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Effects of acquired immunity on co-feeding and systemic transmission of the Lyme disease bacterium, *Borrelia afzelii*.

Thesis presented to the Faculty of Sciences of the University of Neuchâtel for the degree of Doctor of Sciences by

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"Effects of acquired immunity on co-feeding and systemic transmission of the Lyme disease bacterium, *Borrelia afzelii*."

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« The important thing is not to stop questioning. Curiosity has its own reason for existing. »

Albert Einstein

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Résumé

La borréliose de Lyme est une zoonose transmise par les tiques. Les bactéries qui en sont la cause, des spirochètes, se trouvent principalement chez des hôtes réservoirs sauvages tels que des rongeurs ou des petits oiseaux terrricoles. *Borrelia afzelii* est l'un des pathogènes responsables de la borréliose de Lyme les plus importants en Europe et est transmis par la tique du mouton *Ixodes ricinus*. Pendant le repas sanguin de la tique, le pathogène peut infecter l'hôte. La bactérie change l'expression des protéines de surfaces externes (Osp) pendant l'infection pour pouvoir s'adapter efficacement à l'hôte ou à la tique. L'une de ces protéines, OspC, joue un rôle important dans le développement de l'infection chez l'hôte.

Pendant le développement de l'infection systémique dans l'hôte vertébré, ce dernier va développer une immunité acquise (ex : anticorps) contre diverses protéines des spirochètes incluant OspC. Une des stratégies développée par le pathogène pour contourner les anticorps anti-OspC d'un hôte déjà infecté par une autre souche de *Borrelia* est de porter un allèle différent pour *ospC*. Nous pouvons ainsi distinguer des souches de *B. afzelii* en fonction de l'*ospC* dont elles disposent.

Borrelia afzelii dispose de deux modes de transmission: la transmission systémique (de l'hôte à la tique) et la transmission par co-feeding (de la tique à la tique). La transmission systémique requiert le développement d'une infection très répandue, multi-systémique, dans l'hôte vertébré dans le but de faciliter la transmission à de nouvelles tiques. Pendant la transmission par co-feeding, l'hôte crée simplement un pont qui va amener la tique infectée et la tique non-infectée à proximité l'une de l'autre. La transmission par co-feeding peut ainsi être une stratégie pour les spirochètes d'éviter le système immunitaire acquis et d'infecter de nouvelles tiques naïves.

Pour tester cette hypothèse, nous avons immunisé des souris de laboratoire avec l'une des deux protéines recombinantes OspC (rOspC) qui correspondent à deux souches différentes de *B. afzelii* : A3 et A10. Les anticorps contre un antigène OspC particulier bloquent la souche ciblée (homologue) mais pas la souche non-ciblée (hétérologue). L'immunisation réduit aussi drastiquement l'efficacité de la transmission par co-feeding. Chez les souris non-immunisées (contrôles) et les homologues, la transmission par co-feeding a atteint une prévalence de 51,6 % tandis que pour les souris homologues, ce taux n'a atteint que 3.3 %, correspondant à une baisse de 15,6 fois.

Nous avons recherché les effets de l'immunité acquise croisée en comparant les phénotypes d'infection entre les hétérologues et les souris contrôles. Les souris hétérologues ont une première expérience avec le 'mauvais' antigène OspC, et ces souris sont donc suspectées de répondre plus efficacement à l'infection avec *B. afzelii* que les souris contrôles naïves. Nous avons trouvé que l'immunité croisée avait un effet sur la charge en spirochètes des nymphes qui se sont nourries à l'état de larve sur les souris 1 mois après l'infection. La charge en spirochète moyenne dans les nymphes était 2,1 fois plus haute dans le groupe contrôle que dans le groupe hétérologue.

Il y avait aussi un grand effet du mode de transmission sur la charge en spirochètes des nymphes. La charge en spirochètes était en moyenne 4 fois plus basse dans les tiques de co-feeding que dans les tiques infectées par transmission systémique. Finalement, nous avons vu un effet important du vieillissement de la tique sur la charge en spirochètes des nymphes. Les nymphes qui ont été tuées 4 mois après la mue de la larve à la nymphe avaient une charge en spirochètes qui étaient 6,3 à 15,3 fois plus basse que les nymphes qui ont été tuées 3 mois plus tôt. Cette baisse de la charge en spirochètes à mesure que l'infection vieillit dans la nymphe pourrait avoir un effet sur la fitness de *B. afzelii*.

Cette thèse de doctorat a démontré que la transmission par co-feeding ne permet pas à *B. afzelii* d'échapper aux anticorps spécifiques anti-OspC et que la réponse immunitaire de l'hôte avait un effet négatif sur la fitness de *Borrelia*. Ce travail fournit de nouvelles informations sur les mécanismes de la transmission par co-feeding, sur les interactions hôte-parasite d'un pathogène responsable de la maladie de Lyme, et sur les effets de l'immunité acquise d'un hôte vertébré sur la transmission du pathogène.

Mots clés : *Borrelia afzelii* ; co-feeding ; immunité acquise ; transmission systémique ; OspC ; *Ixodes ricinus*.

Abstract

Lyme borreliosis is a tick-borne zoonotic disease and the causative spirochete bacteria are predominantly found in wildlife reservoirs such as rodents and ground-dwelling birds. *Borrelia afzelii* is one of the most common Lyme borreliosis pathogens in Europe, and is transmitted by the sheep tick *Ixodes ricinus*. During the tick blood meal, the pathogen can infect the host. The bacteria change the expression of their outer surface proteins (Osp) during the infection to adapt efficiently to the vertebrate host or the tick vector. One of these proteins, OspC, plays an important role in the development of host infection.

The systemically infected vertebrate host develops an acquired immune response (e.g. antibodies) against various spirochete proteins including OspC. One of the strategies developed by the pathogen to avoid the OspC-specific antibodies of a host already infected by a given *Borrelia* strain is to carry a different *ospC* allele. The *ospC* gene is therefore a useful genetic marker for classifying *B. afzelii* into different strains.

Borrelia afzelii has two modes of transmission: systemic (host-to-tick) transmission and co-feeding (tick-to-tick) transmission. Systemic transmission requires the development of a widespread, multi-system infection in the vertebrate host to facilitate transmission to new vector ticks. During co-feeding transmission, the host merely forms the bridge that brings the infected and uninfected ticks in close proximity to each other. Co-feeding transmission could therefore be a strategy for the spirochete to avoid the host immune system and to infect new naïve ticks.

To test this hypothesis, we immunized lab mice with one of two recombinant OspC (rOspC) proteins that belonged to two different strains of *B. afzelii*: A3 and A10. Antibodies against a particular OspC antigen blocked infection of the targeted (homologous) strain but not the non-targeted (heterologous) strain. Immunization also drastically reduced the efficacy

of co-feeding transmission. In non-immunized or heterologous mice, the co-feeding transmission rate was 51.6 % whereas in homologous mice, this rate was 3.3%, corresponding to a 15.6-fold decrease.

We investigated the effects of cross-reactive acquired immunity by comparing the infection phenotypes between heterologous and control mice. The heterologous mice had previous experience with the heterologous OspC antigen, and we therefore predicted that these mice would respond more efficiently to infection with *B. afzelii* than the completely naive control mice. We found that cross-immunity had an effect on the spirochete load in the nymphal ticks that had fed on the mice one month post-infection. The mean nymphal spirochete load was 2.1 times higher in the control group than in the heterologous group.

There was also a large effect of the mode of transmission on the nymphal spirochete load. The spirochete load was on average 6 times lower in co-feeding ticks than in ticks infected via systemic transmission. Finally, there was an important effect of nymphal ageing on the spirochete load inside the nymph. Nymphs that were examined four months after the larva-to-nymph molt had a spirochete load that was 6.3 to 15.3 times lower than nymphs that were examined 3 months earlier. This decrease in spirochete load as the infection ages inside the nymphal tick could have an effect on the fitness of *B. afzelii*.

This PhD thesis demonstrated that co-feeding transmission does not allow *B. afzelii* to escape the OspC-specific antibodies and that host acquired immunity had a negative effect on the fitness of *Borrelia* spirochetes. This work provides new information on the mechanism of co-feeding transmission, on the host-parasite interactions of an important Lyme disease pathogen, and on the effect of acquired immunity in the vertebrate host on pathogen transmission.

Keywords: *Borrelia afzelii* ; co-feeding ; acquired immunity ; systemic transmission ; OspC ; *Ixodes ricinus*.

1. Introduction

1.1. General background:

1.1.1. History of Lyme disease:

Lyme borreliosis or Lyme disease is the most common vector-borne disease in the Northern hemisphere. This tick-borne disease was discovered by the physician Allen C. Steere in 1972 in the city of Old Lyme (hence the name Lyme disease), in Connecticut, USA (Steere et al., 1977). A number of children and adults had been diagnosed with arthritis and a link was made with the clinical symptoms that follow a tick bite. In 1982, William Burgdorfer isolated the first spirochetes from the deer tick *Ixodes scapularis* (Burgdorfer et al., 1982). In 1984, after the isolation of spirochetes from the blood of patients (Benach and Coleman, 1987) and the discovery of spirochetes in the sheep tick *Ixodes ricinus* in Europe (Burgdorfer and Keirans, 1983), the spirochete was named *Borrelia burgdorferi* (Hyde and Johnson, 1984) in honor of its discoverer, William Burgdorfer.

1.1.2. The tick-borne pathogen *Borrelia burgdorferi* sensu lato:

Borrelia burgdorferi sensu lato (s. l.) forms a genospecies complex that includes 12 described genospecies. In Europe, the three main genospecies that cause Lyme disease are *B. burgdorferi* sensu stricto (the only species that infects humans in North America), *B. afzelii* and *B. garinii* (Baranton et al., 1992; Wang et al., 1999a; Rudenko et al., 2011). The different genospecies of the *Borrelia burgdorferi* s. l. genospecies complex are specialized on different vertebrate hosts (Kurtenbach et al., 2002). For example, *Borrelia afzelii* and *Borrelia garinii* are specialized on rodents and birds, respectively (Kurtenbach et al., 1998b; Humair et al., 1999; Kurtenbach et al., 2002; Hanincova et al., 2003a; Hanincova et al., 2003b). This host specificity appears to be mediated by the tolerance of the pathogen to the complement system of the vertebrate host (Kurtenbach et al., 1998a). Some vertebrate hosts such as roe deer and

sheep appear to be completely resistant to infection with *B. burgdorferi* s. l. pathogens (Jaenson and Talleklint, 1992; Ogden et al., 1997).

1.1.3. Diagnosis and treatment of Lyme disease in humans

The diversity of *B. burgdorferi* s. l. genospecies that cause Lyme disease in humans leads to a variety of clinical manifestations (van Dam et al., 1993). Some of the typical manifestations of the early stage of the infection are the erythema migrans skin lesion, Lyme neuroborreliosis, and Lyme carditis (Stanek et al., 2012). The late stage of the disease includes symptoms such as arthritis, skin disorders, and neurological problems (Stanek et al., 2012). One of the difficulties with diagnosing Lyme disease is the number of clinical manifestations that can be confused with other diseases, leading sometimes to the wrong diagnosis. Detection of pathogen exposure depends on serological tests such as the enzyme linked immuno-sorbent assay (ELISA) or by western blot (Stanek et al., 2012). In the laboratory, ELISA, polymerase chain reaction (PCR), and quantitative PCR (qPCR) can be used to detect *B. burgdorferi* s. l. in vertebrate hosts or ticks. If the disease is diagnosed early, a three-week antibiotic treatment (e.g. doxycycline) usually cures the infection (Stanek et al., 2012).

1.1.4. The life cycle of *Ixodes* ticks:

Borrelia burgdorferi s. l. pathogens are transmitted by ticks of the *Ixodes* species complex, which include *I. ricinus* in Europe, *I. persulcatus* in Eurasia, *I. scapularis* in eastern North America, and *I. pacificus* in western North America (Steere and Malawista, 1979; Piesman and Gern, 2004; Gern, 2009; Mannelli et al., 2011). The sheep tick *Ixodes ricinus* belongs to the class Arachnida (spiders, scorpions, ticks, and mites), the order Ixodida, the family of *Ixodidae* (hard ticks), and finally the genus of *Ixodes*.

The life cycle of *Ixodes* ticks has four stages: the egg, the larva, the nymph and the adult. The larvae, nymphs, and adult female ticks require a single blood meal from a vertebrate host to develop into the next stage (Wall and Shearer, 1997). *Ixodes* ticks engage in questing behavior to find a host, which consists of climbing up the vegetation (e.g., grass) and waiting for a passing host (Gigon, 1985). While questing, the tick's front-legs are stretched out to grab fur, feathers, or clothes depending on the type of host. Ticks are very sensitive to dehydration and must frequently return to the humid leaf litter in order to rehydrate (Gigon, 1985). Ticks are ectotherms and are therefore also sensitive to changes in temperature. For this reason, tick questing activity peaks in spring and late summer when the temperature and the relative humidity are suitable (Figure 1) (Kurtenbach et al., 2006).

After grabbing a host, the tick searches for an appropriate place to attach and take a blood meal. Ticks are often aggregated on the heads of rodents or birds, as this area is more difficult to groom (Randolph, 1975; Craine et al., 1995; Schmidt et al., 1999). This aggregation behavior can facilitate co-feeding transmission of tick-borne pathogens (see point 1.1.6.). The blood meal lasts around 3 days for larvae, 5 days for nymphs and 7 days for adult females (Gigon, 1985). After obtaining a full blood meal, the ticks drop off their host and reside on or near the soil surface, where they need a minimum relative humidity of 80% to survive (Gigon, 1985). Depending on the stage and the environmental conditions, development into the next stage can take from 4 weeks to several months. Adult female ticks die after laying a single clutch of eggs that contains as many as 2,000 offspring. The tick life cycle can last between 2 to 6 years depending on the environmental conditions (Gigon, 1985).

The life stages prefer different species of vertebrate host. The larvae are mainly found on small rodents and ground-dwelling birds but can also feed on larger mammals. Nymphs are also found on small mammals and birds but prefer to feed on larger mammals such as roe deer (Matuschka et al., 1991). Finally, adult ticks are found only on large mammals.

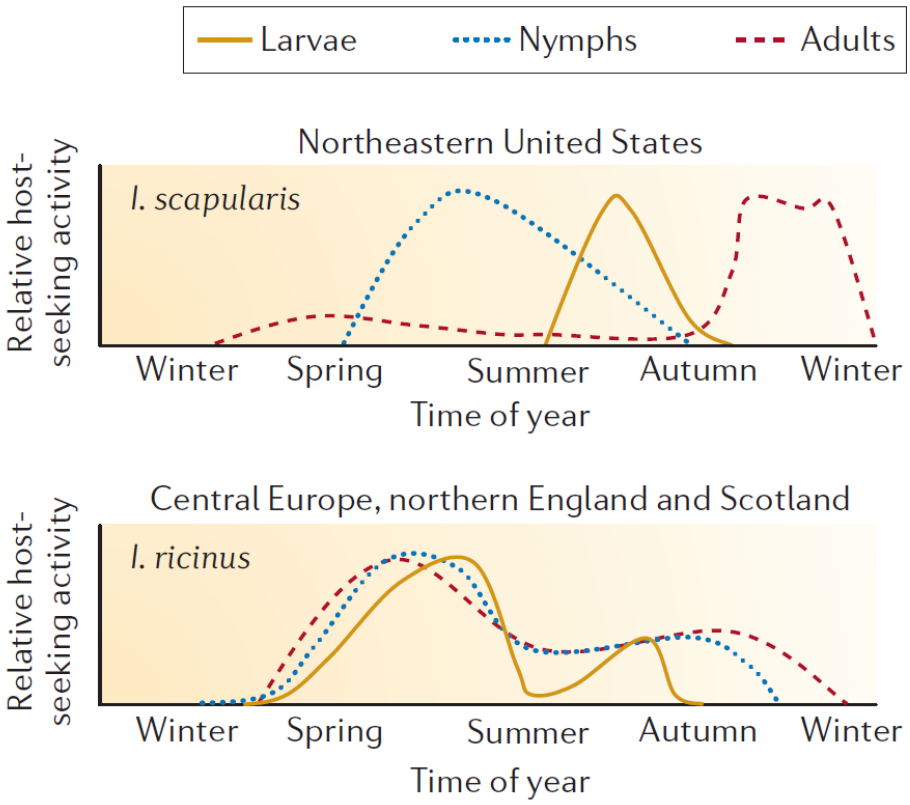

Figure 1: The relative host-seeking activity depends on the season for the three stages (larva, nymph, and adult) of the two main tick vectors of *B. burgdorferi* sensu lato: *I. scapularis* in the northeastern United States and *I. ricinus* in Central Europe, northern England and Scotland (Kurtenbach et al., 2006).

1.1.5. The life cycle of Lyme disease pathogens:

Lyme borreliosis is a zoonotic tick-borne disease that is maintained in nature by transmission cycles involving immature larval ticks and competent vertebrate reservoir hosts (Barbour and Hayes, 1986). Larval ticks acquire the spirochetes after taking a blood meal from an infected vertebrate host. The blood-engorged larva molts into a nymph and maintains the infection via transstadial transmission (Nadelman and Wormser, 1998). The following year the infected nymph can transmit the pathogen to a competent reservoir host during its next blood meal. Adult ticks can acquire *B. burgdorferi* s. l. pathogens during the larval or nymphal blood meal (Barbour and Hayes, 1986; Nadelman and Wormser, 1998). Adult ticks

do not make a direct contribution to the maintenance of *B. burgdorferi* s. l. pathogens because they mainly feed on incompetent vertebrate hosts such as deer (Figure 2) (Telford et al., 1988; Jaenson and Talleklint, 1992). However, adult ticks can transmit Lyme disease pathogens to humans. There is no transovarial transmission of *B. burgdorferi* s. l. pathogens from infected female adult ticks to their offspring (Rollend et al., 2013).

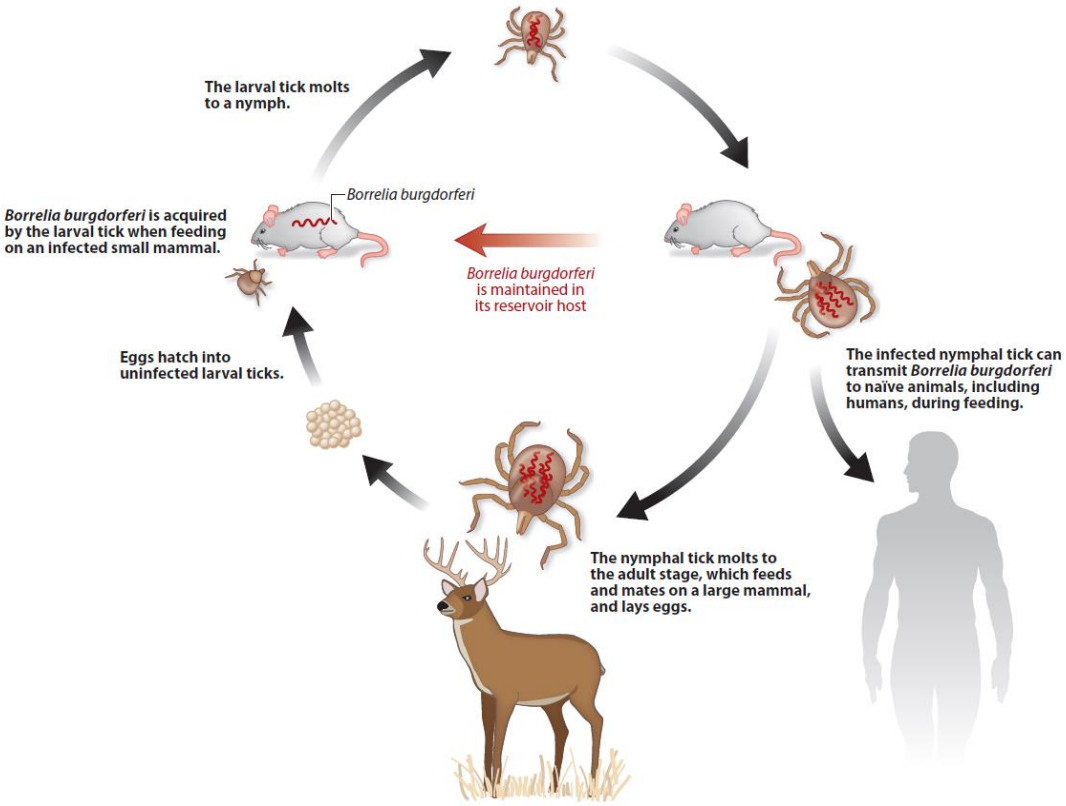

Figure 2: Enzootic cycle of *Borrelia burgdorferi* sensu lato (Brisson et al., 2012).

1.1.6. Systemic and co-feeding transmission:

Many tick-borne pathogens have two distinct modes of transmission: systemic transmission and co-feeding transmission. Systemic transmission requires the development of a systemic infection inside the vertebrate competent host. After the bite of an infected tick, the host (e.g. a rodent) develops a systemic infection within two to three weeks (Donahue et al.,

1987). The host is now infectious and can transmit the spirochete to feeding ticks. The problem with systemic transmission is that there is a temporal delay while the infection becomes patent inside the vertebrate host during which transmission is not possible.

This temporal delay is avoided by co-feeding transmission, which has been reported in a number of tick-borne pathogens including *Borrelia afzelii* (Gern and Rais, 1996). Co-feeding transmission occurs when infected and uninfected ticks feed close to each other in space and time. The spirochete will then use the host as a bridge to go from an infected tick to an uninfected tick (Randolph et al., 1996; Randolph, 2011; Voordouw, 2015).

This alternative mode of pathogen transmission was first discovered in tick-borne viruses including Thogoto virus (Jones et al., 1987), tick-borne encephalitis virus (TBEV) (Labuda et al., 1993), and Bunyavirus (Labuda et al., 1997a). Gern and Rais (1996) were the first to show that co-feeding transmission occurs among *Borrelia* pathogens. Effective co-feeding transmission requires a particular set of ecological conditions. One very important condition is that nymphal ticks and larval ticks search for hosts at the same time of the year (Randolph et al., 1999). In Europe, larval and nymphal host searching behavior is more synchronized whereas in North America, the peak questing activity of the two immature tick stages is separated by several months (Figure 1) (Kurtenbach et al., 2006). The difference in tick phenology between Europe and North America may be why co-feeding transmission is more important for European than North American *Borrelia* genospecies (Voordouw, 2015).

Theoretical models have shown that co-feeding transmission makes a modest contribution to the fitness of *Borrelia* pathogens (Hartemink et al., 2008; Harrison et al., 2011; Harrison and Bennett, 2012). Recent work on *B. afzelii* has shown that there are differences in the efficacy of co-feeding transmission between strains (Tonetti et al., 2015). Some strains of *B. afzelii* are efficiently transmitted by co-feeding and systemic transmission whereas other

strains do not have co-feeding transmission (Tonetti et al., 2015). A recent study by Durand et al. (2015) found a correlation between the prevalence of *B. afzelii* in wild ticks and the ability of the spirochetes to use the two types of transmission. The strains that were most common in wild ticks had the highest levels of co-feeding and systemic transmission in laboratory mice (Durand et al., 2015; Tonetti et al., 2015). This result suggests that co-feeding transmission is associated with an infection phenotype that has high fitness and reaches high frequency in nature.

