Borrelia burgdorferi strain and host sex influence pathogen prevalence and abundance in the tissues of a laboratory rodent host

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Abstract
Experimental infections with different pathogen strains give insight into pathogen life history traits. The purpose of the present study was to compare variation in tissue infection prevalence and spirochete abundance among strains of Borrelia burgdorferi in a rodent host (Mus musculus, C3H/HeJ). Male and female mice were experimentally infected via tick bite with one of 12 strains. Ear tissue biopsies were taken at days 29, 59 and 89 postinfection, and seven tissues were collected at necropsy. The presence and abundance of spirochetes in the mouse tissues were measured by quantitative polymerase chain reaction. To determine the frequencies of our strains in nature, their multilocus sequence types were matched to published data sets. For the infected mice, 56.6% of the tissues were infected with B. burgdorferi. The mean spirochete load in the mouse necropsy tissues varied 4.8-fold between the strains. The mean spirochete load in the ear tissue biopsies decreased rapidly over time for some strains. The percentage of infected tissues in male mice (65.4%) was significantly higher compared to female mice (50.5%). The mean spirochete load in the seven tissues was 1.5 times higher in male mice compared to female mice; this male bias was 15.3 times higher in the ventral skin. Across the 11 strains, the mean spirochete load in the infected mouse tissues was positively correlated with the strain-specific frequencies in their tick vector populations. The study suggests that laboratory-based estimates of pathogen abundance in host tissues can predict the strain composition of this important tick-borne pathogen in nature.

KEYWORDS
Borrelia burgdorferi, Lyme borreliosis, pathogen abundance, pathogen life history, tick-borne disease
1 | INTRODUCTION

Many pathogen species consist of multiple genetically distinct strains. Pathogen strains often differ in fundamental life history traits such as abundance in host tissues, host immune response stimulation, virulence and transmission (Balmer & Tanner, 2011; Cobey, 2014; De Roode et al., 2008; Forbes et al., 2008). Evolutionary theory suggests that there are trade-offs between pathogen transmission and virulence (Acevedo et al., 2019; Anderson & May, 1982), which are often mediated by pathogen abundance in host tissues (De Roode et al., 2008; Massad, 1987). Pathogen strains with higher abundance in host tissues have a higher probability of transmission, but they also consume more host resources resulting in higher virulence compared to strains with lower abundance in host tissues (Schmid-Hempel, 2021). Thus, to fully understand the variation in pathogen life history traits, it is crucial to study strain-specific variation in pathogen abundance within host tissues.

Pathogen life history traits are also impacted by host factors, such as host sex. In vertebrate hosts, females tend to have a stronger immune response than males (Klein & Flanagan, 2001), the underlying mechanism being the greater abundance of immunosuppressive androgens (e.g., testosterone) in males (Hughes & Randolph, 2001; Klein, 2004; Trigunaite et al., 2015). These sex-specific differences in immunocompetence predict that pathogens will have higher abundance in the tissues of male hosts compared to female hosts.

Lyme borreliosis (LB) is the most common vector-borne disease in Europe and North America (Steere et al., 2016). This disease is caused by spirochete bacteria belonging to the Borrelia burgdorferi sensu lato (s.l.) complex, which includes more than 20 genospecies (Margos et al., 2011; Stanek & Reiter, 2011). The three genospecies responsible for most of the disease burden are Borrelia afzelii and B. garinii in Europe, and B. burgdorferi sensu stricto (hereafter B. burgdorferi) in North America. These Borrelia genospecies are transmitted among vertebrate reservoir hosts (e.g., rodents and birds) primarily by the immature stages (larvae and nymphs) of Ixodid ticks (Kurtenbach et al., 2006; Piesman & Gern, 2004). The most important tick vectors in eastern North America and western Europe are Ixodes scapularis and I. ricinus, respectively.

