search that included 19 publications that report on *Borrelia* genospecies in bird-derived ticks. *One study did not report on the total number of larvae that were screened, therefore this number is an under-estimation. **Study did not report on the total number of captured birds.

laboratory mice[38]. However, an alternative explanation for this study is that this strain actually belonged to the closely related but rodent-adapted *B. bavariensis*, as this species was recently shown to be widespread in Asia, including Japan[39].

The purpose of the present study was to test whether *B. afzelii* can use co-feeding transmission to infect *I. ricinus* larval ticks on two different species of songbird: the blackbird (*Turdus merula*) and the great tit (*Parus major*). We chose these two songbird species because they are common in Europe, are often exposed to immature *I. ricinus* ticks in nature, and they are highly competent reservoir hosts for bird-adapted *Borrelia* genospecies. The blackbird can amplify *B. garinii*, *B. valaisiana* and *B. turdi*[24,40,41] and the great tit can amplify *B. garinii*[12]. In addition, we performed a literature review to determine how often songbirds carry *B. afzelii*-infected immature *I. ricinus* ticks in nature.

## Results

**Blackbird experiment.** In the blackbird experiment, each of the four birds was infested with 11–12 nymphs before being infested with 40–50 co-feeding larvae 24 hours later. The challenge nymphs had been randomly selected from a population where the percentage of infected nymphs was 68.1% (47 infected/69 total). For the blackbirds, the nymphal and larval attachment rates (mean ± standard deviation) were 93.7 ± 12.5% per bird and 96.5 ± 4.7% per bird, respectively. A total of 20 engorged challenge nymphs and 128 engorged co-feeding larvae were recovered (mean ± standard deviation: 5.0 ± 0.8 nymphs per bird and 32 ± 12 larvae per bird). The engorged challenge nymphs were allowed to moult into adult ticks, which were tested using qPCR to determine whether the birds had been exposed to *B. afzelii*. A total of 17 challenge nymphs and 90 co-feeding larvae were tested for the four blackbirds (Table 2).

Two of the four blackbirds produced 2 and 4 infected adult ticks (Table 2) indicating that they were properly challenged. The presence of *B. afzelii* in 6 adult ticks suggests that there was nymph-to-adult transtadial transmission but we do not know whether these spirochetes were dead or alive. The other two birds produced 2 and 4 uninfected adult ticks (Table 2). Given that the estimated proportion of infected challenge nymphs was 0.681, the probability that these two birds would produce 6 uninfected adult ticks is $(1–0.681)^6 = 0.001$. Our method of estimating nymphal attachment suggests that 9 and 11 challenge nymphs attached to these two birds. The probability that these two birds were infested with at least one *B. afzelii*-infected nymph is therefore very high (0.9999659 and 0.9999965, respectively). Thus we are confident that all four birds encountered at least one *B. afzelii*-infected nymph. However, none of the 90 xenodiagnostic larval ticks (tested as engorged larvae or as flat nymphs) that had co-fed with the challenge nymphs tested positive for *B. afzelii* (Table 2).

All ticks that had fed on the blackbirds and that had tested positive for *B. afzelii* on the qPCR were sequenced with respect to the *ospC* gene and the 5S-23S (rrfA-rrlB) intergenic spacer (IGS) region gene. We obtained 3 *ospC*

| Species | Bird N° | Nymphs | | | Larvae | |
|---|---|---|---|---|---|---|
| | | Engorged | Moulted | | Engorged | Moulted |
| | | infect./total | infect./total | Attached** | infect./total | infect./total |
| *T. merula* | 1 - ♂ | N.A. | 0/2 | 9 | 0/10 | 0/7 |
| *T. merula* | 2 - ♀ | N.A. | 4/5 | 12 | 0/10 | 0/15 |
| *T. merula* | 3 - ♀ | N.A. | 0/4 | 11 | 0/9 | 0/6 |
| *T. merula* | 4 - ♂ | N.A. | 2/6 | 12 | 0/10 | 0/23 |
| *P. major* | 1 - ♀ | 2/2 | 0/1* | 4 | 0/14 | N.A. |
| *P. major* | 2 - ♀ | 2/2 | 1/1* | 8 | 0/24 | 0/9 |
| *P. major* | 3 - ♂ | 5/5 | 3/3* | 10 | 2/22 | 0/2 |
| *P. major* | 4 - ♂ | 2/2 | N.A. | 6 | 1/16 | 0/3 |

**Table 2. *Borrelia afzelii* infection status is shown for the *Ixodes ricinus* ticks that had co-fed on two species of songbird, the blackbird (*Turdus merula*) and the great tit (*Parus major*).** The blood-engorged nymphs and larvae were either placed in ethanol following drop-off or allowed to moult into the next stage (adult and nymph, respectively). All engorged and moulted ticks were screened for *B. afzelii* infection using qPCR. Adult ticks were also cultured in BSKII-medium to test for nymph-to-adult transtadial transmission of viable *B. afzelii* spirochetes. *Engorged nymphs were allowed to moult into adult ticks and were cut in half. One half was screened for *B. afzelii* using qPCR and the other half was cultured in BSK II-medium to test for viable spirochetes. None of them yielded spirochete cultures; therefore *B. afzelii* is not capable of transstadial transmission in the presence of bird blood. **Attached = total number of nymphs placed on the bird minus the number of nymphs left in the bag.

sequences and 5 IGS sequences and all of them belonged to *B. afzelii*. This sequencing work confirms that the nymphs used to challenge the blackbirds were infected with *B. afzelii*.

**Great tit experiment.** In the great tit experiment, each of the four birds was infested with 11–12 nymphs before being infested with 40–50 co-feeding larvae 24 hours later. The challenge nymphs had been randomly selected from a population where the percentage of infected nymphs was 91.5% (130 infected/142 total). For the great tits, the nymphal and larval attachment rates (mean ± standard deviation) were 58.3 ± 21.5% per bird and 85.6 ± 9.8% per bird, respectively. A total of 16 engorged challenge nymphs and 115 engorged co-feeding larvae were recovered (mean ± standard deviation: 4.0 ± 2.7 nymphs per bird and 28.8 ± 6.8 larvae per bird). The engorged challenge nymphs were either tested directly or were allowed to moult into adult ticks. A total of 16 challenge nymphs and 90 co-feeding larvae were tested for the four great tits (Table 2).

Analysis of the challenge ticks showed that all four great tits had been exposed to *B. afzelii* (2, 3, 8, and 2 infected ticks per bird; Table 2). Three of the 76 xenodiagnostic larval ticks (tested as engorged larvae) that had co-fed with the challenge nymphs tested positive for *B. afzelii*, but the pathogen was not detected in any of the 14 nymphs (moulted from the engorged larvae) (Table 2). Four of the five adult ticks obtained from three birds tested positive for *B. afzelii* based on the qPCR (Table 2), but the culture of these ticks in BSK-II medium did not yield any viable spirochetes.

**Summary of the infection experiments.** Overall, the *B. afzelii*-infection rates in co-feeding larvae were low in both blackbirds (0.00% = 0/90) and great tits (3.33% = 3/90). In summary, we found limited co-feeding transmission of *B. afzelii* for the two bird species used in this study. We emphasize that our sample size was limited with only 4 individuals for each bird species.

**Literature review.** Our review of the literature found 13 of 19 studies in which *B. afzelii* has been reported in songbird-derived *I. ricinus* ticks. Seven species of songbird could play a role in the transmission of *B. afzelii* to larval *I. ricinus* ticks (Table 1). The hosts that were most often reported to have *B. afzelii*-infected larvae were the European robin (*Erithacus rubecula*) and the great tit (2 studies). When considering birds that carried *B. afzelii*-infected nymphal ticks, we found 20 different bird species, of which the blackbird (7 studies), songthrush (*Turdus philomenos*) (6 studies), dunnock (*Prunella modularis*) (5 studies), European robin (5 studies), and great tit (4 studies) were most often reported.

## Discussion

Our study suggests that the rodent-adapted Lyme disease pathogen, *B. afzelii*, cannot use co-feeding transmission as an efficient strategy to infect naive ticks on two species of songbird. There was no co-feeding transmission of *B. afzelii* on the four blackbirds and only three larval ticks acquired *B. afzelii* via co-feeding transmission on the four great tits. The efficiency of co-feeding transmission of *B. afzelii* on the great tit was therefore low (3/90 = 3.33%). In contrast, the isolate of *B. afzelii* used in the great tit experiment (isolate NE4049; also referred to as *ospC* strain A10) has high co-feeding transmission (> 50.00%) on competent rodent reservoir hosts, and in these hosts there is successful trans-stadial transmission[32,34]. We acknowledge that one limitation of the current study is the small sample size (n = 8 birds). However, we point out that studies with similar sample sizes have detected co-feeding transmission of *B. afzelii* on rodents[28,32,33]. Recent theoretical studies have shown that co-feeding transmission makes a modest contribution to the reproductive number ($R_0$) of *B. burgdorferi*

pathogens[42–44]. Specifically, a co-feeding transmission efficiency of 50.0% increases the $R_0$ value by 2.07–6.68% depending on a variety of ecological factors[42]. These analyses suggest that a co-feeding transmission efficiency of 3.33% would have a negligible effect on the $R_0$ of *B. afzelii*. In summary, *B. afzelii* is transmitted efficiently via co-feeding transmission on rodent hosts but not on the two bird species investigated. Studies on *B. afzelii* in laboratory rodents have shown that strains differ in the efficacy of co-feeding transmission[32,34]. Studies on *B. burgdorferi* in North American passerines have shown that reservoir competence can vary widely between bird species[45–47]. We therefore emphasize that we cannot generalize these results to other strains of *B. afzelii* and other songbird species.

Our study also found evidence that avian blood is borreliacidal for *B. afzelii*. For the blackbirds, the probability that two birds would produce six uninfected adult ticks was highly unlikely ($p = 0.001$), given that an independent sample suggested that 68.1% (47 infected/69 total) of the challenge nymphs were infected with *B. afzelii* before feeding on these birds. Our results are similar to a previous study where *B. afzelii* was cleared from *I. ricinus* challenge nymphs after they had fed on pheasants, whereas bird-adapted *Borrelia* species were not cleared from the challenge nymphs[13]. Additional evidence for the borreliacidal effects of avian blood on *B. afzelii* was our demonstration using BSK-II cultures that none of the qPCR-positive adult ticks that had fed as nymphal ticks on the great tits contained viable spirochetes. Previous work has shown that the ability to detect *Borrelia* infections by culturing ticks in BSK media is similar to PCR-based methods[48]. This result suggests that our qPCR assay is detecting dead spirochetes in the adult ticks and shows the limitations of using DNA-based methods to infer the reservoir competence of a particular host species. Further studies using other combinations of pathogen strains and songbird species should investigate the generality of whether avian blood kills *B. afzelii* in *I. ricinus* during tick blood feeding.

Numerous field studies have shown the association of *B. afzelii* with rodent reservoir hosts[4–6,49,50] and of *B. garinii* and *B. valaisiana* with avian reservoir hosts[7,12,13,21,24,40,41,51–53]. The cycling of *B. afzelii* and *B. garinii* in different classes of vertebrate hosts is also supported by studies on wild *I. ricinus* nymphs, which have shown that these two sympatric *Borrelia* species rarely co-occur in the same nymphal tick[54–56]. The host-specificity of *B. afzelii* for rodents and *B. garinii* for birds is believed to be mediated by the complement system of the vertebrate host[15,16,55,56]. *In vitro* assays have shown that *B. afzelii* is tolerant to rodent complement but is lysed by bird complement, and vice versa for bird-adapted *Borrelia* species like *B. garinii* and *B. valaisiana*[15,16]. However, as mentioned previously, there are very few *in vivo* studies showing that *B. afzelii* spirochetes are killed in nymphs that feed on avian hosts[13]. Two recent studies that quantified the abundance of rodent- and bird-adapted *Borrelia* species in wild questing *I. ricinus* nymphs provided indirect evidence for the complement hypothesis[54,57]. In the first study, the spirochete load of nymphs co-infected with rodent- and bird-adapted *Borrelia* species was significantly lower than the additive expectation of when the species occurred alone[54]. In the second study, co-infections between *B. afzelii* and *B. garinii* were surprisingly common in wild nymphs, however, the spirochete load of the dominant *Borrelia* species was always an order of magnitude higher than the sub-dominant species[57]. Taken together, these two studies provide indirect evidence that some component of the vertebrate blood meal (e.g. complement) was reducing the spirochete load of the mal-adapted *Borrelia* species[54,57]. Thus co-infections between rodent- and bird-adapted *Borrelia* species in *I. ricinus* nymphs may be much more common than previously thought but the spirochete population of one of the two species is probably dead.