1.1.7. Cofeeding transmission and acquired immunity:

Co-feeding transmission could be a strategy for vector-borne pathogens to escape the negative effects of the host immune system. Labuda et al. (1997b) showed that antibodies developed against TBEV viremia did not prevent co-feeding transmission of this pathogen. Previously exposed mice have life-long immunity against TBEV suggesting that co-feeding transmission is one method by which tick-borne pathogens avoid the adaptive immune response of resistant hosts (Labuda et al., 1997b). Recent work on *B. afzelii* has found that there is substantial variation in the efficacy of co-feeding transmission among strains (strains were defined by their genotype at the *ospC* locus) (Tonetti et al., 2015). Wild rodents are often infected with multiple *ospC* strains indicating that mice are repeatedly exposed to infected ticks (Pérez et al., 2011; Andersson et al., 2013). This scenario suggests that *ospC* strains in questing nymphs commonly encounter mice that have developed protective OspC-specific antibodies because of an earlier exposure to that same strain. Under these circumstances (i.e. frequent re-exposure to the same *ospC* strain), we expect *Borrelia* pathogens to be under strong selection to evade the acquired immune response in the vertebrate host. Co-feeding transmission may be a strategy that allows *Borrelia* pathogens to

escape sterilizing antibodies in the vertebrate host. However, whether *Borrelia* pathogens are capable of co-feeding transmission on immunized hosts is currently unknown.

1.1.8. The role of the OspC protein:

Pal et al. (2004a) and Fingerle et al. (2007) found that *Borrelia* mutants lacking the *ospC* gene were unable to migrate from the tick midgut to the tick salivary glands. In contrast, the research by the group of Patricia Rosa showed that mutants lacking the *ospC* gene were able to invade the tick salivary glands but were not able to establish infection in the rodent host (Tilly et al., 2006; Tilly et al., 2009). There is also controversy regarding the identity of the ligand of the OspC protein. The study by Ramamoorthi et al. (2005) suggests that the OspC antigen binds the tick salivary gland protein Salp15, which allows the spirochete to evade the host immune system. Another study found that the OspC antigen binds the plasminogen protein of the vertebrate host, which allows the spirochete to degrade the extracellular matrix in the connective tissue and disseminate from the site of the tick bite to the other tissues of the vertebrate host (Onder et al., 2012). In summary, there is a controversy about when the OspC protein is important (within the tick or within the vertebrate host) and what ligand it binds (Radolf and Caimano, 2008; Earnhart et al., 2010). What is certain is that the OspC antigen plays a critical role in the tick-to-host transmission of *Borrelia* spirochetes.

After establishing a systemic infection in the host, *Borrelia* pathogens down-regulate expression of the *ospC* gene. The OspC-specific IgG antibody response therefore does not allow the mouse to clear the *Borrelia* infection. In contrast, this mouse is still susceptible to being infected by a different *ospC* strain (Probert et al. 1997). Probert et al. (1997), using a rOspC immunization trial and three different strains of *B. burgdorferi* s. s., were the first to show that antibodies developed against a particular *ospC* strain do not provide cross-protection against strains carrying a different *ospC* allele.

1.1.9. Multiple strain infections in ticks and hosts:

An interesting aspect of the biology of *B. burgdorferi* s. l. is the high diversity of strains within each genospecies. Each of the three known Lyme disease pathogens (*B. burgdorferi* s. s., *B. afzelii*, and *B. garinii*) consists of multiple strains that are often differentiated by the single-copy, highly polymorphic *ospC* gene (Wilske et al., 1986; Theisen et al., 1993; Wilske et al., 1993; Wang et al., 1999a; Qiu et al., 2002; Brisson and Dykhuizen, 2004; Earnhart and Marconi, 2007c; Pérez et al., 2011; Andersson et al., 2013; Durand et al., 2015; Strandh and Raberg, 2015). OspC induces a strong antibody response in the vertebrate host (Dressler et al., 1993; Fung et al., 1994; Engstrom et al., 1995).

Selection by the immune system of the vertebrate host on the OspC protein explains why this antigen is so diverse. The spirochete expresses the OspC protein during the tick-to-host lifecycle transition. During tick-to-host infection, the vertebrate host is exposed to and develops antibodies against the OspC protein. This antibody response takes time to develop and does not allow the host to clear the first infection. However, the OspC-specific antibodies do protect the host from reinfection with *Borrelia* strains carrying the same *ospC* gene (Gilmore et al., 1996). In the case where the secondary strain carries a different *ospC* gene from the primary strain, the antibodies are not protective and multiple infection is possible.

In the wild, multiple infections are common in both rodents and ticks (Wang et al., 1999b; Brisson and Dykhuizen, 2004; Swanson and Norris, 2008; Pérez et al., 2011; Andersson et al., 2013; Durand et al., 2015; Tonetti et al., 2015). The frequency of mixed infections shows the importance of carrying a different *ospC* gene to be able to super-infect hosts already infected with another *Borrelia* strain.

1.1.10. Vaccination against Lyme disease:

Following the commercial failure of the Lymerix vaccine for humans in the United-States (Nigrovic and Thompson, 2007), scientists became interested in developing vaccines that target the reservoir hosts (Cross et al., 2007). Vaccination of reservoir hosts has been used to reduce the incidence of other zoonotic diseases such as rabies (Pastoret and Brochier, 1998). In one of the two field vaccination trial conducted to date, Tsao et al. (2004) found that immunization of the white-footed mouse (*Peromyscus leucopus*), one of the most important reservoir host of *B. burgdorferi* sensu stricto in North America (Anderson et al., 1987; Bunikis et al., 2004a), produced a modest reduction in the prevalence of infected nymphs and thus the human risk of Lyme disease. In Europe, few studies have been done on this subject, which is partially due to a higher complexity of the European Lyme disease system (Kurtenbach et al., 1994). Both the number of *Borrelia* species and the strain diversity within each species (Derdakova and Lencakova, 2005) are higher in Europe, which makes it more complicated to develop effective vaccines.

The anti-Lyme disease vaccines are based on the outer surface proteins (Osp) of *B. burgdorferi* such as OspC, which was previously described. In fact, there are six different Osps in *Borrelia* labeled OspA to OspF. Each Osp has its own form and function and a vaccine targeting OspA will not induce an immune response against, for example, OspC (Simon et al., 1999; Wallich et al., 2001). The outer surface proteins are up or down-regulated by the bacteria depending on the stage of the spirochete life cycle (i.e. unfed tick, feeding tick, rodent, etc.) (Bockenstedt et al., 1997; Wang et al., 1999a; Liang et al., 2004; Battisti et al., 2008). OspA and OspC have been the most studied outer surface proteins in *B. burgdorferi* s. l. pathogens. The OspA protein in particular, has been the focus of many vaccination studies (Fikrig et al., 1990; Fikrig et al., 1992a; De Silva et al., 1996; Tsao et al., 2001; Tsao et al., 2004). This protein is an interesting candidate for a Lyme disease vaccine because it is highly

conserved between *Borrelia* genospecies and strains (Fikrig et al., 1992b; Probert and Lefebvre, 1994; Kurtenbach et al., 1997; Tsao et al., 2001). This protein is expressed inside the tick where it functions to anchor the spirochete to the tick midgut (De Silva et al., 1996; Schwan and Piesman, 2000; Schwan, 2003; Pal et al., 2004b; Hodzic et al., 2005). There is little genetic variation in the *ospA* gene within each *Borrelia* genospecies and a given OspA vaccine therefore targets all the strains of that species (i.e. the vaccine is said to be multi-competent) (Probert et al., 1997). Antibodies directed against recombinant OspA proteins have been shown to kill the spirochete directly inside the tick. Thus hosts immunized with rOspA are never actually exposed to the OspA antigen on the spirochete. Studies on the Lymerix vaccine found that humans would have to be vaccinated repeatedly to maintain protective antibodies against the Lyme disease pathogen.

The other vaccine candidate that has been investigated is OspC (Probert and Lefebvre, 1994; Wallich et al., 2001; Gilmore et al., 2003; Brown et al., 2005; Earnhart and Marconi, 2007b). The OspC protein is expressed when the spirochete invades the vertebrate host from the tick vector (Schwan et al., 1995; Gilmore and Piesman, 2000; Grimm et al., 2004; Pal et al., 2004a; Tilly et al., 2006; Fingerle et al., 2007). As previously described, and in contrast to *ospA*, the *ospC* gene is highly genetically variable and this variability has likely evolved in response to the host immune system (Wang et al., 1999b). A vaccine consisting of a specific recombinant OspC protein will be protective only against the strain carrying this particular *ospC* allele (Probert et al., 1997; Jacquet et al., 2015). The possibility of using a multivalent OspC vaccine has been studied but the number of *ospC* strains is high, which complicates the task (Earnhart et al., 2005; Earnhart and Marconi, 2007b).

Vaccination with a specific recombinant OspC protein can be a way to target only one strain and study the effects of the acquired immune response on the transmission of *B. afzelii*.

1.1.11. Objectives of the PhD project:

The purpose of the present work was to test whether co-feeding transmission allows the *B. afzelii* pathogen to evade the acquired immune response of the rodent reservoir host. We used immunization trials with recombinant OspC proteins against different *ospC* strains of *B. afzelii* to test hypotheses about the evolutionary ecology of *ospC* strains in nature in addition to providing complementary information on the feasibility of rOspC-based vaccines (Earnhart and Marconi, 2007b, c, a). From an evolutionary perspective, we are interested to test whether previous immune experience with the OspC antigen is effective at reducing systemic transmission and co-feeding transmission of *B. afzelii*. In addition, we are interested in understanding how vaccination of reservoir hosts with rOspC-based vaccines will affect the epidemiology and evolution of the targeted Lyme disease pathogens (Tsao, 2009; Balmer and Tanner, 2011).

In this study, we tested whether immunization with recombinant OspC proteins prevented co-feeding transmission of that particular *ospC* strain of *B. afzelii*. If rOspC immunization of reservoir hosts reduces systemic but not co-feeding transmission, a rOspC-based vaccine targeting reservoir hosts might favor the evolution of those strains capable of co-feeding transmission. This vaccine-induced change in the selective landscape may result in rapid fixation of co-feeding *ospC* strains with potentially unknown outcomes for human Lyme disease risk. We also tested the effects of cross-immunity on the spirochete load of infected ticks. In addition, we investigated how the age of the *B. afzelii* infection in the rodent host influenced the rate of systemic transmission and the spirochete load in ticks fed on mice. Finally, we investigated how aging in the ticks influenced the spirochete persistence and spirochete load in *B. afzelii*-infected nymphs.

2. Protocol of the experiment:

2.1. Principle of the experiment:

The purpose of this experiment was to test whether adaptive immunity in rodent reservoir hosts prevents co-feeding transmission of *B. afzelii*. Our experiment essentially simulated the scenario where a particular *B. afzelii ospC* strain encounters a host that has been previously exposed to the same strain and has developed a strong antibody response to it. To induce adaptive immunity without infecting the mice, we immunized the mice with recombinant OspC (rOspC). To test whether *ospC* strains capable of co-feeding transmission have a fitness advantage in immune-competent hosts, we compared the transmission success between *ospC* strains that were previously shown to differ in their efficacy of co-feeding transmission on laboratory rodents (Tonetti et al., 2015). In addition, we tested whether immunization with recombinant OspC provides cross-protection against strains carrying different *ospC* alleles. Importantly, we challenged immunized mice with ticks (i.e. the natural mode) rather than needles to determine the efficacy of protection.

2.2. Timeline of the work conducted during the PhD thesis:

From September to December 2011, we established the goals of this PhD project.

From January 2012 to May 2013, we developed the recombinant OspC proteins that were used for the immunization.

From 24 April 2012 to 26 July 2012, we created the “challenge” nymphs infected with either strain A3 or strain A10. Ten mice were infected via tick bite with one of the two strains. The mice were infested with two batches of xenodiagnostic larvae. The blood-engorged larvae were collected and allowed to molt into nymphs. These “challenge” nymphs were used to

challenge the mice in the immunization trial in 2013. Ticks were checked every month and sampled twice to determine their infection status on 9 August 2012 and 7 February 2013.

For the main immunization experiment, the 42 mice were received on 30 April 2013 and were sacrificed on 14 November 2013 (7.5 months). Four immunization treatments, one infectious challenge and four xenodiagnoses were conducted on these mice. Ticks collected from those xenodiagnoses were allowed to molt and then killed at different period. The last tick sampling was done on 24 January 2014.

ELISA analysis of serum samples and DNA extraction of ticks followed by qPCR were done during 2014 and the first 6 months of 2015. The last qPCR was done on 10 June 2015.

The statistical analysis of the data and the writing of this manuscript were done between February 2014 and September 2015.

During this PhD thesis, I also gave some practical courses to 1st and 3rd year Bachelor students in histology (spring semester) and in statistics (autumn semester). We also developed a course on molecular biology techniques for the Master students in parasitology (autumn 2013).

2.3. General methods:

2.3.1 *B. afzelii ospC* strains:

We used *B. afzelii* isolates E61 and NE4049. These isolates were originally obtained from xenodiagnostic ticks that had been sampled from field-captured mice (Pérez et al., 2011; Tonetti et al., 2015). Using the nomenclature of Bunikis et al. (2004b), isolates E61 and NE4049 carried the major *ospC* groups A3 (GenBank accession number: L42890) and A10 (GenBank accession number: JX103488), respectively (Tonetti et al., 2015). Isolates were

grown in BSK culture at 32°C and stored at -80°C. 454-sequencing analysis shows that our strains were essentially monogenic for the major *ospC* group allele of interest. The co-feeding and systemic transmission success of these two strains was previously described by Tonetti et al. (2015). Strain A3 has medium systemic transmission (50% of xenodiagnostic larvae were infected) but no co-feeding transmission whereas strain A10 has both high systemic transmission (85%) and co-feeding transmission (65%).

2.3.2 Production of *B. afzelii*-infected nymphs and *B. afzelii* antiserum:

B. afzelii isolates E61 and NE4049 (*ospC* strains A3 and A10) were inoculated into BALB/c mice (Tonetti et al., 2015). Mice were infested with xenodiagnostic larval ticks and blood-engorged larvae were allowed to molt into nymphs. These nymphs were used to infect five BALB/c mice for each of the two strains. Each mouse was infested with ~100 xenodiagnostic larval ticks at two and four months after the nymphal challenge and blood-engorged larvae were allowed to molt into nymphs. When the nymphs were two months old, we randomly selected 10 nymphs for each mouse (total of 50 nymphs for each strain) and used qPCR to estimate the nymphal infection prevalence. We repeated this assay of the nymphal infection prevalence when the nymphs were 8 months old. For strain A3, the infection prevalence was 76.7% (23/30) for the 2-month-old nymphs and 80.0% (16/20) for the 8-month-old nymphs. For strain A10, the infection prevalence was 90.0% (27/30) for the 2-month-old nymphs and 70.0% (14/20) for the 8-month-old nymphs. All mice were exsanguinated three months after infection. The sera from these mice were used to test whether our rOspC proteins contained the same epitopes as native OspC protein in the *B. afzelii* pathogen. The nymphs that remained from the first xenodiagnostic batch were used to challenge the immunized mice when the nymphs were 11 months old.

2.3.3 Production of the recombinant OspC Proteins:

We produced recombinant OspC proteins for major *ospC* groups A3 and A10. The DNA for these two *ospC* strains came from isolates E61 and NE4049. The *ospC* gene sequences were amplified with primers modified from Earnhart et al. (2005), which contained restriction sites and a stop codon on the reverse primer:

OspC forward + 5' BamHI site (green):

5' – GTATAGGATCCAATAATTCAGGGAAAGGTGG – 3'

OspC reverse + 5' HincII site (green) + Stop codon (red):

5' – CATGGTCGACTTAAGGTTTTTTTGGGGTTTCTGC – 3'

The PCR amplicon of the *ospC* was inserted into the pGEM-t easy vector (PROMEGA). The *ospC* gene + plasmid complex was digested with BamHI and HincII restriction enzymes and the digestion product was ligated into the pQE-30 Xa plasmid (QIAGEN). We sequenced the recombinant plasmid to ensure that the *ospC* gene was in-frame. The pQE-30 Xa plasmid carries a 6xHis tag to facilitate protein purification. The *ospC*-pQE-30 Xa construct was transformed into *Escherichia coli* (strain JM109). Recombinant *E. coli* cells were grown in 200 ml of LB media to a density of 10^9 cells/ml and rOspC-expression was induced by adding IPTG (250 µM) for one hour. Proteins were purified using the QIAGEN Ni-NTA protein purification kit and was followed by a dialysis in PBS. We also sent the *ospC*-pQE-30 Xa construct to ImmBiomed Company (Germany) who produced and purified the rOspC proteins. Purification was done using His-Tag affinity chromatography followed by gel filtration.

2.3.4 Experimental Design:

Female BALB/c mice were randomly assigned to one of three immunization treatments: (1) control immunization with phosphate-buffered solution (PBS), (2) immunization with rOspC-A3, and (3) immunization with rOspC-A10. All mice were subsequently challenged via tick bite with either strain A3 or A10. Thus there were a total of 6 combinations of immunization treatment and *B. afzelii* strain as listed below:

Control A3: Immunized with PBS, challenged with *B. afzelii ospC* strain A3

Control A10: Immunized with PBS, challenged with *B. afzelii ospC* strain A10

Homologous A3: Immunized with rOspC type A3, challenged with strain A3

Homologous A10: Immunized with rOspC type A10, challenged with strain A10

Heterologous A3: Immunized with rOspC type A10, challenged with strain A3

Heterologous A10: Immunized with rOspC type A3, challenged with strain A10

The groups control A3 and control A10 each contained 5 mice whereas the other groups contained 8 mice for a total of 42 mice in the experiment. Following immunization, each mouse was challenged with ten *B. afzelii*-infected nymphs to test whether immunization with rOspC protected mice from infection with *B. afzelii*.

2.3.5 Immunization of mice with recombinant OspC:

Each mouse was immunized subcutaneously once per week for four weeks. A first dose of 20 μg of rOspC mixed with Freund's Complete Adjuvant (FCA) was followed by 3 booster doses of 10 μg of rOspC mixed with Freund's Incomplete Adjuvant (FIA). Control mice were inoculated with PBS and adjuvant only. Mice were bled two days before the first

immunization and 10 days after the fourth immunization to determine seroconversion. The mouse sera were used in a subsequent ELISA to test whether the mice had developed OspC-specific IgG antibodies against the OspC antigen.

2.3.6 Nymphal Challenge:

Immunized and control mice were challenged with *B. afzelii*-infected nymphs two weeks after the last immunization. Each mouse was infested with 10 nymphs that were infected with the appropriate strain of *B. afzelii*. Nymphs were placed in a plastic capsule (diameter of 1 cm) that had been attached to the skin of the mouse to protect the ticks from mouse grooming behavior during the attachment phase. Infested mice were placed in cages with a metal grill floor that facilitated collection of blood-engorged ticks. After dropping off the mice, the blood-engorged ticks fell through the grill floor into the collecting trays below. The blood-engorged nymphs were immediately frozen at -20°C for future analysis.

2.2.6 Xenodiagnosis to measure mouse-to-tick transmission success:

We infested mice with pathogen-free larval ticks from our laboratory colony of *Ixodes ricinus* to measure co-feeding and systemic transmission of each strain. To measure co-feeding transmission, mice were infested with larval ticks 48 hours after attachment of nymphal ticks. The 50 to 100 larvae were placed in the plastic capsule to ensure that the two tick stages fed in close proximity to each other, which enhances the probability of co-feeding transmission. To measure systemic transmission of each strain, all mice were infested with 50 to 100 larval ticks at 34, 66, 94, and 128 days (corresponding to 1, 2, 3, and 4 months) after the nymphal challenge. Infested mice were placed in special cages that facilitated the collection of blood-engorged larval ticks. Blood-engorged larvae were placed in 1.7 ml tubes

with a piece of moist filter paper and allowed to molt into nymphs (~ 30 days). Random samples of ticks were frozen at -20°C at 1, 2, 3, or 4 months after molting.

2.2.7 qPCR to detect *Borrelia* infection in ticks and mouse tissue:

We used qPCR to determine *B. afzelii* infection status of the challenge nymphs and xenodiagnostic larvae. The qPCR protocol targets a 132 bp fragment of the highly conserved *flagellin* gene using primers and a probe previously described by Schwaiger et al. (2001).

2.4. Output data:

The experiment collected the following output data:

1) ELISA on blood samples three weeks after the last immunization to show that immunized mice developed an OspC-specific IgG antibody response against the rOspC antigen.

2) *Borrelia*-infection status of nymphs used to challenge the mice. For the logic of the experiment, it is important to confirm that all mice were challenged with at least one *B. burgdorferi*-infected nymph. If we do not recover any infected nymphs or xenodiagnostic larvae (molted into nymphs) for a given mouse, we cannot be sure that this mouse was ever challenged with *B. burgdorferi*. A conservative approach would exclude such individuals from the statistical analysis.

3) Ear biopsy from the mice four weeks after the infectious challenge. The ear biopsy allowed us to establish whether the mice were systemically infected with *B. afzelii* or not.

4) *Borrelia* infection status of the xenodiagnostic larvae (molted into nymphs). The infection prevalence of the xenodiagnostic ticks allows us to estimate the rates of co-feeding transmission and systemic transmission for the two different strains of *B. afzelii*. The

xenodiagnostic ticks from the infestation 1 month after the infectious challenge also allowed us to test whether the mice were systemically infected with *B. afzelii* or not.

5) Xenodiagnostic larval ticks were fed on mice at 1, 2, 3, and 4 months after the infectious challenge. This data allows us to test whether the age of the infection inside the mouse influences the systemic transmission rate and the spirochete load of the nymphal ticks.

6) Xenodiagnostic larval ticks that had molted into nymphs were killed at 1, 2, 3, and 4 months after the larva-to-nymph molt. This data allows us to test whether the spirochete load changes over time as the nymph and the *Borrelia* infection age together.

2.5. Maintenance of the laboratory colony of *Ixodes ricinus* ticks:

The laboratory colony of *Ixodes ricinus* ticks at the University of Neuchatel was created over 30 years ago. No ticks from the wild were added to the colony during this period to avoid contaminating the colony with tick-borne pathogens. Below, I give a brief description of how the laboratory colony of *I. ricinus* ticks is maintained.