In nature, populations of each B. burgdorferi s.l. genospecies consist of multiple strains (Brisson & Dykhuizen, 2004; Durand et al., 2017; Margos et al., 2012; Ogden et al., 2011, 2015; Råberg et al., 2017; Wang et al., 1999). Previous studies have shown that strains of the same genospecies (e.g., B. burgdorferi and B. afzelii) differ in their spirochete abundance in host tissues (Baum et al., 2012; Genné et al., 2021; Jacquet et al., 2015; Wang et al., 2001, 2002) and in their transmission from infected hosts to immature ticks (hereafter host-to-tick transmission; Brisson & Dykhuizen, 2004; Derdáková et al., 2004; Genné et al., 2018, 2019; Hanincová et al., 2008; Jacquet et al., 2016; Rynkiewicz et al., 2017; Tonetti et al., 2015). Other studies have found that infection phenotypes from experimental studies, such as spirochete abundance in host tissues and host-to-tick transmission success, are predictive of the strain frequencies observed in nature (Baum et al., 2012; Durand et al., 2017). Thus, investigating the phenotypes of these strains in a controlled laboratory setting may help us understand which strains are dominant in areas where LB is endemic and which strains will spread in areas where LB is emerging.

Borrelia burgdorferi s.l. genospecies have a wide tissue tropism and invade a variety of organs including the skin, joints, bladder and heart (Belli et al., 2017; Wooten & Weis, 2001; Zhong et al., 2019). Studies on B. afzelii and I. ricinus suggest that the skin is the critical source for the host-to-tick transmission of this pathogen (Genné et al., 2021; Jacquet et al., 2015; Råberg, 2012). Studies on B. burgdorferi have suggested that colonization of internal organs with a large proportion of connective tissue (e.g., joints) represents an immune evasion strategy that facilitates persistent infections (Embers et al., 2004; Liang et al., 2004; Lin et al., 2020). Importantly, B. burgdorferi s.l. genospecies do not persist in the host bloodstream, although they do use the blood to disseminate from the tick bite site to organs and tissues (Hyde, 2017; Shih et al., 1992; Wang et al., 2001). Tissue tropism can vary among strains (Baum et al., 2012; Brisson et al., 2011), and may influence strain-specific fitness in nature.

This paper represents part of a larger study that investigated strain-specific variation in important pathogen life history traits including pathogen tissue abundance, virulence and host-to-tick transmission. Male and female Mus musculus mice were experimentally infected via nymphal tick bite with 12 different strains of B. burgdorferi. Here we explore differences in tissue tropism and tissue spirochete burden. We predict that strains will differ in their prevalence and abundance in the necropsy tissues of the rodent host (i.e., strain-specific tissue tropism). Given possible impacts of testosterone on immunity in adult male mice (e.g., Hughes & Randolph, 2001), we predict that the percentage of infected tissues and the tissue spirochete abundance will be higher in male mice compared to female mice. Finally, we predict that strains that establish a higher prevalence and/or abundance in mouse tissues will have higher frequencies in nature.

2 | MATERIALS AND METHODS

2.1 | B. burgdorferi, I. scapularis ticks and C3H/HeJ mice

Twelve low-passage strains of B. burgdorferi used in this study came from the isolate collection of the Public Health Agency of Canada (PHAC). The isolates were obtained from I. scapularis ticks collected during field surveillance in Canada and were cultured on semisolid agar to obtain single-strain colonies. These clones were sequenced using whole genome sequencing (Tyler et al., 2018) and are hereafter referred to as strains. The 12 strains were selected based on their multilocus sequence type (MLST), ospC type and region of origin (Midwestern Canada, n = 7; Eastern Canada, n = 5; Table 1). These two regions were included because previous work has shown substantial differences in strain diversity between Midwestern Canada and Eastern Canada (Margos et al., 2012; Mechal et al., 2015; Ogden et al., 2017).
Previous studies have shown that the mode of infection, tick bite versus needle inoculation, can influence the infection phenotype (Gern et al., 1993; Roehrig et al., 1992; Sertour et al., 2018). For this reason, we created a tick infection model by feeding naïve I. scapularis larvae on uninfected C3H/HeJ mice. These larvae were maintained in humidity chambers and allowed to molt into nymphs. A random subset of 10 nymphs per mouse were tested for their B. burgdorferi infection status using qPCR (quantitative polymerase chain reaction). All strains were present in 80%–100% of nymphs tested. Uninfected control nymphs were generated by feeding naïve I. scapularis larvae on uninfected C3H/HeJ mice. These nymphs were used for the nymphal infestations to infect the mice with the B. burgdorferi strains (see below). To minimize plasmid loss during culture, the B. burgdorferi strains had undergone only four passages from the time of isolation from field-collected I. scapularis ticks to needle inoculation into our mice.

