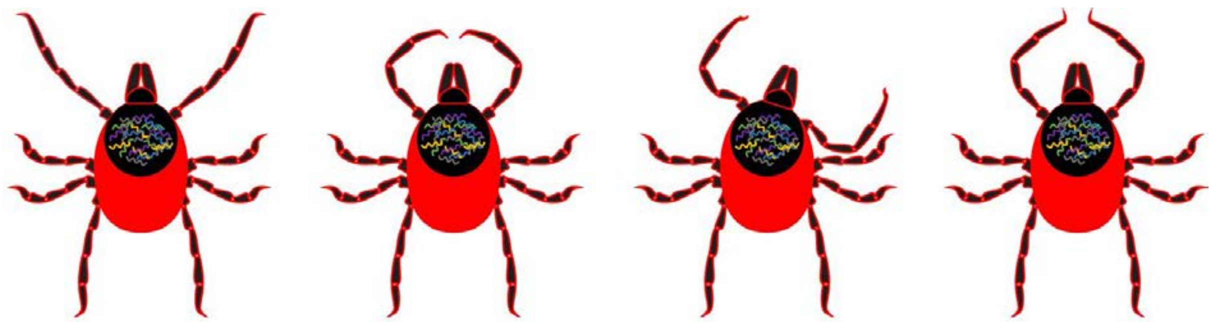
University of Neuchâtel – Faculty of Sciences

Laboratory of Ecology and Evolution of Parasites

Evolution of a local population of a multiple-strain pathogen in its vector



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"Evolution of a local population of a multiple-strain pathogen in its vector"

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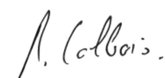

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1. Abstract

Mixed-strain infections are the rule rather than the exception in most infectious diseases, and have important implication for the ecology and evolution of pathogens. The presence of multiple strains results in competitive interactions that can have a strong impact on the population structure of the pathogen. In vector-borne diseases, most of the research on competition between pathogen strains has focused on the vertebrate host, and there is a lack of information about what happens inside the arthropod vector.

Lyme borreliosis is the most common vector-borne disease in the northern hemisphere. The causative agents are spirochete bacteria that belong to the group *Borrelia burgdorferi* sensu lato and that are transmitted among vertebrate host by hard ticks of the genus *Ixodes*. In Europe, the two most common species are *Borrelia afzelii* and *Borrelia garinii*, which are specialized on different classes of reservoir hosts: small mammals and birds, respectively. Each of these two *Borrelia* species can be further subdivided into genetically distinct strains. Mixed-strain infections are common in both the vertebrate host and the tick vector. Strains are often defined using the highly polymorphic *ospC* gene, which has a discrete pattern of genetic variation. A major *ospC* group is a cluster of *ospC* alleles that is more than 8% divergent in nucleotide sequence from other such clusters, whereas alleles within a major *ospC* group are less than 2% divergent from each other. The *Borrelia* species that have been studied to date contain about 20 different major *ospC* groups.

In the literature, there are currently two explanations for the maintenance of the *ospC* polymorphism: negative frequency-dependent selection (NFDS) or multiple host-as-niche polymorphism (MNP). NFDS states that rare alleles have a fitness advantage over common ones and should therefore invade the population. MNP states that the strains carrying the different major *ospC* groups are specialized on different host species, and that it is the host species richness that maintains the *ospC* diversity. We propose a third explanation that is

based on published theoretical models by Gupta and colleagues on how cross-reactive acquired immunity in the vertebrate host can drive the population structure of multiple-strain pathogens. This theory states that strong immune selection against immunodominant antigens will cause the pathogen strains to organize themselves into a set of unique serotypes that minimizes cross-reactive acquired immunity. These sets of antigenically distinct strains can remain stable over long periods of time and the frequency of each strain depends on its intrinsic fitness.

In a first study, we used 454-sequencing to characterize the *ospC* strain structure of *B. afzelii* and *B. garinii* in a local population of questing *Ixodes ricinus* nymphs over a period of 11 years. We also used estimates of the intrinsic fitness of six strains of *B. afzelii* from a previous experimental infection study that used laboratory mice.

We did not find any intermediately divergent alleles in the population, which suggested that cross-immunity from the vertebrate host prevents invasion by intermediate strains. In both *Borrelia* species the community of strains was stable over 11 years. Our laboratory estimates of fitness explained 63% of the variation in the frequencies between the different strains. Our results were consistent with the theory of Gupta and colleagues that explains how strong cross-reactive acquired immunity can structure the community of a multiple strain pathogen, but not with the NFDS or MNP theories.

In the second study, we used 454 sequencing and qPCR estimates of the total spirochete load to estimate the *ospC* strain-specific spirochete load per tick. We analysed questing nymphs infected by either *B. afzelii* or *B. garinii* that had been captured over a period of 3 years in the same location as the previous study.

We found that ticks had a fixed carrying capacity for spirochetes resulting in strong competition between strains. In *B. afzelii*, strains with the highest spirochete load in the nymphal tick were the most common strains in the tick population. In *B. garinii*, strains

whose spirochete load was least affected by competition with other strains were the most common strains in the tick population. In both *Borrelia* species, the spirochete load in the tick is an important life history trait. Competition between *Borrelia* strains in the tick vector plays a critical role in the community structure of this multiple-strain, tick-borne pathogen.

In both studies, we found that nymphs infected with a single *Borrelia* species often carried ‘exotic’ major *ospC* groups that belonged to other *Borrelia* species. This result was surprising and reveals the importance of using de novo clustering methods to analyze high-throughput sequencing data. We propose two explanations for the presence of these exotic major *ospC* groups. One explanation is frequent horizontal transfer of the *ospC* groups between the different *Borrelia* species. The other explanation is that co-infections with *B. afzelii* and *B. garinii* are an order of magnitude more common than previously suspected.

This work provides new insights on the evolution of multiple-strain *Borrelia* populations and on the importance of competitive interactions between pathogen strains in the arthropod vector.

Keywords: *Borrelia afzelii*, *Borrelia garinii*, *ospC*, *Ixodes ricinus*, Lyme borreliosis, mixed infection, vector-borne disease, evolutionary epidemiology.

2. Résumé

Dans la plupart des maladies infectieuses, les infections sont causées par différentes souches de la même espèce de pathogène. Ces infections multiples ont des implications importantes pour l'écologie et l'évolution de ces agents pathogènes. La présence de plusieurs souches au même endroit peut en effet conduire à des interactions compétitives qui peuvent avoir un impact fort sur la structure de la population de pathogènes. Dans les maladies à transmission vectorielle, la plupart des recherches sur les interactions entre les souches d'un pathogène se sont concentrées sur l'hôte vertébré, et il y a un manque d'information sur ce qui se passe à l'intérieur du vecteur.

La Borréliose de Lyme est la maladie vectorielle la plus courante dans l'hémisphère nord. Les agents pathogènes sont des bactéries spirochètes qui appartiennent au groupe *Borrelia burgdorferi* sensu lato et sont transmis entre hôtes vertébrés par les tiques dures du genre *Ixodes*. En Europe, les deux espèces les plus communes sont *Borrelia afzelii* et *Borrelia garinii*, qui sont spécialisés sur différentes classes d'hôtes réservoirs: les petits mammifères et les oiseaux. Chacune de ces deux espèces de *Borrelia* peut être subdivisées en différentes souches génétiquement distinctes. Les infections multiples causées par plusieurs souches sont communes à la fois chez l'hôte vertébré et chez la tique vectrice. Les souches sont souvent définies en utilisant le gène hautement polymorphe *ospC*, qui présente un motif de variation génétique particulier. En effet, un groupe majeur d'*ospC* est un groupe d'allèles d'*ospC* qui est plus de 8% différent dans sa séquence nucléotidique des autres groupes, alors que les allèles au sein d'un groupe majeur d'*ospC* sont moins de 2% différentes les unes des autres. Les espèces de *Borrelia* qui ont été étudiés à ce jour contiennent environ 20 différents groupes majeurs d'*ospC*.

Dans la littérature, il existe actuellement deux explications pour le maintien du polymorphisme sur le gène *ospC* : la sélection fréquence-dépendante négative (SFDN) ou le

polymorphisme de niches multiples (PNM). La SFDN prédit que les allèles rares ont une meilleure valeur adaptative que les plus communes et devraient donc envahir la population. La PNM prédit que les souches portant les différents groupes majeurs d'*ospC* sont spécialisées sur différentes espèces d'hôtes, et que c'est la richesse en espèces d'hôtes qui maintient la diversité d'*ospC*. Nous proposons une troisième explication qui est basée sur des modèles théoriques publiés par Gupta et ses collègues, sur la façon dont une forte immunité acquise croisée chez l'hôte vertébré peut structurer une communauté de multiples souches de pathogène. Cette théorie prédit qu'une forte sélection immunitaire contre des antigènes immuno-dominants obligera les souches pathogènes à s'organiser en un ensemble de sérotypes uniques qui minimisent l'immunité acquise croisée. Ces ensembles de souches distinctes au niveau antigénique peuvent rester stables pendant de longues périodes de temps et la fréquence de chaque souche dépend de sa valeur adaptative intrinsèque.

Dans une première expérience, nous avons utilisé le séquençage 454 afin de caractériser les communautés de souches *ospC* de *B. afzelii* et *B. garinii* dans une population locale de nymphes *Ixodes ricinus* en quête, pendant une période de 11 ans. Nous avons également utilisé des estimations de la valeur adaptative intrinsèque de six souches de *B. afzelii* provenant d'une étude précédente d'infection expérimentale de souris de laboratoire. Nous n'avons pas trouvé d'allèles divergents intermédiairement (entre 2 et 8%) dans la population, ce qui suggère que l'immunité acquise croisée de l'hôte vertébré empêche l'invasion de la population par des souches intermédiaires. Chez les deux espèces de *Borrelia* la communauté des souches était stable pendant 11 ans. Nos estimations expérimentales de la valeur adaptative expliquent 63% de la variation dans les fréquences entre les différentes souches. Nos résultats étaient cohérents avec la théorie de Gupta et ses collègues qui explique comment une forte immunité acquise croisée peut structurer une communauté de multiples souches de pathogènes, mais pas avec les théories SFDN ou PNM.

Dans la deuxième expérience, nous avons utilisé le séquençage 454 et des estimations de la charge en spirochète totale pour estimer la charge en spirochète spécifique de chaque souche *ospC* dans chaque tique. Nous avons analysé les nymphes en quête infectées soit par *B. afzelii*, soit par *B. garinii*, qui avaient été collectées pendant une période de 3 ans dans le même endroit que l'étude précédente.

Nous avons constaté que les tiques ont une capacité de charge fixe entraînant une forte compétition entre les souches. Chez *B. afzelii*, les souches à la charge en spirochètes la plus élevée dans la tique étaient les souches les plus courantes dans la population de tiques. Chez *B. garinii*, les souches dont la charge en spirochètes était la moins affectée par la compétition avec d'autres souches étaient les souches les plus courantes dans la population de tiques. Chez les deux espèces de *Borrelia*, la charge en spirochètes dans la tique est un important trait d'histoire de vie. La compétition entre les souches de *Borrelia* dans la tique vectrice joue un rôle essentiel dans la structuration de la communauté de ce pathogène.

Dans les deux études, nous avons constaté que les nymphes infectées par une seule espèce de *Borrelia* contenaient souvent des groupes majeurs d'*ospC* « exotiques » qui appartenaient à d'autres espèces de *Borrelia*. Ce résultat est surprenant et révèle l'importance de l'utilisation de méthodes de classification *de novo* pour analyser les données de séquençage à haut débit. Nous proposons deux explications à la présence de ces groupes majeurs d'*ospC* exotiques. Il peut y avoir des transferts horizontaux fréquents d'allèles d'*ospC* entre les différentes espèces de *Borrelia*, ou les co-infections avec *B. afzelii* et *B. garinii* sont d'un ordre de grandeur plus fréquent qu'on ne le pensait.

Ce travail apporte un nouvel éclairage sur l'évolution des communautés de souches multiples de *Borrelia* et sur l'importance des interactions compétitives entre les souches pathogènes dans l'arthropode vecteur.

Mots-clés : *Borrelia afzelii*, *Borrelia garinii*, *ospC*, *Ixodes ricinus*, maladie de Lyme, infections multiples, maladie vectorielles, épidémiologie évolutive

3. General Introduction

3.1. Mixed infections

Infections are frequently caused by multiple strains or genotypes of the same pathogen species (Read and Taylor 2001, Balmer and Tanner 2011). Mixed-strain infections create a complex challenge for the host immune system and can increase pathology and disease. In malaria for example, an increasing number of co-infecting strains is correlated with a higher disease risk (Zwetyenga et al. 1998, Basco and Ringwald 2007). From a public health perspective, mixed infections represent a major problem because they complicate the development of treatments and vaccines (Balmer and Tanner 2011, Johnson et al. 2015). Mixed infections are also of great interest to evolutionary biologists. For example theory suggests that they can select for more virulent pathogen genotypes (Van Valen 1973, Nowak and May 1994, May and Nowak 1995, de Roode et al. 2005, Schmid-Hempel 2011), but the contrary is also possible (Brown et al. 2002, Choisy and de Roode 2010, Staves and Knell 2010, Schmid-Hempel 2011). Pathogens that maintain a high density inside their host are more likely to be transmitted to the next host (or vector) and are less likely to be cleared by the host immune system. However, such virulent pathogens are also more likely to damage host tissues and reduce host fitness (Van Valen 1973, Nowak and May 1994, May and Nowak 1995, Schmid-Hempel 2011).

Mixed infections generally result in competitive interactions between pathogen strains (Read and Taylor 2001, Mideo 2009). These interactions are characterized as competition because the performance of a focal strain is reduced in the presence of other strains compared to its performance in single-strain infections. We can distinguish three different types of intraspecific competition: exploitation competition, interference competition, and apparent competition. In exploitation competition, pathogen strains compete with each other for limited

host resources. In malaria infections for example, red blood cells are a limited resource that can produce strong competition between strains of *Plasmodium* (Mideo et al. 2008). Interference competition is the result of direct interactions between strains that can inhibit the growth, transmission or reproduction of their competitors. For example, strains of the pathogenic bacterium *Haemophilus influenza* produce highly specific toxins that are active against other strains but not against individuals from the same strain (Venezia and Robertson 1975). Apparent competition is mediated by the host immune system where a given strain induces a nonspecific or cross-reactive host immune response that has a negative impact on the other strains. This phenomenon has been demonstrated in the rodent malaria parasite *Plasmodium chabaudi* (Råberg et al. 2006). However, for any demonstration of competition between pathogen strains, it is often difficult to know which type of competition is at play (Mideo 2009).

In vector-borne diseases, mixed-strain infections are common and occur in both the vertebrate reservoir host and the arthropod vector (Schmid-Hempel 2011). Studies on competition between strains of vector-borne pathogens typically focus on the vertebrate reservoir host (Mercereau-Puijalon 1996, Smith et al. 1999, Read and Taylor 2001, De Roode et al. 2003, de Roode et al. 2005, Råberg et al. 2006, Mideo et al. 2008). To date, only a few studies have investigated mixed infections and inter-strain competition in the vector and its importance for the strain structure of the pathogen (Araújo et al. 2007, Peacock et al. 2007, Rego et al. 2014, Reif et al. 2014, Pollitt et al. 2015). All these studies suggest that interactions between pathogen strains in the arthropod vector can be dramatically different than the ones observed in the vertebrate host. For example, in the kissing bug *Triatoma brasiliensis*, when two strains of *Trypanosoma cruzi* colonize the same individual vector they occupy different parts of the insect gut (Araújo et al. 2007). Exploitation competition is avoided, and it is actually facilitation that is observed, with one strain more abundant in the

presence of the other. Another study on the tick-borne pathogen *Francisella novicida* found that intraspecific interactions within the tick vector *Dermacentor andersoni* can result in severe population bottlenecks and greatly influence the strain diversity in the pathogen population (Reif et al. 2014).

3.2. *Borrelia* species

Borrelia burgdorferi sensu lato (Adeolu and Gupta 2014), is a complex of bacteria that contains different species of diderm spirochete bacteria, some of which cause Lyme borreliosis (LB) in humans. To date, up to 19 different species of *Borrelia* have been described in the world (Pritt et al. 2016). In Europe, the most common species are *Borrelia afzelii* and *Borrelia garinii* (Rauter and Hartung 2005, Estrada-Peña et al. 2011). These two *Borrelia* species are both transmitted by the same generalist hard tick vector, *Ixodes ricinus*, which feeds on a large range of vertebrate hosts. However, these *Borrelia* species are adapted to different vertebrate hosts: *B. afzelii* is specialized on small mammals (Humair and Gern 1998, Kurtenbach et al. 1998, Kurtenbach et al. 2002, Hanincova et al. 2003), whereas *B. garinii* is specialized on birds (Kurtenbach et al. 1998, Kurtenbach et al. 2002, Hanincová et al. 2003). This specificity is based on the fact that *B.* afzelii spirochetes are mostly found in small mammals, and that these small mammals are able to transmit the spirochetes to uninfected larval ticks (Humair et al. 1995, Humair and Gern 1998, Kurtenbach et al. 1998, Huegli et al. 2002, Kurtenbach et al. 2002, Hanincova et al. 2003). Conversely, *B. garinii* spirochetes are mostly found in birds, and these birds are able to transmit the spirochetes to uninfected larval ticks (Humair et al. 1998, Kurtenbach et al. 1998, Kurtenbach et al. 2002, Hanincová et al. 2003, Taragel'ová et al. 2008). This specificity is mediated by the complement system of the vertebrate host (Kurtenbach et al. 1998, Kurtenbach et al. 2002).

3.2.1. Lyme borreliosis (LB)

Lyme borreliosis (LB) is the most common vector-borne disease in Europe and North America. The first symptom is usually a rash at the site of the tick bite and is called an erythema migrans. If left untreated, LB patients can develop arthritis, joint pain, chronic fatigue, neurological problems, and skin disorders, depending on the species or strain of *Borrelia* involved. The spirochetes are transmitted among vertebrate hosts by hard-bodied ticks belonging to the genus *Ixodes*. *Ixodes* ticks have three obligate blood-feeding stages: larva, nymph, and adult. Lyme disease exists in nature because the two immature tick stages (larvae and nymphs) feed on the same set of susceptible vertebrate hosts (small mammals and birds). In contrast, the adult ticks are a dead-end for *B. burgdorferi* because they feed on larger hosts (e.g. deer) that are resistant to the spirochete (Telford 3rd et al. 1988, Jaenson and Tälleklint 1992, Matuschka et al. 1992, Ogden et al. 1997, Kurtenbach et al. 2006). Transovarial transmission of *B. burgdorferi* is believed to be rare in *Ixodes ricinus* (Bellet-Edimo et al. 2005, Richter et al. 2012, Rollend et al. 2013). However, a recent study has shown that *B. afzelii*-infected larvae, even if rare, are able to infect rodent hosts (van Duijvendijk et al. 2016). A more detailed description of the Lyme disease life cycle is as follows. In the early summer, infected nymphs transmit *B. burgdorferi* to susceptible hosts, which subsequently transmit the pathogen to the next generation of larval ticks in the late summer. These larval ticks molt into infected nymphs that overwinter and become active the following year when they infect the next generation of hosts and larval ticks. Humans are accidental hosts and are not part of the natural life cycle of *Borrelia* spirochetes.

3.2.2. Multiple infections in the ticks and the hosts

In *Borrelia* pathogens, multiple strain infections are common in both the tick vector and the vertebrate reservoir hosts. On average, 1.8 different strains are found in both questing

ticks (up to 11 different strains in one tick; Appendix Table 1), ticks feeding on the host (up to 7 strains in one tick; Appendix Table 2), and in the hosts (up to 6 different strains; Appendix Table 3). The observed strain richness is higher in studies that use next-generation sequencing (Durand et al. 2015, Strandh and Råberg 2015). A field study of *B. afzelii* strains in the wild rodent reservoir host found evidence of apparent competition (Andersson et al. 2013). This study found that *B. afzelii* strains that were genetically similar at the *ospC* locus (this gene codes for an immunodominant surface protein that induces a strong antibody response in the vertebrate host) were less likely to be found together in the same rodent reservoir host. A recent study on *B. afzelii* in wild reservoir hosts found evidence for competition because the strain-specific spirochete abundance was lower in mixed infections than in single infections (Strandh and Råberg 2015). In summary, *Borrelia* pathogens consist of multiple strains that compete with each other inside the vertebrate reservoir host.

3.2.3. Effect of the infection on the reservoir hosts and the vector

To date, almost no negative effect of *Borrelia* infection has been observed in reservoir hosts. An early experimental infection study of the white-footed mouse *Peromyscus leucopus* found that infant mice but not adult mice developed carditis and arthritis (Moody et al. 1994). *P. leucopus* mice that had been experimentally infected with *B. burgdorferi* via tick bite showed no changes in running behavior over a period of six weeks post-infection compared to uninfected control mice (Schwanz et al. 2011). Capture-mark-recapture (CMR) studies on populations of *P. leucopus* and *Rissa tridactyla* have found no evidence that infection with *B. burgdorferi* reduces the survival of these wild reservoir hosts (Hofmeister et al. 1999, Chambert et al. 2012, Voordouw et al. 2015).

In the tick vector, the effects of *Borrelia* infections are usually positive. Recent studies on *B. afzelii*-infected nymphs have shown that infected ticks have better survival under thermo-hygrometric stresses (Herrmann and Gern 2010), reduced horizontal movement along

a humidity gradient (Herrmann and Gern 2012), higher energy reserves (Herrmann et al. 2013), and better survival at cold temperatures (Herrmann and Gern 2013).

3.2.4. Infection cycles of *Borrelia* spirochetes

Host-to-larval tick transmission: The success of host-to-larval tick transmission depends on the spirochete abundance in the host (Råberg 2012, Jacquet et al. 2015). Larvae start to acquire *Borrelia* spirochetes during the first 12 hours of attachment (Pal and Fikrig 2010). The spirochetes migrate from the host dermis to the tick where they attach to the midgut epithelium using *Borrelia* outer surface protein A (OspA). In the weeks following engorgement, the spirochetes multiply rapidly, but their abundance can drop five-fold during the larva-to-nymph molt (Piesman et al. 1990). *Borrelia* pathogens spend a long period of time (~8 months) in the midgut of the nymphal tick until the next blood meal (Tälleklint and Jaenson 1995, Lindsay et al. 1997). Spirochetes depend on the tick blood meal for nutrients (Dunham-Ems et al. 2009, Radolf et al. 2012). During the winter, the ticks produce glycerol as a putative natural antifreeze, which the spirochetes use as an energy source (Pappas et al. 2011). There is currently much scientific interest in identifying the genes that allow the spirochetes to persist inside the midgut of flat, unfed nymphs (Pappas et al. 2011, Kung et al. 2013, Fazzino et al. 2015). This part of the life cycle must be particularly challenging, as the bacteria suffer from nutritional stress and extreme changes in temperature. Spirochete persistence in the nymph midgut is probably a game of attrition, which the most abundant strains are more likely to win. Spirochete load inside the nymphal tick can decrease by as much as 90% over 8 months (Jacquet et al. 2015). A similar phenomenon was observed in *I. scapularis* nymphs infected with *B. burgdorferi* (Voordouw et al. 2013). Both of these studies involved laboratory populations of *Ixodes* ticks that were kept under laboratory conditions, but a decline in spirochete abundance has also been observed in wild adult ticks after winter (Sharon et al. 1992).

Nymph-to-host transmission: During the nymphal blood meal, only a few spirochetes are able to complete the migration from the tick midgut to the salivary glands (Piesman 1993, Ohnishi et al. 2001, Piesman et al. 2001, Dunham-Ems et al. 2009). *Borrelia* spirochetes start to multiply rapidly in the tick midgut following tick attachment. At this stage, the spirochetes are still non-motile, but they penetrate more deeply into the midgut epithelium by dividing and forming networks that advance toward the basement membrane (Dunham-Ems et al. 2009). Only a few spirochetes manage to cross the midgut epithelium and enter the hemolymph (Coleman et al. 1997, Dunham-Ems et al. 2009). In the hemolymph, the spirochetes are motile but they are attacked by the hemocytes (immune cells) of the tick immune system (Johns et al. 2001, Hajdusek et al. 2013). The number of spirochetes that reaches the salivary glands is therefore very low (Spielman et al. 1987, Piesman 1993, Grimm et al. 2004, Fisher et al. 2005, Dunham-Ems et al. 2009). A qPCR study on *B. burgdorferi* in *I. scapularis* suggested that less than 100 spirochetes reached the salivary glands of nymphal ticks. These data are consistent with studies showing that only a small inoculum of spirochetes from the tick salivary glands is required to establish infection in a competent vertebrate host (Ohnishi et al. 2001, Lima et al. 2005). Spirochetes are usually detected in the tick salivary glands after 60 hours (Ohnishi et al. 2001, Dunham-Ems et al. 2009), but nymph-to-host transmission can start as early as 24 hours after attachment (Hodzic et al. 2002, Cook 2015). The large decline in spirochete abundance during the migration from the midgut to the salivary glands creates a population bottleneck that reduces the strain richness of the inoculum (Rego et al. 2014). *Borrelia* strains that start the journey with a higher abundance in the midgut are more likely to reach their final destination. A recent study used genetically tagged strains of *B. burgdorferi* (that were otherwise identical in fitness) to show that the more abundant strains in the nymphal tick had higher levels of nymph-to-host transmission (Rego et al. 2014).

At the site of attachment, ticks inject their saliva to modulate the hemostatic, inflammatory, and immune responses of the vertebrate host and thereby optimize blood uptake (Ribeiro et al. 1985, Brossard and Wikel 2004, Francischetti et al. 2009). Ticks create a zone of immunosuppression around the feeding lesion that is beneficial to both the tick and *Borrelia*. Salivary gland extracts suppress both the innate and the acquired immune response in the rodent host (Ribeiro 1987, Ribeiro et al. 1990, Mejri et al. 2002, Pechová et al. 2002, Guo et al. 2009). In addition, *Borrelia* spirochetes interact with the Salp15 protein from the tick saliva to evade antibody-mediated killing (Ramamoorthi et al. 2005). The spirochetes can remain at the inoculation site for several days before disseminating into the host (Shih et al. 1992). *Borrelia* pathogens have been recovered from numerous tissues and organs in the vertebrate host including the skin, blood, joints, spleen, heart, liver, urinary bladder, kidney, and nervous system (Gray et al. 2002).

Co-feeding transmission: *Borrelia* spirochetes can be transmitted from an infected nymph to larvae via co-feeding transmission without necessarily establishing a systemic infection in the host (Gern and Rais 1996, Randolph et al. 1996, Voordouw 2015). Co-feeding transmission occurs when two ticks are feeding at the same time and in close proximity on the same host. This mode of transmission is particularly important in Europe where the peak questing activity of nymphs and larvae are synchronized (Kurtenbach et al. 2006). Co-feeding transmission may allow *Borrelia* pathogens to obtain some fitness benefits from vertebrate reservoir hosts that are otherwise refractory to systemic infection (Voordouw 2015). In the UK for example, Lyme disease was maintained via co-feeding transmission in a population of sheep, which is an incompetent host for spirochetes (Ogden et al. 1997). Other studies have shown that some species of *Borrelia* can be transmitted via co-feeding while feeding on incompetent hosts (Sato and Nakao 1997, Hu et al. 2003, Hasle 2013). A recent study on *B. afzelii* in laboratory mice found that acquired immunity against a given strain reduced co-

feeding transmission success (Jacquet et al. 2015). This study also showed that the mode of transmission influenced the spirochete abundance in the nymphal tick. Nymphs that had acquired the spirochetes as larvae via co-feeding transmission had a spirochete load that was six times lower than nymphs that had acquired the infection as larvae via systemic transmission. As the spirochete abundance is important for nymph-to-host transmission, this result suggests that co-feeding nymphs are less infectious to vertebrate hosts than systemic nymphs.

The genome of *Borrelia* bacteria is very complex and consists of a linear chromosome (~910 kb) and a large number of circular and linear plasmids (~610 kb) (Fraser et al. 1997, Casjens et al. 2000, Casjens et al. 2012). The number of plasmids differs between *Borrelia* strains (Casjens et al. 2012). Plasmids can be horizontally transferred between strains (Livey et al. 1995, Wang et al. 1999, Qiu et al. 2004) and lost during in vitro growth (Barbour 1988, Schwan et al. 1988, Labandeira-Rey and Skare 2001). However, the gene repertoire remains surprisingly consistent between the different strains (Casjens et al. 2012). A *Borrelia* species is often considered as a complex of clones or strains, and strong linkage disequilibrium has been observed between different genes (Bunikis et al. 2004, Qiu et al. 2004, Hellgren et al. 2011, Brisson et al. 2012). The plasmids contain genes coding for various surface proteins that are expressed at different phases during the spirochete life cycle (Kenedy et al. 2012).

3.2.5. Outer-surface protein C (OspC)

OspC is a 22-kDa immunodominant protein (Fuchs et al. 1992, Marconi et al. 1993), and is highly polymorphic in a variety of *Borrelia* species. Some studies have suggested that OspC allows the spirochetes to migrate from the tick midgut to the tick salivary glands (Pal et al. 2004, Fingerle et al. 2007). However, the balance of evidence suggests that the OspC protein is essential for establishing infection in the vertebrate host (Grimm et al. 2004, Tilly et al. 2006). Two mechanisms have been described by which OspC infects the vertebrate host:

(1) OspC binds Salp 15, a tick salivary gland protein (Ramamoorthi et al. 2005), which allows it to hide from the host immune system and (2) OspC binds to host plasminogen, which allows it to cut through host tissue and reach the circulatory system (Lagal et al. 2006, Onder et al. 2012). The OspC protein is expressed during the early stages of the infection and induces a strong antibody response in the infected host (Schwan et al. 1995, Schwan and Piesman 2000, Ohnishi et al. 2001, Liang et al. 2002, Liang et al. 2004). This pathogen protein is therefore under strong selection pressure by the vertebrate immune system (Theisen et al. 1993, Fung et al. 1994). The *ospC* gene is encoded on the circular plasmid Cp26 (Sadziene et al. 1993) and has a large amount of sequence variation that can be classified into discrete major *ospC* groups within each *Borrelia* species. A major *ospC* group is defined as a group of *ospC* alleles that are >8% (average difference of ≈20%) divergent in their DNA sequence from other such groups and <2% divergent (average difference of ≈1%) within the same major group (Wang et al. 1999). These major *ospC* groups can be considered as different strains, especially in local populations in which linkage disequilibrium is very high (Hellgren et al. 2011). Experimental infections of rodents with genetically defined strains of *Borrelia* have found substantial variation in fitness between strains (Derdáková et al. 2004, Hanincová et al. 2008, Tonetti et al. 2015).

The *ospC* gene exhibits a discrete pattern of genetic variation and sequences that are intermediately divergent (2–8%) have not been found (Baranton et al. 2001). This pattern of divergence suggests two conflicting selection pressures. On the one hand, a given OspC protein is under strong divergent selection to escape into an immune-free space where it cannot be recognized by the antibodies developed against other OspC variants. On the other hand, the OspC protein is also under stabilizing selection to maintain its ability to establish infection in the vertebrate host. The diversity of major o*spC* groups suggests that there are many o*spC* variants that are functional but the lack of divergence within a major o*spC* group

suggest that there are functional constraints. Alternatively, cross-reactive antibodies developed against the common major *ospC* groups prevent the mutant intermediately divergent *ospC* alleles from increasing to a detectable frequency. The *ospC* gene can be subdivided into regions that are more or less conserved (Livey et al. 1995, Theisen et al. 1995, Wang et al. 1999). These regions present different rates of recombination (Livey et al. 1995) and different ratios of synonymous (ds) to non-synonymous substitutions (dn) (Theisen et al. 1995, Wang et al. 1999), suggesting that they do not evolve at the same speed, and are not under the same selection pressure.

There is much interest in determining the factors that maintain the *ospC* polymorphism in *Borrelia* species. Two alternative but non-exclusive explanations are negative frequency-dependent selection (NFDS) (Qiu et al. 1997, Wang et al. 1999) and multiple-niche polymorphism (MNP) (Brisson and Dykhuizen 2004). Strong selection pressure from the vertebrate immune response (Gilmore et al. 1996) could be sufficient to create NFDS. This theory is supported by the high rates of recombination and the low ds/dn ratio in this gene (Theisen et al. 1995, Wang et al. 1999, Haven et al. 2011). According to the Lyme disease literature, NFDS should lead to fluctuations in the frequencies of the major *ospC* groups (Kurtenbach et al. 2006, Tsao 2009, Brisson et al. 2012). However, it is important to point out that NFDS can also produce stable polymorphisms where there are no cycles in genotype frequencies (Ayala and Campbell 1974). To date, only Wang et al. (1999) have proposed an immunological mechanism that could actually drive NFDS of the *ospC* gene. Wang et al. (1999) stated that, "anti-OspC antibodies can prevent reinfection with the same strain and clear present infections" and that, "The most abundant *ospC* population will, on average, be the group that a host responds to immunologically both first and most strongly. Thus a rare type is more likely to establish a persistent infection than a common one and be passed onto the next generation of ticks." The idea that frequent exposure to the same OspC

antigen will clear the infection from the rodent reservoir host is unlikely to be true for a number of reasons. First, the general consensus is that *Boreliella* pathogens establish chronic, life-long infections in their rodent reservoir hosts (Donahue et al. 1987, Gern et al. 1994, Richter et al. 2004). Second, expression of the OspC antigen is down regulated once the *Boreliella* pathogen has established a disseminated infection in the vertebrate host (Schwan et al. 1995, Schwan and Piesman 2000, Ohnishi et al. 2001, Liang et al. 2002, Liang et al. 2004), and OspC-specific antibodies therefore cannot clear chronic infections. Third, a recent study in our group found that there was no difference in spirochete transmission to larval ticks between mice that were exposed once or twice to the same *ospC* strain via tick bite (unpublished data). This experiment suggests that repeated exposure to the same strain does not have a negative effect on host-to-tick transmission.

Brisson and Dykhuizen (2004) proposed the MNP explanation for *B. burgdorferi* in the eastern United States after showing that the major *ospC* groups were associated with certain host species. For example, major *ospC* group B was found in the white-footed mouse, *Peromyscus leucopus*, but not in the other three host species. In contrast, major *ospC* group I was found in *P. leucopus* and the eastern chipmunk, *Tamias striatus*, but not in the other host species. They concluded that some *B. burgdorferi ospC* strains were unable to infect certain vertebrate host species. The strongest evidence for the MNP hypothesis in the United States is the fact that there are some major *ospC* groups that have been found in ticks but that have never been isolated from the common vertebrate reservoir hosts. This observation suggests that these *ospC* strains are circulating in other vertebrate reservoir host species.

Divergent selection by the acquired immune system of the vertebrate host has driven the diversification and adaptive radiation of the *ospC* gene (Theisen et al. 1993). Immunization studies with recombinant OspC proteins have shown that OspC-specific antibodies provide strain-specific protection against super-infection with strains carrying the

same but not different major *ospC* group alleles (Probert et al. 1997, Earnhart et al. 2005, Jacquet et al. 2015). Thus any immunological theory for the *ospC* polymorphism must consider the role of the adaptive arm of the vertebrate immune system. In the late 1990s, Gupta and colleagues developed a body of theoretical work that explained how cross-reactive acquired immunity in the vertebrate host could structure pathogen populations into discrete strains. This theory showed that strong immune selection against immunodominant antigens can cause the pathogen population to organize into antigenically distinct strains or serotypes that induce strain-specific immunity (Gupta et al. 1996, Gupta and Anderson 1999). These sets of antigenically distinct strains can remain stable over long periods of time and the frequency of each strain depends on its intrinsic fitness (Gupta et al. 1998, Gupta and Anderson 1999). Such systems are difficult to invade by mutant pathogen strains because the host population has been saturated with antigenic variants so that there is no immune-free space left for the mutant to occupy (Gupta et al. 1996). When the host immune response is not strong enough to induce a stable community of antigenically distinct strains, the frequencies of the strains can exhibit cyclical or chaotic fluctuations (Gupta et al. 1998, Gupta and Anderson 1999). Thus studies that monitor the frequencies of pathogen strains over time can provide insight into how acquired immunity in the host population shapes the epidemiology of multiple-strain pathogens (Plummer et al. 1989, Brunham et al. 1996, Bambini et al. 2013).

4. Aims of the PhD project

The purpose of the present work was to investigate how the *ospC* strain diversity of *Borrelia* pathogens could be maintained in a local population of *I. ricinus* ticks. We decided to study the two most common *Borrelia* species in Europe: *B. afzelii* and *B. garinii*.

In the first paper, we studied the influence of genetic variation in the transmission success of *B. afzelii* spirochetes. We measured the transmission rate of nymph-to-host, host-to-larvae, and cofeeding transmission for six strains of *B. afzelii*. We used next generation matrix models to combine these transmission components into a single estimate of the reproductive number (R_0) for each *B. afzelii* strain. We also tested whether these strain-specific estimates of R_0 were correlated with the strain-specific frequencies in the field obtained from a previous study (Pérez et al. 2011).

In the second paper, we characterized the community of major *ospC* group alleles for *B. afzelii* and *B. garinii* in a local population of *I. ricinus* ticks. Pérez et al (2011) had previously used cold single-strand conformation polymorphism (SSCP) to characterize the *ospC* strain diversity of *B. afzelii* in *I. ricinus* ticks and in wild rodent reservoir hosts at two different sites in Switzerland over three years. In contrast, we wanted to examine whether the community of *ospC* strains changed over time in a local population of *I. ricinus* ticks. In addition, we wanted to use next generation sequencing to characterize the *ospC* strain diversity to a much higher resolution. This approach produced a large number of *ospC* gene sequences (240,000 reads) and allowed us to screen for intermediately divergent major *ospC* groups (divergence of 2–8% in the DNA sequence). In addition, we wanted to test whether there was a positive relationship between the pairwise genetic distance between the major *ospC* groups and their degree of association in the *I. ricinus* nymphs, as had been previously shown in the rodent reservoir host (Andersson et al. 2013).

In the third paper, we studied the evolution of *ospC* diversity in our local population of *I. ricinus* ticks over a period of 11 years. Specifically, we wanted to test whether the frequencies of the major *ospC* groups would fluctuate over the duration of our study. We also tested the theory by Gupta and colleagues that states that strong cross-reactive acquired immunity in the vertebrate host can cause the pathogen population to diverge into a set of non-overlapping serotypes that can remain stable over long periods of time (Gupta et al. 1996, Gupta and Anderson 1999). In this scenario, the pathogen is a complex of independent strains and the frequency of each strain should depend on its intrinsic fitness (Gupta et al. 1998, Gupta and Anderson 1999). We used laboratory estimates of fitness of six different strains of *B. afzelii* from the first paper to test the prediction that the frequency of each strain should depend on its intrinsic fitness (Tonetti et al. 2015).

In the fourth paper, we studied competition between *ospC* strains in the tick vector separately for each of the two *Borrelia* genospecies: *B. afzelii* and *B. garinii*. We used 454-sequencing to characterize the *ospC* strain diversity of *I. ricinus* nymphs infected with either *B. afzelii* or *B. garinii*. These nymphs had been sampled over a period of 3 years from the same site as the previous 11-year study. The spirochete abundance of each nymphal tick had been determined using qPCR in previous studies (Herrmann and Gern 2010, Herrmann and Gern 2012, Herrmann and Gern 2013, Herrmann et al. 2013). We combined the spirochete load data and the *ospC* strain community data to test whether there was competition between strains in the tick vector.

5. 1st paper

Genetic variation in transmission success of the Lyme borreliosis pathogen *Borrelia afzelii*

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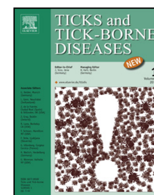

Original article

Genetic variation in transmission success of the Lyme borreliosis pathogen *Borrelia afzelii*

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ABSTRACT

The vector-to-host and host-to-vector transmission steps are the two critical events that define the life cycle of any vector-borne pathogen. We expect negative genetic correlations between these two transmission phenotypes, if parasite genotypes specialized at invading the vector are less effective at infecting the vertebrate host and vice versa. We used the tick-borne bacterium *Borrelia afzelii*, a causative agent of Lyme borreliosis in Europe, to test whether genetic trade-offs exist between tick-to-host, systemic (host-to-tick), and a third mode of co-feeding (tick-to-tick) transmission. We worked with six strains of *B. afzelii* that were differentiated according to their *ospC* gene. We compared the three components of transmission among the *B. afzelii* strains using laboratory rodents as the vertebrate host and a laboratory colony of *Ixodes ricinus* as the tick vector. We used next generation matrix models to combine these transmission components into a single estimate of the reproductive number (R_0) for each *B. afzelii* strain. We also tested whether these strain-specific estimates of R_0 were correlated with the strain-specific frequencies in the field. We found significant genetic variation in the three transmission components among the *B. afzelii* strains. This is the first study to document genetic variation in co-feeding transmission for any tick-borne pathogen. We found no evidence of trade-offs as the three pairwise correlations of the transmission rates were all positive. The R_0 values from our laboratory study explained 45% of the variation in the frequencies of the *B. afzelii ospC* strains in the field. Our study suggests that laboratory estimates of pathogen fitness can predict the distribution of pathogen strains in nature.
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Introduction

The ability to establish an infection in a naive host and transmission to secondary hosts are the critical fitness components of the parasite life cycle. Parasite populations often exhibit genetic variation in life history traits despite the fact that these characters are expected to be under strong selection. Life history theory suggests that negative genetic correlations (trade-offs) between different components of the parasite life cycle can influence the evolution of the optimal parasite phenotype (Stearns, 1992). Previous work has shown trade-offs among a variety of parasite life history traits including within- and among-host transmission, ability to avoid clearance by the host immune system, and parasite life expectancy (de Roode et al., 2008; Ebert, 1998; Fraser et al., 2007; Mackinnon et al., 2008; Mackinnon and Read, 1999). These trade-offs are of considerable interest because they drive the evolution of virulence, which is the level of parasite-induced harm to the host (Ebert and Bull, 2003).

Life history trade-offs might be particularly prevalent in vector-borne pathogens that are adapted to live in two very different environments: an arthropod vector and a vertebrate host. The life cycle of all vector-borne pathogens contains two critical transmission events: vector-to-host transmission and host-to-vector transmission (Randolph, 1998). Vector-to-host transmission requires the pathogen to colonize the transmission tissues of the vector (often the salivary glands) and avoid clearance by the vertebrate immune system. Host-to-vector transmission requires ingestion of the pathogen by the vector from the host tissues (blood, skin) and resistance against the arthropod immune system. The genetic and physiological mechanisms underlying these two

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transmission events are likely to be very different (Tsao, 2009). For example, pathogen interactions with vector saliva influence the efficacy of vector-to-host transmission (Titus and Ribeiro, 1988; Wikel, 1999) whereas pathogen load in the vertebrate host determines host-to-vector transmission success (de Roode et al., 2005; Raberg, 2012). Given the difficulty of adapting to both vector and host, we expect vector-borne pathogens to exhibit trade-offs between vector-to-host and host-to-vector transmission success. In the present study, we test this hypothesis using the tick-borne pathogen, *Borrelia afzelii*.

The tick-borne spirochete bacterium, *B. afzelii*, is one of the most important causes of Lyme borreliosis in Europe (Piesman and Gern, 2004). This pathogen uses the tick, *Ixodes ricinus*, as its vector and a variety of rodent species as its reservoir hosts (Humair and Gern, 1998; Humair et al., 1995; Kurtenbach et al., 1998). *B. afzelii* establishes a chronic and systemic infection inside rodent hosts. Studies on *B. afzelii* have found that host-to-tick transmission rates were high over the lifetime of the infection (Gern et al., 1994; Humair et al., 1999). In contrast, studies on *B. burgdorferi* sensu stricto (s. s.) have found that host-to-tick transmission can decline rapidly over short time periods (i.e. one month) (Derdakova et al., 2004; Hanincova et al., 2008; Lindsay et al., 1997). In addition to the classic mode of systemic transmission, *B. afzelii* is also capable of co-feeding transmission (Gern and Rais, 1996; Hu et al., 2003; Richter et al., 2002). Co-feeding transmission occurs when the pathogen is transmitted directly between vectors that are feeding on the same host at the same time and can occur in the absence of systemic infection (Randolph et al., 1996; Voordouw, 2015). A recent field study suggested that strains of *B. afzelii* may differ in their efficacy of co-feeding transmission (Pérez et al., 2011). These findings motivated us to test whether the efficacy of co-feeding transmission differed among strains of *B. afzelii* in the present study.

Local populations of *Borrelia* pathogens often contain a number of genetically diverse strains (Pérez et al., 2011; Qiu et al., 2002). Previous genetic work has shown that there is very little horizontal gene transfer and that *Borrelia* strains are essentially clonal (Bunikis et al., 2004; Dykhuizen and Baranton, 2001; Hellgren et al., 2011; Qiu et al., 2004). The *ospC* gene, which codes for outer surface protein C (OspC), is a commonly used genetic marker to differentiate among strains (Wang et al., 1999). Strains differing at their *ospC* genotype have been compared with respect to a number of different phenotypes expressed in the vertebrate host including spirochete load, pathology, antibody profiles, and host-to-tick transmission (Baum et al., 2012; Derdakova et al., 2004; Hanincova et al., 2008; Wang et al., 2001, 2002). In the present study, the *ospC* gene is of particular interest because the OspC protein plays a critical role during the tick-to-host transmission event (Radolf and Caimano, 2008; Tilly et al., 2008). Mutant strains lacking the *ospC* gene are unable to colonize the tick salivary glands (Fingerle et al., 2007; Pal et al., 2004) and/or the vertebrate host (Grimm et al., 2004; Tilly et al., 2006). Thus in the present study, the *ospC* gene is both a potential virulence factor and a strain-specific genetic marker.

The purpose of this study was to test whether there was genetic variation and covariation among strains of *B. afzelii* in the three canonical fitness components of any tick-borne pathogen: tick-to-host transmission, host-to-tick (systemic) transmission, and tick-to-tick (co-feeding) transmission. As *B. afzelii* is a species that is specialized on rodents (Piesman and Gern, 2004), we used laboratory mice as an approximate model of the wild rodent reservoir host. Our laboratory colony of *I. ricinus* was used as the tick vector. For each *B. afzelii* strain, we combined the transmission components into a single estimate of the reproductive number (R_0) using recently developed next generation matrix models (Harrison and Bennett, 2012; Harrison et al., 2011; Hartemink et al., 2008). R_0 measures the ability of a pathogen to invade and persist in the host population and provides a convenient framework for comparing fitness between pathogen strains. Theory predicts that in the absence of inter-strain competition, each strain will rise to a frequency that is commensurate with its R_0 value (Gupta et al., 1998). Recent work by our group estimated the frequencies of *B. afzelii* strains (using the *ospC* gene as a marker) in wild *I. ricinus* ticks at two sites in Switzerland (Pérez et al., 2011). In the present study, we tested whether our strain-specific estimates of R_0 in laboratory mice were predictive of the frequencies of the *B. afzelii* strains in the field.

Materials and methods

Mice and ticks

Pathogen-free, one month-old, male BALB/c mice were used in this study and were housed in separate cages. Prior to any invasive procedures (inoculation of spirochetes, tick infestations, and ear biopsies), mice were anaesthetized with a mix of xylazine (Xylasol®, 10 mg/kg) and ketamine (Narketan®, 100 mg/kg) to minimize discomfort. Tick infestations and ear biopsies were attempted only after the loss of toe pinch reflexes. Larval and nymphal ticks came from the laboratory colony of spirochete-free *I. ricinus* ticks of the University of Neuchâtel and were reared according to Graf (1978). Infected nymphal ticks were used one month after moulting and xenodiagnostic larval ticks were used at least one month after hatching. All experiments involving mice respected the Swiss legislation on animal experimentation and were authorized by the Veterinary Service of the Canton of Neuchâtel (Authorization numbers 1/2006 and 2/2009).

Borrelia afzelii *isolates*

Most of the *B. afzelii* isolates used in this study came from the isolate collection of the University of Neuchâtel (Table 1). These isolates were obtained from ticks and rodent ear biopsies that had been collected at two different sites in Switzerland: Glütschbachtal (Thoune, Bern, Switzerland) and Bois de l'Hôpital (Neuchâtel, Switzerland). All isolates had been passaged fewer than five times to avoid the loss of the virulence genes that are critical for infection. However, it is possible that our low-passage treatment reduced the infectiousness of the cultured isolates (Ebert, 1998). Strains E61 and P/sto were obtained from the Pasteur Institute, Paris because our Swiss collection of *B. afzelii* isolates did not contain *ospC* groups A3 and A4 (Table 1).

The *B. afzelii* isolates were selected to represent a diversity of *ospC* groups. The nomenclature of the *B. afzelii ospC* groups used in the present study was developed by Lagal et al. (2003) and was also used in the study by Pérez et al. (2011). We note that Bunikis et al. (2004) developed a different nomenclature for the *B. afzelii ospC* groups that is also used in the literature (Table 1). All *ospC* groups used in the present study are known to infect wild rodents (Pérez et al., 2011). The isolates with *ospC* group YU were of particular interest because they appear to have high co-feeding transmission (Pérez et al., 2011). We used experimental infection via needle inoculation (see below) to determine which isolates were infectious for BALB/c mice and retained eight isolates belonging to six *ospC* groups: NE4053 (A1.v1), NE5046 (A1.v2), NE36 (A2), E61 (A3), P/sto (A4), NE4054 (ME), NE4049 (YU.v1), and NE4051 (YU.v2) (Table 1). Isolates NE4053 and NE5046 both had *ospC* group A1 and were therefore designated A1 variant 1 (A1.v1) and A1 variant 2 (A1.v2). Similarly, isolates NE4049 and NE4051 both had *ospC* group YU and were therefore designated YU variant 1 (YU.v1) and YU variant 2 (YU.v2). The *ospC* group of each isolate was determined via DNA sequencing (see below).

Table 1
The eight *Borrelia afzelii* isolates used in this study are shown. The isolates are differentiated according to their *ospC* gene group for which there are two different nomenclatures. For each isolate, the country and site of origin, the source of the isolate, and the Genbank accession number of the *ospC* gene sequence are shown.

Isolate	*ospC* Lagal[a]	*ospC* Bunikis[b]	Country	Site	Source	Genbank #
NE4053	A1.v1	A9.v1	Switzerland	Neuchâtel	Tick	JX103490
NE5046	A1.v2	A9.v2	Switzerland	Neuchâtel	Tick	JX103493
NE36	A2	A1	Switzerland	Thune	Mouse	KC207939
E61[c]	A3	A3	Austria		Human	L42890
P/sto[c]	A4	A12	Germany		Human	AY150205
NE4054	ME	A2	Switzerland	Neuchâtel	Tick	JX103491
NE4049	YU.v1	A10.v1	Switzerland	Neuchâtel	Tick	JX103488
NE4051	YU.v2	A10.v2	Switzerland	Neuchâtel	Tick	JX103489

[a] Nomenclature of *B. afzelii ospC* groups developed by Lagal et al. (2003) and by Pérez et al. (2011). This terminology is used in the present study.
[b] Nomenclature of *B. afzelii ospC* groups developed by Bunikis et al. (2004).
[c] Obtained from G. Baranton, Institute Pasteur, Paris.

Obtaining experimentally infected nymphal ticks

Experimentally infected nymphal ticks were used to measure tick-to-host transmission and were created as follows. Each of the eight *B. afzelii* isolates was cultured in 5 ml tubes containing BSK-H medium (Sinsky and Piesman, 1989) at 34 °C and spirochete densities were estimated using a Helber counting chamber. For each of the eight *B. afzelii* isolates, at least two naïve BALB/C mice were inoculated subcutaneously in the neck with 200 μl of BSK medium containing 2.0×10^6 spirochetes/ml. Thirty days post-inoculation, mice were infested with approximately 100 *I. ricinus* larvae. Infested mice were placed in special cages to facilitate the collection of blood-engorged larvae. Blood-engorged larvae were placed in collecting tubes and were allowed to moult to the nymphal stage (Graf, 1978). One month after moulting, a haphazardly selected subset of 'sentinel' nymphs (n = 13/mouse) was screened to determine the proportion of infected ticks for each isolate. For the eight isolates, the proportion of infected nymphs ranged from 0.154 (2/13) to 1.000 (13/13). The remaining nymphal ticks that had not been used for screening and that had fed on the same mice as the sentinel nymphs will be referred to as the 'challenge' nymphs. These challenge nymphs, for which the strain-specific probability of infection had been estimated from the sentinel nymphs, were used to challenge the mice and measure the strain-specific tick-to-host transmission rates (see below).

DNA extraction, amplification and detection of spirochetes

PCR was used to determine whether *I. ricinus* nymphs were infected with *B. afzelii*. DNA was extracted from ticks using the boiling ammonium hydroxide method described by Morán Cadenas et al. (2007a). The PCR targeted the variable region between the 23S and the 5S ribosomal gene following the protocol described by Morán Cadenas et al. (2007a). This gene is variable among *Borrelia* genospecies but not within a *Borrelia* genospecies (Morán Cadenas et al., 2007a). We therefore assumed that the sensitivity of our detection assay was the same for all eight *B. afzelii* isolates. To detect spirochete DNA, the amplicons from the PCR were used in a reverse line blot (RLB) assay that contained specific probes for *B. afzelii* (Morán Cadenas et al., 2007a).

Sequencing of the ospC *gene*

Part of the *ospC* gene from spirochete DNA extractions was amplified by PCR with primers ospC20 m (5′-AAT AAT TCA GGG AAA GGT GG-3′) and ospC210 (modified from Earnhart et al., 2005). Spirochete DNA was obtained from culture thermolysates following the protocol described by Jouda et al. (2004). PCR reactions were then performed in a 50 μl volume containing 10 μl of the extracted DNA, 1× PCR buffer with 1.5 mM $MgCl_2$, 50 μM of each dNTP, 0.2 μM of each primer, 125 μM $MgCl_2$, and 1.5 units of Taq polymerase. Reactions were performed under the following conditions: initial denaturation step at 94 °C for 180 seconds (s), 35 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, elongation at 72 °C for 60 s and final annealing step at 72 °C for 420 s. PCR products were purified using a commercial purification kit (QIAquick® PCR Purification Kit, QIagen). For each isolate, 1 to 3 amplicons were sent to Microsynth AG (Balgach, Switzerland) for sequencing.

Isolates of *B. burgdorferi* sensu lato (s. l.) often contain multiple *ospC* strains. As part of another study, we recently used 454-sequencing to test the purity of four isolates that were used in the present study. For isolates A2, A3, A4, and YU.v1 this approach obtained 873, 977, 1105, and 1313 sequences that were 99.20%, 100.0%, 99.70%, and 100.00% pure for *ospC* groups A2, A3, A4, and YU, respectively. This deep sequencing approach showed that these four isolates were dominated by a single *ospC* strain.

For each of the four remaining isolates, A1.v1, A1.v2, ME, YU.v2, we checked the purity by sequencing 20 clones containing the amplicons from a nested *ospC* PCR reaction following the protocol described by Bunikis et al. (2004). The cloning reactions were performed using the TOPO® TA cloning kit for sequencing with chemically competent cells (Invitrogen). This work showed that the four isolates were pure at this admittedly low level of resolution. For genetic material, we used the sentinel nymphs that tested positive for *B. afzelii* on the RLB. For isolate A1.v1, we were unable to amplify the *ospC* gene from four RLB-positive sentinel nymphs and we therefore used the culture thermolysate to determine strain purity and re-confirm the identity of the *ospC* gene. We then confirmed that the RLB-positive sentinel nymphs of isolate A1.v1 were infected with *B. afzelii* by amplifying and sequencing the *recA* gene as described in Richter et al. (2006). The presence of the *ospC* gene in the isolate but not the sentinel nymphs suggests that isolate A1.v1 lost the *ospC* plasmid during the transmission cycle from needle to mouse to sentinel nymph. In the results, we therefore excluded isolate A1.v1 from all of the statistical tests because the loss of the *ospC* plasmid was an artefact of culturing spirochetes.

Transmission dynamics experiments

Challenge of mice with B. afzelii*-infected nymphs and tick-to-host transmission*

To compare tick-to-host transmission among the eight *B. afzelii* isolates, four to ten pathogen-free mice were each infested with experimentally infected challenge nymphs. The protocol for infection by tick-bite followed Crippa et al. (2002). The proportion of infected sentinel nymphs for each isolate (range = 0.154–1.000) was used to calculate the number of challenge nymphs (20–3) to ensure that each mouse was infested with an average of three infected challenge nymphs. For example, if the proportion of infected sentinel nymphs for a given isolate was 0.5, each mouse was infected with 3/0.5 = 6 challenge nymphs. Experimentally infected nymphs

were placed in plastic capsules that had been attached to the shaved backs of the mice with wax (Mbow et al., 1994). Capsules were checked on a daily basis to confirm that nymphs had actually attached and additional nymphs were added when this was not the case. Infection status of mice was subsequently determined using xenodiagnosis and ear biopsy (see below).

The nymphal infestation protocol was designed so that each mouse would be infested with an average of three infected challenge nymphs. By chance some mice may have only been infested with uninfected ticks. The number of challenge nymphs and the proportion of infected sentinel nymphs were used to calculate the probability that each mouse was challenged with at least one infected challenge nymph. The geometric mean probability that each mouse was challenged with at least one infected challenge nymph was 0.993 (range = 0.967–0.999). The experiment-wide probability that all 45 mice were challenged with at least one infected challenge nymph was 0.736. Thus we are relatively confident (73.6%) that all 45 mice were challenged with at least one infected challenge nymph and very confident (96.4%) that at least 44 of the 45 mice were truly challenged.

Co-feeding transmission and systemic transmission

To measure co-feeding transmission between experimentally infected challenge nymphs and uninfected larvae, ~100 pathogen-free *I. ricinus* larvae from our laboratory colony were added to the capsules two days after the infestation with the challenge nymphs. To measure systemic transmission between infected mice and uninfected larvae, mice were additionally infested with ~100 xenodiagnostic larvae 30 days after the nymphal challenge. All blood-engorged larvae were collected, allowed to moult into nymphs, and tested for spirochete infection as described above. For each mouse we used 13 haphazardly selected nymphs to measure co-feeding transmission and 13 haphazardly selected nymphs to measure systemic transmission.