Larval ticks are fed on mice. These mice are anesthetized using a mix of ketamine, xylazine and PBS (ratio 1:2:9, 1µl/g of animal). The larval ticks are deposited on the head of the mice allowing them to attach for the duration of the anesthetic (30 to 45 minutes). Larvae-infested, anesthetized mice are placed in type 2 cages. Each type 2 cage is placed inside a type 3 cage containing a thin layer of water (2 cm). When ticks drop off the mice at the end of the blood meal, they are looking for a moist place to molt. The blood-engorged larval ticks climb up the walls of the type 2 cage and fall into the type 3 cage containing the water. The floating ticks are easily collected. Using entomological forceps, ticks are dried on filter paper and then placed in glass tubes in order to molt. The tube is closed with a perforated cap that allows the

air to circulate. The glass tubes are placed in a plastic box with a small container of water that maintains a high relative humidity to facilitate efficient molting of the ticks.

Nymphs and adult ticks are fed on rabbits. Tick-infested rabbits are placed in a containment box adapted to this purpose. Sock-like covers are placed over the ears of the rabbit to prevent the ticks from escaping during the blood meal. A neck brace is used to prevent the rabbit from removing the socks. After the first 3 days, the socks are checked twice per day to collect ticks that would have died or dropped off. After collecting all the blood-engorged ticks (7 or 8 days), the rabbit is returned to the communal rabbit pen. Engorged nymph and adult ticks are kept the same way as the larvae. Male and female adult ticks are put together in the socks on the rabbit ears allowing them to mate. Blood-engorged females are placed in individual glass tubes (sometime with the mating male) to lay eggs. Once the larvae hatch the cycle starts again.

3. Paper 1

Cross-reactive acquired immunity influences transmission success of the Lyme disease pathogen, *Borrelia afzelii*.

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Cross-reactive acquired immunity influences transmission success of the Lyme disease pathogen, *Borrelia afzelii*

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ABSTRACT

Cross-reactive acquired immunity in the vertebrate host induces indirect competition between strains of a given pathogen species and is critical for understanding the ecology of mixed infections. In vector-borne diseases, cross-reactive antibodies can reduce pathogen transmission at the vector-to-host and the host-to-vector lifecycle transition. The highly polymorphic, immunodominant, outer surface protein C (OspC) of the tick-borne spirochete bacterium *Borrelia afzelii* induces a strong antibody response in the vertebrate host. To test how cross-immunity in the vertebrate host influences tick-to-host and host-to-tick transmission, mice were immunized with one of two strain-specific recombinant OspC proteins (A3, A10), challenged via tick bite with one of the two *B. afzelii ospC* strains (A3, A10), and infested with xenodiagnostic ticks. Immunization with a given rOspC antigen protected mice against homologous strains carrying the same major *ospC* group allele but provided little or no cross-protection against heterologous strains carrying a different major *ospC* group allele. There were cross-immunity effects on the tick spirochete load but not on the probability of host-to-tick transmission. The spirochete load in ticks that had fed on mice with cross-immune experience was reduced by a factor of two compared to ticks that had fed on naive control mice. In addition, strain-specific differences in mouse spirochete load, host-to-tick transmission, tick spirochete load, and the OspC-specific IgG response revealed the mechanisms that determine variation in transmission success between strains of *B. afzelii*. This study shows that cross-immunity in infected vertebrate hosts can reduce pathogen load in the arthropod vector with potential consequences for vector-to-host pathogen transmission.

1. Introduction

Cross-reactive acquired immunity occurs when the antibodies developed against one pathogen strain interfere with the fate of another pathogen strain. Antibodies developed against an earlier, primary infection may prevent the establishment of a later, secondary infection or reduce the density of the secondary strain in the host tissues. Cross-reactive acquired immunity (or cross-immunity) induces indirect competition between strains and is critical for structuring the ecology of mixed infections (Frank, 2002; Read and Taylor, 2001). In vector-borne infections, acquired immunity can reduce pathogen transmission success at two critical steps in the pathogen life cycle: vector-to-host transmission and host-to-vector transmission. Previous work has shown that host-to-vector transmission success often depends on the density of the pathogen in the host tissues at the time of vector attachment (de Roode et al., 2005; Mackinnon et al., 2008; Raberg, 2012). Thus cross-immunity, by reducing the density of competing pathogen strains inside the host, might have important consequences for host-to-vector transmission success.

Borrelia burgdorferi sensu lato (s. l.) is a genospecies complex of tick-borne spirochete bacteria that includes the causative agents of Lyme disease in Europe and North America (Kurtenbach et al., 2006). This zoonotic pathogen is maintained in nature by cycles involving *Ixodes* ticks and vertebrate reservoir hosts such as birds and small mammals. Each *Borrelia* genospecies, in turn, consists of multiple strains that are often differentiated by the single copy, highly polymorphic *ospC* gene (Andersson et al., 2013b; Brisson and Dykhuizen, 2004; Durand et al., 2015; Earnhart and Marconi, 2007c; Perez et al., 2011; Qiu et al., 2002; Strandh and Raberg, 2015; Theisen et al., 1993; G. Wang et al., 1999; Wilske et al., 1986, 1993). The *ospC* gene codes for the immunodominant outer surface protein C (OspC), which induces a strong antibody response in the vertebrate host (Dressler et al., 1993; Engstrom et al., 1995; Fung et al., 1994). The anti-OspC IgG response provides protection against secondary infection (Gilmore et al., 1996; Preac-Mursic et al., 1992; Probert and Lefebvre, 1994). A study on the North American genospecies of *B. burgdorferi* sensu stricto (s. s.) showed that immunization with OspC provides protection only against strains carrying that particular *ospC* allele suggesting that there is no cross-protective immunity (Probert et al., 1997). Similarly, a sequential infection experiment with two strains of *B. burgdorferi* s. s. carrying different *ospC* alleles found no evidence for cross-protective immunity (Derdakova et al., 2004). In contrast, a recent study on the

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European genospecies of *Borrelia afzelii* in wild rodents found a pattern of co-occurrence between *ospC* strains suggesting that cross-immunity was shaping the community of multiple infections in the rodent reservoir host (Andersson et al., 2013b). Thus despite the fact that the OspC antigen has received extensive study, the pattern of protective cross-immunity between the different *ospC* strains is not well understood for most members of the *B. burgdorferi* s. l. genospecies complex.

Acquired immunity against *Borrelia* pathogens can reduce the efficacy of host-to-tick transmission. Immunization of infected mice with outer surface protein A (OspA) reduced the transmission rate of *B. burgdorferi* s. s. (Bhattacharya et al., 2011; Gomes-Solecki et al., 2006; Richer et al., 2014; Tsao et al., 2001; Voordouw et al., 2013). However, this transmission-blocking acquired immunity does not occur under natural conditions because the spirochetes rarely express the OspA antigen inside the vertebrate host (De Silva and Fikrig, 1997; De Silva et al., 1996). In contrast, the OspC antigen is expressed inside the vertebrate host (Crother et al., 2004; Liang et al., 2004; Zhong et al., 1997) and so OspC-specific antibodies could potentially reduce host-to-tick transmission. In particular, hosts with previous immune experience with the OspC antigen may develop a faster and more effective anti-OspC IgG response against secondary infections carrying a different *ospC* allele. In *B. burgdorferi* s. s., shared epitopes between different OspC antigens can create cross-reactive antibodies (Ivanova et al., 2009). Thus the purpose of the present study was to test whether antibodies against a given OspC antigen can influence the host-to-tick transmission success and tick pathogen load of a strain carrying a different *ospC* allele. To isolate the effect of cross-immunity and avoid direct competition between strains, we used recombinant OspC (rOspC) proteins to induce an OspC-specific antibody response, thereby removing the confounding effect of a resident primary infection. We predicted that immunization with the rOspC antigen would protect mice against infectious challenge (via tick bite) with strains carrying the same *ospC* allele (homologous strain) but not against strains carrying a different *ospC* allele (heterologous strain). We also predicted that cross-immunity would reduce the host-to-tick transmission rate and the tick spirochete load. Specifically, we predicted that these two spirochete phenotypes would be lower in infected mice that had immune experience with the heterologous rOspC antigen compared to infected mice that had no immune experience with the rOspC antigen.

2. Materials and methods

2.1. Mice and ticks

Four-week-old, pathogen-free, female *Mus musculus* BALB/cByJ mice (Charles River, l'Arbresle, France) were housed in groups of four or five with ad libitum access to food and water (Protector, Switzerland). The animals were allowed to adjust to their new surroundings for seven days before the start of the experiment. Mice were housed individually following infectious challenge with *B. afzelii* to avoid any direct transmission between animals. The mice were euthanized 28 weeks after entering our animal care facility. The commission that is part of the 'Service de la Consommation et des Affaires Vétérinaires (SCAV)' of Canton Vaud, Switzerland evaluated and approved the ethics of this study. The Veterinary Service of the Canton of Neuchâtel, Switzerland issued the animal experimentation permit used in this study (NE2/2012). *Ixodes ricinus* ticks came from our pathogen-free, laboratory colony that has been maintained for over 33 years at the Institute of Biology, University of Neuchâtel. To ensure that this *I. ricinus* colony remains pathogen-free, no wild-caught ticks have been introduced into the colony since its establishment.

Host-to-tick transmission was recently compared between laboratory and wild *I. ricinus* ticks infected with one of the two strains of *B. afzelii* used in this study (A10) and BALB/c mice. Host-to-tick transmission of strain A10 was 85.5% for the laboratory ticks (Tonetti et al., 2015) and 64.0% (64 infected/100 total) for the wild ticks (unpublished data). This comparison suggests that laboratory ticks are more competent at acquiring *B. afzelii* than wild ticks. One explanation for this difference is that the laboratory ticks have a reduced microbial symbiont community compared to wild *I. ricinus* ticks (Lo et al., 2006). *Ixodes* ticks with experimentally reduced microbial symbiont communities are more susceptible to infection with *B. burgdorferi* s. l. pathogens (Narasimhan et al., 2014).

2.2. B. afzelii isolates and the major ospC group allele

B. afzelii isolates E61 and NE4049 were chosen for this study because both isolates are highly infectious to laboratory mice via tick bite (Tonetti et al., 2015). The origins of these isolates and their capacity for tick-to-host transmission and systemic (host-to-tick) transmission were described in a previous study (Tonetti et al., 2015). Both isolates had been passaged fewer than five times to avoid the loss of the virulence genes that are critical for infection (Tonetti et al., 2015). The *ospC* alleles of a given *Borrelia* species are often clustered into what are called major *ospC* groups that are defined as being more than 8% divergent at the DNA sequence level from all other such groups (I.N. Wang et al., 1999). *B. afzelii* contains at least 19 different major *ospC* groups (Strandh and Raberg, 2015). There are currently two different systems of nomenclature for the major *ospC* groups of *B. afzelii*: one developed by Lagal et al. (2003) and the other developed by Bunikis et al. (2004). Using the nomenclature of Bunikis et al. (2004), isolates E61 and NE4049 carried the major *ospC* groups A3 (GenBank accession number: L42890) and A10 (GenBank accession number: JX103488), respectively (Durand et al., 2015; Tonetti et al., 2015). The genetic distance between major *ospC* groups A3 and A10 is intermediate (20.7%) compared to other such pairs (8.9–26.4%; Durand et al., 2015). Thus if cross-immunity effects occur for this intermediately divergent pair of major *ospC* groups, it is likely to exist for pairs that are genetically more similar. Hereafter, we refer to isolates E61 and NE4049 as *B. afzelii ospC* strains A3 and A10, respectively.

Isolates of *B. burgdorferi* s. l. often contain multiple *ospC* strains (Durand et al. 2015; Perez et al., 2011; Qiu et al., 2002). We recently used deep sequencing to confirm that isolates E61 and NE4049 were 100.0% pure for major *ospC* groups A3 and A10, respectively (Tonetti et al., 2015). In the present study, we also used the *ospC* gene as a strain-specific marker to differentiate between strains as numerous other studies have done (Durand et al., 2015; Andersson et al., 2013b; Baum et al., 2012; Brisson and Dykhuizen, 2004; Perez et al., 2011; Tonetti et al., 2015; I.N. Wang et al., 1999). Previous genetic work has shown that the *ospC* locus is in linkage disequilibrium with many other loci in the *Borrelia* genome (Brisson et al., 2012; Bunikis et al., 2004; Hellgren et al., 2011; Qiu et al., 2004). We therefore emphasize that any phenotypic differences between strains A3 and A10 may be due to genetic variation at these other loci.

2.3. Creation of nymphs infected with B. afzelii ospC strains A3 and A10

Five mice were infected via nymphal tick bite for each of the two strains of *B. afzelii* (total of 10 mice). The nymphal ticks used to infect the mice were obtained from a previous experiment (Tonetti et al., 2015). Four weeks after infection, each mouse was infested with ~100 larval ticks. Blood-engorged larvae were placed in individual tubes (1.7 ml Eppendorf tubes containing a moistened piece of paper towel) and were allowed to molt into nymphs. These flat pre-challenge nymphs were tested for *B. afzelii* infection using a quantitative polymerase chain reaction (qPCR) at 1 month and 7 months post-molt. The infection prevalence of the 7-month-old nymphs was 80.0% (16 infected/20 total) and 70.0% (14 infected/20 total) for strains A3 and A10, respectively (Table 1).

2.4. Production of recombinant OspC proteins

DNA was isolated from ticks infected with *B. afzelii ospC* strains A3 or A10 using the QIAGEN DNeasy® Blood & Tissue kit according to the

Table 1
The geometric mean spirochete loads are shown for the subset of *Borrelia afzelii*-infected *Ixodes ricinus* nymphs that were used to challenge the immunized mice.

Nymphal state[a]	Nymphal age (months)[b]	*B. afzelii* strain	rOspC immunogen	Immunization treatment	Infected nymphs/total nymphs	Spirochete load[c] Geometric mean (95% C. L.)[d]
Flat	1	A3	N. A.[e]	N. A.	23/30	1406 (584–3382)
Flat	1	A10	N. A.	N. A.	27/30	11,344 (6912–18,619)
Flat	7	A3	N. A.	N. A.	16/20	743 (375–1472)
Flat	7	A10	N. A.	N. A.	14/20	1537 (471–5014)
Engorged	11	A3	PBS	Control	20/34	3530 (1437–8667)
Engorged	11	A3	rOspC A10	Hetero	18/29	1521 (769–3007)
Engorged	11	A3	rOspC A3	Homo	31/58	3159 (1799–5546)
Engorged	11	A10	PBS	Control	21/38	2896 (1478–5675)
Engorged	11	A10	rOspC A3	Hetero	31/57	2723 (1468–5050)
Engorged	11	A10	rOspC A10	Homo	37/51	2907 (1750–4861)

[a] The nymphal state refers to whether the nymphs were flat (pre-challenge) or blood-engorged (post-challenge).
[b] The nymphal age is the number of months after the larva-to-nymph molt that the nymphs were killed to check their infection status for *B. afzelii*.
[c] The spirochete load is the number of spirochetes per nymph.
[d] 95% confidence limits of the geometric mean.
[e] N. A. = not applicable.

manufacturer's instructions. The *ospC* gene, corresponding to the full OspC protein without its leader peptide, was amplified using primers modified from Earnhart et al. (2005). The forward primer contained a BamH1 restriction site (underlined) in the 5′ end (5′-GT ATA GGA TCC AAT AAT TCA GGG AAA GGT GG-3′) and the reverse primer contained a HincII restriction site (underlined) in the 5′ end (5′-C ATG GTC GAC TTA AGG TTT TTT TGG ACT TTC TGC-3′). DNA was ligated by T/A cloning to a pGEM-T plasmid (PROMEGA) and then digested with BamH1 and HincII restriction enzymes. Digested blunt-ended DNA was ligated to the BamH1 and HincII sites of the bacterial expression vector pQE30Xa. ImmBiomed GmbH (Pfungstadt, Germany) performed the expression and purification of the rOspC proteins using His-Tag chromatography and gel filtration. The rOspC proteins were dissolved in PBS (pH 7.0) and their concentrations were determined using a Bradford assay.

2.5. Immunization treatments and infectious challenge

Forty-two mice were randomly assigned to one of three immunization treatments: rOspC A3 (n = 16), rOspC A10 (n = 16), or PBS (n = 10). Each mouse was immunized subcutaneously four times at weekly intervals (days 1, 8, 15, and 22). The first immunization contained 20 μg of rOspC mixed with Freund's complete adjuvant (total volume = 100 μl). The second, third and fourth immunizations contained 10 μg of rOspC mixed with Freund's incomplete adjuvant (total volume = 100 μl per immunization). Control mice were inoculated with 100 μl of PBS and adjuvant. Immunized mice were randomly assigned to infectious challenge via tick bite with one of two *B. afzelii ospC* strains: A3 or A10. Thus mice immunized with rOspC A3 were challenged with the homologous A3 strain (n = 8 mice) and the heterologous A10 strain (n = 8 mice) and vice versa for the mice immunized with rOspC A10 (Table 2). The control mice were challenged with strain A3 (n = 5) or strain A10 (n = 5). One of the mice belonging to the rOspC A10/strain A3 group died during the experiment so that the final sample size was 41 mice.

2.6. Infectious challenge with *B. afzelii*-infected ticks

To test whether immunization was protective, we challenged the mice with *B. afzelii* via tick bite two weeks after the last immunization (day 34). To ensure infectious challenge, each mouse was infested with ten randomly selected, putatively infected nymphs. To prevent the challenge nymphs from escaping, they were placed in a plastic cap (15 mm diameter) that was glued to the shaved backs of the mice using a mix of resin and honey wax (4:1). Mice were anesthetized with a mix of xylazine, ketamine and PBS (1:2:9; 5 μl per gram of mouse) during this procedure. The mice were checked daily and any detached, blood-engorged nymphal ticks were removed from the cap and frozen at −20 °C for further analysis.

2.7. Mouse ear skin biopsies

Ear skin biopsies were taken to test whether the immunization treatments had protected the mice from infectious challenge. Ear tissue samples were taken from each mouse four weeks after the nymphal challenge (day 68) and again seven days later (day 75) using a forceps type punch (2 mm in diameter). With respect to another important event in the pathogen life cycle, the two tissue samples were taken on the day of and one week after the infestation with the xenodiagnostic larvae. For simplicity, these two biopsies will be referred to as the pre-xenodiagnosis and the post-xenodiagnosis ear tissue samples.

Table 2
The status of *B. afzelii* infection is shown for the six combinations of the rOspC immunogen and *B. afzelii* strain.

rOspC immunogen	*B. afzelii* Strain	Immunization treatment	Ear tissue sample[a]	VlsE ELISA[b]	Systemic transmission[c]	Infected ticks All mice[d]	Infected ticks Infected mice[e]
PBS	A3	Control	5/5 (100.0%)	5/5 (100.0%)	5/5 (100.0%)	39/50 (78.0%)	39/50 (78.0%)
rOspC A10	A3	Heterologous	5/7 (71.4%)	5/7 (71.4%)	5/7 (71.4%)	36/70 (51.4%)	36/50 (72.0%)
rOspC A3	A3	Homologous	0/8 (0.0%)	0/8 (0.0%)	0/8 (0.0%)	0/79 (0.0%)	NA
PBS	A10	Control	5/5 (100.0%)	5/5 (100.0%)	5/5 (100.0%)	45/50 (90.0%)	45/50 (90.0%)
rOspC A3	A10	Heterologous	8/8 (100.0%)	8/8 (100.0%)	8/8 (100.0%)	73/80 (91.3%)	73/80 (91.3%)
rOspC A10	A10	Homologous	0/8 (0.0%)	0/8 (0.0%)	0/8 (0.0%)	0/80 (0.0%)	NA

[a] Proportion of mice that tested positive for *B. afzelii* infection according to the qPCR of the ear tissue sample at four weeks post-infection.
[b] Proportion of mice that tested positive for *B. afzelii* infection according to the ELISA using the VlsE protein at seven weeks post-infection.
[c] Proportion of mice that produced at least one *B. afzelii*-infected tick via systemic transmission at four weeks post-infection.
[d] Systemic transmission rate for all mice (n = 41). Number of infected ticks/total number of ticks (% of infected ticks).
[e] Systemic transmission rate for the subset of infected mice (n = 23). Number of infected ticks/total number of ticks (% of infected ticks).

2.8. Systemic transmission assay

The systemic transmission rate refers to the proportion of xenodiagnostic larval ticks that acquire the spirochete from an infected mouse. To measure systemic transmission, each mouse was infested with 50 to 100 xenodiagnostic larvae four weeks after the nymphal challenge (day 68). The mice were anesthetized during this procedure as described above. Infested mice were placed in individual cages that facilitated the collection of blood-engorged larvae. Blood-engorged larval ticks were placed in individual tubes and were allowed to molt into nymphs. These tubes were stored in plastic cryoboxes at room temperature and high humidity. Four weeks after molting, ten nymphs were randomly selected for each mouse and frozen at −20 °C for further analysis (total of 410 nymphs).

2.9. Serum sampling

One week before (day 28) and seven weeks after (day 83) the infectious challenge with *B. afzelii*, blood samples were collected from the tail vein of each mouse. Blood samples were spun at 1500 G for 10 min and the serum was transferred to a new tube.

2.10. Enzyme-linked immunosorbent assay (ELISA)

To determine the specificity of the anti-OspC IgG response, the mice serum samples were tested for their ability to bind both the homologous and the heterologous rOspC antigen. The details for the ELISA protocol are given in the supplementary material. To test whether the mice were systemically infected with *B. afzelii*, an ELISA targeting the VlsE protein was performed on the serum samples taken seven weeks after the infectious challenge (day 83). The VlsE protein is expressed by *B. burgdorferi* s. l. pathogens during systemic infection and is one of the classical antigens used to determine the infection status of a vertebrate host. The full-length VlsE antigen used in this study was a gift from Reinhard Wallich and had been derived from *B. burgdorferi* s. s. strain B31-5A3 (Lawrenz et al., 1999). The ELISA protocol for the VlsE antigen was the same as the one for the OspC antigen.

2.11. DNA extraction of nymphs and mouse ear tissue biopsies

All xenodiagnostic ticks analyzed in this study were killed four weeks after molting into the nymphal stage. Ticks were crushed using the TissueLyser II by shaking them with a stainless steel bead (1.4 mm in diameter) at a frequency of 30 Hz for 1 min. Total DNA was extracted for each tick using the DNeasy 96 Blood & Tissue kit well plates (QIAGEN) and following the manufacturer's instructions. Each DNA extraction plate contained 94 ticks and two negative DNA extraction controls (*Anopheles gambiae* mosquitoes). DNA from the mouse ear tissue samples was extracted using the DNeasy Blood & Tissue kit mini spin column according to the manufacturer's instructions. We measured the DNA concentration of all mouse ear tissue samples using a Nanodrop.

