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Colonization and pathology of *Borrelia afzelii* in its natural hosts

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ABSTRACT

Studies of *Borrelia burgdorferi* sensu lato in laboratory mice and humans have shown that spirochaetes disseminate from the site of infection (skin) to internal tissues, and cause various pathological effects. However, less is known about colonization and pathology of Lyme borreliosis spirochaetes in their natural hosts. In the present study, we assessed the colonization and manifestations during *B. afzelii* infection in reservoir hosts (yellow-necked mouse, *Apodemus flavicollis*; bank vole, *Myodes glareolus*; common shrew, *Sorex araneus*) infected in the wild. The infection prevalence and bacterial load was measured in skin (ear), joints and heart by quantitative PCR, and pathology in infected joints was evaluated by histology. The prevalence of *B. afzelii* was higher in skin than in joints and heart, but most animals that were positive in skin were also positive in internal tissues, and there was no difference between species in tissue-specific prevalence. Thus, spirochaetes disseminated from skin to other tissues in a similar way in all species. The bacterial load varied among host species and among different tissues within the same host species. In the case of skin and joints, bank voles and common shrews had higher bacterial loads than yellow-necked mice. In hearts, voles had higher bacterial loads than shrews and mice. Histological analyses showed no inflammation in joints of infected animals when compared to controls. We conclude that *B. afzelii* disseminates to internal tissues in natural hosts, but that levels of colonization vary between both species and tissues. There is as yet little evidence for pathological effects in natural hosts.

1. Introduction

Lyme borreliosis is a common human vector-borne disease in North America and Eurasia. It is caused by spirochaete bacteria belonging to the species complex *Borrelia burgdorferi* sensu lato (s.l.), of which *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, and *B. garinii* are the major causative species (Kurtenbach et al., 2006; Stanek and Reiter, 2011). Lyme borreliosis spirochaetes are transmitted between a variety of vertebrate hosts by hard ticks in the genus *Ixodes*. *B. burgdorferi* s.s. occurs in North America and Europe and has a wide spectrum of reservoir hosts, including both rodents and birds. *B. afzelii* and *B. garinii* occur in Eurasia and are more host-specific, with rodents and birds, respectively, as principal reservoirs (Gern et al., 1998; Kurtenbach et al., 1998, 2001, 2002; Humair and Gern, 2000; Hanincova et al., 2003; Becker et al., 2016)

In humans, a typical early clinical symptom of Lyme borreliosis is a characteristic skin lesion (erythema migrans) at the tick bite site. When the infection is left untreated, spirochaetes can disseminate throughout the blood to multiple organs, including the central nervous system, joints and the heart, and cause diverse clinical manifestations including neuroborreliosis, Lyme arthritis and carditis (Stanek et al., 2012). Interestingly, different *Borrelia* species are associated with different symptoms in humans. *B. burgdorferi* s.s. infection is often associated with Lyme arthritis, whereas *B. garinii* infection is mostly linked to neuroborreliosis, and *B. afzelii* is primarily associated with chronic skin condition called acrodermatitis (Balmelli and Piffaretti, 1995; Wang et al., 1999; Coipan et al., 2016). Lyme carditis is one of the rarer organ manifestations and was seen in only 4–10% of all untreated patients with Lyme borreliosis, and the association with *Borrelia* species was not very clear (Rudenko et al., 2011; Scheffold et al., 2015).

Some inbred strains of the laboratory mouse *Mus musculus* infected with *B. burgdorferi* s.s. have been found to show similar symptoms to those of human Lyme borreliosis. For example, when C3H and BALB/c mice are infected with *B. burgdorferi* s.s, they develop both ankle joint arthritis and carditis. In contrast, other mouse strains, such as C57BL/6 and DBA, display minimal inflammation in the heart and joints (Barthold et al., 1990; Wooten and Weis, 2001; Brown et al., 2004; Radolf et al., 2012; Lin et al., 2014). Unlike humans, mice do not develop neuroborreliosis, unless the spirochaetes are injected intracerebrally (Li et al., 2006; Pachner and Steiner, 2007). Infection experiments with *B. afzelii* in laboratory animals (gerbil and laboratory mice) showed that this *Borrelia* species also disseminates from skin to

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internal tissues, including heart, bladder and joints (Preac-Mursic et al., 1992; Pechová et al., 2002; Belli et al., 2017; Bhatia et al., 2018).

Studies of *B. burgdorferi* s.s infections in a natural host, the white-footed mouse (*Peromyscus leucopus*), in the field and laboratory showed that spirochaetes disseminate to joint and heart (like in laboratory mice and humans), but there was no observable pathology in adults, while juveniles developed carditis and multifocal arthritis, similar to what was observed in laboratory mice (Moody et al., 1994; Barbour, 2017). Less is known about tissue colonization and pathology of *B. afzelii* in its natural hosts. Previous studies have found that *B. afzelii* infection intensities (number of *Borrelia* spirochaetes/unit host tissue) as measured in skin biopsies from ears differ considerably between natural host species, with bank voles (*Myodes glareolus*) and common shrews (*Sorex araneus*) having ten-fold higher infection intensities than yellow-necked mice (*Apodemus flavicollis*) (Råberg, 2012; Strandh and Råberg, 2015). However, it is not known if there are also differences in tissue colonization and pathology between species.

In the current study, we investigated the tissue colonization and pathology of *B. afzelii* in four natural host species (*M. glareolus*, *A. flavicollis*, *S. araneus* and *S. minutus*). Specifically, we investigated: (i) Whether the degree of dissemination differs between host species, where dissemination is measured as the prevalence (proportion of individuals that are positive) of spirochaetes in different tissues. (ii) If the bacterial load differs between species and tissues, where load is measured as abundance of spirochaetes in infected animals. (iii) Whether *B. afzelii* infection in natural hosts causes pathology in the form of inflammation in the affected tissues.