### 2.2 Creation of I. scapularis nymphs infected with each of the 12 strains

The I. scapularis nymphs infected with one of the 12 strains were created as follows. All strains were cultured in BSK-H media at 37°C for 9–14 days. Mice (C3H/HeJ, n = 2–4) were needle-inoculated with a single strain (10^6–10^5 spirochetes) and infected with naïve I. scapularis larvae at 2–4 weeks postinfection (PI). Larvae were maintained in humidity chambers and allowed to molt into nymphs. A random subset of 10 nymphs per mouse were tested for their B. burgdorferi infection status using qPCR (quantitative polymerase chain reaction). All strains were present in 80%–100% of nymphs tested. Uninfected control nymphs were generated by feeding naïve I. scapularis larvae on uninfected C3H/HeJ mice. These nymphs were used for the nymphal infestations to infect the mice with the B. burgdorferi strains (see below). To minimize plasmid loss during culture, the B. burgdorferi strains had undergone only four passages from the time of isolation from field-collected I. scapularis ticks to needle inoculation into our mice.

### 2.3 Experimental infection of mice with strains of B. burgdorferi via tick bite

Prior to needle inoculation, naïve C3H/HeJ mice were fed upon by uninfected I. scapularis nymphs. As expected, these 20 mice all tested negative for the three B. burgdorferi infection criteria.
reason, all the mice used in this study were infected via the bite of an infected nymph to reproduce a natural infection rather than a needle inoculation. A total of 120 specific-pathogen-free C3H/HeJ mice (60 male, 60 female) aged 6–8 weeks were used in this study. For all 12 strains, eight mice (four male, four female) were used for a total of 96 mice in the infected group, with an additional 24 (12 male, 12 female) uninfected control mice. To manage the workload, the experiment was run in two orthogonal temporal blocks (A and B: separated by ~6 months; each block contained 48 infected mice and 12 uninfected control mice). At 6–8 weeks, experimental mice were infected with three *I. scapularis* nymphs putatively infected with the strain of interest (see Section S2 in Appendix S1), whereas control mice were infected with three uninfected nymphs. To confirm the infectious challenge for each mouse, engorged nymphs were recovered and tested for infection using qPCR targeting the 23S rRNA gene of *B. burgdorferi* (see below and Section S2 in Appendix S1).

Another objective of this study was to compare transmission of the 12 *B. burgdorferi* strains from infected mice to *I. scapularis* ticks over the first 90 days of the infection. For this, mice were infested with 50–100 naïve *I. scapularis* larval ticks at days 30, 60 and 90 PI. The host-to-tick transmission data of the 12 different *B. burgdorferi* strains are not be discussed in this paper. However, it is important to mention this aspect of the experimental design for two reasons. First, it explains why our sample collections were chosen. Second, we unexpectedly discovered that the infestations with *I. scapularis* larvae reduced the presence and abundance of *B. burgdorferi* in the mouse tissues (see Results and Discussion), which is relevant to the main objective of the present study.