2.12. qPCR to determine spirochete infection

A qPCR amplifying a 132 base pair fragment of the *flagellin* gene (Schwaiger et al., 2001) was used to detect and quantify *Borrelia* DNA. The 20 μl qPCR mixture consisted of 10 μl of 2× Master Mix (FastStart Essential DNA Probes Master, Roche Applied Science), 3 μl of water, 0.4 μl of 20 μM primer FlaF1A, 0.4 μl of 20 μM primer FlaR1, 0.2 μl of 10 μM Flaprobe1, and 5 μl of DNA template. The thermocycling conditions included a denaturation step at 95 °C for 10 min followed by 55 cycles of 60 °C for 30 s and 95 °C for 10 s using a LightCycler® 96 (Roche Applied Science, Switzerland). Each sample (tick or mouse ear biopsy) was run in triplicate. Each qPCR plate contained 28 samples, 3 standards, and one negative control (all in triplicate) for a total of 96 qPCR reactions. The three standards contained 27,780, 2778 and 278 copies of the *flagellin* gene in 5 μl, respectively (see supplementary material for details). The LightCycler® 96 software (Roche Applied Science, Switzerland) calculated the standard curves and the absolute number of spirochetes present in each positive sample. The total spirochete load for each tick was calculated by multiplying the spirochete load in 5 μl of tick DNA template by the appropriate correction factor.

2.13. Statistical methods

All statistical analyses were done in R version 3.1.0. (R Development Core Team, 2013).

2.13.1. Quantification of the OspC-specific IgG antibody response

To obtain a reliable measure of OspC-specific or VlsE-specific antibody activity, the area under the curve of absorbance versus time was integrated over the first 28 min of measurement (hereafter referred to as the $Absorb_{28}$ value). The specificity of the anti-OspC IgG antibody response to immunization with one of the two rOspC antigens and to infection with one of the two *B. afzelii ospC* strains is presented in the supplementary material.

2.13.2. Definition of B. afzelii infection status for mice and ticks

Mice or ticks were considered infected if at least two of the three qPCR runs tested positive for *B. afzelii*. All mice and the vast majority of ticks were either definitively positive (all three runs tested positive) or definitively negative (all three runs tested negative). Ticks with ambiguous qPCR results (one or two positive runs) were rare (5.3% = 90/1697) and the classification of their infection status did not influence the results.

2.13.3. Effect of rOspC immunization on the mouse-specific systemic transmission rate

The systemic transmission rate was calculated for each infected mouse (n = 23 infected mice). The homologous mice were excluded from this analysis because they were not infected. A GLM with binomial errors was used to test whether the immunization treatment (control, heterologous), *B. afzelii ospC* strain (A3, A10), and their interaction had an effect on the mouse-specific systemic transmission rate. As the rodent spirochete load can influence the probability of host-to-tick transmission (Raberg, 2012), the above analysis was repeated using the spirochete load of the pre-xenodiagnosis ear tissue samples as a covariate. Mouse ear spirochete load was divided by the DNA concentration of the ear tissue sample and this ratio was subsequently log-transformed (see supplementary material for more details). This variable is hereafter referred to as the mouse ear spirochete load.

2.13.4. Effect of cross-immunity on spirochete load inside xenodiagnostic ticks infected via systemic transmission

The spirochete load of each xenodiagnostic tick was calculated as the geometric mean of the three replicate runs (negative runs were excluded). Similarly, the average xenodiagnostic tick spirochete load for each infected mouse (n = 23) was calculated as the geometric mean of the infected ticks (negative ticks were excluded). This variable was log-transformed to improve normality and then modeled as a linear function of immunization treatment (control, heterologous), *B. afzelii ospC* strain (A3, A10), and their interaction. The homologous mice were excluded from this analysis because they were not infected. The above analysis was repeated using the mouse ear spirochete load as a covariate.

3. Results

In what follows below, the tick spirochete load refers to the total number of *B. afzelii* spirochetes inside a tick. The mouse spirochete load refers to the number of spirochetes inside the ear tissue biopsy.

All means are reported with their standard errors unless otherwise indicated.

3.1. Immunization with rOspC induced a strong IgG response against the rOspC antigen

Immunization with the rOspC antigen induced a strong IgG response in the mice one week after the last immunization (Fig. S1; Supplementary material). For the pre-infection serum samples, the mean $Absorb_{28}$ value of the mice immunized with rOspC A3 (2105 ± 119.3 units) was 26 times higher than that of the control mice (81 ± 2.8 units). Similarly, the mean $Absorb_{28}$ value of the mice immunized with rOspC A10 (2942 ± 99.9 units) was 33 times higher than that of the control mice (89 ± 2.4 units).

3.2. Infection status of the challenge nymphs

An average of 6.5 blood-engorged nymphs were recovered per mouse (range = 1–10). For strains A3 and A10, each mouse was challenged with an average of 3.5 infected ticks (range = 2–10) and 4.2 infected ticks (range = 1 to 9), respectively. Analysis of the blood-engorged nymphs confirmed that all the mice in the study had been challenged with at least one *B. afzelii*-infected nymph. The mean spirochete load inside the pre-challenge flat nymphs decreased over time (compare month 1 versus month 7 in Table 1). For strains A3 and A10, the mean spirochete load decreased by 47.2% ($p = 0.283$) and 86.5% ($p < 0.001$), respectively. The spirochete load inside the challenge nymphs increased over the blood meal (compare pre-challenge flat nymphs at 7 months versus post-challenge engorged nymphs fed on the control mice at 11 months in Table 1). Blood feeding increased the spirochete load of the challenge nymphs for strains A3 and A10 by 375.1% ($p = 0.444$) and 88.4% ($p = 0.067$), respectively. We note here that a previous study on *B. burgdorferi* s. s. in *I. scapularis* found that the nymphal spirochete load increased six-fold over the blood meal (Piesman et al., 2001). There was no effect of immunization treatment ($p = 0.681$), strain ($p = 0.399$), and their interaction ($p = 0.342$) on the mean spirochete load inside the post-challenge engorged nymphs (Table 1).

3.3. Infection status of mice following the infectious challenge

Of the 41 mice, 18 individuals (16 homologous, 2 heterologous) were protected from the infectious challenge with *B. afzelii* (Table 2). The remaining 23 individuals (10 controls, 13 heterologous) became infected with one of the two strains of *B. afzelii* (Table 2). The infection status of the mice was determined using three independent tests: (1) the ear tissue biopsies one month after infectious challenge, (2) the VlsE ELISA seven weeks after infectious challenge (Fig. S3; supplementary material), and (3) the xenodiagnostic assay one month after infectious challenge (Table 2). Importantly, there was 100% agreement between these three independent lines of evidence (Table 2).

3.4. Antibodies against rOspC provides specific protection against B. afzelii

All of the ten control mice immunized with PBS became infected with either strain A3 or strain A10 following the infectious challenge (Table 2). This result shows that the challenge nymphs were infectious to immunologically naive mice. The effect of the immunization treatment was highly significant (GLM with binomial errors, $p < 0.001$). Immunization with rOspC induced strong protection against infectious challenge with the homologous strain but not the heterologous strain. All of the 16 homologous mice were protected from infectious challenge (Table 2) whereas only 2 of the 15 heterologous mice were protected from infectious challenge (Table 2). These two mice had been immunized with rOspC A10 and challenged with strain A3. The cross-protective immunity of the rOspC A10 antigen against strain A3 (28.6% = 2/7) was therefore broader than that of the rOspC A3 antigen against strain A10 (0.0% = 0/8) but the difference was not significant.

3.5. Antibodies against rOspC had no effect on the mouse-specific systemic transmission rate

For the subset of infected mice (n = 23), the GLM analysis of the mouse-specific systemic transmission rate found a significant effect of strain ($p = 0.001$; Fig. 1) but not of the immunization treatment (control versus heterologous, $p = 0.678$; Fig. 1) or the interaction ($p = 0.545$). The systemic transmission rate of strain A10 (90.7% = 118/130 ticks; n = 13 mice) was 1.2 times higher than strain A3 (75.0% = 75/100 ticks; n = 10 mice).

The previous analysis was repeated using mouse ear spirochete load as a covariate. The main effect of strain remained statistically significant ($p = 0.019$). There was a significant interaction between immunization treatment and mouse ear spirochete load ($p = 0.033$). The relationship between mouse ear spirochete load and systemic transmission was therefore examined separately for the control and heterologous mice (Fig. 2). There was a significant positive relationship between mouse ear spirochete load and systemic transmission in the heterologous mice ($p = 0.035$) but not in the control mice ($p = 0.667$; Fig. 2).

3.6. Effect of immunization treatment and B. afzelii ospC strain on the mouse ear spirochete load

The repeatability of the mouse ear spirochete load was 0.513 (see supplementary material for details). For the subset of infected mice (n = 23 mice), a two-way ANOVA found no significant interaction between immunization treatment and strain on the mouse ear spirochete load ($p = 0.065$). The immunization treatment was not statistically significant ($p = 0.918$) but there was a significant effect of strain ($p = 0.004$). The mean mouse ear spirochete load (in a 2 mm diameter biopsy) for strain A10 (34,716 ± 4732 spirochetes) was 1.9 times higher than strain A3 (18,172 ± 3300 spirochetes).

3.7. Effect of cross-immunity on spirochete load of xenodiagnostic ticks infected via systemic transmission

The repeatability of the log-transformed spirochete load inside the xenodiagnostic ticks was 0.972 (see supplementary material for details). The linear model of the log-transformed spirochete load of the xenodiagnostic ticks found a significant effect of immunization treatment ($p = 0.009$) and of strain ($p = 0.040$) but not for the interaction ($p = 0.535$). For strain A3, the mean spirochete load of the

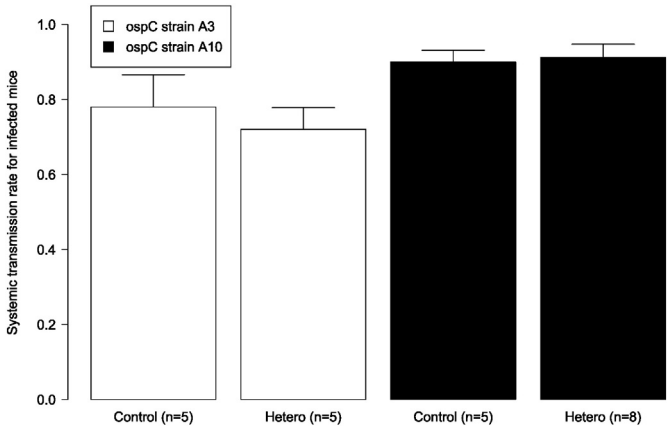

Fig. 1. Cross-reactive acquired immunity in the mouse had no effect on the systemic transmission rate of *B. afzelii*. Strain A10 had significantly higher systemic transmission than strain A3. The sample size was the subset of infected mice (n = 10 control and 13 heterologous). Shown are the means and the standard errors.

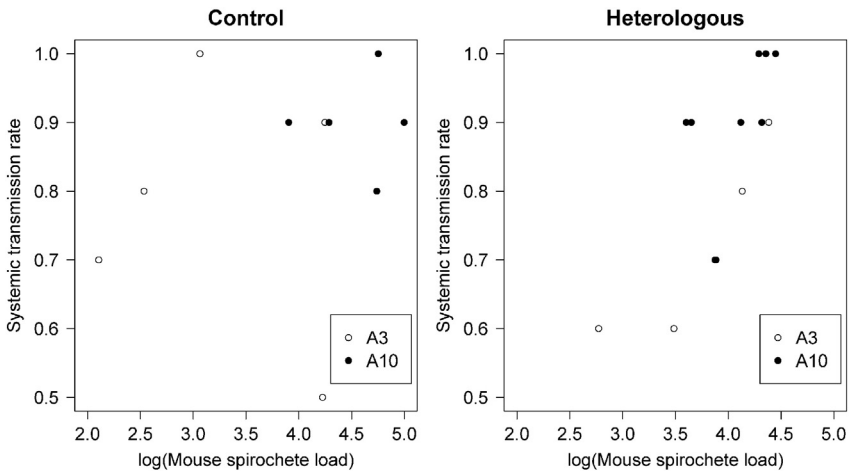

Fig. 2. The systemic transmission rate of *Borrelia afzelii* increases with the spirochete load in the mouse ear tissues. The sample size was the subset of infected mice (n = 10 control and 13 heterologous) and each data point represents a single mouse.

xenodiagnostic ticks infected by the control mice (24,284 ± 7384 spirochetes/nymph) was 2.3 times higher than the heterologous mice (10,348 ± 5044 spirochetes/nymph). For strain A10, the mean spirochete load of the xenodiagnostic ticks infected by the control mice (32,552 ± 4589 spirochetes/nymph) was 1.9 times higher than the heterologous mice (16,809 ± 3133 spirochetes/nymph). Thus acquired cross-immunity (in the heterologous mice) reduced by half the spirochete load inside the xenodiagnostic ticks for both strains of *B. afzelii* (Fig. 3). Strain A10 established a mean spirochete load in the xenodiagnostic ticks that was 1.34 times higher than strain A3 (for the control mice in Fig. 3).

Including mouse ear spirochete load as a covariate did not change the conclusions of the previous analysis. None of the 3- or 2-way interactions between immunization treatment, strain, and mouse ear spirochete load had a significant effect on the xenodiagnostic tick spirochete load. The mouse ear spirochete load itself had no significant effect on the xenodiagnostic tick spirochete load ($p = 0.953$).

4. Discussion

4.1. Antibodies against rOspC provides specific protection against B. afzelii

Immunization with rOspC antigen protected mice from infection with the matching homologous *ospC* strain. Our results are consistent with previous studies on *B. afzelii* and *B. burgdorferi* s. s., which showed that immunization with rOspC protects mice from infection (Gilmore et al., 1996; Preac-Mursic et al., 1992; Probert and Lefebvre, 1994). Our study is the first demonstration in *B. afzelii* that immunization with a given rOspC antigen provided little or no cross-protection against a strain carrying a different major *ospC* group allele. There are surprisingly few studies showing the pattern of cross-protection of the anti-OspC antibody response against strains carrying different major *ospC* group alleles (Earnhart and Marconi, 2007a; Probert et al., 1997). The study by Probert et al. (1997) demonstrated the absence of cross-protection of the anti-OspC antibody response in *B. burgdorferi* s. s. by showing that immunization with the rOspC antigen from strain SON188 protected mice from homologous challenge but not heterologous challenge (strains CA4 and 297). Infection experiments that demonstrate that mice can be sequentially infected with strains carrying different major *ospC* group alleles also demonstrate the specificity of the anti-OspC antibody response (Derdakova et al., 2004). More generally, the observation that wild reservoir hosts are frequently infected with multiple *ospC* strains is further evidence that there is limited cross-immunity between the major *ospC* groups (Anderson and Norris, 2006; Andersson et al., 2013b; Brisson and Dykhuizen, 2004; Perez et al., 2011; Strandh and Raberg, 2015).

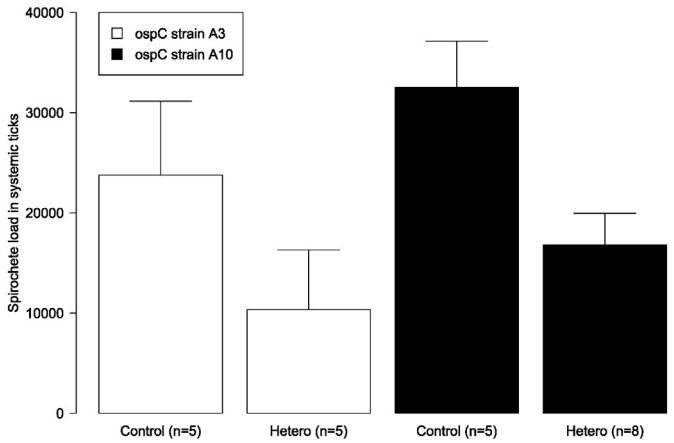

Fig. 3. Cross-reactive acquired immunity reduced the mean spirochete load of *Borrelia afzelii* inside the xenodiagnostic ticks. Strain A10 had a significantly higher mean tick spirochete load than strain A3. The sample size was the subset of infected mice (n = 10 control and 13 heterologous). Shown are the means and the standard errors.

4.2. Limited cross-immunity favors strain A10 over strain A3

We found evidence of some cross-protective acquired immunity between the two strains of *B. afzelii*. Previous studies on North American strains of *B. burgdorferi* s. s. found no evidence of cross-protection between rOspC antigens (Earnhart and Marconi, 2007a; Probert et al., 1997). A recent field study suggested that cross-immunity was structuring the community of *B. afzelii ospC* strains in a population of wild rodents (Andersson et al., 2013b). That study found a positive relationship between the genetic distance between two major *ospC* groups and their degree of association in the rodent host (Andersson et al., 2013b). Our study found evidence of asymmetric cross-immunity because previous immune experience with rOspC type A10 protected 28.6% (2/7) of the mice from infection with strain A3 but the reverse was not true. Asymmetric cross-immunity gives the dominant strain a two-fold competitive advantage over the weaker strain (Frank, 2002; Read and Taylor, 2001). First, the dominant strain induces an acquired immune response that blocks the weaker strain from super-infecting the same host. Second, the dominant strain is not affected by cross-immunity and is therefore capable of super-infecting hosts carrying the weaker strain. The genetic distance between major *ospC* groups A3 and A10 is intermediate (20.7%) with respect to the range of genetic distances

(8.9–26.4%) between other pairs of major *ospC* groups (Durand et al., 2015). Thus the limited cross-protective immunity observed in this study might exist for other pairs of major *ospC* groups. Whether the observed cross-immunity effect also occurs under natural conditions remains to be determined.

4.3. Mechanism of how OspC-specific antibodies protect mice from infection

The mechanism of how OspC-specific antibodies protect mice from infection is not completely understood. We found that the immunization treatment had no effect on the load of spirochetes inside the blood-engorged challenge nymphs. This result is consistent with previous work showing that OspC-specific antibodies are not borreliacidal inside the challenge nymphs (Gilmore et al., 1996). In contrast, OspA-specific antibodies are known to reduce the prevalence and load of spirochetes inside the tick vector (Fikrig et al., 1992). Expression of the OspC protein is controlled during spirochete transmission from the tick vector to the vertebrate host (De Silva and Fikrig, 1997; Tilly et al., 2008). Following tick attachment to the host, the spirochetes in the tick midgut start expressing OspC (Fingerle et al., 1998; Ohnishi et al., 2001; Schwan and Piesman, 2000; Schwan et al., 1995). Some studies suggest that OspC is critical for spirochetes to migrate from the tick midgut to the tick salivary glands (Fingerle et al., 2007; Pal et al., 2004). Other studies have shown that OspC is critical for dissemination inside the vertebrate reservoir host (Grimm et al., 2004; Seemanapalli et al., 2010; Tilly et al., 2006). Gilmore et al. (1996) proposed that OspC-specific antibodies could act in either the tick vector or the vertebrate host to protect the latter from infection. The OspC-specific antibodies can act inside the tick vector to block the migration of the spirochetes from the tick midgut to the tick salivary glands (Gilmore and Piesman, 2000). Alternatively, the vertebrate immune system can kill the spirochetes once they are injected into the host tissues by the tick vector. Heterogeneous expression of the OspC protein suggests that spirochetes will be targeted at different times during their transition from the tick vector to the vertebrate host (Ohnishi et al., 2001) and so the two mechanisms are not mutually exclusive.

4.4. Acquired cross-immunity reduces spirochete load in xenodiagnostic ticks

There was no effect of acquired cross-immunity on systemic (host-to-tick) transmission (Fig. 1). In contrast, we found cross-reactive acquired immunity effects on the tick spirochete load. The spirochete load of the ticks that had fed on the infected heterologous mice was two-fold lower than the ticks that had fed on the infected control mice (Fig. 3). This result suggests that previous immune experience with the OspC antigen allowed the heterologous mice to develop a more effective antibody response, which ultimately reduced the spirochete load inside the xenodiagnostic ticks, compared to the PBS-immunized control mice. The OspC antigen is not believed to play an important role in host-to-tick transmission because its expression is generally suppressed inside the vertebrate reservoir host to facilitate long-term persistence (Crother et al., 2004; Liang et al., 2004; Zhong et al., 1997). However, the regulation of gene expression is not 100% perfect (Gilmore and Piesman, 2000; Ohnishi et al., 2001) and OspC-specific antibodies could clear any spirochetes that accidentally expressed the OspC antigen. We found no effect of the immunization treatment on mouse ear spirochete load suggesting that this infection phenotype did not mediate the observed cross-immunity effect on tick spirochete load. This result suggests that the OspC-specific antibodies transmitted with the blood meal reduced the spirochete load inside the tick vector. Previous work has shown that the spirochete load increases inside the larval tick following the blood meal before declining dramatically during the molt from larva to nymph (Piesman et al., 1990). Given these dynamic changes in spirochete abundance, we were surprised to find an effect of the anti-OspC IgG antibodies two months after the host-to-tick transmission event. A recent field study suggested that the innate immune system of the vertebrate reservoir host plays an important role in structuring the spirochete load inside *I. ricinus* nymphs (Herrmann et al., 2013). The present study extends this work by showing that the acquired immune system of the vertebrate host can also influence the spirochete load inside *I. ricinus*.

Cross-immunity effects on tick spirochete load are only relevant if they influence spirochete fitness. Higher spirochete load might increase the probability of spirochete persistence in the tick vector and/or the probability of tick-to-host transmission in the next step of the Lyme disease life cycle. A recent study on *I. scapularis* ticks infected with *B. burgdorferi* s. s. found that the proportion of infected ticks decreased from 90% to 15% as the spirochete infection aged inside the ticks over a period of six months under laboratory conditions (Voordouw et al., 2013). In the present study, we found that the spirochete load of *B. afzelii* decreased dramatically over a period of 6 months in the flat pre-challenge *I. ricinus* nymphs for both strains A3 (47.2% decrease) and A10 (86.5% decrease). In contrast, the proportion of infected nymphs over the same period was stable: from 90% to 70% for strain A10 and from 77% to 80% for strain A3. Thus the spirochete population declines over time inside the nymphal midgut under laboratory conditions and future studies should investigate whether this phenomenon occurs under natural conditions.

4.5. Mechanism underlying fitness variation between strains of B. afzelii

We found a positive relationship between the spirochete load inside the mouse ear tissues and the systemic transmission rate (heterologous mice in Fig. 2). A positive relationship between the spirochete load in the mouse tissues and the probability of host-to-tick transmission makes intuitive sense and was previously shown in a study on two species of wild rodents (Raberg, 2012). Strains of *B. afzelii* are probably under strong selection to maintain a high density in transmission-relevant tissues like the skin of the ears where ticks are likely to feed and acquire spirochetes.

Strain A10 outperformed strain A3 on the three infection phenotypes. The mouse ear spirochete load, the systemic transmission rate, and the spirochete load inside the ticks were 1.9, 1.2, and 1.34 times higher for strain A10 than for strain A3. Interestingly, a field study on *B. afzelii* in populations of wild rodents and *I. ricinus* in Switzerland found that A10 was one of the most common strains (Durand et al., 2015; Perez et al., 2011; Tonetti et al., 2015). In a previous experimental infection study, we estimated the reproductive number (R_0) for six *ospC* strains of *B. afzelii* including strains A3 and A10 (Tonetti et al., 2015). This study showed that strain A10 had one of the highest R_0 values, which was 1.6 times higher than that of strain A3 (Tonetti et al., 2015). The present study suggests that strain A10 is more successful than strain A3 because it maintains a higher spirochete density in both the rodent host and the tick vector. This study has therefore enhanced our understanding of the mechanisms that determine variation in fitness between strains of *B. afzelii* (Tonetti et al., 2015). However, we emphasize that most of the phenotypic differences between strains A3 and A10 are not necessarily caused by the *ospC* gene but by other loci that are in linkage disequilibrium with the *ospC* locus (Brisson et al., 2012; Bunikis et al., 2004; Hellgren et al., 2011; Qiu et al., 2004).