Migratory songbirds have a great capacity to disperse ticks and tick-borne pathogens to new geographic locations[58]. Interestingly, phylogenetic studies have shown that *B. afzelii* has much more spatial genetic structure than *B. garinii*, which may reflect the migratory potential of their rodent and bird hosts[59,60]. Our literature review found that ground-dwelling birds such as the blackbird, song thrush, European robin and dunnock were common carriers of *B. afzelii*-infected immature *I. ricinus* ticks. These studies have led to speculation that *B. afzelii* can use bird hosts to achieve transmission and is not as restricted to rodent hosts as previously thought[61]. However, all of these studies used PCR-based methods to determine *Borrelia* infection and none of these studies used culture-based methods to show that the spirochetes are actually alive. The present study shows that nymph-to-adult transtadial transmission of *B. afzelii* DNA can occur on birds but that the spirochetes are not necessarily viable. We suggest that PCR-based studies demonstrating that birds can amplify *B. afzelii* or that rodents can amplify *B. garinii* should be interpreted with great caution.

We propose three alternative explanations for the observation that *B. afzelii*-positive larval ticks are regularly collected from wild birds (Table 1). First, the larval ticks could have acquired *B. afzelii* via vertical transmission. There is a general consensus that vertical transmission in *Ixodes* ticks is rare for *B. burgdorferi* s. l. pathogens but common for the relapsing fever spirochete *B. miyamotoi*[25,26]. A second explanation is partial blood feeding where larval ticks take multiple meals from different vertebrate hosts. Host blood meal analysis of wild *I. ricinus* ticks in Switzerland suggests that 9.5–19.5% of larval ticks feed on multiple hosts[62,63]. An early study on *B. burgdorferi* s. s. in *I. scapularis* showed that partially fed larval ticks could acquire spirochetes[64]. Thus larval ticks could acquire *B. afzelii* from a partial blood meal on a rodent and then attach to a bird to feed to repletion. A recent study in the Netherlands reported that wild *I. ricinus* larvae carried *B. afzelii* (prevalence was 0.62%), and these larvae were able to infect pathogen-free rodents[27]. The authors suggested that their data were consistent with both vertical transmission and partial blood meals[27]. A third explanation involves variation in the efficiency of co-feeding transmission between strains of *B. afzelii*. Like many vector-borne pathogens, populations of *B. afzelii* consist of multiple strains[57,65–68]. Two recent studies found that some *B. afzelii* strains are much more efficient at co-feeding transmission than other strains[32,34]. The *B. afzelii* strains in the blackbird experiment were derived from naturally infected *Apodemus* mice, and their genetic identity and co-feeding transmission efficiency on rodent hosts are currently unknown. For this reason, we used *B. afzelii* isolate NE4049 in the great tit experiment because it has a high efficiency of co-feeding transmission (>50%) on lab mice[32,34].

We conclude that blackbirds and great tits do not allow efficient co-feeding transmission of viable *B. afzelii* spirochetes. The present study supports the hypothesis that the bird complement system inhibits the rodent-adapted *B. afzelii* from exploiting avian hosts for spirochete transmission. The generality of our results for other combinations of *B. afzelii* strains and bird species remains to be investigated.

## Methods

**Birds.** Eurasian blackbirds and great tits are two abundant bird species in Europe. The Eurasian blackbird is frequently infested with tens of immature *I. ricinus* ticks[24,69,70]. The great tits in our Belgian study population frequently carry high burdens of immature *I. ricinus* ticks (maximum number of larvae = 40; nymphs = 17)[71,72]. Both bird species are competent reservoir hosts for bird-adapted *B. burgdorferi* s. l. pathogens. Blackbirds transmit *B. garinii*, *B. valaisiana*, and *B. turdi*[24,40,41], whereas great tits transmit *B. garinii*[12,73–75].

Four pathogen-free blackbirds and four pathogen-free great tits were obtained, respectively, from a certified Belgian breeder and a laboratory colony at the Netherlands Institute of Ecology (NIOO-KNAW)[76]. Environmental conditions consisted of a 12 h light: 12 h dark cycle (7:00 to 19:00) and ambient temperature varied with outdoor conditions. Birds were given food and water *ad libitum*, and had access to a fresh water bath. Birds were kept in individual cages and were allowed to habituate to the lab environment for at least four days before the start of the experiment.

***Ixodes ricinus* ticks.** Pathogen-free *I. ricinus* larval ticks from the laboratory colony at the University of Neuchâtel were fed on *B. afzelii*-infected rodents and were allowed to moult into *B. afzelii*-infected nymphs (hereafter referred to as the challenge nymphs). The creation of the challenge nymphs was different for the blackbirds and great tits (see below). The pathogen-free *I. ricinus* larvae that were used for co-feeding with the infected challenge nymphs were obtained from a German laboratory colony (IS Insect Services GmbH, Berlin).

For the blackbirds, the challenge nymphs had been fed as larval ticks on 7 field-captured and naturally infected wood mice (*Apodemus sylvaticus* L.). Infection with *B. burgdorferi* s. l. of each wood mouse was confirmed with a commercial Lyme borreliosis ELISA assay and qPCR on an ear tissue sample, using protocols described elsewhere[77]. All challenge nymphs were kept in individual Eppendorf tubes to facilitate random sampling. We randomly selected 9–10 nymphs from each of the 7 *Apodemus* mice and tested them for *B. afzelii* infection using qPCR. The infection prevalence of the challenge nymphs used in the black bird experiment was 68.1% (47 infected/69 total).

For the great tits, the challenge nymphs had been fed as larval ticks on 15 *Mus musculus* BALB/c mice that had been experimentally co-infected via tick bite with *B. afzelii* isolates NE4049 and Fin-Jyv-A3. Infection with *B. afzelii* of each mouse was confirmed with a commercial Lyme borreliosis ELISA assay and qPCR on an ear tissue sample, using protocols described elsewhere[77]. Isolates Fin-Jyv-A3 and NE4049 were obtained from a bank vole (*Myodes glareolus*) in Finland and an *I. ricinus* nymph in Switzerland. Isolate Fin-Jyv-A3 carries *ospC* major group (oMG) A3. Isolate NE4049 has multi-locus sequence type 679, oMG A10, and strain ID number 1887 in the *Borrelia* MLST database[11,32,34,77]. We used isolate NE4049 (also referred to as *ospC* strain A10) because it has very efficient co-feeding transmission in lab mice[11,32,34]. All challenge nymphs were kept in individual Eppendorf tubes to facilitate random sampling. We randomly selected 7–10 nymphs from each of the 15 mice and tested them for *B. afzelii* infection using a previously described qPCR protocol[77]. The infection prevalence of the challenge nymphs used in the great tit experiment was 91.5% (130 infected/142 total), of which 75.4% (107/142) and 59.9% (85/142) were infected with isolates NE4049 and Fin-Jyv-A3, respectively.

**Ethics statement and animal experimentation permits.** Experiments on the birds were carried out at the University of Antwerp, Belgium in accordance with national environmental legislation and university regulations. The Ethics Committee for Animal Experiments of the University of Antwerp approved the tick infestation procedure (Dossier 2009-32) and the transmission experiment (Dossier 2014-49). Experiments to create the *I. ricinus* nymphs infected with *B. afzelii* were carried out at the University of Neuchâtel, Switzerland. The commission that is part of the 'Service de la Consommation et des Affaires Vétérinaires (SCAV)' of Canton Vaud, Switzerland evaluated and approved the ethics of this part of the study. The Veterinary Service of the Canton of Neuchâtel, Switzerland issued the animal experimentation permits (NE1/2014 and NE4/2016).

**Study design.** The infestation experiments for the blackbirds and great tits were conducted in November 2015 and February 2016, respectively. For each bird species, four individuals were infested with 11–12 *B. afzelii*-infected *I. ricinus* nymphs that had been randomly selected from the pool of available nymphs. These tick loads are within the range observed in field-captured birds[24,69–72]. Nymphs were placed underneath the crown feathers on the right side of the head above the eye using moistened tweezers, as described in ref. 72 (Fig. 1). After each infestation, birds were kept for 1 h in an air-permeable cotton bag (size: 25 cm × 20 cm for blackbirds; 20 cm × 15 cm for great tits) inside a darkened cage to keep them inactive and to facilitate tick attachment[72]. Twenty-four hours after nymphal exposure, the blackbirds and great tits were additionally infested with 40–50 xenodiagnostic larvae, following the same protocol as for the challenge nymphs. The larvae were placed near the nymphs to facilitate co-feeding transmission[32–34]. After each infestation, the cotton bags were checked for ticks to determine the number of nymphs and larvae that had attached to each bird. Birds were not checked for the number of attached nymphs to avoid disturbing these ticks. Following infestation, birds were returned to their individual cages (40 cm × 80 cm) that had a wire mesh floor to facilitate the daily collection of engorged ticks. Most of the engorged ticks were placed in 80% ethanol and stored at −20 °C. The remaining engorged ticks were allowed to moult to the next stage to study transstadial transmission of *B. afzelii* DNA. These ticks were kept in individual tubes under summer conditions (16 h light at 25 °C, 8 h at dark at 16 °C) and with a relative humidity >90%. For the great tit experiment, we further tested whether the *B. afzelii* spirochetes in the adult ticks were

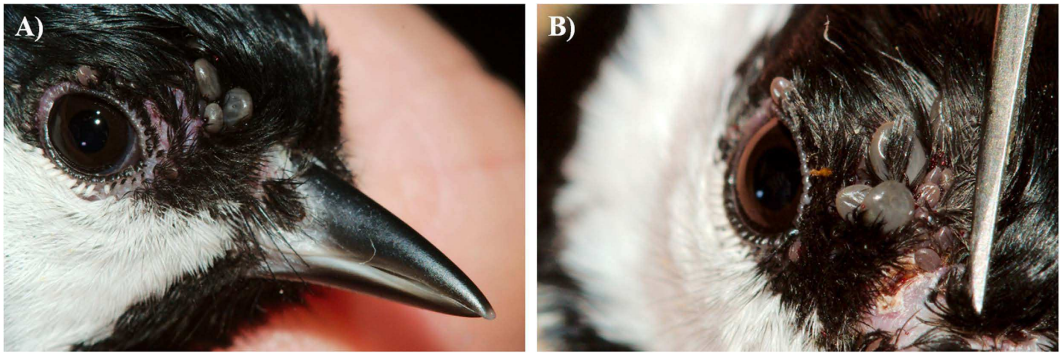


**Figure 1. Naïve *I. ricinus* larvae co-feed with *B. afzelii*-infected nymphs on the head of a great tit.** The larvae (small) and nymphs (large) were placed underneath the crown-feathers on the right side of the head (**A**: lateral; **B**: frontal view). By feeding in close spatial and temporal proximity, the *B. afzelii* spirochetes can migrate directly from the infected nymphs to the naïve larvae via co-feeding transmission. Dr. Frank Adriaensen took the photos.

actually viable. Each of five adult ticks that had fed as challenge nymph on three great tits, were cut into two halves using sterile scissors. One tick half was screened for *B. afzelii* infection using qPCR, the other tick half was cultured in tubes containing BSK-II medium[78], incubated at 34 °C, and examined by dark-field microscopy every 10 days for 40 days.