Ear tissue biopsies were taken from each mouse prior to their experimental infection (~2 to ~1 weeks PI), and before each larval infestation (days 29, 59 and 89 PI). Mice were anesthetized by isoflurane prior to collection of a 2-mm ear tissue biopsy by punch. The pre-infection tissue biopsy was taken from the left ear, and the three post-infection tissue biopsies were taken from the right ear. Blood samples were taken from each mouse at pre-infection (~2 to ~1 weeks PI), day 28 PI, and at euthanasia (day 97 PI). Pre-euthanasia blood samples were taken by submandibular bleeding with Goldenrod lancets (Gold et al., 2005) and 10–50 μl of blood was collected. At day 97 PI, all mice were killed via isoflurane overdose followed by cervical dislocation, cardiac puncture and exsanguination.

2.4 Necropsy and tissue sample collection

Mice were necropsied immediately following euthanasia, and seven organs/tissues were collected including the kidney, left ear, right ear, ventral skin (from the belly of each mouse), right rear tibiotarsal joint, heart and bladder. Each mouse was processed with sterile equipment disinfected with Virkon between uses. Tissues were kept at 4°C before being trimmed and weighed to the nearest 0.1 mg within 24h of their collection. Final samples were held at −80°C prior to DNA extraction.

2.5 Homogenization and DNA extraction of tissue samples

Necropsy and ear biopsy samples were homogenized by a microprobe or by 3.6-mm stainless steel beads with the Qiagen TissueLyser II. For all organs, extractions were done on partial samples, hereafter referred to as tissue. DNA was extracted from homogenized tissue samples using the Qiagen DNAPure and Tissue kit individual spin columns following the manufacturer’s instructions (see Section S2 in Appendix S1).

2.6 qPCR to measure the abundance of *B. burgdorferi* in mouse organs

To test for the presence and quantity of *B. burgdorferi*, a probe and primer assay targeting the 23S rRNA intergenic spacer gene was used as described previously (Courtney et al., 2004; see Section S2 in Appendix S1). Quantification cycle (Cq) values were transformed using a synthetic gene standard (IDTDNA, gBlock; see Section S3 in Appendix S1). The repeatability of the sample Cq for the 23S rRNA intergenic spacer gene was 85.3% and was based on 70 necropsy samples.

A second qPCR was performed on each sample to quantify the *M. musculus* housekeeping gene Beta-actin using a previously described protocol (Dai et al., 2009; see Section S2 in Appendix S1). Cq values were transformed using a synthetic gene standard (IDTDNA, gBlock; see Section S3 in Appendix S1). The repeatability of the sample Cq for the mouse Beta-actin gene was 85.8% and was based on 70 necropsy samples.

2.7 Statistical methods

We analysed four infection phenotypes: (i) prevalence of *B. burgdorferi* in the ear biopsies over the course of the infection, (ii) abundance of *B. burgdorferi* in the subset of infected ear biopsies over the course of the infection, (iii) prevalence of *B. burgdorferi* in the tissues at euthanasia and (iv) abundance of *B. burgdorferi* in the subset of infected tissues at euthanasia.

2.8 Analysis of presence of *B. burgdorferi* in ear tissues and necropsy tissues

For all tissues, the presence of *B. burgdorferi* infection was a binomial variable (0 = absent = uninfected, 1 = present = infected). These response variables were analysed by generalized linear mixed effect
models (GLMMs) with binomial errors. For the analysis of *B. burgdorferi* infection presence in the ear tissues over time, the fixed factors included ear punch (four levels: days 29, 59, 89 and 97 PI), strain (11 levels; see Table 1), sex (two levels: female, male) and their interactions. Temporal block (two levels: A, B) was included but not in the interaction terms. The right ear necropsy sample from day 97 PI was included in this analysis because all post-infection biopsies were from the right ear. For the analysis of *B. burgdorferi* infection presence in the tissues at euthanasia, the fixed factors included tissue (seven levels: kidney, left ear, right ear, ventral skin, tibiotarsal joint, heart and bladder), strain, sex, their interactions and temporal block. Mouse identity was included as a random factor in both analyses. Both analyses were restricted to samples from the subset of 84 mice that became infected following the infectious nymphal challenge (see below). Nonsignificant interaction terms were sequentially removed to generate the final models. Factor significance was estimated using type II Wald tests, post hoc analyses were performed and estimated marginal means were calculated.