Assessment of mouse infection status

Ear biopsy was used as a second, independent measure of mouse infection status. Ear biopsies were taken from anesthetized mice at day 30 post-infection using surgical scissors after cleaning the skin with 70% ethanol. Skin samples were placed into 5 ml BSK-H medium at 34 °C to allow isolation of *B. afzelii*. Cultures were screened for the presence of spirochetes using dark-field microscopy each week for one month. Mice were considered as systemically infected if mouse-to-larva systemic transmission produced at least one infected nymph and/or if the ear biopsy tested positive for spirochetes.

Statistical methods

The level of statistical significance used in this study was 0.05.

Tick-to-host transmission

The isolate A1.v1 was excluded from the statistical analysis of tick-to-host transmission. Generalized linear models with binomial error functions were used to compare the tick-to-host transmission rate among the seven remaining *B. afzelii* isolates (A1.v2, A2, A3, A4, ME, YU.v1, YU.v2). We used the glm() function in R.

Systemic (host-to-tick) transmission

The isolate A1.v1 was excluded from the statistical analysis of systemic transmission. Generalized linear mixed effects models with binomial error functions were used to compare the systemic transmission rate among the seven *B. afzelii* isolates that had this mode of transmission. We used the glmer() function in R. The analysis was done on the subset of mice (n = 36 mice) that produced at least one infected tick via systemic transmission or that had a spirochete-positive ear biopsy. Isolate and *ospC* group were treated as fixed factors whereas mouse identity was treated as a random factor.

Co-feeding (tick-to-tick) transmission

The isolate A1.v1 was excluded from the statistical analysis of co-feeding transmission. Generalized linear mixed effects models with binomial error functions were used to compare the co-feeding transmission rate among the five *B. afzelii* isolates (A1.v2, A2, ME, YU.v1, YU.v2) that had this mode of transmission. We used the glmer() function in R. The analysis was done on the subset of mice (n = 25 mice) that produced at least one co-infected tick. Isolate and *ospC* group were treated as fixed factors whereas mouse identity was treated as a random factor.

Correlations between tick-to-host, systemic and co-feeding transmission

We tested for correlations among the three transmission components: (1) tick-to-host transmission, (2) systemic transmission, and (3) co-feeding transmission, using isolate as the unit of replication. We calculated two sets of correlation coefficients, which differed in how the systemic and co-feeding transmission rates were calculated for the isolates. In the first set, the isolate-specific estimates of systemic and co-feeding transmission were calculated over all seven isolates and all 45 mice were included regardless of their infection status. In the second set, the isolate-specific estimates of systemic transmission were based on the subset of systemically infected mice (n = 36 mice). Similarly, in the second set, the isolate-specific estimates of co-feeding transmission were based on the subset of mice that produced at least one co-infected tick (n = 25 mice), which excluded two isolates (A3, A4).

Estimation of reproductive number (R_0) for B. afzelii *isolates*

Recent theoretical developments have allowed the estimation of the reproductive number (R_0) for complex disease systems like tick-borne infections (Hartemink et al., 2008). These so-called next generation matrices describe the pathogen life cycle by keeping track of infected ticks of different stages (larva, nymph, adult) and their ability to transition into the next stage (via survival, development, and reproduction). The largest eigenvalue of this matrix estimates R_0, which determines whether the pathogen can invade the host population. If $R_0 > 1$, the pathogen invades the population whereas if $R_0 < 1$, the pathogen declines towards extinction. These standardized estimates of R_0 facilitate comparison between different tick-borne pathogens and the testing of epidemiological hypotheses. For example, recent studies using this approach have shown that co-feeding transmission and aggregation of ticks on hosts make a critical contribution to the epidemiology of tick-borne encephalitis (Harrison and Bennett, 2012; Harrison et al., 2011; Hartemink et al., 2008).

In the present study, we used the next generation matrix approach to estimate R_0 for each of the eight isolates of *B. afzelii* (including isolate A1.v1) using the equations in the Appendix of Harrison et al. (2011). For *I. ricinus*, we used all the parameters from Table 1 in Harrison et al. (2011). For *B. afzelii*, we used the systemic duration of infection (i = 120 days), and the efficiency of vertical transmission (r_A = 0.1) from Table 2 in Harrison et al. (2011). For each of the eight isolates, we set co-feeding transmission (θ), host-to-larva transmission (p_L), and nymph-to-host transmission (q_N) to the isolate-specific parameter estimates in

the present study (Table 2). The model by Harrison et al. (2011) contained stage-specific host-to-tick (p_L, p_N, p_A), and stage-specific tick-to-host transmission parameters (q_L, q_N, q_A) for all three tick stages (larva, nymph, adult). We assumed that $p_L = p_N = p_A$ and that $q_L = q_N = q_A$ for the eight isolates. We set the proportion of competent hosts (h_c) at 0.50 because blood meal analysis of questing *I. ricinus* in Switzerland has shown that 40% to 60% of ticks feed on spirochete-incompetent hosts such as artiodactyls and carnivores (Morán Cadenas et al., 2007b). In summary, we used the next generation matrix method to estimate R_0 for each of the eight isolates of *B. afzelii*. To obtain a single estimate of R_0 for *ospC* strain YU, we took the geometric mean of the R_0 estimates of the two variants (YU.v1 and YU.v2). As a point of comparison, we calculated R_0 for *B. burgdorferi* s. l. using the parameters in Harrison et al. (2011): ($\theta = 0.56$, $p_L = p_N = p_A = 0.5$, $q_L = q_N = q_A = 0.8$).

Correlation between R_0 *and the frequency of the* B. afzelii ospC *strains*

The R_0 value of each *B. afzelii ospC* strain should determine its frequency in nature. A recent study by Pérez et al. (2011) estimated the frequency of ten *B. afzelii ospC* strains, of which six occurred in the present study (A1–A4, ME, and YU). Thus the study by Pérez et al. (2011) allowed us to test whether the R_0 value of each *B. afzelii ospC* strain measured in laboratory mice is predictive of its frequency in nature. The authors sampled ticks from two sites in Switzerland: Portes–Rouges (PR), Neuchâtel, Canton Neuchâtel and Staatswald (SW), Ins, Canton Bern. Isolates of *B. afzelii* were cultured from two distinct sources of ticks: questing nymphs and ticks that had been removed from rodents. For the PR site, 81 isolates from questing ticks and 89 isolates from rodent-derived ticks were genotyped. For the SW site, 51 isolates from questing ticks and 171 isolates from rodent-derived ticks were genotyped. The authors used cold single-strand conformational polymorphism analysis to identify the *ospC* group of each isolate. We used Pearson's correlation to test whether there was a significant correlation between the R_0 value and the frequency of the six *B. afzelii ospC* strains in the questing ticks and the rodent-derived ticks for the two sites combined and for each site separately.

Results

Correlation between xenodiagnosis and ear biopsy detection methods

The infection status of the mice was determined using two different methods: (1) PCR of xenodiagnostic ticks and (2) microscope screening of ear biopsies cultured in BSK media. Of the 52 mice (including the seven mice for isolate A1.v1), 37 tested positive for infection with *B. afzelii* according to the xenodiagnosis assay and 30 tested positive according to the ear biopsy. Thus xenodiagnosis (37/52) was more sensitive than the ear biopsy (30/52) but this difference was not statistically significant (proportion test, $p = 0.219$). Of the 30 mice that tested positive for the ear biopsy, 29 also tested positive for the PCR. Thus there was a strong correlation in infection status between the two methods ($r = 0.658$, $t = 6.17$, df = 50, $p < 0.0001$). The strong correlation between the two detection methods shows that our PCR results were not biased by differential sensitivity between strains. The only mouse that tested positive via ear biopsy but not xenodiagnoses had been challenged with isolate A1.v1.

Variation in tick-to-host transmission among B. afzelii *isolates*

All seven *B. afzelii* isolates succeeded in infecting mice via tick bite. The average tick-to-host transmission rate was 82.2% (37 infected mice/45 challenged mice) over the seven isolates (Table 2). The tick-to-host transmission rate of strain ME (40.0% = 4/10) was lower than the other six isolates combined (94.3% = 33/35 mice) and this difference was statistically significant ($\chi^2 = 13.33$, df = 1, $p < 0.0003$).

Variation in systemic (host-to-larva) transmission among B. afzelii *isolates*

All seven *B. afzelii* isolates had systemic transmission (Fig. 1; Table 2). Systemic transmission occurred in 80.0% (36/45) of the mice and 65.5% (305/466) of the xenodiagnostic ticks from these mice were infected. For the subset of systemically infected mice ($n = 36$), there were significant differences in systemic transmission among the seven isolates ($\chi^2 = 32.86$, df = 6, $p < 0.0001$), among the six *ospC* strains ($\chi^2 = 26.92$, df = 5, $p < 0.0001$), and between variants belonging to the same *ospC* strain ($\chi^2 = 5.94$, df = 1, $p = 0.015$). Post hoc model simplification allowed us to combine the seven isolates with systemic transmission into low (A4, ME; range = 15.4–30.8%), medium (A2, A3, YU.v2; range = 53.8–70.4%), and high (A1.v2, YU.v1; range = 85.5–92.3%) transmission groups. The medium transmission group was significantly different from the low ($\chi^2 = 19.66$, df = 5, $p < 0.002$) and the high transmission group ($\chi^2 = 13.41$, df = 5, $p = 0.020$).

Table 2
The three transmission components are shown for the eight *B. afzelii* isolates. The proportion of infected mice is the tick-to-host transmission rate. An infected mouse was defined as having systemic transmission and/or a spirochete-positive ear biopsy. Co-feeding mice refers to the proportion of mice that produced at least one infected tick via co-feeding. The systemic transmission rates show the proportion of ticks that acquired spirochetes from the subset of 36 systemically infected mice. The co-feeding transmission rates show the proportion of ticks that acquired spirochetes via co-feeding from the subset of 25 mice with at least one co-feeding event.

ospC (Lagal)	*B. afzelii* isolate	Infected mice	Co-feeding mice	Systemic transmission	Co-feeding transmission
B. b. s. l.		0.80[a]		0.50[a]	0.56[a]
A1.v1 [b]	NE4053	2/7 (28.6)	1/7 (14.3)	1/26 (3.8)	2/13 (15.4)
A1.v2 [b]	NE5046	4/4 (100.0)	4/4 (100.0)	48/52 (92.3)	17/52 (32.7)
A2	NE36	4/4 (100.0)	2/4 (50.0)	35/52 (67.3)	8/26 (30.8)
A3	E61	3/4 (75.0)	0/4 (0.0)	21/39 (53.8)	0/52[c] (0.0)
A4	P/sto	4/4 (100.0)	0/4 (0.0)	8/52 (15.4)	0/52[c] (0.0)
ME	NE4054	4/10 (40.0)	1/10 (10.0)	12/39 (30.8)	1/26 (3.8)
YU.v1 [b]	NE4049	8/9 (88.9)	8/9 (88.9)	100/117 (85.5)	129/195 (66.2)
YU.v2 [b]	NE4051	9/10 (90.0)	10/10 (100.0)	81/115 (70.4)	103/221 (46.6)
Total		36/45 (80.0)	25/45 (55.6)	305/466 (65.5)	258/520 (49.6)

[a] Parameter estimates of *Borrelia burgdorferi* sensu lato (*B. b.* s. l.) were taken from Table 2 in Harrison et al. (2011).
[b] The suffixes v1 and v2 refer to variant 1 and variant 2.
[c] These nymphs were not included in the calculation of the total co-feeding transmission rate (258/520 = 49.6%).

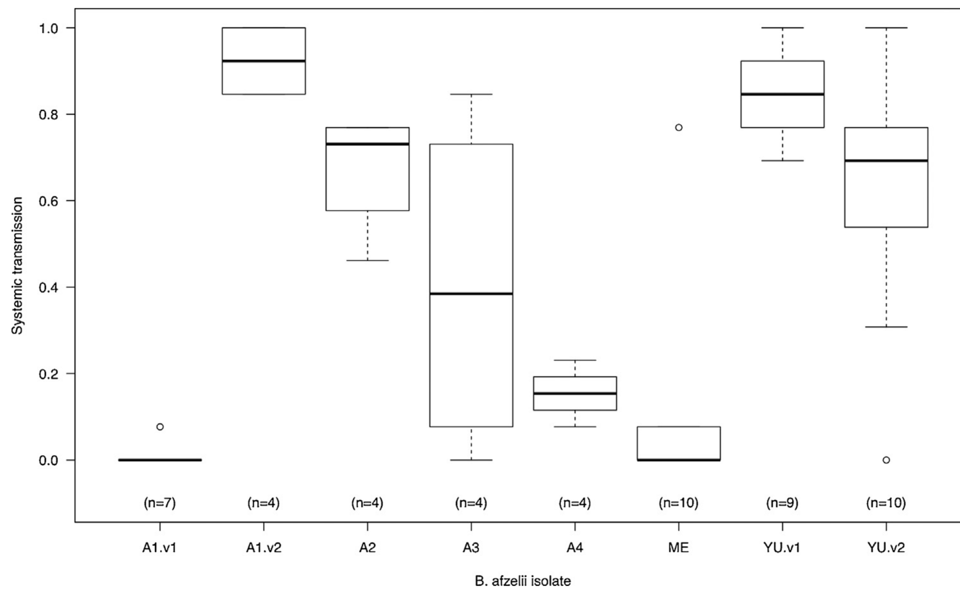

Fig. 1. Systemic transmission differs among the eight isolates of *B. afzelii*. Each data point represents the systemic transmission rate for one mouse and is based on 13 ticks. The numbers of mice (n) used for each isolate are shown in brackets above the x-axis. Shown are the median (black line), 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and outliers (circles).

Variation in co-feeding (nymph-to-larva) transmission among B. afzelii *isolates*

Three isolates (A3, A4, ME) had little or no co-feeding transmission, two isolates (A1.v2, A2) had intermediate co-feeding transmission, and two isolates (YU.v1, YU.v2) had high co-feeding transmission (Fig. 2; Table 2). Co-feeding transmission occurred on 55.6% (25/45) of the mice and 49.6% (258/520) of the larvae on those mice were infected via this mode of transmission (Table 2). There were 11 mice that had systemic transmission but no co-feeding transmission. Interestingly, one mouse infected with isolate YU.v2 had co-feeding transmission (46.2% = 6/13) but no systemic transmission (0.0% = 0/13). For the subset of mice with co-feeding transmission (n = 25 mice), there were significant differences in co-feeding transmission among the five isolates (χ^2 = 10.23, df = 4, p = 0.037) and among the four *ospC* strains (χ^2 = 7.95, df = 3, p = 0.047) but no differences between variants belonging to the same *ospC* strain (χ^2 = 2.28, df = 1, p = 0.131). Post hoc model simplification allowed us to combine the five isolates with co-feeding transmission into low (ME; mean = 3.8%), medium (A1.v2, A2; range = 30.8–32.7%), and high (YU.v1, YU.v2; range = 46.6–66.2%) transmission groups. The medium transmission group was not significantly different from either the low (χ^2 = 4.65, df = 3, p = 0.200) or the high transmission group (χ^2 = 5.94, df = 3, p = 0.115). The difference between the low and high transmission group was most pronounced when the medium group was combined with the low transmission group (A1, A2, ME versus YU; χ^2 = 5.58, df = 1, p = 0.018).

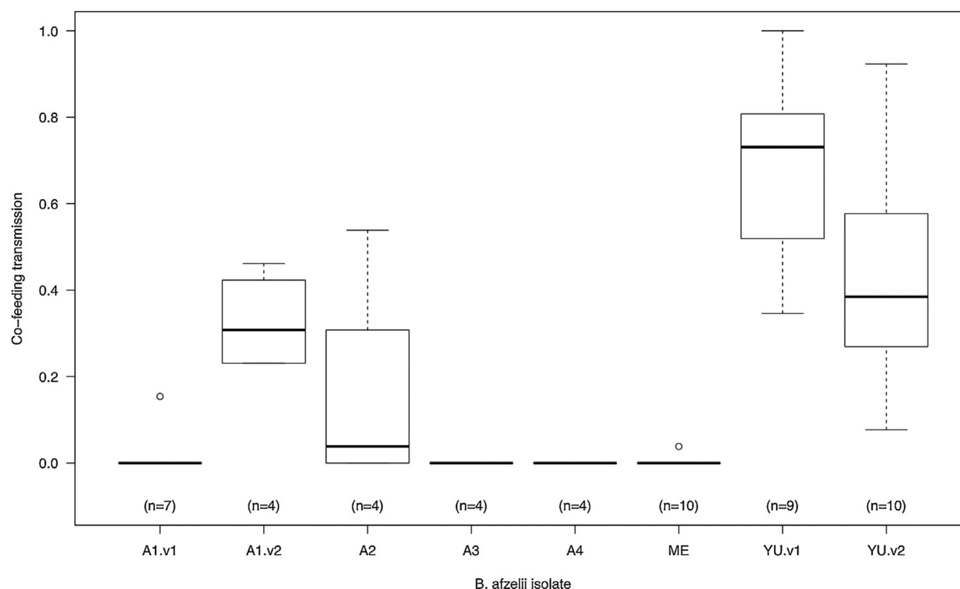

Fig. 2. Co-feeding transmission differs among the eight isolates of *B. afzelii*. Each data point represents the co-feeding transmission rate for one mouse and is based on 13–26 ticks. The numbers of mice (n) used for each isolate are shown in brackets above the x-axis. Shown are the median (black line), 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and outliers (circles).

Table 3
Correlations between the three transmission components are shown. The first set of correlations was based on all eight isolates and all 45 mice. In the second set of correlations, the strain-specific estimates of systemic and co-feeding transmission were based on a subset of mice ($n = 36$ and 25, respectively), which resulted in the exclusion of some strains. Shown are the Pearson's correlation coefficient (r), the t-statistic (t), the degrees of freedom (df), and the p-value (p).

Trait 1	Trait 2	Isolates	Mice	r	t	df	p
Tick-to-host	Systemic	7	45	0.651	1.916	5	0.113
Tick-to-host	Co-feeding	7	45	0.530	1.399	5	0.221
Systemic	Co-feeding	7	45	0.793	2.916	5	0.033
Tick-to-host	Systemic	7	36	0.474	1.2043	5	0.282
Tick-to-host	Co-feeding	5	25	0.786	2.2017	3	0.115
Systemic	Co-feeding	5	25	0.760	2.0251	3	0.136

Correlations between tick-to-host, systemic, and co-feeding transmission

The correlations between the three transmission components were all positive (Table 3). When all the seven *B. afzelii* isolates and all the 45 mice were included, the correlation between systemic and co-feeding transmission was statistically significant ($r = 0.793$, $p = 0.033$) but the correlations between tick-to-host transmission and systemic transmission ($r = 0.651$, $p = 0.113$) and between tick-to-host transmission and co-feeding transmission ($r = 0.530$, $p = 0.221$) were not significant. When the isolate-specific estimates of systemic and co-feeding transmission were restricted to the subset of systemically infected mice ($n = 36$) and the subset of mice that had at least one co-feeding event ($n = 25$), the correlations remained positive but were not significant (Table 3). In general, strains that were highly infectious for mice had high systemic and co-feeding transmission. Conversely, strains that were less infectious for mice had lower systemic and co-feeding transmission.

The reproductive number (R_0) of the B. afzelii *isolates*

The reproductive number (R_0) was greater than 2.0 for all *B. afzelii* isolates except A1.v1 ($R_0 = 0.878$; supplementary material, Table S1). Thus seven isolates of *B. afzelii* were capable of invading and persisting in a Lyme borreliosis system where half of the hosts ($h_c = 0.50$) have a reservoir competence similar to the laboratory mice in this study. Of the seven isolates capable of invasion and persistence, R_0 ranged from 2.036 (strain ME) to 5.519 (strain YU.v1) (supplementary material, Table S1). As a point of comparison, the value of R_0 for *B. burgdorferi* s. l. was 4.052 using the parameter estimates from Table 2 in Harrison et al. (2011).

Correlation between R_0 and the frequency of the B. afzelii ospC *groups*

We tested whether the reproductive number (R_0) of each *B. afzelii ospC* strain measured in laboratory mice was predictive of its frequency in nature using data from a study by Pérez et al. (2011). The correlation between R_0 and the frequency in the questing nymphs (for the two sites combined) of the six *B. afzelii ospC* strains was positive but not statistically significant ($r = 0.670$, $t = 1.80$, df = 4, $p = 0.146$; Fig. 3; Table 4). Despite the lack of statistical significance, it is worth pointing out that the R_0 values from a laboratory study accounted for 44.9% of the variation in the frequencies of the six *B. afzelii ospC* strains in questing nymphs in the field (Fig. 3). The results were similar when the correlation test was conducted separately for each site (Table 4). Finally, none of the correlations between R_0 and the *ospC* strain frequencies in the rodent-derived nymphs were significant (Table 4).

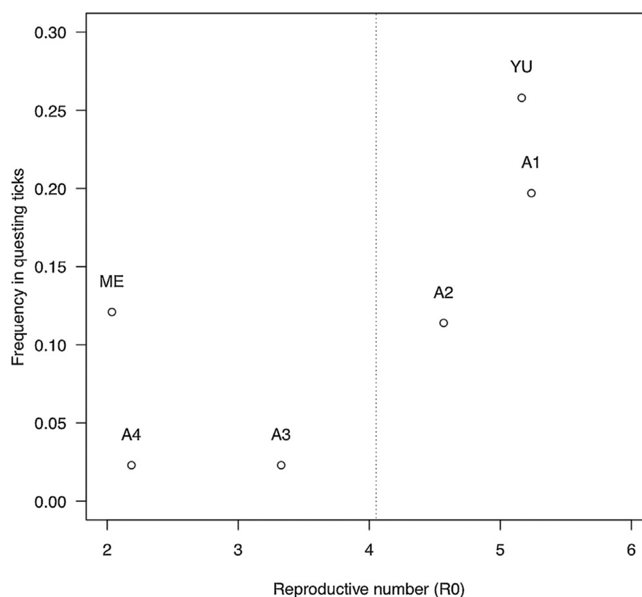

Fig. 3. The relationship between the reproductive number (R_0) and the frequency of the six *B. afzelii ospC* strains in questing ticks is shown. The R_0 value for YU was calculated as the geometric mean of YU.v1 and YU.v2. The stippled line is an estimate of R_0 for *B. burgdorferi* sensu lato from the literature.

Table 4
The correlation between the log-transformed reproductive number (R_0) of the *B. afzelii ospC* strains and the strain-specific frequencies in the field are shown. The frequencies of the *ospC* strains in the field were calculated either from the questing nymphs ($F_{questing}$) or the rodent-derived nymphs (F_{rodent}), respectively. The correlations are shown separately for the Portes–Rouges site (PR), the Staatswald site (SW), and the two sites combined (Comb). Shown are Pearson's correlation coefficient (r), the t-statistic (t), the degrees of freedom (df), and the p-value (p).

Frequency	Site	r	t	df	p
$F_{questing}$	PR	0.426	0.941	4	0.400
$F_{questing}$	SW	0.768	2.401	4	0.074
$F_{questing}$	Comb	0.670	1.804	4	0.146
F_{rodent}	PR	0.198	0.405	4	0.706
F_{rodent}	SW	0.786	2.545	4	0.064
F_{rodent}	Comb	0.660	1.759	4	0.153

Discussion

The most interesting result from this study was the observation that *B. afzelii* populations contain some strains that are highly successful at all three transmission components of the Lyme borreliosis cycle. Strains YU.v1, YU.v2, A1.v2, and A2 all had high rates of tick-to-host, systemic (Fig. 1), and co-feeding transmission (Fig. 2), and consequently these four isolates all had an R_0 value greater than 4.0 (Fig. 3). In contrast, *B. afzelii* strains A3, A4, and ME were less successful at all three components of transmission and consequently these three isolates had an R_0 less than 4.0. With respect to the trade-off hypothesis, the correlation coefficients of the three pairs of transmission components were always positive (Table 3). Thus in contrast to our prediction that *B. afzelii* strains would be specialized on different aspects of the life cycle, we found no evidence of trade-offs among the three canonical transmission components.

In the absence of competition between pathogen strains, theory predicts that the reproductive number (R_0) of each strain will determine its prevalence in the pathogen population (Gupta et al., 1998). Assuming that performance in BALB/c mice is correlated with performance in wild rodents, we expected a positive correlation between the R_0 values of the *B. afzelii* strains in laboratory mice and their frequencies in questing ticks as measured in a recent

field study (Pérez et al., 2011). The R_0 values estimated for the six *B. afzelii ospC* strains in the present study explained 45% of the variation in the strain-specific frequencies in the field study by Pérez et al. (2011) (Fig. 3). Despite the high r^2 value, the positive relationship between R_0 and strain frequency in the field was not statistically significant because of the small sample size (Table 4). However, the relationship between R_0 and the strain-specific frequency provides a useful framework for investigating the ecology of the different *B. afzelii ospC* strains. Strain YU had the highest R_0 value and the highest prevalence whereas other strains such as A2 and A3 had high R_0 values that were not commensurate with their low frequencies in the field (Fig. 3). These strains suggest that we are missing other important ecological factors that determine strain performance in nature. One obvious explanation is that high performance in laboratory mice may not translate to the relevant reservoir hosts in the field. An experimental infection study on two strains of *B. burgdorferi* s. s. found that host-to-tick transmission was considerably lower in natural rodent reservoir hosts than in laboratory mice (Hanincova et al., 2008). According to the multiple niche hypothesis, the different *ospC* strains of *B. burgdorferi* s. s. are associated with different mammalian reservoir hosts (Brisson and Dykhuizen, 2004). This explanation may apply to strain ME, which had the lowest fitness in laboratory mice (R_0 = 2.036) but which reached the third highest frequency in questing ticks (0.121) at the two sites combined (Fig. 3; supplementary material, Table S1). Alternative reservoir hosts for *B. afzelii* in Switzerland include birds, wild boar, and small carnivores, as shown by host blood meal analysis of questing nymphs (Morán Cadenas et al., 2007b).

Our study greatly condensed the tick component of the spirochete life cycle. Thus another explanation for the low field prevalence of some strains is that they perform poorly in the tick vector. In nature, *Borrelia* spends most of the year, including the winter, inside the nymphal tick. By contrast, in the present study, the spirochetes spent less than two months inside the ticks before infecting the mice, and the ticks were kept at room temperature. This explanation may be appropriate for strain A3, which had a low frequency in questing ticks (0.023) at the two sites combined in the study by Pérez et al. (2011). In contrast, strain A2 had an appreciable frequency in questing nymphs (0.114) at the two sites combined, suggesting that this strain does not suffer from poor survival in overwintering nymphs.

Competition among strains in multiply infected hosts or ticks is another factor that can influence the strain-specific frequencies in the field. Previous work has shown that infections with multiple *Borrelia ospC* strains are common in both the tick vector (Pérez et al., 2011; Qiu et al., 2002; Wang et al., 1999) and the rodent reservoir (Andersson et al., 2013; Brisson and Dykhuizen, 2004; Pérez et al., 2011). We used single-strain infections in the present study and so we did not consider how competitive interactions between strains might affect R_0. We have found substantial competition among *Borrelia* genospecies inside the tick (Herrmann et al., 2013) and similar phenomena might occur among *Borrelia ospC* strains. With respect to the vertebrate host, there is some evidence for *B. burgdorferi* s. s. that multiple infections change the temporal dynamics of systemic transmission (Derdakova et al., 2004). A recent field study on multiple infections of *B. afzelii* in wild rodents found that strains with genetically similar OspC antigens were less likely to co-occur in the same host than *ospC* strains that were genetically different (Andersson et al., 2013). This study suggests that a given OspC antigen induces an antibody response that reduces the probability of tick-to-host transmission of closely related *ospC* strains. Thus cross-reactive antibodies in the vertebrate host can play an important role in mediating indirect competition between strains of *Borrelia*.

There was significant genetic variation among the *B. afzelii ospC* strains in all three components of transmission: tick-to-host, systemic, and co-feeding transmission. To date, most studies investigating fitness differences among strains of *Borrelia* only measure systemic transmission (Derdakova et al., 2004; Hanincova et al., 2008). Other studies comparing strains of *B. burgdorferi* s. s. in laboratory mice have investigated the relationship between spirochete load in mouse tissues and measures of pathology such as arthritis, carditis, and ankle swelling (Dolan et al., 2004; Wang et al., 2001, 2002; Zeidner et al., 2001). These studies often find a positive relationship between spirochete load, ability to disseminate to different tissues, and pathology, and strains that score high on all these phenotypes are often labelled as invasive (Dolan et al., 2004; Wang et al., 2001, 2002; Zeidner et al., 2001). However, as none of these studies measured tick-to-host or host-to-tick transmission, the relationships between spirochete load, pathology, and *Borrelia* fitness (R_0) remain unknown.

The mechanisms that determine transmission success in *Borrelia* pathogens are probably related to the spirochete loads inside the host tissues and the tick salivary glands. A recent study on wild rodents found that the load of *B. afzelii* spirochetes inside host tissues determined the probability of systemic transmission but not the spirochete load inside these same ticks after they had moulted into nymphs (Raberg, 2012). A study on *B. burgdorferi* s. s. found that strains that are common in questing ticks have higher spirochete loads when inoculated into their natural rodent hosts than strains that are rare in questing ticks (Baum et al., 2012). Thus we expect the *B. afzelii* strains with high values of R_0 to have higher spirochete loads in their rodent reservoir hosts. Genetic variation in tick-to-host transmission may likewise depend on the density of spirochetes inside the tick salivary glands. During the tick blood meal, spirochetes migrate from the tick midgut to the salivary glands from where they are injected into the feeding lesion (de Silva and Fikrig, 1995; Gern et al., 1990, 1996; Ohnishi et al., 2001; Piesman et al., 2001). Thus genetic variation in tick-to-host transmission may depend on the speed at which the spirochetes colonize the tick salivary glands following attachment to the vertebrate host (Crippa et al., 2002).

Another important result from this study was the first demonstration of genetic variation in co-feeding transmission in a tick-borne pathogen. To date, all theoretical studies investigating R_0 of *B. burgdorferi* s. l. assume that the co-feeding transmission rate is 56% (Harrison and Bennett, 2012; Harrison et al., 2011; Hartemink et al., 2008). The present study found that co-feeding transmission rates range from 0.0% to 66.2% among strains of *B. afzelii* (Table 2). The existence of genetic variation for co-feeding transmission implies that this trait can evolve by natural selection. While co-feeding is the main mode of transmission for tick-borne viruses (Randolph, 2011; Randolph et al., 1996), its importance for *Borrelia* pathogens is controversial (Richter et al., 2002; Voordouw, 2015). Co-feeding transmission is believed to be less important to *Borrelia* pathogens because systemic infections can be so long-lived in the rodent reservoir hosts (Gern et al., 1994; Humair et al., 1999). Recent theoretical work has shown that co-feeding transmission makes a modest contribution to the fitness of *Borrelia* pathogens and is not necessary for invading naive tick populations (Harrison et al., 2011; Hartemink et al., 2008). However, these models ignore the fact that multiple infections are ubiquitous in Lyme borreliosis systems and that strains of *Borrelia* may be in constant competition with each other. Under these circumstances, the modest contribution of co-feeding transmission might provide a decisive fitness advantage. In the present study, the four strains with the highest values of R_0 (A1.v2, A2, YU.v1, YU.v2) also had the highest values of co-feeding transmission (32.7%, 30.8%, 46.6% and 62.2%). In contrast, the three strains with the lowest values of R_0 (A3, A4, ME) had very little co-feeding transmission (<4.0%). In summary, the present study found substantial variation in co-feeding transmission among strains of *B. afzelii* and that this mode of transmission is associated with high values of R_0.

The field study by Pérez et al. (2011) suggested that *B. afzelii ospC* strain YU was highly efficient at co-feeding transmission and this observation was one of the motivating factors for the present study. A critical condition for co-feeding transmission is coincident feeding of nymphal and larval ticks on the same rodent reservoir host (Randolph et al., 1999). Pérez et al. (2011) compared the community of *B. afzelii ospC* strains between two different field sites where the wild rodent populations differed in the frequency of coincident feeding and hence the potential for co-feeding transmission. Their study found that *B. afzelii ospC* strain YU was much more common in the site with high potential for co-feeding transmission (Pérez et al., 2011). The present study confirms that *ospC* strain YU is indeed highly efficient at co-feeding transmission.

The OspC protein plays a critical role in the tick-to-host transmission step of *B. burgdorferi* s. l. pathogens (Fingerle et al., 2007; Grimm et al., 2004; Pal et al., 2004; Tilly et al., 2006). In the present study, the importance of the *ospC* gene was shown by the difference in fitness between the two variants of the A1 strain. Variant A1.v1 had lost its *ospC* gene and was much less efficient at infecting mice than variant A1.v2 (Table 2). Interestingly, variant A1.v1 maintained some co-feeding transmission (Table 2). The OspC protein allows the spirochetes to disseminate from the tick bite and establish a systemic infection inside the vertebrate host (Grimm et al., 2004; Tilly et al., 2006). As tick-to-tick transmission occurs before systemic infection, the co-feeding transmission success of the A1.v1 variant was consistent with the functional role of the OspC protein (Radolf and Caimano, 2008).

Due to its role in host invasion, the *ospC* gene has received much interest from a public health perspective. Studies on *B. burgdorferi* s. s. (Seinost et al., 1999) and *B. afzelii* (Baranton et al., 2001) have shown that only a limited set of *ospC* groups are associated with disseminated infections in humans, although this concept is not without controversy (Alghaferi et al., 2005; Earnhart et al., 2005; Lagal et al., 2003). Studies using more sophisticated measures of invasiveness have largely confirmed that the *ospC* locus remains a useful marker for studying the human pathogenicity of *Borrelia* strains (Dykhuizen et al., 2008; Wormser et al., 2008). The present study found that *B. afzelii ospC* strains A1, A2, A3, A4, and YU were highly infectious to laboratory mice when delivered via tick bite. Two of these three *ospC* strains (A1 and A4) are known to cause disseminated infections in humans (Baranton et al., 2001; Lagal et al., 2003).

In summary, the present study found genetic variation among *B. afzelii* strains in all three components of transmission. We found positive correlations between tick-to-host transmission, systemic transmission, and co-feeding transmission and thus no evidence of trade-offs between these pathogen life history traits. Certain strains of *B. afzelli* were successful in all three components of transmission and consequently these strains had high values of R_0. Our laboratory estimates of R_0 explained an important (but not statistically significant) percentage of the variation in the strain-specific frequencies measured in another field study. The present study shows the value of estimating R_0 in a well-chosen animal model to understand the strain composition of a pathogen population in the field.

Author's contributions

LG conceived and designed the study. NT and SM conducted the experimental work. The sequencing work to determine the purity of the isolates was done by JD. MJV conducted the statistical analyses and the next generation matrix models. MJV and NT wrote the manuscript. LG and JD edited the manuscript.

Competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.02.007.

References

Alghaferi, M.Y., Anderson, J.M., Park, J., Auwaerter, P.G., Aucot, J.N., Norris, D.E., Dumler, J.S., 2005. *Borrelia burgdorferi OspC* heterogeneity among human and murine isolates from a defined region of northern Maryland and southern Pennsylvania: lack of correlation with invasive and noninvasive genotypes. J. Clin. Microbiol. 43, 1874–1879.

Andersson, M., Scherman, K., Raberg, L., Råberg, L., 2013. Multiple-strain infections of *Borrelia afzelii*: a role for within-host interactions in the maintenance of antigenic diversity? Am. Nat. 181, 545–554.

Baranton, G., Seinost, G., Theodore, G., Postic, D., Dykhuizen, D., 2001. Distinct levels of genetic diversity of *Borrelia burgdorferi* are associated with different aspects of pathogenicity. Res. Microbiol. 152, 149–156.

Baum, E., Hue, F., Barbour, A.G., 2012. Experimental infections of the reservoir species *Peromyscus leucopus* with diverse strains of *Borrelia burgdorferi*, a Lyme disease agent. mBio 3, e00434–512.

Brisson, D., Dykhuizen, D.E., 2004. *ospC* diversity in *Borrelia burgdorferi*: different hosts are different niches. Genetics 168, 713–722.

Bunikis, J., Garpmo, U., Tsao, J., Berglund, J., Fish, D., Barbour, A.G., 2004. Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. Microbiology 150, 1741–1755.

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

de Roode, J.C., Pansini, R., Cheesman, S.J., Helinski, M.E.H., Huijben, S., Wargo, A.R., Bell, A.S., Chan, B.H.K., Walliker, D., Read, A.F., 2005. Virulence and competitive ability in genetically diverse malaria infections. Proc. Natl. Acad. Sci. U. S. A. 102, 7624–7628.

de Roode, J.C., Yates, A.J., Altizer, S., 2008. Virulence-transmission trade-offs and population divergence in virulence in a naturally occuring butterfly parasite. Proc. Natl. Acad. Sci. U. S. A. 105, 7489–7494.

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

Derdakova, M., Dudioak, V., Brei, B., Brownstein, J.S., Schwartz, I., Fish, D., 2004. Interaction and transmission of two *Borrelia burgdorferi* sensu stricto strains in a tick-rodent maintenance system. Appl. Environ. Microbiol. 70, 6783–6788.

Dolan, M., Piesman, J., Schneider, B., Schriefer, M., Brandt, K., Zeidner, N., 2004. Comparison of disseminated and nondisseminated strains of *Borrelia burgdorferi* sensu stricto in mice naturally infected by tick bite. Infect. Immun. 72, 5262–5266.

Dykhuizen, D.E., Baranton, G., 2001. The implications of a low rate of horizontal transfer in *Borrelia*. Trends Microbiol. 9, 344–350.

Dykhuizen, D.E., Brisson, D., Sandigursky, S., Wormser, G.P., Nowakowski, J., Nadelman, R.B., Schwartz, I., 2008. Short report: The propensity of different *Borrelia burgdorferi* sensu stricto genotypes to cause disseminated infections in humans. Am. J. Trop. Med. Hyg. 78, 806–810.

Earnhart, C.G., Buckles, E.L., Dumler, J.S., Marconi, R.T., 2005. Demonstration of OspC type diversity in invasive human Lyme disease isolates and identification of previously uncharacterized epitopes that define the specificity of the OspC murine antibody response. Infect. Immun. 73, 7869–7877.

Ebert, D., 1998. Evolution – experimental evolution of parasites. Science 282, 1432–1435.

Ebert, D., Bull, J.J., 2003. Challenging the trade-off model for the evolution of virulence: is virulence management feasible? Trends Microbiol. 11, 15–20.

Fingerle, V., Goettner, G., Gern, L., Wilske, B., Schulte-Spechtel, U., 2007. Complementation of a *Borrelia afzelii* OspC mutant highlights the crucial role of OspC for dissemination of *Borrelia afzelii* in *Ixodes ricinus*. Int. J. Med. Microbiol. 297, 97–107.

Fraser, C., Hollingsworth, T.D., Chapman, R., de Wolf, F., Hanage, W.P., 2007. Variation in HIV-1 set-point viral load: Epidemiological analysis and an evolutionary hypothesis. Proc. Natl. Acad. Sci. U. S. A. 104, 17441–17446.

Gern, L., Lebet, N., Moret, J., 1996. Dynamics of *Borrelia burgdorferi* infection in nymphal *Ixodes ricinus* ticks during feeding. Exp. Appl. Acarol. 20, 649–658.

Gern, L., Rais, O., 1996. Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). J. Med. Entomol. 33, 189–192.

Gern, L., Siegenthaler, M., Hu, C.M., Leuba-Garcia, S., Humair, P.F., Moret, J., 1994. *Borrelia burgdorferi* in rodents (*Apodemus flavicollis* and *A. sylvaticus*): duration and enhancement of infectivity for *Ixodes ricinus* ticks. Eur. J. Epidemiol. 10, 75–80.

Gern, L., Zhu, Z., Aeschlimann, A., 1990. Development of *Borrelia burgdorferi* in *Ixodes ricinus* females during blood feeding. Ann. Parasitol. Hum. Comp. 65, 89–93.

Graf, J.-F., 1978. Copulation, nutrition et ponte chez *Ixodes ricinus* L. (Ixodoidea: Ixodidae) – 1e partie. Bull. Soc. Entomol. Suisse 51, 89–97.

Grimm, D., Tilly, K., Byram, R., Steward, P.E., Krum, J.G., Bueschel, D.M., Schwan, T.G., Policastro, P.F., Elias, A.F., Rosa, P.A., 2004. Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. Proc. Natl. Acad. Sci. U. S. A. 101, 3142–3147.

Gupta, S., Ferguson, N., Anderson, R., 1998. Chaos, persistence, and evolution of strain structure in antigenically diverse infectious agents. Science 280, 912–915.

Hanincova, K., Ogden, N.H., Diuk-Wasser, M., Pappas, C.J., Iyer, R., Fish, D., Schwartz, I., Kurtenbach, K., 2008. Fitness variation of *Borrelia burgdorferi* sensu stricto strains in mice. Appl. Environ. Microbiol. 74, 153–157.

Harrison, A., Bennett, N., 2012. The importance of the aggregation of ticks on small mammal hosts for the establishment and persistence of tick-borne pathogens: an investigation using the R(0) model. Parasitology 139, 1605–1613.

Harrison, A., Montgomery, W.I., Bown, K.J., 2011. Investigating the persistence of tick-borne pathogens via the R-0 model. Parasitology 138, 896–905.

Hartemink, N.A., Randolph, S.E., Davis, S.A., Heesterbeek, J.A.P., 2008. The basic reproduction number for complex disease systems: Defining R-0 for tick-borne infections. Am. Nat. 171, 743–754.

Hellgren, O., Andersson, M., Raberg, L., Råberg, L., 2011. The genetic structure of *Borrelia afzelii* varies with geographic but not ecological sampling scale. J. Evol. Biol. 24, 159–167.

Herrmann, C., Gern, L., Voordouw, M., 2013. Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*. Appl. Environ. Microbiol. 79, 7273–7280.

Hu, C.M., Cheminade, Y., Perret, J.L., Weynants, V., Lobet, Y., Gern, L., 2003. Early detection of *Borrelia burgdorferi* sensu lato infection in Balb/c mice by co-feeding *Ixodes ricinus* ticks. Int. J. Med. Microbiol. 293, 421–426.

Humair, P.F., Gern, L., 1998. Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland. Acta Trop. 69, 213–227.

Humair, P.F., Péter, O., Wallich, R., Gern, L., 1995. Strain variation of Lyme disease spirochetes isolated from *Ixodes ricinus* ticks and rodents collected in two endemic areas in Switzerland. J. Med. Entomol. 32, 433–438.

Humair, P.F., Rais, O., Gern, L., 1999. Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance. Parasitology 118, 33–42.

Jouda, F., Perret, J.L., Gern, L., 2004. Density of questing *Ixodes ricinus* nymphs and adults infected by *Borrelia burgdorferi* sensu lato in Switzerland: spatio-temporal pattern at a regional scale. Vector-Borne Zoonot. Dis. 4, 23–32.

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

Lagal, V., Postic, D., Ruzic-Sabljic, E., Baranton, G., 2003. Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness. J. Clin. Microbiol. 41, 5059–5065.

Lindsay, L.R., Barker, I.K., Surgeoner, G.A., McEwen, S.A., Campbell, G.D., 1997. Duration of *Borrelia burgdorferi* infectivity in white-footed mice for the tick vector *Ixodes scapularis* under laboratory and field conditions in Ontario. J. Wildl. Dis. 33, 766–775.

Mackinnon, M.J., Gandon, S., Read, A.F., 2008. Virulence evolution in response to vaccination: The case of malaria. Vaccine 26, C42–C52.

Mackinnon, M.J., Read, A.F., 1999. Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. Evolution 53, 689–703.

Mbow, M.L., Christe, M., Rutti, B., Brossard, M., 1994. Absence of acquired resistance to nymphal *Ixodes ricinus* ticks in BALB/c mice developing cutaneous reactions. J. Parasitol. 80, 81–87.

Morán Cadenas, F., Rais, O., Jouda, F., Douet, V., Humair, P.-F., 2007a. Phenology of *Ixodes ricinus* and infection with *Borrelia burgdorferi* sensu lato along a North- and South-facing altitudinal gradient on Chaumont Mountain, Switzerland. J. Med. Entomol. 44, 683–693.

Morán Cadenas, F.M., Rais, O., Humair, P.F., Douet, V., Moret, J., Gern, L., 2007b. Identification of host bloodmeal source and *Borrelia burgdorferi* sensu lato in field-collected *Ixodes ricinus* ticks in Chaumont (Switzerland). J. Med. Entomol. 44, 1109–1117.

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

Pal, U., Yang, X.F., Chen, M., Bockenstedt, L.K., Anderson, J.F., Flavell, R.A., Norgard, M.V., Fikrig, E., 2004. OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. J. Clin. Investig. 113, 220–230.

Pérez, D., Kneubühler, Y., Rais, O., Jouda, F., Gern, L., 2011. *Borrelia afzelii ospC* genotype diversity in *Ixodes ricinus* questing ticks and ticks from rodents in two Lyme borreliosis endemic areas: Contribution of co-feeding ticks. Ticks Tick-Borne Dis. 2, 137–142.

Piesman, J., Gern, L., 2004. Lyme borreliosis in Europe and North America. Parasitology 129, S191–S220.

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

Qiu, W.G., Dykhuizen, D.E., Acosta, M.S., Luft, B.J., 2002. Geographic uniformity of the Lyme disease spirochete (*Borrelia burgdorferi*) and its shared history with tick vector (*Ixodes scapularis*) in the northeastern United States. Genetics 160, 833–849.

Qiu, W.G., Schutzer, S.E., Bruno, J.F., Attie, O., Yun, X., Dunn, J.J., Fraser, C.M., Casjens, S.R., Luft, B.J., 2004. Genetic exchange and plasmid transfers in *Borrelia burgdorferi* sensu stricto revealed by three-way genome comparisons and multilocus sequence typing. Proc. Natl. Acad. Sci. U. S. A. 101, 14150–14155.

Raberg, L., 2012. Infection intensity and infectivity of the tick-borne pathogen *Borrelia afzelii*. J. Evol. Biol. 25, 1448–1453.

Radolf, J.D., Caimano, M.J., 2008. The long strange trip of *Borrelia burgdorferi* outer-surface protein C. Mol. Microbiol. 69, 1–4.

Randolph, S.E., 1998. Ticks are not insects: consequences of contrasting vector biology for transmission potential. Parasitol. Today 14, 186–192.

Randolph, S.E., 2011. Transmission of tick-borne pathogens between co-feeding ticks: Milan Labuda's enduring paradigm. Ticks Tick-Borne Dis. 2, 179–182.

Randolph, S.E., Gern, L., Nuttall, P.A., 1996. Co-feeding ticks: epidemiological significance for tick-borne pathogen transmission. Parasitol. Today 12, 472–479.

Randolph, S.E., Miklisova, D., Lysy, J., Rogers, D.J., Labuda, M., 1999. Incidence from coincidence: patterns of tick infestations on rodents facilitate transmission of tick-borne encephalitis virus. Parasitology 118, 177–186.

Richter, D., Allgower, R., Matuschka, F.R., 2002. Co-feeding transmission and its contribution to the perpetuation of the Lyme disease spirochete *Borrelia afzelii*. Emerg. Infect. Dis. 8, 1421–1425.

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

Seinost, G., Dykhuizen, D.E., Dattwyler, R.J., Golde, W.T., Dunn, J.J., Wang, I.N., Wormser, G.P., Schriefer, M.E., Luft, B.J., 1999. Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans. Infect. Immun. 67, 3518–3524.

Sinsky, R.J., Piesman, J., 1989. Ear punch biopsy method for detection and isolation of *Borrelia burgdorferi* from rodents. J. Clin. Microbiol. 27, 1723–1727.

Stearns, S.C., 1992. The evolution of life-histories. Oxford University Press, Oxford.

Tilly, K., Krum, J.G., Bestor, A., Jewett, M.W., Grimm, D., Bueschel, D., Byram, R., Dorward, D., VanRaden, M.J., Steward, P., Rosa, P., 2006. *Borrelia burgdorferi* ospC protein required exclusively in a crucial early stage of mammalian infection. Infect. Immun. 74, 3554–3564.

Tilly, K., Rosa, P.A., Stewart, P.E., 2008. Biology of infection with *Borrelia burgdorferi*. Infect. Dis. Clin. North Am. 22, 217–234.

Titus, R.G., Ribeiro, J.M.C., 1988. Salivary gland lysates from the sand fly *Lutzomyia longipalpis* enhance leishmania infectivity. Science 239, 1306–1308.

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

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

Wang, G., Ojaimi, C., Iyer, R., Saksenberg, V., McClain, S.A., Wormser, G.P., Schwartz, I., 2001. Impact of genotypic variation of *Borrelia burgdorferi* sensu stricto on kinetics of dissemination and severity of disease in C3H/HeJ mice. Infect. Immun. 69, 4303–4312.

Wang, G.Q., Ojaimi, C., Wu, H.Y., Saksenberg, V., Iyer, R., Liveris, D., McClain, S.A., Wormser, G.P., Schwartz, I., 2002. Disease severity in a murine model of Lyme borreliosis is associated with the genotype of the infecting *Borrelia burgdorferi* sensu stricto strain. J. Infect. Dis. 186, 782–791.

Wang, I.N., Dykhuizen, D.E., Qiu, W., Dunn, J.J., Bosler, E.M., Luft, B.J., 1999. Genetic diversity of *ospC* in a local population of *Borrelia burgdorferi* sensu stricto. Genetics 151, 15–30.

Wikel, S.K., 1999. Tick modulation of host immunity: an important factor in pathogen transmission. Int. J. Parasitol. 29, 851–859.

Wormser, G.P., Brisson, D., Liveris, D., Hanincova, K., Sandigursky, S., Nowakowski, J., Nadelman, R.B., Ludin, S., Schwartz, I., 2008. *Borrelia burgdorferi* genotype predicts the capacity for hematogenous dissemination during early Lyme disease. J. Infect. Dis. 198, 1358–1364.

Zeidner, N.S., Schneider, B.S., Dolan, M.C., Piesman, J., 2001. An analysis of spirochete load, strain, and pathology in a model of tick-transmitted Lyme borreliosis. Vector Borne Zoonot. Dis. 1, 35–44.

Supplementary material

Table S1. Reproductive numbers (R_0) of the *B. afzelii* isolates are shown. The frequencies of the *ospC* strains in the rodent-derived ticks (F_{rodent}) and the questing ticks ($F_{questing}$) are from Table 1 of the study by Pérez *et al*. (2011). The frequencies are shown separately for the Portes-Rouges site (PR), the Staatswald site (SW), and the two sites combined (Comb). The sample sizes in the column headers (n) refer to the number of isolates on which each frequency is based.

		F_{rodent}			$F_{questing}$		
ospC group (Lagal) [a]	R_0	PR (n=89)	SW (n=171)	Comb (n=260)	PR (n=81)	SW (n=51)	Comb (n=132)
A1.v1	0.878						
A1.v2	5.237	0.225	0.433	0.362	0.123	0.314	0.197
A2	4.568	0.000	0.053	0.035	0.136	0.078	0.114
A3	3.328	0.000	0.076	0.050	0.000	0.059	0.023
A4	2.185	0.022	0.000	0.008	0.012	0.039	0.023
ME	2.036	0.303	0.012	0.112	0.160	0.059	0.121
YU	5.163[b]	0.404	0.462	0.442	0.210	0.333	0.258
B. b. s. l.	4.052[c]						

[a] Nomenclature of *B. afzelii ospC* groups developed by Lagal *et al*. (2003) and Pérez *et al*. (2011). This terminology is used in the present study.

[b] R_0 of strain YU (5.163) is the geometric average of strains YU.v1 (R_0 = 5.519) and YU.v2 (R_0 = 4.830).

[c] R_0 of *Borrelia burgdorferi* sensu lato (*B. b.* s. l.) was calculated using the parameter estimates from Table 2 in Harrison *et al*. (2011).

References

Harrison, A., Montgomery, W.I., Bown, K.J., 2011. Investigating the persistence of tick-borne pathogens via the R-0 model. Parasitology 138, 896-905.

Lagal, V., Postic, D., Ruzic-Sabljic, E., Baranton, G., 2003. Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness. Journal of Clinical Microbiology 41, 5059-5065.

Pérez, D., Kneubühler, Y., Rais, O., Jouda, F., Gern, L., 2011. *Borrelia afzelii ospC* genotype diversity in *Ixodes ricinus* questing ticks and ticks from rodents in two Lyme borreliosis endemic areas: Contribution of co-feeding ticks. Ticks and Tick-borne Diseases 2, 137-142.

6. 2nd Paper

Cross-immunity and community structure of a multiple-strain pathogen in the tick vector

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Cross-Immunity and Community Structure of a Multiple-Strain Pathogen in the Tick Vector

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Many vector-borne pathogens consist of multiple strains that circulate in both the vertebrate host and the arthropod vector. Characterization of the community of pathogen strains in the arthropod vector is therefore important for understanding the epidemiology of mixed vector-borne infections. *Borrelia afzelii* and *B. garinii* are two species of tick-borne bacteria that cause Lyme disease in humans. These two sympatric pathogens use the same tick, *Ixodes ricinus*, but are adapted to different classes of vertebrate hosts. Both *Borrelia* species consist of multiple strains that are classified using the highly polymorphic *ospC* gene. Vertebrate cross-immunity against the OspC antigen is predicted to structure the community of multiple-strain *Borrelia* pathogens. *Borrelia* isolates were cultured from field-collected *I. ricinus* ticks over a period spanning 11 years. The *Borrelia* species of each isolate was identified using a reverse line blot (RLB) assay. Deep sequencing was used to characterize the *ospC* communities of 190 *B. afzelii* isolates and 193 *B. garinii* isolates. Infections with multiple *ospC* strains were common in ticks, but vertebrate cross-immunity did not influence the strain structure in the tick vector. The pattern of genetic variation at the *ospC* locus suggested that vertebrate cross-immunity exerts strong selection against intermediately divergent *ospC* alleles. Deep sequencing found that more than 50% of our isolates contained exotic *ospC* alleles derived from other *Borrelia* species. Two alternative explanations for these exotic *ospC* alleles are cryptic coinfections that were not detected by the RLB assay or horizontal transfer of the *ospC* gene between *Borrelia* species.

Many vector-borne pathogens consist of multiple genetically distinct strains (1–4). The adaptive arm of the vertebrate immune system plays a key role in generating and maintaining this diversity of pathogen strains (5–7). Genetic diversity is often the highest at loci coding for surface-exposed pathogen molecules that function during the invasion and infection of host tissues (8, 9). The study of these highly polymorphic pathogen molecules is important for understanding how cross-reactive acquired immunity can mediate indirect competition and superinfection in the vertebrate host (10, 11). In addition, these pathogen outer surface proteins are often used to characterize pathogen strains because they provide an upper estimate of pathogen strain richness.

In vector-borne diseases, the community of pathogen strains can be studied in both the vertebrate host and the arthropod vector. The vertebrate immune system creates nonrandom associations between pathogen strains (1, 12) that are subsequently transmitted to the arthropod vector. Conversely, the study of mixed infections in the arthropod vector can provide information on the processes that structure the community of multiple pathogen strains in the vertebrate host (13, 14). In addition, estimates of strain richness in the arthropod vector are important for understanding the frequency with which vertebrate hosts are exposed to infections with multiple strains (13, 15). In summary, studying the diversity of pathogen strains in the arthropod vector is important for understanding the epidemiology of vector-borne diseases.

Borrelia afzelii and *B. garinii* are two species of tick-borne spirochete bacteria that cause Lyme borreliosis (LB) in Europe (16). These two sympatric pathogens use the same tick vector, *Ixodes ricinus*, but are adapted to different classes of vertebrate hosts (17–19). *Borrelia afzelii* cycles in rodents (20–25), whereas *B. garinii* cycles in birds (24, 26–29), and this host specificity is mediated by the vertebrate complement system (30, 31). Previous work has shown that the ecological separation is not 100% complete and that double infections with these two *Borrelia* species do occur inside ticks (14, 32–34). The tick vector, *I. ricinus*, has three stages, larva, nymph, and adult, that take a single blood meal to develop into the next stage. The reservoir hosts are infected by nymphal ticks, which acquired the spirochete in the previous year during the larval blood meal (vertical transmission is rare [35, 36]). In summary, *B. afzelii* and *B. garinii* occupy distinct ecological niches in the vertebrate reservoir host community, but this ecological separation is not 100% complete.

The three most-studied *Borrelia* species (*B. burgdorferi sensu stricto*, *B. afzelii*, and *B. garinii*) all contain a highly polymorphic, single-locus *ospC* gene that codes for outer surface protein C (OspC) (46, 47, 49, 72). The OspC protein is critical for establishing infection inside the vertebrate host (37, 38). This antigen induces a protective antibody response in the vertebrate host (39–41) that is highly specific for strains carrying that particular *ospC* allele (42–44). The *ospC* gene has a large amount of sequence variation that allows *ospC* alleles to be classified into discrete major *ospC* groups (45). A major *ospC* group is defined as a cluster of

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ospC alleles that is more than 8% divergent in DNA sequence from other such major groups and less than 2% divergent within the same major group (45). The absence of intermediately divergent *ospC* alleles (2% to 8%) suggests that cross-reactive acquired immunity has structured the community of *Borrelia* pathogens into discrete OspC serotypes (45–47). As *Borrelia* strains are essentially clonal (48–51), the *ospC* gene is a commonly used genetic marker for studying the ecology and evolution of multiple strains of *Borrelia* pathogens (1, 13, 45, 52–55).