**Probability that each bird was challenged by at least one *B. afzelii*-infected nymph.** If avian blood clears spirochetes from feeding nymphs, the post-hoc analysis of such ticks is not a reliable indicator as to whether the bird was challenged or not. For example, after feeding *B. afzelii*-infected *I. ricinus* nymphs on pheasants, 0 of the 56 engorged nymphs tested positive for *B. afzelii*[13]. In this case, it is critical to know the prevalence of *B. afzelii* infection in the flat nymphs (q) before they are placed on the birds, and the number of nymphs that attached to the bird (n). With this information one can calculate the probability (P) that each bird was bitten by at least one *B. afzelii*-infected challenge nymph as follows: $P = 1 - (1 - q)^n$. The exact value of n is often unknown: the maximum is the number of nymphs that attached to the bird ($n_{max}$) and the minimum is the number of blood-engorged nymphs that were recovered ($n_{min}$). For example, for a bird that was infested with 12 challenge nymphs with an expected prevalence of infection of 0.681 and for which 4 engorged challenge nymphs were recovered, the probability that at least one of the challenge nymphs was infected with *B. afzelii* ranges from $P_{max} = 0.9999989$ to $P_{min} = 0.9896447$.

**PCR-based detection of *B. afzelii*.** Total tick DNA was purified using the DNeasy Blood & Tissue Kit following the protocol for the purification of total DNA from ticks. All ticks were screened for the presence of *B. burgdorferi* s. l. using a duplex qPCR that was designed based on existing qPCR protocols that target fragments of the *ospA* gene[79] and the *flagellin* gene[80]. A detailed description of primers, probes and the qPCR protocol is given in an earlier study[75]. For the subsample of qPCR-positive ticks that had fed on the blackbirds, the *B. burgdorferi* s. l. genospecies was determined by PCR amplification and sequencing of the *ospC* gene[81] and the variable 5S-23S (rrfA-rrlB) intergenic spacer (IGS) region gene[75]. For each PCR and multiplex qPCR, positive controls, negative controls, and blank samples were included. To minimize contamination, the three steps of the PCR protocol were performed in separate rooms. The DNA extraction room was kept at negative pressure, whereas the reagent setup and sample addition rooms were kept at positive pressure. All rooms had airlocks.

**Literature review.** We used an extensive systematic literature search that is described in Hofmeester *et al.* (2016)[82]. The search strings and selection procedure as well as the dataset are provided in the supplementary material of that study (URL: http://iopscience.iop.org/article/10.1088/1748-9326/11/4/043001/meta). The search was done using PubMed, Web of Science and Scopus to review the occurrence of *B. burgdorferi* s. l. pathogens in Europe, in songbird hosts and their *I. ricinus* ticks. The last literature search was carried out in January 2015 and used the years 1945–2014. We added one more study to that dataset[22]. Only studies that identified the *Borrelia* genospecies in infected larvae and nymphs derived from songbirds were included, which resulted in 19 usable studies.

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## Author Contributions

D.H., M.J.V. and H.S. conceived and designed the study. D.H., A.K. and N.V. performed the experiments. D.G. and A.G.-C. created the *B. afzelii*-infected nymphal ticks. M.J.V., H.S., K.V. and D.H. provided funding. D.H., H.S., and M.J.V. wrote the manuscript. All authors reviewed the manuscript.

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# Competition between strains of *Borrelia afzelii* inside the rodent host and the tick vector

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Multiple-strain pathogens often establish mixed infections inside the host that result in competition between strains. In vector-borne pathogens, the competitive ability of strains must be measured in both the vertebrate host and the arthropod vector to understand the outcome of competition. Such studies could reveal the existence of trade-offs in competitive ability between different host types. We used the tick-borne bacterium *Borrelia afzelii* to test for competition between strains in the rodent host and the tick vector, and to test for a trade-off in competitive ability between these two host types. Mice were infected via tick bite with either one or two strains, and these mice were subsequently used to create ticks with single or mixed infections. Competition in the rodent host reduced strain-specific host-to-tick transmission and competition in the tick vector reduced the abundance of both strains. The strain that was competitively superior in host-to-tick transmission was competitively inferior with respect to bacterial abundance in the tick. This study suggests that in multiple-strain vector-borne pathogens there are trade-offs in competitive ability between the vertebrate host and the arthropod vector. Such trade-offs could play an important role in the coexistence of pathogen strains.

## 1. Introduction

Many populations of pathogens consist of genetically distinct strains. As a result of this strain diversity, hosts are often infected with multiple strains, a phenomenon known as co-infection, mixed infection or multiple-strain infection [1–3]. Co-infection implies that the strains can interact with each other, which can result in cooperation or competition [3–5]. Empirical evidence for inter-strain competition typically shows that the performance (e.g. abundance, transmission) of a given strain in a co-infection is reduced compared with when it occurs alone [6–10]. Pathogen strains with similar ecological niche requirements will experience intense competition, which can result in competitive exclusion and loss of strain diversity [11–13]. Thus a fundamental task is to understand the mechanisms that can maintain strain diversity in the face of strong within-host competition, and one potential explanation is life-history trade-offs [5,14].

A commonly assumed life-history trade-off for pathogens is between transmission and virulence [15,16], which is essentially a trade-off between current and future reproduction [14]. In mixed infections, competition between strains should select for fast-growing virulent strains that can monopolize the limited host resources and thereby outcompete slow-growing avirulent strains [6,8,17–20]. At the pathogen population level, a diversity of strains can be maintained

because virulent strains outcompete avirulent strains in mixed infections, but the reverse is true in single-strain infections [21,22]. Other life-history trade-offs that have been reported for pathogens include trade-offs between multiplication rate (and presumably competitive ability) and persistence in the abiotic environment [23,24], and between competitiveness and ability to colonize new hosts [25]. Given the variety of pathogen life cycles, we should expect a similar diversity of trade-offs between competitive ability and other life-history traits.

Trade-offs in competitive ability might be particularly common in parasites that must infect more than one host type to complete their life cycle [2]. In these complex or vector-borne life cycles, the genes and phenotypes that lead to competitive success in one host type (e.g. vertebrate host) may have little bearing on within-host competition in another host type (e.g. arthropod vector). Despite the plausibility of this idea, there are few studies that have compared the strain-specific competitive ability between different host types [26]. In the case of vector-borne pathogens, numerous studies have investigated interactions between strains in the vertebrate host [6,10,12,27–31], but similar studies in the arthropod vector are rare [32–35], and we are not aware of any studies that have investigated both host types. To investigate these questions, we used *Borrelia afzelii*, a spirochaete bacterium that requires both a vertebrate host and an arthropod vector to complete its life cycle, as a model system.

*Borrelia afzelii* belongs to the *Borrelia burgdorferi sensu lato* (sl) genospecies complex, which includes the aetiological agents of Lyme borreliosis in North America and Eurasia [36,37]. In Europe, *Borrelia afzelii* is a common genospecies [36,38,39], which is transmitted by the hard tick *Ixodes ricinus* and uses rodents as reservoir hosts [38,39]. The life cycle of *I. ricinus* consists of three stages: larva, nymph and adult. The larvae acquire *B. afzelii* from infected rodents during the larval blood meal, and develop into infected nymphs that transmit the pathogen back to the reservoir host population the following year. The engorged larva and the resultant nymph are therefore the two stages where interactions between strains are most ecologically important. Populations of *B. burgdorferi* sl consist of multiple strains and mixed infections are common in both the vertebrate host [10,29,40,41] and the tick vector [41–44]. Competition between strains of *B. burgdorferi* sl in the vertebrate host has been shown in field studies [10,29] and experimental infections [9,45,46]. Field studies on our local *I. ricinus* population found that approximately 80% of *B. afzelii*-infected nymphs were infected with multiple strains and that the mean strain richness was 2.4–2.9 strains per nymph [42,44]. In co-infected nymphs, the spirochaete load per strain decreased with increasing strain richness, and this result provides indirect evidence for competition [44]. However, to date there is no direct experimental evidence that competition between strains of *B. burgdorferi* sl can occur in the tick vector.

The purpose of this study was to test whether strains of *B. burgdorferi* sl compete inside their rodent host and their tick vector, and whether there was a trade-off in strain-specific competitive ability between the two host types. Mice were infected via tick bite with either one or two strains of *B. afzelii*. The infected mice were infested with larval ticks and these were allowed to moult into nymphal ticks that carried either single-strain infections or co-infections. The mouse-to-tick transmission success and the spirochaete load in the nymph were used as measures of competitive success in the rodent host and the tick vector, respectively. We predicted that inter-strain competition in the mouse and the nymph would reduce the strain-specific mouse-to-tick transmission success and the strain-specific spirochaete load, respectively. We also predicted that the strain that was competitively superior in the rodent host would be competitively inferior in the tick vector, and vice versa.

## 2. Material and methods

### (a) Mice, ticks, and strains of *Borrelia afzelii*

Forty female, pathogen-free *Mus musculus* BALB/c mice aged five weeks were used as the rodent reservoir host. All *I. ricinus* ticks came from the *Borrelia*-free laboratory colony that has been maintained at the University of Neuchâtel since 1978. *Borrelia afzelii* isolates Fin-Jyv-A3 and NE4049 were used in this study, which were obtained from a bank vole (*Myodes glareolus*) in Finland and an *I. ricinus* nymph in Switzerland, respectively. We had originally started the study with two Swiss strains, but one of the strains failed and we used strain Fin-Jyv-A3 as a back-up solution. Fin-Jyv-A3 has *ospC* major group (oMG) A3, multi-locus sequence type (MLST) 676, and strain ID number 1961 in the *Borrelia* MLST database. Isolate NE4049 has oMG A10, MLST 679, and strain ID number 1887 in the *Borrelia* MLST database. The purity of these isolates with respect to the oMG allele has been assessed using 454-sequencing. For isolates Fin-Jyv-A3 and NE4049, 137 and 1313 *ospC* gene sequences were obtained, respectively, and all but one belonged to the correct oMG. We are confident that these isolates are genetically homogeneous and will hereafter refer to them as strains Fin-Jyv-A3 and NE4049.

*I. ricinus* nymphs infected with either strain Fin-Jyv-A3 or strain NE4049 were created as follows. Female BALB/c mice ($n = 5$) were infected with one of the two strains via needle inoculation. At four weeks post-infection, *Borrelia*-free larval ticks from our laboratory colony of *I. ricinus* were fed on these mice. Engorged larval ticks were placed in individual eppendorf tubes and were allowed to moult into nymphs. At four weeks after the larva-to-nymph moult, a random sample of nymphs was selected for each strain and tested for *B. afzelii* infection using qPCR. The percentage of nymphs infected with *B. afzelii* was 70% (7/10) for strain Fin-Jyv-A3 and 71.4% (10/14) for strain NE4049.

### (b) Infection of mice via tick bite with one or two strains

The study consisted of experiments 1 and 2, where the focal strains were Fin-Jyv-A3 and NE4049, respectively. In experiment 1, mice were randomly assigned to infection with strain Fin-Jyv-A3 ($n = 10$ mice) or to co-infection with strains Fin-Jyv-A3 and NE4049 ($n = 10$ mice). In experiment 2, mice were randomly assigned to infection with strain NE4049 ($n = 10$ mice) or to co-infection with strains NE4049 and Fin-Jyv-A3 ($n = 10$ mice). All mice were infected via tick bite. Mice in the co-infection treatments were infested with 5 Fin-Jyv-A3-infected nymphs and 5 NE4049-infected nymphs. Mice in the single-strain infection treatments were infested with 5 Fin-Jyv-A3-infected nymphs or 5 NE4049-infected nymphs. Each mouse in the single-strain infection treatment was also infested with five uninfected nymphs. When nymphs take a blood meal, they secrete saliva that contains immunosuppressive molecules [47]; for this reason, each mouse in the study was infested with the same number of nymphs. Details of the tick infestation procedure have been described elsewhere [48].

Four weeks after the nymphal challenge, ear tissue biopsies and blood samples were taken from each mouse to confirm their *B. afzelii* infection status [48]. A *Borrelia*-specific qPCR assay was used on the ear tissue samples to determine the presence of *B. afzelii* spirochaetes. A Serion Elisa classic *Borrelia burgdorferi* IgG/IgM immunoassay was used on the blood samples to determine the presence of *Borrelia*-specific IgG antibodies. The two infection phenotypes were 100% congruent.