2. Materials and methods

2.1. Field work

All samples used in this study were collected from bank voles (*M. glareolus;* n = 69), yellow-necked mice (*A. flavicollis;* n = 32), common shrews (*S. araneus;* n = 15), and pygmy shrews, (*S. minutus;* n = 11). These animals had been trapped in the Revinge area in southern Sweden during August - September in 2015 and 2016. These four species are the most common hosts of *B. afzelii* in this area (Hellgren et al., 2011). All animals were caught with live traps (Ugglan special, Grahn AB, Sweden) that had been baited with grains and apple. Animals were weighed and sacrificed before collecting ear biopsies, the heart and two rear ankles. In 2015, we collected the whole heart, left and right joint, and ear biopsies (two from each ear) for DNA extraction; in 2016, collection of ear biopsies and heart was performed as in 2015, but only one joint was collected for DNA extraction; the other joint was preserved for histological analysis. Tissue samples for DNA extraction were stored in 70% ethanol, while tissues for histological analysis were stored in formalin.

2.2. Detection and quantification of B. afzelii

Total DNA was extracted from the ear biopsies and hearts following the protocol of Laird et al. (1991). The method applied for DNA extraction from joints was similar, but samples were homogenized with the TissueLyser II (Qiagen). Two samples of each tissue from individuals were prepared for DNA extraction. The quantity and quality of DNA for each tissue sample was assessed by measuring the DNA concentration and the ratio of the UV absorption at 260 to 280 nm by Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA). For the PCR, the DNA was diluted to a concentration of 25 ng/µl. Reactions were performed with SYBR Green–based detection (Platinum SYBR Green qPCR SuperMIX-UDG; Invitrogen, Carlsbad, CA) in an Mx3000 thermocycler (Stratagene), using primers targeting the *flaB* gene of *B. afzelii* (Fla5F 5'-CACCAGCATCACTTTCAGGA-3' and Fla6R 5'-CTCC CTCACCAGCAAAAAGA-3'), as described in Råberg (2012). Each PCR reaction contained a total volume of 25 µl with 4 µl of DNA template. A 2-minute initial incubation at 50 °C and a 2-minute denaturation at 95 °C was followed by 42 cycles of 95 °C for 15 s, 59 °C for 30 s, and 72 °C for 30 s. At least eight negative controls were included on each plate to check for contamination. To quantify the number of *B. afzelii* spirochaetes in a sample, a standard was prepared by amplifying a longer part of *flaB* (Bunikis et al., 2004). The obtained product was purified, diluted and run as a serially diluted standard (in steps of 1: 5) on each plate. The number of *flaB* copies in the most concentrated standard well was estimated to be 16,600. Each sample was run in duplicates on separate plates. Samples with a melting temperature between 78.15 °C and 78.85 °C and a Ct value corresponding to greater than or equal to 1 gene copy were considered positive. Bacterial loads were expressed as number of spirochaetes (as measured by qPCR targeting to the *flaB* gene) per nanogram of host DNA (as measured by Nanodrop). Bacterial loads were log-transformed ($\log_{10}$(bacterial load + 1)) to meet the requirements of parametric tests.

The repeatability (intra-class correlation coefficient, calculated as the variance between samples divided by the total variance; Sokal and Rohlf, 1995) of the quantification of bacterial load (of positive individuals) between different analyses of the same sample was 0.94, while the repeatability between different samples of the same tissue from an individual was 0.90.

2.3. Evaluation of inflammation in joints

For histological evaluations of joints, one rear ankle from each animal was collected from bank voles and yellow-necked mice. Joints were fixed in 4% phosphate-buffered formaldehyde, demineralized and processed for hematoxylin and eosin staining by routine histologic techniques. Joint tissue sections were blindly examined without knowledge of infection. Overall signs of arthritis were based on a combined assessment of histological parameters of *B. burgdorferi*–induced inflammation, such as immune cell infiltration into the joint cavity, alteration in the thickness of tendons or ligament sheaths, and hypertrophy of the synovium (Lin et al., 2014).

2.4. Statistical analyses

The concordance of infection status in different tissues was calculated as (no. of individual positive in both tissues + no. of individuals negative in both tissues)/all individuals, where 100% means perfect agreement in infection status between two tissues.

To test for differences in prevalence of *B. afzelii* among host species and tissues, we performed generalized linear mixed models with binomial error distribution, using proc glimmix in SAS 9.3 (SAS Inc, NC, USA) (dependent variable: presence/absence of *B. afzelii*; fixed factors: host species, tissue, year and their interactions; random effect: host individual). Parameters were estimated by Laplace approximation and statistical significance of the random effect was determined by a likelihood ratio test, as recommended by Bolker (Bolker et al., 2009).

To test for differences in bacterial loads of *B. afzelii* among host species and tissues, we performed general linear mixed models, using proc mixed in SAS 9.3 (dependent variable: bacterial load; fixed factors: host species, tissue, year and their interactions; random effect: host individual). Denominator degrees of freedom were determined by Satterthwaite approximation.

To test if bacterial load in skin (ear) predicted bacterial load in other tissues, we performed ANCOVAs with bacterial load in joint or heart against bacterial load in ear, species, and their interaction, using proc glm in SAS 9.3.

In all analyses, non-significant terms ($p > 0.1$) were deleted in a stepwise manner (interactions first). P values for fixed effects were determined by F tests (type 3).

Table 1
Numbers and body mass of trapped animals of the different species, and number of *B. afzelii*-positive animals per species and tissue. (No. = number).