In the present study, we used next-generation sequencing (NGS) methods to characterize the community of genetically distinct entities characterized by a particular major *ospC* group (referred to here as "*ospC* strains") of *B. afzelii* and *B. garinii* that were found in the same local community of *I. ricinus* ticks over a period of 11 years. As previous studies of the *ospC* polymorphism in European *Borrelia* species have used more traditional genotyping methods (46, 47, 49, 55), we expected that a larger sequencing effort by the NGS approach would change our understanding of the *ospC* gene polymorphism (52, 54). We predicted that infections with multiple *ospC* strains in *I. ricinus* nymphs would be common. We predicted that genetically similar major *ospC* groups would be less likely to occur in the same tick, as was previously demonstrated for *B. afzelii* in wild rodents (1). We predicted that our NGS approach would detect the intermediately divergent *ospC* alleles that must periodically appear, despite strong selection by the vertebrate immune system. Finally, we predicted that the genetic divergence between the *ospC* alleles of *B. garinii* and *B. afzelii* could take on intermediate divergence values (2% to 8%) because the two *Borrelia* species occur in different vertebrate hosts and their OspC antigens are therefore not subject to cross-immunity.

(This study is part of the Ph.D. thesis of Jonas Durand.)

MATERIALS AND METHODS

Tick collection and *Borrelia* detection. *Ixodes ricinus* ticks were sampled monthly from the Bois de l'Hôpital site in a deciduous forest near Neuchâtel, Switzerland (47°00′55.6″N, 6°94′16.7″E; surface area of 1 ha), over a period of 11 years (2000 to 2010). We collected questing ticks by dragging a white flag (surface area of 1 m^2) over the vegetation along four transects of 100 m. After collection, the species and stage of each tick were identified. In the laboratory, each tick was cut into two halves using sterile scissors. One tick half was used to determine spirochete infection via immunofluorescence microscopy (56). The other tick half was used to grow *Borrelia* isolates as follows. Half-ticks were placed in culture tubes containing BSK II medium (57), incubated at 34°C, and examined by dark-field microscopy every 10 days for 2 months. DNA was extracted from all spirochete-positive BSK II medium cultures. Briefly, 1 ml of culture medium was centrifuged and washed two times with phosphate-buffered saline before the pellet was suspended in 50 ml of ultrafiltered water. Pellets were incubated at 100°C for 10 min, and the thermolysates were stored at −20°C. *Borrelia* species were identified using a PCR-reverse line blot (RLB) assay following the method of Schouls et al. (58). The PCR protocol amplifies the 23S-5S spacer gene of *B. burgdorferi sensu lato* and was previously described by Alekseev et al. (59), and the RLB assay probes were described by Burri et al. (60).

As of 2012, the isolate archive at the University of Neuchâtel contained ~3,000 tick-derived spirochete isolates for which the *Borrelia* species had been identified by RLB assay. In this region of Switzerland, *I. ricinus* ticks are commonly infected with the following *Borrelia* species: *B. afzelii*, *B. bavariensis*, *B. burgdorferi sensu stricto*, *B. garinii*, and *B. valaisiana* (14). In this study, we focused on *B. afzelii* and *B. garinii* because they are the two most common species in the isolate archive at the University of Neuchâtel and because they are the most common etiological agents of LB in Europe. We included only *Borrelia* isolates derived from questing nymphs because this stage takes only one blood meal during the previous larval stage. We included only those isolates that, according to the RLB assay, were infected with a single *Borrelia* species (either *B. afzelii* or *B. garinii*) so that we could assign major *ospC* groups with confidence to the correct *Borrelia* species. We established a database of all the nymph-derived spirochete isolates of either *B. afzelii* or *B. garinii* obtained from 2000 to 2010. For each of the two *Borrelia* species, we randomly sampled a maximum of 20 nymph-derived isolates for each of the 11 years. Using this sampling strategy, we obtained 193 isolates of *B. afzelii* and 190 isolates of *B. garinii* (Table 1).

TABLE 1 Number of nymph-derived spirochete isolates used for each of the 22 combinations of *Borrelia* species and year[a]

	No. of nymph-derived isolates tested (total no.)			
	B. afzelii		*B. garinii*	
Yr	Infected nymphs	Uninfected nymphs	Infected nymphs	Uninfected nymphs
2000	20 (51)[b]	234 (599)[c]	20 (41)	297 (609)
2001	17 (37)[d]	295 (643)	18 (37)	312 (643)
2002	16 (69)	153 (661)	19 (39)	336 (691)
2003	12 (21)	261 (458)	14 (19)	338 (460)
2004	13 (21)	298 (448)	14 (16)	396 (453)
2005	19 (88)	104 (459)	19 (29)	339 (518)
2006	20 (57)	171 (490)	15 (33)	249 (514)
2007	20 (41)	275 (564)	20 (62)	175 (543)
2008	18 (31)	347 (598)	20 (54)	212 (575)
2009	20 (51)	211 (540)	13 (14)	535 (577)
2010	18 (58)	156 (503)	18 (32)	297 (529)

[a] We randomly selected a maximum of 20 nymph-derived isolates for NGS (the total number of infected nymphs is shown in parentheses) for each combination of *Borrelia* species and year. To calculate the absolute frequency of each strain (F3 in Tables 2 and 3), we used a subsample of uninfected nymphs (the total number of uninfected nymphs is shown in parentheses) for each combination of *Borrelia* species and year. This subsample of uninfected nymphs is proportional to the subsample of infected nymphs.
[b] In the year 2000, 51 nymph-derived cultures tested positive for *B. afzelii*. We randomly selected 20 of these 51 isolates of *B. afzelii* and determined the community of *ospC* strains using NGS.
[c] In the year 2000, 599 nymph-derived cultures did not test positive for *B. afzelii*. We calculated the absolute frequencies of the *B. afzelii ospC* strains (F3 in Tables 2 and 3), using a subsample of 234 cultures [$(20/51) \times 599 = 234$] not infected with *B. afzelii*.
[d] In the year 2001, 37 nymph-derived cultures tested positive for *B. afzelii*, but only 17 of these 37 isolates of *B. afzelii* had sufficient material for NGS.

Deep sequencing of the *ospC* strain community in nymph-derived spirochete isolates. The *ospC* gene was amplified using a nested PCR method consisting of two consecutive amplification steps that was described by Bunikis et al. (49). We modified the oligonucleotides for the second PCR to include 454 Life Science's A or B sequencing adapter, the key, and the template-specific primers (see the supplemental material). The forward primer was tagged for forward sequencing. The reaction was performed in an automatic thermocycler (Eppendorf, USA). We checked the success of the PCRs by visualizing the amplicons on a 1% agarose gel stained with Midori green (Labgene, Switzerland). The PCR products were purified using a DNA purification kit (Wizard SV Gel and PCR cleanup system; Promega, Switzerland). We created eight different pools of *Borrelia* isolates for the 454 sequencing run (40 to 50 isolates per pool). Isolates were randomly assigned to pools subject to the constraint that each pool contained a balanced number of isolates from each year. Sequencing was done on a 454 Roche GS FLX sequencing apparatus and was outsourced to Microsynth AG (Balgach, Switzerland).

Bioinformatics. (i) Cleaning of *ospC* gene sequences. The 454 sequencing run produced 352,882 sequences of the *ospC* gene. After cleaning the data set (see the supplemental material), we retained 240,410 *ospC* gene sequences that were each 521 bp long.

(ii) Clustering analysis of major *ospC* groups. We defined an *a priori* set of seed sequences prior to conducting the cluster analysis. Seed sequences were defined as those sequences that occurred at least 50 times in our cleaned data set. The seed sequences were selected using the cd-hit-dup function of CD-HIT software (61, 62) with an error rate of 1%, which produced a set of 331 seed sequences. These sequences were used as seeds to perform a cluster analysis of the data from the cleaned data set with similarity thresholds ranging from 98% to 91% using the software CD-HIT. In addition, we created a phylogenetic tree (1,000 bootstraps) of the 331 seed sequences using the PhyML program (63). This tree confirmed the reliability of the clustering of the seeds. To have a measure of genetic similarity of the major *ospC* groups, we calculated the mean pairwise genetic distances using the distmat program (64) with a Kimura model. Each mean pairwise genetic distance was based on 30 sequences of each major *ospC* group in the pair. We also calculated the focal pairwise genetic distance for each major *ospC* group. This coefficient measures the average genetic distance between a focal major *ospC* group and all other major *ospC* groups.

(iii) Nature of genetic variation within major *ospC* groups. The nature of the genetic variation observed within each major *ospC* group was investigated. Only the first 350 nucleotides of the *ospC* gene sequences were analyzed because the end of the forward sequences contained too much sequencing error. For each major *ospC* group, 30 sequences were selected from the seed sequences that had been used for the clustering (a total of 690 sequences). For major *ospC* groups with less than 30 seed sequences, nonseed sequences (sequences that occurred less than 50 times in our data set) were added. Sequences were aligned manually, and the numbers of synonymous and nonsynonymous substitutions, insertions, and deletions were recorded.

Nomenclature of major *ospC* groups. Several papers have dealt with the nomenclature of the major *ospC* groups in *B. burgdorferi sensu lato* (46, 47, 49, 54, 65, 66). In the United States, workers have developed a well-established system of names for the major *ospC* groups of *B. burgdorferi sensu stricto* (45). In Europe, in contrast, there is no universally adopted naming system for the major *ospC* groups of *B. afzelii* and *B. garinii* (47, 49). In the current study, we present a summary of all the different names that have been used depending on the author (see Tables S1 and S2 in the supplemental material). In addition, we suggest that future work on the major *ospC* groups of these two *Borrelia* species follow the nomenclature developed by Bunikis et al. (49), Hellgren et al. (50), and Strandh and Råberg (54) for *B. afzelii* and that developed by Lagal et al. (47) for *B. garinii*. Here, we use "*ospC* strain" to refer to a genetically distinct entity characterized by a particular major *ospC* group.

Confirmation of *Borrelia* species classification by RLB assay. To confirm the reliability of the RLB assay, we performed additional sequencing of a subset of 120 isolates. According to the RLB assay, 60 of these isolates were singly infected with *B. afzelii* and 60 were singly infected with *B. garinii*. For each of these 120 samples, we used PCR to amplify the *recA* gene and the *hbb* gene according to the protocol described by Richter et al. (67). The amplicons were purified using a Qiagen purification kit. Purified amplicons were sent to Microsynth AG (Balgach, Switzerland) for Sanger sequencing.

Statistical methods. All statistical analyses were performed using R (68).

(i) Prevalence of *ospC* strains inside ticks. The absolute prevalence of a pathogen strain is its frequency in the entire population (uninfected and infected individuals). The relative prevalence of a pathogen strain is its frequency in the subpopulation of infected individuals. In the present study, the absolute prevalence of infection was determined for each *Borrelia* species by the RLB assay. We then used 454 sequencing to estimate the relative prevalence of each major *ospC* group in a randomly selected subset of *Borrelia* isolates. Some analyses of multiple-strain pathogen communities require estimates of the absolute prevalence. We therefore converted our relative prevalences to absolute prevalences by including the fraction of uninfected ticks that corresponded to the fraction of infected ticks that had been processed by 454 sequencing for each of the 22 combinations of *Borrelia* species and year (Table 1). For example, if we subsampled half of the infected ticks for a particular combination of *Borrelia* species and year, then we included half of the uninfected ticks for that combination of species and year. In this way, our data set was a random and representative sample of the populations of infected and uninfected ticks (Table 1). All analyses described below were conducted separately for each *Borrelia* species.

(ii) Aggregation of *ospC* strains inside ticks. Strain richness refers to the number of different *ospC* strains that were found within a single tick (including uninfected ticks). To test whether strains were aggregated inside ticks, we compared the observed distribution of strain richness to the Poisson distribution, where the variance is equal to the mean (69). Aggregation occurs when the number of ticks with multiple strains is greater than the Poisson expectation (variance/mean > 1). We used a bootstrap analysis to calculate the 95% confidence limits of the variance-to-mean ratio.

(iii) Association of *ospC* strains inside ticks. The strength and the direction of each pairwise association were estimated using the association coefficient described by Pielou (70). We used the absolute frequencies to calculate this pairwise association coefficient, which has a range from -1 (a complete negative association) to $+1$ (a complete positive association) and is undefined if all hosts are uninfected or infected with both strains. For each pair of strains, the statistical significance of the pairwise association was assessed using a two-tailed Fisher exact test. The significance level was adjusted for multiple comparisons using the Bonferroni correction (for *B. afzelii*, $P = 0.05/171$ pairs $= 0.000292$; for *B. garinii*, $P = 0.05/153$ pairs $= 0.000327$).

We also calculated the focal pairwise association coefficient for each major *ospC* group. This coefficient is a measure of whether a focal major *ospC* group is more or less likely to co-occur with other *ospC* strains. Strains with high focal pairwise association coefficients ($+1$) are more likely to be found in multiple infections than strains with low focal pairwise association coefficients (-1).

Finally, we tested whether strains with genetically dissimilar major *ospC* groups were more likely to occur together inside the same tick. Specifically, we tested for a correlation between the pairwise associations and the pairwise genetic distances using the Mantel test in the vegan package in R (71).

Nucleotide sequence accession numbers. The *ospC* gene sequence data have been deposited in the Sequence Read Archive under BioProject PRJNA293785 with the accession number SRP063760. A type sequence of each major *ospC* group of *B. afzelii* and *B. garinii* is available in GenBank (see the supplemental material for the GenBank accession numbers). The GenBank accession numbers for major *ospC* groups V1 of *B. valaisiana* and Q of *B. burgdorferi sensu stricto* are AF093483 and JQ253799, respectively.

RESULTS

Clustering analysis. For each of the two *Borrelia* species, we randomly sampled a maximum of 20 nymph-derived isolates for each of the 11 years. Using this sampling strategy, we obtained 193 isolates of *B. afzelii* and 190 isolates of *B. garinii* (Table 1). The 454 sequencing run produced 352,882 sequences of the *ospC* gene. After cleaning the data set (see the supplemental material), we retained 240,410 *ospC* gene sequences that were each 521 bp long. There were 23 different major *ospC* groups in our local population of *I. ricinus* ticks, and this number was stable across a range of similarity thresholds (93% to 98%; see Fig. S1 in the supplemental material). According to BLAST analysis of the 23 major *ospC* groups, 10 belonged to *B. afzelii* (*ospC* groups A1, A2, A3, A5, A7, A9, A10, A11, A12, and A14), 11 belonged to *B. garinii* (*ospC* groups G2, G4, G6, G7, G8, G9, G10, G11, G13, G14, and G15), 1 belonged to *B. valaisiana* (*ospC* group V1), and 1 belonged to *B. burgdorferi sensu stricto* (*ospC* group Q). All of the 23 major

TABLE 2 Frequencies of major *ospC* groups in the *B. afzelii* isolates

ospC allele	*ospC* status	Frequency (%)		
		F1[a]	F2[b]	F3[c]
A10	Native[d]	43.35 (49,601)	54.40 (105)	3.90 (105)
A9	Native[d]	14.31 (16,372)	31.09 (60)	2.23 (60)
A1	Native[d]	12.28 (14,056)	24.35 (47)	1.75 (47)
A14	Native[d]	11.02 (12,613)	24.87 (48)	1.78 (48)
A11	Native[d]	2.31 (2,642)	10.36 (20)	0.74 (20)
A12	Native	4.61 (5,270)	10.36 (20)	0.74 (20)
A3	Native	2.69 (3,080)	4.66 (9)	0.33 (9)
A5	Native	2.90 (3,318)	7.77 (15)	0.56 (15)
A2	Native	2.64 (3,023)	8.81 (17)	0.63 (17)
A7	Native	2.02 (2,307)	7.25 (14)	0.52 (14)
G9	Exotic[e]	0.49 (560)	7.77 (15)	0.56 (15)
G11	Exotic[e]	0.39 (449)	4.15 (8)	0.30 (8)
Q	Exotic	0.36 (409)	1.04 (2)	0.07 (2)
V1	Exotic	0.27 (314)	0.52 (1)	0.04 (1)
G13	Exotic[e]	0.26 (302)	10.88 (21)	0.78 (21)
G8	Exotic[e]	0.04 (45)	8.81 (17)	0.63 (17)
G14	Exotic[e]	0.02 (27)	7.77 (15)	0.56 (15)
G2	Exotic[e]	0.02 (24)	6.74 (13)	0.48 (13)
G7	Exotic[e]	0.02 (20)	5.70 (11)	0.41 (11)

[a] Frequency 1 (F1) refers to the frequency of the major *ospC* group in the sample of sequences (n = 114,432 sequences), and values in parentheses are numbers of sequences.
[b] Frequency 2 (F2) refers to the frequency of the major *ospC* group in the sample of infected nymphs (n = 193 nymphs), and values in parentheses are numbers of infected nymphs.
[c] Frequency 3 (F3) refers to the frequency of the major *ospC* group in the sample of infected and uninfected nymphs (n = 2,500 nymphs), and values in parentheses are numbers of infected nymphs.
[d] These native major *ospC* groups were horizontally transferred from *B. afzelii* to *B. garinii*.
[e] These exotic major *ospC* groups were horizontally transferred from *B. garinii* to *B. afzelii*.

TABLE 3 Frequencies of major *ospC* groups in the *B. garinii* isolates

ospC allele	*ospC* status	Frequency (%)		
		F1[a]	F2[b]	F3[c]
G8	Native[d]	25.19 (31,737)	42.11 (80)	2.18 (80)
G14	Native[d]	16.85 (21,223)	30.00 (57)	1.55 (57)
G9	Native[d]	11.71 (14,748)	22.63 (43)	1.17 (43)
G13	Native[d]	10.34 (13,030)	27.89 (53)	1.44 (53)
G2	Native[d]	10.68 (13,457)	21.05 (40)	1.09 (40)
G7	Native[d]	9.57 (12,057)	31.05 (59)	1.61 (59)
G11	Native[d]	4.28 (5,389)	14.74 (28)	0.76 (28)
G4	Native	3.99 (5,025)	14.74 (28)	0.76 (28)
G6	Native	3.14 (3,952)	11.58 (22)	0.60 (22)
G15	Native	0.61 (774)	3.68 (7)	0.19 (7)
G10	Native	0.55 (689)	1.58 (3)	0.08 (3)
A10	Exotic[e]	1.75 (2,209)	31.58 (60)	1.63 (60)
A1	Exotic[e]	0.74 (930)	12.11 (23)	0.63 (23)
V1	Exotic	0.41 (512)	1.58 (3)	0.08 (3)
Q	Exotic	0.10 (128)	2.63 (5)	0.14 (5)
A9	Exotic[e]	0.04 (50)	11.05 (21)	0.57 (21)
A14	Exotic[e]	0.03 (36)	10.53 (20)	0.54 (20)
A11	Exotic[e]	0.03 (32)	5.26 (10)	0.27 (10)

[a] Frequency 1 (F1) refers to the frequency of the major *ospC* group in the sample of sequences (n = 125,978 sequences), and values in parentheses are numbers of sequences.
[b] Frequency 2 (F2) refers to the frequency of the major *ospC* group in the sample of infected nymphs (n = 190 nymphs), and values in parentheses are numbers of infected nymphs.
[c] Frequency 3 (F3) refers to the frequency of the major *ospC* group in the sample of infected and uninfected nymphs (n = 3,486 nymphs), and values in parentheses are numbers of infected nymphs.
[d] These native major *ospC* groups were horizontally transferred from *B. garinii* to *B. afzelii*.
[e] These exotic major *ospC* groups were horizontally transferred from *B. afzelii* to *B. garinii*.

ospC groups had been previously described in the literature (1, 46, 47, 72).

There were a number of major *ospC* groups that were found in both *Borrelia* species. Five of the 10 *B. afzelii* major *ospC* groups (A1, A9, A10, A11, A14) were found in isolates that were singly infected with *B. garinii* according to the RLB assay. Conversely, 7 of the 11 *B. garinii* major *ospC* groups (G2, G7, G8, G9, G11, G13, G14) were found in isolates that were singly infected with *B. afzelii* according to the RLB assay. In what follows, we use the terms "native" and "exotic" to distinguish between these two types of major *ospC* groups. Thus, *B. afzelii* had 10 native and 9 exotic *ospC* groups (7 *B. garinii*-derived *ospC* groups and the V1 and Q *ospC* groups) for a total of 19 major *ospC* groups (Table 2), and *B. garinii* had 11 native and 7 exotic *ospC* groups (5 *B. afzelii*-derived *ospC* groups and the V1 and Q *ospC* groups) for a total of 18 major *ospC* groups (Table 3).

Description of the *Borrelia ospC* strain communities in *I. ricinus*. The major *ospC* groups were more evenly distributed in *B. garinii* than *B. afzelii*. In *B. afzelii*, the strain of *ospC* group A10 (referred to here as strain A10) was found in 54.40% of the infected nymphs (Table 2). Three strains (A1, A9, A14) were found in 24.35% to 31.09% of the infected nymphs, and the remaining 15 strains were found in less than 11% of the infected nymphs (Table 2). In *B. garinii*, strain G8 was found in 42.11% of the infected nymphs, and six other strains were found in 21.05% to 31.58% of the infected nymphs (Table 3). Six strains were found in 10.53% to 14.74% of the infected nymphs, whereas the five remaining strains were found in less than 6% of the infected nymphs. In *B. garinii*, the exotic major *ospC* group A10 was very common and occurred in 31.58% of the infected nymphs.

Pairwise genetic distances within the native major *ospC* groups. For *B. afzelii*, the mean pairwise genetic distance within each of the 10 native major *ospC* groups was 1.06% (n = 44,250 pairwise comparisons, range = 0.42% to 2.16%). For *B. garinii*, the mean pairwise genetic distance within each of the 11 native major *ospC* groups was 1.14% (n = 54,285 pairwise comparisons, range = 0.66% to 1.63%).

Nature of genetic variation within major *ospC* groups. Across the 690 sequences belonging to the 23 major *ospC* groups, there were 140 nucleotide substitutions, of which 58.9% were synonymous (83/141) and 41.1% were nonsynonymous (58/141). Single nucleotide insertions and deletions and codon deletions were common, whereas double nucleotide insertions or deletions were rare (see Table S3 in the supplemental material).

Pairwise genetic distances between the native major *ospC* groups. We calculated the pairwise genetic distances between the 21 native major *ospC* groups (i.e., the exotic major *ospC* groups were excluded). The conspecific or allospecific pairwise genetic distance refers to whether the two native major *ospC* groups belonged to the same *Borrelia* species or to different *Borrelia* species, respectively. The mean conspecific pairwise genetic distances of *B. afzelii* (n = 45 pairwise comparisons, mean = 17.84%, range = 8.86% to 23.59%; Fig. 1) and *B. garinii* (n = 55 pairwise compar-

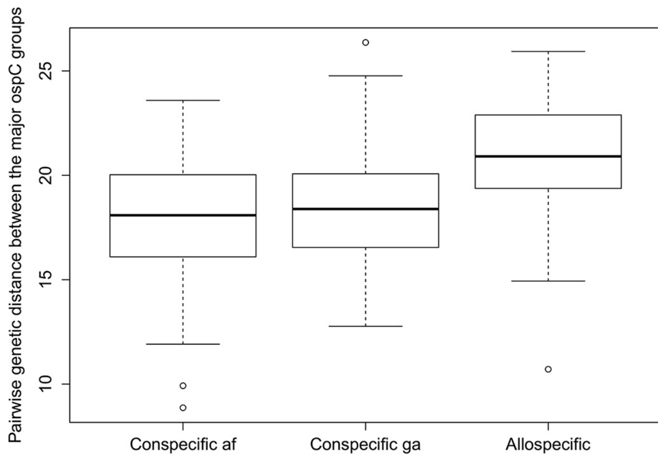

FIG 1 Distribution of pairwise genetic distances between major *ospC* groups. The genetic distances for three different pairwise comparisons are shown: conspecific *B. afzelii* (af; $n = 55$), conspecific *B. garinii* (ga; $n = 55$), and allospecific (*B. afzelii* versus *B. garinii*; $n = 121$). Shown are the medians (heavy black lines), the 25th and 75th percentiles (edges of the boxes), the minimum and maximum values (whiskers), and the outliers (circles).

isons, mean = 18.54%, range = 12.77% to 26.36%; Fig. 1) were not significantly different ($t = -1.13$, degrees of freedom [df] = 98, $P = 0.261$). The mean allospecific pairwise genetic distance ($n = 110$ comparisons, mean = 20.94%, range = 10.71% to 25.93%; Fig. 1) was significantly larger than the mean conspecific pairwise genetic distance ($t = 6.921$, df = 208, $P < 0.001$).

Strain richness in nymphal ticks. The strain richness of the infected nymphal ticks was calculated using both the native and exotic major *ospC* groups for each *Borrelia* species. Most nymphal ticks were infected with multiple *ospC* strains. For *B. afzelii* (19 major *ospC* groups), 78.76% (152/193) of the infected nymphal ticks carried multiple *ospC* strains. For *B. garinii* (18 major *ospC* groups), 84.74% (161/190) of the infected nymphal ticks carried multiple *ospC* strains. The mean strain richness of *B. garinii* (2.96 strains per infected tick, range = 1 to 11 strains per tick) was 25% higher than that of *B. afzelii* (2.37 strains per infected tick, range = 1 to 7 strains per tick), and this difference was statistically significant ($t = -3.764$, df = 381, $P < 0.001$).

Aggregation of strains in nymphal ticks. The variance-to-mean ratio of strain richness was calculated using the absolute frequencies of both the native and exotic major *ospC* groups for each *Borrelia* species. The variance-to-mean ratio of strain richness was significantly greater than 1.0 for both *Borrelia* species, indicating that the *ospC* strains were highly aggregated in the nymphal ticks (Fig. 2). The variance-to-mean ratio of strain richness was 47.4% higher in *B. garinii* (mean = 3.98, 95% confidence interval [CI] = 3.44 to 4.51) than in *B. afzelii* (mean = 2.70, 95% CI = 2.50 to 2.91).

Pairwise association index. The pairwise association coefficients within each *Borrelia* species were calculated using the absolute frequencies of both the native and exotic major *ospC* groups. For *B. afzelii* (19 major *ospC* groups), 66.7% (114/171) of the pairwise association coefficients were positive (mean = 0.08, range = −0.01 to 0.35). All of the 39 statistically significant ($P <$ 0.05/171) pairwise associations were positive (mean = 0.20, range = 0.11 to 0.35). For *B. garinii* (18 major *ospC* groups), 77.8% (119/153) of the pairwise association coefficients were positive (mean = 0.15, range = −0.004 to 0.48). Again, all of the 78 statistically significant ($P <$ 0.05/153) pairwise associations were positive (mean = 0.25, range = 0.11 to 0.48). The mean pairwise association coefficient of *B. garinii* was significantly greater than that of *B. afzelii* ($t = 6.10$, df = 322, $P < 0.001$). The results were the same for the subset of 117 statistically significant pairwise association coefficients ($t = 3.54$, df = 115, $P < 0.001$).

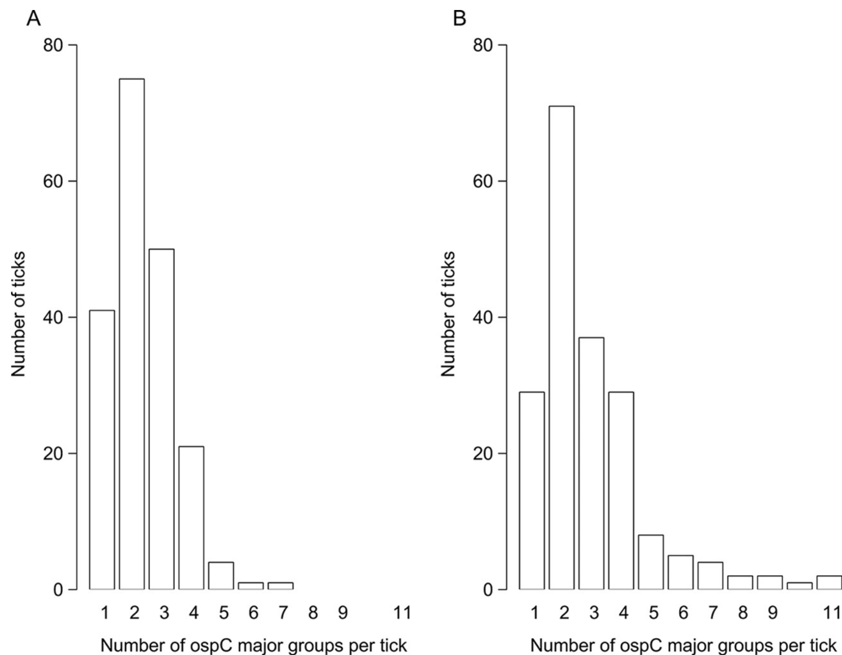

FIG 2 Distribution of strain richness in nymphs infected with *B. afzelii* (A) and nymphs infected with *B. garinii* (B). The numbers of ticks infected with zero strains are not shown for scaling purposes (i.e., uninfected nymphs in Table 1).

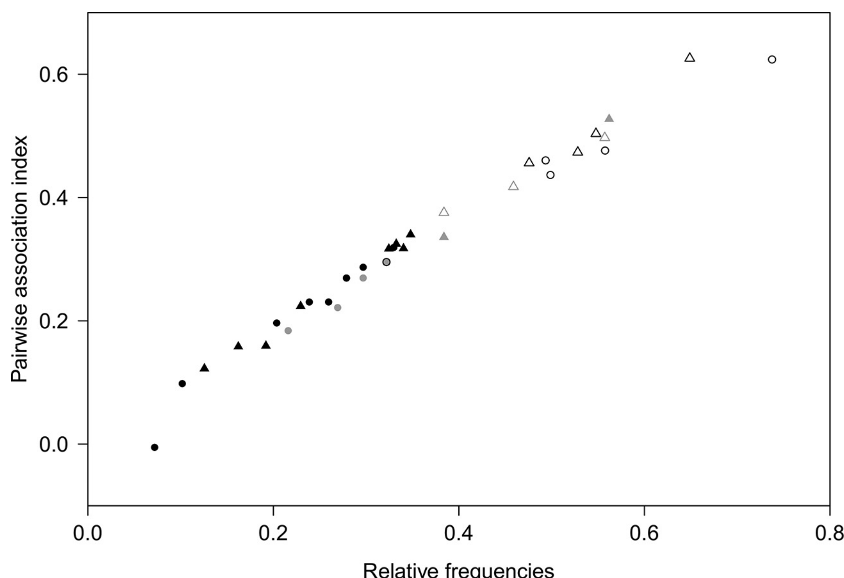

FIG 3 Positive relationship between the focal pairwise association index and the square root-transformed relative frequency of each major *ospC* group inside the tick. Results are combined for *B. afzelii* (circles) and *B. garinii* (triangles). The native major *ospC* groups that were horizontally transferred to the other *Borrelia* species (white symbols; $n = 12$) had much higher relative frequencies and pairwise association indices in the native *Borrelia* species than the native major *ospC* groups that were not transferred (gray symbols; $n = 9$). The exotic major *ospC* groups ($n = 16$) are shown in black symbols.

The mean focal pairwise association index of *B. garinii* (n = 18 major *ospC* groups, mean = 0.35, range = 0.12 to 0.63) was 20.7% higher than that of *B. afzelii* (n = 19 major *ospC* groups, mean = 0.29, range = −0.005 to 0.62), but this difference was not significant ($t = 1.35$, df = 35, $P = 0.187$). Some *ospC* strains, such as *B. afzelii ospC* strain A10 and *B. garinii ospC* strains G8, G14, and A10, had very high focal pairwise association coefficients (>0.50).

Relationship between relative frequency and the focal pairwise association index. The relative frequency (F2 in Tables 2 and 3) of each major *ospC* group (transformed by the square root) was a highly significant predictor of the focal pairwise association index in *B. afzelii* (n = 19 major *ospC* groups, $F_{1,\ 17} = 565.7$, $P < 0.001$, $r^2 = 0.971$; Fig. 3) and in *B. garinii* (n = 18 major *ospC* groups, $F_{1,\ 16} = 1{,}688.0$, $P < 0.001$, $r^2 = 0.991$; Fig. 3). An analysis of covariance found no significant interaction between *Borrelia* species and relative frequency on the pairwise association index ($F_{1,\ 33} = 0.9$, $P = 0.354$). The slope of the relationship between the focal pairwise association index and the square root-transformed relative frequency was therefore the same for each *Borrelia* species (0.90 ± 0.044). After controlling for the relative frequency, the focal pairwise association index remained significantly higher for *B. garinii* than *B. afzelii* ($F_{1,\ 34} = 4.5$, $P = 0.041$).

Relationship between pairwise genetic distance and pairwise association index. The correlation between the pairwise genetic distance between the major *ospC* groups and the pairwise association index of the major *ospC* groups inside the ticks was not statistically significant for either *B. afzelii* (n = 171 pairwise elements; Mantel test, $r = -0.041$, $P = 0.630$; Fig. 4) or *B. garinii* (n = 153 pairwise elements; Mantel test, $r = -0.053$, $P = 0.664$; Fig. 4). Similarly, the correlation between the focal pairwise genetic distance and the focal pairwise association index was not statistically significant for either *B. afzelii* (n = 19 major *ospC* groups; Pearson correlation test, $r = 0.032$, $t = 0.13$, df = 17, P = 0.897; Fig. 5) or *B. garinii* (n = 18 major *ospC* groups; Pearson correlation test, $r = -0.175$, $t = -0.71$, df = 16, $P = 0.485$; Fig. 5).

Exotic major *ospC* groups were widespread in both *Borrelia* species. For the *B. afzelii*-infected nymphs (as determined by the RLB assay), 42.0% (81/193) of the infections contained exotic major *ospC* groups. Conversely, for the *B. garinii*-infected nymphs (as determined by the RLB assay), 55.3% (105/190) of the infections contained exotic major *ospC* groups. In *B. afzelii*, 1.88% of the *ospC* gene sequences (2,150/114,432) were of exotic origin. In *B. garinii*, 3.09% of the sequences (3,897/125,978) were of exotic origin.

Exotic major *ospC* groups were common in their native *Borrelia* species. The five native *B. afzelii* major *ospC* groups that had undergone horizontal transfer (to *B. garinii*) accounted for 84.62% of the native *ospC* gene sequences of *B. afzelii* (95,598/112,596). Similarly, the seven native *B. garinii* major *ospC* groups that had undergone horizontal transfer (to *B. afzelii*) accounted for 91.45% of the native *ospC* gene sequences of *B. garinii* (111,641/122,081). For the two *Borrelia* species combined, we compared the mean relative frequency between native major *ospC* groups that had experienced horizontal transfer or not (Tables 2 and 3). The 12 native major *ospC* groups that had experienced horizontal transfer had a significantly higher relative frequency ($t = 4.89$, df = 19, $P < 0.001$; Fig. 3) than the 9 native major *ospC* groups that had not been transferred. For the 12 major *ospC* groups that had experienced horizontal transfer, the relative frequency in the native species was always higher than the frequency in the exotic species (paired t test, $t = 8.13$, df = 11, $P < 0.001$). There was a significant correlation between the relative frequencies of these 12 major *ospC* groups in their native species and in their exotic species (Pearson correlation test, $r = 0.783$, $t = 3.99$, df = 10, $P = 0.002$).

Sanger sequencing of *Borrelia* genes to confirm *Borrelia* species classification by RLB assay. For a subsample of 120 *Borrelia*

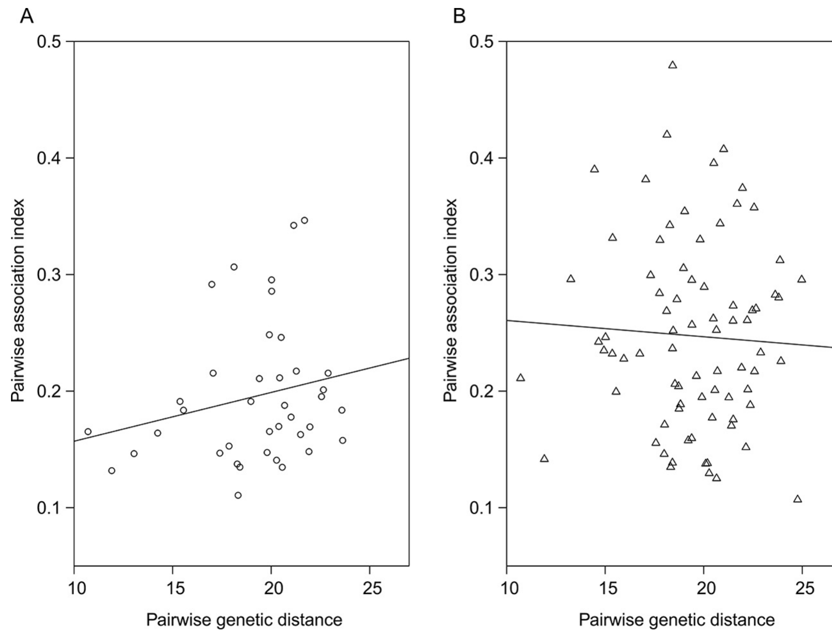

FIG 4 Relationship between the pairwise association index of the major *ospC* groups inside the tick versus the pairwise genetic distance. The relationship is shown for *B. afzelii* (A) and *B. garinii* (B). The regression was done on the subset of the statistically significant pairwise associations indices but was not statistically significant for either *Borrelia* species.

isolates, we obtained high-quality sequences for both the *recA* gene and the *hbb* gene for 110 isolates. This genetic analysis confirmed the *Borrelia* species classification of the RLB assay in 95.5% (105/110) of our samples. In general, this additional analysis showed that the RLB assay provided a reliable classification of the *Borrelia* species.

DISCUSSION

Cross-immunity and co-occurrence of *Borrelia ospC* strains. Cross-reactive acquired immunity should reduce the co-occurrence of antigenically similar strains in the same reservoir host (5, 73, 74). Assuming that the genetic similarity between major *ospC* groups predicts the probability of cross-reactive acquired immunity between their respective OspC antigens, we expected a positive relationship between the pairwise genetic distance and the pairwise association index. A study of multiple *B. afzelii* infections in a population of wild voles found that dissimilar major *ospC* groups were more likely to be found together in the same host than similar major *ospC* groups (1). In contrast, we found no relationship between the pairwise genetic distance and the pairwise association index inside the ticks. A simple explanation for the difference between these two studies is that one looked at the reservoir

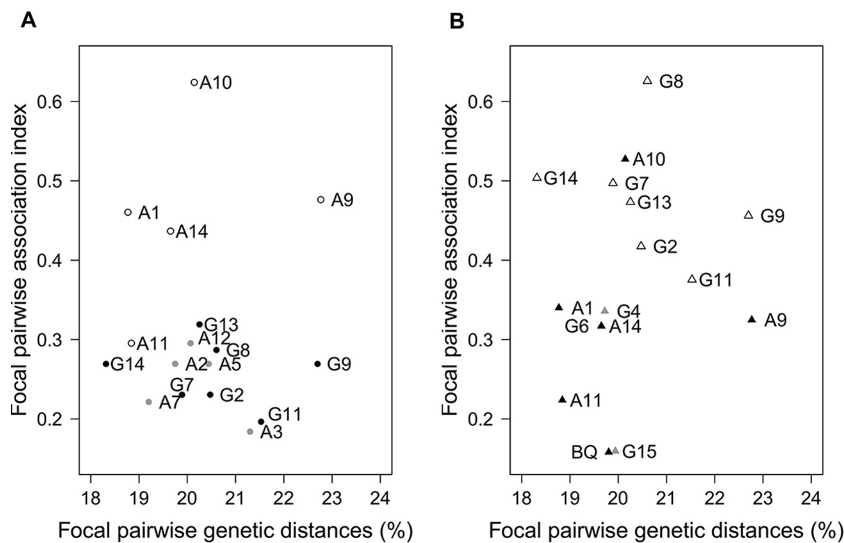

FIG 5 Relationship between the focal pairwise association index and the focal pairwise genetic distance of each major *ospC* group inside the tick. The relationship is shown for *B. afzelii* (A) and *B. garinii* (B). All data points are labeled with the name of the major *ospC* group. White and gray symbols indicate native major *ospC* groups that had and that had not been transferred to the other *Borrelia* species, respectively; black symbols indicate exotic major *ospC* groups.

host, whereas the other looked at the tick vector. The vertebrate immune system structures the community of *ospC* strains inside the reservoir hosts, and the tick is expected to acquire a subsample of this strain community (13, 75). However, the community structure of the colonizing strains might be modified by subsequent events inside the tick. The spirochete load can change dramatically following the blood meal and during the larva-to-nymph molt, and these changes in abundance may alter the community of strains inside the tick (14, 76). There is currently very little information as to how *Borrelia* strains interact inside the tick over time (75).

Strong selection against *ospC* alleles with intermediate divergence. Despite a large sequencing effort (240,410 sequences from 389 ticks over a period of 11 years), our study did not find any *ospC* alleles that were intermediately divergent (2% to 8%) from the community of major *ospC* groups. The average pairwise genetic distance between conspecific major *ospC* groups was always greater than 8%. Thus, our study confirmed the highly discontinuous pattern of genetic variation at the *ospC* locus, as was reported in previous studies of *B. burgdorferi sensu lato* (45–47, 49). The original studies demonstrating the 8% divergence cutoff between the major *ospC* groups in *B. afzelii* and *B. garinii* were based on a total of 140 sequences (46, 47, 49). Thus, it was reasonable to expect that a larger sequencing effort would discover the missing intermediate major *ospC* groups. The present study suggests that strains carrying *ospC* alleles that are 2% to 8% divergent rarely coexist in the same *Borrelia* species. Interestingly, our results also suggest that such intermediately divergent *ospC* alleles rarely coexist in *Borrelia* species occupying different niches in the vertebrate host community. The allospecific pairwise genetic distances between *B. afzelii* and *B. garinii* had the same discontinuous pattern, suggesting that the evolution of the *ospC* allele is not independent in each *Borrelia* species. One explanation is that frequent horizontal transfer of the *ospC* gene (see below) essentially synchronizes the community of major *ospC* groups between these two *Borrelia* species. Thus, a new *ospC* mutant can rise to a detectable frequency only if it is at least 8% divergent from all the major *ospC* groups of all the *Borrelia* pathogens in the local community.

Purifying selection by cross-reactive antibodies is the most plausible explanation for the missing intermediate major *ospC* groups. Mutant major *ospC* groups that are genetically similar and thus antigenically similar to their conspecific counterparts are vulnerable to cross-reactive antibodies, which prevent these mutants from invading the local population (11, 74, 77). Indirect evidence for this hypothesis comes from studies of *B. burgdorferi sensu stricto* that have shown that antibodies against a given OspC antigen provide protection against strains belonging to the same major *ospC* group but not against strains belonging to a different major *ospC* group (42, 44). Thus, in *B. burgdorferi sensu stricto*, we see the end result of divergent selection on the *ospC* antigen to escape from the cross-reactive antibodies directed against its neighbors. However, immune escape between strains carrying different major *ospC* group alleles is not always perfect. A recent study of *B. afzelii* found that OspC antigens belonging to different major *ospC* groups (that were 20.68% divergent in DNA sequence) could still interfere with one another via the cross-reactive antibodies of the rodent host (43). In summary, cross-reactive antibodies are the most plausible source of purifying selection that removes intermediately divergent major *ospC* groups from the local population of *Borrelia* pathogens.

Each major *ospC* group contains a cluster of *ospC* alleles that are less than 2% divergent from each other with respect to their DNA sequence. An earlier study of the *ospC* polymorphism found that there was very little sequence variation within the major *ospC* groups (9 of the 11 major *ospC* groups were isosequential) and concluded that the effective population size of each group was very small (45). The present study found that strain richness within the major *ospC* groups was high (331 unique seed sequences clustered into 23 major *ospC* groups, giving a mean richness of 14.4 seed sequences per group). Examination of the sequence variation within the major *ospC* groups found that the majority (~60%) of the nucleotide substitutions were synonymous. Sequencing error was the likely cause of all insertions and deletions that resulted in a frameshift, as the downstream parts of the OspC protein were highly conserved. The present study suggests that most of the sequence variation observed within a major *ospC* group does not affect the amino acid sequence and/or the function of the OspC protein. Under this scenario, natural selection cannot discriminate among the different allelic variants, and the frequencies of these silent alleles depend on genetic drift alone (78). Thus, the observation that the major *ospC* groups contain allelic variants does not contradict the hypothesis that cross-immunity selects against insufficiently divergent *ospC* alleles.

Richness of the major *ospC* groups. We found that the *B. afzelii* and *B. garinii* isolates in our local area contained 10 and 11 native major *ospC* groups, respectively (strain richness increased to 19 and 18, respectively, after the exotic major *ospC* groups were included). These findings were similar to those of previous studies of the *ospC* polymorphism in local tick populations (45, 53, 55, 79). In the United States, local populations of *B. burgdorferi sensu stricto* have between 13 and 16 different major *ospC* groups (13, 45, 53, 79). In Europe, local populations of *B. afzelii* have 10 different major *ospC* groups (55). Why do populations of *B. burgdorferi sensu lato* contain 12 and not 120 different major *ospC* groups? The relatively small length of the *ospC* gene (~600 bp) in combination with the constraints of remaining functional and of avoiding cross-reactive acquired immunity with all of its neighbors must set an upper limit to the number of major *ospC* groups that can exist in a single, local population.

Aggregation of *Borrelia ospC* strains in the tick vector. *Ixodes ricinus* nymphs infected with *B. afzelii* or *B. garinii* contained a mean richness of 2.37 and 2.97 different *ospC* strains per nymph, respectively. Our estimates of the mean *ospC* strain richness in questing *I. ricinus* nymphs were considerably higher than those from previous studies (1.07 to 1.44 strains per nymph) of *B. burgdorferi sensu lato* infections of questing nymphs in *I. scapularis*, *I. pacificus*, and *I. ricinus* (53, 55, 80). However, our estimates of the mean *ospC* strain richness are similar to those from previous studies (1.0 to 3.7 strains per tick) that sampled larval ticks feeding on infected hosts (13, 55, 81, 82) or questing adults (45, 53). We found a very high prevalence of multiple *ospC* strains in both *B. afzelii* (78.76%) and *B. garinii* (84.74%). Our estimates of the prevalence of multiple *ospC* strain infections were much higher than those in previous studies (0.0% to 50.0%) (1, 45, 55, 81–85). These earlier studies estimated the mean *ospC* strain richness and the prevalence of multiple strain infections by using molecular methods, such as the RLB assay, cold single-strand conformation polymorphism (SSCP) analysis, and Sanger sequencing. A previous study showed that the RLB assay was more sensitive at detecting infections with multiple strains than SSCP analysis (45, 53).

Thus, differences in strain richness among studies are partially confounded by differences in the sensitivity of the molecular methods. In the present study, we used next-generation sequencing (NGS) to analyze an average of 618 *ospC* gene sequences per tick (240,410 sequences, 389 ticks). This high level of coverage for each tick allowed us to detect major *ospC* groups that represented a tiny fraction of that particular amplicon (<0.2%). Thus, NGS methods estimate higher levels of strain diversity than the older molecular methods because these approaches are more sensitive at detecting rare strains.

The *ospC* strains were highly aggregated in *I. ricinus* ticks. There are a number of explanations for this pattern of aggregation. First, vertebrate hosts vary in their competence in acquiring infections and transmitting spirochetes to vector ticks (18, 30, 86, 87). If a substantial proportion of ticks feed on incompetent hosts and the remaining ticks feed on infected reservoir hosts, the *Borrelia* strains will appear to be aggregated inside the ticks when these two subpopulations of ticks are analyzed together. Second, the aggregation of ticks on reservoir hosts can also cause the aggregation of *Borrelia* strains in the tick vector. Numerous studies have observed that a minority of wild rodent hosts feeds a majority of the *I. ricinus* ticks (88, 89). There are a variety of ecological factors that cause aggregation of ticks in wild rodents (90). Third, the immune system of the vertebrate host might have more difficulty controlling and/or clearing infections with multiple strains than infections with a single strain (74, 91). Under this hypothesis, infections with multiple strains have a higher probability of establishing infection in the reservoir host and/or have a higher host-to-tick transmission success than infections with a single strain.

Differences between *B. garinii* and *B. afzelii*. The mean *ospC* strain richness of *B. garinii* (2.96 strains per nymph) was 25% higher than that of *B. afzelii* (2.37 strains per nymph). *Borrelia garinii ospC* strains were more often found in multiple infections and had higher pairwise association indices than their *B. afzelii* counterparts. The higher mean *ospC* strain richness for *B. garinii* was not caused by differences in total strain richness, which was the same between *B. afzelii* ($n = 19$ *ospC* strains) and *B. garinii* ($n = 18$ *ospC* strains). *Borrelia garinii* is mostly found in avian hosts, whereas *B. afzelii* is mostly found in rodent hosts (16, 20–22, 24, 25). Thus, one explanation is that the richness of *B. garinii ospC* strains in birds is higher than the richness of *B. afzelii ospC* strains in rodents. To date, most of the studies investigating *ospC* strain richness in vertebrate reservoir hosts have focused on mammals (1, 13, 50, 52, 55, 66, 82, 83). The two studies that investigated birds (81, 85) found a mean *ospC* strain richness (1.00 to 1.76 strains per tick) that was low compared to that found in the studies with mammals, but this could be an artifact of the relatively inefficient molecular methods that were used in the bird studies (RLB assay and Sanger sequencing). A recent study characterized the spirochete loads of different *Borrelia* species inside questing *I. ricinus* nymphs (14). This study found that the median spirochete load of *B. garinii* (5,080 spirochetes per nymph) was 1.6 times higher than that of *B. afzelii* (3,140 spirochetes per nymph) (14). Thus, another explanation for the discrepancy in strain richness between the two species is that the tick vector has a higher carrying capacity for *B. garinii* than for *B. afzelii*.

Presence of exotic major *ospC* group alleles in nymphs that, according to the RLB assay, were infected with a single *Borrelia* species. In the present study, we used only nymph-derived isolates that, according to the RLB assay (which targeted the 23S-5S spacer gene), were singly infected with either *B. afzelii* or *B. garinii*, and we therefore excluded those isolates that, according to the RLB assay, were coinfected with multiple *Borrelia* species. A surprising result was therefore that 42% of the *B. afzelii*-infected ticks and 55% of the *B. garinii*-infected ticks contained exotic major *ospC* groups from other *Borrelia* species. While the exotic major *ospC* groups were widespread, they were not abundant. For *B. afzelii* and *B. garinii*, the exotic major *ospC* groups accounted for 1.88% and 3.09% of all the *ospC* sequences, respectively. Two alternative explanations for these results are the coinfection explanation and the horizontal gene transfer explanation.

Coinfection explanation for the presence of exotic major *ospC* group alleles. In the coinfection explanation, the presence of native and exotic *ospC* groups in the same isolate represents a true coinfection with *B. afzelii* and *B. garinii* that was not detected by the RLB assay. In this scenario, the RLB assay detected the highly abundant *Borrelia* species (corresponding to the abundant native *ospC* alleles from the 454 sequencing) but not the *Borrelia* species of low abundance (corresponding to the low-abundance exotic *ospC* alleles from the 454 sequencing). Although we have recently shown that the sensitivity of our RLB assay is similar to that of a widely used quantitative PCR assay for detecting *Borrelia* infections in ticks (14, 92), we cannot exclude this possibility. Previous studies have shown that nymphs and adult ticks carry coinfections with *B. afzelii* and *B. garinii* (14, 32–34). In our local *I. ricinus* population, the RLB assay found that 0.75% (13/1,731) of the nymphs are coinfected with *B. afzelii* and *B. garinii* (14). If the coinfection explanation is true, the implication is that the true frequency of coinfections with *B. afzelii* and *B. garinii* has been underestimated by a factor of 56 to 73 (42% to 55% in the present study versus 0.75% in the study by Herrmann et al. [14]). Interestingly, we recently showed that the spirochete load of *B. afzelii* and *B. garinii* coinfections was much lower than the additive expectation (14). We suggested that the complement system of the vertebrate host reduced the spirochete load of the maladapted *Borrelia* species inside the nymphal tick (14). Thus, the observation that the exotic *ospC* groups had low abundances (1.88% and 3.09%) is consistent with our understanding of how the vertebrate complement system would interact with coinfections containing bird- and rodent-adapted *Borrelia* species inside the same tick.

If the coinfection hypothesis is true, the observation that >50% of our nymphs had coinfections with *B. afzelii* and *B. garinii* requires an additional explanation, given that these two *Borrelia* species are adapted to different vertebrate hosts (24, 32). We recently presented four mechanisms by which *B. afzelii* and *B. garinii* could coinfect the same tick (14). The specificity of *B. afzelii* for rodents and of *B. garinii* for birds is not 100% complete. Studies in France (93) and England (94) found that introduced species of rodents were infected with the bird-adapted *B. garinii*. Similarly, other studies found that birds can transmit the rodent-adapted *B. afzelii* (95, 96). Rodents or birds that are coinfected with *B. afzelii* and *B. garinii* could transmit both species to feeding ticks. Larval ticks could also acquire coinfections of *B. afzelii* and *B. garinii* by taking multiple blood meals from different hosts (43, 44), by a combination of cofeeding and systemic transmission, or by a combination of vertical transmission and systemic transmission (14). For example, a larval tick cofeeding next to a *B. afzelii*-infected nymph on a *B. garinii*-infected bird would acquire both *Borrelia* species. Cofeeding transmission has been observed in both *B. afzelii* and *B.*

garinii (90). In contrast, vertical transmission of *Borrelia* pathogens is believed to be rare (36).

Horizontal gene transfer explanation for the presence of exotic major *ospC* group alleles. In the horizontal gene transfer explanation, the RLB assay result is correct (all nymphs were infected with a single *Borrelia* species) and the exotic major *ospC* group alleles were horizontally transferred into the recipient *Borrelia* species. Previous studies have found that major *ospC* group alleles can be transferred between *Borrelia* species (46, 97, 98). In the present study, we found that 12 of the 21 native *ospC* major groups occurred as exotic major *ospC* groups in the other *Borrelia* species. If the horizontal gene transfer explanation is true, we make the following three observations. First, the present study suggests that horizontal transfer of the *ospC* gene is much more common than previous reports in the literature would suggest (45, 72, 97–100). Second, horizontal gene transfer was more likely for major *ospC* groups that were common in the donor *Borrelia* species. Third, some of the exotic *ospC* alleles increased to an appreciable frequency in the recipient *Borrelia* species. For example, the major *ospC* group A10, which is native to *B. afzelii*, had a relative frequency of 31.58% (60/190 infected nymphs) in *B. garinii* (Table 3). If the horizontal gene transfer explanation is true, one puzzling observation is why the exotic major *ospC* groups are so much less abundant (1.88% to 3.09%) than the native *ospC* groups (98.12% and 96.91%) in the nymph-derived isolates. This observation suggests that the exotic strains are underperforming relative to the native strains. Future studies should perform deep sequencing of other *Borrelia* genes that differ between *B. afzelii* and *B. garinii* (e.g., *recA* or *hbb*) in order to differentiate between the coinfection hypothesis and the horizontal gene transfer hypothesis.

In conclusion, despite a large sequencing effort (240,410 sequences), our study was unable to find any *ospC* alleles that were intermediately divergent (>2% to <8%) from the community of major *ospC* groups. Our study suggests that cross-reactive antibodies prevent the invasion of intermediately divergent *ospC* alleles. In contrast, we found no evidence that cross-immunity in the vertebrate host was structuring the community of *ospC* strains in the tick vector. Deep sequencing found that more than 50% of our *Borrelia* isolates contained exotic major *ospC* group alleles that had a low abundance (1.88% to 3.09% of all *ospC* gene sequences) and that had been derived from other *Borrelia* species. Two alternative explanations for these exotic major *ospC* group alleles are coinfections, where only the numerically dominant *Borrelia* species was detected by the RLB assay, or horizontal transfer of the *ospC* gene between *Borrelia* species.