### (c) Host-to-tick transmission

Five weeks after the nymphal challenge, each mouse was infested with approximately 100 *Borrelia*-free xenodiagnostic larvae from our laboratory colony of *I. ricinus*. Blood-engorged larvae were kept in individual Eppendorf tubes, and were allowed to moult into xenodiagnostic nymphs under standard laboratory conditions (20–25°C, 12 h light : 12 h dark). To maintain high humidity, each tube contained a piece of moistened paper towel. Four weeks after the larva-to-nymph moult, 10 live nymphs were randomly selected from each mouse and frozen at −20°C. To test whether the spirochaetes were viable, we placed up to three xenodiagnostic nymphs in BSK medium for all *B. afzelii*-infected mice that had sufficient numbers of ticks. The cultures were checked using a dark field microscope on a weekly basis for the presence of live spirochaetes.

### (d) DNA extraction

Four-week-old nymphs were crushed in the TissueLyser II using a previously described protocol [48]. The crushed nymphs were digested with proteinase K at 56°C overnight. The DNA of the nymphs was extracted using Qiagen DNeasy 96 Blood and Tissue kit well plates and following the Qiagen protocol. Each plate contained two negative DNA extraction controls (*Anopheles gambiae* mosquitoes). DNA from the mouse ear samples was extracted using Qiagen DNeasy Blood & Tissue mini spin columns and following the Qiagen protocol. Ear tissue DNA and nymph DNA were eluted into 65 µl of water.

### (e) General and strain-specific qPCR assays

Each tick was tested with three independent qPCR assays. A *Borrelia*-diagnostic qPCR assay that targets a 132 bp fragment of the highly conserved *flagellin* gene was used to determine infection and quantify the total spirochaete load in each nymph. We developed two strain-specific qPCRs that allowed us to detect and quantify the oMG allele A3 of strain Fin-Jyv-A3 or the oMG allele A10 of strain NE4049. Each strain-specific qPCR used the same primers to amplify a 143 bp fragment of the *ospC* gene, but used a different strain-specific probe to detect the two different oMG alleles (see electronic supplementary material for details). To validate the *ospC* qPCR assays, we created communities that differed in the percentage of the A3 and A10 alleles. This independent validation experiment showed that the *ospC* qPCR assays were highly reliable at estimating the frequencies of each of the two oMG alleles (see electronic supplementary material for details).

The qPCRs were done using a LightCycler 96 Multiwell Plate white (Roche). All the plates contained negative controls for the DNA extraction (mosquito DNA), negative controls for the qPCR (water), and four standards containing $10^2$, $10^3$, $10^4$, and $10^5$ gene copies. All the controls (and standards) were run in duplicate (or triplicate) in each plate. The quantification of copy gene numbers in the samples was done using the LightCycler 96 Software (Roche). For each qPCR assay, a sample of 81 ticks was tested twice to determine the repeatability of the assay. The repeatability of the log10-transformed gene copy number for each of the three independent qPCR assays was very high (see electronic supplementary material). The estimates of the *ospC* gene copy numbers were also highly correlated with the estimates of the *flagellin* gene copy numbers (see electronic supplementary material). These results indicate that our methods of estimating the strain-specific spirochaete load in the nymphal ticks are highly reliable.

## 3. Statistical analyses

All the statistical analyses were done using R v. 3.4.2.

### (a) General statistical approach

The two measures of strain-specific fitness include host-to-tick transmission and the spirochaete load inside the nymphal tick. The experimental design of the study contains two fixed factors that are orthogonal to each other: focal strain (two levels: Fin-Jyv-A3, NE4049) and infection treatment (two levels: single, co-infection). To determine the effect of competition, the performance of the focal strain (host-to-tick transmission and spirochaete load in the nymphal tick) was compared between the single strain infection and the co-infection. A significant interaction between the focal strain and the infection treatment indicates that each focal strain is affected differently by the presence of the co-infecting strain.

Generalized linear mixed effects (GLME) models with binomial errors or linear mixed effects (LME) models with normal errors were used to analyse the response variables. The focal strain (two levels: Fin-Jyv-A3, NE4049), the infection treatment (two levels: single, co-infection) and their interaction were fixed factors, and mouse identity was included as a random factor. To determine statistical significance, models that differed with respect to the fixed factor of interest were compared using a log-likelihood ratio test (LLR).

### (b) Host-to-tick transmission of *Borrelia afzelii*-infected nymphs

Host-to-tick transmission refers to the percentage of nymphs that acquired *B. afzelii* during their larval blood meal. The *flagellin* qPCR was used to determine the infection status of the ticks. Of the 346 ticks, 22 ticks (distributed over 17 different mice) were excluded from the analysis because they had contradictory results between the *flagellin* qPCR and the *ospC* qPCR. The strain-specific *ospC* qPCR was used to determine the presence of the focal strain in the ticks. Tick infection status with the focal strain was modelled as GLME model with binomial errors.

### (c) Spirochaete loads of the nymphs

The analyses were done on the subset of infected nymphs. The gene copy number estimated from the *flagellin* qPCR assay was adjusted to give an estimate of the total spirochaete load for the entire nymph. For nymphs that were co-infected with strains Fin-Jyv-A3 and NE4049, the estimates of the strain-specific spirochaete loads from the *ospC* qPCR assays were constrained to sum to the total spirochaete load estimated by the *flagellin* qPCR assay (see the electronic supplementary material for details). The strain-specific spirochaete loads were log10-transformed to improve their fit to the normal distribution. The log10-transformed spirochaete load of the focal strain was modelled as an LME model with normal errors.

**Table 1.** The proportion of *B. afzelii*-infected nymphs is shown for each of the 33 mice in the study. For each mouse, the geometric mean spirochaete load for the subset of infected nymphs and the 95% confidence interval (95% CI) are also shown. Here A3 and A10 refer to strains Fin-Jyv-A3 and NE4049, respectively.

| exp | strains | mouse ID | infected nymphs/total nymphs (%) | spiro load mean | spiro load 95% CI |
|---|---|---|---|---|---|
| 1 | A3 | S01 | 8/8 (100.0%) | 18 408 | 6613–51 235 |
| 1 | A3 | S02 | 9/9 (100.0%) | 4885 | 1861–12 824 |
| 1 | A3 | S03 | 9/9 (100.0%) | 9309 | 3546–24 436 |
| 1 | A3 | S04 | 6/8 (75.0%) | 9513 | 2917–31 023 |
| 1 | A3 | S06 | 6/7 (85.7%) | 1672 | 513–5454 |
| 1 | A3 | S07 | 8/8 (100.0%) | 6722 | 2415–18 710 |
| 1 | A3 | S08 | 8/9 (88.9%) | 9523 | 3421–26 504 |
| 1 | A3 | S09 | 6/7 (85.7%) | 8643 | 2650–28 185 |
| 1 | A3 | S10 | 7/8 (87.5%) | 4771 | 1597–14 251 |
| 1 | A3 + A10 | S11 | 8/10 (80.0%) | 2188 | 786–6089 |
| 1 | A3 + A10 | S13 | 10/10 (100.0%) | 8730 | 3494–21 809 |
| 1 | A3 + A10 | S14 | 7/9 (77.8%) | 1963 | 657–5863 |
| 1 | A3 + A10 | S15 | 9/10 (90.0%) | 4786 | 1823–12 565 |
| 1 | A3 + A10 | S16 | 10/10 (100.0%) | 4898 | 1961–12 236 |
| 1 | A3 + A10 | S18 | 10/10 (100.0%) | 5117 | 2048–12 783 |
| 1 | A3 + A10 | S19 | 8/10 (80.0%) | 6839 | 2457–19 036 |
| 2 | A10 | S31 | 6/8 (75.0%) | 5888 | 1806–19 202 |
| 2 | A10 | S32 | 8/10 (80.0%) | 9094 | 3267–25 312 |
| 2 | A10 | S33 | 10/10 (100.0%) | 9162 | 3667–22 890 |
| 2 | A10 | S34 | 8/10 (80.0%) | 2563 | 921–7134 |
| 2 | A10 | S35 | 6/10 (60.0%) | 2247 | 689–7329 |
| 2 | A10 | S36 | 9/9 (100.0%) | 4986 | 1900–13 090 |
| 2 | A10 | S38 | 6/10 (60.0%) | 614 | 188–2003 |
| 2 | A10 | S39 | 7/9 (77.8%) | 1820 | 609–5436 |
| 2 | A10 | S40 | 7/10 (70.0%) | 2138 | 716–6387 |
| 2 | A10 + A3 | S21 | 7/9 (77.8%) | 4169 | 1396–12 453 |
| 2 | A10 + A3 | S22 | 10/10 (100.0%) | 5808 | 2325–14 509 |
| 2 | A10 + A3 | S23 | 8/9 (88.9%) | 6978 | 2507–19 423 |
| 2 | A10 + A3 | S24 | 10/10 (100.0%) | 6223 | 2491–15 547 |
| 2 | A10 + A3 | S26 | 10/10 (100.0%) | 7907 | 3165–19 753 |
| 2 | A10 + A3 | S27 | 7/7 (100.0%) | 7268 | 2433–21 712 |
| 2 | A10 + A3 | S28 | 8/9 (88.9%) | 6550 | 2353–18 232 |
| 2 | A10 + A3 | S30 | 8/9 (88.9%) | 2447 | 879–6813 |

# 4. Results

## (a) Infection success

Three of the 40 mice in the study were excluded: two mice (S5 and S12) died during the study, and one mouse (S37) did not become infected with *B. afzelii* following the nymphal challenge. In the co-infection treatment, four mice (S17, S20, S25 and S29) were only infected with strain Fin-Jyv-A3. Two of these four mice had been challenged with at least one NE4049-infected nymph. These four mice and their nymphs were excluded from the analyses because the aim of our study was to test whether strains in co-infected mice and co-infected nymphs experienced competition. Including these four mice in the statistical analyses made the results more statistically significant (see the electronic supplementary material). The final analysis therefore included 301 nymphs from 33 *B. afzelii*-infected mice (table 1). For a subsample of 29 infected mice, we obtained live cultures of *B. afzelii* from one or more nymphs; this result indicates that the mice transmitted live spirochaetes to the nymphs.

## (b) Comparison of fitness between strains Fin-Jyv-A3 and NE4049

For the mice infected with one strain, the host-to-tick transmission of Fin-Jyv-A3 (91.8% = 67/73) was significantly

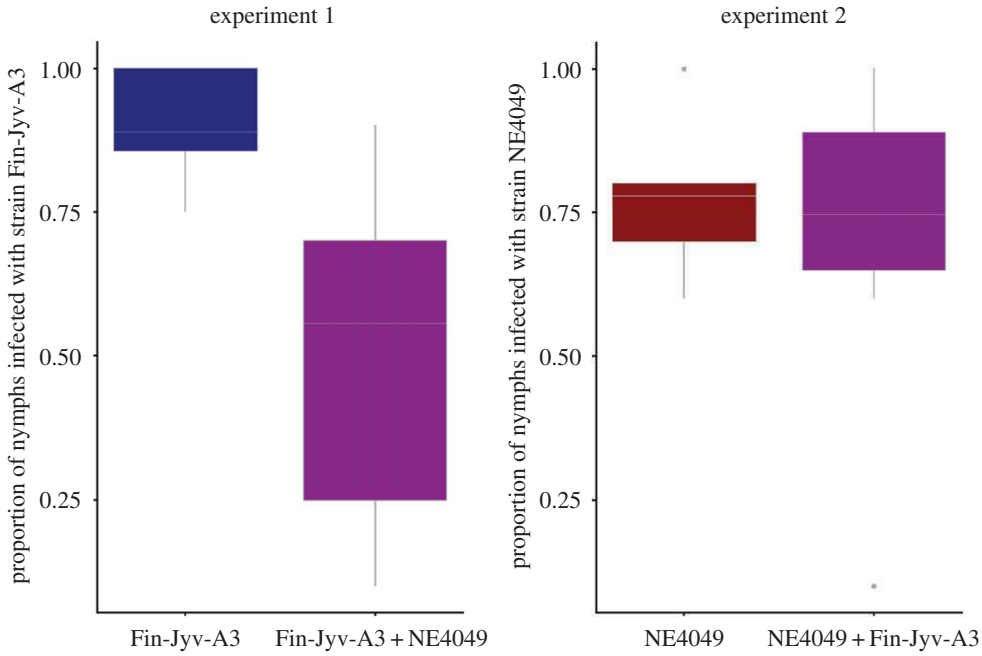


**Figure 1.** Co-infection reduces host-to-tick transmission for two strains of *B. afzelii*. In experiments 1 and 2, the focal *B. afzelii* strains are Fin-Jyv-A3 and NE4049, respectively. Experiment 1 shows that host-to-tick transmission of strain Fin-Jyv-A3 is reduced in the presence of strain NE4049. Experiment 2 shows that host-to-tick transmission of strain NE4049 is not affected in the presence of strain Fin-Jyv-A3. Each data point represents the mean for a single mouse ($n = 33$ mice). Shown are the medians (grey line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (solid circles). (Online version in colour.)

higher than NE4049 (77.9% = 67/86; GLME LLR: $p = 0.021$). The nymphal spirochaete load of strain Fin-Jyv-A3 ($n = 67$, mean = 7109, 95% CI = 4947–10 216) was twice that of strain NE4049 ($n = 67$, mean = 3497, 95% CI = 2434–5026; LME LLR: $p = 0.038$). Thus, in single-strain infections, strain Fin-Jyv-A3 outperformed strain NE4049 in both phenotypes.