Species	No.	Body mass mean ± SD(g)	Tissue-specific prevalence			Overall prevalence
			ear	heart	joint	
bank vole (*M. glareolus*)	69	20.25 ± 4.58	22/69 (32%)	13/69 (19%)	15/69 (22%)	23/69 (33%)
yellow-necked mouse (*A. flavicollis*)	32	32.44 ± 6.52	18/32 (56%)	11/31 (36%)	15/32 (47%)	19/32 (59%)
common shrew (*S. araneus*)	15	7 ± 0.8	7/15 (47%)	4/15 (27%)	7/15 (47%)	8/15 (53%)
pygmy shrew (*S. minutus*)	11	3 ± 0	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)

3. Results

3.1. Overall infection prevalence in different species

In total, we obtained samples from 127 animals from four species (Table 1). The concordance of infection prevalence in different tissues was high: ear vs joint: 93.0%; ear vs heart: 81.9%; heart vs joint: 82.7% (Fig. S1). The prevalence of infection (where an individual animal was scored as infected if it was positive in ≥ 1 tissue) varied between species (Fisher's exact test: $p < 0.001$). The prevalence was highest in the yellow-necked mice (59% = 19/32) and common shrews (53% = 8/15), lower in the bank voles (33% = 23/69) and zero in the pygmy shrews (0% = 0/11). Since the infection prevalence was zero in this sample of pygmy shrews, the analyses below focus exclusively on the other three species, that is, yellow-necked mouse, bank vole, and common shrew.

3.2. Dissemination

There was no significant difference in prevalence of *B. afzelii* between yellow-necked mice, bank voles and common shrews ($F_{2, 229} = 0.36$, $p = 0.7$), and no tissue-by-species interaction ($F_{4, 225} = 1.62$, $p = 0.17$). The prevalence of *B. afzelii* in these three species was 40.5% in skin, 31.9% in joints, and 24.4% in heart. The difference in prevalence between tissues was statistically significant ($F_{2, 229} = 7.79$, $p = 0.0005$; Tukey post hoc tests: ear vs heart: $p = 0.0003$; ear vs joint: $p = 0.043$; heart vs joint: $p = 0.076$). The random effect of individual was highly significant ($p < 0.0001$).

3.3. Bacterial load

For the subsample of infected individuals, the bacterial load differed between host species ($F_{2, 93} = 18.32$, $p < 0.0001$; Fig. 1). The bacterial load also differed between tissues ($F_{2, 93} = 40.52$, $p < 0.0001$), with ear and joint having more spirochaetes than heart (Tukey: ear vs heart: $p < 0.0001$; joint vs heart: $p < 0.0001$). The random effect of individual was significant (variance estimates: individual = 0.062 ± 0.037, $p = 0.047$, residual = 0.31 ± 0.045), showing that there were consistent differences in bacterial load between individual hosts (after controlling for the effect of host species). The species × tissue interaction was also significant ($F_{4, 93} = 6.29$, $p = 0.0002$). Separate analyses for each tissue showed that this was a result of bank voles and common shrews having higher bacterial load than yellow-necked mice in ear and joint (ear: $F_{2, 47} = 8.54$, $p < 0.0007$; joint: $F_{2, 47} = 14.83$, $p < 0.0001$), whereas bank voles have higher bacterial load than shrews and mice in heart ($F_{2, 46} = 3.79$, $p = 0.03$).

To test if bacterial load in ear predicted bacterial load in heart and joint, we performed ANCOVAs with bacterial load in heart or joint against bacterial load in ear, species, and their interaction. There was a positive but not significant relationship between bacterial load in ear and heart (ear: $F_{1, 48} = 2.70$, $p = 0.11$; species: $F_{2, 48} = 2.66$, $p = 0.08$; the ear × species interaction was not significant and therefore excluded from the model: $F_{2, 48} = 0.78$, $p = 0.46$)) (Fig. 2a). There was a significant positive relationship between bacterial load in ear and joint (ear: $F_{1, 49} = 4.26$, $p = 0.044$; species: $F_{2, 49} = 7.08$, $p = 0.002$; the ear × species interaction was not significant and therefore excluded from the model: ear × species: $F_{2, 49} = 1.79$, $p = 0.18$) (Fig. 2b).

3.4. Disease manifestations

To determine whether the observed *B. afzelii* infection in joints of wild rodents resulted in inflammation, bank voles and yellow-necked mice were subjected to histological analysis. We evaluated 24 tibiotarsal joints from bank voles (8 infected and 16 uninfected) and 19 tibiotarsal joints from yellow-necked mice (5 infected and 14 uninfected). No inflammatory cells and pathological signs were observed in surrounding tendons and in the synovium when comparing the infected and uninfected groups (Fig. 3).

4. Discussion

The prevalence of *B. afzelii* spirochaetes was higher in skin (ear) than in joints and heart, but most animals that were positive in skin also had spirochaetes in internal tissues. Moreover, the prevalence in different tissues was similar across species. Thus, *B. afzelii* readily disseminates from the skin to joint and heart tissue in its main natural hosts, and there are no differences between host species in dissemination patterns.

In all three host species, bacterial loads in joints were as high as in the skin, whereas bacterial loads were lower in heart. The bacterial loads also differed between host species. As in previous studies (Råberg, 2012; Råberg et al., 2017), yellow-necked mice had > 10-fold lower spirochaetes load than voles and shrews in skin. The present study showed a similar difference between species in joint bacterial loads. This contrasts with the pattern in heart, where both shrews and mice had significantly lower bacterial loads than voles. One explanation for the species-specific difference in bacterial loads between tissues is that the ability of *B. afzelii* to replicate in and attach to different tissues depends on which host species it infects. An alternative explanation is that the immune response (and thereby the clearance rate of *B. afzelii*) varies between species in a tissue-specific way.