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REFERENCES

1. **Andersson M, Scherman K, Raberg L.** 2013. Multiple-strain infections of *Borrelia afzelii*: a role for within-host interactions in the maintenance of antigenic diversity? Am Nat **181:**545–554. http://dx.doi.org/10.1086/669905.
2. **Futse JE, Brayton KA, Dark MJ, Knowles DP, Palmer GH.** 2008. Superinfection as a driver of genomic diversification in antigenically variant pathogens. Proc Natl Acad Sci U S A **105:**2123–2127. http://dx.doi.org/10.1073/pnas.0710333105.
3. **Juliano JJ, Porter K, Mwapasa V, Sem R, Rogers WO, Ariey F, Wongsrichanalai C, Read A, Meshnick SR.** 2010. Exposing malaria in-host diversity and estimating population diversity by capture-recapture using massively parallel pyrosequencing. Proc Natl Acad Sci U S A **107:**20138–20143. http://dx.doi.org/10.1073/pnas.1007068107.
4. **Lorono-Pino M, Cropp C, Farfan J, Vorndam A, Rodriguez-Angulo E, Rosado-Paredes E, Flores-Flores L, Beaty B, Gubler D.** 1999. Common occurrence of concurrent infections by multiple dengue virus serotypes. Am J Trop Med Hyg **61:**725–730.
5. **Frank SA.** 2002. Immunology and evolution of infectious disease. Princeton University Press, Princeton, NJ.
6. **Gog JR, Grenfell BT.** 2002. Dynamics and selection of many-strain pathogens. Proc Natl Acad Sci U S A **99:**17209–17214. http://dx.doi.org/10.1073/pnas.252512799.
7. **Gupta S, Anderson R.** 1999. Population structure of pathogens: the role of immune selection. Parasitol Today **15:**497–501. http://dx.doi.org/10.1016/S0169-4758(99)01559-8.
8. **Brunham RC, Plummer FA, Stephens RS.** 1993. Bacterial antigenic variation, host immune-response, and pathogen-host coevolution. Infect Immun **61:**2273–2276.
9. **Conway D, Polley S.** 2002. Measuring immune selection. Parasitology **125:**S3–S16.
10. **Fairlie-Clarke KJ, Shuker DM, Graham AL.** 2009. Why do adaptive immune responses cross-react? Evol Appl **2:**122–131. http://dx.doi.org/10.1111/j.1752-4571.2008.00052.x.
11. **Read AF, Taylor LH.** 2001. The ecology of genetically diverse infections. Science **292:**1099–1102. http://dx.doi.org/10.1126/science.1059410.
12. **Gilbert SC, Plebanski M, Gupta S, Morris J, Cox M, Aidoo M, Kwiatkowski D, Greenwood BM, Whittle HC, Hill AV.** 1998. Association of malaria parasite population structure, HLA, and immunological antagonism. Science **279:**1173–1177. http://dx.doi.org/10.1126/science.279.5354.1173.
13. **Brisson D, Dykhuizen DE.** 2004. *ospC* diversity in *Borrelia burgdorferi*: different hosts are different niches. Genetics **168:**713–722. http://dx.doi.org/10.1534/genetics.104.028738.
14. **Herrmann C, Gern L, Voordouw MJ.** 2013. Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*. Appl Environ Microbiol **79:**7273–7280. http://dx.doi.org/10.1128/AEM.02158-13.
15. **Imwong M, Nakeesathit S, Day NP, White NJ.** 2011. A review of mixed malaria species infections in anopheline mosquitoes. Malar J **10:**253. http://dx.doi.org/10.1186/1475-2875-10-253.
16. **Piesman J, Gern L.** 2004. Lyme borreliosis in Europe and North America. Parasitology **129:**S191. http://dx.doi.org/10.1017/S0031182003004694.
17. **Gern L, Humair P.** 2002. Ecology of *Borrelia burgdorferi* sensu lato in Europe, p 149–174. *In* Lyme borreliosis biology, epidemiology and control. Centre for Agriculture and Biosciences International (CABI), Wallingford, United Kingdom.
18. **Gern L, Estrada-Peña A, Frandsen F, Gray JS, Jaenson TGT, Jongejan F, Kahl O, Korenberg E, Mehl R, Nuttall PA.** 1998. European reservoir hosts of *Borrelia burgdorferi* sensu lato. Zentralbl Bakteriol **287:**196–204. http://dx.doi.org/10.1016/S0934-8840(98)80121-7.
19. **Humair P-F, Gern L.** 2000. The wild hidden face of Lyme borreliosis in Europe. Microbes Infect **2:**915–922. http://dx.doi.org/10.1016/S1286-4579(00)00393-2.
20. **Hanincova K, Schäfer S, Etti S, Sewell H-S, Taragelova V, Ziak D, Labuda M, Kurtenbach K.** 2003. Association of *Borrelia afzelii* with rodents in Europe. Parasitology **126:**11–20. http://dx.doi.org/10.1017/S0031182002002548.
21. **Huegli D, Hu C, Humair P-F, Wilske B, Gern L.** 2002. *Apodemus* species mice are reservoir hosts of *Borrelia garinii* OspA serotype 4 in Switzerland. J Clin Microbiol **40:**4735–4737. http://dx.doi.org/10.1128/JCM.40.12.4735-4737.2002.
22. **Humair P, Gern L.** 1998. Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland. Acta Trop **69:**213–227. http://dx.doi.org/10.1016/S0001-706X(97)00126-5.
23. **Humair P-F, Peter O, Wallich R, Gern L.** 1995. Strain variation of Lyme disease spirochetes isolated from *Ixodes ricinus* ticks and rodents col-

lected in two endemic areas in Switzerland. J Med Entomol **32:**433–438. http://dx.doi.org/10.1093/jmedent/32.4.433.
24. **Kurtenbach K, Peacey M, Rijpkema SG, Hoodless AN, Nuttall PA, Randolph SE.** 1998. Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. Appl Environ Microbiol **64:**1169–1174.
25. **Humair P, Rais O, Gern L.** 1999. Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance. Parasitology **118:**33–42. http://dx.doi.org/10.1017/S0031182098003564.
26. **Hanincová K, Taragelová V, Koci J, Schäfer SM, Hails R, Ullmann AJ, Piesman J, Labuda M, Kurtenbach K.** 2003. Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. Appl Environ Microbiol **69:**2825–2830. http://dx.doi.org/10.1128/AEM.69.5.2825-2830.2003.
27. **Humair P, Postic D, Wallich R, Gern L.** 1998. An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes. Zentralbl Bakteriol **287:**521–538.
28. **Taragel'ová V, Koči J, Hanincová K, Kurtenbach K, Derdáková M, Ogden NH, Literák I, Kocianová E, Labuda M.** 2008. Blackbirds and song thrushes constitute a key reservoir of *Borrelia garinii*, the causative agent of borreliosis in Central Europe. Appl Environ Microbiol **74:**1289–1293. http://dx.doi.org/10.1128/AEM.01060-07.
29. **Olsén B, Jaenson TG, Noppa L, Bunikis J, Bergström S.** 1993. A Lyme borreliosis cycle in seabirds and *Ixodes uriae* ticks. Nature **362:**340–342. http://dx.doi.org/10.1038/362340a0.
30. **Kurtenbach K, De Michelis S, Etti S, Schäfer SM, Sewell H-S, Brade V, Kraiczy P.** 2002. Host association of *Borrelia burgdorferi* sensu lato—the key role of host complement. Trends Microbiol **10:**74–79. http://dx.doi.org/10.1016/S0966-842X(01)02298-3.
31. **Kurtenbach K, Sewell H-S, Ogden NH, Randolph SE, Nuttall PA.** 1998. Serum complement sensitivity as a key factor in Lyme disease ecology. Infect Immun **66:**1248–1251.
32. **Kurtenbach K, De Michelis S, Sewell H-S, Etti S, Schäfer SM, Hails R, Collares-Pereira M, Santos-Reis M, Hanínçová K, Labuda M.** 2001. Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies found in individual questing ticks from Europe. Appl Environ Microbiol **67:**4926–4929. http://dx.doi.org/10.1128/AEM.67.10.4926-4929.2001.
33. **Gern L, Douet V, López Z, Rais O, Cadenas FM.** 2010. Diversity of *Borrelia* genospecies in *Ixodes ricinus* ticks in a Lyme borreliosis endemic area in Switzerland identified by using new probes for reverse line blotting. Ticks Tick-Borne Dis **1:**23–29. http://dx.doi.org/10.1016/j.ttbdis.2009.11.001.
34. **Rauter C, Hartung T.** 2005. Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis. Appl Environ Microbiol **71:**7203–7216. http://dx.doi.org/10.1128/AEM.71.11.7203-7216.2005.
35. **Richter D, Debski A, Hubalek Z, Matuschka F-R.** 2012. Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks. Vector-Borne Zoonotic Dis **12:**21–27. http://dx.doi.org/10.1089/vbz.2011.0668.
36. **Rollend L, Fish D, Childs JE.** 2013. Transovarial transmission of *Borrelia* spirochetes by *Ixodes scapularis*: a summary of the literature and recent observations. Ticks Tick-Borne Dis **4:**46–51. http://dx.doi.org/10.1016/j.ttbdis.2012.06.008.
37. **Grimm D, Tilly K, Byram R, Stewart PE, Krum JG, Bueschel DM, Schwan TG, Policastro PF, Elias AF, Rosa PA.** 2004. Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. Proc Natl Acad Sci U S A **101:**3142–3147. http://dx.doi.org/10.1073/pnas.0306845101.
38. **Tilly K, Krum JG, Bestor A, Jewett MW, Grimm D, Bueschel D, Byram R, Dorward D, VanRaden MJ, Stewart P.** 2006. *Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection. Infect Immun **74:**3554–3564. http://dx.doi.org/10.1128/IAI.01950-05.
39. **Gilmore RD, Kappel KJ, Dolan MC, Burkot TR, Johnson B.** 1996. Outer surface protein C (OspC), but not P39, is a protective immunogen against a tick-transmitted *Borrelia burgdorferi* challenge: evidence for a conformational protective epitope in OspC. Infect Immun **64:**2234–2239.
40. **Preac-Mursic V, Wilske B, Jauris S, Will G, Reinhardt S, Lehnert G, Patsouris E, Mehraein P, Soutschek E, Klockmann U.** 1992. Active immunization with OspC protein of *Borrelia burgdorferi* protects gerbils against *B. burgdorferi* infection. Infection **20:**342–349. http://dx.doi.org/10.1007/BF01710681.
41. **Probert WS, LeFebvre RB.** 1994. Protection of C3H/HeN mice from challenge with *Borrelia burgdorferi* through active immunization with OspA, OspB, or OspC, but not with OspD or the 83-kilodalton antigen. Infect Immun **62:**1920–1926.
42. **Probert WS, Crawford M, Cadiz RB, LeFebvre RB.** 1997. Immunization with outer surface protein (Osp) A, but not OspC, provides cross-protection of mice challenged with North American isolates of *Borrelia burgdorferi*. J Infect Dis **175:**400–405. http://dx.doi.org/10.1093/infdis/175.2.400.
43. **Jacquet M, Durand J, Rais O, Voordouw MJ.** 2015. Cross-reactive acquired immunity influences transmission success of the Lyme disease pathogen, *Borrelia afzelii*. Infect Genet Evol **36:**131–140. http://dx.doi.org/10.1016/j.meegid.2015.09.012.
44. **Earnhart CG, Buckles EL, Dumler JS, Marconi RT.** 2005. Demonstration of OspC type diversity in invasive human Lyme disease isolates and identification of previously uncharacterized epitopes that define the specificity of the OspC murine antibody response. Infect Immun **73:**7869–7877. http://dx.doi.org/10.1128/IAI.73.12.7869-7877.2005.
45. **Wang IN, Dykhuizen DE, Qin WG, Dunn JJ, Bosler EM, Luft BJ.** 1999. Genetic diversity of *ospC* in a local population of *Borrelia burgdorferi* sensu stricto. Genetics **151:**15–30.
46. **Baranton G, Seinost G, Theodore G, Postic D, Dykhuizen D.** 2001. Distinct levels of genetic diversity of *Borrelia burgdorferi* are associated with different aspects of pathogenicity. Res Microbiol **152:**149–156. http://dx.doi.org/10.1016/S0923-2508(01)01186-X.
47. **Lagal V, Postic D, Ruzic-Sabljic E, Baranton G.** 2003. Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness. J Clin Microbiol **41:**5059–5065. http://dx.doi.org/10.1128/JCM.41.11.5059-5065.2003.
48. **Brisson D, Drecktrah D, Eggers CH, Samuels DS.** 2012. Genetics of *Borrelia burgdorferi*. Annu Rev Genet **46:**515–536. http://dx.doi.org/10.1146/annurev-genet-011112-112140.
49. **Bunikis J, Garpmo U, Tsao J, Berglund J, Fish D, Barbour AG.** 2004. Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. Microbiology **150:**1741–1755. http://dx.doi.org/10.1099/mic.0.26944-0.
50. **Hellgren O, Andersson M, Råberg L.** 2011. The genetic structure of *Borrelia afzelii* varies with geographic but not ecological sampling scale. J Evol Biol **24:**159–167. http://dx.doi.org/10.1111/j.1420-9101.2010.02148.x.
51. **Qiu W-G, Schutzer SE, Bruno JF, Attie O, Xu Y, Dunn JJ, Fraser CM, Casjens SR, Luft BJ.** 2004. Genetic exchange and plasmid transfers in *Borrelia burgdorferi* sensu stricto revealed by three-way genome comparisons and multilocus sequence typing. Proc Natl Acad Sci U S A **101:**14150–14155. http://dx.doi.org/10.1073/pnas.0402745101.
52. **Jacquot M, Bisseux M, Abrial D, Marsot M, Ferquel E, Chapuis J-L, Vourc'h G, Bailly X.** 2014. High-throughput sequence typing reveals genetic differentiation and host specialization among populations of the *Borrelia burgdorferi* species complex that infect rodents. PLoS One **9:**e88581. http://dx.doi.org/10.1371/journal.pone.0088581.
53. **Qiu W-G, Dykhuizen DE, Acosta MS, Luft BJ.** 2002. Geographic uniformity of the Lyme disease spirochete (*Borrelia burgdorferi*) and its shared history with tick vector (*Ixodes scapularis*) in the northeastern United States. Genetics **160:**833–849.
54. **Strandh M, Råberg L.** 2015. Within-host competition between *Borrelia afzelii ospC* strains in wild hosts as revealed by massively parallel amplicon sequencing. Philos Trans R Soc Lond B Biol Sci **370:**20140293. http://dx.doi.org/10.1098/rstb.2014.0293.
55. **Pérez D, Kneubühler Y, Rais O, Jouda F, Gern L.** 2011. *Borrelia afzelii ospC* genotype diversity in *Ixodes ricinus* questing ticks and ticks from rodents in two Lyme borreliosis endemic areas: contribution of co-feeding ticks. Ticks Tick-Borne Dis **2:**137–142. http://dx.doi.org/10.1016/j.ttbdis.2011.06.003.
56. **Gern L, Hu CM, Kocianova E, Vyrostekova V, Rehacek J.** 1999. Genetic diversity of *Borrelia burgdorferi* sensu lato isolates obtained from *Ixodes ricinus* ticks collected in Slovakia. Eur J Epidemiol **15:**665–669. http://dx.doi.org/10.1023/A:1007660430664.
57. **Sinsky RJ, Piesman J.** 1989. Ear punch biopsy method for detection and

isolation of *Borrelia burgdorferi* from rodents. J Clin Microbiol **27:**1723–1727.
58. **Schouls LM, Van De Pol I, Rijpkema SG, Schot CS.** 1999. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. J Clin Microbiol **37:**2215–2222.
59. **Alekseev AN, Dubinina HV, Van De Pol I, Schouls LM.** 2001. Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. J Clin Microbiol **39:**2237–2242. http://dx.doi.org/10.1128/JCM.39.6.2237-2242.2001.
60. **Burri C, Cadenas FM, Douet V, Moret J, Gern L.** 2007. Ixodes ricinus density and infection prevalence of *Borrelia burgdorferi* sensu lato along a north-facing altitudinal gradient in the Rhône Valley (Switzerland). Vector-Borne Zoonotic Dis **7:**50–58. http://dx.doi.org/10.1089/vbz.2006.0569.
61. **Fu L, Niu B, Zhu Z, Wu S, Li W.** 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics **28:**3150–3152. http://dx.doi.org/10.1093/bioinformatics/bts565.
62. **Li W, Godzik A.** 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics **22:**1658–1659. http://dx.doi.org/10.1093/bioinformatics/btl158.
63. **Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O.** 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol **59:**307–321. http://dx.doi.org/10.1093/sysbio/syq010.
64. **Rice P, Longden I, Bleasby A.** 2000. EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet **16:**276–277. http://dx.doi.org/10.1016/S0168-9525(00)02024-2.
65. **Theisen M, Borre M, Mathiesen MJ, Mikkelsen B, Lebech A-M, Hansen K.** 1995. Evolution of the *Borrelia burgdorferi* outer surface protein OspC. J Bacteriol **177:**3036–3044.
66. **Anderson JM, Norris DE.** 2006. Genetic diversity of *Borrelia burgdorferi* sensu stricto in *Peromyscus leucopus*, the primary reservoir of Lyme disease in a region of endemicity in southern Maryland. Appl Environ Microbiol **72:**5331–5341. http://dx.doi.org/10.1128/AEM.00014-06.
67. **Richter D, Postic D, Sertour N, Livey I, Matuschka FR, Baranton G.** 2006. Delineation of *Borrelia burgdorferi* sensu lato species by multilocus sequence analysis and confirmation of the delineation of *Borrelia spielmanii* sp. nov. Int J Syst Evol Microbiol **56:**873–881. http://dx.doi.org/10.1099/ijs.0.64050-0.
68. **Core Team R.** 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.
69. **Lord C, Barnard B, Day K, Hargrove J, McNamara J, Paul R, Trenholme K, Woolhouse M.** 1999. Aggregation and distribution of strains in microparasites. Philos Trans R Soc Lond B Biol Sci **354:**799–807. http://dx.doi.org/10.1098/rstb.1999.0432.
70. **Pielou EC.** 1977. Mathematical ecology. John Wiley & Sons, New York, NY.
71. **Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H.** 2013. Package 'vegan.' Community ecology package, version 2.0-10. https://cran.r-project.org/web/packages/vegan/vegan.pdf.
72. **Wang G, Dam AP, Dankert J.** 1999. Evidence for frequent OspC gene transfer between *Borrelia valaisiana* sp. nov. and other Lyme disease spirochetes. FEMS Microbiol Lett **177:**289–296. http://dx.doi.org/10.1111/j.1574-6968.1999.tb13745.x.
73. **Gupta S, Ferguson N, Anderson R.** 1998. Chaos, persistence, and evolution of strain structure in antigenically diverse infectious agents. Science **280:**912–915. http://dx.doi.org/10.1126/science.280.5365.912.
74. **Balmer O, Tanner M.** 2011. Prevalence and implications of multiple-strain infections. Lancet Infect Dis **11:**868–878. http://dx.doi.org/10.1016/S1473-3099(11)70241-9.
75. **Rego RO, Bestor A, Stefka J, Rosa PA.** 2014. Population bottlenecks during the infectious cycle of the Lyme disease spirochete *Borrelia burgdorferi*. PLoS One **9:**e101009. http://dx.doi.org/10.1371/journal.pone.0101009.
76. **Piesman J, Oliver JR, Sinsky RJ.** 1990. Growth kinetics of the Lyme disease spirochete (*Borrelia burgdorferi*) in vector ticks (*Ixodes dammini*). Am J Trop Med Hyg **42:**352–357.
77. **Gupta S, Maiden MCJ, Feavers IM, Nee S, May RM, Anderson RM.** 1996. The maintenance of strain structure in populations of recombining infectious agents. Nat Med **2:**437–442. http://dx.doi.org/10.1038/nm0496-437.
78. **Grenfell BT, Pybus OG, Gog JR, Wood JL, Daly JM, Mumford JA, Holmes EC.** 2004. Unifying the epidemiological and evolutionary dynamics of pathogens. Science **303:**327–332. http://dx.doi.org/10.1126/science.1090727.
79. **States SL, Brinkerhoff RJ, Carpi G, Steeves TK, Folsom-O'Keefe C, DeVeaux M, Diuk-Wasser MA.** 2014. Lyme disease risk not amplified in a species-poor vertebrate community: similar *Borrelia burgdorferi* tick infection prevalence and OspC genotype frequencies. Infect Genet Evol **27:**566–575. http://dx.doi.org/10.1016/j.meegid.2014.04.014.
80. **Girard YA, Travinsky B, Schotthoefer A, Fedorova N, Eisen RJ, Eisen L, Barbour AG, Lane RS.** 2009. Population structure of the Lyme borreliosis spirochete *Borrelia burgdorferi* in the western black-legged tick (*Ixodes pacificus*) in Northern California. Appl Environ Microbiol **75:**7243–7252. http://dx.doi.org/10.1128/AEM.01704-09.
81. **Ogden NH, Lindsay LR, Hanincova K, Barker IK, Bigras-Poulin M, Charron DF, Heagy A, Francis CM, O'Callaghan CJ, Schwartz I, Thompson RA.** 2008. Role of migratory birds in introduction and range expansion of *Ixodes scapularis* ticks and of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in Canada. Appl Environ Microbiol **74:**1780–1790. http://dx.doi.org/10.1128/AEM.01982-07.
82. **Ogden NH, Margos G, Aanensen DM, Drebot MA, Feil EJ, Hanincova K, Schwartz I, Tyler S, Lindsay LR.** 2011. Investigation of genotypes of *Borrelia burgdorferi* in *Ixodes scapularis* ticks collected during surveillance in Canada. Appl Environ Microbiol **77:**3244–3254. http://dx.doi.org/10.1128/AEM.02636-10.
83. **Fedorova N, Kleinjan JE, James D, Hui LT, Peeters H, Lane RS.** 2014. Remarkable diversity of tick or mammalian-associated borreliae in the metropolitan San Francisco Bay Area, California. Ticks Tick-Borne Dis **5:**951–961. http://dx.doi.org/10.1016/j.ttbdis.2014.07.015.
84. **Hamer SA, Hickling GJ, Walker ED, Tsao JI.** 2014. Increased diversity of zoonotic pathogens and *Borrelia burgdorferi* strains in established versus incipient *Ixodes scapularis* populations across the midwestern United States. Infect Genet Evol **27:**531–542. http://dx.doi.org/10.1016/j.meegid.2014.06.003.
85. **Mathers A, Smith RP, Cahill B, Lubelczyk C, Elias SP, Lacombe E, Morris SR, Vary CP, Parent CE, Rand PW.** 2011. Strain diversity of *Borrelia burgdorferi* in ticks dispersed in North America by migratory birds. J Vector Ecol **36:**24–29. http://dx.doi.org/10.1111/j.1948-7134.2011.00137.x.
86. **Ginsberg HS, Buckley P, Balmforth MG, Zhioua E, Mitra S, Buckley FG.** 2005. Reservoir competence of native North American birds for the Lyme disease spirochete, *Borrelia burgdorferi*. J Med Entomol **42:**445–449.
87. **LoGiudice K, Ostfeld RS, Schmidt KA, Keesing F.** 2003. The ecology of infectious disease: effects of host diversity and community composition on Lyme disease risk. Proc Natl Acad Sci U S A **100:**567–571. http://dx.doi.org/10.1073/pnas.0233733100.
88. **Perkins SE, Cattadori IM, Tagliapietra V, Rizzoli AP, Hudson PJ.** 2003. Empirical evidence for key hosts in persistence of a tick-borne disease. Int J Parasitol **33:**909–917. http://dx.doi.org/10.1016/S0020-7519(03)00128-0.
89. **Randolph S, Miklisova D, Lysy J, Rogers D, Labuda M.** 1999. Incidence from coincidence: patterns of tick infestations on rodents facilitate transmission of tick-borne encephalitis virus. Parasitology **118:**177–186. http://dx.doi.org/10.1017/S0031182098003643.
90. **Voordouw MJ.** 2015. Co-feeding transmission in Lyme disease pathogens. Parasitology **142:**290–302. http://dx.doi.org/10.1017/S0031182014001486.
91. **Read A, Taylor L.** 2000. Within-host ecology of infectious diseases: patterns and consequences, p 59–75. *In* Molecular epidemiology of infectious diseases. Arnold, London, United Kingdom.
92. **Berret J, Voordouw MJ.** 2015. Lyme disease bacterium does not affect attraction to rodent odour in the tick vector. Parasites Vectors **8:**249. http://dx.doi.org/10.1186/s13071-015-0856-8.
93. **Marsot M, Sigaud M, Chapuis J, Ferquel E, Cornet M, Vourc'h G.** 2011. Introduced Siberian chipmunks (*Tamias sibiricus barberi*) harbor more-diverse *Borrelia burgdorferi* sensu lato genospecies than native bank voles (*Myodes glareolus*). Appl Environ Microbiol **77:**5716–5721. http://dx.doi.org/10.1128/AEM.01846-10.
94. **Millins C, Magierecka A, Gilbert L, Edoff A, Brereton A, Kilbride E, Denwood M, Birtles R, Biek R.** 2015. An invasive mammal (the gray squirrel, *Sciurus carolinensis*) commonly hosts diverse and atypical genotypes of the zoonotic pathogen *Borrelia burgdorferi* sensu lato. Appl Environ Microbiol **81:**4236–4245. http://dx.doi.org/10.1128/AEM.00109-15.
95. **Dubska L, Literak I, Kocianova E, Taragelova V, Sychra O.** 2009.

Differential role of passerine birds in distribution of *Borrelia* spirochetes, based on data from ticks collected from birds during the postbreeding migration period in Central Europe. Appl Environ Microbiol **75:**596–602. http://dx.doi.org/10.1128/AEM.01674-08.

96. **Franke J, Moldenhauer A, Hildebrandt A, Dorn W.** 2010. Are birds reservoir hosts for *Borrelia afzelii*? Ticks Tick-Borne Dis **1:**109–112. http://dx.doi.org/10.1016/j.ttbdis.2010.03.001.
97. **Livey I, Gibbs C, Schuster R, Dorner F.** 1995. Evidence for lateral transfer and recombination in OspC variation in Lyme disease *Borrelia*. Mol Microbiol **18:**257–269. http://dx.doi.org/10.1111/j.1365-2958.1995.mmi_18020257.x.
98. **Barbour AG, Travinsky B.** 2010. Evolution and distribution of the *ospC* gene, a transferable serotype determinant of *Borrelia burgdorferi*. mBio **1:**e00153-10. http://dx.doi.org/10.1128/mBio.00153-10.
99. **Dykhuizen DE, Baranton G.** 2001. The implications of a low rate of horizontal transfer in *Borrelia*. Trends Microbiol **9:**344–350. http://dx.doi.org/10.1016/S0966-842X(01)02066-2.
100. **Jauris-Heipke S, Liegl G, Preac-Mursic V, Rössler D, Schwab E, Soutschek E, Will G, Wilske B.** 1995. Molecular analysis of genes encoding outer surface protein C (OspC) of *Borrelia burgdorferi* sensu lato: relationship to *ospA* genotype and evidence of lateral gene exchange of *ospC*. J Clin Microbiol **33:**1860–1866.

Supplementary Material

Forward: *CCATCTCATCCCTGCGTGTCTCCGAC***TCAG-***MID*-
TATTAATGACTTTATTTTTATTTATATCT

Reverse:
*CCTATCCCCTGTGTGCCTTGGCAGTC***TCAG**TTGATTTTAATTAAGGTTTTTTTGG

1. Description of the primers used in the second PCR. The forward and the reverse primers both had the 454 Life Science's A or B sequencing adapter (blue italic font) and the key (bold black font). The forward primer also included a 10 bp Multiplex Identifier (MID) to individually tag each sample (red italic font). The template-specific sequences target the *ospC* gene (green font).

2. Description of the method used to clean the sequences

Preliminary cleaning of the *ospC* gene sequences: The 454 sequencing run produced 352,882 sequences. The *split_library.py* function of *QIIME* (Caporaso et al. 2010) was used to do a preliminary cleaning on each pool of sequences with the following parameters: a maximum of four mismatches for the primers, a minimum length of 300 bp, maximum homopolymers of eight bp, and with primers retained. During this step, sequences were assigned to their sample name according to their ten bp tag.

Removing indels and chimeric sequences: After the preliminary cleaning, we checked our sequences for methodological errors such as insertions or deletions (indels) produced by the PCR or the sequencing technique. For each pool, the nucleotide sequences were aligned individually to an OspC amino acid sequence using *Exonerate* (Slater and Birney 2005). This software can align genomic sequences to a protein sequence and takes frame shifts into account. Indels were soft-masked (written in lower case) and all sequences were aligned together using *psa2msa*. Soft-masked nucleotides were coded as gaps in sequences that did not contain that particular indel. Resulting sequences contained many gaps and were very long (1697 bp). We therefore deleted all positions for which more than 60% of the sequences had a gap using *TrimAl* (Capella-Gutiérrez et al. 2009). After deleting these uninformative positions, the resultant sequences were 521 bp long.

The last cleaning step removed chimeric and other artificial sequences that can arise during either PCR or 454-sequencing. In the *Exonerate* software, sequences that align poorly will contain a large number of gaps. We therefore erased all sequences that contained fewer than 350 bp and more than 171 gaps, basing our criteria on the distribution of the sequence length. In addition, some poorly aligned sequences were split into two separate sequences by the software but were assigned the same name. We therefore deleted all sequences for which the name was repeated twice. The purpose of the previous cleaning steps was to eliminate low

quality sequences and sequences that were artificially created by the molecular methods. The resultant cleaned data set of 240,410 sequences (521 bp long) is expected to contain only those sequences that are biologically real.

References

1. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7: 335-336.
2. Slater GS, Birney E (2005) Automated generation of heuristics for biological sequence comparison. BMC bioinformatics 6: 31.
3. Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T (2009) trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics 25: 1972-1973.

3. Table S1. Comparison of the nomenclature of the different major *ospC* groups of *B. afzelii* between the present study and four earlier studies (Theisen et al. 1995, Baranton et al. 2001, Lagal et al. 2003, Pérez et al. 2011). We based the new names of the major *ospC* groups on the classification by Bunikis et al. (Bunikis et al. 2004). The studies of Baranton et al. (2001) and Theisen et al. (1995) did not name the major *ospC* groups. We therefore used the order of appearance in the original paper to rename the major *ospC* groups as unknown 1 (Unk1), unknown 2 (Unk2), and so on.

New Names	Lagal 2003	Baranton 2001	Theisen 1995	Genbank Accession number
A1	A2	Unk2	Unk1	AY363710
A2	ME*	Unk14	Unk3	FJ750334
A3	A3	Unk8		AY363712
A4	A8	Unk13		AY363713
A5		Unk3	Unk2	AY363714
A6				AY363715
A7	A6	Unk11		AY363718
A8				AY150201
A9	A1	Unk5	Unk4	AY363719
A10	YU*			AY363720
A11		Unk7		AY363721
A12	A4	Unk9		AY150205
A13				FJ750336
A14	A5	Unk4		AY150203
A15		Unk12		AB000348
A16				FJ546555
A17				X83552
A18		Unk10		AY491403
A19		Unk6		AF230184
A20				AY491407

*YU and ME were named in Pérez et al. (Pérez et al. 2011).

References

1. Baranton G, Seinost G, Theodore G, Postic D, Dykhuizen D (2001) Distinct levels of genetic diversity of *Borrelia burgdorferi* are associated with different aspects of pathogenicity. Research in microbiology 152: 149-156.
2. Lagal V, Postic D, Ruzic-Sabljic E, Baranton G (2003) Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness. Journal of Clinical Microbiology 41: 5059-5065.

3. Theisen M, Borre M, Mathiesen MJ, Mikkelsen B, Lebech A-M, et al. (1995) Evolution of the *Borrelia burgdorferi* outer surface protein OspC. Journal of bacteriology 177: 3036-3044.
4. Pérez D, Kneubühler Y, Rais O, Jouda F, Gern L (2011) *Borrelia afzelii ospC* genotype diversity in *Ixodes ricinus* questing ticks and ticks from rodents in two Lyme borreliosis endemic areas: Contribution of co-feeding ticks. Ticks and tick-borne diseases 2: 137-142.
5. Bunikis J, Garpmo U, Tsao J, Berglund J, Fish D, et al. (2004) Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. Microbiology 150: 1741-1755.

4. Table S2. Comparison of the nomenclature of the different major *ospC* groups of *B. garinii* between the present study and three earlier studies (Theisen et al. 1995, Baranton et al. 2001, Lagal et al. 2003). We based the new names of the major *ospC* groups on the classification by Lagal et al. (2003). The studies of Baranton et al. (2001) and Theisen et al. (1995) did not name the major *ospC* groups. We therefore used the order of appearance in the original paper to rename the major *ospC* groups as unknown 1 (Unk1), unknown 2 (Unk2), and so on.

New Names	Lagal 2004	Baranton 2001	Theisen 1995	Genbank Accession number
G1	G1		Unk3	L42879
G2	G2	Unk4	Unk2	X81526
G3	G3	Unk9		L42870
G4	G4	Unk21	Unk1	AJ132797
G5	G5	Unk8		AY150185
G6	G6	Unk16		AY150186
G7	G7			AY150199
G8	G8	Unk19		AY150193
G9	G9	Unk20		AY150191
G10	G10	Unk11		AY150189
G11	G11	Unk18		AY150195
G12		Unk17		L42886
G13		Unk6		L42875
G14		Unk10		L42863
G15		Unk13		D49376
G16		Unk1		X83556
G17		Unk2		D49509
G18		Unk3		AF098941
G19		Unk5		D49505
G20		Unk7		D49504
G21		Unk12		D49381
G22		Unk14		D49499
G23		Unk15		D49508
G24		Unk22	Unk4	X84773

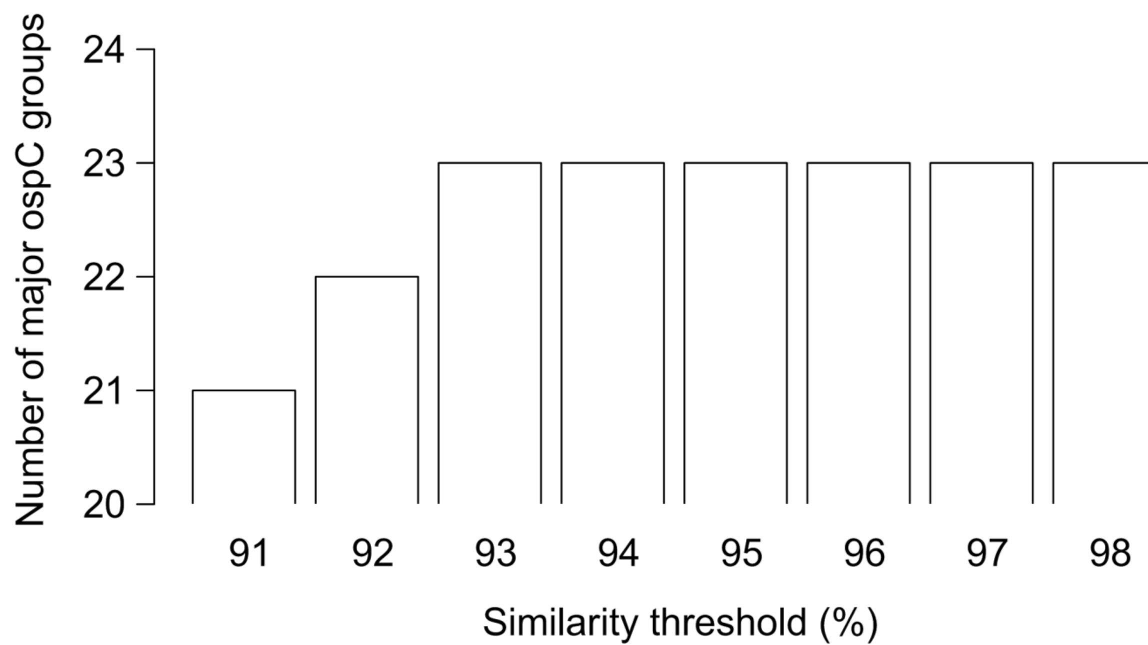
References

1. Baranton G, Seinost G, Theodore G, Postic D, Dykhuizen D (2001) Distinct levels of genetic diversity of *Borrelia burgdorferi* are associated with different aspects of pathogenicity. Research in microbiology 152: 149-156.
2. Lagal V, Postic D, Ruzic-Sabljic E, Baranton G (2003) Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC*

gene and its association with invasiveness. Journal of Clinical Microbiology 41: 5059-5065.
3. Theisen M, Borre M, Mathiesen MJ, Mikkelsen B, Lebech A-M, et al. (1995) Evolution of the *Borrelia burgdorferi* outer surface protein OspC. Journal of bacteriology 177: 3036-3044.

5. Table S4. Total number of substitutions, non-synonymous substitutions and deleted codons for 30 sequences of each major ospC groups. The sequences included the first 350 bp of the *ospC* gene.

	Substitutions			Insertions			Deletions		
ospC	Total	S	NS	1nt	2nt	3nt	1nt	2nt	3nt
A1	7	4	3	1	0	0	5	0	2
A2	13	9	4	10	0	0	4	1	5
A3	3	3	0	2	0	0	2	2	3
A5	5	1	4	8	0	0	2	0	0
A7	10	7	3	6	0	0	8	4	7
A9	9	4	5	5	0	0	3	0	2
A10	1	1	0	0	0	0	0	0	4
A11	7	4	3	1	0	0	1	0	3
A12	5	3	2	0	0	0	3	0	3
A14	7	3	4	1	0	0	1	1	2
V1	10	5	5	2	0	0	4	1	4
Q	10	7	3	6	0	0	11	1	7
G2	3	1	2	1	0	0	3	0	4
G4	5	5	0	4	0	0	5	0	6
G6	6	3	3	3	0	0	3	1	5
G7	3	1	2	4	0	0	4	1	3
G8	0	0	0	0	0	0	2	1	3
G9	2	1	1	0	0	0	3	1	4
G10	13	8	5	0	0	0	4	0	4
G11	5	4	1	2	0	0	1	0	2
G13	4	1	3	3	0	0	1	2	4
G14	1	1	0	0	0	0	3	0	1
G15	12	7	5	6	0	0	5	4	1



6. Figure S1. The number of major *ospC* groups found by the clustering analysis is stable across a range of similarity thresholds (93% to 98%). In this analysis, the *ospC* gene sequences were combined for *B. afzelii* and *B. garinii*.

7. 3rd Paper

The evolution of a multiple-strain tick-borne pathogen in the field over one decade

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Abstract

Immunodominant antigens play a key role in structuring the populations of multiple-strain pathogens. Strong immune selection against these antigens will cause the pathogen strains to organize themselves into a set of unique serotypes that minimizes cross-reactive acquired immunity. These sets of antigenically distinct strains can remain stable over long periods of time and the frequency of each strain depends on its intrinsic fitness.

Two sympatric multiple-strain tick-borne pathogens, *Borrelia afzelii* and *B. garinii*, that use the same tick vector, *Ixodes ricinus*, but different vertebrate reservoir hosts were studied. 454-sequencing of the immunodominant, polymorphic *ospC* gene was used to characterize the community of *Borrelia* strains in a local population of *I. ricinus* ticks over a period of 11 years. Estimates of the intrinsic fitness of six *B. afzelii ospC* strains were obtained from a previous experimental infection study using laboratory mice.

The frequency distributions of the *ospC* strains were stable over the duration of the study in both *Borrelia* species. In *B. afzelii*, the estimates of strain fitness in laboratory mice explained over 63% of the variation in the frequencies of the strains in our local population of ticks. We found evidence for a selective sweep as the major *ospC* groups that are currently the most common had the lowest number of genetic variants within the major *ospC* group. Our results are consistent with theoretical models on how acquired immunity in the vertebrate host structures multiple-strain pathogen populations.

Keywords: *Borrelia afzelii*, *Borrelia garinii*, *Ixodes ricinus*, Lyme borreliosis, mixed infection, *ospC*, outer surface protein C, pathogen strain, tick-borne pathogen, vector-borne disease

Introduction

In endemic infectious diseases, the host population may develop high levels of acquired immunity because of frequent encounters with the pathogen (Brunham et al. 1993, Lena et al. 2005). Strong immune selection against immunodominant antigens will cause the pathogen population to organize into antigenically distinct strains or serotypes that induce strain-specific immunity (Gupta et al. 1996, Gupta and Anderson 1999). These sets of antigenically distinct strains can remain stable over long periods of time and the frequency of each strain depends on its intrinsic fitness (Gupta et al. 1998, Gupta and Anderson 1999). Such systems are difficult to invade by mutant pathogen strains because the host population has been saturated with antigenic variants so that there is no immune-free space left for the mutant to occupy (Gupta et al. 1996). When the host immune response is not strong enough to induce a stable community of antigenically distinct strains, the frequencies of the strains can exhibit cyclical or chaotic fluctuations (Gupta et al. 1998, Gupta and Anderson 1999). Thus studies that monitor the frequencies of pathogen strains over time can provide insight into how acquired immunity in the host population shapes the epidemiology of multiple-strain pathogens (Plummer et al. 1989, Brunham et al. 1996, Bambini et al. 2013). In the present study, we used a tick-borne bacterium to study how a community of pathogen strains can change over time.

Borrelia burgdorferi sensu lato (s. l.) is a complex of tick-borne spirochete bacteria that causes Lyme borreliosis (LB) in humans (Kurtenbach et al. 2006). In Europe, the two most common etiological agents of LB, *B. afzelii* and *B. garinii*, are both transmitted by the castor bean tick, *Ixodes ricinus*, which has three obligate blood-feeding stages: larva, nymph, and adult (Piesman and Gern 2004). Larval ticks acquire spirochetes after feeding on an infected reservoir host and develop into infected nymphs that transmit the pathogen the following year to the next generation of reservoir hosts. *B. afzelii* and *B. garinii* are

specialized on different classes of vertebrate reservoir hosts, rodents and birds, respectively (Gern and Humair 2002, Kurtenbach et al. 2002, Piesman and Gern 2004). Because of this ecological specialization, these two sympatric pathogens rarely occur together in the same tick (Kurtenbach et al. 2001, Rauter and Hartung 2005, Gern et al. 2010, Herrmann et al. 2013). Adult ticks do not contribute directly to transmission because they feed on larger hosts (e.g. deer) that are resistant to the spirochete (Telford 3rd et al. 1988, Jaenson and Tälleklint 1992, Matuschka et al. 1993). Humans are accidental hosts and are not part of the natural life cycle of *Borrelia* pathogens.

The epidemiology of a multiple strain pathogen will depend on those antigens that induce the strongest immune response in the host (Gupta et al. 1998). *B. burgdorferi* s. l. outer surface protein C (OspC) is a highly polymorphic virulence factor that is critical for establishing infection inside the vertebrate host (Grimm et al. 2004, Tilly et al. 2006). This antigen induces a strong antibody response (Dressler et al. 1993, Fung et al. 1994, Engstrom et al. 1995) that protects the host from secondary infection (Barthold 1999, Mbow et al. 1999). Genetic studies have shown that *ospC* gene sequences can be clustered into the so-called major *ospC* groups (Wang et al. 1999). A major *ospC* group is a cluster of *ospC* alleles that is more than 8% divergent in DNA sequence from other such clusters, whereas alleles within a major *ospC* group are less than 2% divergent from each other (Wang et al. 1999, Baranton et al. 2001, Lagal et al. 2003, Bunikis et al. 2004, Durand et al. 2015). The conspicuous absence of *ospC* alleles that are 2%–8% divergent from each other suggests that there is strong selection by the vertebrate immune system against such intermediately divergent major *ospC* groups (Durand et al. 2015). Immunization with a given OspC protein induces antibodies that protect the vertebrate host against strains carrying the same major *ospC* group allele (Preac-Mursic et al. 1992, Probert and LeFebvre 1994, Gilmore et al. 1996) but not against strains carrying a different major *ospC* group allele (Probert et al. 1997, Earnhart et al. 2005, Jacquet

et al. 2015). These immunization studies suggest that the major *ospC* groups have diverged from each other to minimize competition mediated by cross-reactive antibodies in the vertebrate host. Numerous genetic studies have shown that the *ospC* locus is in linkage disequilibrium with many other loci in the *Borrelia* genome (Bunikis et al. 2004, Qiu et al. 2004, Hellgren et al. 2011, Brisson et al. 2012). This observation supports the idea that OspC is an immunodominant antigen that plays a critical role in structuring the community of strains in *Borrelia* species (Gupta et al. 1996, Gupta and Anderson 1999). In summary, the OspC protein is an example of a highly polymorphic, single-locus antigen (Conway and Polley 2002) that enhances between-host transmission by permitting super-infection of hosts with previous immune experience to other strains (Brunham et al. 1993).

Long-term studies on changes in the frequencies of multiple-strain pathogens are rare but valuable (Plummer et al. 1989, Brunham et al. 1996, Bambini et al. 2013). A previous review on the evolutionary ecology of LB pathogens emphasized the importance of studying temporal variation in the frequencies of *Borrelia ospC* strains (Kurtenbach et al. 2006). The purpose of the present study is to examine the frequencies of the major *ospC* groups in two sympatric *Borrelia* species, *B. afzelii* and *B. garinii*, that have been sampled from the same local population of *I. ricinus* nymphal ticks over a period of 11 years. Importantly, the discrete nature of the Lyme disease life cycle is such that the *Borrelia* strain composition of the questing nymphs in a given year is a complete snapshot of the strain community that was acquired by the larval ticks the previous year. Under the theory of Gupta and colleagues, strong immune selection on the OspC antigen creates a limited set of antigenically distinct major *ospC* groups (Gupta et al. 1996, Gupta and Anderson 1999). The fitness of each major *ospC* group strain does not depend on whether it is common or rare. The frequencies of the major *ospC* groups can remain stable over time and depend on the intrinsic fitness or reproductive number (R_0) of each *ospC* strain in a common vertebrate host. We used estimates

of R_0 for six *B. afzelii ospC* strains in laboratory mice (Tonetti et al. 2015) to test this prediction in *B. afzelii*.

Finally, to better understand the processes governing the community structure of these multiple-strain tick-borne pathogens, we studied the amount of genetic variation within each of the major *ospC* groups. We recently showed that most of the nucleotide substitutions within a major *ospC* group are synonymous, indicating that these 'minor' *ospC* alleles produce the same OspC antigen (Durand et al. 2015). Natural selection by the host immune system cannot discriminate among identical phenotypes and neutral processes such as genetic drift are expected to govern the frequencies of these 'minor' *ospC* alleles (Gog and Grenfell 2002). In the absence of selection, large populations maintain more genetic variants than small populations (Hartl and Clark 1997). We therefore predicted that the common major *ospC* groups in our local Lyme disease system should have more 'minor' *ospC* alleles than the rare major *ospC* groups. We tested this prediction by examining the relationship between the frequencies of the major *ospC* groups and the number of nucleotide substitutions within each of the major *ospC* groups.

Results

Evolution of the relative frequencies of the major *ospC* groups over time in *B. afzelii* and *B. garinii*: The relative frequencies of the major *ospC* groups were stable over time in both *B. afzelii* and *B. garinii* (Figure 1 and Figures S3 and S4; for each year, the data points are based on a maximum of 20 nymphs so there is binomial variation in the frequencies). The relative frequency distribution of the major *ospC* groups for the first six years of the study was significantly correlated with the relative frequency distribution for the last five years of the study in both *B. afzelii* (Pearson correlation test: $r = 0.874$, $p < 0.001$; Figure 2) and *B. garinii* (Pearson correlation test: $r = 0.916$, $p < 0.001$; Figure 2). In addition,

with respect to the annual relative frequency distribution of the major *ospC* groups, the magnitude of the Pearson's correlation coefficient between years remained stable as the time interval increased between the years in the pair in both *B. afzelii* (Linear regression: $r^2 = 0.018$, $F = 0.018$, $p = 0.894$; Figure 3) and *B. garinii* (Linear regression: $r^2 = 0.015$, $F = 0.203$, $p = 0.654$; Figure 3). Of the 209 proportion tests (19 most common *ospC* groups*11 years), only 11 frequencies were significantly different ($\alpha = 0.05$) from the average frequency over the duration of the study for that particular major *ospC* group (see supplementary material). These 11 significant frequencies occurred in 6 different years and were distributed over 9 different strains. The observed number of significant frequencies (11) was equal to the expected number of type I errors (0.05*209 = 10.45), indicating that the major *ospC* group strains did not fluctuate over time.

Relationship between the R_0-values and the relative frequencies of the major *ospC* groups in *B. afzelii*: For the six *B. afzelii ospC* strains for which we had data (Tonetti et al. 2015), there was a positive relationship between the strain-specific reproductive number (R_0) in laboratory mice and the strain-specific relative frequency in the questing *I. ricinus* nymphs ($F_{1, 4} = 2.63$, $p = 0.058$; Figure 4). The relationship between R_0 and the relative frequency (averaged over the entire duration of the study) was almost statistically significant for the two-tailed test ($p = 0.058$) and statistically significant ($p = 0.029$) under the a priori prediction that the strain-specific relative frequency should increase with the strain-specific R_0 value. The strain-specific R_0 value explained 63.34% of the variation in the strain-specific relative frequency (Figure 4). Thus estimates of R_0 using laboratory mice were a good predictor of the relative frequencies of the *B. afzelii ospC* strains in a wild population of *I. ricinus* nymphs.

Relationship between the relative frequency of the major *ospC* group and the amount of genetic variation within each major *ospC* group: For the 21 native major *ospC*

groups of *B. afzelii* and *B. garinii* pooled together, the relative frequencies of the native major *ospC* groups were negatively correlated with the amount of genetic variation within each native major *ospC* group. A significant negative relationship was found for each of the four measures of genetic variation: total number of substitutions (Pearson correlation test: r = -0.633, p = 0.002; Figure 5), number of synonymous substitutions (r = -0.644, p = 0.002), number of non-synonymous substitutions (r = -0.435, p = 0.049), and number of single indels (r = -0.474, p = 0.030). Thus common major *ospC* groups contained fewer genetic variants than rare major *ospC* groups. The relationship remained negative when analyzed separately for the two *Borrelia* species, but was only statistically significant for *B. garinii* (r = -0.900, p < 0.001; Figure S1).

For example, *B. afzelii* major *ospC* group A10 was found in 54.40% of all infected ticks but the 30 randomly selected seed sequences of this major *ospC* group contained only one nucleotide substitution. In contrast, *B. afzelii* major *ospC* groups A2, A3, A5, and A7 were found in 28.49% of all infected ticks but together they contained 31 nucleotide substitutions (Figure 5; Durand et al. 2015). Similarly, *B. garinii* major *ospC* group G8 was found in 42.11% of all infected ticks and contained zero nucleotide substitutions, whereas *B. garinii* major *ospC* groups G6, G10, and G15 were found in 16.84% of all infected ticks and contained 31 nucleotide substitutions (Figure 5; Durand et al. 2015).

Effect of the origin of the DNA extract on the composition of the community *ospC* strains: There was a strong correlation in the frequencies of the major *ospC* group strains (data were combined for 2009 and 2010) between the nymph-derived *Borrelia* isolates (Durand et al. 2015) and the nymph-derived DNA extracts in both *B. afzelii* (Pearson correlation test: r = 0.845, t = 7.056, df = 20, p < 0.001; Figure S2) and *B. garinii* (r = 0.516, t = 2.559, df = 18, p = 0.019; Figure S2). Thus the step of culturing nymph-derived isolates in

BSK medium did not induce strong selection on the composition of the community of *ospC* strains.

Discussion

The most interesting results from this study was our demonstration that the community of *ospC* strains was stable over time and that some strains were consistently common whereas other strains were consistently rare (Figures 1, 2, 3, S3, S4). This result was particularly striking in *B. afzelii* where *ospC* strain A10 was the most common in 10 of the 11 years of the study (Figure 1). Previous studies on *B. burgdorferi* s. s. in *I. scapularis* ticks in the northeastern United States have documented rapid shifts in the frequency distribution of the major *ospC* groups (Qiu et al. 1997, Qiu et al. 2002, MacQueen et al. 2012). However, these studies were either done in tick populations where *B. burgdorferi* s. s. was emerging (MacQueen et al. 2012) or over shorter time periods (3 years) using less reliable methods (cold single-strand conformation polymorphism analysis) for detecting multiple *ospC* strain infections in ticks (Qiu et al. 1997, Qiu et al. 2002). The present 11-year study in two *Borrelia* genospecies is therefore the most complete temporal survey of any multiple-strain tick-borne pathogen.

Another remarkable result was that our laboratory estimates of fitness for six *ospC* strains of *B. afzelii* explained a substantial portion of the differences in frequencies (63.34%) among these strains in the field (Figure 4). This result suggests that the laboratory *Mus musculus* mouse is a good model for the wild reservoir hosts of *B. afzelii*. Host blood meal analysis at our field site in Switzerland has found that immature *I. ricinus* ticks feed on birds (16.6%), artiodactyls (40.0%), carnivores (15.5%), and rodents (28.0%) (Humair et al. 2007, Morán Cadenas et al. 2007). The first three vertebrate groups can be ignored because they are not competent reservoir hosts for *B. afzelii* (Jaenson and Tälleklint 1992, Matuschka et al. 1993, Kurtenbach et al. 1998, Kurtenbach et al. 2002). At our field site, the rodent species that

fed the most immature *I. ricinus* ticks were *Apodemus* mice, the bank vole (*Myodes glareolus*), and the red squirrel (*Sciurus vulgaris*). Previous work in Switzerland and elsewhere in Europe has shown that these species are the most important reservoir hosts of *B. afzelii* (Matuschka et al. 1992, Gern et al. 1994, Tälleklint and Jaenson 1994, Gern et al. 1998, Humair and Gern 1998, Humair et al. 1999, Pérez et al. 2011, Råberg 2012, Andersson et al. 2013, Strandh and Råberg 2015). Experimental infection studies with *B. afzelii* have shown that the host-to-tick transmission dynamics over the duration of the infection are similar between laboratory rodents and the wood mouse, *A. sylvaticus* (Richter et al. 2004). Thus our experimental Lyme disease system involving laboratory mice was able to capture the relevant fitness differences between the six tested *B. afzelii ospC* strains. We are not aware of any other vector-borne disease studies where estimates of strain-specific fitness in the laboratory where predictive of strain-specific performance in the field.

Our results are consistent with theoretical models on how the acquired immune system of vertebrate hosts interacts with immunodominant pathogen antigens like the OspC protein (Gupta et al. 1996, Gupta et al. 1998, Gupta and Anderson 1999). Strong immune selection against the OspC antigen in a common reservoir host will cause alleles at the *ospC* locus to organize into a set of unique serotypes that minimizes cross-reactive acquired immunity (Gupta et al. 1996). In such systems, the community of strains can be stable over long periods of time and the frequency of each strain depends on its intrinsic fitness or reproductive number (R_0) (Gupta et al. 1998, Gupta and Anderson 1999). With respect to another vector-borne parasite, the human malaria parasite *Plasmodium falciparum*, Gupta and colleagues suggested that, "many features of its epidemiology can be explained by assuming that it is a construct of 'independently transmitted strains'" (Gupta et al. 1994, Gupta and Anderson 1999). We suggest that this concept is also true for multiple-strain tick-borne pathogens like *B. afzelii* and *B. garinii*.

The acquired immune response in the vertebrate reservoir host plays a critical role in the development of strain-specific immunity against *Borrelia* pathogens, as required by the theory of Gupta and colleagues (Gupta et al. 1996, Gupta et al. 1998, Gupta and Anderson 1999). We believe, as others have suggested (Theisen et al. 1995, Wang et al. 1999), that the *ospC* gene has diversified in response to the vertebrate antibody response and that the highly polymorphic OspC antigen facilitates super-infection of the vertebrate reservoir host. In wild rodent populations, the proportion of individuals with OspC-specific antibodies increases over the course of the summer as the mice are exposed to infected nymphs (Bunikis et al. 2004). Experimental infection studies have shown that the OspC antigen induces strain-specific immunity that protects rodents from infectious challenge with strains carrying a homologous but not a heterologous major *ospC* group allele (Probert et al. 1997, Earnhart et al. 2005, Jacquet et al. 2015). Wild reservoir hosts are frequently infected with multiple strains of *B. burgdorferi* s. l. that carry different major *ospC* group alleles (Brisson and Dykhuizen 2004, Swanson and Norris 2008, Pérez et al. 2011, Andersson et al. 2013, Strandh and Råberg 2015). In addition, experimental infections with *B. burgdorferi* s. s. have shown that wild rodents challenged via tick bite can be super-infected with strains carrying a different major *ospC* group allele (Derdáková et al. 2004). Taken together, these studies suggest that *Borrelia* strains carrying different major *ospC* group alleles do not compete with each other via cross-reactive antibodies (Andersson et al. 2013). This pattern is consistent with the prediction of Gupta and colleagues that strong selection on immunodominant antigens causes the pathogen population to diverge into a set of discrete serotypes (Gupta et al. 1996, Gupta et al. 1998, Gupta and Anderson 1999).

Many models of host-pathogen interactions assume that there is negative frequency-dependent selection (NFDS) where rare genotypes have an inherent fitness advantage over common genotypes (Peters and Lively 1999, Schmid-Hempel and Jokela 2002, Engelstädter

and Bonhoeffer 2009). However, in the presence of cross-reactive immune responses, common pathogen genotypes can prevent rare pathogen genotypes from invading the host population. This is true even when the intrinsic fitness (i.e. the R_0 value) of the rare pathogen genotype is much higher than that of the common pathogen genotype (Gupta et al. 1996). Thus cross-reactive acquired immunity against rare, recombinant genotypes plays a critical role in the stability of the community of pathogen strains. The estimates of the *ospC* strain frequencies in the present study were based on the analysis of over 240,000 *ospC* gene sequences. In a previous study, we showed that all of the major *ospC* group alleles in our study population were at least 8% divergent from each other at the DNA sequence level (Durand et al. 2015). Remarkably, we did not find a single recombinant *ospC* gene sequence that was intermediately divergent (2–8%). This result suggests that there is strong selection by the vertebrate immune system against *ospC* alleles that are intermediately divergent from the community of major *ospC* group alleles (Durand et al. 2015). Again, these results are consistent with the prediction of Gupta and colleagues that sets of discrete non-overlapping serotypes can prevent the invasion and establishment of rare, recombinant strains (Gupta et al. 1996, Gupta et al. 1998, Gupta and Anderson 1999).

Researchers in the Lyme disease community have advanced two hypotheses to explain how the *ospC* gene polymorphism is maintained in nature: negative frequency-dependent selection (NFDS) (Wang et al. 1999) and multiple niche polymorphism (MNP) (Brisson and Dykhuizen 2004, Brisson et al. 2012). Numerous authors have suggested that the *ospC* gene polymorphism is maintained by NFDS (Qiu et al. 1997, Wang et al. 1999, Qiu et al. 2002). NFDS suggests that the immune system of the vertebrate host targets the common rather than the rare *ospC* strains (Qiu et al. 1997, Wang et al. 1999, Brisson et al. 2012). For example, vertebrate hosts that are repeatedly exposed to a common strain (via tick bite) might develop a stronger strain-specific immune response, which reduces the spirochete density in the host

tissues and host-to-tick transmission success. However, we recently tested this hypothesis in *B. afzelii* and found that there was no effect of the number of infective tick bites on the strength of the *Borrelia*-specific IgG response or on the host-to-tick transmission (unpublished data). Thus the NFDS hypothesis lacks a plausible mechanism by which a competent reservoir host would develop a stronger antibody response against a common versus a rare *ospC* strain. In addition, there is a misunderstanding in the Lyme disease literature that NFDS should lead to fluctuations in the frequencies of the major *ospC* groups (Kurtenbach et al. 2006, Tsao 2009, Brisson et al. 2012). Under NFDS, the frequencies of the parasite and host genotypes can be stable or cycle over time (Lively and Apanius 1995). Future studies should indicate the immunological mechanism by which NFDS favors rare *Borrelia* strains over common ones.

Under MNP, the different *ospC* strains of the *Borrelia* pathogen are specialized on different vertebrate host species, which represent different ecological niches (Brisson and Dykhuizen 2004, Hanincová et al. 2006, Brisson et al. 2012). We believe that the MNP hypothesis is unlikely in our system for a number of reasons. First, host blood meal analysis of questing *I. ricinus* nymphs in Switzerland has found a limited diversity of relevant reservoir hosts (Humair et al. 2007, Morán Cadenas et al. 2007). Second, host specificity of *B. afzelii* and *B. garinii* for rodents and birds, respectively, is mediated by the vertebrate complement system (Kurtenbach et al. 1998, Kurtenbach et al. 2002) and additional *ospC*-mediated specificity seems unnecessary. Third, a study on *B. afzelii* in Switzerland found that 8 of the 10 major *ospC* groups in questing *I. ricinus* nymphs were found in just 19 individuals belonging to three different rodent species: *Apodemus sylvaticus*, *A. flavicollis*, and *Myodes glareolus* (Pérez et al. 2011). Fourth, if the major *ospC* group alleles circulate in different reservoir hosts, it begs the question as to why the pairwise genetic divergences are constrained to be >8% and why the OspC antigen induces strain-specific immunity in a common rodent

reservoir host. In our view, these data are not compatible with the MNP hypothesis of the *ospC* polymorphism of *B. burgdorferi* s. l. pathogens.