For the co-infected mice, 91.5% of the nymphs (130/142) were infected with *B. afzelii*. Of the 130 infected nymphs, 17.7% (23/130) carried strain Fin-Jyv-A3 alone, 34.6% (45/130) carried strain NE4049 alone and 47.7% (62/130) carried both strains. For these 142 nymphs, host-to-tick transmission was 59.9% (85/142) for strain Fin-Jyv-A3 and 75.4% (107/142) for strain NE4049. Thus in co-infected mice, strain NE4049 was the superior competitor because it had higher host-to-tick transmission than strain Fin-Jyv-A3.

## (c) Effect of competition on host-to-tick transmission

The effect of co-infection on host-to-tick transmission was analysed separately for each focal strain (figure 1) because the interaction between the focal strain and infection treatment was significant (GLME LLR: $p = 0.0134$). In experiment 1, host-to-tick transmission of strain Fin-Jyv-A3 was significantly lower for the co-infected mice (49.3% = 34/69), compared to the single strain mice (91.8% = 67/73; figure 1; GLME LLR: $p < 0.001$). In experiment 2, host-to-tick transmission of strain NE4049 was lower but not significantly so for the co-infected mice (69.9% = 51/73) compared to the single strain mice (77.9% = 67/86; figure 1; GLME LLR: $p = 0.487$). There was a significant negative effect of competition on the host-to-tick transmission of strain NE4049 when all 10 mice in the co-infection treatment were included in the analysis (see electronic supplementary material).

## (d) Comparison of the total nymphal spirochaete load between single strain and co-infection treatments

In experiments 1 and 2, there was no significant difference in the total spirochaete load (as estimated by the *flagellin* qPCR) between the nymphs that had fed as larvae on the co-infected mice and the nymphs that had fed as larvae on the mice infected with a single strain (see the electronic supplementary material for details).

## (e) Effect of competition on the nymphal spirochaete load

The nymphal spirochaete load of the focal strain is the number of spirochaetes of that strain in the nymph. With respect to the log10-transformed spirochaete load, focal strain (LME LLR: $p = 0.009$) and infection treatment (LME LLR: $p = 0.003$) were significant, but their interaction was not (LME LLR: $p = 0.715$; figure 2). For focal strain Fin-Jyv-A3, the nymphal spirochaete load in the co-infection group ($n = 34$, mean = 3257, 95% CI = 1918–5531) was reduced by more than half compared to the single strain infection group (figure 2; $n = 67$, mean = 7109, 95% CI = 4879–10 368). For focal strain NE4049, the nymphal spirochaete load in the co-infection group ($n = 51$, mean = 1929, 95% CI = 1252–2972) was reduced by almost half compared to the single strain infection group (figure 2; $n = 67$, mean = 3497, 95% CI = 2396–5094). The parameter estimates of the LME model also show a 50% reduction in the nymphal spirochaete load of the focal strain when the mouse was co-infected with another strain.

For the 62 nymphs that were co-infected with both strains, the mean spirochaete load of Fin-Jyv-A3 (mean = 3286, 95% CI = 2217–4872) was significantly higher than that of NE4049 (mean = 1543, 95% CI = 1041–2287; LME LLR:

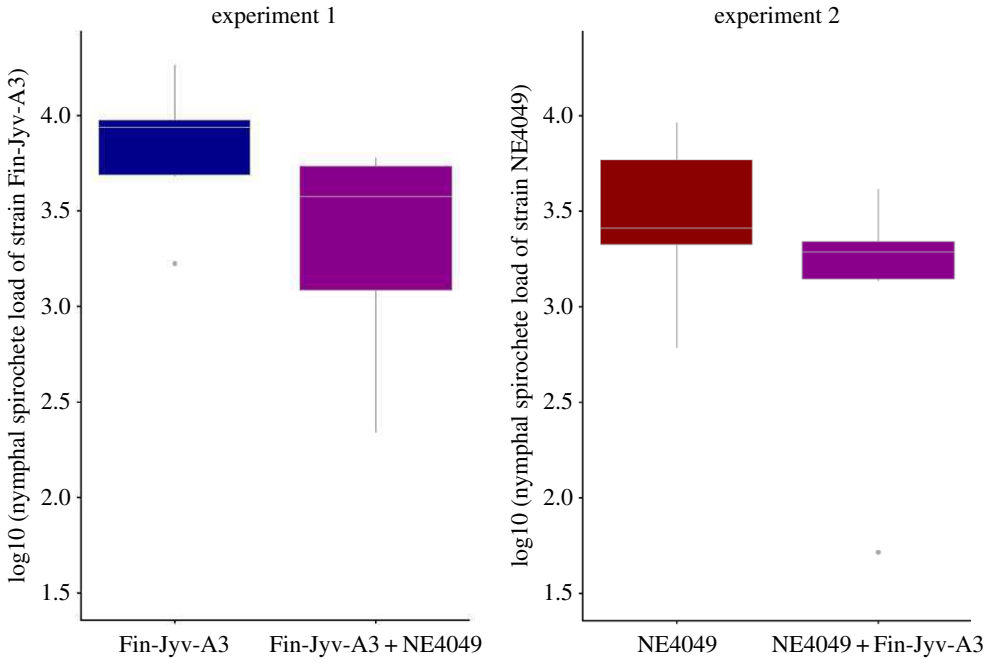


**Figure 2.** Co-infection reduces the spirochaete load in *I. ricinus* nymphs for two strains of *B. afzelii*. In experiments 1 and 2, the focal *B. afzelii* strains are Fin-Jyv-A3 and NE4049, respectively. Experiment 1 shows that the nymphal spirochaete load of strain Fin-Jyv-A3 is reduced by 50% in the presence of strain NE4049. Experiment 2 shows that the nymphal spirochaete load of strain NE4049 is reduced by 50% in the presence of strain Fin-Jyv-A3. Each data point represents the mean for a single mouse ($n = 33$ mice). Shown are the medians (grey line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (solid circles). (Online version in colour.)

$p = 0.0002$). Thus in co-infected ticks, strain Fin-Jyv-A3 was the superior competitor because it had a higher spirochaete load than strain NE4049.

## 5. Discussion

Our study found that the competitive ability of each strain differed depending on the host type in the life cycle of this important tick-borne pathogen. In co-infected mice, strain NE4049 was the superior competitor because it had higher host-to-tick transmission than strain Fin-Jyv-A3. In contrast, in co-infected ticks, strain Fin-Jyv-A3 was the superior competitor because its spirochaete load was higher than strain NE4049. To our knowledge, this study is the first demonstration that in multi-strain pathogens with a vector-borne life cycle, the winner of inter-strain competition in the vertebrate host can be the loser of inter-strain competition in the arthropod vector.

One of the central questions in ecology is to understand the factors that allow a community of species (or strains) to persist over time [5,49–52]. We and others have previously shown that a dozen strains of *B. afzelii* can coexist at the spatial scale of a soccer field [41,42,44,53]. Two independent long-term studies on *B. afzelii* in ticks and reservoir hosts have shown that the community of strains is stable over a time period of at least a decade [53,54]. Thus, the central question is how a dozen strains of *B. afzelii* can persist in the same local Lyme disease system [42,44,53]. A general explanation for coexistence is the presence of trade-offs where the competitive hierarchy between genotypes is reversed between different states [14]. Below, we give three examples of how the competitive hierarchy could be reversed between two or more strains. First, trade-off between performance when alone versus co-infection; for example, in single infections, strain Fin-Jyv-A3 had higher host-to-tick transmission than strain NE4049, but in mixed infections the relationship was reversed (figure 1). Second, trade-off between different host types in the life cycle; for example, strain NE4049 outcompeted strain Fin-Jyv-A3 in the rodent host but the relationship was reversed in the tick vector. Third, intransitive competition relationships between strains; for example, strain A beats B, B beats C and C beats A. In summary, there are different trade-offs that could stabilize a community of a dozen *B. afzelii* strains coexisting in the same local Lyme disease system.

To our knowledge, this study is also one of the first experimental demonstrations that strains of a vector-borne pathogen experience competitive interactions inside the arthropod vector. Our study adds to a growing literature on interactions between strains of vector-borne pathogens within their arthropod vectors [33,35]. A study on mixed infections of rodent malaria parasites in mosquitoes found that malaria strains had a greater chance to establish infection and reach a high density if the mosquito was already infected by another strain, which is an example of cooperation or facilitation [33]. A study on the tick-borne bacterium *Francisella novicida* found that a wild-type strain excluded other strains from establishing infection in the tick vector [35]. Our results are in agreement with our previous study, which found indirect evidence for competition between strains of *B. afzelii* in wild *I. ricinus* nymphal ticks [44]. That study found that the number of spirochaetes per strain decreased as the strain richness increased inside the nymphs [44]. Negative associations between the spirochaete abundances of some strains inside the nymphal tick were also shown [44]. The present study found a large effect size of inter-strain competition in the tick vector; co-infection in the tick reduced the spirochaete abundance of each strain by 50%. A critical question is whether competition between

strains in the nymph influences the strain-specific nymph-to-host transmission success.

Competition between strains in the tick vector could have important consequences if the observed reductions in spirochaete load influence strain-specific nymph-to-host transmission. During the nymphal blood meal, spirochaetes migrate from the midgut to the salivary glands [55,56], and the nymph inoculates about 100 spirochaetes into the vertebrate host [57]. This small inoculum size suggests that competition between strains in the nymphal tick could influence the strain-specific nymph-to-host transmission success. We have recently shown that *B. afzelii* strains that establish high spirochaete loads in wild *I. ricinus* nymphs are more common in nature [44]. This observation suggests that strains with high nymphal spirochaete loads are more competitive and have higher nymph-to-host transmission success [44]. Previous studies on other vector-borne pathogens have shown that the arthropod vector can limit the genetic diversity of strains that is transmitted to the vertebrate host [35,58]. For example, Rego *et al.* [58] used a set of genetically tagged clones of *B. burgdorferi sensu stricto* (ss) to show that co-infected ticks transmit a subset of clones to the rodent host. This result shows that the arthropod vector can act as a genetic bottleneck when they transmit mixed infections to the vertebrate host. The vector bottleneck would be even more important, if some strains are better than others at achieving vector-to-host transmission from co-infected vectors.