The patterns of dissemination and tissue-specific bacterial loads of *B. afzelii* in its natural hosts are similar to patterns observed from *B. burgdorferi* s.s. in the white-footed mouse (Baum et al., 2012); in both cases high spirochaetal loads in skin and joints, but much lower in heart. The pattern for *B. afzelii* and *B. burgdorferi* s.s. in natural host species differ somewhat from those observed in laboratory mice infected with *B. burgdorferi* s.s., where several studies have found that there is not much difference (less than 10-fold) in spirochaetal loads between skin, heart and joints (Wang et al., 2001; Hodzic et al., 2013; Imai et al., 2013; Lin et al., 2014).

The variation in bacterial loads among host species could be a result of host species differing in resistance to *B. afzelii,* and/or that different host species are infected by different *B. afzelii* genotypes. In the case of *B. burgdorferi* s.s., at least 25 different *ospC* (outer surface protein C) genotypes have been recognized in ticks, and they vary in dissemination ability and bacterial load in both humans and laboratory mice (Jones et al., 2006; Dykhuizen et al., 2008; Chan et al., 2012). Like *B. burgdorferi* s.s., *B. afzelii* has several *ospC* genotypes (Lagal et al., 2003; Bunikis et al., 2004; Hellgren et al., 2011; Durand et al., 2015). In laboratory mice, *B. afzelii* strains differed in bacterial loads in ear tissues (Jacquet et al., 2015). However, a recent field study of the same rodent populations as in the present study found no difference in prevalence of different *ospC* strains (in skin biopsies) among host species (Råberg et al., 2017). Hence, there is no indication that the difference in

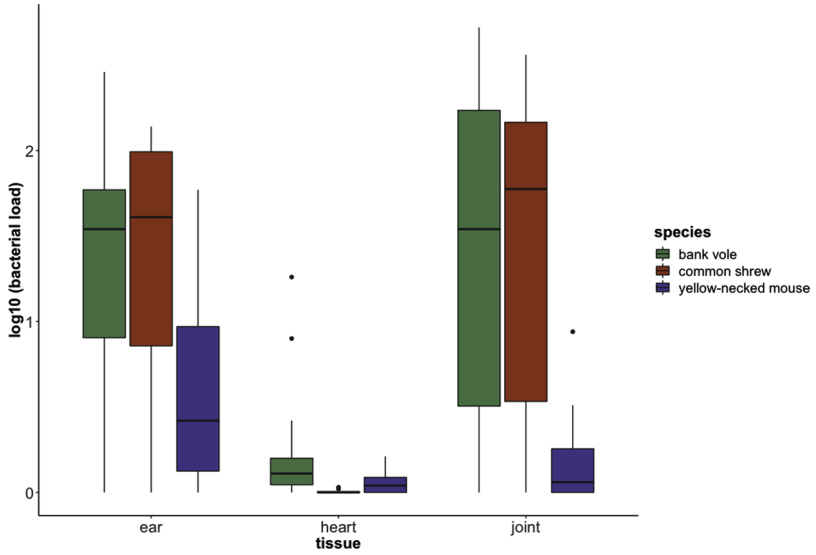

Fig. 1. Box plot of bacterial load in different tissues in three small mammal species. The box plots indicate the median, first and third quartiles, and range of the data. Bacterial load was estimated with qPCR of *B. afzelii flaB*.

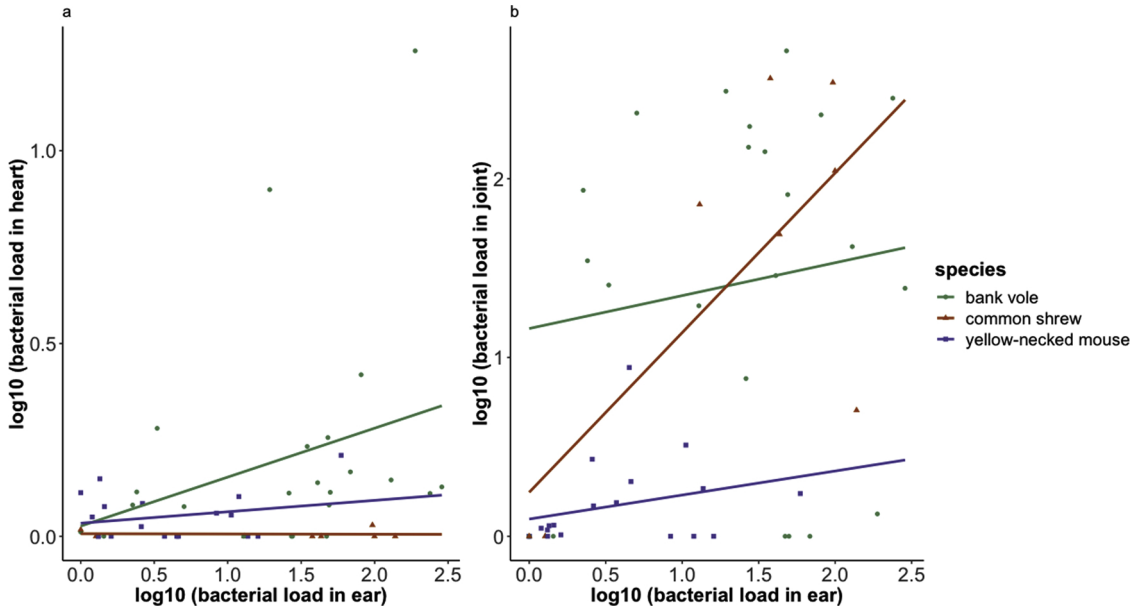

Fig. 2. Correlation between tissue-specific bacterial loads measured from bank vole, yellow-necked mouse and common shrew. (a) Correlation of bacterial load between ear and heart; (b) correlation of bacterial load between ear and joint.

bacterial loads between host species is caused by that host species are infected by different *B. afzelii* strains, although we cannot rule out such an effect without data on the composition of *ospC* strains in different tissues. Still, we favor the interpretation that differences in bacterial loads are a result of variation in resistance among host species. This interpretation is consistent with the previous finding that *Apodemus* mice have stronger antibody responses to experimental *B. burgdorferi* s.s. infection than bank voles (Kurtenbach et al., 1994).