In the present study, we used nymph-derived *Borrelia* isolates that were grown up in BSK culture. One concern was therefore that the BSK culture step induced selection on the community of *ospC* strains. To address this concern, we analyzed the community of *ospC* strains in an additional sample of 253 *Borrelia*-infected nymphs where the DNA was extracted directly. This additional work showed that the BSK culture step did not change the composition of the community of *ospC* strains. We also note that previous work had shown that the ability to detect *B. burgdorferi* s. l. infections in ticks is actually higher for the method of BSK culture followed by DNA extraction than direct DNA extraction of ticks (Morán Cadenas et al. 2007).

A surprising result of this study was the negative correlation between the relative frequency of a major *ospC* group and the amount of genetic variation within a major *ospC* group (Figure 5). This result contradicted our prediction that the most common major *ospC* groups should have the highest number of 'minor' *ospC* alleles. The signature of a common genotype with a low amount of neutral genetic variation is indicative of a selective sweep (Stotz et al. 2000, Ford 2002). Thus one potential explanation is that strain A10 and strain G8 recently invaded their respective communities (i.e. before 2000 when monitoring began) and that they have not had enough time to mutate and build up a population of neutral genetic variants. The observation of a selective sweep followed by a period of stability is consistent with the model of Gupta et al (1998) that predicts the "large amplitude chaos" state mentioned above.

Due to its critical role in host invasion (Grimm et al. 2004, Pal et al. 2004, Tilly et al. 2006, Fingerle et al. 2007), the *ospC* gene has received much interest from a public health perspective. Genetic analysis of human isolates of *B. burgdorferi* s. l. revealed that only a

subset of major *ospC* groups is capable of infecting and causing disease in humans (Seinost et al. 1999, Dykhuizen et al. 2008, Wormser et al. 2008). In the United States, the major *ospC* groups A, B, K, I, and N are most commonly associated with disseminated infections in humans but these strains are also the most common in questing *I. scapularis* ticks (Dykhuizen et al. 2008). In the present study, strains carrying *ospC* major group A10 dominated the *B. afzelii* population in Neuchatel over the last decade (>50% of *B. afzelii*-infected ticks contained strain A10). Recent studies have shown that *ospC* strain A10 is common in other parts of Switzerland (Pérez et al. 2011) and Sweden (Andersson et al. 2013). In contrast, genetic screening of human isolates has never recovered *B. afzelii ospC* strain A10 from a human patient (Baranton et al. 2001, Lagal et al. 2003, Bunikis et al. 2004). However, these genetic screens were based on a limited number of tissue biopsies (245 sequences) that may have been sampled from areas in Europe where strain A10 was not locally common. Future studies should screen human isolates of *B. afzelii* to test whether strains carrying this major *ospC* group are infectious to humans.

In conclusion, our study on two common LB pathogens in a local population of *I. ricinus* ticks showed that the community of *Borrelia ospC* strains was stable over a period of 11 years. In both *B. afzelii* and *B. garinii*, some *ospC* strains were consistently common whereas other *ospC* strains were consistently rare. In *B. afzelii*, the strain-specific estimates of fitness in laboratory rodents explained over 63% of the variation in the strain-specific frequencies in the field. Our results are consistent with the theoretical models of Gupta and colleagues (Gupta et al. 1996, Gupta et al. 1998, Gupta and Anderson 1999). The observation that the most common major *ospC* groups (A10 and G8) had the least amount of genetic variation, suggests a recent selective sweep of these two strains in the communities of their *Borrelia* genospecies. The present study shows the importance of studying local pathogen populations over long periods of time to better understand their epidemiology.

Materials and Methods

Field sampling and molecular methods: The sampling of the *I. ricinus* ticks in the field, the testing for *Borrelia* infection, and the 454-sequencing of the *ospC* gene was described in a previous study (Durand et al. 2015). Briefly, *I. ricinus* nymphs were sampled at the same location in a deciduous forest near Neuchâtel (47°00’55.6”N, 6°94’16.7”E; surface of ~1 ha) over a period of 11 years (2000 to 2010). Nymphs were screened for spirochete infection using immunofluorescence microscopy and *Borrelia*-infected nymphs were incubated in BSK II medium at 34°C. DNA was extracted from all spirochete-positive BSK cultures and the *Borrelia* species was identified using a PCR-Reverse Line Blot (RLB) assay that targets the 23S-5S spacer gene (Burri et al. 2007). Only those isolates that were singly infected with *B. afzelii* or *B. garinii* were selected for 454-sequencing of the *ospC* gene. For each *Borrelia* species, a maximum of 20 isolates was randomly selected for each of the 11 years of the survey for a total of 193 *B. afzelii* isolates and 190 *B. garinii* isolates. For each of these 383 isolates, the *ospC* gene was amplified using the PCR protocol of Bunikis et al. (2004). 454-sequencing of the amplicons in the forward direction produced 240,410 useable *ospC* gene sequences (reads) and each sequence was 521 bp. For each nymphal-tick derived isolate, the community of major *ospC* groups was based on an average of 632 *ospC* gene sequences (reads).

The *ospC* gene sequences clustered into 23 distinct major *ospC* groups: 10 for *B. afzelii* (A1, A2, A3, A5, A7, A9, A10, A11, A12, and A14), 11 for *B. garinii* (G2, G4, G6, G7, G8, G9, G10, G11, G13, G14, and G15), 1 for *B. burgdorferi* s. s. (Q), and 1 for *B. valaisiana* (V1) (Durand et al. 2015). A number of major *ospC* groups were shared suggesting horizontal transfer between *Borrelia* species. The *B. afzelii* isolates contained 19 major *ospC* groups: 10 native and 9 exotic (derived from the other *Borrelia* species). The *B. garinii* isolates contained

18 major *ospC* groups: 11 native and 7 exotic (derived from the other *Borrelia* species) (Durand et al. 2015).

Effect of BSK culture step on the community of major *ospC* group alleles: One concern was that the BSK culture step induced selection on the community of major *ospC* group alleles. We therefore analyzed an additional 253 *I. ricinus* nymphal ticks of which, 152 and 101 were infected with *B. afzelii* and *B. garinii*, respectively. These nymphs had been collected from the same field site over a period of three years (2009 to 2011) as part of the PhD thesis of Coralie Herrmann. Details of the field sampling and subsequent molecular methods have been described previously (Herrmann and Gern 2010, Herrmann and Gern 2012, Herrmann and Gern 2013, Herrmann et al. 2013, Herrmann et al. 2013). Critically for the purpose of the present study, the DNA had been extracted directly from the nymphal ticks with no intervening BSK culture step. We used the same 454-sequencing protocol to determine the community of *ospC* strains in each nymph. To test if there was an effect of the origin of the DNA extract (cultured isolate versus nymphal tick) on the community of *ospC* strains, we focused on the two years of overlap between the two studies (2009, 2010). We used Pearson correlations to compare the relative frequencies of the *ospC* strains between the two studies for each of the two *Borrelia* species.

Evolution of the relative frequencies of the major *ospC* groups over time: The community of major *ospC* group alleles was determined for each of the 383 nymph-derived spirochete isolates. A major *ospC* group was considered as present as long as the isolate contained a single sequence belonging to that group. For each *Borrelia* species, the annual relative frequency of each major *ospC* group was calculated as the proportion of isolates that carried that particular major *ospC* group each year (Durand et al. 2015). We performed two analyses to test whether the relative frequencies of the major *ospC* groups changed over time. First, we calculated the relative frequencies of the major *ospC* groups for the first six years

(2000 to 2005) and for the last five years of the survey (2006 to 2010). We then used a Pearson's correlation test to determine whether there was a correlation in the relative frequency distribution of the major *ospC* groups between these two periods of time. Second, we calculated the Pearson's correlation of the relative frequency distribution of the major *ospC* groups for each of the 55 pairs of years. We then tested whether the magnitude of this pairwise correlation decreased with the time interval between the two years in each pair. In addition, for each strain we used proportion tests to determine if the frequency in a given year was significantly different from the frequency averaged over the duration of the study (results are shown in the supplementary material).

Relationship between R_0 and the relative frequency of the major *ospC* group: In the absence of immunity-mediated competition between strains, theory predicts that the R_0 value of each strain will determine its frequency in nature (Gupta et al. 1998). We recently conducted a study in which laboratory mice were experimentally infected via tick bite with one of six *B. afzelii ospC* strains: A1, A2, A3, A9, A10, and A12 (Tonetti et al. 2015). Tonetti et al. (2015) used the nomenclature developed by Lagal et al. (2003) and Pérez et al. (2011) and the six *B. afzelii ospC* strains in that study are therefore referred to as A2, ME, A3, A1, YU, and A4, respectively. The purity of these six strains was recently confirmed by 454-sequencing of the *ospC* gene (Table S1). For each of the six strains, the following three fitness components were measured: tick-to-host transmission, systemic (host-to-tick) transmission, and co-feeding transmission. We used next generation matrix methods (Hartemink et al. 2008, Harrison et al. 2011, Harrison and Bennett 2012) to combine these transmission components into a single estimate of fitness, the reproductive number (R_0), for each of the six *B. afzelii ospC* strains (Tonetti et al. 2015). The study of Tonetti et al. (2015) assumed that the efficiency of vertical transmission (r_A) was 0.10 and that the proportion of competent hosts (h_c) was 0.50. Recent work suggests that transovarial transmission of *B. burgdorferi* s. l. does

not occur in *Ixodes* ticks (Richter et al. 2012, Rollend et al. 2013) and the value of r_A was therefore set to 0.00 in the present study. Host blood meal analysis in our local Lyme disease system suggests that only 28.0% of questing immature *I. ricinus* ticks obtained their blood meal from *B. afzelii*-competent rodent reservoir hosts (Morán Cadenas et al. 2007), and the value of h_c was therefore set to 0.28 in the present study. Thus the estimates of R_0 for the six strains of *B. afzelii* are similar but not identical between Tonetti et al. (2015) and the present study. We used linear regression to test whether there was a positive relationship between the R_0 values of the six *B. afzelii ospC* strains and the strain-specific relative frequencies in the local *I. ricinus* population (averaged over the entire course of the study). We used a one-tailed test because we had an a priori expectation that strains with higher R_0 values should have higher relative frequencies.

Relationship between the relative frequency of the major *ospC* group and the amount of genetic variation within each major *ospC* group: The approach for estimating the amount of genetic variation among 'minor' *ospC* alleles within each major *ospC* group was described in a previous study (Durand et al. 2015). Briefly, we randomly selected 30 seed sequences for each of the 23 major *ospC* groups (total of 690 seed sequences). The seed sequences were those 521 bp *ospC* gene sequences that occurred at least 50 times in our sequence library. These seed sequences were eventually clustered into the 23 different major *ospC* groups. For each major *ospC* group, we aligned the 30 seed sequences manually and counted the numbers of synonymous and non-synonymous substitutions, insertions, and deletions. These counts of genetic changes are estimates of the amount of genetic variation within each major *ospC* group. The 454-sequencing run had sequenced the *ospC* gene in the forward direction only and the quality of the sequences deteriorated after 350 bp. To avoid sequencing errors, the counts of the genetic changes were restricted to the first 350 bp of the *ospC* gene. It is important to point out that the 30 randomly selected seed sequences of a

major *ospC* group can be genetically identical over the first part of the gene (bp 1 to bp 350) but genetically distinct in the second part of the gene (bp 350 to bp 521) that was not included for quantifying genetic variation within a major *ospC* group. We tested whether there was a correlation between the relative frequency of each major *ospC* group and our measures of genetic variation within a major *ospC* group using Pearson correlation tests.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JD and MJV designed the study, analyzed the data, and wrote the manuscript. LG designed the tick sampling survey, screened the isolates for *Borrelia* infection, and edited the manuscript. OR sampled the ticks in the field and determined *Borrelia* infection via the RLB assay. JD performed the library preparation and the clustering analysis of the *ospC* gene sequences into the major *ospC* groups. MJ participated in the library preparation of the samples. All authors read and approved the manuscript.

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References

Andersson, M., K. Scherman, L. Raberg and L. Råberg (2013). "Multiple-strain infections of *Borrelia afzelii*: a role for within-host interactions in the maintenance of antigenic diversity?" American Naturalist, The **181**(4): 545-554.

Bambini, S., J. Piet, A. Muzzi, W. Keijzers, S. Comandi, L. De Tora, M. Pizza, R. Rappuoli, D. van de Beek and A. van der Ende (2013). "An analysis of the sequence variability of meningococcal fHbp, NadA and NHBA over a 50-year period in the Netherlands." PLoS One **8**(5): e65043.

Baranton, G., G. Seinost, G. Theodore, D. Postic and D. Dykhuizen (2001). "Distinct levels of genetic diversity of *Borrelia burgdorferi* are associated with different aspects of pathogenicity." Research in Microbiology **152**(2): 149-156.

Barthold, S. W. (1999). "Specificity of infection-induced immunity among *Borrelia burgdorferi* sensu lato species." Infection and Immunity **67**(1): 36-42.

Brisson, D., D. Drecktrah, C. H. Eggers and D. S. Samuels (2012). "Genetics of *Borrelia burgdorferi*." Annual Review of Genetics **46**.

Brisson, D. and D. E. Dykhuizen (2004). "*ospC* diversity in *Borrelia burgdorferi*: Different hosts are different niches." Genetics **168**(2): 713-722.

Brunham, R. C., J. Kimani, J. Bwayo, G. Maitha, I. Maclean, C. L. Yang, C. X. Shen, S. Roman, N. J. D. Nagelkerke, M. Cheang and F. A. Plummer (1996). "The epidemiology of *Chlamydia trachomatis* within a sexually transmitted diseases core group." Journal of Infectious Diseases **173**(4): 950-956.

Brunham, R. C., F. A. Plummer and R. S. Stephens (1993). "Bacterial antigenic variation, host immune-response, and pathogen-host coevolution." Infection and Immunity **61**(6): 2273-2276.

Bunikis, J., U. Garpmo, J. Tsao, J. Berglund, D. Fish and A. G. Barbour (2004a). "Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe." Microbiology **150**(Pt 6): 1741-1755.

Bunikis, J., J. Tsao, C. J. Luke, M. G. Luna, D. Fish and A. G. Barbour (2004b). "*Borrelia burgdorferi* infection in a natural population of *Peromyscus leucopus* mice: a longitudinal study in an area where Lyme borreliosis is highly endemic." Journal of Infectious Diseases **189**: 1515-1523.

Burri, C., F. Morán Cadenas, V. Douet, J. Moret and L. Gern (2007). "Ixodes ricinus density and infection prevalence of *Borrelia burgdorferi* sensu lato along a north-facing altitudinal gradient in the Rhône Valley (Switzerland)." Vector-Borne and Zoonotic Diseases **7**(1): 50-58.

Conway, D. and S. Polley (2002). "Measuring immune selection." Parasitology **125**(07): S3-S16.

Derdáková, M., V. Dudiòák, B. Brei, J. S. Brownstein, I. Schwartz and D. Fish (2004). "Interaction and transmission of two *Borrelia burgdorferi* sensu stricto strains in a tick-rodent maintenance system." Applied and Environmental Microbiology **70**(11): 6783-6788.

Dressler, F., J. A. Whalen, B. N. Reinhardt and A. C. Steere (1993). "Western blotting in the serodiagnosis of Lyme disease." Journal of Infectious Diseases **167**(2): 392-400.

Durand, J., M. Jacquet, L. Paillard, O. Rais, L. Gern and M. J. Voordouw (2015). "Cross-immunity and community structure of a multiple-strain pathogen in the tick vector." Applied and Environmental Microbiology **81**(22): 7740-7752.

Dykhuizen, D. E., D. Brisson, S. Sandigursky, G. P. Wormser, J. Nowakowski, R. B. Nadelman and I. Schwartz (2008). "Short report: The propensity of different *Borrelia burgdorferi* sensu stricto genotypes to cause disseminated infections in humans." The American Journal of Tropical Medicine and Hygiene **78**(5): 806-810.

Earnhart, C. G., E. L. Buckles, J. S. Dumler and R. T. Marconi (2005). "Demonstration of OspC type diversity in invasive human Lyme disease isolates and identification of previously uncharacterized epitopes that define the specificity of the OspC murine antibody response." Infection and Immunity **73**(12): 7869-7877.

Engelstädter, J. and S. Bonhoeffer (2009). "Red Queen dynamics with non-standard fitness interactions." PLoS Comput Biol **5**(8): e1000469.

Engstrom, S. M., E. Shoop and R. C. Johnson (1995). "Immunoblot interpretation criteria for serodiagnosis of early Lyme disease." Journal of Clinical Microbiology **33**(2): 419-427.

Fingerle, V., G. Goettner, L. Gern, B. Wilske and U. Schulte-Spechtel (2007). "Complementation of a *Borrelia afzelii* OspC mutant highlights the crucial role of OspC for dissemination of *Borrelia afzelii* in *Ixodes ricinus*." International Journal of Medical Microbiology **297**(2): 97-107.

Ford, M. J. (2002). "Applications of selective neutrality tests to molecular ecology." Molecular Ecology **11**(8): 1245-1262.

Fung, B. P., G. L. McHugh, J. M. Leong and A. C. Steere (1994). "Humoral immune response to outer surface protein C of *Borrelia burgdorferi* in Lyme disease: role of the immunoglobulin M response in the serodiagnosis of early infection." Infection and Immunity **62**(8): 3213-3221.

Gern, L., V. Douet, Z. López, O. Rais and F. Morán Cadenas (2010). "Diversity of *Borrelia* genospecies in *Ixodes ricinus* ticks in a Lyme borreliosis endemic area in Switzerland identified by using new probes for reverse line blotting." Ticks and Tick-Borne Diseases **1**(1): 23-29.

Gern, L., A. Estrada-Peña, F. Frandsen, J. S. Gray, T. G. T. Jaenson, F. Jongejan, O. Kahl, E. Korenberg, R. Mehl and P. A. Nuttall (1998). "European reservoir hosts of *Borrelia burgdorferi* sensu lato." Zentralblatt für Bakteriologie **287**(3): 196-204.

Gern, L. and P. Humair (2002). "Ecology of *Borrelia burgdorferi* sensu lato in Europe." Lyme Borreliosis Biology, Epidemiology and Control. CABI International: 149-174.

Gern, L., M. Siegenthaler, C. Hu, S. Leuba-Garcia, P. Humair and J. Moret (1994). "Borrelia burgdorferi in rodents (*Apodemus flavicollis* and *A. sylvaticus*): Duration and enhancement of infectivity for *Ixodes ricinus* ticks." European Journal of Epidemiology **10**(1): 75-80.

Gilmore, R. D., K. J. Kappel, M. C. Dolan, T. R. Burkot and B. Johnson (1996). "Outer surface protein C (OspC), but not P39, is a protective immunogen against a tick-transmitted *Borrelia burgdorferi* challenge: evidence for a conformational protective epitope in OspC." Infection and Immunity **64**(6): 2234-2239.

Gog, J. R. and B. T. Grenfell (2002). "Dynamics and selection of many-strain pathogens." Proceedings of the National Academy of Sciences **99**(26): 17209-17214.

Grimm, D., K. Tilly, R. Byram, P. E. Stewart, J. G. Krum, D. M. Bueschel, T. G. Schwan, P. F. Policastro, A. F. Elias and P. A. Rosa (2004). "Outer-surface protein C of the Lyme disease spirochete: A protein induced in ticks for infection of mammals." Proceedings of the National Academy of Sciences of the United States of America **101**(9): 3142-3147.

Gupta, S. and R. Anderson (1999). "Population structure of pathogens: the role of immune selection." Parasitology Today **15**(12): 497-501.

Gupta, S., N. Ferguson and R. Anderson (1998). "Chaos, persistence, and evolution of strain structure in antigenically diverse infectious agents." Science **280**(5365): 912-915.

Gupta, S., M. C. J. Maiden, I. M. Feavers, S. Nee, R. M. May and R. M. Anderson (1996). "The maintenance of strain structure in populations of recombining infectious agents." Nature Medicine **2**(4): 437-442.

Gupta, S., K. Trenholme, R. M. Anderson and K. P. Day (1994). "Antigenic diversity and the transmission dynamics of *Plasmodium falciparum*." Science **263**(5149): 961-963.

Hanincová, K., K. Kurtenbach, M. Diuk-Wasser, B. Brei and D. Fish (2006). "Epidemic spread of Lyme borreliosis, northeastern United States." Emerging Infectious Diseases **12**(4): 604.

Harrison, A. and N. C. Bennett (2012). "The importance of the aggregation of ticks on small mammal hosts for the establishment and persistence of tick-borne pathogens: an investigation using the R(0) model." Parasitology **139**(12): 1605-1613.

Harrison, A., W. I. Montgomery and K. J. Bown (2011). "Investigating the persistence of tick-borne pathogens via the R(0) model." Parasitology **138**(7): 896-905.

Hartemink, N., S. Randolph, S. Davis and J. Heesterbeek (2008). "The basic reproduction number for complex disease systems: defining R(0) for tick-borne infections." The American Naturalist **171**(6): 743-754.

Hartl, D. L. and A. G. Clark (1997). Principles of population genetics, Sinauer associates Sunderland.

Hellgren, O., M. Andersson and L. Råberg (2011). "The genetic structure of *Borrelia afzelii* varies with geographic but not ecological sampling scale." Journal of Evolutionary Biology **24**(1): 159-167.

Herrmann, C. and L. Gern (2010). "Survival of *Ixodes ricinus* (Acari: Ixodidae) under challenging conditions of temperature and humidity is influenced by *Borrelia burgdorferi* sensu lato infection." Journal of Medical Entomology **47**(6): 1196-1204.

Herrmann, C. and L. Gern (2012). "Do the level of energy reserves, hydration status and *Borrelia* infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks?" Parasitology **139**(03): 330-337.

Herrmann, C. and L. Gern (2013). "Survival of *Ixodes ricinus* (Acari: Ixodidae) nymphs under cold conditions is negatively influenced by frequent temperature variations." Ticks and Tick-Borne Diseases **4**(5): 445-451.

Herrmann, C., L. Gern and M. J. Voordouw (2013a). "Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*." Applied and Environmental Microbiology **79**(23): 7273-7280.

Herrmann, C., M. Voordouw and L. Gern (2013b). "*Ixodes ricinus* ticks infected with the causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, have higher energy reserves." International Journal for Parasitology **43**(6): 477-483.

Humair, P.-F., V. Douet, F. M. Cadenas, L. M. Schouls, I. Van De Pol and L. Gern (2007). "Molecular identification of bloodmeal source in *Ixodes ricinus* ticks using 12S rDNA as a genetic marker." Journal of Medical Entomology **44**(5): 869-880.

Humair, P. and L. Gern (1998). "Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland." Acta Tropica **69**(3): 213-227.

Humair, P., O. Rais and L. Gern (1999). "Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance." Parasitology **118**(01): 33-42.

Jacquet, M., J. Durand, O. Rais and M. J. Voordouw (2015). "Cross-reactive acquired immunity influences transmission success of the Lyme disease pathogen, *Borrelia afzelii*." Infection, Genetics and Evolution **36**: 131-140.

Jaenson, T. G. T. and L. Tälleklint (1992). "Incompetence of roe deer as reservoirs of the Lyme borreliosis spirochete." Journal of Medical Entomology **29**(5): 813-817.

Kurtenbach, K., S. De Michelis, S. Etti, S. M. Schäfer, H.-S. Sewell, V. Brade and P. Kraiczy (2002). "Host association of *Borrelia burgdorferi* sensu lato–the key role of host complement." Trends in Microbiology **10**(2): 74-79.

Kurtenbach, K., S. De Michelis, H.-S. Sewell, S. Etti, S. M. Schäfer, R. Hails, M. Collares-Pereira, M. Santos-Reis, K. Hanincová and M. Labuda (2001). "Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies found in individual questing ticks from Europe." Applied and Environmental Microbiology **67**(10): 4926-4929.

Kurtenbach, K., K. Hanincová, J. I. Tsao, G. Margos, D. Fish and N. H. Ogden (2006). "Fundamental processes in the evolutionary ecology of Lyme borreliosis." Nature Reviews Microbiology **4**(9): 660-669.

Kurtenbach, K., M. Peacey, S. G. Rijpkema, A. N. Hoodless, P. A. Nuttall and S. E. Randolph (1998). "Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England." Applied and Environmental Microbiology **64**(4): 1169-1174.

Lagal, V., D. Postic, E. Ruzic-Sabljic and G. Baranton (2003). "Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness." Journal of Clinical Microbiology **41**(11): 5059-5065.

Lena, S., B. Pourbohloul and R. C. Brunham (2005). "Effect of immune response on transmission dynamics for sexually transmitted infections." Journal of Infectious Diseases **191**: S78-S84.

Lively, C. and V. Apanius (1995). "Genetic diversity in host-parasite interactions." Ecology of Infectious Diseases in Natural Populations **7**: 421.

MacQueen, D. D., C. Lubelczyk, S. P. Elias, B. K. Cahill, A. J. Mathers, E. H. Lacombe, P. W. Rand and R. P. Smith Jr (2012). "Genotypic diversity of an emergent population of *Borrelia burgdorferi* at a coastal Maine island recently colonized by *Ixodes scapularis*." Vector-Borne and Zoonotic Diseases **12**(6): 456-461.

Matuschka, F.-R., P. Fischer, M. Heiler, D. Richter and A. Spielman (1992). "Capacity of European animals as reservoir hosts for the Lyme disease spirochete." Journal of Infectious Diseases **165**(3): 479-483.

Matuschka, F.-R., M. Heiler, H. Eiffert, P. Fischer, H. Lotter and A. Spielman (1993). "Diversionary role of hoofed game in the transmission of Lyme disease spirochetes." The American journal of tropical medicine and hygiene **48**(5): 693-699.

Mbow, M. L., R. D. Gilmore and R. G. Titus (1999). "An OspC-specific monoclonal antibody passively protects mice from tick-transmitted infection by *Borrelia burgdorferi* B31." Infection and Immunity **67**(10): 5470-5472.

Morán Cadenas, F., O. Rais, P.-F. Humair, V. Douet, J. Moret and L. Gern (2007a). "Identification of host bloodmeal source and *Borrelia burgdorferi* sensu lato in field-collected *Ixodes ricinus* ticks in Chaumont (Switzerland)." Journal of Medical Entomology **44**(6): 1109-1117.

Morán Cadenas, F., H. Schneider, E. Lommano, C. Burri, J. Moret and L. Gern (2007b). "A comparison of two DNA extraction approaches in the detection of *Borrelia*

burgdorferi sensu lato from live *Ixodes ricinus* ticks by PCR and reverse line blotting." Vector-Borne and Zoonotic Diseases **7**(4): 555-561.

Pal, U., X. F. Yang, M. Chen, L. K. Bockenstedt, J. F. Anderson, R. A. Flavell, M. V. Norgard and E. Fikrig (2004). "OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands." Journal of Clinical Investigation **113**(2): 220-230.

Pérez, D., Y. Kneubühler, O. Rais, F. Jouda and L. Gern (2011). "*Borrelia afzelii ospC* genotype diversity in *Ixodes ricinus* questing ticks and ticks from rodents in two Lyme borreliosis endemic areas: Contribution of co-feeding ticks." Ticks and Tick-Borne Diseases **2**(3): 137-142.

Peters, A. and C. Lively (1999). "The Red Queen and fluctuating epistasis: a population genetic analysis of antagonistic coevolution." The American Naturalist **154**(4): 393-405.

Piesman, J. and L. Gern (2004). "Lyme borreliosis in Europe and North America." Parasitology-Cambridge **129**: S191.

Plummer, F. A., J. N. Simonsen, H. Chubb, L. Slaney, J. Kimata, M. Bosire, J. O. Ndinyaachola and E. N. Ngugi (1989). "Epidemiologic evidence for the development of serovar-specific immunity after gonococcal-infection." Journal of Clinical Investigation **83**(5): 1472-1476.

Preac-Mursic, V., B. Wilske, S. Jauris, G. Will, S. Reinhardt, G. Lehnert, E. Patsouris, P. Mehraein, E. Soutschek and U. Klockmann (1992). "Active immunization with OspC protein of *Borrelia burgdorferi* protects gerbils against *B. burgdorferi* infection." Infection **20**(6): 342-349.

Probert, W. S., M. Crawford, R. B. Cadiz and R. B. LeFebvre (1997). "Immunization with outer surface protein (Osp) A, but not OspC, provides cross-protection of mice challenged with North American isolates of *Borrelia burgdorferi*." Journal of Infectious Diseases **175**(2): 400-405.

Probert, W. S. and R. B. LeFebvre (1994). "Protection of C3H/HeN mice from challenge with *Borrelia burgdorferi* through active immunization with OspA, OspB, or OspC, but not with OspD or the 83-kilodalton antigen." Infection and Immunity **62**(5): 1920-1926.

Qiu, W.-G., D. E. Dykhuizen, M. S. Acosta and B. J. Luft (2002). "Geographic uniformity of the Lyme disease spirochete (*Borrelia burgdorferi*) and its shared history with tick vector (*Ixodes scapularis*) in the northeastern United States." Genetics **160**(3): 833-849.

Qiu, W.-G., S. E. Schutzer, J. F. Bruno, O. Attie, Y. Xu, J. J. Dunn, C. M. Fraser, S. R. Casjens and B. J. Luft (2004). "Genetic exchange and plasmid transfers in *Borrelia burgdorferi* sensu stricto revealed by three-way genome comparisons and multilocus sequence typing." Proceedings of the National Academy of Sciences of the United States of America **101**(39): 14150-14155.

Qiu, W. G., E. M. Bosler, J. R. Campbell, G. D. Ugine, I. N. Wang, B. J. Luft and D. E. Dykhuizen (1997). "A population genetic study of *Borrelia burgdorferi* sensu stricto from eastern Long Island, New York, suggested frequency-dependent selection, gene flow and host adaptation." Hereditas **127**(3): 203-216.

Råberg, L. (2012). "Infection intensity and infectivity of the tick-borne pathogen Borrelia afzelii." Journal of Evolutionary Biology **25**(7): 1448-1453.

Rauter, C. and T. Hartung (2005). "Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis." Applied and Environmental Microbiology **71**(11): 7203-7216.

Richter, D., A. Debski, Z. Hubalek and F.-R. Matuschka (2012). "Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks." Vector-Borne and Zoonotic Diseases **12**(1): 21-27.

Richter, D., B. Klug, A. Spielman and F.-R. Matuschka (2004). "Adaptation of diverse Lyme disease spirochetes in a natural rodent reservoir host." Infection and Immunity **72**(4): 2442-2444.

Rollend, L., D. Fish and J. E. Childs (2013). "Transovarial transmission of *Borrelia* spirochetes by *Ixodes scapularis*: A summary of the literature and recent observations." Ticks and Tick-Borne Diseases **4**(1): 46-51.

Schmid-Hempel, P. and J. Jokela (2002). "Socially structured populations and evolution of recombination under antagonistic coevolution." The American Naturalist **160**(3): 403-408.

Seinost, G., D. E. Dykhuizen, R. J. Dattwyler, W. T. Golde, J. J. Dunn, I. N. Wang, G. P. Wormser, M. E. Schriefer and B. J. Luft (1999). "Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans." Infection and Immunity **67**(7): 3518-3524.

Stotz, H. U., J. G. Bishop, C. W. Bergmann, M. Koch, P. Albersheim, A. G. Darvill and J. M. Labavitch (2000). "Identification of target amino acids that affect interactions of fungal polygalacturonases and their plant inhibitors." Physiological and Molecular Plant Pathology **56**(3): 117-130.

Strandh, M. and L. Råberg (2015). "Within-host competition between *Borrelia afzelii ospC* strains in wild hosts as revealed by massively parallel amplicon sequencing." Phil. Trans. R. Soc. B **370**(1675): 20140293.

Swanson, K. I. and D. E. Norris (2008). "Presence of multiple variants of *Borrelia burgdorferi* in the natural reservoir *Peromyscus leucopus* throughout a transmission season." Vector-Borne and Zoonotic Diseases **8**(3): 397-406.

Tälleklint, L. and T. G. Jaenson (1994). "Transmission of *Borrelia burgdorferi* sl from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari: Ixodidae), in Sweden." Journal of Medical Entomology **31**(6): 880-886.

Telford 3rd, S., T. N. Mather, S. I. Moore, M. L. Wilson and A. Spielman (1988). "Incompetence of deer as reservoirs of the Lyme disease spirochete." The American journal of tropical medicine and hygiene **39**(1): 105-109.

Theisen, M., M. Borre, M. J. Mathiesen, B. Mikkelsen, A. M. Lebech and K. Hansen (1995). "Evolution of the *Borrelia burgdorferi* outer surface protein OspC." Journal of Bacteriology **177**(11): 3036-3044.

Tilly, K., J. G. Krum, A. Bestor, M. W. Jewett, D. Grimm, D. Bueschel, R. Byram, D. Dorward, M. J. VanRaden, P. Stewart and P. Rosa (2006). "*Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection." Infection and Immunity **74**(6): 3554-3564.

Tonetti, N., M. J. Voordouw, J. Durand, S. Monnier and L. Gern (2015). "Genetic variation in transmission success of the Lyme borreliosis pathogen *Borrelia afzelii*." Ticks and Tick-Borne Diseases **6**(3): 334-343.

Tsao, J. I. (2009). "Reviewing molecular adaptations of Lyme borreliosis spirochetes in the context of reproductive fitness in natural transmission cycles." Veterinary Research **40**(2): 1.

Wang, I. N., D. E. Dykhuizen, W. G. Qin, J. J. Dunn, E. M. Bosler and B. J. Luft (1999). "Genetic diversity of *ospC* in a local population of *Borrelia burgdorferi* sensu stricto." Genetics **151**(1): 15-30.

Wormser, G. P., D. Brisson, D. Liveris, K. Hanincova, S. Sandigursky, J. Nowakowski, R. B. Nadelman, S. Ludin and I. Schwartz (2008). "*Borrelia burgdorferi* genotype predicts the capacity for hematogenous dissemination during early Lyme disease." Journal of Infectious Diseases **198**(9): 1358-1364.

Figures

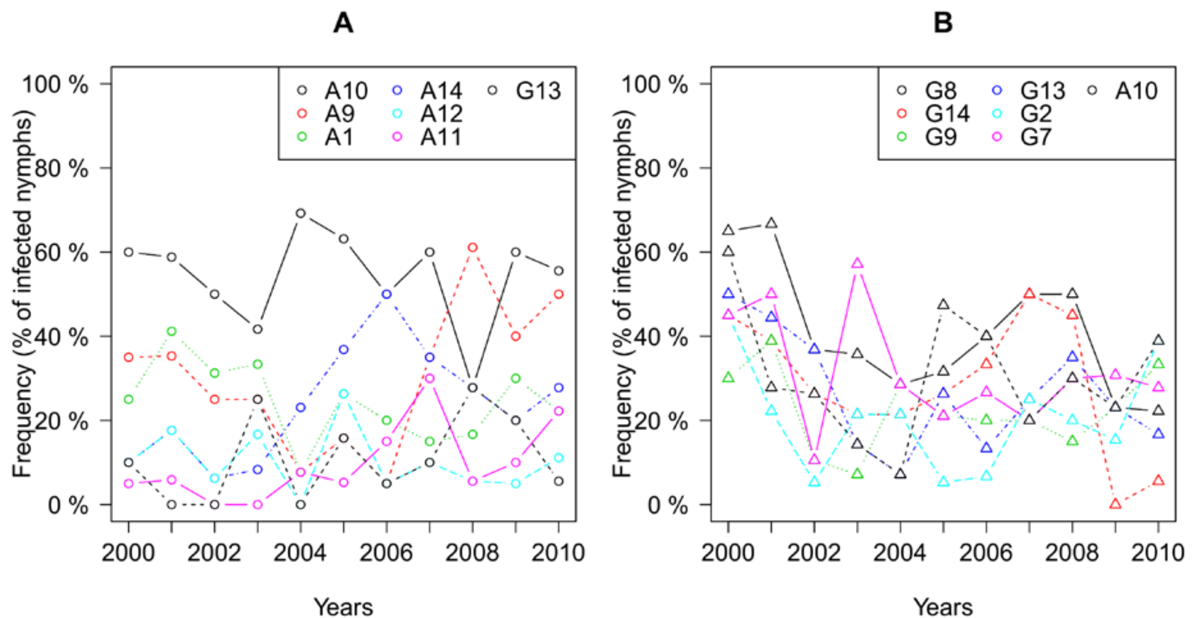

Figure 1. Relative frequencies of the major *ospC* groups are shown for *Borrelia afzelii* and *B. garinii* over the eleven years of the study. The relative frequencies are shown for the seven most common major *ospC* groups of (A) *B. afzelii* and (B) *B. garinii*. The relative frequencies were calculated as the proportion of nymphal tick-derived isolates infected with a particular major *ospC* group. The community of *B. afzelii* was dominated by strains carrying major *ospC* group A10 for the duration of the study.

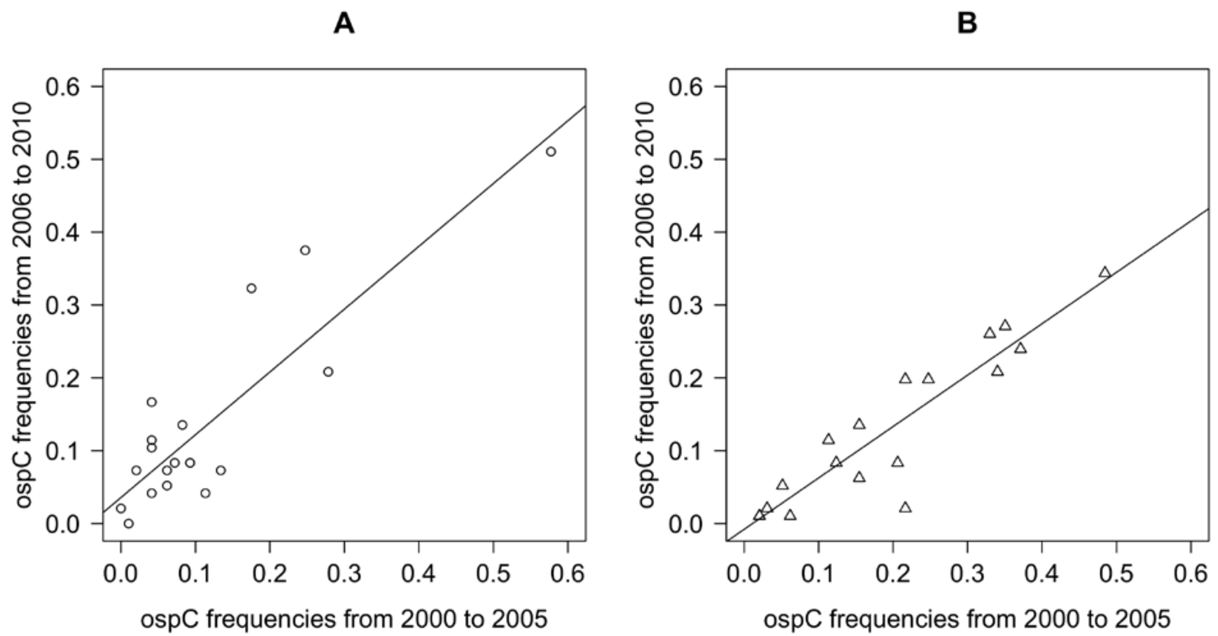

Figure 2. The community of *ospC* strains is stable over time. The relationship between the relative frequencies of the major *ospC* groups of the first 6 years versus the last 5 years of the survey is shown for both (A) *B. afzelii* and (B) *B. garinii*. The Pearson correlation of the relative frequency distribution of the *ospC* strains between the two time periods was highly significant for both *Borrelia* species.

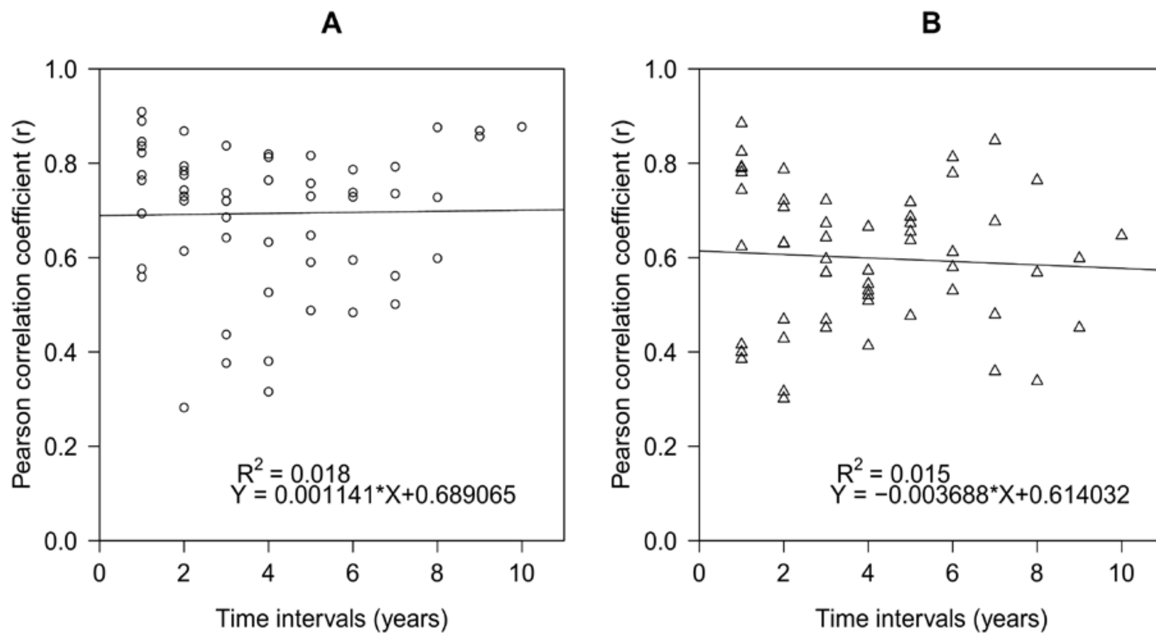

Figure 3. The relative frequency distribution of the major *ospC* groups is stable over time. The Pearson correlation coefficient (r) of the relative frequency distribution of the major *ospC* groups was calculated for each pair of years (n = 55 pairs). The relationship between these r-values and the time interval between the years in the pair is shown for both (A) *B. afzelii* and (B) *B. garinii*. For both *Borrelia* species, there is no significant relationship between r and the time interval showing that the frequencies of the different major *ospC* groups are stable over time. The r^2-value and the equation of the linear regression are indicated on each plot.

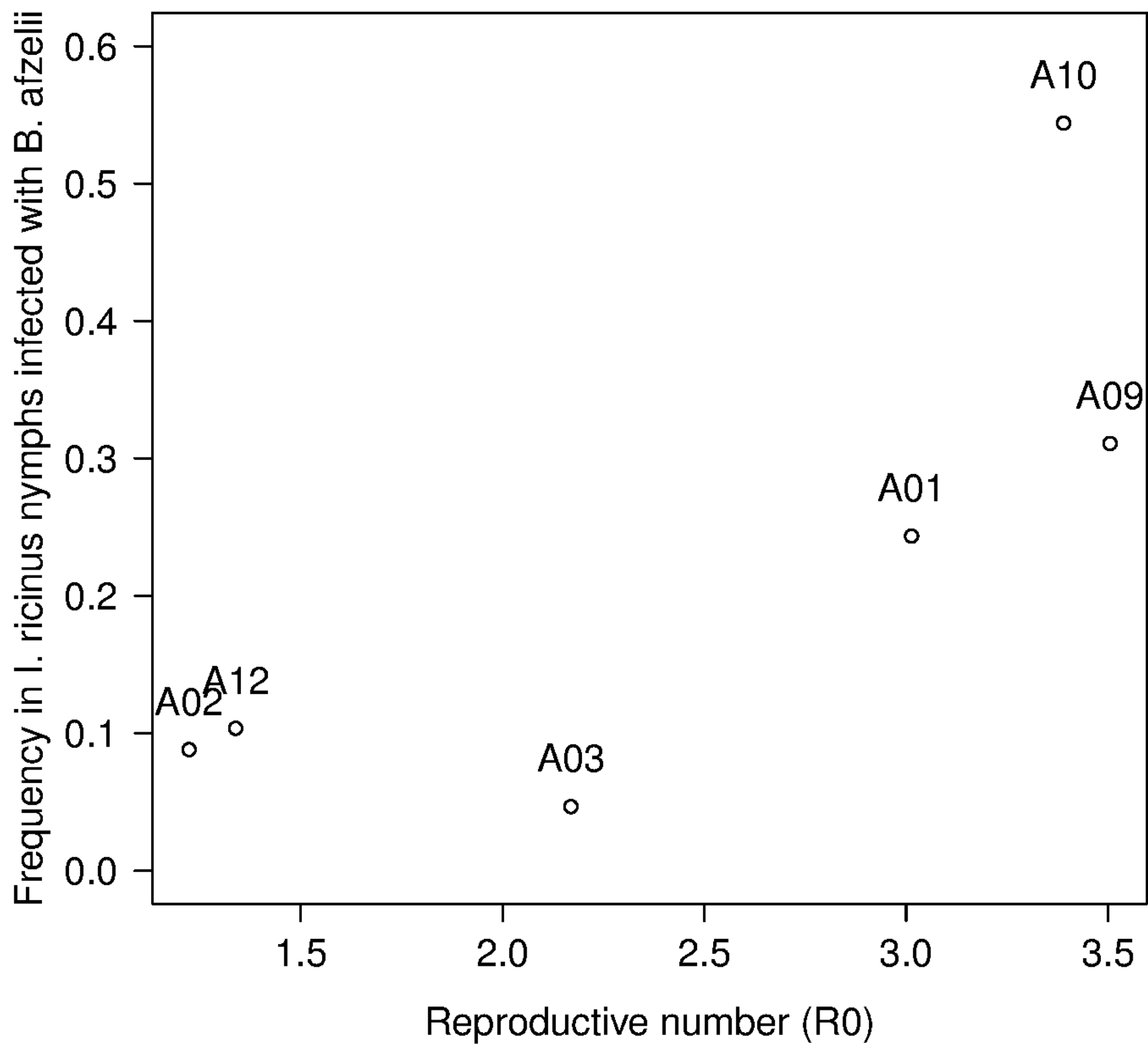

Figure 4. The reproductive numbers (R_0) of six *B. afzelii ospC* strains determine the strain-specific relative frequencies in a population of wild *I. ricinus* nymphs. Each data point is labeled with the name of the major *ospC* group of the *B. afzelii* strain. The strain-specific R_0 values were estimated from an experimental infection study using laboratory mice (Tonetti et al. 2015). The strain-specific relative frequencies were estimated over the 11-year duration of the present study.

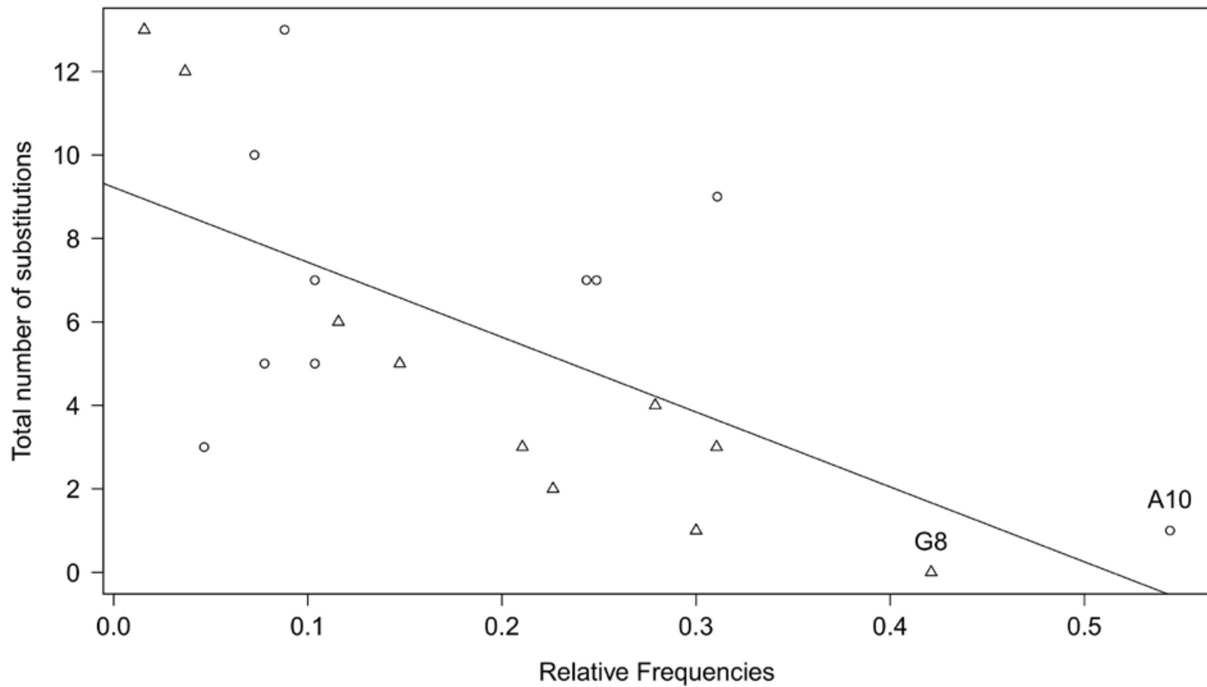

Figure 5. The relative frequencies of the major *ospC* groups are negatively correlated with the total number of substitutions within the major *ospC* groups. Common major *ospC* groups have fewer nucleotide substitutions than rare ones. The major *ospC* groups of *B. afzelii* and *B. garinii* are shown with circles and triangles, respectively. The most common major *ospC* groups of *B. afzelii* and *B. garinii* are A10 and G8, respectively, and are labeled in Figure 5.

Supplementary material:

Evolution of the relative frequencies of the major *ospC* groups over time: For each of the 10 *B. afzelii* native major *ospC* groups (A1, A2, A3, A5, A7, A9, A10, A11, A12, and A14), we used a proportion test to determine whether the relative frequency of a given year was significantly different from the average relative frequency of that strain over the duration of the study. We did the same for each of the 9 most common *B. garinii* native major *ospC* groups (G2, G4, G6, G7, G8, G9, G11, G13, and G14). The other two *B. garinii* major *ospC* groups (G10 and G15) were too rare and were not analyzed. There were 110 proportion tests for *B. afzelii* (10 native major *ospC* groups*11 years) and 99 proportion tests for *B. garinii* (9 native major *ospC* groups*11 years). Assuming a type I error rate of 0.05, we expect that *B. afzelii* and *B. garinii* should have 5.50 (0.05*110 proportion tests) and 4.95 (0.05*99 proportion tests) proportion tests that are significantly different. For *B. afzelii*, only 6 of 110 frequencies were significantly different ($\alpha = 0.05$). For *B. garinii*, only 5 of 99 frequencies were significantly different ($\alpha = 0.05$). Thus the observed number of significant frequencies for both genospecies (6 + 5 = 11) was equal to the expected type I error rate (5.50 + 4.95 = 10.45). This result supports the idea that the major *ospC* strains did not fluctuate over time. The trajectories of the 5 most common *B. afzelii ospC* groups and the 5 most common *B. garinii ospC* groups are shown in Figures S3 and S4, respectively.

Table S1: Purity of the six *B. afzelii* isolates used in the experimental infection study by Tonetti et al. (2015). These six *B. afzelii* isolates were used to calculate the strain-specific fitness (R_0) in laboratory mice. The *ospC* gene of each of the six isolates was sequenced using 454 sequencing.

Major *ospC* group	Total number of sequences	Purity
A1	873	99.20%
A2	141	99.29%
A3	977	100.00%
A9	131	99.24%
A10	162	100.00%
A10	1313	100.00%
A12	1105	99.70%

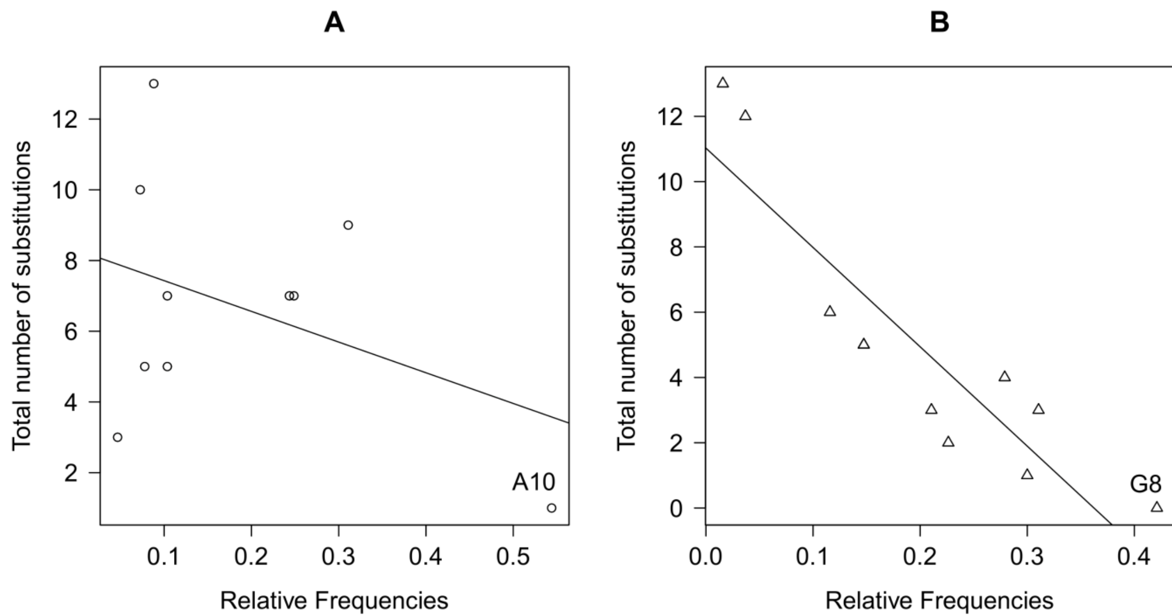

Figure S1. The relative frequencies of the major *ospC* groups are negatively correlated with the total number of substitutions within the major *ospC* groups. Common major *ospC* groups have fewer nucleotide substitutions than rare ones. The relationship is shown for both (A) *B. afzelii* and (B) *B. garinii*. The most common major *ospC* groups of *B. afzelii* and *B. garinii* are A10 and G8, respectively, and are labeled in Figure S1.

Figure S2. The origin of the *Borrelia* DNA extraction has no effect on the relative frequencies of the major *ospC* group strains. The relationship between the relative frequencies of the major *ospC* groups (combined for 2009 and 2010) using DNA from nymph-derived isolates cultured in BSK medium versus DNA directly extracted from the nymphs is shown for both (A) *B. afzelii* and (B) *B. garinii*. The Pearson correlation of the relative frequency distribution of the *ospC* strains between the two DNA extraction methods was highly significant in both *B. afzelii* ($r = 0.845$, $t = 7.056$, $df = 20$, $p < 0.001$) and *B. garinii* ($r = 0.516$, $t = 2.559$, $df = 18$, $p = 0.019$).

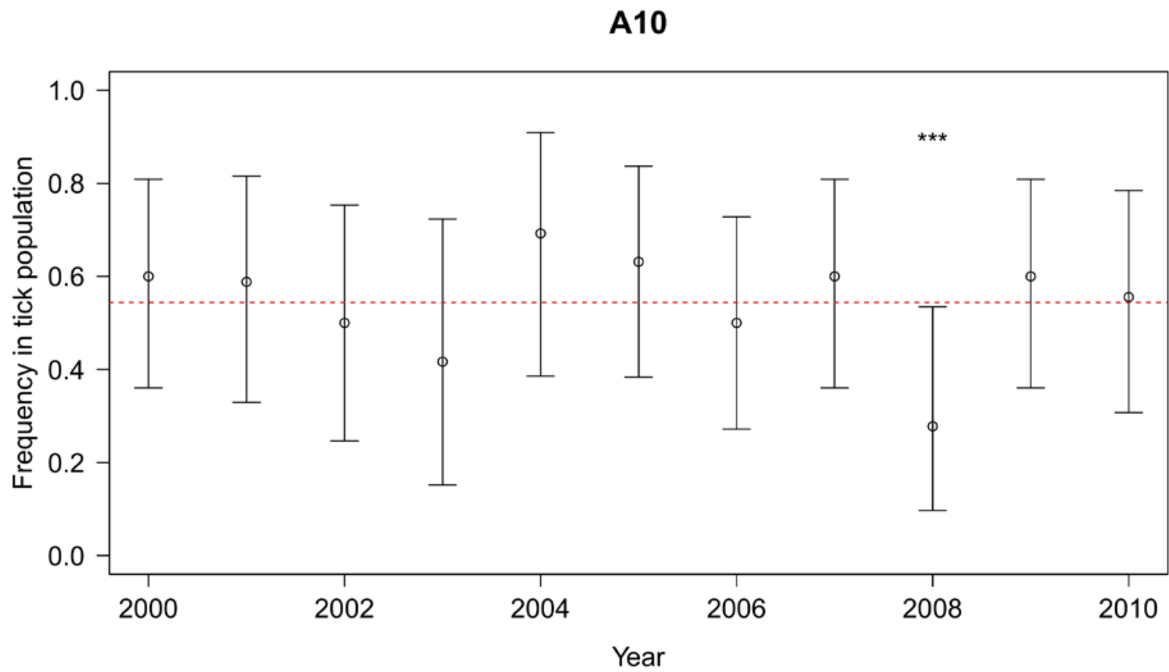

Figure S3a. The relative frequencies of *Borrelia afzelii* major *ospC* group A10 over the 11 years of the study. The relative frequencies were calculated as the proportion of nymphal tick-derived *B. afzelii* isolates infected with major *ospC* group A10. The bars indicate the 95% confidence interval and the red dotted line shows the mean relative frequency of group A10 over the duration of the study. The asterisks indicate the years where the frequencies were significantly different (α = 0.05) from the long-term mean.