Competition between microbial species or strains can occur by three different mechanisms: interference, exploitation and apparent competition [4,59–62]. In interference competition, microbes produce toxic substances that weaken the performance (transmission, growth, reproduction) of other species or strains [63–65]. This mechanism is unlikely because *B. burgdorferi* sl does not produce toxic substances [66]. Exploitation competition happens when pathogen strains compete over limited host resources such as nutrients or space [8,67]. For example, many species of free-living and pathogenic bacteria compete over iron [68,69]. A recent theoretical study of the microbiome found that rapid population expansion of bacteria followed by extrinsic resource limitation by the host exacerbates competitive interactions and enhances community stability [52]. This condition is likely to be met in ixodid ticks where a single resource pulse (the blood meal) is followed by a rapid expansion of the microbial community [70–72]. Others and we have shown that following the larval blood meal, the population of *B. burgdorferi* sl expands rapidly from an initial inoculum of about 100 bacteria to a spirochaete population size inside the nymph that ranges from 2000 to 32 000 cells [73–76]. We have also shown that the spirochaete loads in the nymphs decrease dramatically over time, further suggesting that the resources inside the nymphal midgut are limiting [74]. Thus, exploitation competition over limited resources probably underlies the competitive interactions between the two strains of *B. afzelii* observed in this study. Finally, in apparent competition, the host immune response triggered by one strain affects the fitness of another strain [31,77]. Ticks contain immune defences such as antimicrobial peptides and phagocytic cells [78–81]. The observation that the total spirochaete load inside the nymph is low, suggests that the tick immune system restricts the spirochaete population inside the nymphal midgut to certain limits.

Our study found direct evidence for competitive interactions between strains of *B. afzelii* in the rodent reservoir host. This result is in agreement with other studies on the North American Lyme disease system of *B. burgdorferi* ss, *I. scapularis* ticks and *Peromyscus leucopus* mice [9,45] that have shown that co-infection reduces host-to-tick transmission success. In addition, we found evidence for asymmetric competition: co-infection reduced host-to-tick transmission of strain Fin-Jyv-A3 by approximately 50%, but had no effect on the host-to-tick transmission of strain NE4049. This result is not novel, as one of the studies on *B. burgdorferi* ss also found evidence of asymmetric competition [9]. If we include the four mice in the co-infected group that became infected with strain Fin-Jyv-A3 alone, competition significantly reduced host-to-tick transmission by approximately 30% in both strains (see electronic supplementary material for details). In summary, there is strong evidence that co-infection of strains of *B. burgdorferi* sl in the rodent host reduces host-to-tick transmission success.

Competitive exclusion occurs when one pathogen strain prevents another strain from establishing infection in the host [11–13]. In the present study, four of the 20 mice in the co-infection treatment became infected with strain Fin-Jyv-A3 but not strain NE4049. This result suggests competitive exclusion: strain Fin-Jyv-A3 prevented strain NE4049 from establishing infection in the host. There is some evidence that competitive exclusion is important in the field [29]. A field study on mixed strain infections of *B. afzelii* in bank voles found that strains carrying genetically similar *ospC* alleles were less likely to co-occur in the same host, a pattern that is consistent with competitive exclusion [29]. We have shown that the majority of *I. ricinus* nymphs carry mixed strain infections [42,44], which means that simultaneous exposure to multiple strains is common in populations of wild reservoir hosts. Thus our observation that competitive exclusion occurred in 20.0% of mice simultaneously exposed to two strains, suggests that this phenomenon could be important in the field.

In nature, *Ixodes* nymphs are frequently infected with multiple strains of a given species of *B. burgdorferi* sl [40–44]. Our work on a wild population of *I. ricinus* in Neuchâtel, Switzerland found that approximately 80% of nymphs infected with *B. afzelii* carried more than one strain [42,44]. In the present study, the rodents were infected with a maximum of two strains whereas in the field, rodents are often infected with more than two strains [10,29,54]. These differences in strain richness in the rodent host explain why the percentage of co-infected nymphs in our experiment (43.7%) was lower than what we have observed in nature. Our results are in agreement with a recent experimental study using the North American Lyme disease system, which found that 24.5% of nymphs that had fed as larvae on co-infected mice acquired both strains [9]. A field study in North America also showed that larval *I. scapularis* ticks that feed on wild reservoir hosts often acquire multiple strain infections [40]. A remaining question is why some nymphs acquire a subset of strains present in the vertebrate host whereas other nymphs acquire the complete set of strains?

In the present study, competition between strains of *B. afzelii* was shown in both the rodent host and the tick vector. Competition in the rodent host reduced host-to-tick transmission of strain Fin-Jyv-A3 but not strain NE4049. Competition in the tick vector reduced the bacterial abundance of both strains by 50%, but strain Fin-Jyv-A3 had a higher abundance than strain NE4049. Thus, strain NE4049 was the superior competitor in the rodent host, whereas

strain Fin-Jyv-A3 was the superior competitor in the tick vector. Future studies should investigate whether inter-strain competition in the tick has important consequences for the strain-specific tick-to-host transmission success.

Ethics. The commission that is part of the 'Service de la Consommation et des Affaires Vétérinaires (SCAV)' of Canton Vaud, Switzerland evaluated and approved the ethics of this study. The Veterinary Service of the Canton of Neuchâtel, Switzerland issued the animal experimentation permit used in this study (NE04/2016).

Data accessibility. All data and code used in this study are available in the electronic supplementary material.

Authors' contributions. D.G. and M.J.V. conceived and designed the study. D.G. and A.S. conducted the experiment and performed the molecular work. A.S., A.G.-C. and J.D. helped develop the *ospC*-specific qPCR. J.D. did 454-sequencing on isolates Fin-Jyv-A3 and NE4049 to confirm their purity. C.C. isolated Fin-Jyv-A3 and created the nymphs infected with this isolate. O.R. helped with the experimental infections. D.G. conducted the statistical analyses. D.G. and M.J.V. wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests. The authors declare no competing interests.

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# *Borrelia afzelii* alters reproductive success in a rodent host

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The impact of a pathogen on the fitness and behaviour of its natural host depends upon the host–parasite relationship in a given set of environmental conditions. Here, we experimentally investigated the effects of *Borrelia afzelii*, one of the aetiological agents of Lyme disease in humans, on the fitness of its natural rodent host, the bank vole (*Myodes glareolus*), in semi-natural conditions with two contrasting host population densities. Our results show that *B. afzelii* can modify the reproductive success and spacing behaviour of its rodent host, whereas host survival was not affected. Infection impaired the breeding probability of large bank voles. Reproduction was hastened in infected females without alteration of the offspring size at birth. At low density, infected males produced fewer offspring, fertilized fewer females and had lower mobility than uninfected individuals. Meanwhile, the infection did not affect the proportion of offspring produced or the proportion of mating partner in female bank voles. Our study is the first to show that *B. afzelii* infection alters the reproductive success of the natural host. The effects observed could reflect the sickness behaviour due to the infection or they could be a consequence of a manipulation of the host behaviour by the bacteria.

## 1. Introduction

The impact of pathogens on the physiology, behaviour and fitness of their natural hosts is a key determinant for the coevolution between the pathogen and the host [1–4]. Identifying the effect of a pathogen on all components of host fitness is also essential for predicting the population dynamics of a host–pathogen association and is fundamental for anticipating zoonotic outbreaks [5–8]. However, the study of the impact of parasites on their natural hosts often focuses on host survival [3,9–11], despite the recognition that host reproduction is an important component of host fitness [12–14]. Indeed, subtle effects of an endemic pathogen on the reproduction of its natural host can influence the population dynamics of the wild host [15,16] and ultimately the population dynamics of the pathogen [17].

Numerous studies have shown that pathogen virulence depends on ecological factors such as temperature and nutrition [18–21]. Another important ecological factor is host population density because it generates intra-specific competition for limited resources such as space, food and mating partners [22–24]. High host density is, therefore, expected to exacerbate pathogen virulence. Fluctuations in population density are typical in many small mammal species such as rodents [25]. However, experimental studies on density-dependent costs of infection in rodents are rare because it is often difficult to manipulate host density in an ecologically relevant way (but see [10,26,27]).

Spirochaete bacteria belonging to the *Borrelia burgdorferi sensu lato* (*s.l.*) complex cause Lyme borreliosis in humans, which is the most common vector-borne disease in the Northern Hemisphere [28,29]. *Borrelia afzelii*, which is transmitted by *Ixodes* ticks and hosted by rodents, is the most common aetiological agent of human Lyme borreliosis in Europe [28,30]. While Lyme borreliosis causes serious morbidity in humans [31,32], there is currently no clear evidence that *B. burgdorferi s.l.* reduces the fitness of the rodent or avian reservoir hosts [6,9,33–36]. However, most studies that have investigated the virulence of *B. burgdorferi s.l.* pathogens were correlational and focused on host survival and, to date, the potential effects on host reproductive success have been ignored (but see [37,38] for physiological cost and effect on host behaviour, respectively).

We conducted a field experiment to test whether *B. afzelii* reduces the survival and reproductive success of its rodent host, the bank vole (*Myodes glareolus*). Rodent populations are often strongly influenced by density-dependent effects [25]. We, therefore, hypothesized that the detrimental effects of *B. afzelii* infection on the fitness of bank voles would be more pronounced at high population density. Here, we show that while *B. afzelii* did not affect the host survival, the infection impaired the reproduction of large bank voles, and unexpectedly, that male bank voles had lower reproductive performances at low population density.

## 2. Material and methods

### (a) Experimental design

The schedule of the experimental procedure is shown in electronic supplementary material, figure S1, and all methods are detailed in the electronic supplementary material. Male and female bank voles (*M. glareolus*) from the laboratory colony at the University of Jyväskylä were measured and assigned to either the *B. afzelii* infection group (injected with a local strain of *B. afzelii*) or the uninfected control group (injected with phosphate-buffered saline (PBS)). All infected and uninfected voles (total of 136 individuals, 68 females and 68 males) were released in 12 large outdoor vegetated enclosures (each 0.2 ha) that were assigned to 'high' density (16 individuals per enclosure, 8 females and 8 males, half of each sex infected, 5 enclosures) and 'low' density (8 individuals per enclosure, 4 females and 4 males, half of each sex infected, 7 enclosures) treatments. In the enclosures, the bank voles could move and reproduce freely for 18 days, which is the minimum gestation length in females. During this period, spacing behaviour was monitored using live trapping. At the end of this period, all trapped individuals were taken to the laboratory for measurements and monitoring of parturition. Male reproductive success was determined by paternity analyses conducted on the offspring born in the laboratory.

### (b) Measurements

Before the enclosure period, individuals were weighed, and the head width was measured with a calliper ruler (Electronic Digital Caliper, Scala). These measurements were taken into account when experimental animals were assigned to treatments and enclosures. An ear tissue sample was taken for paternity analysis. A blood sample was taken for an ELISA (enzyme-linked immunosorbent assay) targeting *B. burgdorferi s.l.*-specific IgG antibodies [39] (electronic supplementary material).

After the enclosure period, the body measurements and blood sampling were carried out as described above. Males were processed shortly after the trapping day, gravid females were processed after parturition, and females that were not gravid were processed at the end of the experiment. Pups were measured (body mass and head width) within 24 h of parturition. All measurements were performed blind regarding the infection treatment and density treatment.

### (c) Statistical analysis

All statistical analyses were carried out using the statistical software R v. 3.1.1 [40]. Survival of bank voles in the enclosures and individual breeding probability are binary variables. For survival, individuals were assigned 1 or 0 depending on whether they were trapped at the end of the experiment or not. For the assessment of the breeding probability, individuals were assigned 0 or 1 depending on whether their number of produced offspring was zero or at least one. Moreover, two response variables of reproductive success were calculated: (1) 'relative number of offspring' is the proportion of offspring produced in an enclosure by a given individual, (2) 'relative number of partners' is the proportion of partners with which a given individual produced offspring. Eventually, space trapping data allowed calculation of two different home range variables: home range perimeter (m) and home range surface ($m^2$) (electronic supplementary material, table S1).

In the statistical analyses, the injection (*B. afzelii* versus PBS) was used to define 'infection' treatment (infected versus uninfected), and the population density in the enclosure defined the 'density' treatment ('low' versus 'high'). The explanatory variables of the full models always included the two experimentally manipulated factors, i.e. the infection treatment and the population density in the enclosure, sex, body mass before injection (BM) and relevant two- and three-way interactions. Enclosure ID was included as a random effect in all models. Three-way interactions involving vole sex were expected in models assessing bank vole reproductive success because the drivers of reproductive success differ between male and female bank voles [41–44]. When these three-way interactions were significant in the full model (see electronic supplementary material, table S1), separate analyses were conducted for males and females to ease the interpretation of the interactions. Otherwise, reductions of the full models were carried out starting from the non-significant interactions (see electronic supplementary material).