### 2.9 Analysis of abundance of *B. burgdorferi* in ear tissues and necropsy tissues

For the subset of infected tissue samples, we standardized the abundance of *B. burgdorferi* (measured as the number of *B. burgdorferi* 23S rRNA copies) relative to three estimates of the amount of mouse tissue: (i) mass of tissue used in the DNA extraction, (ii) DNA concentration of the resultant DNA extraction and (iii) number of mouse Beta-actin gene copies. We found that all three of these methods of standardizing the tissue spirochete load were highly correlated with each other and that method (iii) yielded the best quality results (see Section S4 in Appendix S1). For this reason, we present the abundance of *B. burgdorferi* (also referred to as spirochete load) as the log$_{10}$-transformed ratio of the number of *B. burgdorferi* 23S rRNA copies per one million mouse Beta-actin copies.

Analyses of the spirochete loads were run on the subset of infected tissues using linear mixed effect models (LMMs). For the analysis of spirochete loads in the ear biopsies over time, the fixed factors included ear punch, strain, sex, their interactions and temporal block. For the analysis of spirochete loads in the necropsy tissues, the fixed factors included tissue, strain, sex, their interactions and temporal block. Mouse identity was included as a random factor in both analyses. As with the analyses on *B. burgdorferi* infection presence, nonsignificant interactions were sequentially removed to generate the final simplified models. Post hoc analyses were done in the same manner as for the analysis of the tissue infection presence.

### 2.10 Effect of region on the four infection phenotypes

For each of the four infection phenotypes, a separate model was run to analyse the effect of region (Midwestern Canada vs. Eastern Canada). As the strains are nested within region, region and strain could not both be included as fixed factors and the strain was therefore included in the random effects for these analyses.

### 2.11 Correlation analyses

We performed a correlation analysis across the set of 11 strains of *B. burgdorferi* to determine whether there were correlations among the four measures of infection: (i) infection presence in third ear biopsy, (ii) spirochete abundance in third ear biopsy, (iii) infection presence in the seven necropsy tissues and (iv) spirochete abundance in the seven necropsy tissues. The estimated marginal means of these four infection phenotypes were calculated for each of the 11 *B. burgdorferi* strains and the pairwise correlations were tested using Pearson's correlation test. As mouse sex had a significant effect on the four infection phenotypes, we repeated the previous analysis for the 22 combinations of mouse sex and strain. For the sample of infected mice (n = 84), a separate correlation analyses were done to determine whether the presence of infection or spirochete loads were correlated among the seven necropsy tissues.

### 2.12 Estimates of the frequencies of *B. burgdorferi* strains in nature

To determine whether our laboratory estimates of the strain-specific spirochete infection prevalence and/or abundance in the mouse tissues predict the frequencies of these *B. burgdorferi* strains in nature, we used the PubMLST database and the published literature to estimate the latter. The PubMLST database for *B. burgdorferi* s.l. contains almost 2500 MlST profiles that include samples from wildlife, ticks and patients (Jolley et al., 2018). We used two published studies that investigated the frequencies of *B. burgdorferi* MLSTs in I. scapularis ticks in the USA (n = 741) and Canada (n = 153; Ogden et al., 2011; Travinsky et al., 2010). For each of our strains, we determined the number of times its MLST occurred in the database. These counts were converted to frequencies by dividing them by the total count for the MLSTs in our set of strains (see Section S5 in Appendix S1). We used generalized linear models (GLMs) with binomial errors to test the relationship between these two estimates of the strain-specific frequency in nature (response variable) and three different estimates of strain-specific abundance in the mouse tissues (explanatory variables): (i) proportion of infected mouse tissues, (ii) mean spirochete load in the subset of infected mouse tissues and (iii) mean spirochete load in all mouse tissues (see Section S5 in Appendix S1).