One potential problem with the present study is that it is based on naturally infected wild animals, and that the time of infection therefore is not known. Previous infection experiments with laboratory mice have shown that the bacterial loads varies during the course of an infection, and that this variation is not well synchronized across tissues (Hodzic et al., 2003). Hence, one explanation for the difference in bacterial loads between tissues in our study, in particular the lower spirochaetes load in heart, is that *B. afzelii* has not yet had time to establish infection in this tissue. Another potential problem is that the majority of naturally infected animals have “mixed infections”, that is infections that consist of more than one *OspC* strain (Strandh and Råberg, 2015). Thus, the difference in bacterial loads between tissues could be a result of that different *ospC* strains preferentially colonize different tissues, as has been observed in infection experiments with *B. burgdorferi* in laboratory mice (Wang et al., 2001, 2002). Ultimately, controlled infection experiments are therefore required to confirm the pattern we found here.

Lyme borreliosis spirochaetes transmit to ticks directly from skin, and the spirochaetes load in the skin is positively related with host-to-tick transmission (Råberg, 2012; Jacquet et al., 2015). Thus, it is critical for the fitness of *B. burgdorferi* s.l. to colonize and persist in the skin of the vertebrate host, so why do spirochaetes disseminate from the skin to internal tissues like joint and heart in the natural hosts? To disseminate from the site of tick bite, *B. burgdorferi* expresses a variety of proteins that mediate attachment to host cells and tissues. Some of these proteins, for example BBK32 and DbpA, have been shown to preferentially

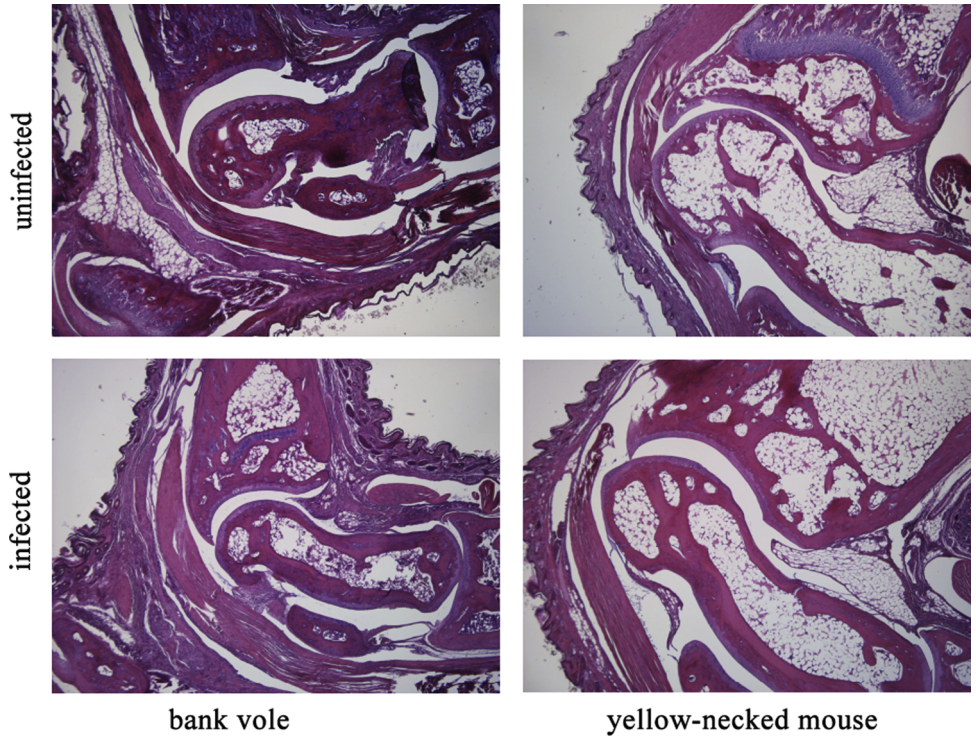

Fig. 3. Representative demonstration of joint histology in bank voles and yellow-necked mice infected by *B. afzelii*.

bind to host proteins expressed in joint tissue (Lin et al., 2014, 2015). Thus, it seems dissemination to internal tissues is not just a side effect of dissemination through the skin, but that *Borrelia* has adaptations that specifically enhance this kind of dissemination. One possible explanation is that joint tissue, specifically the synovial fluid, represents an immune privileged site (Embers et al., 2003). Thus, spirochaetes in joints and perhaps other tissues might represent a reservoir population from which spirochaetes can migrate back to skin and be transmitted to ticks. Support for this scenario comes from a study of *B. burgdorferi* s.s. in antibiotic-treated mice, which found that even though spirochaetes were only detectable in internal tissues such as heart and joint, xenodiagnosis showed transmission to ticks (Hodzic et al., 2008). Further studies on these persisting spirochaetes in internal tissues are required to explore the roles they play in the life cycle of *B. burgdorferi* s.l. in nature.