4.6. Specificity of the anti-OspC IgG response differs between OspC antigens

Infection with *B. afzelii* produced an anti-OspC IgG response that was highly specific for that particular OspC antigen (Fig. S2; Supplementary material). The OspC-specific IgG antibodies of the infected control mice were 3.5–9.8 times more likely to bind the homologous rOspC antigen than the heterologous rOspC antigen (Fig. S2). A previous study on *B. burgdorferi* s. s. used a panel of seven rOspC proteins (major *ospC* groups A, B, C, D, H, K, N) to show that the antiserum developed against infection with one of three major *ospC* group strains (A, B, or D) was

specific for that particular rOspC protein (Earnhart et al., 2005). Interestingly, *B. afzelii* strain A10 induced an OspC-specific IgG response that was twice as strong as strain A3 (Fig. S2). Strain A10 had a spirochete load in the mouse tissues that was almost twice as high as strain A3. Thus one possible explanation is that the higher density of strain A10 in the mouse tissues induced a stronger OspC-specific IgG antibody response. Another explanation for the difference in the strength of the OspC-specific immune response is that strain A10 produces more OspC on its surface than strain A3.

The structure of the OspC protein and the locations of the protective epitopes are critical for understanding how the pattern of cross-protective acquired immunity can influence the community structure of *B. afzelii ospC* strains in the field. The OspC protein is a dimer where each monomer consists of five α-helices (α1, α2, α3, α4, α5) and two β-strands (β1, β2) (Eicken et al., 2001; Kumaran et al., 2001). Most of the variable regions are found on the β-strands and the two loops (L4, L5) connecting helix α2 with α3 and helix α3 with α4. Earnhart et al. (2005) found linear epitopes on the α5 helix (residues 168 to 203) and on loop 5 (residues 136 to 150) of the rOspC protein of *B. burgdorferi* s. s. strain B31. Subsequent work showed that antibodies developed against the α5 helix and loop 5 epitopes were bactericidal (Earnhart et al., 2007). Gilmore and Mbow (1999) using the same strain found a conformational epitope involving either the N- or C-terminal of the rOspC protein. Mathiesen et al. (1998) found one linear epitope within the C-terminal seven residues of the OspC protein of *Borrelia garinii*. Future studies should investigate whether the protective epitopes of the OspC antigen in *B. afzelii* are the same as the ones found in *B. burgdorferi* s. s. and *B. garinii*.

The diversity of the *ospC* gene and the lack of cross-protection between the different OspC antigens complicate the development of an OspC-based vaccine. In the United States, researchers have developed a multivalent vaccine that combines the epitopes of up to eight different OspC antigens (Earnhart et al., 2007; Earnhart and Marconi, 2007b). However, an octavalent vaccine would not be sufficient in Europe where a single population of *I. ricinus* ticks can carry as many as 22 different major *ospC* group alleles (Durand et al., 2015). In addition, there are concerns regarding the public interest in a Lyme disease vaccine given the previous failure of the OspA-based Lymerix vaccine in the United States (Embers and Narasimhan, 2013; Nardelli et al., 2009; Plotkin, 2011). In summary, an OspC-based Lyme disease vaccine for humans faces both technical and sociological hurdles.

4.7. The diversity and complexity of tick-borne infections in nature

The present experimental infection study is an oversimplification of the situation in nature. In the field, infections with multiple *ospC* strains are common in both ticks and reservoir hosts (Andersson et al., 2013b; Brisson and Dykhuizen, 2004; Durand et al., 2015; Heylen et al., 2014; Perez et al., 2011; Strandh and Raberg, 2015; I.N. Wang et al., 1999). The present study investigated indirect competition between *ospC* strains mediated by the host immune system but did not consider direct competition between strains over limited tick or host resources (Derdakova et al., 2004; Strandh and Raberg, 2015). In addition to the *ospC* strain diversity within a *Borrelia* genospecies, ticks and reservoir hosts are often infected with multiple *Borrelia* genospecies (Gern et al., 2010; Herrmann et al., 2013; Hovius et al., 2007; Perez et al., 2011; Rauter and Hartung, 2005) and with different species of tick-borne pathogens (Alekseev et al., 2003; Andersson et al., 2013a, 2014; Burri et al., 2014; Levin and Fish, 2000). Mixed infections can result in facilitation or inhibition where one pathogen strain or species has positive or negative effects on the transmission of another pathogen strain or species (Ginsberg, 2008; Macaluso et al., 2002; Mixson et al., 2006). The potential number of interactions between multiple tick-borne pathogen strains and species is therefore overwhelming. However, a recent study on the *ospC* strains of *B. afzelii* found that laboratory estimates of strain fitness could explain a surprisingly large amount of the variation in the strain-specific frequencies in the field (Tonetti et al., 2015). Thus there is hope that studies that ignore most of the interspecific diversity of tick-borne pathogens can still shed light on the factors that maintain a complex of pathogen strains (Tonetti et al., 2015).

5. Conclusions

In summary, our study found that acquired immunity against a given OspC antigen provides limited cross-protection against *B. afzelii* strains carrying a different major *ospC* group allele. Cross-reactive acquired immunity in the vertebrate host influenced the spirochete load in ticks that fed on those hosts with potentially important consequences for spirochete persistence inside the tick vector and tick-to-host transmission. The spirochete load in the rodent host influenced the probability of host-to-tick transmission, thereby illuminating the mechanisms underlying the variation in fitness between strains of *B. afzelii*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2015.09.012.

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4. Paper 2

Strain-specific antibodies reduce co-feeding transmission of the Lyme disease pathogen, *Borrelia afzelii*.

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Running head: OspC-antibodies reduce co-feeding of *Borrelia*.

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Abstract:

Vector-borne pathogens use a diversity of strategies to evade the vertebrate immune system. Co-feeding transmission is a potential immune evasion strategy because the vector-borne pathogen minimizes the time spent in the vertebrate host. We tested whether the Lyme disease pathogen, *Borrelia afzelii*, can use co-feeding transmission to escape the acquired immune response in the vertebrate host. We induced a strain-specific, protective antibody response by immunizing mice with one of two variants of OspC (A3 and A10), the highly variable outer surface protein C of *Borrelia* pathogens. Immunized mice were challenged via tick bite with *B. afzelii* strains A3 or A10 and infested with larval ticks at days 2 and 34 post-infection to measure co-feeding and systemic transmission, respectively. Antibodies against a particular OspC variant significantly reduced co-feeding transmission of the targeted (homologous) strain but not the non-targeted (heterologous) strain. Cross-immunity between OspC antigens had no effect in co-feeding ticks but reduced the spirochete load two-fold in ticks infected via systemic transmission. In summary, OspC-specific antibodies reduced co-feeding transmission of a homologous but not a heterologous strain of *B. afzelii*. Co-feeding transmission allowed *B. afzelii* to evade the negative consequences of cross-immunity on the tick spirochete load.

Keywords: acquired immunity; *Borrelia afzelii*; co-feeding transmission; cross-immunity; *Ixodes ricinus*; Lyme borreliosis; outer surface protein C; spirochete load; tick-borne pathogen

INTRODUCTION

Pathogens have evolved many strategies to avoid being cleared by the immune system of their hosts (Schmid-Hempel, 2008). Evasion of the host immune system is particularly important for vector-borne pathogens that establish long-lived systemic infections inside vertebrate hosts (Brunham et al., 1993). Many vector-borne pathogens use antigenic variation to stay one step ahead of the vertebrate antibody response (Bloom, 1979; Blaxter et al., 1992; Roberts et al., 1992; Damian, 1997; van der Woude and Baumler, 2004; Frank and Barbour, 2006). Another strategy by which vector-borne pathogens can avoid the vertebrate immune system is to spend less time in the vertebrate host and more time in the arthropod vector. This strategy is most developed in vector-borne pathogens that are capable of co-feeding transmission. In co-feeding transmission, vector-borne pathogens are transmitted between infected and uninfected vectors feeding next to each other on the same vertebrate host at the same time (Randolph et al., 1996; Nuttall and Labuda, 2004; Tsao, 2009; Randolph, 2011; Voordouw, 2015). In systemic transmission by contrast, there is a latent phase during which the pathogen establishes a widespread (systemic) infection inside the vertebrate host before achieving host-to-vector transmission. Thus the main difference between co-feeding and systemic transmission is that the former is local and immediate whereas the latter is from anywhere on the host body and delayed (Randolph, 2011; Voordouw, 2015). These two modes of transmission are not exclusive and many vector-borne pathogens use both. Co-feeding transmission has been reported in a variety of vector-borne pathogens including the vesicular stomatitis virus in black flies (Mead et al., 2000), the West Nile Virus in mosquitoes (Higgs et al., 2005), and a number of tick-borne pathogens including Thogoto virus (Jones et al., 1987), Bunyavirus (Labuda et al., 1997a), tick-borne encephalitis virus (TBEV) (Alekseev and Chunikhin, 1990; Labuda et al., 1993a; Labuda et al., 1993b; Labuda et al., 1993c), *Anaplasma phagocytophilum* (Levin and Fish, 2000), and *Borrelia burgdorferi* sensu lato (s.

l.), the species complex of tick-borne spirochete bacteria that includes the etiological agents of human Lyme disease (Gern and Rais, 1996; Sato and Nakao, 1997; Piesman and Happ, 2001; Crippa et al., 2002; Richter et al., 2002; Hu et al., 2003; Tonetti et al., 2015).

Co-feeding transmission allows vector-borne pathogens to evade the innate and acquired immune system of their vertebrate hosts (Voordouw 2015). TBEV causes a short-term viremia in mice that induces lifelong sterilizing immunity against future infection (Labuda et al., 1997b). However, rodents with acquired immunity against TBEV are still capable of transmitting the virus via co-feeding transmission (Labuda et al., 1997b). Thus co-feeding transmission allows TBEV to evade the antibody response of resistant vertebrate hosts (Labuda et al., 1997b). Similarly, a study on the intracellular tick-borne bacterium, *A. phagocytophilum*, found that acquired immunity in rodents reduced but did not completely block co-feeding transmission (Levin and Fish, 2000). Co-feeding transmission also allows *B. burgdorferi* s. l. pathogens to obtain some fitness benefits from incompetent vertebrate hosts (Randolph et al., 1996; Gern et al., 1998; Voordouw, 2015). Ungulate hosts do not develop a systemic infection because their complement system kills *Borrelia* spirochetes (Kurtenbach et al., 1998a; Kurtenbach et al., 2002). However, a number of field studies suggest that deer and sheep can amplify *Borrelia* pathogens via co-feeding transmission (Kimura et al., 1995; Ogden et al., 1997; Pichon et al., 2000). Thus co-feeding transmission allows *Borrelia* pathogens to evade clearance by the hostile innate immune system of incompetent reservoir hosts (Voordouw, 2015). The purpose of the present study was to investigate whether the Lyme disease pathogen, *B. afzelii,* can use co-feeding transmission to evade pre-existing acquired immunity in the vertebrate host.

Borrelia afzelii is one of the most common causes of Lyme disease in Europe (Piesman and Gern, 2004; Kurtenbach et al., 2006). This tick-borne spirochete bacterium is vectored by the hard tick *Ixodes ricinus* and the main reservoir hosts are wild rodents (Humair

et al., 1995; Humair and Gern, 1998; Kurtenbach et al., 1998b; Humair et al., 1999; Hanincova et al., 2003). *Borrelia afzelii* can establish long-lived infections in its rodent reservoir hosts with a high rate of systemic (host-to-tick) transmission (Gern et al., 1994; Humair et al., 1999). This tick-borne pathogen is also capable of co-feeding transmission (Gern and Rais, 1996; Crippa et al., 2002; Richter et al., 2002; Hu et al., 2003; Tonetti et al., 2015). We have recently shown that there is genetic variation in the efficacy of co-feeding transmission among strains of *B. afzelii* suggesting that this trait can evolve under natural selection (Tonetti et al., 2015). In nature, rodent reservoir hosts are repeatedly exposed to infected ticks and studies in the United States have shown that wild rodent populations develop high levels of *Borrelia*-specific antibodies (Hofmeister et al., 1999; Bunikis et al., 2004a). Under these circumstances, co-feeding transmission may allow *Borrelia* pathogens to escape acquired immunity in the rodent host (Voordouw, 2015).

Acquired immunity in the vertebrate host plays an important role in the epidemiology of Lyme disease (Johnson et al., 1986a, b; Kurtenbach et al., 1994; Piesman et al., 1997; Liang et al., 2004). One *Borrelia* antigen that is particularly important for the pathogen's interaction with the vertebrate immune system is outer surface protein C (OspC) (Radolf and Caimano, 2008). OspC is expressed during the transmission of *Borrelia* spirochetes from the tick vector to the vertebrate host (Schwan et al., 1995; Gilmore and Piesman, 2000; Grimm et al., 2004; Pal et al., 2004; Tilly et al., 2006; Fingerle et al., 2007). The single-copy *ospC* gene is highly polymorphic and this variability has likely evolved in response to the acquired immune system of the vertebrate host (Wang et al., 1999; Baranton et al., 2001). For the three *Borrelia* species that have been studied (*B. burgdorferi* s. s., *B. afzelii*, and *B. garinii*), the *ospC* alleles cluster into 14 to 22 major *ospC* groups, which are defined as > 8% divergent at the DNA sequence level from all other such groups (Wang et al., 1999; Baranton et al., 2001; Lagal et al., 2003; Brisson and Dykhuizen, 2004; Bunikis et al., 2004b; Durand et al., 2015;

Strandh and Raberg, 2015). Each OspC antigen induces a strong IgG antibody response that is protective against strains carrying that particular major *ospC* group allele (Preac-Mursic et al., 1992; Probert and Lefebvre, 1994; Gilmore et al., 1996) but not against strains carrying different major *ospC* group alleles (Probert et al., 1997; Earnhart et al., 2005; Jacquet et al., 2015). In nature, wild rodents and *Ixodes* ticks are often infected with multiple *ospC* strains of a given *B. burgdorferi* s. l. pathogen (Wang et al., 1999; Qiu et al., 2002; Brisson and Dykhuizen, 2004; Anderson and Norris, 2006; Pérez et al., 2011; Andersson et al., 2013; Durand et al., 2015; Strandh and Raberg, 2015). A recent study on *B. afzelii* suggested that cross-immunity between OspC antigens determined the pattern of multiple strain infections in wild rodents (Andersson et al., 2013). In summary, the OspC protein is a highly polymorphic immunodominant antigen that plays a key role in structuring the strain community of *Borrelia* pathogens in the field.

In a previous study, we showed that immunization with recombinant OspC protein (rOspC) protected mice from a homologous infectious challenge with *B. afzelii* strains carrying the same major *ospC* group allele but not from a heterologous infectious challenge with *B. afzelii* strains carrying a different major *ospC* group allele (Jacquet et al., 2015). While there was no cross-immunity effect on systemic transmission, there was a cross-immunity effect on the spirochete load in "systemic" ticks (i.e. ticks that had acquired the infection via systemic transmission). The purpose of the present study was to test whether co-feeding transmission allowed *B. afzelii* to evade the negative effects of strain-specific antibodies developed against the homologous or the heterologous rOspC antigen. We predicted that co-feeding transmission would occur on the homologous mice but that transmission success would be reduced compared to the heterologous and control mice. We also predicted that the cross-immunity effect on the spirochete load in the "systemic" ticks, which depends on an enhanced secondary antibody response to *B. afzelii* infection, would not

occur in the co-feeding ticks. Co-feeding spirochetes evade this cross-immunity effect because transmission occurs before the secondary antibody response has time to develop. Of the two strains used in the immunization trial, strain A10 but not A3 is highly competent at co-feeding transmission (Tonetti et al., 2015). We chose these two strains to highlight that *B. afzelii* strains capable of co-feeding transmission have an important fitness advantage when faced with hosts that have protective, sterilizing antibodies.

RESULTS

Definitions: Mice that were immunized with a rOspC antigen that matched or did not match the major *ospC* group of the subsequent challenge strain are referred to as "homologous" or "heterologous" mice, respectively. Larval ticks that had the opportunity to acquire the *B. afzelii* infection via co-feeding or systemic transmission and then molted into nymphs are referred to as "co-feeding" or "systemic" ticks, respectively.

Prevalence of B. afzelii in co-feeding challenge nymphs: There was no evidence for co-feeding transmission among the blood-engorged challenge nymphs. For strain A3, the prevalence of infection in the blood-engorged challenge nymphs was similar between the control (58.8% = 20/34), heterologous (62.1% = 18/29), and homologous (53.4% = 31/58) groups. For strain A10, the prevalence of infection in the blood-engorged challenge nymphs was also similar between the control (55.3% = 21/38), heterologous (54.4% = 31/57), and homologous (72.5% = 37/51) groups. There was no effect of immunization treatment (GLM: Δ df = 1, $\Delta \chi^2 = 0.972$, p = 0.615), strain (GLM: Δ df = 1, $\Delta \chi^2 = 0.608$, p = 0.436), and their interaction (GLM: Δ df = 2, $\Delta \chi^2 = 4.225$, p = 0.121) on the proportion of blood-engorged challenge nymphs that were infected with *B. afzelii*.

Correspondence between mice that had co-feeding and systemic transmission: There was a statistically significant association between the two modes of transmission across the 40

mice (χ^2 = 4.812, df = 1, p = 0.028). Sixteen mice had both modes of transmission and 12 mice had neither. There were 5 homologous mice that had co-feeding but no systemic transmission: two challenged with strain A3 and three challenged with strain A10. There were 7 *B. afzelii*-infected mice that had systemic but no co-feeding transmission: six infected with strain A3 (three control, three heterologous) and one infected with strain A10 (heterologous).

Antibodies against rOspC reduced the mouse-specific co-feeding transmission rate: There was no difference in the mouse-specific co-feeding transmission rate between the control and heterologous mice (GLM: Δ df = 2, $\Delta \chi^2$ = 0.24, p = 0.889; Figure 1) and these two groups were therefore combined (Table 1). In contrast, there was a highly significant difference in the mouse-specific co-feeding transmission rate between the homologous mice and the combined group of control and heterologous mice (GLM: Δ df = 1, $\Delta \chi^2$ = 83.74, p < 0.001; Figure 1). For strain A10, the co-feeding transmission rate of the control and heterologous mice combined (51.6% = 98/190 ticks; 13 mice; Table 1) was 15.6 times higher than the homologous mice (3.3% = 3/90 ticks; 7 mice; Table 1). For strain A3, the co-feeding transmission rate of the control and heterologous mice combined (11.1% = 14/126 ticks; 12 mice; Table 1) was 6.2 times higher than the homologous mice (1.8% = 2/111 ticks; 8 mice; Table 1). Thus in both strains, co-feeding transmission was drastically reduced but not completely eliminated by antibodies directed against the homologous but not the heterologous rOspC antigen.

There was also a significant effect of *B. afzelii* strain on the mouse-specific co-feeding transmission rate (GLM: Δ df = 1, $\Delta \chi^2$= 58.16, p < 0.001; Figure 1). For the control and heterologous mice combined (n = 25), the co-feeding transmission rate of strain A10 (51.6% = 98/190 ticks; summed over 5 control and 8 heterologous mice; Table 1) was 4.6 times higher than that of strain A3 (11.1% = 14/126 ticks; summed over 5 control and 7 heterologous mice; Table 1).

Efficacy of co-feeding versus systemic transmission: Larval ticks were more likely to acquire spirochetes via systemic transmission than co-feeding transmission. For strain A10, systemic transmission (90.7% = 118/130; summed over 5 infected control and 8 infected heterologous mice) was 1.75 times higher than co-feeding transmission (51.6% = 98/190) and this difference was statistically significant (paired t-test: $t = 4.67$, $df = 12$, $p < 0.001$). For strain A3, systemic transmission (75% = 75/100; summed over 5 infected control and 5 infected heterologous mice) was 5.8 times higher than co-feeding transmission (13.3% = 14/105) and this difference was statistically significant (paired t-test: $t = 8.58$, $df = 9$, $p < 0.001$). Thus systemic transmission was more efficient than co-feeding transmission for both strains.

Effect of co-feeding versus systemic transmission on the tick spirochete load: Nymphs infected as larvae via co-feeding transmission had significantly lower spirochete loads than nymphs infected as larvae via systemic transmission (analysis was restricted to the subset of mice that had both modes of transmission for strain A10 (n = 12); paired t-test: $t = 3.30$, $df = 11$, $p = 0.007$; Figure 2). For the control mice (n = 5), the spirochete load of the systemic ticks (32,557 ± 4,590 spirochetes per nymph) was 6.1 times higher than the co-feeding ticks (5,337 ± 1,221 spirochetes per nymph). For the heterologous mice (n = 7), the spirochete load of the systemic ticks (16,809 ± 3,133 spirochetes per nymph) was 1.9 times higher than the co-feeding ticks (8,940 ± 2,267 spirochetes per nymph). There was no difference in the spirochete load of co-feeding ticks between control and heterologous mice (independent samples t-test: $t = 1.52$, $df = 10$, $p = 0.161$).

Correlations between co-feeding transmission rate, systemic transmission rate, co-feeding tick spirochete load, and the systemic tick spirochete load: None of the six pairwise correlations were statistically significant between the following four variables: the co-feeding

transmission rate, the systemic transmission rate, the co-feeding tick spirochete load, and the systemic tick spirochete load (Table S1).

DISCUSSION

OspC-antibodies reduced co-feeding transmission of B. afzelii: OspC-specific antibodies in laboratory rodents greatly reduced the efficacy of co-feeding transmission of the homologous but not the heterologous strain of *B. afzelii*. For strain A10, immunization with the homologous rOspC A10 antigen reduced the co-feeding transmission rate 15-fold compared to the control and heterologous groups. There were a number of homologous mice that infected larval ticks via co-feeding transmission despite being protected from systemic infection. This important result shows that co-feeding transmission can occur independently from and is not inevitably followed by systemic infection. However, the co-feeding transmission rate of *B. afzelii* on these homologous mice was so low that this strategy is unlikely to make a significant contribution to pathogen fitness (Hartemink et al., 2008). Other studies have shown that acquired immunity in the vertebrate host can reduce the efficacy of co-feeding transmission of tick-borne pathogens (Jones and Nuttall, 1989; Labuda et al., 1997b; Levin and Fish, 2000). For the tick-borne bacterium *A. phagocytophilum*, acquired immunity reduced the co-feeding transmission rate ten-fold (10.8% versus 1.1%) in a natural rodent host (Levin and Fish, 2000). For TBEV, acquired immunity reduced the co-feeding transmission rate three-fold (72% versus 24%) in field mice and 1.4-fold (42% versus 29%) in bank voles (Labuda et al., 1997b). Finally, acquired immunity against the Thogoto virus in guinea pigs completely eliminated co-feeding transmission (Jones and Nuttall, 1989). Thus acquired immunity in the vertebrate host generally reduces co-feeding transmission of vector-borne pathogens but there is substantial variation in the magnitude of the effect size. In

summary, co-feeding transmission did not allow *B. afzelii* to escape the protective strain-specific antibody response of the vertebrate host.