### 2.13 Statistical software

We used R version 4.0.4 for all statistical analyses (R Core Team, 2021). The list of R packages and R functions we used is given in the Section S2 in Appendix S1.
FIGURE 1 Spirochete load in the right ear tissue samples over the course of the infection separated by strain. The spirochete loads were calculated as the log$_{10}$-transformed ratio of the number of Borrelia burgdorferi 23S rRNA copies/10$^6$ mouse Beta-actin copies. The right ear was sampled on days 29, 59, 89 and 97 post-infection (PI). The spirochete loads are estimated marginal means (EMMs) based on a total of 256 infected ear samples from the 84 infected mice. Only six of 11 strains had positive ear samples at day 97 PI (euthanasia). The spirochete loads in right ear tissue biopsies (days 29, 59 and 89 PI) were generally consistent for most strains except for strains 66, 57 and 54, which showed significant decreases between strains (p = 0.004), sex (p = 0.019) and punch (p = 3.004 × 10$^{-10}$) were significant. In general, the presence of B. burgdorferi in the right ear at euthanasia (day 97 PI) was significantly lower compared to the three right ear biopsies (days 29, 59 and 89 PI), and males had a higher infection presence in the right ear compared to females.

3.2 | Presence of B. burgdorferi in the ear tissue biopsies

Ear tissue biopsies were taken prior to the nymphal infestation (pre-infection), prior to each larval infestation (days 29, 59 and 89 PI) and a final necropsy sample was taken at euthanasia (day 97 PI). For the subset of the 84 infected mice, the mean prevalence of B. burgdorferi infection at pre-infection, days 29, 59, 89 and 97 PI were 0.0% (0/84), 98.9% (83/84), 100.0% (84/84), 91.7% (77/84) and 14.3% (12/84), respectively. Only six of 11 strains were detected in the terminal ear tissue samples from day 97 PI. For the subset of the 28 uninfected mice (20 uninfected control mice plus eight mice with failed infections), all 112 ear tissue biopsies tested negative for B. burgdorferi.

The infection presence of B. burgdorferi in the post-infection right ear tissue samples was analysed with a GLMM with the fixed factors of strain, sex, punch (days 29, 59, 89 and 97 PI) and temporal block. There were no significant interactions among the fixed factors, and temporal block was not significant (see Section S8 in Appendix S1). Strain (p = 0.004), sex (p = 0.019) and punch (p = 3.004 × 10$^{-10}$) were significant. In general, the presence of B. burgdorferi in the right ear at euthanasia (day 97 PI) was significantly lower compared to the three right ear biopsies (days 29, 59 and 89 PI), and males had a higher infection presence in the right ear compared to females.

3.3 | Abundance of B. burgdorferi in the ear tissue biopsies

The abundance of B. burgdorferi in the right ear biopsies was expressed as the log$_{10}$-transformed ratio of the number of B. burgdorferi 23S rRNA copies per million mouse Beta-actin copies. For the subset of infected ear biopsies (n = 256), an LMM was used to test the tissue spirochete load. Strain (p = 0.004), sex (p = 0.027),
punch (p = 2.2 × 10^{-16}), and the interactions between strain and punch (p = 3.467 × 10^{-8}), and between sex and punch (p = .017) were significant. The other interactions, and temporal block, were not significant (see Section S8 in Appendix S1). For the six strains that had positive terminal right ear samples at day 97 PI, the spirochete load was significantly lower compared to the ear biopsies on days 29, 59 and 89 PI (Figure 1). After excluding the terminal ear sample on day 97 PI, we found that the spirochete loads did not differ between the three ear biopsies (days 29, 59 and 89 PI) for most strains (Figure 1). The exceptions were strains 66, 57 and 54, which had significantly higher spirochete loads on day 29 PI compared to days 59 and 89 PI (Figure 1). Strain 66 had the greatest decrease in spirochete load, with the ear biopsy at day 59 PI having 0.03× the number of spirochetes as the ear biopsy at day 29 PI (day 29 PI ear biopsy: mean = 13,182.6 23S rRNA/10^6 Beta-actin; day 59 PI ear biopsy: mean = 389.0 23S rRNA/10^6 Beta-actin; p < .001).