Our finding that *B. afzelii* infections did not cause any measurable inflammation in the joints of wild rodents is consistent with previous studies of *B. burgdorferi* s.s. in *Apodemus* mice and bank voles (Kurtenbach et al., 1994) and white-footed mice (Schwanz et al., 2011; Barbour, 2017). The apparently minimal pathology, at least in adult hosts, also fits with the finding that *B. burgdorferi* s.s. infections had no effect on the survival of its reservoir host (Hofmeister et al., 1999; Voordouw et al., 2015). Nevertheless, a recent field experiment found that *B. afzelii* infection impairs reproduction of bank voles (Cayol et al., 2018), but the mechanisms behind this effect were not investigated. Clearly, the nature and extent of *Borrelia* pathology in natural hosts requires further investigation.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.03.017.

References

Balmelli, T., Piffaretti, J.C., 1995. Association between different clinical manifestations of Lyme disease and different species of *Borrelia burgdorferi* sensu lato. Res. Microbiol. 146, 329–340. https://doi.org/10.1016/0923-2508(96)81056-4.

Barbour, A.G., 2017. Infection resistance and tolerance in *Peromyscus* spp., natural reservoirs of microbes that are virulent for humans. Semin. Cell Dev. Biol. 61, 115–122. https://doi.org/10.1016/j.semcdb.2016.07.002.

Barthold, S.W., Beck, D.S., Hansen, G.M., Terwilliger, G.A., Moody, K.D., 1990. Lyme borreliosis in selected strains and ages of laboratory mice. J. Infect. Dis. 162, 133–138. https://doi.org/10.1093/infdis/162.1.133.

Baum, E., Hue, F., Barbour, A.G., 2012. Experimental infections of the reservoir species *Peromyscus leucopus* with diverse strains of *Borrelia burgdorferi*, a Lyme disease agent. MBio 3, 1–11. https://doi.org/10.1128/mBio.00434-12.

Becker, N.S., Margos, G., Blum, H., Krebs, S., Graf, A., Lane, R.S., Castillo-Ramírez, S., Sing, A., Fingerle, V., 2016. Recurrent evolution of host and vector association in bacteria of the *Borrelia burgdorferi* sensu lato species complex. BMC Genomics 17, 734. https://doi.org/10.1186/s12864-016-3016-4.

Belli, A., Sarr, A., Rais, O., Rego, R.O.M., Voordouw, M.J., 2017. Ticks infected via co-feeding transmission can transmit Lyme borreliosis to vertebrate hosts. Sci. Rep. 7, 5006. https://doi.org/10.1038/s41598-017-05231-1.

Bhatia, B., Hillman, C., Carracoi, V., Cheff, B.N., Tilly, K., Rosa, P.A., 2018. Infection history of the blood-meal host dictates pathogenic potential of the Lyme disease spirochete within the feeding tick vector. PLoS Pathog. 14, e1006959. https://doi.org/10.1371/journal.ppat.1006959.

Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H.H., White, J.S.S., 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends Ecol. Evol. https://doi.org/10.1016/j.tree.2008.10.008.

Brown, C.R., Blaho, V.A., Loiacono, C.M., 2004. Treatment of mice with the neutrophil-depleting antibody RB6-8C5 results in early development of experimental lyme arthritis via the recruitment of Gr-1- polymorphonuclear leukocyte-like cells. Infect. Immun. 72, 4956–4965. https://doi.org/10.1128/IAI.72.9.4956-4965.2004.

Bunikis, J., Garpmo, U., Tsao, J., Berglund, J., Fish, D., Barbour, A.G., 2004. Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. Microbiology 150, 1741–1755. https://doi.org/10.1099/mic.0.26944-0.

Cayol, C., Giermek, A., Gomez-Chamorro, A., Hytönen, J., Kallio, E.R., Mappes, T., Salo, J., Voordouw, M.J., Koskela, E., 2018. *Borrelia afzelii* alters reproductive success in a