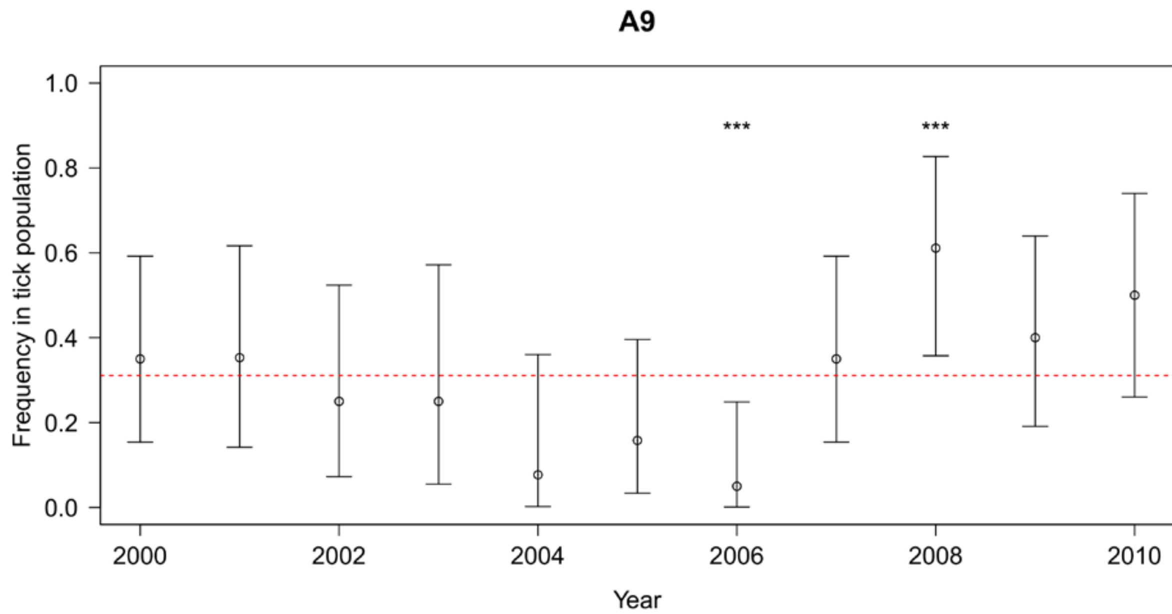

Figure S3b. The relative frequencies of *Borrelia afzelii* major *ospC* group A9 over the 11 years of the study. The relative frequencies were calculated as the proportion of nymphal tick-derived *B. afzelii* isolates infected with major *ospC* group A9. The bars indicate the 95% confidence interval and the red dotted line shows the mean relative frequency of group A9 over the duration of the study. The asterisks indicate the years where the frequencies were significantly different ($\alpha = 0.05$) from the long-term mean.

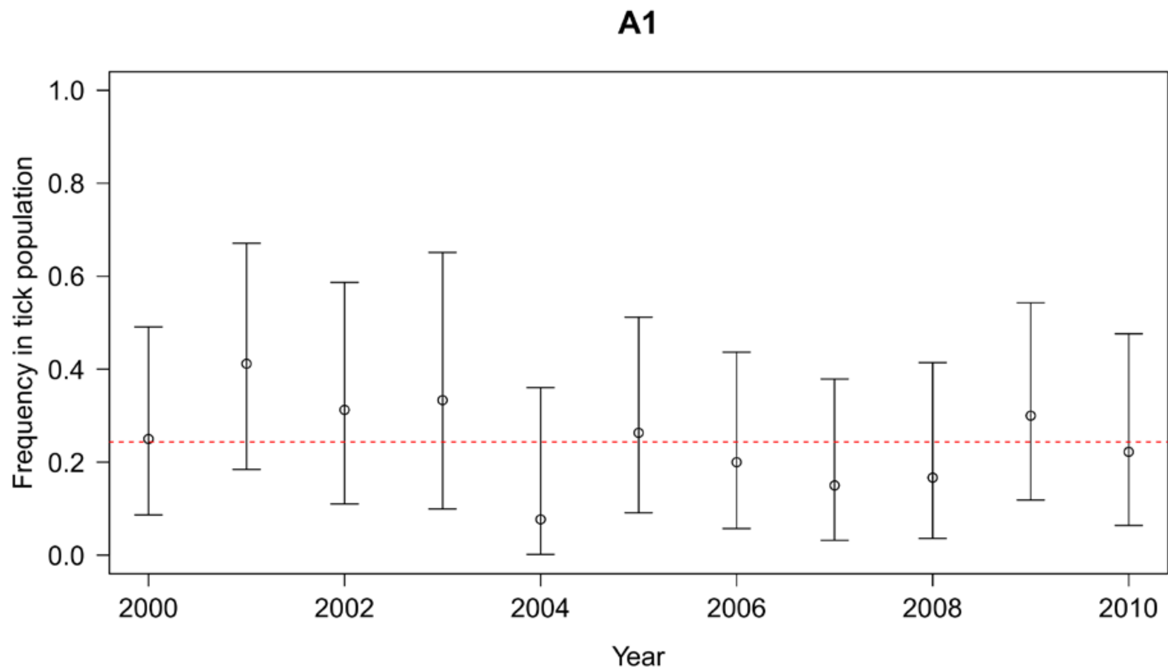

Figure S3c. The relative frequencies of *Borrelia afzelii* major *ospC* group A1 over the 11 years of the study. The relative frequencies were calculated as the proportion of nymphal tick-derived *B. afzelii* isolates infected with major *ospC* group A1. The bars indicate the 95% confidence interval and the red dotted line shows the mean relative frequency of group A1 over the duration of the study. The asterisks indicate the years where the frequencies were significantly different ($\alpha = 0.05$) from the long-term mean.

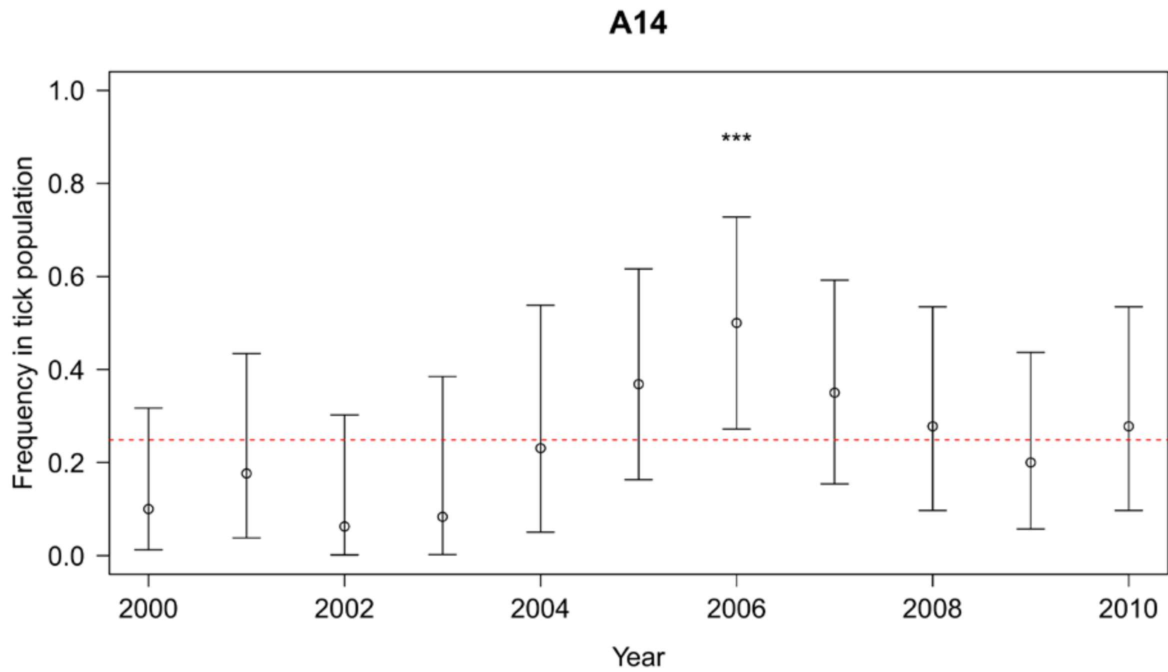

Figure S3d. The relative frequencies of *Borrelia afzelii* major *ospC* group A14 over the 11 years of the study. The relative frequencies were calculated as the proportion of nymphal tick-derived *B. afzelii* isolates infected with major *ospC* group A14. The bars indicate the 95% confidence interval and the red dotted line shows the mean relative frequency of group A14 over the duration of the study. The asterisks indicate the years where the frequencies were significantly different (α = 0.05) from the long-term mean.

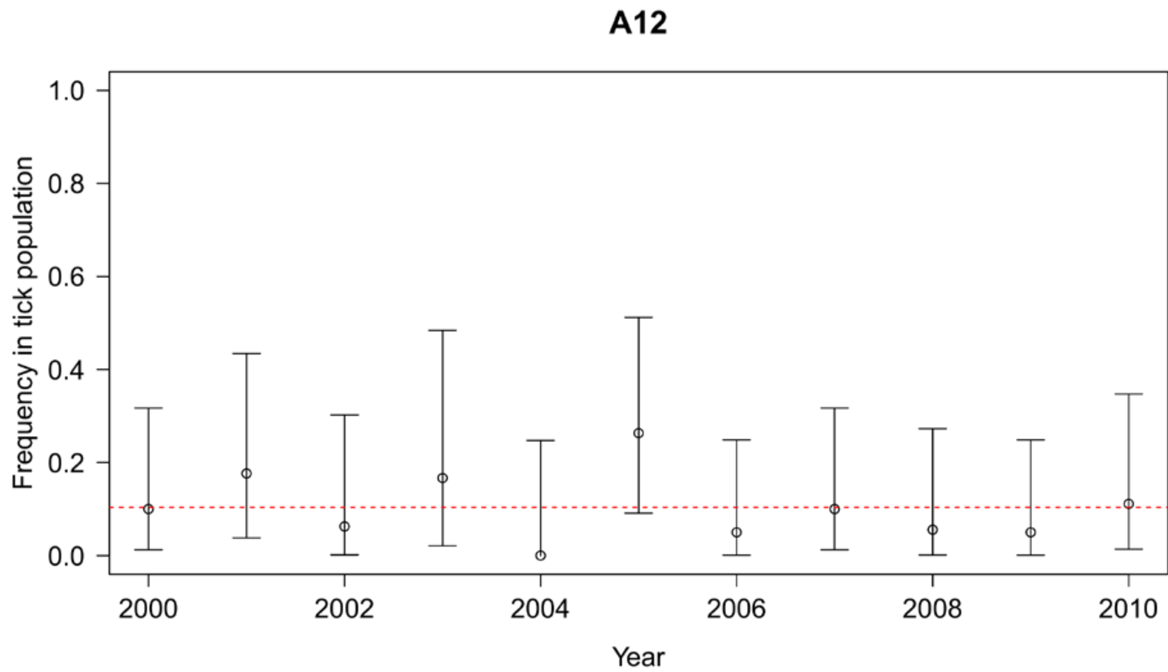

Figure S3e. The relative frequencies of *Borrelia afzelii* major *ospC* group A12 over the 11 years of the study. The relative frequencies were calculated as the proportion of nymphal tick-derived *B. afzelii* isolates infected with major *ospC* group A12. The bars indicate the 95% confidence interval and the red dotted line shows the mean relative frequency of group A12 over the duration of the study. The asterisks indicate the years where the frequencies were significantly different ($\alpha = 0.05$) from the long-term mean.

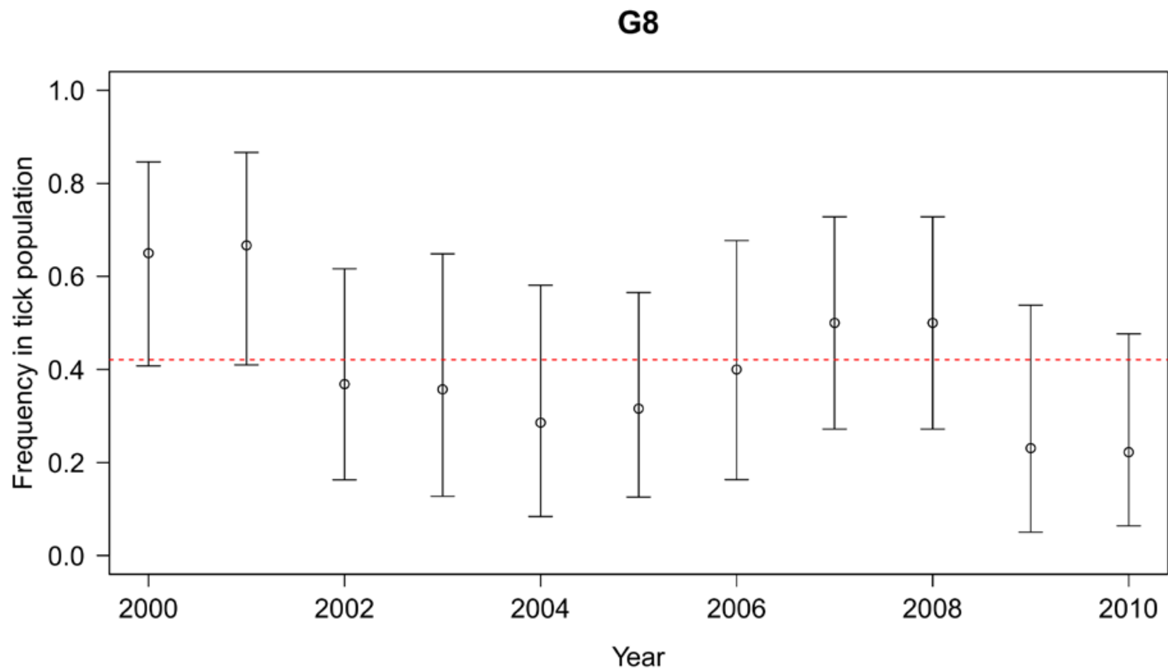

Figure S4a. The relative frequencies of *Borrelia garinii* major *ospC* group G8 over the 11 years of the study. The relative frequencies were calculated as the proportion of nymphal tick-derived *B. garinii* isolates infected with major *ospC* group G8. The bars indicate the 95% confidence interval and the red dotted line shows the mean relative frequency of group G8 over the duration of the study. The asterisks indicate the years where the frequencies were significantly different ($\alpha = 0.05$) from the long-term mean.

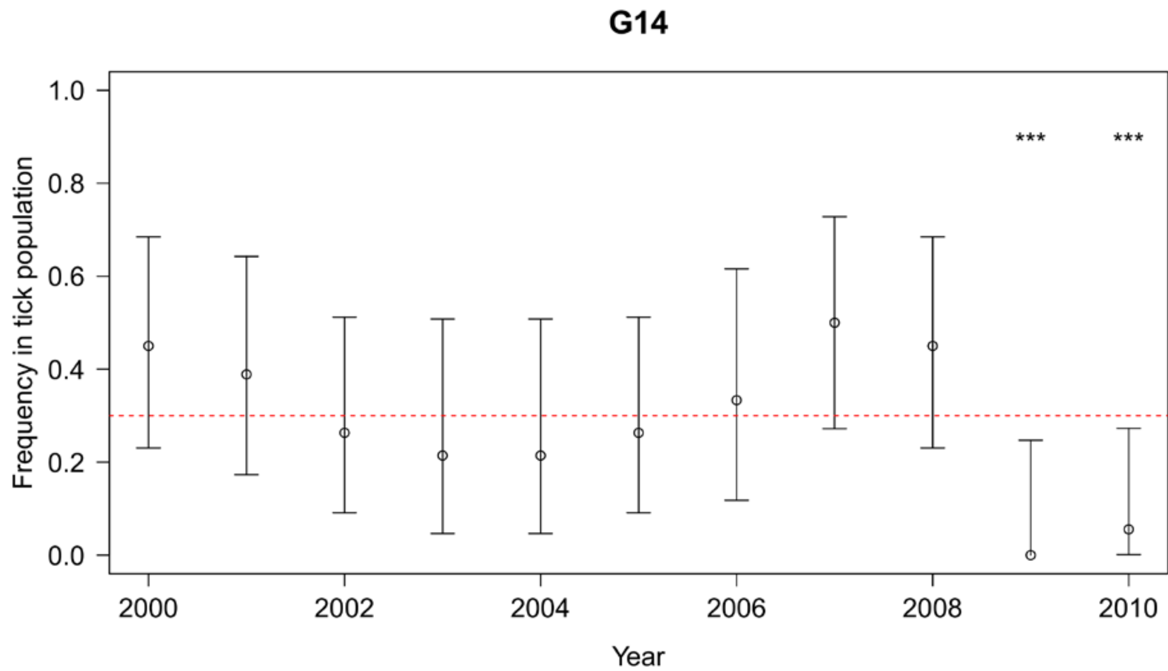

Figure S4b. The relative frequencies of *Borrelia garinii* major *ospC* group G14 over the 11 years of the study. The relative frequencies were calculated as the proportion of nymphal tick-derived *B. garinii* isolates infected with major *ospC* group G14. The bars indicate the 95% confidence interval and the red dotted line shows the mean relative frequency of group G14 over the duration of the study. The asterisks indicate the years where the frequencies were significantly different ($\alpha = 0.05$) from the long-term mean.

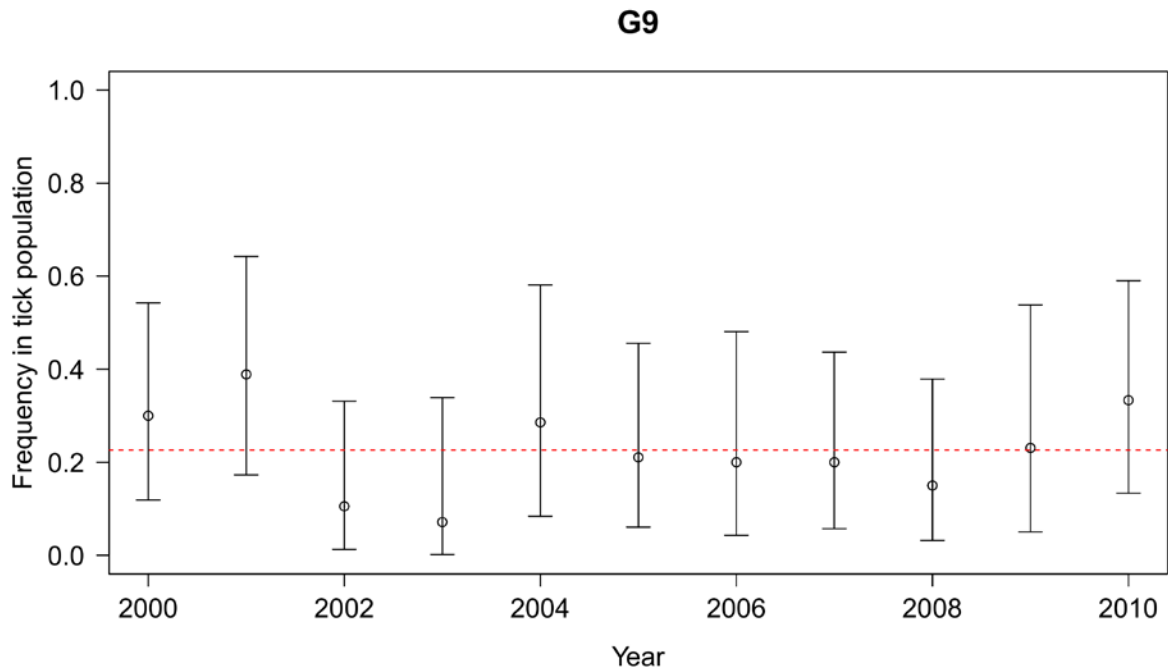

Figure S4c. The relative frequencies of *Borrelia garinii* major *ospC* group G9 over the 11 years of the study. The relative frequencies were calculated as the proportion of nymphal tick-derived *B. garinii* isolates infected with major *ospC* group G9. The bars indicate the 95% confidence interval and the red dotted line shows the mean relative frequency of group G9 over the duration of the study. The asterisks indicate the years where the frequencies were significantly different ($\alpha = 0.05$) from the long-term mean.

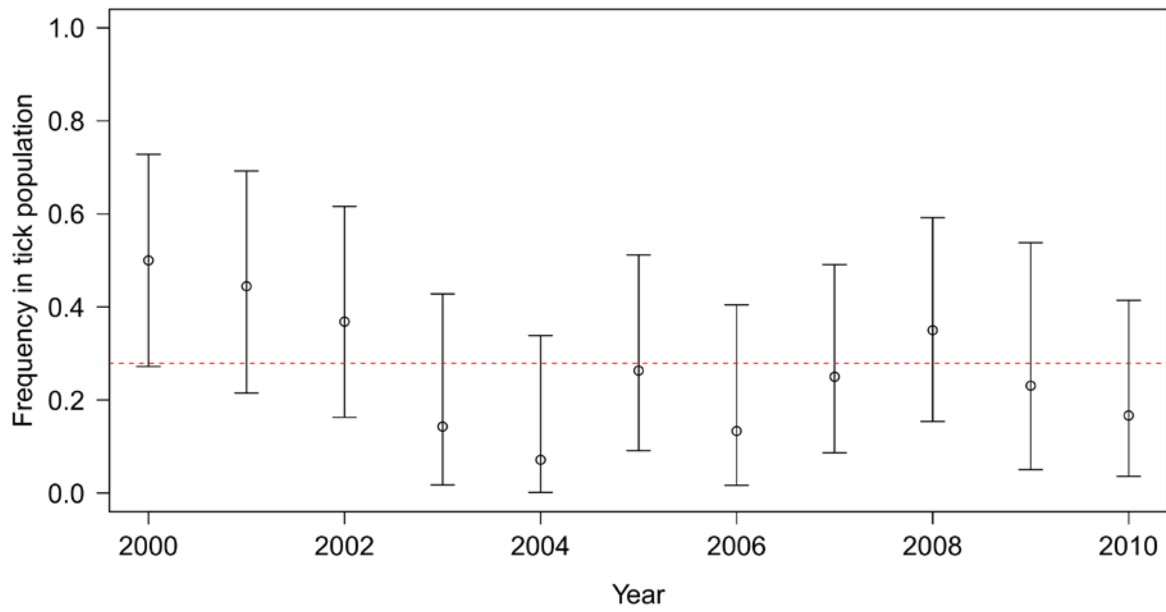

Figure S4d. The relative frequencies of *Borrelia garinii* major *ospC* group G13 over the 11 years of the study. The relative frequencies were calculated as the proportion of nymphal tick-derived *B. garinii* isolates infected with major *ospC* group G13. The bars indicate the 95% confidence interval and the red dotted line shows the mean relative frequency of group G13 over the duration of the study. The asterisks indicate the years where the frequencies were significantly different ($\alpha = 0.05$) from the long-term mean.

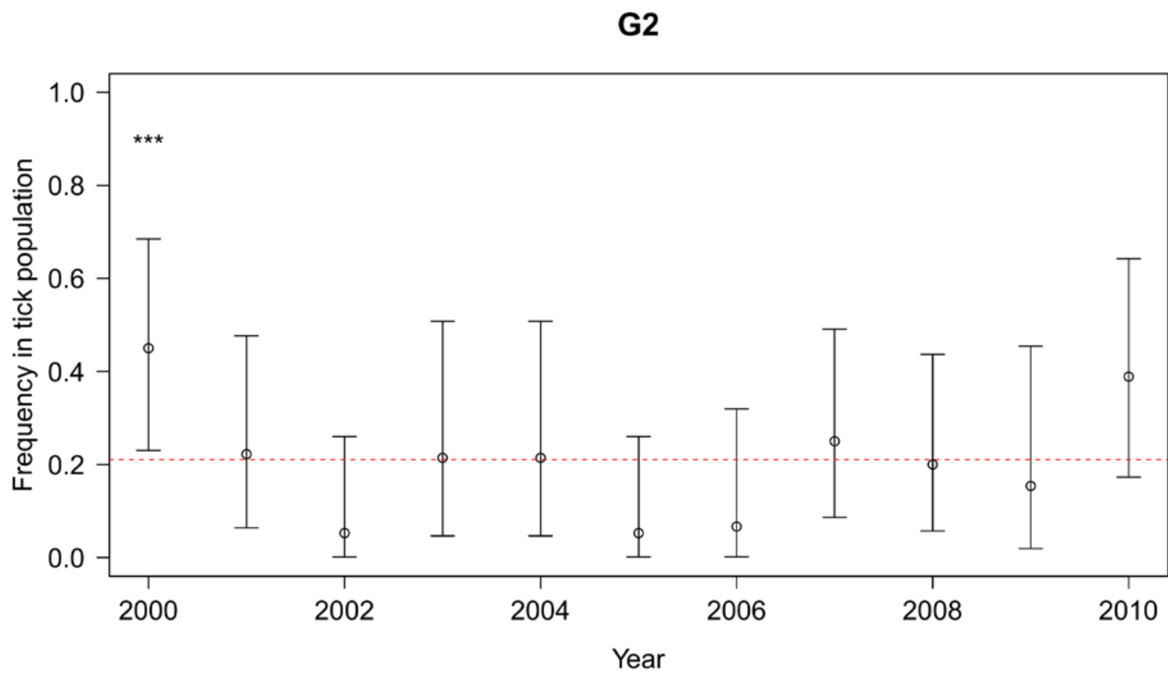

Figure S4e. The relative frequencies of *Borrelia garinii* major *ospC* group G2 over the 11 years of the study. The relative frequencies were calculated as the proportion of nymphal tick-derived *B. garinii* isolates infected with major *ospC* group G2. The bars indicate the 95% confidence interval and the red dotted line shows the mean relative frequency of group G2 over the duration of the study. The asterisks indicate the years where the frequencies were significantly different (α = 0.05) from the long-term mean.

8. 4th Paper

Competition between strains of the Lyme borreliosis pathogen in the tick vector

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Abstract

Mixed or multiple-strain infections are common in vector-borne diseases and have important implications for the epidemiology of these pathogens. Previous studies have mainly focused on interactions between pathogen strains in the vertebrate host, but little is known about what happens in the arthropod vector.

The spirochete bacteria *Borrelia afzelii* and *B. garinii* are the two most common causes of Lyme borreliosis in Europe. For each of these two tick-borne pathogens, we studied inter-strain competition in their common tick vector, *Ixodes ricinus*. We analyzed *I. ricinus* nymphal ticks that had been collected from the same field site over a period of 3 years. We used qPCR to estimate the spirochete load and 454-sequencing of the polymorphic *ospC* gene to characterize the community of *Borrelia* strains within each tick.

Ticks have a fixed limit for spirochetes resulting in strong competition between strains. In both *Borrelia* species, strains with the highest spirochete load in the nymphal tick were the most common strains in the tick population. The spirochete load in the tick is an important life history trait for Lyme disease pathogens. Competition between *Borrelia* strains in the tick vector plays a critical role in the community structure of this multiple-strain, tick-borne pathogen.

Introduction

Genetically diverse pathogens often establish mixed infections in their hosts that consist of multiple strains or genotypes of the same pathogen species (Read and Taylor 2001, Mideo 2009, Balmer and Tanner 2011). Mixed infections represent a major problem for public health because they complicate the development of treatments and vaccines (Balmer and Tanner 2011, Johnson et al. 2015). Some pathogen strains are more infective or do more harm to certain host species (Seinost et al. 1999, Lagal et al. 2003). The strain composition of the mixed infection can therefore greatly influence disease pathology (Louhi et al. 2015). In addition, competition between pathogen strains can lead to the evolution of increased virulence (Alizon et al. 2013), transmission (Susi et al. 2015), or infectivity (Karvonen et al. 2011).

In vector-borne diseases, mixed strain infections are common and occur in both the vertebrate reservoir host and the arthropod vector (Schmid-Hempel 2011). Studies on interactions between strains of vector-borne pathogens typically focus on the vertebrate reservoir host (Mercereau-Puijalon 1996, Smith et al. 1999, Read and Taylor 2001, De Roode et al. 2003, de Roode et al. 2005, Råberg et al. 2006, Mideo et al. 2008). To date, only a few studies on vector-borne pathogens have investigated the importance of inter-strain interactions in the arthropod vector (Araújo et al. 2007, Peacock et al. 2007, Rego et al. 2014, Reif et al. 2014, Pollitt et al. 2015). Some of these studies have shown that mixed infections can enhance or facilitate colonization of the arthropod vector (Araújo et al. 2007, Pollitt et al. 2015). Other studies have shown that the arthropod vector is an important population genetic bottleneck that reduces the diversity of pathogen strains (Rego et al. 2014, Reif et al. 2014). Very few studies have shown the existence of competitive interactions between strains in the arthropod vector (Reif et al. 2014). To date, no studies have investigated whether the ability to compete

and maintain a high abundance inside the arthropod vector influences the strain-specific prevalence in a natural vector-borne disease system.

Borrelia burgdorferi sensu lato (s. l.) is a complex of spirochete bacteria that causes Lyme borreliosis (LB), the most common vector-borne disease in Europe and North America (Kurtenbach et al. 2006). In Europe, the two most common etiological agents of LB are *B. afzelii* and *B. garinii*. These two *Borrelia* genospecies are sympatric in distribution but are adapted to different classes of vertebrate reservoir hosts: rodents and birds, respectively (Gern and Humair 2002, Kurtenbach et al. 2002, Piesman and Gern 2004). Their principal vector is the castor bean tick, *Ixodes ricinus*, which has three obligate blood-feeding stages: larva, nymph, and adult. Larval ticks acquire spirochetes while feeding on infected reservoir hosts and subsequently molt into infected nymphs that transmit the pathogen to the next generation of reservoir hosts the following year. Vertical transmission *B. burgdorferi* s. l. is absent or rare in *Ixodes* ticks (Richter et al. 2012, Rollend et al. 2013). Due to their adaptation to different reservoir hosts, *B. afzelii* and *B. garinii* rarely co-infect the same tick (Kurtenbach et al. 2001, Rauter and Hartung 2005, Gern et al. 2010, Herrmann et al. 2013).

LB pathogens establish mixed-strain infections in both the vertebrate host (Brisson and Dykhuizen 2004, Swanson and Norris 2008, Andersson et al. 2013, Strandh and Råberg 2015) and the tick vector (Wang et al. 1999, Qiu et al. 2002, Brisson and Dykhuizen 2004, Pérez et al. 2011, Durand et al. 2015). Strains are often defined by the highly polymorphic, single-locus, *ospC* gene (Wang et al. 1999, Baranton et al. 2001, Lagal et al. 2003, Bunikis et al. 2004, Pérez et al. 2011, Andersson et al. 2013, Durand et al. 2015, Strandh and Råberg 2015). This gene codes for outer surface protein C (OspC), which is a critical virulence factor for the infection of the vertebrate host (Grimm et al. 2004, Tilly et al. 2006). The *ospC* alleles can be clustered into major *ospC* groups, which are more than 8% divergent in DNA sequence from other such clusters (Wang et al. 1999, Baranton et al. 2001, Lagal et al. 2003, Bunikis et

al. 2004, Durand et al. 2015). Each of the three most important LB pathogens, *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto (s. s.), has approximately 20 different major *ospC* groups (Baranton et al. 2001, Durand et al. 2015). In the present study, we use the *ospC*-typing system to study mixed-strain infections within each of the two *Borrelia* species, as others have done previously (Hellgren et al. 2011, Pérez et al. 2011, Andersson et al. 2013, Durand et al. 2015, Strandh and Råberg 2015).

The purpose of the present study was to investigate whether inter-strain competition and strain-specific abundance in the tick vector influenced the strain fitness (measured as strain frequency) in two independent Lyme disease systems. For each of the two *Borrelia* genospecies, we determined the frequencies and the abundance of the different major *ospC* groups in a local population of *I. ricinus* nymphs over a period of three years. We predicted that a fixed carrying capacity in the nymphal tick would induce strong inter-strain competition, where each additional strain would reduce the spirochete load per strain. We also predicted that the *Borrelia ospC* strains that maintain the highest spirochete load inside the tick vector would be the most common strains in our local population of *I. ricinus* ticks. This is the first study to show that mixed-strain populations of Lyme disease pathogens experience strong competition in the tick vector. In addition, we show that the spirochete abundance in the tick vector is a key predictor of whether a given strain is common or rare.

Materials and methods

Tick collection and *Borrelia* detection: *I. ricinus* nymphal ticks were sampled from the Bois de l'Hôpital site in a deciduous forest near Neuchâtel (47°00'55.6'' N, 6°94'16.7'' E) over a period of three years (2009 to 2011). These ticks were sampled as part of the PhD thesis of Coralie Herrmann and details of the field sampling and subsequent molecular

methods have been described previously (Herrmann and Gern 2010, Herrmann and Gern 2012, Herrmann and Gern 2013, Herrmann et al. 2013, Herrmann et al. 2013).

Briefly, total DNA from nymphal ticks was extracted using NH_4OH. Ticks were tested for infection with *B. burgdorferi* s. l. using a qPCR assay that targeted the *flagellin* gene. The use of standards in this qPCR assay allowed us to estimate the spirochete load in each nymphal tick. Nymphs that tested positive for *Borrelia* infection were processed using a PCR-Reverse Line Blot (RLB) assay that targets the 23S-5S spacer gene (Burri et al. 2007). The RLB allowed us to identify each of the five *B. burgdorferi* s. l. genospecies present at our study site: *B. burgdorferi* s. s., *B. afzelii*, *B. garinii*, *B. valaisiana*, and *B. bavariensis*. Of the 7,400 nymphs that were collected, 1,741 tested positive for infection with one or more *Borrelia* genospecies, and 788 and 290 nymphs were singly infected with *B. afzelii* and *B. garinii*, respectively (Herrmann et al. 2013).

Sampling strategy of *Borrelia*-infected nymphs: We only included the questing nymphs that, according to the RLB, were singly infected with either *B. afzelii* or *B. garinii*. For each of the two *Borrelia* species, we randomly sampled a maximum of 52 infected nymphs for each of the three years of the study. Using this sampling strategy, we obtained 153 infected nymphs for *B. afzelii* (49, 51, and 52 for 2009, 2010, and 2011, respectively) and 100 infected nymphs for *B. garinii* (12, 42, 47 for 2009, 2010, and 2011, respectively).

Characterization of the community of major *ospC* group strains via 454-sequencing: For each of the 253 *Borrelia*-infected nymphs, the *ospC* gene (~600 base pairs) was amplified using the PCR protocol of Bunikis et al. (2004). 454-sequencing in the forward direction only was performed on a 454 Roche GS FLX sequencing apparatus and was outsourced to Microsynth AG (Balgach, Switzerland) as previously described in Durand et al. (2015). The 454-sequencing run produced 130,948 sequences of the *ospC* gene. After cleaning the data set (Durand et al. 2015), we retained 92,645 *ospC* gene sequences that were

each 585 base pairs long. For each nymphal tick, the community of major *ospC* groups was based on an average of 366 *ospC* gene sequences (165 reads for the geometric mean). A nymph was considered as infected with a given *ospC* strain as soon as we recovered a single read for that major *ospC* group.

Statistical analyses

Estimate of the spirochete load for the *Borrelia*-infected nymphal ticks: The original qPCR assay estimated the spirochete load for a volume of 10 µl of DNA extract (Herrmann et al. 2013). The spirochete loads were therefore multiplied by a correction factor to calculate the total spirochete load for each tick (see supplementary material for details).

Relative frequencies of the major *ospC* group strains: In the present study, we report the relative frequencies of the major *ospC* group strains. The relative frequency is the frequency of each major *ospC* group strain in the subset of nymphs infected with that particular *Borrelia* genospecies (see supplementary material for details).

Calculation of the spirochete abundance for each major *ospC* group strain in the mixed-strain infection in the tick: To calculate the abundance of each major *ospC* group strain in each tick, we used the same approach as Strandh and Råberg (2015). For each tick, we calculated the abundance of each *ospC* strain by multiplying the spirochete load of the tick (as estimated by qPCR) by the frequencies of the *ospC* strains (as estimated by 454-sequencing). For example, the *B. afzelii*-infected tick B1N014 had a spirochete load of 21,164 spirochetes, and we obtained 651 *ospC* gene sequences that belonged to 3 major *ospC* groups: A7, A9, and G4, with the following frequencies: 0.00153 (1/651), 0.99693 (649/651), and 0.00153 (1/651), respectively. Thus tick B1N014 contained 33, 21,098, and 33 spirochetes for major *ospC* group strains: A7, A9, and G4, respectively.

Relationship between spirochete abundance within the tick and the relative frequency of the major *ospC* group strain: We predicted that major *ospC* group strains that are abundant inside the tick (have a high spirochete load) would be more common (found in many ticks) compared to major *ospC* group strains that are less abundant. For each major *ospC* group strain, we calculated the geometric mean spirochete load using the subset of nymphs that carried that particular strain (i.e. uninfected nymphs were excluded). For the set of 13 major *ospC* group strains, we used ANCOVA to test the relationship between the geometric mean spirochete load and the relative frequency (averaged over the three years of the study) for the two *Borrelia* genospecies.

The spirochete carrying capacity of the nymphal tick for each *Borrelia* genospecies: We used ANCOVA to test the relationship between the total spirochete load in each tick (log10-transformed) and the strain richness (number of major *ospC* strains inside the nymphal tick), with the *Borrelia* species as a fixed factor. If the slope of this regression is zero, it suggests that there is a limit of the spirochete population inside the nymphal tick.

Competition among *ospC* strains within a tick: The relationship between the total spirochete load in a tick and the number of strains in a tick provides information as to whether there is competition among strains (Strandh and Råberg 2015). If there is no competition inside a tick, the spirochete load should increase additively with each additional strain. For each tick, we calculated the mean number of spirochetes per strain (hereafter referred to as the infection intensity per strain) by dividing the log10-transformed total spirochete load by the strain richness (number of strains for that tick). We used ANCOVA to test whether there was a relationship between the infection intensity per strain and the inverse of strain richness, with the *Borrelia* species as a fixed factor. If the mean total spirochete load is independent of strain richness, the relationship between the mean infection intensity per strain and the inverse of strain richness should be linear.

The effect of inter-strain competition on a focal *ospC* strain: Some strains are more adversely affected by competition than others. For each subset of ticks infected with a focal *ospC* strain, we modeled the log10(spirochete load) of the focal *ospC* strain as a simple linear regression of the strain richness of the tick. The slope of this regression is a measure of how the presence of other strains influences the spirochete load of the focal *ospC* strain. Major *ospC* group strains with slopes that are not different from zero are not affected by the presence of other major *ospC* group strains. In contrast, major *ospC* group strains with negative slopes are adversely affected by the presence of other major *ospC* group strains. Analyses were done separately for the two *Borrelia* species.

Results

Major *ospC* groups of *B. afzelii* and *B. garinii*: We obtained 68,631 *ospC* gene sequences for the 153 *B. afzelii*-infected nymphs and 24,014 *ospC* gene sequences for the 100 *B. garinii*-infected nymphs. The *ospC* alleles clustered into 13 different major *ospC* groups: six belonged to *B. afzelii* (A1, A2, A7, A9, A10, A14), and seven to *B. garinii* (G2, G4, G6, G7, G13, G14, G15). Of the 68,631 reads from the *B. afzelii*-infected nymphs, 113 reads (0.16%) clustered with the *ospC* major groups of *B. garinii* (Supplementary material, Tables S1 and S2). Conversely, of the 24,014 reads from the *B. garinii*-infected nymphs, 1,836 reads (7.65%) clustered with the *ospC* major groups of *B. afzelii* (Supplementary material, Tables S1 and S2). In what follows, we took the conservative approach and removed these 1,949 reads (113 + 1,836) from the statistical analysis. When these reads were included in the statistical analyses the results were more statistically significant (Supplementary material).

Relationship between spirochete abundance within the tick and the relative frequency of the major *ospC* group strain: The interaction between *Borrelia* species and strain-specific spirochete abundance on the strain-specific relative frequency was not

statistically significant (ANCOVA: $F_{1, 9} = 3.487$, $p = 0.095$; Figure 1). We found a positive and significant effect of the strain-specific spirochete abundance in the nymphal tick on the strain-specific relative frequency in the population (ANCOVA: $F_{1, 10} = 6.477$, $p = 0.029$; Figure 1). The slope (0.322 ± 0.126) indicated that for each ten-fold increase in spirochete abundance, the relative frequency of the strain would increase by 0.322 in the population of *I. ricinus* nymphs. We also found that for a given spirochete load, the mean strain-specific relative frequency was 23.5% higher in *B. afzelii* than in *B. garinii* and this difference was statistically significant (ANCOVA: $F_{1, 10} = 9.985$, $p = 0.010$; Figure 1).

***Borrelia* spirochete carrying capacity of the ticks:** The interaction between *Borrelia* species and strain richness on the total spirochete abundance in the nymphal tick was not statistically significant (ANCOVA: $F_{1, 244} = 0.032$, $p = 0.859$; Figure 2). There was no effect of the strain richness on the total spirochete load in the nymph (ANCOVA: $F_{1, 245} = 0.912$, $p = 0.341$; Figure 2), but the total spirochete load in *B. garinii* was significantly higher than in *B. afzelii* (ANCOVA: $F_{1, 245} = 6.217$, $p = 0.013$; Figure 2). The mean spirochete loads were 1,438 spirochetes per nymph for *B. afzelii* (95% confidence interval (CI): 941 to 2,197 spirochetes per nymph) and 4,449 spirochetes per nymph for *B. garinii* (95% CI: 2,419 to 8,183 spirochetes per nymph). These results suggest that there is a fixed limit for each *Borrelia* species inside the nymphal tick.

Competition among *ospC* strains within a tick: The interaction between *Borrelia* species and the inverse of strain richness on the mean infection intensity per strain was not statistically significant (ANCOVA: $F_{1, 244} = 1.117$, $p = 0.291$; Figure 3). There was a highly significant negative effect of the inverse of strain richness on the mean infection intensity per strain (ANCOVA: $F_{1, 245} = 72.082$, $p < 0.001$; Figure 3). In addition, the mean spirochete load per strain in the nymphal tick was 12.6% higher in *B. garinii* than in *B. afzelii* and this difference was statistically significant (ANCOVA: $F_{1, 245} = 8.625$, $p = 0.004$; Figure 3). This

result shows that for each *Borrelia* genospecies, there is competition between the *ospC* strains in the tick vector.

The effect of inter-strain competition on a focal *ospC* strain: For the 6 major *ospC* groups of *B. afzelii*, all the slopes measuring the magnitude of inter-strain competition on a focal major *ospC* group strain were negative and 4 slopes were statistically significant (Supplementary material, Tables S1 and S2). For the 7 major *ospC* groups of *B. garinii*, all the slopes were negative and 2 were statistically significant (Supplementary material, Tables S1 and S2). This result shows that all the strains were negatively impacted by competition and that the negative effect of competition was statistically significant for 6 of the 13 *ospC* strains.

Discussion

Competition among major *ospC* groups: Mixed strain infections are common in the life cycle of *Borrelia* pathogens in both the vertebrate host (Anderson and Norris 2006, Andersson et al. 2013, Jacquot et al. 2014, Strandh and Råberg 2015) and the tick vector (Wang et al. 1999, Qiu et al. 2002, Girard et al. 2009, Durand et al. 2015). A recent study on mixed strain infections of *B. afzelii* found evidence of inter-strain competition in wild rodent and insectivore reservoir hosts (Strandh and Råberg 2015). In the present study, we show that the nymphal stage of *I. ricinus*, the principal vector of tick-borne pathogens in Western Europe, has a fixed limit for spirochetes belonging to *B. afzelii* and *B. garinii*, the two most important LB pathogens in Eurasia. As multiple *ospC* strain infections of LB pathogens are common in nymphal ticks (Durand et al. 2015), this fixed limit inevitably induces strong inter-strain competition in the tick vector.

In systemic transmission, competition between *Borrelia* strains can occur at three critical steps: (1) host-to-larva transmission, (2) transstadial transmission during the larva-to-nymph molt and persistence in the nymphal tick (up to 8 months), and (3) nymph-to-host

transmission. The host-to-larva transmission success depends on the spirochete density in the skin of the rodent reservoir host (Råberg 2012, Jacquet et al. 2015). Strains that establish higher spirochete loads in the tissues of the reservoir host (e.g. *B. afzelii ospC* strain A10) have higher systemic transmission success than strains with lower spirochete loads (e.g. *B. afzelii ospC* strain A3) (Jacquet et al. 2015). Stochastic population bottlenecks (i.e. random events) that occur during the larval blood meal can further reduce the diversity of strains that is acquired by the larval ticks from the rodent reservoir host (Rego et al. 2014).

The spirochete population in the tick changes dramatically during the larva-to-nymph molt (Piesman et al. 1990) suggesting that there is substantial scope for inter-strain competition during transstadial transmission. In addition, spirochetes have to persist in the nymphal tick for a long period of time (~8 months) until the next blood meal (Tälleklint and Jaenson 1995, Lindsay et al. 1997). The midgut of the nymph is believed to be the key organ for spirochete persistence but we note that some *Borrelia* pathogens can establish disseminated infections in *I. ricinus* nymphs (Lebet and Gern 1994). How spirochetes persist inside the midgut of flat, unfed nymphs is currently an active area of research (Pappas et al. 2011, Kung et al. 2013, Fazzino et al. 2015). Spirochetes depend on the tick blood meal for nutrients (Dunham-Ems et al. 2009, Radolf et al. 2012). During the winter, the ticks produce glycerol as a putative natural antifreeze, which the spirochetes can use as an energy resource (Pappas et al. 2011). Spirochete persistence in the nymph midgut is probably a game of attrition, which the most abundant strains are more likely to win. We have recently shown that the spirochete load inside the nymphal tick can decrease by as much as 90% over a time interval (8 months) that reflects the duration between the larval and nymphal blood meal (Jacquet et al. 2015). A similar phenomenon was observed in *I. scapularis* nymphs infected with *B. burgdorferi* s. s. (Voordouw et al. 2013). Both of these studies involved laboratory populations of *Ixodes* ticks that were kept under laboratory conditions. A recent study on a

cohort of wild *I. ricinus* nymphs captured in the fall and spring suggested that the spirochete load can decrease over time in nature (Herrmann et al. 2013). Future studies should test whether wild *Ixodes* ticks exhibit such drastic declines in spirochete load in nature.

Nymph-to-host transmission is another step in the lifecycle where inter-strain competition is likely to be important. During the nymphal blood meal, only a few spirochetes are able to complete the migration from the tick midgut to the salivary glands (Piesman 1993, Ohnishi et al. 2001, Piesman et al. 2001, Dunham-Ems et al. 2009). During this migration, the spirochetes have to traverse the midgut epithelium, pass through the hemolymph where they are targeted by the tick immune system, and cross additional tissue barriers in the salivary glands to finally reach the salivary streams (Coleman et al. 1997, Dunham-Ems et al. 2009). Spirochetes are lost at each of these barriers and strains that start the journey with a higher abundance in the midgut are more likely to reach their final destination. A recent study using genetically tagged strains of *B. burgdorferi* s. s. (that were otherwise identical in fitness) showed that the more abundant strains in the nymphal tick had a higher nymph-to-host transmission success (Rego et al. 2014). Our study found that the *Borrelia ospC* strains with the highest spirochete load in the nymphal ticks were also the strains that were the most common in our local tick population over the duration of the study. This result strongly suggests that a high spirochete load in the tick vector is a critical life history trait for LB pathogens.

Conclusion: Studies on inter-strain competition of vector-borne pathogens have typically focused on the vertebrate host at the expense of the arthropod vector. In the present study, we show that *I. ricinus* nymphs have a fixed limit for *B. afzelii* and *B. garinii*, the two most common LB pathogens in Europe. This fixed limit in the tick vector inevitably induces inter-strain competition with increasing strain richness. Strains with a higher abundance in the nymphal tick were more common in our population of *I. ricinus* ticks. The spirochete load in

the nymphal tick is therefore a critical predictor of strain fitness. The study of mixed strain infections in the arthropod vector is important for understanding the epidemiology of vector-borne pathogens.

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References

Alizon, S., J. C. de Roode and Y. Michalakis (2013). "Multiple infections and the evolution of virulence." Ecology Letters **16**(4): 556-567.

Anderson, J. M. and D. E. Norris (2006). "Genetic diversity of *Borrelia burgdorferi* sensu stricto in *Peromyscus leucopus*, the primary reservoir of Lyme disease in a region of endemicity in southern Maryland." Applied and Environmental Microbiology **72**(8): 5331-5341.

Andersson, M., K. Scherman and L. Raberg (2013). "Multiple-strain infections of *Borrelia afzelii*: a role for within-host interactions in the maintenance of antigenic diversity?" American Naturalist **181**(4): 545-554.

Araújo, C. A., P. H. Cabello and A. M. Jansen (2007). "Growth behaviour of two *Trypanosoma cruzi* strains in single and mixed infections: in vitro and in the intestinal tract of the blood-sucking bug, *Triatoma brasiliensis*." Acta Tropica **101**(3): 225-231.

Balmer, O. and M. Tanner (2011). "Prevalence and implications of multiple-strain infections." The Lancet Infectious Diseases **11**(11): 868-878.

Baranton, G., G. Seinost, G. Theodore, D. Postic and D. Dykhuizen (2001). "Distinct levels of genetic diversity of *Borrelia burgdorferi* are associated with different aspects of pathogenicity." Research in Microbiology **152**(2): 149-156.

Brisson, D. and D. E. Dykhuizen (2004). "*ospC* diversity in *Borrelia burgdorferi*: Different hosts are different niches." Genetics **168**(2): 713-722.

Bunikis, J., U. Garpmo, J. Tsao, J. Berglund, D. Fish and A. G. Barbour (2004). "Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe." Microbiology **150**(Pt 6): 1741-1755.

Burri, C., F. Morán Cadenas, V. Douet, J. Moret and L. Gern (2007). "Ixodes ricinus density and infection prevalence of *Borrelia burgdorferi* sensu lato along a north-facing altitudinal gradient in the Rhône Valley (Switzerland)." Vector-Borne and Zoonotic Diseases **7**(1): 50-58.

Coleman, J. L., J. A. Gebbia, J. Piesman, J. L. Degen, T. H. Bugge and J. L. Benach (1997). "Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice." Cell **89**(7): 1111-1119.

De Roode, J., A. Read, B. Chan and M. Mackinnon (2003). "Rodent malaria parasites suffer from the presence of conspecific clones in three-clone *Plasmodium chabaudi* infections." Parasitology **127**(05): 411-418.

de Roode, J. C., R. Pansini, S. J. Cheesman, M. E. Helinski, S. Huijben, A. R. Wargo, A. S. Bell, B. H. Chan, D. Walliker and A. F. Read (2005). "Virulence and competitive ability in genetically diverse malaria infections." Proceedings of the National Academy of Sciences of the United States of America **102**(21): 7624-7628.

Dunham-Ems, S. M., M. J. Caimano, U. Pal, C. W. Wolgemuth, C. H. Eggers, A. Balic and J. D. Radolf (2009). "Live imaging reveals a biphasic mode of dissemination of *Borrelia burgdorferi* within ticks." The Journal of Clinical Investigation **119**(12): 3652.

Durand, J., M. Jacquet, L. Paillard, O. Rais, L. Gern and M. J. Voordouw (2015). "Cross-immunity and community structure of a multiple-strain pathogen in the tick vector." Applied and environmental microbiology **81**(22): 7740-7752.

Fazzino, L., K. Tilly, D. P. Dulebohn and P. A. Rosa (2015). "Long-term survival of *Borrelia burgdorferi* lacking the hibernation promotion factor homolog in the unfed tick vector." Infection and Immunity **83**(12): 4800-4810.

Gern, L., V. Douet, Z. López, O. Rais and F. Morán Cadenas (2010). "Diversity of *Borrelia* genospecies in *Ixodes ricinus* ticks in a Lyme borreliosis endemic area in Switzerland identified by using new probes for reverse line blotting." Ticks and Tick-Borne Diseases **1**(1): 23-29.

Gern, L. and P. Humair (2002). "Ecology of *Borrelia burgdorferi* sensu lato in Europe." Lyme Borreliosis Biology, Epidemiology and Control. CABI International: 149-174.

Girard, Y. A., B. Travinsky, A. Schotthoefer, N. Fedorova, R. J. Eisen, L. Eisen, A. G. Barbour and R. S. Lane (2009). "Population structure of the Lyme borreliosis spirochete *Borrelia burgdorferi* in the western black-legged tick (*Ixodes pacificus*) in Northern California." Applied and Environmental Microbiology **75**(22): 7243-7252.

Grimm, D., K. Tilly, R. Byram, P. E. Stewart, J. G. Krum, D. M. Bueschel, T. G. Schwan, P. F. Policastro, A. F. Elias and P. A. Rosa (2004). "Outer-surface protein C of the Lyme disease spirochete: A protein induced in ticks for infection of mammals." Proceedings of the National Academy of Sciences of the United States of America **101**(9): 3142-3147.

Hellgren, O., M. Andersson and L. Råberg (2011). "The genetic structure of *Borrelia afzelii* varies with geographic but not ecological sampling scale." Journal of Evolutionary Biology **24**(1): 159-167.

Herrmann, C. and L. Gern (2010). "Survival of *Ixodes ricinus* (Acari: Ixodidae) under challenging conditions of temperature and humidity is influenced by *Borrelia burgdorferi* sensu lato infection." Journal of Medical Entomology **47**(6): 1196-1204.

Herrmann, C. and L. Gern (2012). "Do the level of energy reserves, hydration status and *Borrelia* infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks?" Parasitology **139**(03): 330-337.

Herrmann, C. and L. Gern (2013). "Survival of *Ixodes ricinus* (Acari: Ixodidae) nymphs under cold conditions is negatively influenced by frequent temperature variations." Ticks and Tick-Borne Diseases **4**(5): 445-451.

Herrmann, C., L. Gern and M. J. Voordouw (2013a). "Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*." Applied and Environmental Microbiology **79**(23): 7273-7280.

Herrmann, C., M. Voordouw and L. Gern (2013b). "*Ixodes ricinus* ticks infected with the causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, have higher energy reserves." International Journal for Parasitology **43**(6): 477-483.

Jacquet, M., J. Durand, O. Rais and M. J. Voordouw (2015). "Cross-reactive acquired immunity influences transmission success of the Lyme disease pathogen, *Borrelia afzelii*." Infection, Genetics and Evolution **36**: 131-140.

Jacquot, M., M. Bisseux, D. Abrial, M. Marsot, E. Ferquel, J.-L. Chapuis, G. Vourc'h and X. Bailly (2014). "High-throughput sequence typing reveals genetic differentiation and host specialization among populations of the *Borrelia burgdorferi* species complex that infect rodents." PLoS One **9**(2): e88581.

Johnson, P. T., J. C. De Roode and A. Fenton (2015). "Why infectious disease research needs community ecology." Science **349**(6252): 1259504.

Karvonen, A., C. Rellstab, K.-R. Louhi and J. Jokela (2011). "Synchronous attack is advantageous: mixed genotype infections lead to higher infection success in trematode parasites." Proceedings of the Royal Society of London B: Biological Sciences: rspb20110879.

Kung, F., J. Anguita and U. Pal (2013). "*Borrelia burgdorferi* and tick proteins supporting pathogen persistence in the vector." Future Microbiology **8**(1): 41-56.

Kurtenbach, K., S. De Michelis, S. Etti, S. M. Schäfer, H.-S. Sewell, V. Brade and P. Kraiczy (2002). "Host association of *Borrelia burgdorferi* sensu lato–the key role of host complement." Trends in Microbiology **10**(2): 74-79.

Kurtenbach, K., S. De Michelis, H.-S. Sewell, S. Etti, S. M. Schäfer, R. Hails, M. Collares-Pereira, M. Santos-Reis, K. Hanincová and M. Labuda (2001). "Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies found in individual questing ticks from Europe." Applied and Environmental Microbiology **67**(10): 4926-4929.

Kurtenbach, K., K. Hanincová, J. I. Tsao, G. Margos, D. Fish and N. H. Ogden (2006). "Fundamental processes in the evolutionary ecology of Lyme borreliosis." Nature Reviews Microbiology **4**(9): 660-669.

Lagal, V., D. Postic, E. Ruzic-Sabljic and G. Baranton (2003). "Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness." Journal of Clinical Microbiology **41**(11): 5059-5065.

Lebet, N. and L. Gern (1994). "Histological examination of *Borrelia burgdorferi* infections in unfed *Ixodes ricinus* nymphs." Experimental & Applied Acarology **18**(3): 177-183.

Lindsay, L. R., I. K. Barker, G. A. Surgeoner, S. A. McEwen and G. D. Campbell (1997). "Duration of *Borrelia burgdorferi* infectivity in white-footed mice for the tick vector *Ixodes scapularis* under laboratory and field conditions in Ontario." Journal of Wildlife Diseases **33**(4): 766-775.

Louhi, K.-R., L.-R. Sundberg, J. Jokela and A. Karvonen (2015). "Interactions among bacterial strains and fluke genotypes shape virulence of co-infection." Proceedings of the Royal Society of London B: Biological Sciences **282**(1821).

Mercereau-Puijalon, O. (1996). "Revisiting host/parasite interactions: molecular analysis of parasites collected during longitudinal and cross-sectional surveys in humans." Parasite Immunology **18**(4): 173-180.

Mideo, N. (2009). "Parasite adaptations to within-host competition." Trends in Parasitology **25**(6): 261-268.

Mideo, N., V. C. Barclay, B. H. Chan, N. J. Savill, A. F. Read and T. Day (2008). "Understanding and predicting strain-specific patterns of pathogenesis in the rodent malaria *Plasmodium chabaudi*." The American Naturalist **172**(5): E214-E238.

Ohnishi, J., J. Piesman and A. M. de Silva (2001). "Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks." Proceedings of the National Academy of Sciences **98**(2): 670-675.

Pappas, C. J., R. Iyer, M. M. Petzke, M. J. Caimano, J. D. Radolf and I. Schwartz (2011). "*Borrelia burgdorferi* requires glycerol for maximum fitness during the tick phase of the enzootic cycle." PLoS Pathogens **7**(7): e1002102.