For gravid females, the parturition delay was calculated as the difference in the number of days between the date the first litter was observed and the parturition date for the other pregnant females. This variable was modelled as a function of infection, density, BM and the interaction infection × density. Moreover, offspring body mass at birth and head width at birth were modelled as a function of the infection status of the mother and father, density and all their two- and three-way interactions. Offspring sex and litter size were included as covariates. Enclosure ID, mother ID and father ID were included as random effects.

To analyse the data, we used generalized linear mixed models (GLMMs) with an error distribution that was either normal (home range perimeter, home range surface, body mass and head width of offspring), binomial (survival, breeding probability and variables describing reproductive success: relative number of offspring and relative number of partners) or negative binomial (female parturition delay).

## 3. Results

Out of the 68 female and 68 male bank voles released into the enclosures at the beginning of the experiment, 48 females and 30 males (one of which was found dead in the trap) were recovered, and the remaining 58 individuals were considered as dead. Of these 58 individuals, 56 were never observed

**Table 1.** Selected final models for reproductive success and spacing behaviour in bank voles. BM, centred value of body mass before injection; HW, centred value of head width before injection; low, low population density; inf., infected bank voles; $\sigma 2$ is the variance attributable to random effect; s.d., standard deviation; s.e. standard error. Significant effects are shown in bold.

| | response variable | predictors | estimates (s.e.) | *t*-value | *z*-value | *p*-value | random effect (enclosure) |
|---|---|---|---|---|---|---|---|
| males and females | breeding probability | intercept | 1.40 (0.39) | | 3.60 | **<0.01** | $\sigma 2$: 0.00 |
| | | infection (inf.) | −0.53 (0.38) | | −1.39 | 0.16 | s.d.: 0.00 |
| | | density (low) | −0.93 (0.38) | | −2.42 | **0.02** | |
| | | sex (male) | −0.42 (0.38) | | −1.10 | 0.27 | |
| | | BM | −0.11 (0.15) | | −0.71 | 0.48 | |
| | | infection: BM | −0.33 (0.17) | | −2.00 | **0.05** | |
| | | sex: BM | 0.40 (0.17) | | 2.27 | **0.02** | |
| males | relative number of offspring | intercept | −2.08 (0.31) | −6.80 | | **<0.01** | $\sigma 2$: $1.19 \times 10^{-9}$ |
| | | infection (inf.) | 0.20 (0.42) | 0.493.79 | | 0.63 | s.d.: $3.46 \times 10^{-5}$ |
| | | density (low) | 1.76 (0.47) | 2.31 | | **<0.01** | |
| | | BM | 0.16 (0.07) | −3.06 | | **0.03** | |
| | | infection: density | −2.72 (0.89) | | | **<0.01** | |
| | relative number of partners | intercept | −1.52 (0.28) | −5.51 | | **<0.01** | $\sigma 2$: $1.11 \times 10^{-9}$ |
| | | infection (inf.) | 0.07 (0.39) | 0.18 | | 0.86 | s.d.: $3.33 \times 10^{-5}$ |
| | | density (low) | 1.24 (0.48) | 2.59 | | **0.03** | |
| | | BM | 0.15 (0.06) | 2.31 | | **0.03** | |
| | | infection: density | −1.95 (0.82) | −2.38 | | **0.02** | |
| | home range surface | intercept | 378.18 (78.24) | 4.83 | | **<0.01** | $\sigma 2$: 0.00 |
| | | infection (inf.) | 146.38 (107.54) | 1.36 | | 0.19 | s.d.: 0.00 |
| | | density (low) | 429.71 (126.16) | **3.41** | | **<0.01** | |
| | | infection: density | −594.50 (176.51) | **3.37** | | **<0.01** | |
| females | parturition delay | intercept | 1.70 (0.18) | | 9.49 | **<0.01** | $\sigma 2$: 0.00 |
| | | infection (inf.) | −0.75 (0.25) | | −3.00 | **<0.01** | s.d.: 0.00 |
| | | density (low) | −0.27 (0.26) | | −1.04 | 0.30 | |

during the 14 trapping occasions and 2 were not observed during the six last trapping occasions. As we did not observe any introduction of unmarked wild bank voles in the enclosures, and all trapped animals were found in their original enclosure, we assume that missing animals died, rather than escaped. Of the 78 captured individuals, 39 were from the *B. afzelii* infection group (24 females, 15 males), and 39 were from the control group (24 females, 15 males, including the individual found dead in the trap). There was no effect of *B. afzelii* infection or population density on the survival of bank voles (GLMM: $p > 0.35$, electronic supplementary material, table S4), but females survived better than male bank voles (GLMM: $p < 0.01$, electronic supplementary material, table S4).

## (a) *Borrelia afzelii* infection reduces the breeding probability of large bank voles

Based on the paternity test, 39 of 68 males reproduced during the study (18 of the 30 males that were trapped and 21 of the 38 males that were not trapped). For the analysis of reproductive success, all males were included, regardless of whether they were trapped or not at the end of the study. Out of the 48 captured females, 45 gave birth in the laboratory. We found that the effect of *B. afzelii* infection on bank vole breeding probability was dependent on body size: among small individuals, there was no difference in the breeding probability between infected and uninfected animals. However, uninfected individuals had significantly higher breeding probability than *B. afzelii*-infected individuals among large bank voles (GLMM: body mass × infection, $p = 0.05$, table 1 and figure 1; electronic supplementary material, table S4 and figure S4).

## (b) *Borrelia afzelii* infection reduces male reproductive success at low density

Reproductive success was further explored as the analysis of the relative number of produced offspring and the relative number of partners. The three-way interaction infection × density × sex was significant for the relative number of offspring (GLMM: $p = 0.02$, electronic supplementary material, table S1) and the relative number of partners (GLMM: $p = 0.03$, electronic supplementary material, table S1), providing

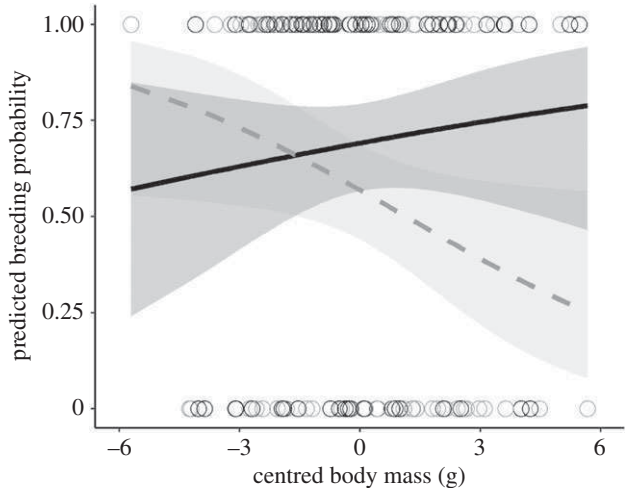


**Figure 1.** The estimated probability of reproduction for a bank vole (±95% CI) depends on their *B. afzelii* infection treatment (uninfected individuals in solid black, $N = 68$, infected individuals in dashed grey, $N = 68$) and their body size (measured as the body mass before injection). In small bank voles, there is no effect of *B. afzelii* infection on breeding probability. In large bank voles, by contrast, uninfected individuals have higher breeding probability than infected individuals. The observed values are shown with open circles.

evidence that infection and breeding density affected these components of reproductive success differently in males and females. In male bank voles, the relative numbers of offspring and partners were associated with *B. afzelii* infection status, but the effect differed between the population density treatments (table 1 and figure 2; electronic supplementary material, table S2). At low density, uninfected control males sired a higher relative number of offspring (0.42) and fertilized a higher relative number of females (0.43) than *B. afzelii*-infected males (0.05 offspring sired and 0.10 female fertilized). Conversely, in high density there was no effect of the infection treatment: the relative numbers of offspring sired by uninfected and infected males were 0.13 and 0.11, and the relative numbers of females fertilized by uninfected and infected males were 0.18 and 0.19, respectively (GLMM: $p = 0.004$ and $p = 0.02$, table 1 and figure 2). For female bank voles, the relative numbers of offspring and partners were not affected by the infection (the proportion of offspring produced by uninfected and infected females was 0.27 and 0.26, respectively; GLMM: $p = 0.79$, electronic supplementary material, table S3). As expected, population density influenced the relative number of offspring produced by a female bank vole (relative number of offspring produced by females from low- and high-density enclosures was 0.41 and 0.18, respectively; GLMM: estimate on the logit scale (s.e.): density = 0.85 (0.25), $p < 0.001$, electronic supplementary material, table S3).

### (c) *Borrelia afzelii* infection reduces male home range at low density

We found evidence that male and female bank voles differ in their spacing behaviour as the three-way interaction infection × density × sex was significant for home range surface and home range perimeter (GLMM: $p < 0.01$, $p = 0.03$, respectively, electronic supplementary material, table S1). For the uninfected male bank voles, the home range surface was significantly larger in the low-density enclosures (808 $m^2$) compared with the high-density enclosures (378 $m^2$) (LMM: $p = 0.003$, table 1 and figure 3). By contrast, the home range surface of the *B. afzelii*-infected male bank voles was not significantly different between the low-density (360 $m^2$) and high-density (524 $m^2$) enclosures (table 1 and figure 3). Female home range surface and perimeter were not affected by the infection or the density treatments (electronic supplementary material, table S3).

### (d) Infection caused early reproduction in female bank voles

Of the 48 females captured from the enclosures, 45 were gravid and produced a total of 226 pups, with a mean number of 5 pups per female (range: 1–7). *Borrelia afzelii*-infected females reproduced on average 3 days earlier than uninfected control females (GLMM: $p = 0.003$, figure 4 and table 1) and this effect was independent of the population density (GLMM: $p = 0.30$, table 1). The size of the offspring at birth was not affected by the infection treatment of the mother or father or population density (LMM for all variables: $p > 0.05$, electronic supplementary material, table S6).

## 4. Discussion

We examined the hypothesis that *B. afzelii* infection reduces the reproductive success of the rodent host and we tested the density-dependence of this effect. We found that *B. afzelii* infection had density-dependent and statistically differing effects on the relative numbers of partners and offspring of male and female bank voles. In males, infected individuals kept at low population density sired a lower proportion of offspring, fertilized a lower proportion of females and displayed smaller home range surface than uninfected males (figures 2 and 3). In females, by contrast, *B. afzelii* infection did not affect the relative offspring number, relative number of partners and home range surface, but infected individuals gave birth approximately 3 days earlier than uninfected individuals. The offspring size (head width and body mass) was not affected by the mother's infection status (figure 4; electronic supplementary material, table S6). Finally, in both sexes, infection reduced the breeding probability of large individuals but did not affect their survival (figure 1; electronic supplementary material, table S4 and table S5).