Co-feeding transmission allows B. afzelii to escape the negative effects of cross-immunity on tick spirochete load: Cross-reactive acquired immunity (or cross-immunity) refers to differences in infection phenotype between the heterologous and control groups. Heterologous mice had previous experience with a different (heterologous) OspC antigen whereas control mice were completely naïve at the time of the infectious challenge. Our study found no effects of cross-immunity on the co-feeding transmission rate or on the co-feeding tick spirochete load (Figures 1 and 2). By contrast, we showed in a previous study that there were strong effects of cross-immunity on the spirochete load of both strains in "systemic" ticks (Jacquet et al., 2015). The mean spirochete load of the systemic ticks that had fed on the infected heterologous mice was half that of the systemic ticks that had fed on the infected control mice (Jacquet et al., 2015). This result suggests that previous immune experience with a different OspC antigen allowed the heterologous mice to develop a faster secondary antibody response against the *B. afzelii* infection than the control mice. The efficacy of this secondary antibody response would have peaked at three to four weeks after the infectious challenge, which is when the mice were infested with the second batch of larval ticks to measure systemic transmission. In contrast, co-feeding transmission was measured 48 hours after the infectious challenge, which was not sufficient time for the heterologous mice to develop the enhanced secondary antibody response. Thus the difference in timing between co-feeding and systemic transmission explains the difference in the cross-immunity effect on tick spirochete load between these two modes of transmission. Co-feeding transmission is instantaneous and therefore escapes the negative consequences of the cross-immunity-enhanced secondary antibody response, which is time-lagged. Systemic transmission is delayed and is therefore vulnerable to this time-lagged, cross-immunity-enhanced secondary

antibody response. In summary, co-feeding transmission allowed *B. afzelii* to evade the negative effects of cross-immunity on tick spirochete load.

The mechanism of co-feeding transmission: The mechanism of co-feeding transmission in *Borrelia* pathogens is not well understood (Voordouw, 2015). During the blood meal, infected nymphs inoculate about 100 spirochetes into the feeding lesion (Kern et al., 2011). These spirochetes replicate locally around the site of the tick bite before disseminating to other host tissues (Shih et al., 1992; Hodzic et al., 2003). Larval ticks attached near the feeding lesion of infected nymphal ticks could subsequently imbibe these locally replicating spirochetes (Randolph et al., 1996; Tsao, 2009). A study on *B. afzelii* in laboratory mice showed that co-feeding transmission has both a spatial and a temporal component (Richter et al., 2002). Co-feeding transmission was most efficient (55.3%) when the larvae fed in close proximity (< 1 cm) to the nymphs and when the larval ticks attached two to three days after the nymphs (Richter et al., 2002). Previous studies have shown that nymph-to-host transmission of *B. afzelii* increases over time and reaches ~100% after 48 hours (Kahl et al., 1998; Crippa et al., 2002). This time delay in nymph-to-host transmission is caused by the migration of the *B. afzelii* spirochetes from the tick midgut to the tick salivary glands. The duration of this spirochete migration explains why co-feeding transmission is highest when the larvae attach >48 hours after the nymphs (Richter et al., 2002).

The saliva of ticks is believed to play an important role in the co-feeding transmission of tick-borne pathogens (Nuttall and Labuda, 2004). Tick saliva contains substances that modulate the inflammatory and immune response of the vertebrate host (Ribeiro et al., 2006; Bowman and Nuttall, 2008; Kazimirova and Stibraniova, 2013). For example, tick saliva inhibits or interferes with the vertebrate complement response (Lawrie et al., 1999; Lawrie et al., 2005), the activity of chemokines and cytokines (Hajnicka et al., 2001; Brossard and Wikel, 2004; Hajnicka et al., 2005), and macrophage function (Kopecky and Kuthejlova,

1998). The immunosuppressive properties of tick saliva help tick-borne pathogens, including *B. burgdorferi* s. l., to evade the host immune system (Kuthejlova et al., 2001; Ramamoorthi et al., 2005). Tick salivary glands also contain substances that stimulate spirochete growth in vitro (Rudolf and Hubalek, 2003; Rudolf et al., 2010) and in laboratory mice (Zeidner et al., 2002; Macháčková et al., 2006). In summary, we expected that co-feeding ticks inside the capsule would create a local immunosuppressed environment in the rodent skin that is propitious for spirochete replication and transmission. However, this local immunosuppression was not sufficient to suppress the protective capacity of the OspC-specific antibodies.

Protection of OspC-specific antibodies: The mechanism by which the OspC-specific antibodies reduced co-feeding transmission is not completely understood. *Borrelia* spirochetes express OspC during their migration from the tick midgut to the tick salivary glands (Schwan et al., 1995; De Silva and Fikrig, 1997). There is some controversy regarding the functional role of the OspC protein during tick-to-host transmission (Radolf and Caimano, 2008). Some studies suggest that the OspC protein allows the *Borrelia* spirochetes to invade the salivary glands of *Ixodes* ticks (Pal et al., 2004; Fingerle et al., 2007). However, the research by Rosa and colleagues shows that the OspC protein allows the spirochete to disseminate from the site of the tick bite and establish infection inside the vertebrate host (Grimm et al., 2004; Stewart et al., 2006; Tilly et al., 2006; Tilly et al., 2008; Seemanapalli et al., 2010; Kenedy et al., 2012). Thus OspC-specific antibodies can target spirochetes in either the tick vector or the vertebrate host (Gilmore et al., 1996; Gilmore and Piesman, 2000). Regardless of the underlying mechanism, the present study clearly shows that OspC-specific antibodies reduce co-feeding transmission of homologous strains of *B. afzelii* carrying the same major *ospC* group allele.

Co-feeding transmission among nymphs: The results do not allow us to conclude whether co-feeding transmission occurs between nymphs or not. In the present study, we found no effect of the immunization treatment on the proportion of infected nymphs, which suggests that co-feeding transmission did not occur between nymphs. However, the blood-engorged nymphs were frozen immediately after dropping off the host. Hence, a likely explanation is that any spirochetes transmitted by co-feeding between nymphs did not have enough time to replicate to a detectable abundance. In contrast, an experimental infection study on songbirds that allowed blood-engorged nymphs to molt into adults showed that nymphs can acquire *Borrelia* pathogens via co-feeding transmission (Heylen et al., 2014). Regardless of its existence or not, theoretical models have shown that nymph-to-nymph co-feeding transmission makes a negligible contribution to the reproductive number (R_0) of *Borrelia* pathogens (Hartemink et al., 2008; Harrison et al., 2011; Harrison and Bennett, 2012). Thus from an epidemiological perspective, nymph-to-larva co-feeding transmission is much more important than nymph-to-nymph transmission (Voordouw, 2015).

The mode of transmission and tick spirochete load: The spirochete load in co-feeding nymphs was up to six times lower than in systemic nymphs two months after the larval blood meal (Figure 2). This result suggests that larval ticks acquire fewer spirochetes via co-feeding transmission than systemic transmission and/or that co-feeding spirochetes are not able to increase their growth rate to reach the same population size as spirochetes acquired via systemic transmission. A study on *B. burgdorferi* s. s. in *I. scapularis* has shown that the spirochete population is highly dynamic during this period (Piesman et al., 1990). The spirochete population grows rapidly after the blood meal and then declines dramatically during the larva-to-nymph molt (Piesman et al., 1990). The detection of spirochetes after the larva-to-nymph molt is therefore proof of transstadial transmission and that the nymph contains a viable population of spirochetes (Richter et al., 2002; Heylen et al., 2014).

Additional evidence of the viability of co-feeding spirochetes comes from studies that have cultured spirochetes from co-feeding ticks (Piesman and Happ, 2001; Hu et al., 2003). Whether ticks infected via co-feeding transmission are capable of infecting competent reservoir hosts is currently unknown and should be addressed in future research (Voordouw, 2015).

Strain-specific differences in co-feeding transmission and fitness: There were strain-specific differences in the efficacy of co-feeding transmission (Table 1). The co-feeding transmission rate of strain A10 was 4.6 times higher than strain A3 confirming our previous study (Tonetti et al., 2015). The rate of systemic transmission of strain A10 is also higher than strain A3 (Jacquet et al., 2015; Tonetti et al., 2015). We recently used next generation matrices to estimate the reproductive number (R_0) for six different *ospC* strains of *B. afzelii* (Tonetti et al., 2015). This analysis found that strain A10 had one of the highest R_0 values, which was 1.6 times higher than that of strain A3 (Tonetti et al., 2015). This strain-specific difference in fitness is associated with strain-specific differences in spirochete load in both the vertebrate host and the tick vector. Compared to strain A3, the spirochete load of strain A10 is 1.9 times higher in the mouse tissues and 1.34 times higher in systemic nymphs (Jacquet et al., 2015). These results suggests that strain A10 has higher co-feeding and systemic transmission success than strain A3 because it establishes a higher spirochete load in the mouse tissues. Importantly, the strain-specific differences in co-feeding and systemic transmission success were not caused by differences in the infectious challenge because the prevalence of infection in the challenge nymphs was the same between strains A3 and A10, both before and after the infectious challenge.

Contribution of co-feeding transmission to fitness of B. afzelii: The importance of co-feeding transmission to *Borrelia* pathogens is controversial (Richter et al., 2002; Randolph and Gern, 2003; Richter et al., 2003; Voordouw, 2015). Theoretical models suggest that co-

feeding transmission makes a modest contribution to the reproductive number of *Borrelia* pathogens and is not necessary for the maintenance of Lyme disease in nature (Hartemink et al., 2008; Harrison et al., 2011; Harrison and Bennett, 2012). However, these models ignore the reality that *Borrelia* infections in the vertebrate host and the tick vector frequently consist of multiple strains (Wang et al., 1999; Qiu et al., 2002; Brisson and Dykhuizen, 2004; Anderson and Norris, 2006; Pérez et al., 2011; Andersson et al., 2013; Durand et al., 2015; Strandh and Raberg, 2015). A recent study showed that *B. afzelii ospC* strains compete with each other inside wild rodent reservoir hosts although the underlying mechanism remains unknown (Strandh and Raberg, 2015). Studies on other vector-borne diseases, namely rodent malaria, have demonstrated that competition between parasite strains inside the rodent host is common and can influence host-to-vector transmission success (de Roode et al., 2005; Bell et al., 2006; Alizon et al., 2013). Assuming that competition exists in multiple-strain infections of *B. afzelii*, strains capable of co-feeding transmission may have an important competitive advantage over strains that are not capable of this mode of transmission.

Previous authors have suggested that co-feeding may allow *Borrelia* pathogens to obtain some transmission on vertebrate hosts that are otherwise refractory to systemic infection (Randolph et al., 1996; Gern et al., 1998). A recent field study suggested that co-feeding transmission enhances the diversity of *ospC* strains in *B. afzelii* (Pérez et al., 2011). The authors speculated that some *ospC* strains are better at the classic lifecycle (systemic infection followed by systemic transmission) whereas other strains are better at co-feeding transmission (Pérez et al., 2011). However, in a recent experimental infection study on six different *ospC* strains of *B. afzelii*, we found no such trade-off between co-feeding transmission and systemic transmission (Tonetti et al., 2015). Instead, strains with high co-feeding transmission also had high systemic transmission, and these strains had the highest values of R_0 (Tonetti et al., 2015). *Borrelia afzelii ospC* strains with high co-feeding

transmission (and thus a high value of R_0) were also the most common strains in a local population of *I. ricinus* ticks over a period of 11 years (Pérez et al., 2011; Durand et al., 2015). Thus co-feeding transmission is correlated with spirochete phenotypes that lead to high fitness in mice and high frequency in tick populations in nature.

CONCLUSIONS

OspC-specific antibodies in the vertebrate host reduced the efficacy of co-feeding transmission of a homologous but not a heterologous strain of *B. afzelii*. Immunization with a heterologous OspC antigen had no effect on co-feeding transmission compared to naive control mice. While co-feeding transmission occurred in homologous mice that were protected from systemic infection, the efficacy was too low to make an epidemiologically relevant contribution to the fitness of *B. afzelii*. Thus *Borrelia* pathogens cannot use co-feeding transmission to evade host antibodies specific for their OspC antigen. However, in comparison with systemic transmission, co-feeding transmission did allow *B. afzelii* to evade the negative consequences of the secondary antibody response on tick spirochete load. The two strains of *B. afzelii* (A3 and A10) differed almost five-fold in their efficacy of co-feeding transmission. Co-feeding ticks had a spirochete load that was six times lower than systemic ticks. Future studies should investigate whether these co-feeding ticks are infectious to vertebrate hosts.

MATERIALS AND METHODS

Immunization trial: We used an immunization trial followed by infectious challenge via tick bite to test whether OspC-specific antibodies in laboratory rodents blocked co-feeding transmission of *B. afzelii*. The details of this immunization trial were previously described in Jacquet et al. (2015). Briefly, BALB/c mice were immunized with adjuvant and one of two

different recombinant OspC (rOspC) proteins: rOspC A3 (n = 16 mice) and rOspC A10 (n = 16 mice). The control mice were immunized with phosphate-buffered solution (PBS) and adjuvant (n = 10 mice). Mice were subsequently challenged via tick bite with one of two *B. afzelii* strains that carried either the A3 or A10 major *ospC* group allele (hereafter referred to as strain A3 and strain A10). Thus there were six combinations of antigen (rOspC A3, rOspC A10, PBS) and infectious challenge (strain A3, strain A10). In what follows, the terms homologous and heterologous refer to whether the major *ospC* allele of the challenge strain matched the rOspC antigen or not (see Table 1). One of the mice belonging to the rOspC A10/strain A3 group died during the experiment so that the final sample size was 41 mice. These 41 mice were distributed as follows: rOspC A3-immunized mice challenged with strain A3 (homologous; n = 8), rOspC A3-immunized mice challenged with strain A10 (heterologous; n = 8), rOspC A10-immunized mice challenged with strain A3 (heterologous; n = 7), rOspC A10-immunized mice challenged with strain A10 (homologous; n = 8), control mice challenged with strain A3 (n = 5), and control mice challenged with strain A10 (n = 5).

In a previous study, Jacquet et al. (2015) showed that the 16 homologous mice were protected from the infectious challenge whereas the 10 control mice became infected with *B. afzelii*. Two of the mice immunized with rOspC A10 were protected from infection with strain A3 whereas the remaining 13 heterologous mice became infected with *B. afzelii*. Thus there were 23 mice that became infected with *B. afzelii*: 5 heterologous mice with strain A3, 8 heterologous mice with strain A10, 5 control mice with strain A3, and 5 control mice with strain A10. The systemic infection status of all 41 mice was determined using three independent criteria: (1) IgG antibody response against the VlsE antigen (blood sample taken 21 days after infectious challenge), (2) qPCR of mouse ear tissue biopsy (taken 34 days after infectious challenge), and (3) qPCR of xenodiagnostic ticks (larval ticks were fed on mice 34

days after infectious challenge). The correspondence between these three independent measures of systemic infection with *B. afzelii* was 100% (Jacquet et al., 2015).

Creation of nymphs infected with B. afzelii ospC strains A3 and A10: The creation of the infected nymphs used in the infectious challenge (hereafter the "challenge" nymphs) was previously described in Jacquet et al. (2015). Briefly, 50–100 larval ticks from our pathogen-free *I. ricinus* colony were fed on each of ten BALB/c mice that had been previously infected via nymphal tick bite with either strain A3 or strain A10. Blood-engorged larval ticks were placed in individual tubes and allowed to molt into the challenge nymphs. For each of the ten mice, we randomly sampled four challenge nymphs and tested them for *B. afzelii* infection using qPCR. The mean proportion of infected challenge nymphs for strain A3 was 80.0% (16/20; 95% confidence interval = 55.7–93.4%) and for strain A10 was 70.0% (14/20; 95% confidence interval = 45.7–87.2%). The remaining challenge nymphs were used in the infectious challenge of the rOspC-immunized and control mice (see below).

Co-feeding transmission assay: With respect to the purpose of the present study, *B. afzelii* strains A3 and A10 were chosen because they differ in the efficacy of co-feeding transmission. Strain A10 has high co-feeding transmission (66.2%) whereas strain A3 has low co-feeding transmission (0.0%) (Tonetti et al., 2015). The infectious challenge consisted of infesting each mouse with ten *B. afzelii*-infected challenge nymphs (Jacquet et al., 2015), which had been randomly selected from a pool of nymphs for which the infection rate of strain A3 (80.0%) and strain A10 (70.0%) was known (see above). These challenge nymphs were placed in a plastic capsule that was glued to the backs of the mice to prevent the nymphs from escaping (Jacquet et al., 2015). To measure co-feeding transmission, mice were infested with 80 larval ticks at 48 hours after the nymphal infestation. To enhance co-feeding transmission, the larvae were placed in the same capsule as the nymphs and the mice were anesthetized with isoflurane during this procedure. The larvae were introduced through a

small hole in the capsule surface that was covered with tape for 48 hours to prevent the ticks from escaping. All nymphal and larval ticks in the capsules were allowed to feed to repletion. Infested mice were placed in individual cages that facilitated the collection of blood-engorged ticks. Blood-engorged nymphs were frozen at –20°C and tested for *B. afzelii* using qPCR to confirm that each mouse had been infested with at least one infected challenge nymph (Jacquet et al., 2015). Blood-engorged larvae were placed in individual tubes and were allowed to molt into nymphs. These tubes were stored at room temperature with high humidity to avoid tick dehydration. Four weeks after molting, the nymphs were frozen at –20°C. One month after the infectious challenge, all the mice were infested with a batch of 50 to 100 xenodiagnostic larvae to measure systemic (host-to-tick) transmission (Jacquet et al., 2015). The nymphs infected as larvae via systemic transmission were processed the same way as the nymphs infected as larvae via co-feeding transmission. These two types of nymphs will hereafter be referred to as co-feeding ticks and systemic ticks. For each mouse, we analyzed a maximum of 20 co-feeding ticks and 10 systemic ticks.

DNA extraction and qPCR to test ticks for spirochete infection: DNA extraction of all the ticks was performed following a protocol described by Jacquet et al. (2015). A quantitative PCR amplifying a 132 base pair fragment of the flagellin gene was used to detect and quantify *Borrelia* DNA following a protocol described by Jacquet et al. (2015). Each qPCR plate contained 28 samples, 3 standards (that also functioned as positive controls), and one negative control (all run in triplicate) for a total of 96 qPCR reactions (see Jacquet et al. (2015) for details).

STATISTICAL METHODS

Effect of rOspC immunization on the mouse-specific co-feeding transmission rate: The co-feeding transmission rate was calculated for each mouse for which we recovered at least

one co-feeding larval tick (mean = 13.9, range = 2–20). There was one mouse for which we did not recover any co-feeding larval ticks so the final sample size was 40 mice. A generalized linear model (GLM) with binomial errors was used to test whether immunization treatment, *B. afzelii ospC* strain, and their interaction had an effect on the mouse-specific co-feeding transmission rate. Model simplification was used to test whether the control and heterologous mice could be combined into a single group.

Efficacy of co-feeding versus systemic transmission: The mouse-specific rates of co-feeding and systemic transmission represent paired data. A paired t-test was therefore used to determine whether co-feeding transmission was less efficient than systemic transmission for the subset of infected mice (n = 23 mice).

Calculation of the nymphal tick spirochete load: The spirochete load refers to the number of spirochetes in the nymph at four weeks after the larva-to-nymph molt (when the nymph was killed by freezing). The spirochete load of each nymphal tick was calculated as the geometric mean of the three replicate runs by the Roche software (negative runs were excluded). Similarly, the average nymphal tick spirochete load for each mouse was calculated as the geometric mean of the ticks that had acquired the infection after feeding on that mouse (negative ticks were excluded). The estimates of tick spirochete load were calculated separately for the co-feeding ticks and the systemic ticks. We had previously shown that the spirochete load in the systemic ticks is a highly repeatable phenotype (Jacquet et al., 2015).

Effect of co-feeding versus systemic transmission on the tick spirochete load: The geometric mean spirochete load of the infected co-feeding ticks and of the infected systemic ticks was calculated for a subset of 16 infected mice that had both modes of transmission. The analysis was subsequently restricted to the 12 mice infected with strain A10 because only 4 mice were infected with strain A3. A paired t-test was used to determine whether the spirochete load of the co-feeding ticks was different from the spirochete load of the systemic

ticks. An independent two-sample t-test was used to test whether the immunization treatment affected the spirochete load in the co-feeding ticks.

Correlations between co-feeding transmission rate, systemic transmission rate, co-feeding tick spirochete load, and systemic tick spirochete load: The six pairwise correlations between the co-feeding transmission rate (proportion of ticks infected via co-feeding transmission), the systemic transmission rate (proportion of ticks infected via systemic transmission), the log-transformed spirochete load in ticks infected via co-feeding transmission, and the log-transformed spirochete load in ticks infected via systemic transmission, were calculated separately for strain A3 (n = 4 mice) and strain A10 (n = 12 mice) and for both strains combined (n = 16). These tests were done on the subset of systemically infected mice that produced at least one tick infected via co-feeding transmission (n = 16 mice).

All statistical analyses were done in R version 3.2.0. (R Development Core Team, 2009).

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TABLES

Table 1. The rate of co-feeding transmission is shown for the six combinations of the antigen used for immunization (rOspC A3, rOspC A10 or PBS) and the *B. afzelii ospC* strain used in the infectious challenge (A3 or A10).

			Co-feeding transmission			
Antigen	*Strain*	*Treatment*	*Infected mice/ Total mice*[a]	*Co-feeding mice/ Total mice*[b]	*Infected ticks/ Total ticks*[c]	*Infected ticks/ Total ticks*[d]
PBS + Adjuv	A3	Control	5/5 (100.0%)	2/5 (40.0%)	5/52 (9.6%)	5/28 (17.9%)
rOspC A10	A3	Heterologous	5/7 (71.4%)	2/7 (28.6%)	9/74 (12.2%)	9/22 (40.9%)
	A3	Control + Hetero	10/12 (83.3%)	4/12 (33.3%)	**14/126 (11.1%)**	14/50 (28.0%)
rOspC A3	A3	Homologous	0/8 (0.0%)	2/8 (25.0%)	**2/111 (1.8%)**	2/29 (6.9%)
PBS + Adjuv	A10	Control	5/5 (100.0%)	5/5 (100.0%)	35/69 (50.7%)	35/69 (50.7%)
rOspC A3	A10	Heterologous	8/8 (100.0%)	7/8 (87.5%)	63/121 (52.1%)	63/119 (52.9%)
	A10	Control + Hetero	13/13 (100.0%)	12/13 (92.3%)	**98/190 (51.6%)**	98/188 (51.1%)
rOspC A10	A10	Homologous	0/8 (0.0%)	3/7 (42.9%)	**3/90 (3.3%)**	3/48 (6.3%)
Total			23/41	21/40		

[a] Proportion of mice that were systemically infected mice (n = 23). Mouse infection status was determined by three independent criteria: (1) qPCR of ear tissue biopsy, (2) ELISA using the VlsE antigen, and (3) qPCR of xenodiagnostic ticks.