rodent host. Proceedings Biol. Sci. 285, 20181056. https://doi.org/10.1098/rspb.2018.1056.
Chan, K., Awan, M., Barthold, S.W., Parveen, N., 2012. Comparative molecular analyses of *Borrelia burgdorferi* sensu stricto strains B31 and N40D10/E9 and determination of their pathogenicity. BMC Microbiol. 12, 157. https://doi.org/10.1186/1471-2180-12-157.
Coipan, E.C., Jahfari, S., Fonville, M., Oei, G.A., Spanjaard, L., Takumi, K., Hovius, J.W.R., Sprong, H., 2016. Imbalanced presence of *Borrelia burgdorferi* s.l. multilocus sequence types in clinical manifestations of Lyme borreliosis. Infect. Genet. Evol. 42, 66–76. https://doi.org/10.1016/j.meegid.2016.04.019.
Durand, J., Jacquet, M., Paillard, L., Rais, O., Gern, L., Voordouw, M.J., 2015. Cross-immunity and community structure of a multiple-strain pathogen in the tick vector. Appl. Environ. Microbiol. 81, 7740–7752. https://doi.org/10.1128/AEM.02296-15.
Dykhuizen, D.E., Brisson, D., Sandigursky, S., Wormser, G.P., Nowakowski, J., Nadelman, R.B., Schwartz, I., 2008. Short report: The propensity of different *Borrelia burgdorferi* sensu stricto genotypes to cause disseminated infections in humans. Am. J. Trop. Med. Hyg. 78, 806–810. https://doi.org/10.4269/ajtmh.2008.78.806.
Embers, M.E., Ramamoorthy, R., Philipp, M.T., 2003. Survival strategies of *Borrelia burgdorferi*, the etiologic agent of Lyme disease. Microbes Infect. 6, 312–318. https://doi.org/10.1016/j.micinf.2003.11.014.
Gern, L., Estrada-Peña, A., Frandsen, F., Gray, J.S., Jaenson, T.G.T., Jongejan, F., Kahl, O., Korenberg, E., Mehl, R., Nuttall, P.A., 1998. European reservoir hosts of *Borrelia burgdorferi* sensu lato. Zentralbl. Bakteriol. https://doi.org/10.1016/S0934-8840(98)80121-7.
Hanincova, K., Schäfer, S.M., Etti, S., Sewell, H.S., Taragelová, V., Ziak, D., Labuda, M., Kurtenbach, K., 2003. Association of *Borrelia afzelii* with rodents in Europe. Parasitology 126, 11–20. https://doi.org/10.1017/S0031182002002548.
Hellgren, O., Andersson, M., Råberg, L., 2011. The genetic structure of *Borrelia afzelii* varies with geographic but not ecological sampling scale. J. Evol. Biol. 24, 159–167. https://doi.org/10.1111/j.1420-9101.2010.02148.x.
Hodzic, E., Feng, S., Freet, K.J., Barthold, S.W., 2003. *Borrelia burgdorferi* population dynamics and prototype gene expression during infection of immunocompetent and immunodeficient mice. Infect. Immun. 71, 5042–5055. https://doi.org/10.1128/IAI.71.9.5042-5055.2003.
Hodzic, E., Feng, S., Holden, K., Freet, K.J., Barthold, S.W., 2008. Persistence of *Borrelia burgdorferi* following antibiotic treatment in mice. Antimicrob. Agents Chemother. 52, 1728–1736. https://doi.org/10.1128/AAC.01050-07.
Hodzic, E., Feng, S., Barthold, S.W., 2013. Assessment of transcriptional activity of *Borrelia burgdorferi* and host cytokine genes during early and late infection in a mouse model. Vector-Borne Zoonotic Dis. 13, 694–711. https://doi.org/10.1089/vbz.2012.1189.
Hofmeister, E.K., Ellis, B.A., Glass, G.E., Childs, J.E., 1999. Longitudinal study of infection with *Borrelia burgdorferi* in a population of *Peromyscus leucopus* at a Lyme disease-enzootic site in Maryland. Am. J. Trop. Med. Hyg.
Humair, P.F., Gern, L., 2000. The wild hidden face of Lyme borreliosis in Europe. Microbes Infect. https://doi.org/10.1016/S1286-4579(00)00393-2.
Imai, D.M., Scott Samuels, D., Feng, S., Hodzic, E., Olsen, K., Barthold, S.W., 2013. The early dissemination defect attributed to disruption of decorin-binding proteins is abolished in chronic murine lyme borreliosis. Infect. Immun. 81, 1663–1673. https://doi.org/10.1128/IAI.01359-12.
Jacquet, M., Durand, J., Rais, O., Voordouw, M.J., 2015. Cross-reactive acquired immunity influences transmission success of the Lyme disease pathogen, Borrelia afzelii. Infect. Genet. Evol. 36, 131–140. https://doi.org/10.1016/J.MEEGID.2015.09.012.
Jones, K.L., Glickstein, L.J., Damle, N., Sikand, V.K., McHugh, G., Steere, A.C., 2006. *Borrelia burgdorferi* genetic markers and disseminated disease in patients with early Lyme disease. J. Clin. Microbiol. 44, 4407–4413. https://doi.org/10.1128/JCM.01077-06.
Kurtenbach, K., Dizij, A., Seitz, H.M., Margos, G., Moter, S.E., Kramer, M.D., Wallich, R., Schaible, U.E., Simon, M.M., 1994. Differential immune responses to *Borrelia burgdorferi* in European wild rodent species influence spirochete transmission to *Ixodes ricinus* L. (Acari: Ixodidae). Infect. Immun. 62, 5344–5352.
Kurtenbach, K., Peacey, M., Rijpkema, S.G.T., Hoodless, A.N., Nuttall, P.A., Randolph, S.E., 1998. Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. Appl. Environ. Microbiol. 64, 1169–1174.
Kurtenbach, K., De Michelis, S., Sewell, H.S., Etti, S., Schäfer, S.M., Hails, R., Collares-Pereira, M., Santos-Reis, M., Hanincová, K., Labuda, M., Bormane, A., Donaghy, M., 2001. Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies found in individual questing ticks from Europe. Appl. Environ. Microbiol. 67, 4926–4929. https://doi.org/10.1128/AEM.67.10.4926-4929.2001.
Kurtenbach, K., De Michelis, S., Etti, S., Schäfer, S.M., Sewell, H.-S., Brade, V., Kraiczy, P., 2002. Host association of *Borrelia burgdorferi* sensu lato-the key role of host complement. Trends Microbiol. 10, 74–79. https://doi.org/10.1016/S0966-842X(01)02298-3.
Kurtenbach, K., Hanincová, K., Tsao, J.I., Margos, G., Fish, D., Ogden, N.H., 2006. Fundamental processes in the evolutionary ecology of Lyme borreliosis. Nat. Rev. Microbiol. 4, 660–669. https://doi.org/10.1038/nrmicro1475.