Previous studies found no evidence that infection with *B. burgdorferi s.l.* reduces the fitness of natural hosts; however, most of them were correlational or focused on another genospecies than *B. afzelii*. For instance, capture–mark–recapture (CMR) studies on wild populations of the white-footed mouse (*Peromyscus leucopus*) or the black-legged kittiwake found no effect of infection with *B. burgdorferi s.l.* on the survival of these hosts [9,34,35]. Similarly, we found that infection with *B. afzelii* did not impair survival of the bank vole. Another study on white-footed mice found no effect of *B. burgdorferi sensu stricto* on the wheel-running behaviour over the six weeks following experimental infection [6]. In our study, by contrast, the effect of infection on home range size may result from altered running behaviour. A recent study reports a trend in increased foraging behaviour in white-footed mice treated with an anti-*B. burgdorferi* vaccine compared with sham-treated individuals, suggesting similarly to our finding, a wider ranging behaviour in individuals with low or with no

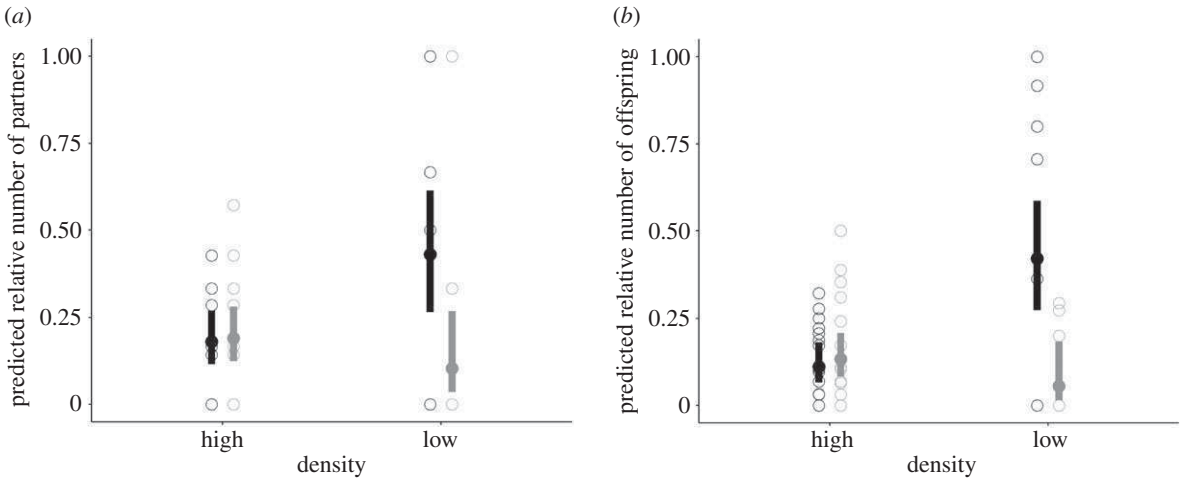


**Figure 2.** The estimated reproductive success of male bank voles depends on the interaction between *B. afzelii* infection (uninfected individuals in black, $N = 34$, infected individuals in grey, $N = 34$) and population density. (*a*) Predicted proportion of females successfully fertilized by a male bank vole ($\pm$95% CI) as a function of infection and density. (*b*) Predicted proportion of offspring sired by a male bank vole ($\pm$95% CI) as a function of infection and density (electronic supplementary material, table S2). The observed values are shown with open circles.

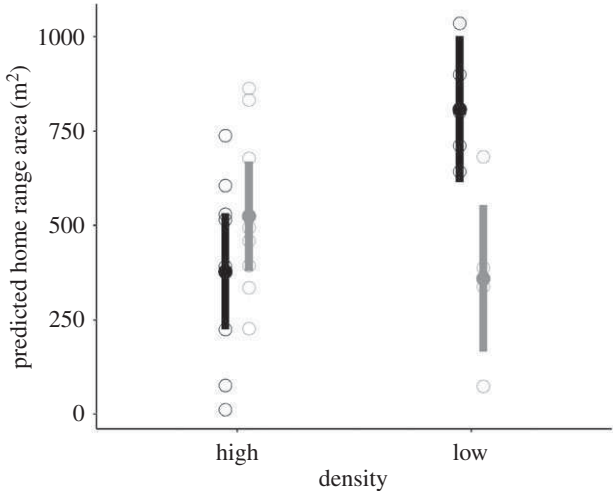


**Figure 3.** The estimated home range (in $m^2$) of male bank voles in the enclosures ($\pm$95% CI) depends on the interaction between *B. afzelii* infection (uninfected individuals in black, $N = 13$, infected individuals in grey, $N = 14$) and population density. At low population density, uninfected males have much larger home ranges than *B. afzelii*-infected males. At high population density, infection with *B. afzelii* does not affect the home range of male bank voles (electronic supplementary material, table S2). The observed values are shown with open circles.

infection burden [38]. To our knowledge, our study is the first to address the effect of *B. afzelii* infection on host reproduction experimentally under field conditions. Studying the effects of infections on host reproduction is challenging in wild rodent populations, and reproduction is often a latent variable inferred from observed variables. Our experimental setting allows controlling for several sources of variation and confounding factors (e.g. age of the host), and we were able to estimate the reproductive success reliably.

The experimental infection was performed by peritoneal injection of the bacteria rather than the natural infection route, which involves *Ixodes* ticks. The infection dose and route were based on the literature [33,36,45–47]. The intraperitoneal route was chosen as it has been shown to give more widely disseminated infection than the subcutaneous route [48]. The use of injection instead of the natural transmission route can be debatable, e.g. due to the lack of tick salivary compounds that enhance the infectivity of *B. burgdorferi s.l.* [49,50]. Molecules present in tick saliva promote the infection by manipulating or depressing the immune system (e.g. salps) [51]. The injection of *B. burgdorferi s.l.* with tick salivary gland extract led to higher infection success with higher bacterial dissemination, so-called saliva-assisted transmission [51,52]. The lack of these molecules could lead to misestimation of the effects of the infection on the host. However, the injection allows the experimenter to control the bacterial dose, and it eliminates the variation linked to the tick vectorial capacity [53], hence ensuring a controlled exposure of the study animals to the bacteria. We acknowledge that needle inoculation mimics only grossly the infection via tick bite. However, we can expect any observed effect to be caused by the *B. afzelii* infection given our controlled experimental conditions.

The demonstration of fitness-related costs caused by *B. burgdorferi s.l.* infection is important for understanding the evolution of resistance in natural hosts. Recent field studies on the bank vole suggested that polymorphism at the Toll-like receptor 2 (*TLR2*) gene, a pathogen recognition receptor of the innate immune system, was associated with variation in susceptibility to *B. afzelii* [54,55]. The prevalence of *B. afzelii* infection in bank voles that were homozygous for the C2 resistance allele was half that of the bank voles that were homozygous for the C1 susceptibility allele [54]. A study of the *TLR2* polymorphism in bank vole populations across Europe found that the resistance allele against *B. afzelii* (C2) was more common in countries with a high incidence of human Lyme disease [56]. This result led Tschirren to suggest that *B. afzelii* was driving the evolution of the resistance allele at the *TLR2* gene in European bank vole populations. However, without clear evidence of reduced fitness in infected rodents, the mechanism of selection was unclear. Our demonstration that infection with *B. afzelii* reduces male reproductive success supports the hypothesis that this pathogen could be driving selection on the *TLR2* gene in bank vole populations.

The effect of the infection on the relative number of offspring sired and the relative number of females fertilized

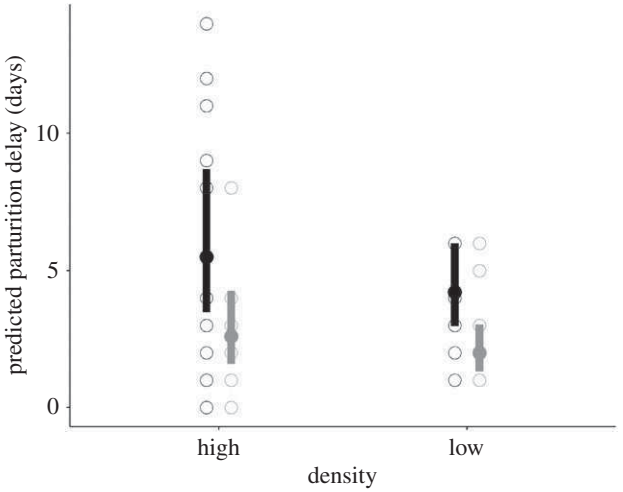


**Figure 4.** Estimated parturition delay in female bank voles (±95% CI) depends on *B. afzelii* infection (uninfected individuals in black, $N = 23$; infected individuals in grey, $N = 22$) and population density (table 1 and electronic supplementary material S4). The observed values are shown with open circles.

by a given male bank vole was density-dependent. In the low-density populations, uninfected control males fertilized more females and fathered more offspring compared with the infected males and males kept in high population density (figure 2). This result was counterintuitive, as we predicted that the negative effects of high population density, such as reduced *per capita* food availability, more aggressive interactions and potentially higher stress levels, would exacerbate the cost of *B. afzelii* infection [10,43,57–59]. Three hypotheses can explain this result. First, several studies have shown that the strength of male–male competition can vary with population density in a nonlinear fashion [see, for instance, 60–62]. For example, males can modify their reproductive strategy in high population density leading to lower rates of aggression and lower reproductive success [60,63]. Second, as estimates of the relative number of partners and the relative number of offspring were based on paternity tests, cryptic female choice (i.e. a female choice that occurs in the reproductive tract of the female, leading to fertilization bias in favour of specific males [64,65]) might have occurred. Thus, a density-dependent female cryptic choice favouring healthy males in low-density populations cannot be excluded. Finally, a spurious effect linked to the length of our experiment, which covers only one reproductive episode, cannot be ruled out [66].

In the low-density populations, uninfected control males had larger home range sizes than infected males whereas, in the high-density enclosures, there was no significant difference in the home range size between uninfected and infected male bank voles (figure 3). One possible explanation for this density-dependent home range reduction is that at high density, males may reduce their exploratory behaviour to avoid encountering other males and having to engage in aggressive male–male interactions. Moreover, at high density, with eight females available in the enclosure, the chance for a male to encounter a receptive female might be higher than in the low-density enclosure where only four females are available. Indeed, female bank voles are territorial and hyperdispersed [42,67]. Consequently, at low density, male bank voles may need to explore a larger home range to search for receptive females than at high density. As expected, the uninfected males had a larger home range in low population density, whereas the infected males presumably allocated resources to their immune response instead of explorative behaviour. In contrast, female bank voles had a smaller home range size than males, which was not affected by population density, reflecting the territorial behaviour of females especially, during late gestation when the space trapping took place [42,67,68].

We found that the cost of infection was more important in large bank voles, which are the most frequently infested with ticks and *B. burgdorferi s.l.* in nature [9,69,70]. Large infected individuals showed reduced reproductive success compared with large healthy individuals. Food resource is generally known to constrain reproduction and food addition has been shown to enhance reproductive success in similar outdoor enclosure setups [43,71,72]. These food constraints might have a more negative effect on the large individuals, which have greater energetic needs [73]. Infected large voles showed altered breeding probability regardless of the population density.

Infected females plastically modified their life history and reproduced approximately 3 days earlier than uninfected females without alteration of the size of the offspring at birth, i.e. without signs of premature birth (figure 4 and table 1; electronic supplementary material, table S3). In nature, reproducing females give birth to 1 or 2 litters per reproductive season [74], and most individuals live only one season. The biological importance of giving birth 3 days earlier is not clear, as concerns population dynamics. At the individual level, early reproduction can be a compensatory strategy if parasites reduce the reproductive success of the adult host later in life via morbidity, mortality or castration [75–77]. According to the terminal investment theory, individuals maximize their fitness by allocating resources to immediate reproduction when the prospects for future reproduction are reduced, for example by chronic infection [27,78–80]. It remains to be estimated whether *B. afzelii* impairs reproduction of female bank vole during the late stage of infection.

In summary, our study shows, for the first time, that the zoonotic pathogen *B. afzelii* can influence the reproductive success of its rodent host. The effect of the infection on the relative numbers of offspring and partners differed between male and female bank voles. Although large body size favoured reproduction in uninfected individuals, this size benefit disappeared if the individual was infected with *B. afzelii*. In males, infected individuals kept at low population density displayed smaller home range surface than uninfected males. Lower mobility can be a consequence of sickness behaviour due to the infection. On the other hand, predation risk by small carnivores generally increases with vole mobility [81]. By reducing home range size, infection with *B. afzelii* could lower the predation risk of male bank voles by small carnivores, enhancing at the same time its own fitness [82]. The hypothesis of manipulation of the rodent host by *B. afzelii* is yet to be explored.

Ethics. The Finnish Animal Experiment Board approved the trapping and handling methods used in this study under the authorizations ESAVI/3834/04.10.03/2011, ESAVI/7256/04.10.07/2014 and ESAVI/3457/04.10.07/2015.

Data accessibility. The dataset analysed during the current study is available in the JYX repository, http://urn.fi/URN:NBN:fi:jyu-201806133148 [83].

Authors' contributions. C.C., E.K., T.M., E.R.K. conceived the study. The fieldwork and laboratory work were carried out by all authors, with the helpers listed in the Acknowledgements. C.C., E.K., T.M., E.R.K., M.J.V. analysed the results, with input from the other authors. All authors contributed to the interpretation and critical revision. C.C. led the writing of the paper. All authors gave final approval for publication.

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