[b] Proportion of mice that produced at least one infected tick via co-feeding transmission (n = 21).

[c] Proportion of ticks that were infected via co-feeding transmission for all mice (n = 40).

[d] Proportion of ticks that were infected for the subset of mice (n = 21) that produced at least one infected tick via co-feeding transmission.

FIGURES

Figure 1. Co-feeding transmission of *B. afzelii* was blocked by the homologous but not the heterologous immunization treatment. There was no difference in co-feeding transmission between the heterologous and control group. The rate of co-feeding transmission of strain A10 was almost five times higher than that of strain A3. The unit of replication is the mouse-specific co-feeding transmission rate. The sample size includes all the mice from which we recovered co-feeding larval ticks (n = 40). Shown are the means and the standard errors.

Figure 2. The mode of transmission (co-feeding or systemic) influenced the spirochete load of *B. afzelii ospC* strain A10 inside *I. ricinus* nymphal ticks. Nymphs infected as larvae via systemic transmission had a higher spirochete load than nymphs infected as larvae via co-feeding transmission. The effect of the immunization treatment (control versus heterologous) depended on the mode of transmission. For the co-feeding nymphs, the immunization treatment had no effect on the tick spirochete load. For the systemic nymphs, immune experience with the heterologous rOspC antigen reduced the tick spirochete load relative to the control group (Jacquet et al., 2015). The unit of replication is the mouse-specific geometric mean spirochete load. The sample size is the subset of systemically infected mice that produced at least one A10-infected tick via co-feeding transmission (n = 12). Shown are the means and the standard errors.

Figure 1.

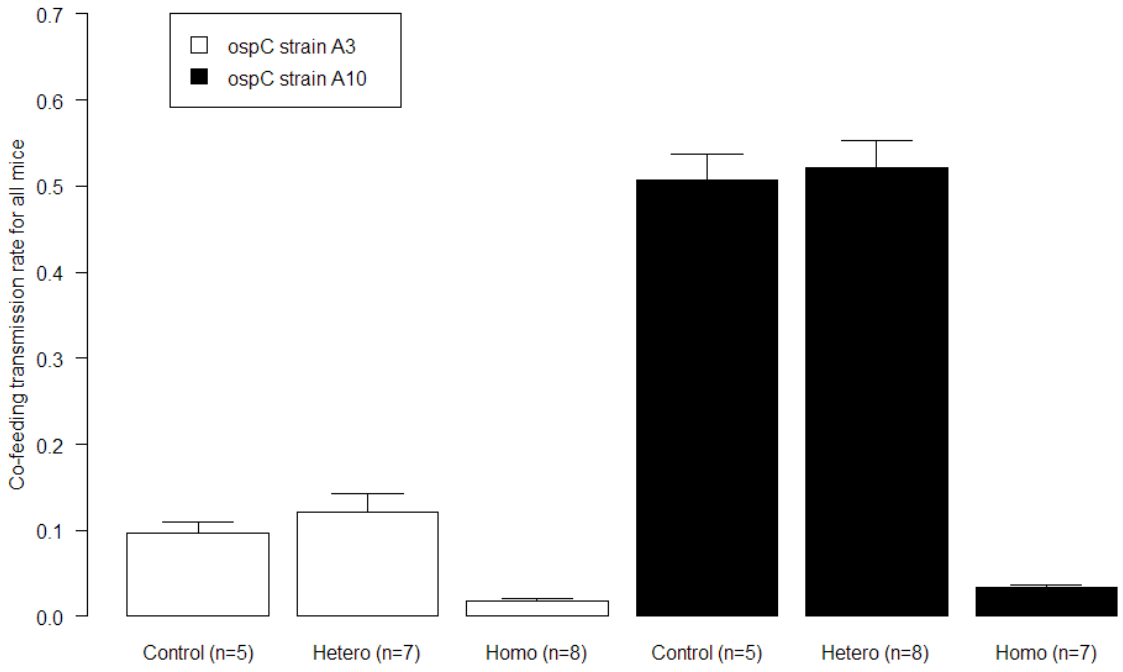
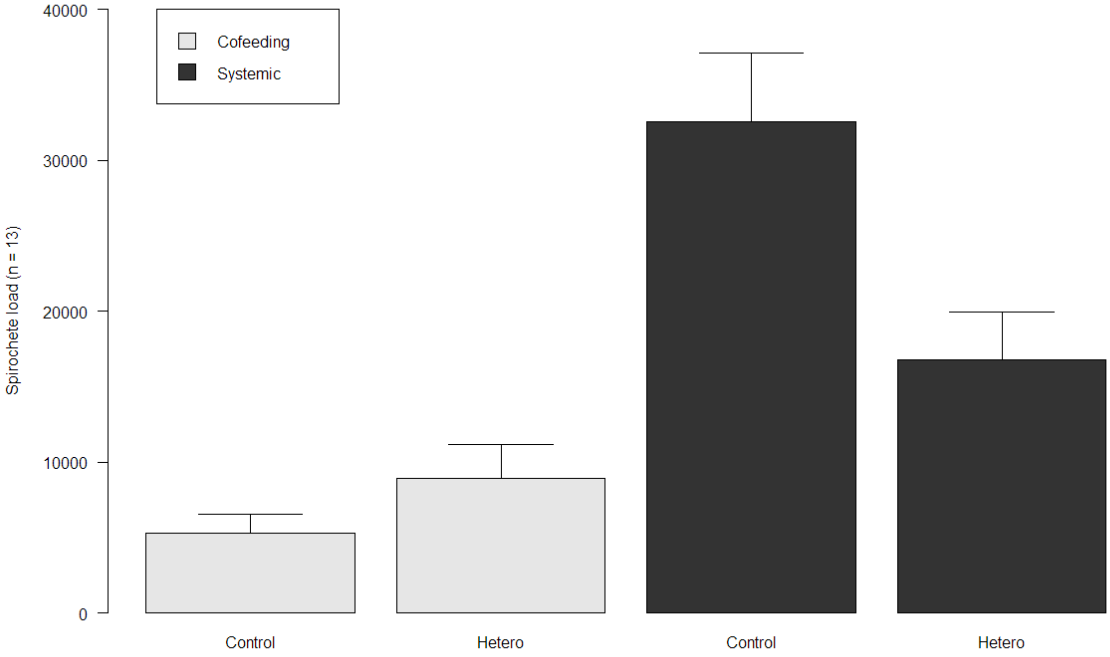

ospC strain A3
ospC strain A10
Co-feeding transmission rate for all mice
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Control (n=5)
Hetero (n=7)
Homo (n=8)
Control (n=5)
Hetero (n=8)
Homo (n=7)

Figure 2.


Cofeeding
Systemic
Spirochete load (n = 13)
40000
30000
20000
10000
0
Control
Hetero
Control
Hetero

5. Paper 3

Systemic transmission and persistence of the Lyme disease pathogen, *Borrelia afzelii* in the tick vector.

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Abstract:

The development of an infection in a susceptible host can depend on the number of infective pathogen cells. In vector-borne diseases, blood-sucking arthropods (e.g. ticks, mosquitoes) transmit the pathogen to the host during the blood meal. For the Lyme disease pathogen, *Borrelia afzelii*, a spirochete bacterium transmitted by the sheep tick (*Ixodes ricinus*), we tested whether the age of infection in the host, the age of infection in the vector, and the strain of the pathogen had an influence on host-to-tick transmission and tick spirochete load. Mice infected via tick bite with one of two strains of *B. afzelii* (A3 or A10) were infested with larval ticks 1, 2, 3, and 4 months post-infection. Those ticks were then analyzed 1 and 4 months after larva-to-nymph molt. Host-to-tick transmission was highest at 1 month post-infection and remained high and stable for the remaining three months. Nymphal aging reduced the *Borrelia* spirochete load in nymphal ticks by a factor of 6.3 to 15.3 over a period of three months. In contrast, nymphal ageing did not influence the probability of detecting the spirochete infection in nymphal ticks. Our study shows that two common strains of *B. afzelii* can persist in rodents over long periods of time and that vector ageing may have important consequences for the fitness of vector-borne pathogens.

Keywords: *Borrelia afzelii*; *Ixodes ricinus*; outer surface protein C; systemic transmission; bacterial load.

INTRODUCTION

Pathogens encounter many critical steps during their life cycle. In vector-borne diseases, the arthropod vector facilitates the step of finding a suitable host for the vector-borne pathogen. During the blood meal, the arthropod vector (e.g. ticks or mosquitoes) helps the pathogen to cross the defensive barrier of the skin. The pathogen will then establish an infection in the vertebrate host that facilitates transmission to new naïve vectors. One of the key factors in this life cycle is the number or dose of pathogen cells that is transmitted to the vertebrate host (Schmid-Hempel 2008).

The infectious dose can vary greatly among vector-borne pathogens. For some mosquito-borne pathogens such as dengue virus, the infective cells are directly injected at the moment of the blood meal (Rosenberg et al. 1990). In contrast, vector-to-host transmission is delayed (~ 24 hours) for the tick-borne spirochete bacterium *Borrelia burgdorferi* sensu lato because the pathogen has to migrate from the tick midgut to the salivary glands during the blood meal (Piesman and Gern 2004). The probability that the spirochete will infect the vertebrate host therefore increases with the duration of the blood meal (Piesman et al. 1987, Kahl et al. 1998, Zhu 1998, Ohnishi et al. 2001, Crippa et al. 2002). The number of spirochetes that reach the tick salivary glands and that infect the host is not 100 % as a number of different factors are involved in this process (De Silva and Fikrig 1995, Schwan and Piesman 2000). The spirochete load in the tick could influence tick-to-host transmission and thus represent a key phenotype of the pathogen life cycle.

Borrelia afzelii is one of the most common causes of Lyme disease in Europe. The life cycle of *B. afzelii* involves the sheep tick *Ixodes ricinus* and wild rodent reservoir hosts (Gern et al. 1998, Piesman and Gern 2004). After tick-to-host transmission, the spirochetes disseminate from the site of the tick bite and establish a widespread, multi-system infection.

Pathogen-free ticks that feed on this infected host can acquire the *B. afzelii* pathogen. An early study on *B. afzelii* found that mouse-to-tick transmission was high over a period of 14 months (Gern et al. 1994). In contrast, studies on *B. burgdorferi* s. s. in wild mice have shown that the efficacy of mouse-to-tick transmission can decrease over time for certain strains (Derdakova et al. 2004, Hanincova et al. 2008).

Aging of ticks can affect the persistence and load of the spirochete infection in ticks. Previous work on *B. burgdorferi* s. s. in *I. scapularis* found that the proportion of infected ticks decreased from 74.0% to 15.5% as the spirochete population aged inside the ticks over a period of 4 months (Voordouw et al. 2013). A study on *B. afzelii* in *I. ricinus* found that the spirochete load decreased by 47.2%–86.5% over a period of 6 months depending on the strain (Jacquet et al. Submitted-b). Hard ticks, such as *I. ricinus*, take only one blood meal for each of their three stages (Piesman and Gern 2004, Gern 2009) and they do not have access to other sources of energy. During the larva-to-nymph molt, there are dramatic changes in the tick spirochete load (Piesman et al. 1990). The off-host phase is long (up to a year) (Gern 2009, Dobson et al. 2011) suggesting that the finite food resources in the tick midgut (including the spirochetes) are important for tick survival. Thus nymphal aging may have a profound effect on the persistence and load of the spirochete infection inside the nymphal tick.

In this study, we wanted to determine whether the age of infection in the host, the age of infection in the tick, and the strain of *B. afzelii* had an influence on host-to-tick transmission and tick spirochete load. In the present study, mice were experimentally infected (via tick bite) with two strains of *B. afzelii*: A3 and A10. To test whether host-to-tick transmission of *B. afzelii* changes over time as the infection ages inside the rodent host, we infested the mice with xenodiagnostic larval ticks at 1, 2, 3, and 4 months post-infection. To test whether the spirochete load changes over time as the infection ages inside the tick, we

allowed the blood-engorged larvae to molt into nymphs and quantified the spirochete load in nymphs at 1 and 4 months post-molt.

MATERIALS AND METHODS

Mice and ticks: Four-week-old, pathogen-free, female *Mus musculus* Balb/cByJ mice (Charles River, l'Arbresle, France) were housed in groups of four or five with ad libitum access to food and water (Protector, Switzerland). The animals were allowed to adjust to their new surroundings for seven days before the start of the experiment. Mice were housed individually following infectious challenge with *B. afzelii* to avoid any direct transmission between animals. The Veterinary Service of the Canton of Neuchâtel, Switzerland, approved the animal experimentation protocol used in this study (NE2/2012). *Ixodes ricinus* ticks came from our pathogen-free, laboratory colony that has been maintained for over 33 years at the Institute of Biology, University of Neuchâtel.

Borrelia afzelii ospC strains: *Borrelia afzelii ospC* strains A3 (isolate E61, GenBank accession number: L42890) and A10 (isolate NE4049, GenBank accession number: JX103488) were chosen because both strains are highly infectious to mice via tick bite and have high systemic (host-to-tick) transmission (85.5% for strain A10 and 53.8% for strain A3) (Tonetti et al. 2015). The origins of these strains and their capacity for tick-to-host and systemic transmission were described in a previous study (Tonetti et al. 2015).

Isolates of *B. burgdorferi* s. l. often contain multiple *ospC* strains. The purity of the two isolates that were used in the present study was tested using 454-sequencing (Tonetti et al. 2015). This approach showed that these two isolates were dominated by a single *ospC* strain (Durand et al. Submitted).

Immunization trial: The details of this immunization trial were previously described in Jacquet et al. (Submitted-b). Briefly, mice were immunized with one of two different rOspC proteins: A3 or A10 and subsequently challenged via tick bite with one of two strains of *B. afzelii*: A3 or A10. In what follows, homologous mice were challenged with the strain that matched their rOspC antigen whereas heterologous mice were challenged with the strain that did not match their rOspC antigen. Control mice were immunized with PBS followed by infectious challenge with one of the two strains. The homologous mice were protected by the rOspC immunization and did not develop any systemic infection (Jacquet et al. Submitted-b). These mice were therefore excluded from the present study. The 23 mice that developed a systemic infection belonged to the following groups: 5 control mice infected with strain A3, 5 control mice infected with strain A10, 5 heterologous mice (immunized with rOspC A10) infected with strain A3, and 8 heterologous mice (immunized with rOspC A3) infected with strain A10.

Systemic transmission assay: To measure systemic transmission, mice were infested with xenodiagnostic larvae on four separate occasions at 34, 66, 94, and 128 days after the nymphal challenge (~1, 2, 3, and 4 months post-infection). Hereafter these infestations will be referred to as mouse age of infection 1, 2, 3, and 4 months. For each of the four infestations, 50 to 100 larvae were placed on the head of each mouse. Mice were anesthetized with a mix of xylazine, ketamine and PBS (1:2:9; 5 µl per gram of mouse) during this procedure. Infested mice were placed in individual cages that facilitated the collection of blood-engorged larval ticks. Blood-engorged larvae were placed in individual tubes, containing a piece of moistened paper towel, and were allowed to molt into nymphs. These tubes were placed in plastic cryoboxes that were stored at room temperature in closed plastic storage boxes that contained 2 cm of water to maintain high humidity and avoid dehydration of ticks. For mouse age of infection 2, 3, and 4 months, the nymphs were frozen at -20°C at 1, 2, 3, and 4 months after

molting into the nymphal stage. For mouse age of infection 1 month, nymphs were frozen at -20°C at 1 month post-molt and the remaining nymphs were used for other experiments. For each of the 13 combinations of mouse age of infection and nymphal age, a maximum of 10 nymphs were frozen.

DNA extraction: In the present study, only the nymphs aged 1 and 4 months were processed. For each of the six combinations of mouse age of infection (2, 3, and 4 months) and nymphal age (1 and 4 months), DNA was extracted from a maximum of 10 nymphs. For mouse age of infection 1 month, there were only 1-month-old nymphs and no 4-month-old nymphs. A total of 1,610 nymphs were processed during the experiment (7 combinations*23 mice/combination*10 nymphs/mouse). Total DNA was extracted using a TissueLyser II and DNeasy 96 Blood & Tissue kit well plates (QIAGEN). The DNA extraction protocol was described in a previous study (Jacquet et al. Submitted-b).

qPCR to determine spirochete infection: A quantitative PCR amplifying a fragment of the *flagellin* gene (Schwaiger et al. 2001) was used to detect and quantify *Borrelia* DNA. The qPCR protocol was described in a previous study (Jacquet et al. Submitted-b).

STATISTICAL ANALYSES

All statistical analyses were done in R version 3.1.0. (R Development Core Team 2009).

Definition of B. afzelii infection status for ticks: Ticks were considered infected if at least two of the three runs of the qPCR assay tested positive for *B. afzelii*, as described in a previous study (Jacquet et al. Submitted-b).

Spirochete loads of xenodiagnostic ticks infected via systemic transmission: The spirochete load of each tick was calculated as the geometric mean of the three replicate runs (negative runs were excluded). Similarly, the average tick spirochete load for each mouse was calculated as the geometric mean of the infected ticks (negative ticks were excluded).

Effect of mouse age of infection, strain, and immunization treatment on the systemic transmission rate of B. afzelii: The systemic (or host-to-tick) transmission rate of each mouse was measured as the proportion of nymphs that tested positive for *B. afzelii* using the qPCR assay. We conducted separate analyses for each nymphal age: 1 and 4 months. A generalized linear model (GLM) with binomial errors was used to model the mouse-specific systemic transmission rate as a function of three fixed factors: the mouse age of infection (1, 2, 3 and 4 months), strain (two levels: A3 and A10), immunization treatment (two levels: control and heterologous), and their interactions.

Effect of mouse age of infection, strain, and immunization treatment on the spirochete load of infected nymphs: For the subset of infected nymphs (i.e. uninfected nymphs were excluded), a linear mixed effects model with normal errors was used to model the log-transformed spirochete load as a function of the mouse age of infection, strain, immunization treatment, and their interactions. The identity of the mouse was included as a random factor. The analyses were conducted separately for the 1-month-old nymphs and the 4-month-old nymphs.

Effect of nymphal age, strain, and immunization treatment on the probability of detecting the B. afzelii *infection in nymphal ticks*: Nymphal ageing may influence the nymphal spirochete load and hence the probability of detecting the *B. afzelii* infection in the nymphal ticks. The probability of detecting spirochete infection in the nymphal ticks was defined as the proportion of nymphs infected with *B. afzelii*. Nymphal age represents paired

data because for each larval infestation, the blood-engorged larvae were split into two groups that were killed at either 1 month or 4 months after the larva-to-nymph molt.

The ticks from the first larval infestation (1 month after the infectious challenge) were excluded from the analysis because this group did not have any four-month-old nymphs. A generalized linear model (GLM) with binomial errors was used to model the probability that a nymph was infected with *B. afzelii* as a function of nymphal age (1 or 4 months), strain (A3 or A10), immunization treatment (control or heterologous), and their interactions. The mouse age of infection was either ignored or the analyses were conducted separately for each mouse age of infection (2, 3, and 4 months).

Effect of nymphal age, strain, and immunization treatment on the spirochete load of infected nymphs: The ticks from the first larval infestation (1 month after the infectious challenge) were excluded from the analysis because this group did not have any four-month-old nymphs. A linear mixed effects model (GLM) with normal errors was used to model the log-transformed spirochete load as a function of nymphal age (1 or 4 months), strain (A3 or A10), immunization treatment (control or heterologous), and their interactions. The mouse age of infection was either ignored or the analyses were conducted separately for each mouse age of infection (2, 3, and 4 months).

RESULTS:

All means are reported with their standard errors unless otherwise indicated.

Effect of mouse infection age on the systemic transmission rate: For the 1-month old nymphs, the systemic transmission rate was highest at 1 month post-infection and then decreased but remained stable for the next three months (Table 1). For the 1-month-old nymphs, there was a significant interaction between mouse age of infection and strain on the

systemic transmission rate ($\Delta \chi^2 = 10.78$, Δ df =3, p=0.013). We therefore analyzed the effect of the mouse age of infection on the systemic transmission rate separately for each strain. The mouse age of infection had a significant effect on the systemic transmission rate for strain A3 ($\Delta \chi^2 = 7.87$, Δ df = 3, p = 0.049) and strain A10 ($\Delta \chi^2 = 41.8$, Δ df = 3, $p < 0.001$). For strain A3, a Tukey HSD test found that the systemic transmission rate at month 1 was significantly higher than month 2 (p = 0.037) but not months 3 and 4. For strain A10, a Tukey HSD test found that the systemic transmission rate at month 1 was significantly higher than months 2, 3, and 4 ($p < 0.001$ for all three contrasts). For the 4-month-old nymphs, there was no significant effect of the mouse age of infection on the systemic transmission rate.

Effect of the mouse age of infection on the spirochete load of infected nymphs: For the 1-month-old nymphs, the effect of the 3-way interaction (mouse age of infection, strain, immunization treatment) on the nymphal spirochete load was almost significant ($\Delta \chi^2 = 7.36$, Δ df = 3, p = 0.0613). The 2-way interaction between mouse age of infection and immunization treatment had a significant effect on the nymphal spirochete load ($\Delta \chi^2 = 15.63$, Δ df = 3, p = 0.0014). The effect of the mouse age of infection on the nymphal spirochete load was therefore analyzed separately for each immunization treatment (control and heterologous). For the ticks fed on control mice (Figure 1), there was a significant effect of the 2-way interaction between mouse age of infection and strain ($\Delta \chi^2 = 9.66$, Δ df = 3, p = 0.022). For the control mice infected with strain A3, there was no effect of the mouse age of infection on the nymphal spirochete load ($\Delta \chi^2 = 4.78$, Δ df = 3, p = 0.188). In contrast, for the control mice infected with strain A10, the nymphal spirochete load appeared to cycle with the mouse age of infection ($\Delta \chi^2 = 22.63$, Δ df = 3, $p < 0.001$). A Tukey HSD post-hoc test found a cyclical pattern in the nymphal spirochete load: month 1 > month 2 (p = 0.0347), month 2 < month 3 ($p < 0.001$), and month 3 > month 4 ($p < 0.001$). For the 1-month-old nymphs fed on the heterologous mice (Figure 1), there was a significant effect of strain ($\Delta \chi^2 = 6.659$, Δ df =

1, p = 0.010). The nymphal spirochete load was consistently higher for strain A10 than strain A3 (Figure 1). We also split the dataset with respect to the mouse age of infection and found that the effect of the immunization treatment was only significant when the infection inside the mice was 1 month old ($\Delta \chi^2 = 8.24$, Δ df = 1, p = 0.004).

For the 4-month old nymphs, there was no effect of mouse age of infection (2, 3, or 4 months), strain (A3 or A10), or the immunization treatment (control or heterologous) on the nymphal spirochete load.