Lagal, V., Postic, D., Ruzic-Sabljic, E., Baranton, G., 2003. Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the ospC gene and its association with invasiveness. J. Clin. Microbiol. 41, 5059–5065. https://doi.org/10.1128/JCM.41.11.5059-5065.2003.
Laird, P.W., Zijderveld, A., Linders, K., Rudnicki, M.A., Jaenisch, R., Berns, A., 1991. Simplified mammalian DNA isolation procedure. Nucleic Acids Res. 19, 4293.
Li, L., Narayan, K., Pak, E., Pachner, A.R., 2006. Intrathecal antibody production in a mouse model of Lyme neuroborreliosis. J. Neuroimmunol. 173, 56–68. https://doi.org/10.1016/j.jneuroim.2005.11.019.
Lin, Y., Benoit, V., Yang, X., Martínez-Herranz, R., Pal, U., Leong, J.M., 2014. Strain-specific variation of the decorin-binding adhesin DbpA influences the tissue tropism of the lyme disease spirochete. PLoS Pathog. 10, e1004238. https://doi.org/10.1371/journal.ppat.1004238.
Lin, Y., Chen, Q., Ritchie, J.A., Dufour, N.P., Fischer, J.R., Coburn, J., Leong, J.M., 2015. Glycosaminoglycan binding by *Borrelia burgdorferi* adhesin BBK32 specifically and uniquely promotes joint colonization. Cell. Microbiol. 17, 860–875. https://doi.org/10.1111/cmi.12407.
Moody, K.D., Terwilliger, G.A., Hansen, G.M., Barthold, S.W., 1994. Experimental *Borrelia burgdorferi* infection in *Peromyscus leucopus*. J. Wildl. Dis. 30, 155–161. https://doi.org/10.7589/0090-3558-30.2.155.
Pachner, A.R., Steiner, I., 2007. Lyme neuroborreliosis: infection, immunity, and inflammation. Lancet Neurol. https://doi.org/10.1016/S1474-4422(07)70128-X.
Pechová, J., Štěpánová, G., Kovář, L., Kopecký, J., 2002. Tick salivary gland extract-activated transmission of *Borrelia afzelii* spirochaetes. Folia Parasitol. (Praha) 49, 153–159. https://doi.org/10.14411/fp.2002.027.
Preac-Mursic, V., Wilske, B., Jauris, S., Will, G., Reinhardt, S., Lehnert, G., Patsouris, E., Mehraein, P., Soutschek, E., Klockmann, U., 1992. Active immunization with pC protein of *Borrelia burgdorferi* protects gerbils against *B. burgdorferi* infection. Infection 20, 342–349. https://doi.org/10.1007/BF01710681.
Råberg, L., 2012. Infection intensity and infectivity of the tick-borne pathogen Borrelia afzelii. J. Evol. Biol. 25, 1448–1453. https://doi.org/10.1111/j.1420-9101.2012.02515.x.
Råberg, L., Hagström, Å., Andersson, M., Bartkova, S., Scherman, K., Strandh, M., Tschirren, B., 2017. Evolution of antigenic diversity in the tick-transmitted bacterium *Borrelia afzelii*: a role for host specialization? J. Evol. Biol. 30, 1034–1041. https://doi.org/10.1111/jeb.13075.
Radolf, J.D., Caimano, M.J., Stevenson, B., Hu, L.T., 2012. Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. Nat. Rev. Microbiol. 10, 87–99. https://doi.org/10.1038/nrmicro2714.Of.
Rudenko, N., Golovchenko, M., Grubhoffer, L., Oliver, J.H., 2011. Updates on *Borrelia burgdorferi* sensu lato complex with respect to public health. Ticks Tick. Dis. 2, 123–128. https://doi.org/10.1016/j.ttbdis.2011.04.002.
Scheffold, N., Herkommer, B., Kandolf, R., May, A.E., 2015. Lyme carditis–diagnosis, treatment and prognosis. Arztebl. Int. 112, 202–208. https://doi.org/10.3238/arztebl.2015.0202.
Schwanz, L.E., Voordouw, M.J., Brisson, D., Ostfeld, R.S., 2011. *Borrelia burgdorferi* has minimal impact on the Lyme disease reservoir host *Peromyscus leucopus*. Vector-Borne Zoonotic Dis. 11, 117–124. https://doi.org/10.1089/vbz.2009.0215.
Sokal, R.R., Rohlf, J.F., 1995. Biometry: the Principles and Practices of Statistics in Biological Research, 3rd ed. WH Freeman and company, New York.
Stanek, G., Reiter, M., 2011. The expanding Lyme *Borrelia* complex-clinical significance of genomic species? Clin. Microbiol. Infect. https://doi.org/10.1111/j.1469-0691.2011.03492.x.
Stanek, G., Wormser, G.P., Gray, J., Strle, F., 2012. Lyme borreliosis. Lancet 379, 461–473. https://doi.org/10.1016/S0140-6736(11)60103-7.
Strandh, M., Råberg, L., 2015. Within-host competition between *Borrelia afzelii* ospC strains in wild hosts as revealed by massively parallel amplicon sequencing. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 370, 20140293. https://doi.org/10.1098/rstb.2014.0293.
Voordouw, M.J., Lachish, S., Dolan, M.C., 2015. The Lyme disease pathogen has no effect on the survival of its rodent reservoir host. PLoS One 1–26. https://doi.org/10.5061/dryad.8bq31.Funding.
Wang, G., van Dam, A.P., Schwartz, I., Dankert, J., 1999. Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. Clin. Microbiol. Rev. 12, 633–653.
Wang, G., Ojaimi, C., Iyer, R., Saksenberg, V., McClain, S.A., Wormser, G.P., Schwartz, I., 2001. Impact of genotypic variation of *Borrelia burgdorferi* sensu stricto on kinetics of dissemination and severity of disease in C3H/HeJ mice. Infect. Immun. 69, 4303–4312. https://doi.org/10.1128/IAI.69.7.4303-4312.2001.
Wang, G., Ojaimi, C., Wu, H., Saksenberg, V., Iyer, R., Liveris, D., McClain, S.A., Wormser, G.P., Schwartz, I., 2002. Disease severity in a murine model of Lyme borreliosis is associated with the genotype of the infecting *Borrelia burgdorferi* sensu stricto strain. J. Infect. Dis. 186, 782–791. https://doi.org/10.1086/343043.
Wooten, R.M., Weis, J.J., 2001. Host-pathogen interactions promoting inflammatory lyme arthritis: Use of mouse models for dissection of disease processes. Curr. Opin. Microbiol. https://doi.org/10.1016/S1369-5274(00)00202-2.