Peacock, L., V. Ferris, M. Bailey and W. Gibson (2007). "Dynamics of infection and competition between two strains of *Trypanosoma brucei brucei* in the tsetse fly observed using fluorescent markers." Kinetoplastid Biology and Disease **6**(1): 4.

Pérez, D., Y. Kneubühler, O. Rais, F. Jouda and L. Gern (2011). "*Borrelia afzelii ospC* genotype diversity in *Ixodes ricinus* questing ticks and ticks from rodents in two Lyme borreliosis endemic areas: Contribution of co-feeding ticks." Ticks and Tick-Borne Diseases **2**(3): 137-142.

Piesman, J. (1993). "Dynamics of *Borrelia burgdorferi* transmission by nymphal *Ixodes dammini* ticks." Journal of Infectious Diseases **167**(5): 1082-1085.

Piesman, J. and L. Gern (2004). "Lyme borreliosis in Europe and North America." Parasitology-Cambridge **129**: S191.

Piesman, J., J. R. Oliver and R. J. Sinsky (1990). "Growth kinetics of the Lyme disease spirochete (*Borrelia burgdorferi*) in vector ticks (*Ixodes dammini*)." The American Journal of Tropical Medicine and Hygiene **42**(4): 352-357.

Piesman, J., B. S. Schneider and N. S. Zeidner (2001). "Use of quantitative PCR to measure density of *Borrelia burgdorferi* in the midgut and salivary glands of feeding tick vectors." Journal of Clinical Microbiology **39**(11): 4145-4148.

Pollitt, L. C., J. T. Bram, S. Blanford, M. J. Jones and A. F. Read (2015). "Existing infection facilitates establishment and density of malaria parasites in their mosquito vector." PLoS Pathogens **11**(7): e1005003.

Qiu, W.-G., D. E. Dykhuizen, M. S. Acosta and B. J. Luft (2002). "Geographic uniformity of the Lyme disease spirochete (*Borrelia burgdorferi*) and its shared history with tick vector (*Ixodes scapularis*) in the northeastern United States." Genetics **160**(3): 833-849.

Råberg, L. (2012). "Infection intensity and infectivity of the tick-borne pathogen Borrelia afzelii." Journal of Evolutionary Biology **25**(7): 1448-1453.

Råberg, L., J. C. de Roode, A. S. Bell, P. Stamou, D. Gray and A. F. Read (2006). "The role of immune-mediated apparent competition in genetically diverse malaria infections." The American Naturalist **168**(1): 41-53.

Radolf, J. D., M. J. Caimano, B. Stevenson and L. T. Hu (2012). "Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes." Nature Reviews Microbiology **10**(2): 87-99.

Rauter, C. and T. Hartung (2005). "Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis." Applied and Environmental Microbiology **71**(11): 7203-7216.

Read, A. F. and L. H. Taylor (2001). "The ecology of genetically diverse infections." Science **292**(5519): 1099-1102.

Rego, R. O., A. Bestor, J. Stefka and P. A. Rosa (2014). "Population bottlenecks during the infectious cycle of the Lyme disease spirochete *Borrelia burgdorferi*." PLoS One **9**(6): e101009.

Reif, K. E., G. H. Palmer, D. W. Crowder, M. W. Ueti and S. M. Noh (2014). "Restriction of *Francisella novicida* genetic diversity during infection of the vector midgut." PLoS Pathogens **10**(11): e1004499.

Richter, D., A. Debski, Z. Hubalek and F.-R. Matuschka (2012). "Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks." Vector-Borne and Zoonotic Diseases **12**(1): 21-27.

Rollend, L., D. Fish and J. E. Childs (2013). "Transovarial transmission of *Borrelia* spirochetes by *Ixodes scapularis*: A summary of the literature and recent observations." Ticks and Tick-Borne Diseases **4**(1): 46-51.

Schmid-Hempel, P. (2011). Evolutionary parasitology : the integrated study of infections, immunology, ecology, and genetics. Oxford, Oxford University Press.

Seinost, G., D. E. Dykhuizen, R. J. Dattwyler, W. T. Golde, J. J. Dunn, I. N. Wang, G. P. Wormser, M. E. Schriefer and B. J. Luft (1999). "Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans." Infection and Immunity **67**(7): 3518-3524.

Smith, T., I. Felger, M. Tanner and H.-P. Beck (1999). "The epidemiology of multiple *Plasmodium falciparum* infections. 11. Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections." Transactions of the Royal Society of Tropical Medicine and Hygiene. Supplement **93**(1): S59-S64.

Strandh, M. and L. Råberg (2015a). "Within-host competition between *Borrelia afzelii ospC* strains in wild hosts as revealed by massively parallel amplicon sequencing." Phil. Trans. R. Soc. B **370**(1675): 20140293.

Strandh, M. and L. Råberg (2015b). "Within-host competition between *Borrelia afzelii ospC* strains in wild hosts as revealed by massively parallel amplicon sequencing." Philosophical Transaction of the Royal Society B **370**(1675): 20140293.

Susi, H., B. Barrès, P. F. Vale and A.-L. Laine (2015). "Co-infection alters population dynamics of infectious disease." Nature Communications **6**.

Swanson, K. I. and D. E. Norris (2008). "Presence of multiple variants of *Borrelia burgdorferi* in the natural reservoir *Peromyscus leucopus* throughout a transmission season." Vector-Borne and Zoonotic Diseases **8**(3): 397-406.

Tälleklint, L. and T. G. Jaenson (1995). "Is the small mammal (*Clethrionomys glareolus*) or the tick vector (*Ixodes ricinus*) the primary overwintering reservoir for the Lyme borreliosis spirochete in Sweden?" Journal of Wildlife Diseases **31**(4): 537-540.

Tilly, K., J. G. Krum, A. Bestor, M. W. Jewett, D. Grimm, D. Bueschel, R. Byram, D. Dorward, M. J. VanRaden, P. Stewart and P. Rosa (2006). "*Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection." Infection and Immunity **74**(6): 3554-3564.

Voordouw, M. J., H. Tupper, Ö. Önder, G. Devevey, C. J. Graves, B. D. Kemps and D. Brisson (2013). "Reductions in human Lyme disease risk due to the effects of oral vaccination on tick-to-mouse and mouse-to-tick transmission." Vector-Borne and Zoonotic Diseases **13**(4): 203-214.

Wang, I. N., D. E. Dykhuizen, W. G. Qin, J. J. Dunn, E. M. Bosler and B. J. Luft (1999). "Genetic diversity of *ospC* in a local population of *Borrelia burgdorferi* sensu stricto." Genetics **151**(1): 15-30.

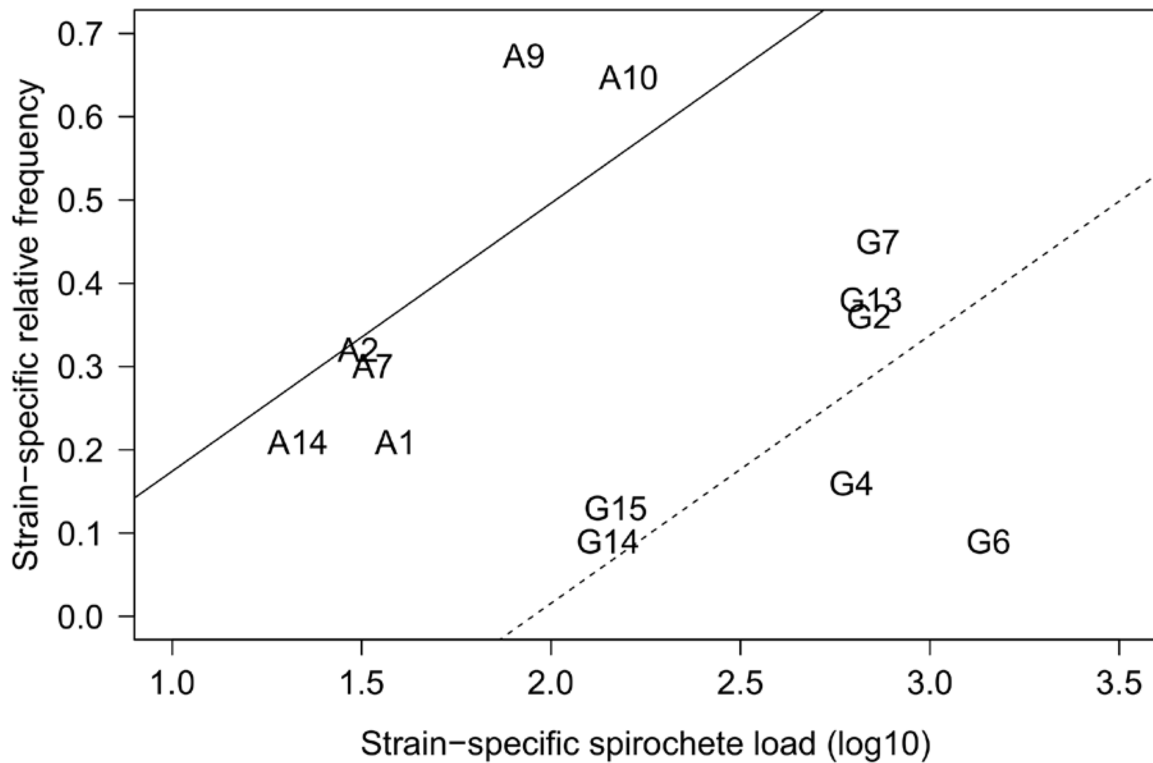

Figure 1. Major *ospC* group strains that are common have a higher spirochete load in the nymphal tick. The relationship between the relative frequencies of each major *ospC* group strain in the population of *I. ricinus* nymphal ticks and their mean spirochete load (log10-transformed) in the nymphal tick is shown for both *B. afzelii* (solid line) and *B. garinii* (dotted line). The effect of the strain-specific spirochete load on the strain-specific relative frequencies was statistically significant (ANCOVA: $p = 0.029$).

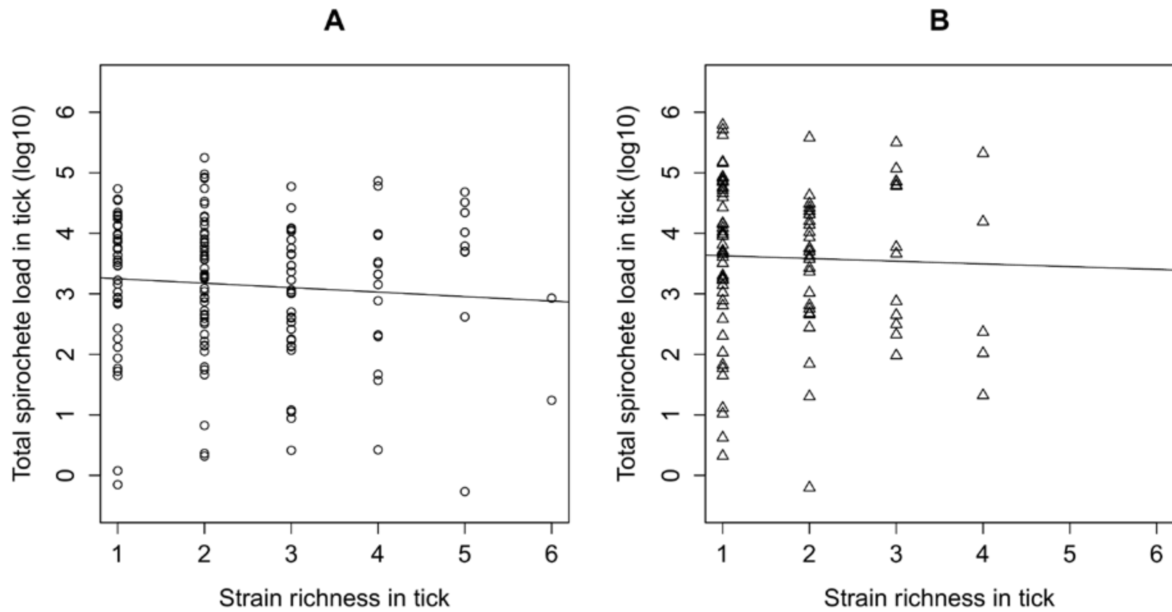

Figure 2. ***Ixodes ricinus*** **nymphs have a fixed limit for** ***Borrelia*** **spirochetes.** The relationship between the total spirochete load (log10-transformed) in the nymph and the strain richness in the nymph is shown for both *B*. *afzelii* (A) and *B*. *garinii* (B). The effect of the strain richness on the total spirochete load was not statistically significant (ANCOVA: $p = 0.341$).

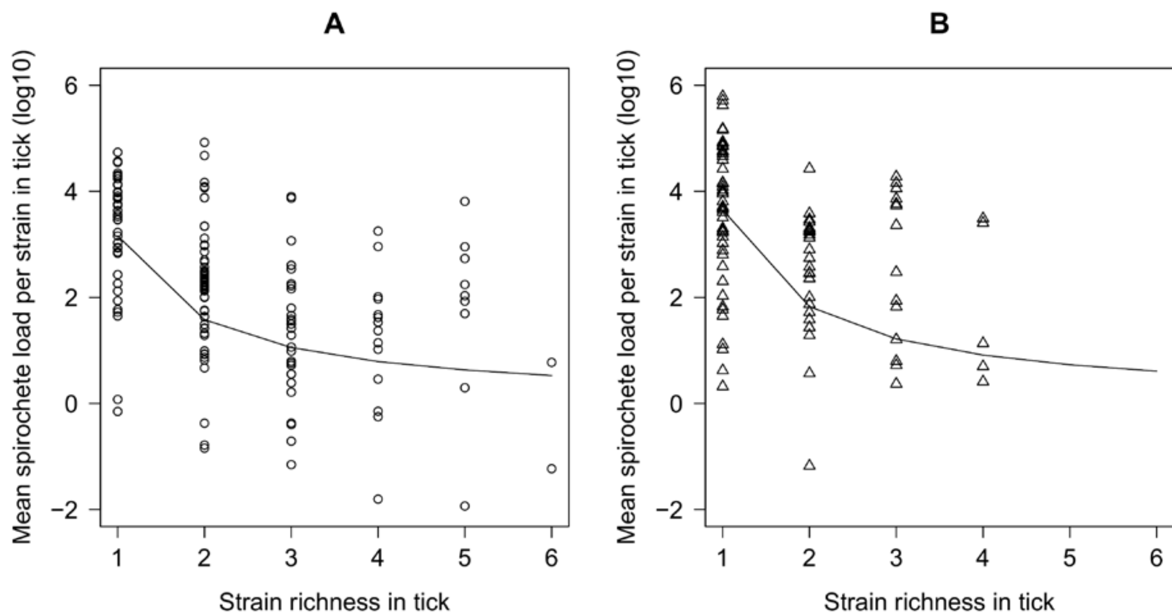

Figure 3. There is a strong competition between *Borrelia* strains within the tick vector. The relationship between the mean nymphal spirochete load per strain (log10-transformed) in the nymph and the strain richness in the nymph is shown for both *B. afzelii* (A) and *B. garinii* (B). The line shows the expected mean nymphal spirochete load per strain when the total number of spirochetes inside the tick is constant.

Supplementary material

Estimate of the spirochete load for the *Borrelia*-infected nymphal ticks: The original qPCR assay estimated the spirochete load for a volume of 10 µl of DNA extract (Herrmann et al. 2013). The nymphal ticks had been extracted in 100 µl of NH_4OH solution but after boiling this solution for 15 minutes, an average volume of 52 µl of DNA extract remained. The spirochete loads were therefore multiplied by a factor of 5.2 to calculate the total spirochete load for each tick. This correction factor was not applied in previous studies and the spirochete loads reported in those studies therefore refer to 10 µl of tick DNA extract and not the whole tick (Herrmann and Gern 2010, Herrmann and Gern 2012, Herrmann and Gern 2013, Herrmann et al. 2013, Herrmann et al. 2013).

Relative frequencies of the major *ospC* group strains: In the present study, we report the relative frequencies rather than the absolute frequencies of the major *ospC* group strains. The relative frequency is the frequency of each major *ospC* group strain in the subset of nymphs infected with that particular *Borrelia* genospecies. For example, the relative frequency of *B. afzelii* major *ospC* group A10 is 0.647 (99 nymphs infected with strain A10/153 nymphs infected with *B. afzelii*). In contrast, the absolute frequency refers to the frequency of each major *ospC* group strain in the population of *I. ricinus* nymphs, which includes both infected and uninfected individuals. The proportion of nymphs infected with *B. afzelii* was 0.118 (872 *B. afzelii*-infected nymphs/7,400 total nymphs) and so the absolute frequency of *B. afzelii* major *ospC* group A10 is 0.118*0.647 = 0.076.

Native and exotic major *ospC* groups: The *Borrelia* genospecies for each nymph was determined using a reverse line blot (RLB) assay that targeted the 23S-5S spacer gene (Herrmann et al. 2013). We had previously used the terms 'native' and 'exotic' to indicate whether a given major *ospC* group corresponded to the *Borrelia* genospecies identification of the RLB assay or not (Durand et al. 2015). For example, the major *ospC* group A10 has been

traditionally associated with *B. afzelii*. Thus major *ospC* group A10 is classified as native when it is recovered from a *B. afzelii*-infected nymph and as exotic when it is recovered from a *B. garinii*-infected nymph. In the manuscript, we removed the exotic major *ospC* groups from the statistical analysis. Here, we re-analyzed the data after including the exotic major *ospC* groups.

Of the 13 major *ospC* groups found in this study, 6 have been traditionally associated with *B. afzelii* (A1, A2, A7, A9, A10, A14), and 7 with *B. garinii* (G2, G4, G6, G7, G13, G14, G15). Of the 68,631 reads from the *B. afzelii*-infected nymphs, 68,518 native reads (99.84%) and 113 exotic reads (0.16%) clustered with the major *ospC* groups of *B. afzelii* and *B. garinii*, respectively. Conversely, of the 24,014 reads from the *B. garinii*-infected nymphs, 22,178 native reads (92.35%) and 1,836 exotic reads (7.65%) clustered with the *ospC* major groups of *B. garinii* and *B. afzelii*, respectively.

Results with the exotic major *ospC* group strains included

Relationship between spirochete abundance within the tick and the relative frequency of the major *ospC* group strain: The interaction between *Borrelia* species and strain-specific spirochete abundance on the strain-specific relative frequency was statistically significant (ANCOVA: $F_{1, 22} = 7.991$, $p = 0.010$) so we analyzed the two genospecies separately. For *B. afzelii*, we found a positive and significant effect of the strain-specific spirochete abundance in the nymphal tick on the strain-specific relative frequency in the population (ANCOVA: $F_{1, 10} = 23.316$, $p < 0.001$). The slope (0.295 ± 0.060) indicated that for each ten-fold increase in spirochete abundance, the relative frequency of the strain would increase by 0.295 in the population of *I. ricinus* nymphs. For *B. garinii*, we did not find a significant effect of the strain-specific spirochete abundance in the nymphal tick on the strain-specific relative frequency in the population (ANCOVA: $F_{1, 10} = 0.816$, $p = 0.386$).

***Borrelia* spirochete carrying capacity of the ticks:** The interaction between *Borrelia* species and strain richness on the total spirochete abundance in the nymphal tick was not statistically significant (ANCOVA: $F_{1, 249} = 0.262$, $p = 0.609$). There was no effect of the strain richness on the total spirochete load in the nymph (ANCOVA: $F_{1, 250} = 0.506$, $p = 0.477$), but the total spirochete load in *B garinii* was significantly higher than in *B. afzelii* (ANCOVA: $F_{1, 250} = 9.600$, $p = 0.002$). These results suggest that there is a fixed limit for each *Borrelia* species inside the nymphal tick.

Competition among *ospC* strains within a tick: The interaction between *Borrelia* species and the inverse of the strain richness on the mean infection intensity per strain was not statistically significant (ANCOVA: $F_{1, 249} = 1.364$, $p = 0.243$). There was a highly significant negative effect of the inverse of the strain richness on the mean infection intensity per strain (ANCOVA: $F_{1, 250} = 70.162$, $p < 0.001$). In addition, the mean spirochete load per strain in the nymphal tick was 17.6% higher in *B. garinii* than in *B. afzelii* and this difference was statistically significant (ANCOVA: $F_{1, 250} = 16.358$, $p < 0.001$). This result shows that for each *Borrelia* genospecies, there is competition between the *ospC* strains in the tick vector.

Table S1. Frequencies and abundances of the major *ospC* groups in the *Borrelia afzelii*-infected *Ixodes ricinus* nymphs are shown.

ospC allele	*ospC* status[a]	F1[b]	F2[c]	Abundance[d]	Mean Abundance[e]	Competition Index[f]
A9	Native	36.58% (25108)	67.32% (103)	606,811	85	-0.204*
A10	Native	37.93% (26035)	64.71% (99)	545,915	161	-0.362*
A2	Native	11.58% (7950)	32.03% (49)	93,839	31	-0.174
A7	Native	3.51% (2408)	30.07% (46)	145,292	34	-0.330*
A14	Native	5.81% (3988)	20.91% (32)	27,746	22	-0.411*
A1	Native	4.41% (3029)	20.91% (32)	199,099	39	-0.167
G13	Exotic	0.05% (33)	13.07% (20)	5,195	7	0.075
G7	Exotic	0.04% (26)	10.46% (16)	177,246	7	-0.119
G2	Exotic	0.03% (19)	10.46% (16)	150	2	-0.528*
G4	Exotic	0.02% (12)	5.23% (8)	9,131	8	0.014
G15	Exotic	0.01% (10)	4.58% (7)	335	5	-0.352
G14	Exotic	0.01% (10)	5.23% (8)	289	2	0.308
G6	Exotic	0.00% (3)	1.31% (2)	108	15	NA
Total	Both	100% (68,631)	100% (153)	1,811,156		
Total	Native	99.84% (68,518)	100% (153)	1,618,702		
Total	Exotic	0.16% (113)	35.95% (55)	192,454		

[a] *ospC* status refers to whether the major *ospC* groups are native to *B. afzelii* or were derived from *B. garinii*.

[b] Frequency 1 (F1) is the proportion of the major *ospC* group in the sample of sequences (n = 68,631 sequences). The number in brackets is the number of reads that belonged to that particular major *ospC* group.

[c] Frequency 2 (F2) is the proportion of the major *ospC* group in the sample of infected nymphs (n = 153 nymphs). The number in brackets is the number of nymphs that were infected with that particular major *ospC* group.

[d] Total spirochete load for each strain in all the ticks (n = 153 nymphs). This number is calculated as follows: multiply the estimate of the spirochete load (qPCR) by the strain-specific frequencies (454-sequencing) and then sum across all 153 nymphs.

[e] Mean spirochete load per strain the subsample of ticks infected with that strain (i.e. nymphs that were not infected with that strain were excluded from the calculation).

[f] Competition index refers to the slope of the linear regression between the log10(spirochete load) of the focal *ospC* strain and the strain richness of the tick.

[*] Statistically significant competition index

Table S2. Frequencies and abundances of the major *ospC* groups in the *Borrelia garinii*-infected *Ixodes ricinus* nymphs are shown.

ospC allele	*ospC* status[a]	F1[b]	F2[c]	Abundance[d]	Mean Abundance[e]	Competition Index[f]
G7	Native	21.06% (5,057)	45.00% (45)	1,099,502	730	-0.123
G13	Native	20.30% (4,875)	38.00% (38)	653,839	699	-0.275
G2	Native	23.04% (5,532)	36.00% (36)	1,213,210	691	-0.424*
G4	Native	6.85% (1,646)	16.00% (16)	724,520	621	-0.389
G15	Native	5.84% (1403)	13.00% (13)	58,523	148	-0.656*
G14	Native	6.36% (1,527)	9.00% (9)	18,120	141	-0.106
G6	Native	8.90% (2,138)	9.00% (9)	528,528	1,431	-0.671
A10	Exotic	4.45% (1,068)	42.00% (42)	146,648	84	-0.162
A9	Exotic	2.92% (701)	31.00% (31)	648,460	69	-0.222
A2	Exotic	0.14% (30)	15.00% (15)	18,713	70	-0.542*
A1	Exotic	0.05% (12)	10.00% (10)	89,942	75	-0.678
A14	Exotic	0.06% (15)	10.00% (10)	404	3	-0.131
A7	Exotic	0.04% (10)	9.00% (9)	98,767	441	-0.513
Total	Both	100% (24,014)	100% (100)	5,299,176		
Total	Native	92.35% (22,178)	97% (97)	4,296,242		
Total	Exotic	7.65% (1,836)	70.30% (71)	1,002,934		

[a] *ospC* status refers to whether the major *ospC* groups are native to *B. garinii* or were derived from *B. afzelii*.

[b] Frequency 1 (F1) refers to the frequency of the major *ospC* group in the sample of sequences (n = 24,014 sequences). The number in brackets is the number of reads that belonged to that particular major *ospC* group.

[c] Frequency 2 (F2) refers to the frequency of the major *ospC* group in the sample of infected nymphs (n = 100 nymphs). The number in brackets is the number of nymphs that were infected with that particular major *ospC* group.

[d] Total spirochete load for each strain in all the ticks (n = 100 nymphs). This number is calculated as follows: multiply the estimate of the spirochete load (qPCR) by the strain-specific frequencies (454-sequencing) and then sum across all 100 nymphs.

[e] Mean spirochete load per strain the subsample of ticks infected with that strain (i.e. nymphs that were not infected with that strain were excluded from the calculation).

[f] Competition index refers to the slope of the linear regression between the log10(spirochete load) of the focal *ospC* strain and the strain richness of the tick.

[*] Statistically significant competition index

References

Durand, J., M. Jacquet, L. Paillard, O. Rais, L. Gern and M. J. Voordouw (2015). "Cross-immunity and community structure of a multiple-strain pathogen in the tick vector." Applied and environmental microbiology **81**(22): 7740-7752.

Herrmann, C. and L. Gern (2010). "Survival of *Ixodes ricinus* (Acari: Ixodidae) under challenging conditions of temperature and humidity is influenced by *Borrelia burgdorferi* sensu lato infection." Journal of Medical Entomology **47**(6): 1196-1204.

Herrmann, C. and L. Gern (2012). "Do the level of energy reserves, hydration status and *Borrelia* infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks?" Parasitology **139**(03): 330-337.

Herrmann, C. and L. Gern (2013). "Survival of *Ixodes ricinus* (Acari: Ixodidae) nymphs under cold conditions is negatively influenced by frequent temperature variations." Ticks and Tick-Borne Diseases **4**(5): 445-451.

Herrmann, C., L. Gern and M. J. Voordouw (2013a). "Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*." Applied and Environmental Microbiology **79**(23): 7273-7280.

Herrmann, C., M. Voordouw and L. Gern (2013b). "*Ixodes ricinus* ticks infected with the causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, have higher energy reserves." International Journal for Parasitology **43**(6): 477-483.

9. Summary and general discussion

9.1. Maintenance of *ospC* diversity

In the second paper, we showed that the discrete pattern of genetic variation at the *ospC* locus was maintained for a very large sampling effort (over 240,000 *ospC* gene sequences). We did not find any *ospC* alleles that were intermediately divergent in DNA sequence (2–8%) from the community of major *ospC* groups. We hypothesized that these missing intermediate *ospC* alleles are removed by purifying selection due to the cross-reactive antibodies of the immune system of the vertebrate host. Immunization experiments with recombinant OspC proteins have shown that protection against a particular *ospC* strain is highly specific (Probert et al. 1997, Earnhart et al. 2005, Jacquet et al. 2015). Thus immunological models that explain the maintenance of the *ospC* polymorphism should be based on acquired immunity.

In the third paper, we showed that frequencies of the *ospC* strains were stable over a period of 11 years. In the rodent-associated *B. afzelii*, we also showed that 63% of the variation in the *ospC* strain frequencies could be explained by laboratory estimates of strain-specific fitness. This result is consistent with the theoretical models proposed by Gupta and colleagues (Gupta et al. 1996, Gupta et al. 1998, Gupta and Anderson 1999). Cross-reactive OspC-specific antibodies produced by the vertebrate host prevented intermediately divergent *ospC* alleles (2–8% of nucleotide difference) from invading the community of major *ospC* groups. Under strong cross-immunity, a dominant set of strains can coexist at stable frequencies over long periods of time. This pattern is what we observed in *B. afzelii* and *B. garinii* where *ospC* strains A10 (relative frequency of 54.40) and G8 (relative frequency of 42.11) dominated the community of *ospC* strains for eleven years. These communities of *ospC* strains can thus be considered as complexes of independent strains that vary in

frequencies according to their intrinsic fitness. Indeed, for the six strains of *B. afzelii* for which we were able to estimate the fitness, there was a positive relationship between fitness and frequency in the wild tick population. The more common strains had a higher fitness than the rare strains. This contradicts a key point of the NFDS theory, in which rare strains have an intrinsic fitness advantage over common ones (Peters and Lively 1999, Schmid-Hempel and Jokela 2002, Engelstädter and Bonhoeffer 2009).

Surprisingly, we also found evidence for a selective sweep as the major *ospC* groups that are currently the most common had the lowest number of genetic variants within the major *ospC* group. One potential explanation is that the most common strains, A10 and G8, have recently invaded their respective communities (i.e. before 2000 when monitoring began) and that they have not had enough time to mutate and build up a population of neutral genetic variants. The observation of a selective sweep followed by a period of stability is consistent with the model of Gupta et al (1998) that predicts a 'large amplitude chaos' state in which chaotic fluctuations are followed by periods of stability. This state implies that the cross-reactive antibodies from the vertebrate immune system are not 100% specific, so that the antibodies against one strain can still influence the fitness (infection and transmission) of another strain. A recent study found that cross-immunity in the host can reduce the spirochete load in the tick vector (Jacquet et al. 2015).

MNP theory: Under MNP, the different *ospC* strains of the *Borrelia* pathogen are specialized on different vertebrate host species, which represent different ecological niches (Brisson and Dykhuizen 2004, Hanincová et al. 2006, Brisson et al. 2012, Mechai et al. 2016). We believe that the MNP hypothesis is unlikely in our system for a number of reasons. First, host blood meal analysis of questing *I. ricinus* nymphs in Switzerland has found a limited diversity of relevant reservoir hosts (Humair et al. 2007, Morán Cadenas et al. 2007). Second, host specificity of *B. afzelii* and *B. garinii* for rodents and birds, respectively, is mediated by

the vertebrate complement system (Kurtenbach et al. 1998, Kurtenbach et al. 2002) and additional *ospC*-mediated specificity seems unnecessary. Third, a study on *B. afzelii* in Switzerland found that 8 of the 10 major *ospC* groups in questing *I. ricinus* nymphs were found in just 19 individuals belonging to three different rodent species: *Apodemus sylvaticus*, *A. flavicollis*, and *Myodes glareolus* (Pérez et al. 2011). Fourth, if the major *ospC* group alleles circulate in different reservoir hosts, it begs the question as to why the pairwise genetic divergences are constrained to be >8% and why the OspC antigen induces strain-specific immunity in a common rodent reservoir host. In our view, these data are not compatible with the MNP hypothesis of the *ospC* polymorphism of *B. burgdorferi* pathogens.

9.2. Coinfection and competition

In both experiments, we found a large number of mixed-strain infections in *I. ricinus* nymphal ticks: *B. afzelii*: 77.78–78.76% and *B. garinii*: 76.00–84.74%. In the fourth paper, we observed that there was a fixed carrying capacity in questing nymphs. This carrying capacity induces strong competition between the different strains in mixed infections. This inter-strain competition could occur during the different steps of the infection cycle of *B. burgdorferi* sensu lato. First, competition can occur during host-to-tick transmission, which depends on the spirochete density in the skin of the rodent reservoir host (Råberg 2012, Jacquet et al. 2015). Second, strains can compete during the transstadial transmission of the larva-to-nymph molt and during the long period of persistence in the nymphal tick when the spirochete load can decrease dramatically (Piesman et al. 1990). Third, strains can interact during tick-to-host transmission where only a small subsample of spirochetes are able to reach the tick salivary gland and achieve transmission to the vertebrate host (Piesman 1993, Ohnishi et al. 2001, Piesman et al. 2001, Dunham-Ems et al. 2009). Competition did not have the same effect on the different strains. In *B. afzelii*, the strains that had a high spirochete load in

the nymphs were also the more common strains in the population. In *B. garinii*, the more common strains in the population were less affected by competition. Strains with a higher spirochete load in the nymphal tick have a higher probability of transmission from the nymph to the host. Spirochete load in the nymphal tick and nymph-to-host transmission are important life history traits that determine the strain-specific fitness. Higher fitness will result in higher frequency of the strain in the population. This study shows that inter-strain competition in the tick vector plays a critical role in the community structure of *Borrelia* strains. There is a need for more studies on the importance of inter-strain competition of vector-borne pathogens in the arthropod vector.

9.3. Presence of exotic *ospC* groups

According to the reverse line blot (RLB) assay, all the nymhal ticks that we used in this study were singly infected with one of two *Borrelia* species: *B. afzelii* or *B. garinii*. One of the more surprising results of this study was the presence of 'exotic' major *ospC* groups that did not belong to the *Borrelia* species indicated by the RLB. For example, nymphal ticks infected with *B. garinii* (according to the RLB) often contained the major *ospC* group A10, which belongs to *B. afzelii*. This result was consistent between the two 454-sequencing runs, indicating that these exotic *ospC* groups were not an error of the DNA extraction method: nymph-derived isolate cultured in BSK versus nymphal tick. In the first experiment (second paper), 42% of the *B. afzelii*-infected ticks and 55% of the *B. garinii*-infected ticks contained exotic major *ospC* groups from other *Borrelia* species. In the second experiment (fourth paper), 36% of the *B. afzelii*-infected ticks and 70% of the *B. garinii*-infected ticks contained exotic major *ospC* groups. While the exotic major *ospC* groups were widespread, they were not abundant. For *B. afzelii*, the exotic major *ospC* groups accounted for 1.88% and 0.16% of all the *ospC* sequences in the first and second experiment, respectively. For *B. garinii*, the

exotic major *ospC* groups accounted for 3.09% and 7.65% of all the *ospC* sequences in the first experiment and second experiment, respectively. Interestingly, in both experiments there were a few nymphs in which the exotic groups were more abundant than the native ones.

This result illustrates the importance of choosing a good method of clustering when analyzing next generation sequencing data. By using de novo clustering (i.e. without a priori expectations), we were able to detect these exotic strains. The importance of de novo clustering also applies to studies on mixed infections in the vertebrate host. For example, Jacquot and colleagues (2014) recently used this approach to show that rodent species can sometimes carry the bird-adapted *B. garinii* species. In contrast, Strandh and Råberg (2015) used a closed-reference clustering method (i.e. they compared their sequences to a database containing only major *ospC* groups from *B. afzelii*) to analyze *ospC* diversity in ear tissue biopsies from different vertebrate hosts and consequently did not find any atypical major *ospC* groups. This example illustrates how the clustering method can limit the observed diversity in the study.

There are two alternative explanations for the presence of exotic major *ospC* groups in our singly-infected nymphs: the co-infection explanation and the horizontal gene transfer explanation.

Co-infection explanation for the presence of exotic major *ospC* groups: In the co-infection explanation, the presence of native and exotic *ospC* groups in the same isolate represents a true co-infection of *B. afzelii* and *B. garinii* that was not detected by the RLB assay. In this scenario, the RLB assay detected the highly abundant *Borrelia* species (corresponding to the abundant 'native' *ospC* alleles from the 454-sequencing) but not the lowly abundant *Borrelia* species (corresponding to the low abundance 'exotic' *ospC* alleles from the 454-sequencing). We recently performed Illumina sequencing on the 16S gene of 21 samples from the first experiment that contained exotic strains. 85.7% (18/21) of them were

actually mixed infections between *B. afzelii* and *B. garinii*. If the co-infection explanation is true, the implication is that the true frequency of co-infections with *B. afzelii* and *B. garinii* has been underestimated by a factor of 70 to 80 (42% to 55% in the first study versus 0.75% in the study by Herrmann et al. (2013). A study on co-infections with different *Borrelia* species suggested that the complement system of the vertebrate host reduced the spirochete load of the maladapted *Borrelia* species inside the nymphal tick (Herrmann et al. 2013). The observation that the exotic *ospC* groups had low abundances is therefore consistent with our understanding of how the vertebrate complement system would affect the spirochete load of co-infections containing bird- and rodent-specialized *Borrelia* species inside the same nymphal tick.

If the co-infection hypothesis is true, the observation that >50% of our nymphs had co-infections with *B. afzelii* and *B. garinii* requires additional explanation given that these two *Borrelia* species are specialized on different vertebrate hosts (Kurtenbach et al. 1998, Kurtenbach et al. 2001). A recent study presented four mechanisms by which *B. afzelii* and *B. garinii* could co-infect the same tick (Herrmann et al. 2013). First, the specificity of *B. afzelii* for rodents and of *B. garinii* for birds is not 100% complete. Studies in France (Marsot et al. 2011, Jacquot et al. 2014, Pisanu et al. 2014) and England (Millins et al. 2015) found that introduced species of rodents were infected with the bird-specialized *B. garinii*. Similarly, other studies found that birds can transmit the rodent-specialized *B. afzelii* (Poupon et al. 2006, Dubska et al. 2009, Franke et al. 2010, Lommano et al. 2014). Rodents or birds that are co-infected with *B. afzelii* and *B. garinii* could transmit both species to feeding ticks. Second, larval ticks could also acquire co-infections of *B. afzelii* and *B. garinii* by taking multiple blood meals from different hosts (Humair et al. 2007, Morán Cadenas et al. 2007). Third, larval ticks could acquire multiple *Borrelia* species via a combination of co-feeding and systemic transmission or fourth, via a combination of vertical transmission and systemic

transmission (Herrmann et al. 2013). For example, a larval tick co-feeding next to a *B. afzelii*-infected nymph on a *B. garinii*-infected bird would acquire both *Borrelia* species. Co-feeding transmission has been observed in both *B. afzelii* and *B. garinii* (Voordouw 2015). In contrast, vertical transmission of *Borrelia* pathogens is believed to be rare (Rollend et al. 2013).

Horizontal gene transfer explanation for the presence of exotic major *ospC* groups: In the horizontal gene transfer explanation, the RLB assay is correct (all nymphs were infected with a single *Borrelia* species) and the exotic major *ospC* group alleles were horizontally transferred into the recipient *Borrelia* species. Previous studies have found that major *ospC* group alleles can be transferred between *Borrelia* species (Livey et al. 1995, Baranton et al. 2001, Barbour and Travinsky 2010). In the two experiments, we found that 17 of the 21 native *ospC* major groups occurred as exotic major *ospC* groups in the other *Borrelia* species. If the horizontal gene transfer explanation is true, we make the following three observations. First, the present study suggests that horizontal gene transfer of the *ospC* gene is much more common than previous reports in the literature (Jauris-Heipke et al. 1995, Livey et al. 1995, Wang et al. 1999, Wang et al. 1999, Dykhuizen and Baranton 2001, Barbour and Travinsky 2010). Second, horizontal gene transfer was more likely for major *ospC* groups that were common in the donor *Borrelia* species. Third, some of the exotic *ospC* alleles increased to an appreciable frequency in the recipient *Borrelia* species. For example, the major *ospC* group A10, which is native to *B. afzelii*, has a relative frequency of 31.58% (60/190 infected nymphs) and 42.00% (42/100) in *B. garinii* in the first and second experiment, respectively. If the horizontal gene transfer explanation is true, one puzzling observation is that the exotic major *ospC* groups are much less abundant than the native *ospC* groups in the nymph. This observation suggests that the exotic strains are underperforming relative to the native strains. Future studies should perform deep sequencing of other *Borrelia*

genes that differ between *B. afzelii* and *B. garinii* (e.g. *recA* or *Hbb*) to differentiate between the co-infection hypothesis and the horizontal gene transfer hypothesis.

9.4. Differences between *Borrelia afzelii* and *Borrelia garinii*.

Surprisingly, we found no important differences between *B. afzelii* and *B. garinii*. For example, we did not find any allospecific *ospC* groups that were intermediately divergent (between 2–8% of nucleotide difference) from each other (Durand et al. 2015 and appendix tables 4-6). These two *Borrelia* species are adapted to different vertebrate hosts (small mammals for *B. afzelii* and birds for *B. garinii*), and should not be found in the same individual. Thus major *ospC* groups that cycle in different vertebrate host populations should not be under selection by the adaptive immune system to create non-overlapping major *ospC* groups. From this perspective, the absence of intermediately divergent major *ospC* groups between the two *Borrelia* species is almost a bigger puzzle than the absence of these groups within the same *Borrelia* species. Different explanations could account for the missing intermediate major *ospC* groups between the two *Borrelia* species. First, horizontal transfer of the *ospC* gene between *Borrelia* species and strains inside the tick could maintain the cohesion between *B. afzelii* and *B. garinii*. Second, mixed-species infections with *B. burgdorferi* could also maintain the cohesion between the strains. *B. burgdorferi* is the third-most prevalent species in Europe (Rauter and Hartung 2005, Estrada-Peña et al. 2011), and can infect both mammals and birds (Kurtenbach et al. 2002). Moreover, no intermediate *ospC* allele between *B. burgdorferi* and *B. afzelii* or *B. garinii* has been described yet (Lagal et al. 2003). This *Borrelia* species could create the cross-reactive antibody responses in both birds and mammals that will maintain these sets of allospecific non-overlapping major *ospC* groups. However, only a small percentage of questing nymphs have been found to carry mixed infections with *B. garinii* and *B. burgdorferi* in Switzerland (Herrmann et al. 2013). Third,

specialization of *B. afzelii* in small mammals and *B. garinii* in birds might be overrated. Different studies have found birds carrying *B. afzelii* (Dubska et al. 2009, Franke et al. 2010, Heylen et al. 2014) and small mammals carrying *B. garinii* (Marsot et al. 2011, Jacquot et al. 2014, Pisanu et al. 2014, Millins et al. 2015). It is critical that these mixed-species infections occur in the same vertebrate host because this is where the OspC protein is expressed. These three explanations are not mutually exclusive, and it is possible that all three contribute to the cohesion of the *ospC* community between different *Borrelia* species. Thus, a new *ospC* mutant can rise to a detectable frequency only if it is at least 8% divergent from all the major *ospC* groups of all the *Borrelia* pathogens in the local community.

Surprisingly, our large sequencing effort did not discover any new major *ospC* groups. This result suggests that the diversity of major *ospC* groups is at saturation. If we set the limits of genetic variation between 8% and 30%, there is a finite number of possible major *ospC* groups. To date, the *ospC* diversity has only been studied in the three most common species: *B. burgdorferi*, *B. afzelii* and *B. garinii* (Baranton et al. 2001, Qiu et al. 2002, Lagal et al. 2003, Bunikis et al. 2004). Approximately 20 different major *ospC* groups have been described for each species. If the different major *ospC* groups are shared between the different *Borrelia* genospecies, it will be interesting to test whether less common *Borrelia* species have new major *ospC* groups or shared 'exotic' major *ospC* groups.

The most important difference between *B. afzelii* and *B. afzelii* was the fixed carrying capacity. *B. garinii* carrying capacity were three time higher than *B. afzelii* (1,438 versus 4,449). The studies by Coralie Herrmann (Herrmann and Gern 2010, Herrmann and Gern 2012, Herrmann and Gern 2013, Herrmann et al. 2013, Herrmann et al. 2013) are the only work that we are aware of that quantifies the spirochete load in *B. afzelii*-infected and *B. garinii*-infected ticks. The difference in spirochete load between the two *Borrelia* species is unlikely to be caused by the tick vector, which is shared by the two *Borrelia* species. So I

propose two explanations for the observed difference in spirochete load between the two *Borrelia* species. First, transmission of *B. garinii* spirochetes from birds to larval *I. ricinus* ticks could be more efficient than transmission of *B. afzelii* spirochetes from rodents to larval ticks. The higher spirochete inoculum of *B. garinii* then grows to a much larger spirochete population in the nymphal tick compared to *B. afzelii*. Second, *B. afzelii* and *B. garinii* spirochetes could differ in their expression of the genes responsible for the persistence of spirochetes in the tick. Several genes coding for different proteins involved in persistence have been identified in *B. burgdorferi* (Kung et al. 2013, Drecktrah et al. 2015) but not in other *Borrelia* species. It would be interesting to test whether differences in the expression of these 'persistence' genes between the most common *Borrelia* species is responsible for the species-specific differences in spirochete load in questing nymphs.

10. Conclusion

The overall purpose of this thesis was to investigate how *ospC* diversity could be maintained in a local population of questing nymphs. Using 454-sequencing, we were able to propose a theory to explain this diversity.

In local populations of *Borrelia* species, cross-reactive antibodies from the vertebrate host prevent the establishment of intermediately divergent major *ospC* groups and drive the community of major *ospC* groups to diverge into a set of non-overlapping strains. These strains have stable frequencies over long periods of time because they are essentially independent of each other (no apparent competition mediated by the host immune system), and their population-wide frequency depends on their intrinsic fitness. One key component of this intrinsic fitness is the nymph-to-host transmission rate, which is linked to the spirochete load in the nymph.

Co-infections are common in questing nymphs, and strains have to compete with each other in the tick midgut and are limited by a fixed carrying capacity. This inter-strain competition may influence strain fitness, as the more abundant strains in the tick are also the most common strains in the population. Future studies on vector-borne pathogens should pay more attention to interactions that occur in the arthropod vector.

A surprising result in this study was the presence of exotic strains in mixed infections. These exotic strains could be caused by undetected mixed-species infection of *B. garinii* and *B. afzelii*, or by horizontal transfer of the *ospC* gene between the two *Borrelia* species. A preliminary experiment with Illumina sequencing on a small number of samples indicates that the first explanation is more likely. Future studies should use multi-locus sequence typing to differentiate between these two hypotheses.

We did not find any important differences between the two *Borrelia* species, which could imply that the two *ospC* communities are synchronized with each other. There was a difference in spirochete abundance, but more studies are needed to confirm if this difference is the same in other populations of *I. ricinus* ticks.

11. References

Adeolu, M. and R. S. Gupta (2014). "A phylogenomic and molecular marker based proposal for the division of the genus *Borrelia* into two genera: the emended genus *Borrelia* containing only the members of the relapsing fever *Borrelia*, and the genus *Borreliella* gen. nov. containing the members of the Lyme disease *Borrelia* (*Borrelia burgdorferi* sensu lato complex)." Antonie Van Leeuwenhoek **105**(6): 1049-1072.

Alizon, S., J. C. de Roode and Y. Michalakis (2013). "Multiple infections and the evolution of virulence." Ecology Letters **16**(4): 556-567.

Anderson, J. M. and D. E. Norris (2006). "Genetic diversity of *Borrelia burgdorferi* sensu stricto in *Peromyscus leucopus*, the primary reservoir of Lyme disease in a region of endemicity in southern Maryland." Applied and Environmental Microbiology **72**(8): 5331-5341.

Andersson, M., K. Scherman and L. Raberg (2013). "Multiple-strain infections of *Borrelia afzelii*: a role for within-host interactions in the maintenance of antigenic diversity?" American Naturalist **181**(4): 545-554.

Andersson, M., K. Scherman, L. Raberg and L. Råberg (2013). "Multiple-strain infections of *Borrelia afzelii*: a role for within-host interactions in the maintenance of antigenic diversity?" American Naturalist, The **181**(4): 545-554.

Araújo, C. A., P. H. Cabello and A. M. Jansen (2007). "Growth behaviour of two *Trypanosoma cruzi* strains in single and mixed infections: in vitro and in the intestinal tract of the blood-sucking bug, *Triatoma brasiliensis*." Acta tropica **101**(3): 225-231.

Ayala, F. J. and C. A. Campbell (1974). "Frequency-dependent selection." Annual Review of Ecology and Systematics **5**: 115-138.

Balmer, O. and M. Tanner (2011). "Prevalence and implications of multiple-strain infections." The Lancet infectious diseases **11**(11): 868-878.

Bambini, S., J. Piet, A. Muzzi, W. Keijzers, S. Comandi, L. De Tora, M. Pizza, R. Rappuoli, D. van de Beek and A. van der Ende (2013). "An analysis of the sequence variability of meningococcal fHbp, NadA and NHBA over a 50-year period in the Netherlands." PloS one **8**(5): e65043.

Baranton, G., G. Seinost, G. Theodore, D. Postic and D. Dykhuizen (2001). "Distinct levels of genetic diversity of *Borrelia burgdorferi* are associated with different aspects of pathogenicity." Research in Microbiology **152**(2): 149-156.

Barbour, A. G. (1988). "Plasmid analysis of *Borrelia burgdorferi*, the Lyme disease agent." Journal of Clinical Microbiology **26**(3): 475-478.

Barbour, A. G. and B. Travinsky (2010). "Evolution and distribution of the *ospC* gene, a transferable serotype determinant of *Borrelia burgdorferi*." MBio **1**(4): e00153-00110.

Barthold, S. W. (1999). "Specificity of infection-induced immunity among *Borrelia burgdorferi* sensu lato Species." Infection and Immunity **67**(1): 36-42.

Basco, L. K. and P. Ringwald (2007). "Molecular epidemiology of malaria in Cameroon. XXIV. Trends of in vitro antimalarial drug responses in Yaounde, Cameroon." The American journal of tropical medicine and hygiene **76**(1): 20-26.

Bellet-Edimo, R., B. Betschart and L. Gern (2005). "Frequency and efficiency of transovarial and subsequent transstadial transmission of Borrelia burgdorferi sensu lato in Ixodes ricinus ticks." Bull. Soc. Neuchâtel Sci. Nat **128**: 117-125.

Brisson, D., D. Drecktrah, C. H. Eggers and D. S. Samuels (2012). "Genetics of *Borrelia burgdorferi*." Annual Review of Genetics **46**.

Brisson, D. and D. E. Dykhuizen (2004). "ospC diversity in Borrelia burgdorferi: Different hosts are different niches." Genetics **168**(2): 713-722.

Brossard, M. and S. Wikel (2004). "Tick immunobiology." Parasitology **129**(S1): S161-S176.

Brown, S. P., M. E. Hochberg and B. T. Grenfell (2002). "Does multiple infection select for raised virulence?" Trends in Microbiology **10**(9): 401-405.

Brunham, R. C., J. Kimani, J. Bwayo, G. Maitha, I. Maclean, C. L. Yang, C. X. Shen, S. Roman, N. J. D. Nagelkerke, M. Cheang and F. A. Plummer (1996). "The epidemiology of *Chlamydia trachomatis* within a sexually transmitted diseases core group." Journal of Infectious Diseases **173**(4): 950-956.

Brunham, R. C., F. A. Plummer and R. S. Stephens (1993). "Bacterial antigenic variation, host immune-response, and pathogen-host coevolution." Infection and Immunity **61**(6): 2273-2276.

Bunikis, J., U. Garpmo, J. Tsao, J. Berglund, D. Fish and A. G. Barbour (2004). "Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe." Microbiology **150**(Pt 6): 1741-1755.

Bunikis, J., J. Tsao, C. J. Luke, M. G. Luna, D. Fish and A. G. Barbour (2004). "*Borrelia burgdorferi* infection in a natural population of *Peromyscus leucopus* mice: a

longitudinal study in an area where Lyme borreliosis is highly endemic." Journal of Infectious Diseases **189**: 1515-1523.

Burri, C., F. Morán Cadenas, V. Douet, J. Moret and L. Gern (2007). "Ixodes ricinus density and infection prevalence of *Borrelia burgdorferi* sensu lato along a north-facing altitudinal gradient in the Rhône Valley (Switzerland)." Vector-Borne and Zoonotic Diseases **7**(1): 50-58.

Capella-Gutiérrez, S., J. M. Silla-Martínez and T. Gabaldón (2009). "trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses." Bioinformatics **25**(15): 1972-1973.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena, J. K. Goodrich and J. I. Gordon (2010). "QIIME allows analysis of high-throughput community sequencing data." Nature Methods **7**(5): 335-336.

Casjens, S., N. Palmer, R. Van Vugt, W. Mun Huang, B. Stevenson, P. Rosa, R. Lathigra, G. Sutton, J. Peterson and R. J. Dodson (2000). "A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*." Molecular Microbiology **35**(3): 490-516.

Casjens, S. R., E. F. Mongodin, W.-G. Qiu, B. J. Luft, S. E. Schutzer, E. B. Gilcrease, W. M. Huang, M. Vujadinovic, J. K. Aron and L. C. Vargas (2012). "Genome stability of Lyme disease spirochetes: comparative genomics of *Borrelia burgdorferi* plasmids." PloS one **7**(3): e33280.

Chambert, T., V. Staszewski, E. Lobato, R. Choquet, C. Carrie, K. D. McCoy, T. Tveraa and T. Boulinier (2012). "Exposure of black-legged kittiwakes to Lyme disease spirochetes: dynamics of the immune status of adult hosts and effects on their survival." Journal of Evolutionary Biology.

Choisy, M. and J. C. de Roode (2010). "Mixed infections and the evolution of virulence: effects of resource competition, parasite plasticity, and impaired host immunity." The American Naturalist **175**(5): E105-E118.

Coleman, J. L., J. A. Gebbia, J. Piesman, J. L. Degen, T. H. Bugge and J. L. Benach (1997). "Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice." Cell **89**(7): 1111-1119.

Conway, D. and S. Polley (2002). "Measuring immune selection." Parasitology **125**(07): S3-S16.

Cook, M. J. (2015). "Lyme borreliosis: a review of data on transmission time after tick attachment." International Journal of General Medicine **8**: 1.

De Roode, J., A. Read, B. Chan and M. Mackinnon (2003). "Rodent malaria parasites suffer from the presence of conspecific clones in three-clone *Plasmodium chabaudi* infections." Parasitology **127**(05): 411-418.

de Roode, J. C., R. Pansini, S. J. Cheesman, M. E. Helinski, S. Huijben, A. R. Wargo, A. S. Bell, B. H. Chan, D. Walliker and A. F. Read (2005). "Virulence and competitive ability in genetically diverse malaria infections." Proceedings of the National Academy of Sciences of the United States of America **102**(21): 7624-7628.

Derdáková, M., V. Dudiòák, B. Brei, J. S. Brownstein, I. Schwartz and D. Fish (2004). "Interaction and transmission of two *Borrelia burgdorferi* sensu stricto strains in a tick-rodent maintenance system." Applied and environmental microbiology **70**(11): 6783-6788.

Donahue, J. G., J. Piesman and A. Spielman (1987). "Reservoir competence of white-footed mice for Lyme disease spirochetes." The American Journal of Tropical Medicine and Hygiene **36**(1): 92-96.

Drecktrah, D., M. Lybecker, N. Popitsch, P. Rescheneder, L. S. Hall and D. S. Samuels (2015). "The *Borrelia burgdorferi* RelA/SpoT homolog and stringent response regulate survival in the tick vector and global gene expression during starvation." PLoS Pathogens **11**(9): e1005160.

Dressler, F., J. A. Whalen, B. N. Reinhardt and A. C. Steere (1993). "Western blotting in the serodiagnosis of Lyme disease." Journal of Infectious Diseases **167**(2): 392-400.

Dubska, L., I. Literak, E. Kocianova, V. Taragelova and O. Sychra (2009). "Differential role of passerine birds in distribution of *Borrelia* spirochetes, based on data from ticks collected from birds during the postbreeding migration period in Central Europe." Applied and environmental microbiology **75**(3): 596-602.

Dunham-Ems, S. M., M. J. Caimano, U. Pal, C. W. Wolgemuth, C. H. Eggers, A. Balic and J. D. Radolf (2009). "Live imaging reveals a biphasic mode of dissemination of *Borrelia burgdorferi* within ticks." The Journal of Clinical Investigation **119**(12): 3652.

Durand, J., M. Jacquet, L. Paillard, O. Rais, L. Gern and M. J. Voordouw (2015). "Cross-immunity and community structure of a multiple-strain pathogen in the tick vector." Applied and Environmental Microbiology **81**(22): 7740-7752.

Dykhuizen, D. E. and G. Baranton (2001). "The implications of a low rate of horizontal transfer in *Borrelia*." Trends in Microbiology **9**(7): 344-350.

Dykhuizen, D. E., D. Brisson, S. Sandigursky, G. P. Wormser, J. Nowakowski, R. B. Nadelman and I. Schwartz (2008). "Short report: The propensity of different *Borrelia*

burgdorferi sensu stricto genotypes to cause disseminated infections in humans." The American Journal of Tropical Medicine and Hygiene **78**(5): 806-810.

Earnhart, C. G., E. L. Buckles, J. S. Dumler and R. T. Marconi (2005). "Demonstration of OspC type diversity in invasive human Lyme disease isolates and identification of previously uncharacterized epitopes that define the specificity of the OspC murine antibody response." Infection and Immunity **73**(12): 7869-7877.

Engelstädter, J. and S. Bonhoeffer (2009). "Red Queen dynamics with non-standard fitness interactions." PLoS Comput Biol **5**(8): e1000469.

Engstrom, S. M., E. Shoop and R. C. Johnson (1995). "Immunoblot interpretation criteria for serodiagnosis of early Lyme disease." Journal of Clinical Microbiology **33**(2): 419-427.

Estrada-Peña, A., C. Ortega, N. Sánchez, L. DeSimone, B. Sudre, J. E. Suk and J. C. Semenza (2011). "Correlation of *Borrelia burgdorferi* sensu lato prevalence in questing *Ixodes ricinus* ticks with specific abiotic traits in the western palearctic." Applied and Environmental Microbiology **77**(11): 3838-3845.

Fazzino, L., K. Tilly, D. P. Dulebohn and P. A. Rosa (2015). "Long-term survival of *Borrelia burgdorferi* lacking the hibernation promotion factor homolog in the unfed tick vector." Infection and Immunity **83**(12): 4800-4810.

Fedorova, N., J. E. Kleinjan, D. James, L. T. Hui, H. Peeters and R. S. Lane (2014). "Remarkable diversity of tick or mammalian-associated *Borreliae* in the metropolitan San Francisco Bay Area, California." Ticks and Tick-borne Diseases **5**(6): 951-961.

Fingerle, V., G. Goettner, L. Gern, B. Wilske and U. Schulte-Spechtel (2007). "Complementation of a *Borrelia afzelii* OspC mutant highlights the crucial role of OspC for dissemination of *Borrelia afzelii* in *Ixodes ricinus*." International Journal of Medical Microbiology **297**(2): 97-107.