Separate linear models were run for each of the three ear biopsies to remove the interaction terms (see Section S8 in Appendix S1). The terminal ear tissue sample on day 97 PI was excluded because there were so few infected samples (12/84). Strain had a significant effect on ear tissue spirochete load for each of the three ear biopsies (day 29 PI: p = 2.547 × 10^{-5}; day 59 PI: p = .002; day 89 PI: p = .029). Sex had a significant effect on ear tissue spirochete load on day 59 PI (p = 2.280 × 10^{-4}) and day 89 PI (p = .030), but not on day 29 PI (p = .408; see Section S8 in Appendix S1). At days 59 and 89 PI, male mice had 1.95× and 1.48× more 23S rRNA/10^6 Beta-actin compared to female mice, respectively.

3.4 | Presence of B. burgdorferi in the mouse necropsy tissues

For the subset of infected mice (n = 84), the mean number of infected necropsy tissues was 3.9 (range = 2–7). Infection presence of necropsy tissues was analysed in the same manner as the infection presence of the ear biopsies. The main effects of strain (p = .004), sex (p = .008), tissue (p = 2.2 × 10^{-15}) and temporal block (p = .013) were significant, and the interactions between strain and sex (p = .033), and between sex and tissue (p = 7.442 × 10^{-5}) were significant. The effect sizes are given below.

The prevalence of B. burgdorferi infection differed among the seven necropsy tissues (Figure 2a): kidney (29.8% = 25/84), left ear (38.1% = 32/84), right ear (14.3% = 12/84), ventral skin (52.4% = 44/84), tibiotarsal joint (71.4% = 60/84), heart (92.9% = 78/84) and bladder (97.6% = 82/84). Mice infected with strain 66 had the lowest number of infected tissues (2.88/7) and mice infected with strain 126 had the highest number of infected tissues (5.50/7; Figure 3a). In general, B. burgdorferi was detected in more tissues in male mice (65.4% = 197/301) compared to female mice (50.5% = 145/287; Figures 2a and 3a). Males had a significantly higher infection prevalence than females in their joints (90.7% vs. 51.2%), ventral skin (93.0% vs. 9.8%) and right ears (23.3% vs. 4.9%), whereas females had a significantly higher infection prevalence than males in their kidneys (61.0% vs. 20.9%; Figure 2a). For 10 of the 11 strains, males had a significantly higher infection prevalence than females (Figure 3a; strain 126 was the exception). Block B had a higher infection prevalence in the mouse tissues compared to block A (61.0% vs. 52.5%).

3.5 | Abundance of B. burgdorferi in the mouse necropsy tissues

For the subset of tissues that were infected with B. burgdorferi (n = 333 infected necropsy tissues from 84 infected mice), the abundance of B. burgdorferi was analysed. There were significant effects of strain (p = .001), sex (p = .005), and tissue (p = 2.2 × 10^{-16}) on the abundance of B. burgdorferi in the necropsy tissues. There were also significant interactions between strain and tissue (p = .010), and between sex and tissue (p = .006). Temporal block was not significant (p = .122). The effect sizes are given below.

Using kidney as the reference tissue, the mean spirochete load in the other six tissues was as follows: left ear (3.27x), right ear (0.90x), ventral skin (12.98x), tibiotarsal joint (6.55x), heart (30.36x) and bladder (18.19x; Figure 2b). A main effects model was used to generate estimates of the mean spirochete load across tissues for each strain (Figure 3b). Strain 66 had a significantly lower spirochete load than the three isolates with the highest spirochete loads (strain 174: p = .013; strain 198: p = .031; strain 150: p = .034). All other strains had similar spirochete loads (Figure 3b).