Effect of nymphal age on the probability of detecting the B. afzelii *infection in nymphal ticks*: The analyses were done separately for each mouse age of infection (2, 3, and 4 months). There was no effect of nymphal age, strain, immunization treatment, and their interactions on the probability of detecting the *B. afzelii* infection in the nymphal ticks (p > 0.050).

Effect of nymphal age on the spirochete load of infected nymphs: There were no significant interactions between nymphal age, immunization treatment, and strain on the nymphal spirochete load. There was a significant effect of strain ($\Delta \chi^2 = 12.35$, Δ df = 1, p < 0.001): nymphs infected with strain A10 had a spirochete load that was 2.2 times higher than nymphs infected with strain A3. There was also a significant effect of nymphal age ($\Delta \chi^2 = 194.25$, Δ df = 1, p < 0.001): 1-month-old nymphs had a mean spirochete load that was 9.2 times higher than 4-month-old nymphs.

DISCUSSION:

The efficiency of systemic (host-to-tick) transmission depends on the age of the infection inside the rodent host and on the strain of *Borrelia afzelii*. Systemic transmission

was highest when the infection inside the mouse was 1 month old, and was lower but stable for the remaining three months. Nymphal age had an important effect on the spirochete load inside the nymphal tick. The spirochete load decreased 9.2-fold as the infection aged inside the nymphs over a period of three months.

The duration of host-to-tick transmission is a critical determinant of the reproductive number and the epidemiology of vector-borne pathogens. For example, TBEV has a short duration of host-to-tick transmission and a reproductive number that is an order of magnitude lower compared to *Borrelia* pathogens (Randolph et al. 1996, Hartemink et al. 2008, Harrison et al. 2011, Harrison and Bennett 2012). Early studies on *B. burgdorferi* s. l. suggested that mice had high systemic transmission over the duration of their infection (Donahue et al. 1987, Gern et al. 1994). Subsequent theoretical models used this paradigm of long-lasting high systemic transmission (50.0% for ~120 days) to show that *B. burgdorferi* s. l. pathogens have a high reproductive number (Hartemink et al. 2008, Harrison et al. 2011, Harrison and Bennett 2012). Later studies on *B. burgdorferi* s. s. found that mouse-to-tick transmission can decrease dramatically over short periods of time (Lindsay et al. 1997) and can differ dramatically between strains (Derdakova et al. 2004, Hanincova et al. 2008). For example, mouse-to-tick transmission declines from 83.3% to 4.1% over a period of 30 days for strain B348 whereas it remains high for strain BL206 (Derdakova et al. 2004). Subsequent modeling efforts showed that natural selection can maintain a diversity of strains with different age-of-infection-related patterns in host-to-tick transmission (Haven et al. 2012).

Kurtenbach et al. (2006) suggested that some *Borrelia* genospecies use a "boom-and-bust" strategy to transmit more efficiently to new larval ticks. This strategy allows the spirochetes to achieve high host-to-tick transmission in a short period of time. After the early "boom" of host-to-tick transmission, the *Borrelia* infection is cleared by the host immune system. The empirical studies by Derdakova et al. (2004) and Hanincova et al. (2008) on *B.*

burgdorferi s. s. and the modeling study by Haven et al. (2012) suggest that there was such a trade-off between 'fast' and 'slow' strains. In contrast, the present study and our previous work have found no evidence of such a trade-off between very early (co-feeding) transmission and later (systemic) transmission in *B. afzelii* (Tonetti et al. 2015, Jacquet et al. Submitted-a).

Studies on strain-specific differences in the relationship between the age of infection and host-to-tick transmission are non-existent for *B. afzelii*. Previous studies have either ignored the effect of strain (Gern et al. 1994, Humair et al. 1999) or have compared host-to-tick transmission between strains at a single age of infection (Tonetti et al. 2015, Jacquet et al. Submitted-a). The present study shows that for two common strains of *B. afzelii*, there is high systemic transmission over the epidemiologically relevant lifespan of the infection inside the rodent host. For both strains, the systemic transmission rate was highest in ticks that acquired spirochetes when the infection was 1 month old in the mouse. Strain A10 had higher systemic transmission than strain A3 as shown in our previous work (Tonetti et al. 2015, Jacquet et al. Submitted-b).

For the control mice infected with strain A10, the nymphal spirochete load fluctuated over the four infestations suggesting the presence of a cyclical immune phenomenon. During host infection, *Borrelia* spirochetes are able to up- or down-regulate surface proteins, or to modify certain surface proteins involved in the infection of the host such as the VlsE protein (Wilske et al. 1988, Ohnishi et al. 2001, Ohnishi et al. 2003, Crother et al. 2004, Connolly and Benach 2005, Graves et al. 2013, Tilly et al. 2013). This immune evasion strategy could cause pathogen abundance in the vertebrate host and host-to-vector transmission to fluctuate as has been observed for the malaria pathogen *Plasmodium falciparum* (Zhang and Norris 1998, Craig and Scherf 2001). Future studies should investigate whether this cyclical phenomenon occurs in other strains and whether it lasts for more than 4 months.

Our study suggests that the host immune system plays an important role in the host-to-tick transmission of *B. afzelii* (Figure 1). In a previous study, we showed that the spirochete load in the nymphs that had fed on the heterologous mice was half that of the nymphs that had fed on the control mice (Jacquet et al. Submitted-b). We interpreted this cross-immunity effect on the nymphal spirochete load as evidence that previous immune experience with the 'wrong' OspC antigen allowed the heterologous mice to develop a faster and more effective antibody response against the spirochete infection than the control mice (Jacquet et al. Submitted-b). However, there was no such cross-immunity effect on the spirochete load for infestations 2, 3, and 4. This result is expected because 2 months post-infection is enough time for the control mice to develop an equally efficient *Borrelia*-specific antibody response. In summary, we observed a transient cross-immunity effect on the nymphal spirochete load that disappeared over time as the acquired immune systems of the control mice caught up with those of the heterologous mice.

Few studies have investigated the dynamics of the spirochete load over long periods of time in infected ticks. Most studies have investigated changes in the spirochete population over the duration of the tick bite (De Silva and Fikrig 1995, Zhu 1998, Piesman et al. 2001), or the duration of the larva-to-nymph molting period (Piesman et al. 1990). Here, we analyzed the effect of nymphal age on the spirochete load over a longer period of time. Nymphs killed at one month after the molt had a spirochete load that was 6.3 to 15.3-fold lower than nymphs killed at four months after the molt. This effect of nymphal ageing on the spirochete load could influence the future infectiousness of the ticks because the host immune system is more likely to eliminate a small dose of pathogen cells (Schmid-Hempel 2008).

Temperature and relative humidity influence the rate at which ticks consume their energetic resources (Randolph and Storey 1999, Herrmann and Gern 2012). In this study, ticks were kept at room temperature and high humidity. In the field, by contrast, ticks

frequently encounter dehydrating conditions that threaten their water balance and survival (Gern 2009). Further studies should investigate whether natural fluctuations in temperature and relative humidity affect the tick spirochete load. The questing behavior of *I. ricinus* consumes the food resources present in the midgut (which may include the spirochetes). The energetic resources available to the tick and the spirochete population are limited by the amount of blood ingested by the tick during the previous stage. Unlike mosquito vectors that feed on flower nectar, ticks obtain all of their energy resources from the blood meal. Thus the tick and the spirochete population are faced with an ever-diminishing supply of energetic resources as the two age over time.

CONCLUSIONS:

Future studies should test whether *Ixodes* ticks can lose their *Borrelia* infections under natural and stressful conditions. The infectiousness of young and older nymphs could be compared to test whether nymphal aging influences the probability of tick-to-host transmission.

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TABLES:

Table 1. The systemic transmission rate and the spirochete load in the infected nymphs are shown for the seven combinations of mouse age of infection (1, 2, 3, 4 months) and nymphal age (1, 4 months) for each of the two strains of *B. afzelii* (A3 or A10). The systemic transmission rate shows the number of infected nymphs divided by the total number of nymphs analyzed, and the corresponding percentage of infected nymphs. The spirochete load presents the mean spirochete load and the 95% confidence limits (C. L.) for the subset of infected nymphs.

		Nymphal age		*Nymphal age*			
		1 month	*4 months*	*1 month*		*4 months*	
		Systemic transmission rate		*Spirochete load*			
Strain	*Mouse age of infection*	*Infected nymphs/ total nymphs (%)*	*Infected nymphs/ total nymphs (%)*	*Mean*	*C. L.*	*Mean*	*C. L.*
A3	1[a]	75/100 (75%)	-	12,154	8,071-18,302	-	-
A3	2	54/95 (56.9%)	46/65 (70.8%)	9,940	6,395-15,451	950	615-1,467
A3	3	57/90 (63.3%)	32/56 (57.1%)	10,423	6,707-16,198	1,533	861-2,729
A3	4	62/90 (68.9%)	17/31 (54.8%)	8,546	5,359-13,628	1,349	562-3,242
A10	1[a]	118/130 (90.8%)	-	19,867	14,914-26,464	-	-
A10	2	73/118 (61.9%)	9/20 (45%)	17,748	12,179-25,862	1,153	304-4,364
A10	3	88/128 (68.9%)	40/62 (64.5%)	35,560	25,141-50,296	3,255	2,193-4,832
A10	4	79/128 (61.8%)	53/78 (67.9%)	19,877	13,945-28,332	2,454	1,679-3,587

[a] For the mouse age of infection at 1 month, no four-month old nymphs were sampled

Figures:

Figure 1:

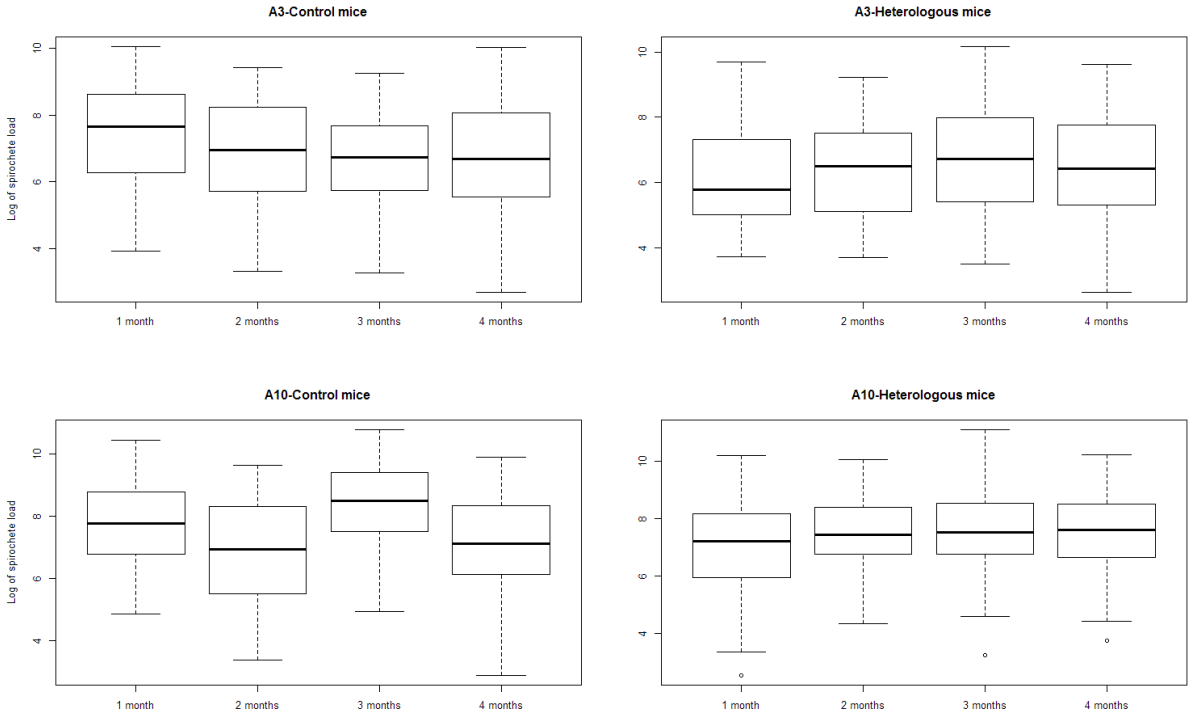

Figure 1. The log-transformed *B. afzelii* spirochete load of the 1-month-old *I. ricinus* nymphal ticks is shown as a function of the age of infection inside the mouse (1, 2, 3, or 4 months) for each of the four combinations of strain and immunization treatment. The two strains of *B. afzelii*, A3 and A10, are shown in the top and bottom rows, respectively. The two immunization treatments, control and heterologous, are shown in the left and right columns, respectively. The control mice were immunized with PBS. The heterologous mice were immunized with the rOspC antigen that did not protect the mice from the subsequent infectious challenge via tick bite. Shown are the median (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (solid circles).

6. General discussion

This PhD thesis, based on one main experiment, allowed us to test a variety of hypotheses of how the rodent immune system interacts with the Lyme disease pathogen *B. afzelii*. In particular, this work addresses three main subjects: (1) the effects of acquired immunity in the vertebrate host on the transmission of *B. afzelii*, (2) the difference in transmission phenotypes between genetically different *B. afzelii* strains, and (3) the maintenance of the pathogen in the vertebrate host and the tick vector.

Immunization with the rOspC antigen protected mice from infection with the matching homologous *ospC* strain. Previous studies on *B. afzelii* and *B. burgdorferi* s. s. had shown that immunization with rOspC protects mice from infectious challenge with strains carrying the same *ospC* allele (Preac-Mursic et al., 1992; Probert and Lefebvre, 1994; Gilmore et al., 1996; Scheiblhofer et al., 2003). However, studies investigating cross-protection to strains carrying different *ospC* alleles are relatively rare (Probert et al. 1997) and have not been conducted in *B. afzelii*. We found that there was limited cross-protection of the OspC-specific antibody response. Mice immunized with rOspC A3 were not completely protected from infectious challenge with strain A10 and vice versa. The highly variable OspC antigen thus allows *Borrelia ospC* strains to super-infect mice that have previous immune experience with other OspC antigens. Evidence that the OspC-specific antibody does not cross-protect rodents against other *ospC* strains in the field comes from the observation that wild rodents are frequently infected with multiple *ospC* strains (Brisson and Dykhuizen, 2004; Anderson and Norris, 2006; Pérez et al., 2011; Andersson et al., 2013). In the present study, we used immunization with the OspC antigen to test whether acquired immunity and cross-immunity influenced other aspects of the epidemiology of *B. afzelii*.

Acquired immunity in laboratory rodents reduced the efficacy of co-feeding transmission of *B. afzelii*. For strain A10, immunizing mice with rOspC A10 reduced the efficacy of co-feeding by 15-fold (from 51.6 % to 3.3 %). Co-feeding transmission was not completely blocked on OspC-immunized rodents. Five mice protected from systemic infection still produced some infected ticks via co-feeding. However, the co-feeding transmission rate on these mice was so low (1.8% and 3.3% for strains A3 and A10, respectively) that this strategy is unlikely to make a significant contribution to pathogen fitness (Hartemink et al., 2008). Thus our prediction that *B. afzelii* uses co-feeding transmission to evade acquired immunity in the vertebrate host was not supported, as the OspC-specific antibody response blocked 93.6 % of the co-feeding transmission of strain A10. Studies on other tick-borne pathogens have also shown that acquired immunity reduces co-feeding transmission but there is much variation in the magnitude of the effect size (Jones and Nuttall, 1989; Labuda et al., 1997b; Levin and Fish, 2000).

We found some cross-protective acquired immunity between the two strains of *B. afzelii*. Of the mice immunized with rOspC A10, 28.6% (2/7) were protected from the infectious challenge with strain A3. In contrast, immunization with rOspC A3 did not protect mice against strain A10. This asymmetric cross-immunity gives the dominant strain a two-fold competitive advantage over the weaker strain (Read and Taylor, 2001; Frank, 2002). First, the dominant strain induces an antibody response that blocks the weaker strain from super-infecting the same host. Second, the dominant strain is not affected by cross-immunity and can therefore super-infect hosts carrying the weaker strain. A recent field study found that A10 was one of the more common *ospC* strains in populations of *I. ricinus* at two different sites in Switzerland (Pérez et al., 2011; Tonetti et al., 2015). One potential explanation for the high frequency of strain A10 is the asymmetric cross-immunity advantage observed in the

present study. Another explanation is that strain A10 has high fitness in rodent hosts compared to other strains of *B. afzelii* (Tonetti et al., 2015).

The present study also allows us to speculate on the mechanism by which OspC-specific antibodies protect mice against infection with *B. afzelii*. The immunization treatment did not influence the prevalence of infection or the spirochete load in the blood-engorged challenge nymphs. This observation suggests that OspC-specific antibodies are not borreliacidal, as previously demonstrated by Gilmore et al. (1996). The OspC-specific antibodies could block migration of the spirochetes from the tick midgut to the tick salivary glands (Gilmore and Piesman, 2000), or kill the spirochetes at the site of the tick bite. These two hypotheses are not mutually exclusive (Ohnishi et al., 2001). Moreover, tick saliva contains a number of substances that suppress and modulate the host immune response (Wikel, 1999; Kuthejlova et al., 2001; Kotál et al., 2015). The feeding lesion of the tick in the skin of the vertebrate host represents an immuno-suppressed area that facilitates growth and replication of the local spirochete population. However, modulation of host immunity by tick saliva was not sufficient to evade the OspC-specific antibodies that ultimately blocked co-feeding transmission.

To test the effect of cross-immunity on the transmission of *B. afzelii*, we compared the heterologous group (mice immunized with the wrong OspC antigen) with the control group (mice that were completely immunologically naive). Cross-immunity had no effect on the rate of systemic transmission or on the rate of co-feeding transmission. However, we found a significant effect of cross-immunity on the tick spirochete load. Previous immune experience with the wrong OspC antigen allowed the heterologous mice to produce a more effective antibody response against the *B. afzelii* infection. This cross-immunity advantage subsequently reduced the spirochete load in the ticks that fed on the heterologous mice. *Borrelia* pathogens down-regulate the expression of the OspC antigen about two weeks after

they have established a systemic infection in the host (Zhong et al., 1997; Crother et al., 2004; Liang et al., 2004). OspC-specific antibodies are therefore not expected to influence host-to-tick transmission. However, the control of gene expression is not 100% perfect (Gilmore and Piesman, 2000; Ohnishi et al., 2001) and OspC-specific antibodies could clear any spirochetes that accidentally expressed the OspC antigen in the rodent host or the tick vector. We found no effect of cross-immunity on the spirochete load in the mouse tissues. Thus the observed cross-immunity effect on the tick spirochete load probably occurred after the host-to-tick transmission event. Perhaps the OspC-specific antibodies in combination with other components of the vertebrate immune system (e.g. the complement system or phagocytic cells) killed some of the spirochetes inside the midgut of the larval tick during the blood meal. A recent study found that the innate immune response of the vertebrate host plays an important role in structuring the spirochete load inside *I. ricinus* nymphs (Herrmann et al., 2013). In the present study, we showed that cross-reactive acquired immunity can also influence spirochete load inside *I. ricinus* nymphs. The 2-fold reduction in spirochete load observed in the present study could have important consequences for spirochete fitness.

In contrast to the cross-immunity effect on the spirochete load of ticks infected via systemic transmission, we found no effect of cross-immunity on the spirochete load in ticks infected via co-feeding transmission. We interpret this result as evidence that co-feeding transmission allowed *B. afzelii* to evade the negative effects of the cross-immunity. Previous immune experience with the ‘wrong’ OspC antigen allowed the heterologous mice to develop a more effective antibody response against the *B. afzelii* infection compared to the control mice. However, this infection-specific antibody response would have taken two or more weeks to develop. Co-feeding occurred during the five days following the infectious challenge and so there was not enough time for the infection-specific antibody response to interfere with co-feeding transmission. In contrast, the mice were infested with the second batch of larval

ticks at four weeks after the infectious challenge at which point in time the infection-specific antibody response was highly developed and capable of interfering with systemic transmission. Thus the instantaneous nature of co-feeding relative to the infectious challenge allowed this mode of transmission to evade the time-lagged, negative effects of cross-immunity.

The capacity of *B. afzelii* to infect the vertebrate host is probably related to the number of spirochetes that are transmitted during the infected tick bite (Schmid-Hempel, 2008). The present study showed that there are many factors that can influence the spirochete load inside the nymphal tick. The mode of transmission had an important effect on the spirochete load in nymphs infected as larvae. The spirochete load was up to six times lower in nymphs infected as larvae via co-feeding than in nymphs infected as larvae via systemic transmission. The strain of *B. afzelii* also affected the nymphal spirochete load, as it was lower for strain A3 than A10. The higher spirochete load of strain A10 in the tick vector may be another explanation as to why this strain is so common in different locations in Europe (Pérez et al., 2011; Andersson et al., 2013). The age of the infection in the vertebrate host also created variation in the nymphal spirochete load.

The age at which the nymphal tick was killed also influenced the spirochete load detected in the nymphal ticks. The spirochete load in nymphs killed four months after the larva-to-nymph molt was 10 times lower than that of nymphs killed one month after the molt, even though both of these nymphs had fed as larvae on the same mice at the same time. This result suggests that the spirochete population inside the nymph declines over time following the larva-to-nymph molt.

OspC is a critical pathogen virulence factor for host infection (Pal et al., 2004a; Stewart et al., 2006; Fingerle et al., 2007; Radolf and Caimano, 2008; Tilly et al., 2009;

Seemanapalli et al., 2010; Tilly et al., 2013). *Borrelia* spirochetes express the OspC antigen in the midgut of the tick, at the site of the tick bite, and during dissemination inside the vertebrate host. The temporal pattern of expression makes the OspC antigen an accessible target for the immune system of the vertebrate host. The present study showed that the OspC-specific antibody response is efficient at blocking both co-feeding and systemic transmission. Selection by the vertebrate immune system has resulted in the diversification of the *ospC* gene to facilitate super-infection of the vertebrate host.

Fast development of a systemic infection in the vertebrate host will lead to earlier transmission to naïve larval ticks that feed on that host. The persistence of the systemic infection in the vertebrate host is important for maintaining high levels of host-to-tick transmission over the duration of the transmission season of Lyme disease. The number of spirochetes acquired by the larval tick may be important because the larval tick will encounter many metabolic changes during its molt into the nymphal stage. A small number of spirochetes acquired by the tick at the larval stage could result in a lower number of spirochetes transmitted during the nymphal blood meal. The present study showed that nymphal aging reduces the tick spirochete load over time.

The spirochete load was lower in ticks infected via co-feeding compared to ticks infected via systemic transmission. Previous studies have investigated the dynamics of the spirochete population inside the tick vector over a short time period (molting phase or the weeks post-challenge). Future studies should investigate whether the nymphal spirochete load and the mode of transmission by which the spirochete infection was acquired influence tick-to-host transmission.

In summary, this PhD thesis provides new information on the host-parasite interactions of an important Lyme disease pathogen, on the effect of acquired immunity in the vertebrate host on pathogen transmission, and on the mechanism of co-feeding transmission.

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