Fisher, M. A., D. Grimm, A. K. Henion, A. F. Elias, P. E. Stewart, P. A. Rosa and F. C. Gherardini (2005). "*Borrelia burgdorferi* σ54 is required for mammalian infection and vector transmission but not for tick colonization." Proceedings of the National Academy of Sciences of the United States of America **102**(14): 5162-5167.

Ford, M. J. (2002). "Applications of selective neutrality tests to molecular ecology." Molecular Ecology **11**(8): 1245-1262.

Francischetti, I. M., A. Sá-Nunes, B. J. Mans, I. M. Santos and J. M. Ribeiro (2009). "The role of saliva in tick feeding." Frontiers in bioscience: a journal and virtual library **14**: 2051.

Franke, J., A. Moldenhauer, A. Hildebrandt and W. Dorn (2010). "Are birds reservoir hosts for *Borrelia afzelii*?" Ticks and Tick-Borne Diseases **1**(2): 109-112.

Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson and E. K. Hickey (1997). "Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*." Nature **390**(6660): 580-586.

Fuchs, R., S. Jauris, F. Lottspeich, V. Preac-Mursic, B. Wilske and E. Soutschek (1992). "Molecular analysis and expression of a *Borrelia burgdorferi* gene encoding a 22kDa protein (pC) in *Escherichia coli*." Molecular Microbiology **6**(4): 503-509.

Fung, B. P., G. L. McHugh, J. M. Leong and A. C. Steere (1994). "Humoral immune-response to outer surface protein-c of *Borrelia burgdorferi* in lyme-disease - role of the immunoglobulin-m response in the serodiagnosis of early infection." Infection and Immunity **62**(8): 3213-3221.

Fung, B. P., G. L. McHugh, J. M. Leong and A. C. Steere (1994). "Humoral immune response to outer surface protein C of *Borrelia burgdorferi* in Lyme disease: role of the immunoglobulin M response in the serodiagnosis of early infection." Infection and Immunity **62**(8): 3213-3221.

Gern, L., V. Douet, Z. López, O. Rais and F. Morán Cadenas (2010). "Diversity of *Borrelia* genospecies in *Ixodes ricinus* ticks in a Lyme borreliosis endemic area in Switzerland identified by using new probes for reverse line blotting." Ticks and Tick-borne Diseases **1**(1): 23-29.

Gern, L., A. Estrada-Peña, F. Frandsen, J. S. Gray, T. G. T. Jaenson, F. Jongejan, O. Kahl, E. Korenberg, R. Mehl and P. A. Nuttall (1998). "European reservoir hosts of *Borrelia burgdorferi* sensu lato." Zentralblatt für Bakteriologie **287**(3): 196-204.

Gern, L. and P. Humair (2002). "Ecology of *Borrelia burgdorferi* sensu lato in Europe." Lyme borreliosis biology, epidemiology and control. CABI International: 149-174.

Gern, L. and O. Rais (1996). "Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae)." Journal of Medical Entomology **33**(1): 189-192.

Gern, L., M. Siegenthaler, C. Hu, S. Leuba-Garcia, P. Humair and J. Moret (1994). "Borrelia burgdorferi in rodents (*Apodemus flavicollis* and *A. sylvaticus*): Duration and enhancement of infectivity for *Ixodes ricinus* ticks." European Journal of Epidemiology **10**(1): 75-80.

Gilmore, R. D., K. J. Kappel, M. C. Dolan, T. R. Burkot and B. Johnson (1996). "Outer surface protein C (OspC), but not P39, is a protective immunogen against a tick-

transmitted *Borrelia burgdorferi* challenge: evidence for a conformational protective epitope in OspC." Infection and Immunity **64**(6): 2234-2239.

Gilmore, R. D., K. J. Kappel, M. C. Dolan, T. R. Burkot and B. J. B. Johnson (1996). "Outer surface protein C (OspC), but not P39, is a protective immunogen against a tick-transmitted Borrelia burgdorferi challenge: Evidence for a conformational protective epitope in OspC." Infection and Immunity **64**(6): 2234-2239.

Girard, Y. A., B. Travinsky, A. Schotthoefer, N. Fedorova, R. J. Eisen, L. Eisen, A. G. Barbour and R. S. Lane (2009). "Population structure of the lyme borreliosis spirochete *Borrelia burgdorferi* in the western black-legged tick (*Ixodes pacificus*) in Northern California." Applied and Environmental Microbiology **75**(22): 7243-7252.

Gog, J. R. and B. T. Grenfell (2002). "Dynamics and selection of many-strain pathogens." Proceedings of the National Academy of Sciences **99**(26): 17209-17214.

Gray, J., O. Kahl, R. Lane and G. Stanek (2002). Lyme borreliosis: biology, epidemiology, and control, CABI.

Grimm, D., K. Tilly, R. Byram, P. E. Stewart, J. G. Krum, D. M. Bueschel, T. G. Schwan, P. F. Policastro, A. F. Elias and P. A. Rosa (2004). "Outer-surface protein C of the Lyme disease spirochete: A protein induced in ticks for infection of mammals." Proceedings of the National Academy of Sciences of the United States of America **101**(9): 3142-3147.

Guo, X., C. J. Booth, M. A. Paley, X. Wang, K. DePonte, E. Fikrig, S. Narasimhan and R. R. Montgomery (2009). "Inhibition of neutrophil function by two tick salivary proteins." Infection and Immunity **77**(6): 2320-2329.

Gupta, S. and R. Anderson (1999). "Population structure of pathogens: the role of immune selection." Parasitology Today **15**(12): 497-501.

Gupta, S., N. Ferguson and R. Anderson (1998). "Chaos, persistence, and evolution of strain structure in antigenically diverse infectious agents." Science **280**(5365): 912-915.

Gupta, S., M. C. J. Maiden, I. M. Feavers, S. Nee, R. M. May and R. M. Anderson (1996). "The maintenance of strain structure in populations of recombining infectious agents." Nature Medicine **2**(4): 437-442.

Gupta, S., K. Trenholme, R. M. Anderson and K. P. Day (1994). "Antigenic diversity and the transmission dynamics of *Plasmodium falciparum*." Science **263**(5149): 961-963.

Hajdusek, O., R. Sima, N. Ayllon, M. Jalovecka, J. Perner, J. De La Fuente and P. Kopacek (2013). "Interaction of the tick immune system with transmitted pathogens." Frontiers in Cellular and Infection Microbiology **3**.

Hamer, S. A., G. J. Hickling, E. D. Walker and J. I. Tsao (2014). "Increased diversity of zoonotic pathogens and *Borrelia burgdorferi* strains in established versus incipient *Ixodes scapularis* populations across the Midwestern United States." Infection, Genetics and Evolution **27**: 531-542.

Hanincová, K., K. Kurtenbach, M. Diuk-Wasser, B. Brei and D. Fish (2006). "Epidemic spread of Lyme borreliosis, northeastern United States." Emerging Infectious Diseases **12**(4): 604.

Hanincová, K., N. H. Ogden, M. Diuk-Wasser, C. J. Pappas, R. Iyer, D. Fish, I. Schwartz and K. Kurtenbach (2008). "Fitness variation of *Borrelia burgdorferi* sensu stricto strains in mice." Applied and Environmental Microbiology **74**(1): 153-157.

Hanincova, K., S. Schäfer, S. Etti, H.-S. Sewell, V. Taragelova, D. Ziak, M. Labuda and K. Kurtenbach (2003). "Association of *Borrelia afzelii* with rodents in Europe." Parasitology **126**(01): 11-20.

Hanincová, K., V. Taragelová, J. Koci, S. M. Schäfer, R. Hails, A. J. Ullmann, J. Piesman, M. Labuda and K. Kurtenbach (2003). "Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia." Applied and Environmental Microbiology **69**(5): 2825-2830.

Harrison, A. and N. C. Bennett (2012). "The importance of the aggregation of ticks on small mammal hosts for the establishment and persistence of tick-borne pathogens: an investigation using the R(0) model." Parasitology **139**(12): 1605-1613.

Harrison, A., W. I. Montgomery and K. J. Bown (2011). "Investigating the persistence of tick-borne pathogens via the R-0 model." Parasitology **138**(7): 896-905.

Harrison, A., W. I. Montgomery and K. J. Bown (2011). "Investigating the persistence of tick-borne pathogens via the R(0) model." Parasitology **138**(7): 896-905.

Hartemink, N., S. Randolph, S. Davis and J. Heesterbeek (2008). "The basic reproduction number for complex disease systems: defining R(0) for tick-borne infections." The American Naturalist **171**(6): 743-754.

Hartl, D. L. and A. G. Clark (1997). Principles of population genetics, Sinauer associates Sunderland.

Hasle, G. (2013). "Transport of ixodid ticks and tick-borne pathogens by migratory birds." Frontiers in Cellular and Infection Microbiology **3**: 48.

Haven, J., L. C. Vargas, E. F. Mongodin, V. Xue, Y. Hernandez, P. Pagan, C. M. Fraser-Liggett, S. E. Schutzer, B. J. Luft, S. R. Casjens and W. G. Qiu (2011). "Pervasive Recombination and Sympatric Genome Diversification Driven by Frequency-Dependent Selection in Borrelia burgdorferi, the Lyme Disease Bacterium." Genetics **189**(3): 951-U341.

Hellgren, O., M. Andersson and L. Råberg (2011). "The genetic structure of *Borrelia afzelii* varies with geographic but not ecological sampling scale." Journal of Evolutionary Biology **24**(1): 159-167.

Herrmann, C. and L. Gern (2010). "Survival of *Ixodes ricinus* (Acari: Ixodidae) under challenging conditions of temperature and humidity is influenced by *Borrelia burgdorferi* sensu lato infection." Journal of Medical Entomology **47**(6): 1196-1204.

Herrmann, C. and L. Gern (2012). "Do the level of energy reserves, hydration status and *Borrelia* infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks?" Parasitology **139**(03): 330-337.

Herrmann, C. and L. Gern (2013). "Survival of *Ixodes ricinus* (Acari: Ixodidae) nymphs under cold conditions is negatively influenced by frequent temperature variations." Ticks and tick-borne diseases **4**(5): 445-451.

Herrmann, C., L. Gern and M. J. Voordouw (2013). "Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*." Applied and Environmental Microbiology **79**(23): 7273-7280.

Herrmann, C., M. Voordouw and L. Gern (2013). "*Ixodes ricinus* ticks infected with the causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, have higher energy reserves." International journal for parasitology **43**(6): 477-483.

Heylen, D., E. Matthysen, M. Fonville and H. Sprong (2014). "Songbirds as general transmitters but selective amplifiers of *Borrelia burgdorferi* sensu lato genotypes in *Ixodes rinicus* ticks." Environmental Microbiology **16**(9): 2859-2868.

Hodzic, E., S. Feng, K. J. Freet, D. L. Borjesson and S. W. Barthold (2002). "*Borrelia burgdorferi* population kinetics and selected gene expression at the host-vector interface." Infection and Immunity **70**(7): 3382-3388.

Hofmeister, E. K., B. A. Ellis, G. E. Glass and J. E. Childs (1999). "Longitudinal study of infection with *Borrelia burgdorferi* in a population of *Peromyscus leucopus* at a Lyme disease-enzootic site in Maryland." American Journal of Tropical Medicine and Hygiene **60**(4): 589-609.

Hu, C. M., Y. Cheminade, J.-L. Perret, V. Weynants, Y. Lobet and L. Gern (2003). "Early detection of *Borrelia burgdorferi* sensu lato infection in Balb/c mice by co-feeding *Ixodes ricinus* ticks." International Journal of Medical Microbiology **293**(6): 421-426.

Huegli, D., C. Hu, P.-F. Humair, B. Wilske and L. Gern (2002). "*Apodemus* species mice are reservoir hosts of *Borrelia garinii* OspA serotype 4 in Switzerland." Journal of Clinical Microbiology **40**(12): 4735-4737.

Humair, P.-F., V. Douet, F. M. Cadenas, L. M. Schouls, I. Van De Pol and L. Gern (2007). "Molecular identification of bloodmeal source in *Ixodes ricinus* ticks using 12S rDNA as a genetic marker." Journal of Medical Entomology **44**(5): 869-880.

Humair, P.-F., O. Peter, R. Wallich and L. Gern (1995). "Strain variation of Lyme disease spirochetes isolated from *Ixodes ricinus* ticks and rodents collected in two endemic areas in Switzerland." Journal of Medical Entomology **32**(4): 433-438.

Humair, P. and L. Gern (1998). "Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland." Acta Tropica **69**(3): 213-227.

Humair, P., D. Postic, R. Wallich and L. Gern (1998). "An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes." Zentralblatt fur Bakteriologie: international journal of medical microbiology **287**(4): 521-538.

Humair, P., O. Rais and L. Gern (1999). "Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance." Parasitology **118**(01): 33-42.

Jacquet, M., J. Durand, O. Rais and M. J. Voordouw (2015). "Cross-reactive acquired immunity influences transmission success of the Lyme disease pathogen, *Borrelia afzelii*." Infection, Genetics and Evolution **36**: 131-140.

Jacquet, M., J. Durand, O. Rais and M. J. Voordouw (2015). "Strain-specific antibodies reduce co-feeding transmission of the Lyme disease pathogen, *Borrelia afzelii*." Environmental microbiology.

Jacquot, M., M. Bisseux, D. Abrial, M. Marsot, E. Ferquel, J.-L. Chapuis, G. Vourc'h and X. Bailly (2014). "High-throughput sequence typing reveals genetic differentiation and host specialization among populations of the *Borrelia burgdorferi* species complex that infect rodents." PloS one **9**(2): e88581.

Jaenson, T. G. T. and L. Tälleklint (1992). "Incompetence of roe deer as reservoirs of the Lyme borreliosis spirochete." Journal of Medical Entomology **29**(5): 813-817.

Jauris-Heipke, S., G. Liegl, V. Preac-Mursic, D. Rössler, E. Schwab, E. Soutschek, G. Will and B. Wilske (1995). "Molecular analysis of genes encoding outer surface protein C (OspC) of *Borrelia burgdorferi* sensu lato: relationship to *ospA* genotype and evidence of lateral gene exchange of *ospC*." Journal of Clinical Microbiology **33**(7): 1860-1866.

Johns, R., J. Ohnishi, A. Broadwater, D. E. Sonenshine, A. M. De Silva and W. L. Hynes (2001). "Contrasts in tick innate immune responses to *Borrelia burgdorferi* challenge: immunotolerance in *Ixodes scapularis* versus immunocompetence in *Dermacentor variabilis* (Acari: Ixodidae)." Journal of Medical Entomology **38**(1): 99-107.

Johnson, P. T., J. C. De Roode and A. Fenton (2015). "Why infectious disease research needs community ecology." Science **349**(6252): 1259504.

Karvonen, A., C. Rellstab, K.-R. Louhi and J. Jokela (2011). "Synchronous attack is advantageous: mixed genotype infections lead to higher infection success in trematode parasites." Proceedings of the Royal Society of London B: Biological Sciences: rspb20110879.

Kenedy, M. R., T. R. Lenhart and D. R. Akins (2012). "The role of *Borrelia burgdorferi* outer surface proteins." FEMS Immunology & Medical Microbiology **66**(1): 1-19.

Kung, F., J. Anguita and U. Pal (2013). "*Borrelia burgdorferi* and tick proteins supporting pathogen persistence in the vector." Future microbiology **8**(1): 41-56.

Kurtenbach, K., S. De Michelis, S. Etti, S. M. Schäfer, H.-S. Sewell, V. Brade and P. Kraiczy (2002). "Host association of *Borrelia burgdorferi* sensu lato–the key role of host complement." Trends in microbiology **10**(2): 74-79.

Kurtenbach, K., S. De Michelis, H.-S. Sewell, S. Etti, S. M. Schäfer, R. Hails, M. Collares-Pereira, M. Santos-Reis, K. Haninçová and M. Labuda (2001). "Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies found in individual questing ticks from Europe." Applied and Environmental Microbiology **67**(10): 4926-4929.

Kurtenbach, K., K. Hanincová, J. I. Tsao, G. Margos, D. Fish and N. H. Ogden (2006). "Fundamental processes in the evolutionary ecology of Lyme borreliosis." Nature Reviews Microbiology **4**(9): 660-669.

Kurtenbach, K., M. Peacey, S. G. Rijpkema, A. N. Hoodless, P. A. Nuttall and S. E. Randolph (1998). "Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England." Applied and Environmental Microbiology **64**(4): 1169-1174.

Kurtenbach, K., H.-S. Sewell, N. H. Ogden, S. E. Randolph and P. A. Nuttall (1998). "Serum complement sensitivity as a key factor in Lyme disease ecology." Infection and Immunity **66**(3): 1248-1251.

Labandeira-Rey, M. and J. T. Skare (2001). "Decreased infectivity in *Borrelia burgdorferi* strain B31 is associated with loss of linear plasmid 25 or 28-1." Infection and Immunity **69**(1): 446-455.

Lagal, V., D. Portnoi, G. Faure, D. Postic and G. Baranton (2006). "Borrelia burgdorferi sensu stricto invasiveness is correlated with OspC-plasminogen affinity." Microbes and Infection **8**(3): 645-652.

Lagal, V., D. Postic, E. Ruzic-Sabljic and G. Baranton (2003). "Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness." Journal of Clinical Microbiology **41**(11): 5059-5065.

Lebet, N. and L. Gern (1994). "Histological examination of *Borrelia burgdorferi* infections in unfed *Ixodes ricinus* nymphs." Experimental & Applied Acarology **18**(3): 177-183.

Lena, S., B. Pourbohloul and R. C. Brunham (2005). "Effect of immune response on transmission dynamics for sexually transmitted infections." Journal of Infectious Diseases **191**: S78-S84.

Liang, F. T., M. B. Jacobs, L. C. Bowers and M. T. Philipp (2002). "An immune evasion mechanism for spirochetal persistence in Lyme borreliosis." The Journal of Experimental Medicine **195**(4): 415-422.

Liang, F. T., J. Yan, M. L. Mbow, S. L. Sviat, R. D. Gilmore, M. Mamula and E. Fikrig (2004). "*Borrelia burgdorferi* changes its surface antigenic expression in response to host immune responses." Infection and Immunity **72**(10): 5759-5767.

Lima, C., N. Zeidner, C. Beard, C. Soares, M. Dolan, G. Dietrich and J. Piesman (2005). "Differential infectivity of the Lyme disease spirochete *Borrelia burgdorferi* derived from *Ixodes scapularis* salivary glands and midgut." Journal of Medical Entomology **42**(3): 506-510.

Lindsay, L. R., I. K. Barker, G. A. Surgeoner, S. A. McEwen and G. D. Campbell (1997). "Duration of *Borrelia burgdorferi* infectivity in white-footed mice for the tick vector *Ixodes scapularis* under laboratory and field conditions in Ontario." Journal of Wildlife Diseases **33**(4): 766-775.

Lively, C. and V. Apanius (1995). "Genetic diversity in host-parasite interactions." Ecology of Infectious Diseases in Natural Populations **7**: 421.

Livey, I., C. P. Gibbs, R. Schuster and F. Dorner (1995). "Evidence for lateral transfer and recombination in ospc variation in lyme-disease B*orrelia*." Molecular Microbiology **18**(2): 257-269.

Lommano, E., C. Dvořák, L. Vallotton, L. Jenni and L. Gern (2014). "Tick-borne pathogens in ticks collected from breeding and migratory birds in Switzerland." Ticks and Tick-Borne Diseases **5**(6): 871-882.

Louhi, K.-R., L.-R. Sundberg, J. Jokela and A. Karvonen (2015). "Interactions among bacterial strains and fluke genotypes shape virulence of co-infection." Proceedings of the Royal Society of London B: Biological Sciences **282**(1821).

MacQueen, D. D., C. Lubelczyk, S. P. Elias, B. K. Cahill, A. J. Mathers, E. H. Lacombe, P. W. Rand and R. P. Smith Jr (2012). "Genotypic diversity of an emergent population of *Borrelia burgdorferi* at a coastal Maine island recently colonized by *Ixodes scapularis*." Vector-Borne and Zoonotic Diseases **12**(6): 456-461.

Marconi, R. T., D. S. Samuels and C. F. Garon (1993). "Transcriptional analyses and mapping of the *ospC* gene in Lyme disease spirochetes." Journal of Bacteriology **175**(4): 926-932.

Marsot, M., M. Sigaud, J. Chapuis, E. Ferquel, M. Cornet and G. Vourc'h (2011). "Introduced Siberian chipmunks (*Tamias sibiricus barberi*) harbor more-diverse *Borrelia burgdorferi* sensu lato genospecies than native bank voles (*Myodes glareolus*)." Applied and environmental microbiology **77**(16): 5716-5721.

Mathers, A., R. P. Smith, B. Cahill, C. Lubelczyk, S. P. Elias, E. Lacombe, S. R. Morris, C. P. Vary, C. E. Parent and P. W. Rand (2011). "Strain diversity of *Borrelia burgdorferi* in ticks dispersed in North America by migratory birds." Journal of Vector Ecology **36**(1): 24-29.

Matuschka, F.-R., P. Fischer, M. Heiler, D. Richter and A. Spielman (1992). "Capacity of European animals as reservoir hosts for the Lyme disease spirochete." Journal of Infectious Diseases **165**(3): 479-483.

Matuschka, F.-R., M. Heiler, H. Eiffert, P. Fischer, H. Lotter and A. Spielman (1993). "Diversionary role of hoofed game in the transmission of Lyme disease spirochetes." The American journal of tropical medicine and hygiene **48**(5): 693-699.

May, R. M. and M. A. Nowak (1995). "Coinfection and the evolution of parasite virulence." Proceedings of the Royal Society of London B: Biological Sciences **261**(1361): 209-215.

Mbow, M. L., R. D. Gilmore and R. G. Titus (1999). "An OspC-specific monoclonal antibody passively protects mice from tick-transmitted infection by *Borrelia burgdorferi* B31." Infection and Immunity **67**(10): 5470-5472.

Mechai, S., G. Margos, E. J. Feil, N. Barairo, L. R. Lindsay, P. Michel and N. H. Ogden (2016). "Evidence for host-genotype associations of *Borrelia burgdorferi* sensu stricto." PLoS One **11**(2): e0149345.

Mejri, N., B. Rutti and M. Brossard (2002). "Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice." Parasitology research **88**(3): 192-197.

Mercereau-Puijalon, O. (1996). "Revisiting host/parasite interactions: molecular analysis of parasites collected during longitudinal and cross-sectional surveys in humans." Parasite Immunology **18**(4): 173-180.

Mideo, N. (2009). "Parasite adaptations to within-host competition." Trends in parasitology **25**(6): 261-268.

Mideo, N., V. C. Barclay, B. H. Chan, N. J. Savill, A. F. Read and T. Day (2008). "Understanding and predicting strain-specific patterns of pathogenesis in the rodent malaria *Plasmodium chabaudi*." The American Naturalist **172**(5): E214-E238.

Millins, C., A. Magierecka, L. Gilbert, A. Edoff, A. Brereton, E. Kilbride, M. Denwood, R. Birtles and R. Biek (2015). "An invasive mammal (grey squirrel, Sciurus carolinensis) commonly hosts diverse and atypical genotypes of the zoonotic pathogen Borrelia burgdorferi sensu lato." Applied and environmental microbiology: AEM. 00109-00115.

Moody, K. D., G. A. Terwilliger, G. M. Hansen and S. W. Barthold (1994). "Experimental *Borrelia burgdorferi* infection in *Peromyscus leucopus*." Journal of Wildlife Diseases **30**(2): 155-161.

Morán Cadenas, F., O. Rais, P.-F. Humair, V. Douet, J. Moret and L. Gern (2007). "Identification of host bloodmeal source and *Borrelia burgdorferi* sensu lato in field-collected *Ixodes ricinus* ticks in Chaumont (Switzerland)." Journal of Medical Entomology **44**(6): 1109-1117.

Morán Cadenas, F., H. Schneider, E. Lommano, C. Burri, J. Moret and L. Gern (2007). "A comparison of two DNA extraction approaches in the detection of *Borrelia burgdorferi* sensu lato from live *Ixodes ricinus* ticks by PCR and reverse line blotting." Vector-Borne and Zoonotic Diseases **7**(4): 555-561.

Nowak, M. A. and R. M. May (1994). "Superinfection and the evolution of parasite virulence." Proceedings of the Royal Society of London B: Biological Sciences **255**(1342): 81-89.

Ogden, N., P. Nuttall and S. Randolph (1997). "Natural Lyme disease cycles maintained via sheep by co-feeding ticks." Parasitology **115**(06): 591-599.

Ogden, N. H., L. R. Lindsay, K. Hanincova, I. K. Barker, M. Bigras-Poulin, D. F. Charron, A. Heagy, C. M. Francis, C. J. O'Callaghan, I. Schwartz and R. A. Thompson (2008). "Role of migratory birds in introduction and range expansion of *Ixodes scapularis* ticks and of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in Canada." Applied and Environmental Microbiology **74**(6): 1780-1790.

Ogden, N. H., G. Margos, D. M. Aanensen, M. A. Drebot, E. J. Feil, K. Hanincova, I. Schwartz, S. Tyler and L. R. Lindsay (2011). "Investigation of genotypes of *Borrelia burgdorferi* in *Ixodes scapularis* ticks collected during surveillance in Canada." Applied and Environmental Microbiology **77**(10): 3244-3254.

Ohnishi, J., J. Piesman and A. M. de Silva (2001). "Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks." Proceedings of the National Academy of Sciences **98**(2): 670-675.

Onder, O., P. T. Humphrey, B. McOmber, F. Korobova, N. Francella, D. C. Greenbaum and D. Brisson (2012). "OspC Is Potent Plasminogen Receptor on Surface of Borrelia burgdorferi." Journal of Biological Chemistry **287**(20): 16860-16868.

Pal, U. and E. Fikrig (2010). Tick Interactions. In: *Borrelia*: molecular biology, host interaction and pathogenesis, Caister Academic Press.

Pal, U., X. F. Yang, M. Chen, L. K. Bockenstedt, J. F. Anderson, R. A. Flavell, M. V. Norgard and E. Fikrig (2004). "OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands." Journal of Clinical Investigation **113**(2): 220-230.

Pappas, C. J., R. Iyer, M. M. Petzke, M. J. Caimano, J. D. Radolf and I. Schwartz (2011). "*Borrelia burgdorferi* requires glycerol for maximum fitness during the tick phase of the enzootic cycle." PLoS Pathogens **7**(7): e1002102.

Peacock, L., V. Ferris, M. Bailey and W. Gibson (2007). "Dynamics of infection and competition between two strains of *Trypanosoma brucei brucei* in the tsetse fly observed using fluorescent markers." Kinetoplastid Biology and Disease **6**(1): 4.

Pechová, J., G. Stepanova, L. Kovar and J. Kopecky (2002). "Tick salivary gland extract-activated transmission of *Borrelia afzelii* spirochaetes." Folia Parasitologica **49**(2): 153-159.

Pérez, D., Y. Kneubühler, O. Rais, F. Jouda and L. Gern (2011). "*Borrelia afzelii ospC* genotype diversity in *Ixodes ricinus* questing ticks and ticks from rodents in two Lyme borreliosis endemic areas: Contribution of co-feeding ticks." Ticks and tick-borne diseases **2**(3): 137-142.

Peters, A. and C. Lively (1999). "The Red Queen and fluctuating epistasis: a population genetic analysis of antagonistic coevolution." The American Naturalist **154**(4): 393-405.

Piesman, J. (1993). "Dynamics of *Borrelia burgdorferi* transmission by nymphal *Ixodes dammini* ticks." Journal of Infectious Diseases **167**(5): 1082-1085.

Piesman, J. and L. Gern (2004). "Lyme borreliosis in Europe and North America." Parasitology-Cambridge **129**: S191.

Piesman, J., J. R. Oliver and R. J. Sinsky (1990). "Growth kinetics of the Lyme disease spirochete (*Borrelia burgdorferi*) in vector ticks (*Ixodes dammini*)." The American Journal of Tropical Medicine and Hygiene **42**(4): 352-357.

Piesman, J., B. S. Schneider and N. S. Zeidner (2001). "Use of quantitative PCR to measure density of *Borrelia burgdorferi* in the midgut and salivary glands of feeding tick vectors." Journal of Clinical Microbiology **39**(11): 4145-4148.

Pisanu, B., J.-L. Chapuis, A. Dozières, F. Basset, V. Poux and G. Vourc'h (2014). "High prevalence of *Borrelia burgdorferi* sl in the European red squirrel *Sciurus vulgaris* in France." Ticks and Tick-Borne Diseases **5**(1): 1-6.

Plummer, F. A., J. N. Simonsen, H. Chubb, L. Slaney, J. Kimata, M. Bosire, J. O. Ndinyaachola and E. N. Ngugi (1989). "Epidemiologic evidence for the development of serovar-specific immunity after gonococcal-infection." Journal of Clinical Investigation **83**(5): 1472-1476.

Pollitt, L. C., J. T. Bram, S. Blanford, M. J. Jones and A. F. Read (2015). "Existing infection facilitates establishment and density of malaria parasites in their mosquito vector." PLoS Pathogens **11**(7): e1005003.

Poupon, M.-A., E. Lommano, P.-F. Humair, V. Douet, O. Rais, M. Schaad, L. Jenni and L. Gern (2006). "Prevalence of *Borrelia burgdorferi* sensu lato in ticks collected from migratory birds in Switzerland." Applied and Environmental Microbiology **72**(1): 976-979.

Preac-Mursic, V., B. Wilske, S. Jauris, G. Will, S. Reinhardt, G. Lehnert, E. Patsouris, P. Mehraein, E. Soutschek and U. Klockmann (1992). "Active immunization with OspC protein of *Borrelia burgdorferi* protects gerbils against *B. burgdorferi* infection." Infection **20**(6): 342-349.

Pritt, B. S., P. S. Mead, D. K. H. Johnson, D. F. Neitzel, L. B. Respicio-Kingry, J. P. Davis, E. Schiffman, L. M. Sloan, M. E. Schriefer and A. J. Replogle (2016). "Identification of a

novel pathogenic *Borrelia* species causing Lyme borreliosis with unusually high spirochaetaemia: a descriptive study." The Lancet Infectious Diseases.

Probert, W. S., M. Crawford, R. B. Cadiz and R. B. LeFebvre (1997). "Immunization with outer surface protein (Osp) A, but not OspC, provides cross-protection of mice challenged with North American isolates of *Borrelia burgdorferi*." Journal of Infectious Diseases **175**(2): 400-405.

Probert, W. S. and R. B. LeFebvre (1994). "Protection of C3H/HeN mice from challenge with *Borrelia burgdorferi* through active immunization with OspA, OspB, or OspC, but not with OspD or the 83-kilodalton antigen." Infection and immunity **62**(5): 1920-1926.

Qiu, W.-G., D. E. Dykhuizen, M. S. Acosta and B. J. Luft (2002). "Geographic uniformity of the Lyme disease spirochete (*Borrelia burgdorferi*) and its shared history with tick vector (*Ixodes scapularis*) in the northeastern United States." Genetics **160**(3): 833-849.

Qiu, W.-G., S. E. Schutzer, J. F. Bruno, O. Attie, Y. Xu, J. J. Dunn, C. M. Fraser, S. R. Casjens and B. J. Luft (2004). "Genetic exchange and plasmid transfers in *Borrelia burgdorferi* sensu stricto revealed by three-way genome comparisons and multilocus sequence typing." Proceedings of the National Academy of Sciences of the United States of America **101**(39): 14150-14155.

Qiu, W. G., E. M. Bosler, J. R. Campbell, G. D. Ugine, I. N. Wang, B. J. Luft and D. E. Dykhuizen (1997). "A population genetic study of *Borrelia burgdorferi* sensu stricto from eastern Long Island, New York, suggested frequency-dependent selection, gene flow and host adaptation." Hereditas **127**(3): 203-216.

Råberg, L. (2012). "Infection intensity and infectivity of the tick-borne pathogen *Borrelia afzelii*." Journal of evolutionary biology **25**(7): 1448-1453.

Råberg, L., J. C. de Roode, A. S. Bell, P. Stamou, D. Gray and A. F. Read (2006). "The role of immune-mediated apparent competition in genetically diverse malaria infections." The American Naturalist **168**(1): 41-53.

Radolf, J. D., M. J. Caimano, B. Stevenson and L. T. Hu (2012). "Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes." Nature Reviews Microbiology **10**(2): 87-99.

Ramamoorthi, N., S. Narasimhan, U. Pal, F. K. Bao, X. F. F. Yang, D. Fish, J. Anguita, M. V. Norgard, F. S. Kantor, J. F. Anderson, R. A. Koski and E. Fikrig (2005). "The Lyme disease agent exploits a tick protein to infect the mammalian host." Nature **436**(7050): 573-577.

Randolph, S., L. Gern and P. Nuttall (1996). "Co-feeding ticks: epidemiological significance for tick-borne pathogen transmission." Parasitology today **12**(12): 472-479.

Rauter, C. and T. Hartung (2005). "Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis." Applied and Environmental Microbiology **71**(11): 7203-7216.

Read, A. F. and L. H. Taylor (2001). "The ecology of genetically diverse infections." Science **292**(5519): 1099-1102.

Rego, R. O., A. Bestor, J. Stefka and P. A. Rosa (2014). "Population bottlenecks during the infectious cycle of the Lyme disease spirochete *Borrelia burgdorferi*." PLoS One **9**(6): e101009.

Reif, K. E., G. H. Palmer, D. W. Crowder, M. W. Ueti and S. M. Noh (2014). "Restriction of *Francisella novicida* genetic diversity during infection of the vector midgut." PLoS Pathogens **10**(11): e1004499.

Ribeiro, J., G. Makoul, J. Levine, D. Robinson and A. Spielman (1985). "Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, Ixodes dammini." The Journal of experimental medicine **161**(2): 332-344.

Ribeiro, J. C. (1987). "*Ixodes dammini*: salivary anti-complement activity." Experimental Parasitology **64**(3): 347-353.

Ribeiro, J. C., J. J. Weis and S. R. Telford (1990). "Saliva of the tick *Ixodes dammini* inhibits neutrophil function." Experimental Parasitology **70**(4): 382-388.

Rice, P., I. Longden and A. Bleasby (2000). "EMBOSS: the European Molecular Biology Open Software Suite." Trends in Genetics **16**(6): 276-277.

Richter, D., A. Debski, Z. Hubalek and F.-R. Matuschka (2012). "Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks." Vector-Borne and Zoonotic Diseases **12**(1): 21-27.

Richter, D., B. Klug, A. Spielman and F.-R. Matuschka (2004). "Adaptation of diverse Lyme disease spirochetes in a natural rodent reservoir host." Infection and Immunity **72**(4): 2442-2444.

Rollend, L., D. Fish and J. E. Childs (2013). "Transovarial transmission of *Borrelia* spirochetes by *Ixodes scapularis*: A summary of the literature and recent observations." Ticks and tick-borne diseases **4**(1): 46-51.

Sadziene, A., B. Wilske, M. Ferdows and A. Barbour (1993). "The cryptic *ospC* gene of *Borrelia burgdorferi* B31 is located on a circular plasmid." Infection and Immunity **61**(5): 2192-2195.

Sato, Y. and M. Nakao (1997). "Transmission of the Lyme disease spirochete, *Borrelia garinii*, between infected and uninfected immature *Ixodes persulcatus* during cofeeding on mice." The Journal of Parasitology **83**(3): 547-550.

Schmid-Hempel, P. (2011). Evolutionary parasitology : the integrated study of infections, immunology, ecology, and genetics. Oxford, Oxford University Press.

Schmid-Hempel, P. and J. Jokela (2002). "Socially structured populations and evolution of recombination under antagonistic coevolution." The American Naturalist **160**(3): 403-408.

Schwan, T., W. Burgdorfer and C. F. Garon (1988). "Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation." Infection and Immunity **56**(8): 1831-1836.

Schwan, T. G. and J. Piesman (2000). "Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice." Journal of Clinical Microbiology **38**(1): 382-388.

Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan and P. A. Rosa (1995). "Induction of an outer surface protein on Borrelia burgdorferi during tick feeding." Proceedings of the National Academy of Sciences **92**(7): 2909-2913.

Schwanz, L. E., M. J. Voordouw, D. Brisson and R. S. Ostfeld (2011). "*Borrelia burgdorferi* has minimal impact on the Lyme Disease reservoir host *Peromyscus leucopus*." Vector-Borne and Zoonotic Diseases **11**(2): 117-124.

Seinost, G., D. E. Dykhuizen, R. J. Dattwyler, W. T. Golde, J. J. Dunn, I. N. Wang, G. P. Wormser, M. E. Schriefer and B. J. Luft (1999). "Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans." Infection and Immunity **67**(7): 3518-3524.

Sharon, M. D., W. A. Rowley, M. G. Novak and K. B. Platf (1992). "Rates of *Borrelia burgdorferi* infection in *lxodes dammini* (Acari: Ixodidae) in Southwestern Wisconsin." Journal of Medical Entomology **29**(2): 314-317.

Shih, C.-M., R. J. Pollack, S. R. Telford and A. Spielman (1992). "Delayed dissemination of Lyme disease spirochetes from the site of deposition in the skin of mice." Journal of Infectious Diseases **166**(4): 827-831.

Slater, G. S. and E. Birney (2005). "Automated generation of heuristics for biological sequence comparison." BMC bioinformatics **6**(1): 31.

Smith, T., I. Felger, M. Tanner and H.-P. Beck (1999). "The epidemiology of multiple *Plasmodium falciparum* infections. 11. Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections." Transactions of the Royal Society of Tropical Medicine and Hygiene. Supplement **93**(1): S59-S64.

Spielman, A., J. Ribeiro, T. Mather and J. Piesman (1987). "Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari: Ixodidae)." Journal of Medical Entomology **24**(2): 201-205.

Staves, P. A. and R. J. Knell (2010). "Virulence and competitiveness: testing the relationship during inter- and intraspecific mixed infections." Evolution **64**(9): 2643-2652.

Stotz, H. U., J. G. Bishop, C. W. Bergmann, M. Koch, P. Albersheim, A. G. Darvill and J. M. Labavitch (2000). "Identification of target amino acids that affect interactions of fungal polygalacturonases and their plant inhibitors." Physiological and Molecular Plant Pathology **56**(3): 117-130.

Strandh, M. and L. Råberg (2015). "Within-host competition between *Borrelia afzelii ospC* strains in wild hosts as revealed by massively parallel amplicon sequencing." Philosophical Transaction of the Royal Society B **370**(1675): 20140293.

Strandh, M. and L. Råberg (2015). "Within-host competition between *Borrelia afzelii ospC* strains in wild hosts as revealed by massively parallel amplicon sequencing." Phil. Trans. R. Soc. B **370**(1675): 20140293.

Susi, H., B. Barrès, P. F. Vale and A.-L. Laine (2015). "Co-infection alters population dynamics of infectious disease." Nature Communications **6**.

Swanson, K. I. and D. E. Norris (2008). "Presence of multiple variants of *Borrelia burgdorferi* in the natural reservoir *Peromyscus leucopus* throughout a transmission season." Vector-Borne and Zoonotic Diseases **8**(3): 397-406.

Tälleklint, L. and T. G. Jaenson (1994). "Transmission of *Borrelia burgdorferi* sl from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari: Ixodidae), in Sweden." Journal of Medical Entomology **31**(6): 880-886.

Tälleklint, L. and T. G. Jaenson (1995). "Is the small mammal (*Clethrionomys glareolus*) or the tick vector (*Ixodes ricinus*) the primary overwintering reservoir for the Lyme borreliosis spirochete in Sweden?" Journal of Wildlife Diseases **31**(4): 537-540.

Taragel'ová, V., J. Koči, K. Hanincová, K. Kurtenbach, M. Derdáková, N. H. Ogden, I. Literák, E. Kocianová and M. Labuda (2008). "Blackbirds and song thrushes constitute a key reservoir of *Borrelia garinii*, the causative agent of borreliosis in Central Europe." Applied and Environmental Microbiology **74**(4): 1289-1293.

Telford 3rd, S., T. N. Mather, S. I. Moore, M. L. Wilson and A. Spielman (1988). "Incompetence of deer as reservoirs of the Lyme disease spirochete." The American Journal of Tropical Medicine and Hygiene **39**(1): 105-109.

Theisen, M., M. Borre, M. J. Mathiesen, B. Mikkelsen, A.-M. Lebech and K. Hansen (1995). "Evolution of the *Borrelia burgdorferi* outer surface protein OspC." Journal of Bacteriology **177**(11): 3036-3044.

Theisen, M., M. Borre, M. J. Mathiesen, B. Mikkelsen, A. M. Lebech and K. Hansen (1995). "Evolution of the *Borrelia burgdorferi* outer surface protein OspC." Journal of Bacteriology **177**(11): 3036-3044.

Theisen, M., B. Frederiksen, A. Lebech, J. Vuust and K. Hansen (1993). "Polymorphism in *ospC* gene of *Borrelia burgdorferi* and immunoreactivity of OspC protein: implications for taxonomy and for use of OspC protein as a diagnostic antigen." Journal of Clinical Microbiology **31**(10): 2570-2576.

Tilly, K., J. G. Krum, A. Bestor, M. W. Jewett, D. Grimm, D. Bueschel, R. Byram, D. Dorward, M. J. VanRaden, P. Stewart and P. Rosa (2006). "Borrelia burgdorferi OspC protein required exclusively in a crucial early stage of mammalian infection." Infection and Immunity **74**(6): 3554-3564.

Tonetti, N., M. J. Voordouw, J. Durand, S. Monnier and L. Gern (2015). "Genetic variation in transmission success of the Lyme borreliosis pathogen *Borrelia afzelii*." Ticks and tick-borne diseases **6**(3): 334-343.

Tsao, J. I. (2009). "Reviewing molecular adaptations of Lyme borreliosis spirochetes in the context of reproductive fitness in natural transmission cycles." Veterinary Research **40**(2): 1.

van Duijvendijk, G., C. Coipan, A. Wagemakers, M. Fonville, J. Ersöz, A. Oei, G. Földvári, J. Hovius, W. Takken and H. Sprong (2016). "Larvae of *Ixodes ricinus* transmit *Borrelia afzelii* and *B. miyamotoi* to vertebrate hosts." Parasites & Vectors **9**(1): 1.

Van Valen, L. (1973). "A new evolutionary law." Evolutionary Theory **1**(1): 1-30.

Venezia, R. and R. Robertson (1975). "Bactericidal substance produced by *Haemophilus influenzae* b." Canadian journal of microbiology **21**(10): 1587-1594.

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

Voordouw, M. J., S. Lachish and M. C. Dolan (2015). "The Lyme disease pathogen has no effect on the survival of its rodent reservoir host." PLoS One **10**(2).

Voordouw, M. J., H. Tupper, Ö. Önder, G. Devevey, C. J. Graves, B. D. Kemps and D. Brisson (2013). "Reductions in human Lyme disease risk due to the effects of oral vaccination on tick-to-mouse and mouse-to-tick transmission." Vector-Borne and Zoonotic Diseases **13**(4): 203-214.

Wang, G., A. P. Dam and J. Dankert (1999). "Evidence for frequent OspC gene transfer between *Borrelia valaisiana* sp. nov. and other Lyme disease spirochetes." FEMS microbiology letters **177**(2): 289-296.

Wang, I. N., D. E. Dykhuizen, W. G. Qin, J. J. Dunn, E. M. Bosler and B. J. Luft (1999). "Genetic diversity of *ospC* in a local population of *Borrelia burgdorferi* sensu stricto." Genetics **151**(1): 15-30.

Wormser, G. P., D. Brisson, D. Liveris, K. Hanincova, S. Sandigursky, J. Nowakowski, R. B. Nadelman, S. Ludin and I. Schwartz (2008). "*Borrelia burgdorferi* genotype predicts the capacity for hematogenous dissemination during early Lyme disease." Journal of Infectious Diseases **198**(9): 1358-1364.

Zwetyenga, J., C. Rogier, A. Tall, D. Fontenille, G. Snounou, J.-F. Trape and O. Mercereau-Puijalon (1998). "No influence of age on infection complexity and allelic distribution in Plasmodium falciparum infections in Ndiop, a Senegalese village with seasonal, mesoendemic malaria." The American journal of tropical medicine and hygiene **59**(5): 726-735.

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13. Appendix

Table 1. Review of mixed infections found in questing ticks (Wang et al. 1999, Qiu et al. 2002, Girard et al. 2009, Pérez et al. 2011, Fedorova et al. 2014, Hamer et al. 2014, Durand et al. 2015). This table contains information on multiple-strain infections of *Borrelia* pathogens in questing *Ixodes* ticks. The columns include the name of the first author of the article and the year of publication (Author), the location of the study (Location), the *Ixodes* tick species and *Borrelia* species (*Ixo*/ *Bor*), the tick stage (Tick stage), the total number of infected ticks (Total N), the mean number of strains per infected tick (Mean strain richness = Mean R), the total number of strains detected (Total strain richness = Total R), the percentage of mixed infections (% Mixed), the method used to analyze the samples (Method), and the maximum number of *ospC* strains found in one sample (Maximum strain richness = Max R).

Author	Location	*Ixo*[a]/ *Bor*[b]	Tick stage	Total N	Mean R	Total R	% Mixed	Method	Max R
Durand 2015	Bois de l'Hôpital, Switzerland	ric/afz	Nymph	193	2.37	18	78.76%	NGS on isolates DNA	7
Durand 2015	Bois de l'Hôpital, Switzerland	ric/gar	Nymph	190	2.96	19	84.74%	NGS on isolates DNA	11
Fedorova 2014	Alameda County, 71 sites	pac/ss	Nymph	144	NA	6	5.56%	PCR on isolates or tick DNA	

Girard 2009	Mendocino County, CA, 78 sites, USA	pac/ss	Nymph	227	1.07	11	NA	PCR on tick DNA	
Pérez 2011	Bois de l'Hôpital, Switzerland	ric/afz	Nymph	81	1.02	7	2.47%	SSCP on isolates DNA	2
Pérez 2011	Staatswald, Switzerland	ric/afz	Nymph	51	1	8	0	SSCP on isolates DNA	
Qiu 2002	Long Island, New York	sca/ss	Nymph	25	1.4	10	NA	PCR on tick DNA	
Hamer 2014	Midwest, 13 sites	sca/ss	Adult	87	NA	18	44.83%	PCR on tick DNA	
Fedorova 2014	Alameda County, 71 sites	pac/ss	Adult	23	NA	6	4.34%	PCR on isolates or tick DNA	
Qiu 2002	Long Island + East Coast, USA	sca/ss	Adult	178	2.8	15	NA	RLB on tick DNA	8
Wang 1999	Shelter Island, USA	sca/ss	Adult	40	1.9	13	50.00%	SSCP on tick DNA	4

14.
15. [a]: pac = *Ixodes pacificus*, ric = *Ixodes ricinus*, sca = *Ixodes scapularis*
16. [b]: afz = *Borrelia afzelii*, gar = *Borrelia garinii*, ss = *Borrelia burgdorferi* sensu stricto

Table 2. Review of mixed infections found in ticks sampled from vertebrate hosts (Brisson and Dykhuizen 2004, Ogden et al. 2008, Mathers et al. 2011, Ogden et al. 2011, Pérez et al. 2011). This table contains information on multiple-strain infections of *Borrelia* pathogens in *Ixodes* ticks sampled from naturally infected vertebrate reservoir hosts. The columns include the name of the first author of the article and the year of publication (Author), the location of the study (Location), the *Ixodes* tick species, the *Borrelia* species and the host species or genus (*Ixo* / *Bor* / host), the tick stage (Tick stage), the total number of infected hosts (Total N), the mean number of strains per infected host (Mean strain richness = Mean R), the total number of strains detected (Total strain richness = Total R), the percentage of mixed infections (% Mixed), the method used to analyze the samples (Method), and the maximum number of *ospC* strains found in one sample (Maximum strain richness = Max R).

Author	Location	*Ixo* [a]/ *Bor* [b]/ host	Tick stage	Total N	Mean R	Total R	% Mixed	Method	Max R
Brisson 2004	Millbrook, New York, USA	sca/ss/*P. leucopus*	Larva	75	3.19	12	NA	RLB on tick DNA	
Brisson 2004	Millbrook, New York, USA	sca/ss/*T. striatus*	Larva	75	3.65	14	NA	RLB on tick DNA	
Brisson 2004	Millbrook, New York, USA	sca/ss/*B. brevicauda*	Larva	75	2.73	11	NA	RLB on tick DNA	
Brisson 2004	Millbrook, New York, USA	sca/ss/*S. carolinensis*	Larva	50	1.75	8	NA	RLB on tick DNA	

Mathers 2011	Appledore Island, Maine, USA	sca/ss/birds	Nymph	24	NA	8	37.50%	PCR on isolates DNA	
Ogden 2008	Long Point, Ontario, Canada	sca/ss/birds	Nymph	17	1.76	8	58.90%	RLB + PCR on direct DNA	4*
Ogden 2008	Prince Edward Point, Ontario, Canada	sca/ss/birds	Nymph	4	1.75	6	25.00%	RLB + PCR on direct DNA	4*
Ogden 2008	Atlantic, Nova Scotia, Canada	sca/ss/birds	Nymph	1	1	1	0	RLB + PCR on direct DNA	4*
Ogden 2011	Canada	sca/ss/humans + dogs + cats	Adults	309	1.59	17	34.90%	RLB on tick DNA	7
Pérez 2011	Bois de l’Hôpital, Switzerland	ric/afz/ rodents	Nymph + larva	47	1.02	4	2.12%	SSCP on isolates DNA	2
Pérez 2011	Staatswald, Switzerland	ric/afz/ rodents	Nymph + larva	79	1.14	7	15.19%	SSCP on isolates DNA	2
Pérez 2011	Bois de l’Hôpital, Switzerland	ric/afz/ rodents	Larva [c]	42	1	5	0	SSCP on isolates DNA	
Pérez 2011	Staatswald, Switzerland	ric/afz/ rodents	Larva [c]	95	1.04	5	2.10%	SSCP on isolates DNA	2

17.

18. [a]: pac = *Ixodes pacificus*, ric = *Ixodes ricinus*, sca = *Ixodes scapularis*

19. [b]: afz = *Borrelia afzelii*, gar = *Borrelia garinii*, ss = *Borrelia burgdorferi* sensu stricto
20. [c]: Xenodiagnostic larvae from a pathogen-free tick colony.
21. *: This value corresponds to the maximum number of strains found in one tick for the whole study, not a particular site.

Table 3. Review of mixed infections found in biopsies from vertebrate hosts (Anderson and Norris 2006, Hellgren et al. 2011, Andersson et al. 2013, Fedorova et al. 2014, Jacquot et al. 2014, Strandh and Råberg 2015). This table contains information on multiple-strain infections of *Borrelia* pathogens in naturally infected vertebrate reservoir hosts. The columns include the name of the first author of the article and the year of publication (Author), the location of the study (Location), the vertebrate host species and *Borrelia* species (Host/ *Bor*), the total number of infected hosts (Total N), the mean number of strains per infected host (Mean strain richness = Mean R), the total number of strains detected (Total strain richness = Total R), the percentage of mixed infections (%Mixed), the method used to analyze the samples (Method), and the maximum number of *ospC* strains found in one sample (Maximum strain richness = Max R).

Author	Location	Host/ *Bor* [a]	Total N	Mean R	Total R	% Mixed	Method	Max R
Anderson 2006	Anne Arundel county, Southern Maryland, USA	*Peromyscus leucopus*/ss	14	1.14	5	24.00%*	SSCP + TOPO TA cloning on biopsy DNA	3*
Anderson 2006	Charles county Southern Maryland, USA	*Peromyscus leucopus*/ss	44	1.11	8	24.00%*	SSCP + TOPO TA cloning on biopsy DNA	3*
Anderson 2006	Calvert county, Southern Maryland, USA	*Peromyscus leucopus*/ss	7	1.43	4	24.00%*	SSCP + TOPO TA cloning on biopsy DNA	3*

Anderson 2006	Prince George’s county, Southern Maryland, USA	*Peromyscus leucopus*/ss	30	1.4	6	24.00%*	SSCP + TOPO TA cloning on biopsy DNA	3*
Anderson 2006	Saint Mary’s county, Southern Maryland, USA	*Peromyscus leucopus*/ss	32	1.31	7	24.00%*	SSCP + TOPO TA cloning on biopsy DNA	3*
Andersson 2013	Revingehed, Sweden	*Myodes glareolus*/ afz	101	2.8	7	54.30%	PCR on biopsy DNA using 7 different ospC primers	6
Strandh 2015	Revingehed, Sweden	*Myodes glareolus*/ afz	50	2.47	7	NA	454 sequencing on biopsy DNA	6
Strandh 2015	Revingehed, Sweden	*Apodemus flavicollis*/ afz	50	1.87	7	NA	454 sequencing on biopsy DNA	4
Strandh 2015	Revingehed, Sweden	*Sorex araneus*/ afz	24	2.98	7	NA	454 sequencing on biopsy DNA	4
Fedorova 2014	Alameda County, 71 sites, USA	*Sciurus niger*/afz	17	NA	5	5.88%	PCR on isolates or biopsy DNA	
Hellgren 2011	Revingehed, Sweden	Rodents/afz	221	NA	9	60.63%	PCR on biopsy DNA	
Hellgren 2011	Revingehed, Sweden	Shrews/afz	15	NA	9	40.00%	PCR on biopsy DNA	

Hellgren 2011	Lister, Sweden	Rodents/afz	59	NA	9	54.24%	PCR on biopsy DNA	
Hellgren 2011	Lister, Sweden	Shrews/afz	0	NA	0	0	PCR on biopsy DNA	
Jacquot 2014	Forest of Sénart, France	*Tamias sibiricus barberi*/afz	94	NA	NA	38.64%	NGS on biopsy DNA	
Jacquot 2014	Forest of Sénart, France	*Myodes glareolus*/ afz	90	NA	NA	31.33%	NGS on biopsy DNA	

22. [a]: afz = *Borrelia afzelii*, gar = *Borrelia garinii*, ss = *Borrelia burgdorferi* sensu stricto
23. *: This value corresponds to the total for the whole study, not a particular site.
24.

Table 4: Genetic distances between and within the different major *ospC* groups of *B. afzelii* found in our local population of questing *I. ricinus* nymphs. Genetic distances were calculated using the distmat program (Rice et al. 2000) with a Kimura model. Each mean pairwise genetic distance was based on 30 sequences of each major *ospC* group in the pair. The mean pairwise genetic distance within each major *ospC* group was based on 30 sequences belonging to that group.

	A1	**A2**	**A3**	**A5**	**A7**	**A9**	**A10**	**A11**	**A12**	**A14**
A1	0.72									
A2	15.83	2.16								
A3	14.33	14.84	0.42							
A5	9.92	16.09	15.85	0.89						
A7	17.08	16.24	17.09	18.5	0.94					
A9	18.12	17.4	16.2	18.07	14.24	1.74				
A10	20.02	16.29	20.68	19.92	19.43	21.69	1.04			
A11	21.27	19.17	20.48	21.15	19.32	21.49	15.55	1.08		
A12	20.03	18.53	8.87	18.09	22.09	21.53	23.59	22.31	0.85	
A14	18.32	15.29	17.96	16.99	13.04	11.91	19.91	16.8	21.28	0.8

Table 5: Genetic distances between and within the different major *ospC* groups of *B. garinii* found in our local population of questing *I. ricinus* nymphs. Genetic distances were calculated using the distmat program with a Kimura model. Each mean pairwise genetic distance was based on 30 sequences of each major *ospC* group in the pair. The mean pairwise genetic distance within each major *ospC* group was based on 30 sequences belonging to that group.

	G2	**G4**	**G6**	**G7**	**G8**	**G9**	**G10**	**G12**	**G13**	**G14**	**G15**
G2	0.67										
G4	20.49	0.94									
G6	20.2	24.64	0.96								
G7	18.64	22.36	14.94	1.32							
G8	20.83	22.23	19.21	17.05	1.2						
G9	19.4	24.76	18.24	15.95	13.26	1.63					
G10	19.95	21.26	14.99	15.49	16.36	15.2	0.88				
G12	15.36	23.8	17.57	19.63	19.82	20.64	18.96	1.23			
G13	19.39	23.92	18.41	17.78	18.13	17.75	17.87	18.45	1.62		
G14	14.46	22.58	15.38	17.3	18.42	16.76	14.3	14.66	19.03	1.48	
G15	21.02	26.36	16.73	17.76	16.08	18.29	12.77	18.39	18.72	17.99	0.66

Table 6: Allospecific genetic distances between the different major *ospC* groups of *B. garinii* and *B. afzelii* found in our local population of questing *I. ricinus* nymphs. Genetic distances were calculated using the distmat program with a Kimura model. Each mean pairwise genetic distance was based on 30 sequences of each major *ospC* group in the pair.

	A1	**A2**	**A3**	**A5**	**A7**	**A9**	**A10**	**A11**	**A12**	**A14**
G2	22.56	22.18	20.23	22.97	22.14	20.56	14.94	15.02	23.62	18.54
G4	22.14	19.35	17.09	22.58	21.4	19.2	23.68	21.5	20.92	20.18
G6	23.97	19.17	19.99	24.14	20.44	23.22	18.74	19.42	25	20.19
G7	21.97	18.66	20.63	23.55	21.52	21.92	18.28	19.39	23.39	20.1
G8	24.97	20.08	19.8	25.93	25	22.21	21.01	18.01	23.62	20.69
G9	23.87	20.89	18.8	24.21	22.9	21.49	18.97	18.82	23.83	20.28
G10	23.26	19.08	20.04	24.65	20.5	21.83	18.24	18.78	24.38	21.56
G12	22.65	19.16	21.91	19.76	21.74	25.53	10.71	15.4	23.94	19.8
G13	20.51	17.86	17.96	20.92	20.39	22.89	18.41	20.65	24.1	20.43
G14	22.47	20.53	19.95	23.24	20.64	23.63	15.38	15.02	24.35	21.4
G15	21.34	19.38	19.13	22.31	22.78	22.42	18.27	19.76	23.37	21.1