The mean spirochete load in the necropsy tissues for strain 174 (659.2 23S rRNA/10^6 Beta-actin) was 4.79× higher compared to strain 66 (137.5 23S rRNA/10^6 Beta-actin). In general, the mean spirochete load in the necropsy tissues was 1.45× higher in males compared to females across tissues and strains (male: 547.1 23S rRNA/10^6 Beta-actin; female: 316.2 23S rRNA/10^6 Beta-actin; p = .005; Figures 2b and 3b).

Separate linear models were run for each necropsy tissue to remove the two interactions (see Section S9 in Appendix S1). Male mice had a spirochete load in their ventral skin that was 15.3× higher compared to female mice (male: 1266 23S rRNA/10^6 Beta-actin; female: 23S rRNA/10^6 Beta-actin; p = .012) and a spirochete load in their tibiotarsal joints that was 3.8× higher compared to females (male: 604; female: 160; p = 4.504 × 10^{-4}). There were significant differences in spirochete load among strains in the heart (p = .012) and bladder (p = .031).

3.6 | Infection phenotypes did not differ by region

There were no significant effects of region (Midwestern Canada vs. Eastern Canada) on the four infection phenotypes and the presentation of these results is restricted to the Section S10 in Appendix S1.
3.7 Correlations among the *B. burgdorferi* infection phenotypes in the mouse tissues

For the 142 combinations of strain, sex and tissue, there was a strong positive correlation between the presence of *B. burgdorferi* in the necropsy tissues and the abundance of *B. burgdorferi* in the necropsy tissues \( r = 0.676, p = 2.2 \times 10^{-16}; \) see Section S11 in Appendix S1. This result shows that *B. burgdorferi* strains with high abundance in the mouse tissues are more likely to be detected than strains with low abundance. For the 22 combinations of mouse sex and *B. burgdorferi* strain, there was also a strong positive correlation between the spirochete load in the necropsy tissues on day 97 PI and...
versus the spirochete load in the third biopsy of the right ear on day 89 PI (Figure 4; $r = .923, p = 9.551 \times 10^{-10}$). This result shows that the third larval infestation did not change the ranking of the strains with respect to the spirochete load in the mouse tissues.

Correlation tests were done to determine whether the infection presence or spirochete load was correlated among pairs of the seven necropsy tissues. Infection presence and spirochete load were strongly and positively correlated for many pairs of necropsy tissues and there were no negative correlations (see Section S11 in Appendix S1). These pairwise correlations were especially strong for the internal tissues of heart, bladder and kidney (see Section S11 in Appendix S1). Heart and bladder were the most strongly correlated (infection presence: $r = .828, p < .001$; spirochete load: $r = .918, p < .001$).

We used GLMs with quasibinomial errors to examine the relationship between three different estimates of strain-specific abundance in the mouse tissues and two estimates of the strain-specific frequency in nature (see Section S5 in Appendix S1 for details). For the proportion of infected mouse tissues and for the mean spirochete load in all mouse tissues, there was no relationship with the strain-specific frequencies in nature. There was a positive relationship between the mean spirochete load in the subset of infected mouse tissues and the strain-specific frequencies in nature for the PubMLST database (see Section S5 in Appendix S1; $p = .055$) and for the I. scapularis ticks (Figure 5; $p = .045$).

4 | DISCUSSION

4.1 | Strain-specific estimates of spirochete abundance in laboratory mice are related to strain-specific frequencies in nature

We found significant differences among the 11 B. burgdorferi strains in the percentage of infected tissues at necropsy and in the mean abundance of spirochetes in the necropsy tissues. These results indicate that some strains are present in more mouse tissues and establish higher abundance in those mouse tissues compared to

**FIGURE 4** Correlation between the spirochete load in the necropsy tissues versus the spirochete load in the last right ear biopsy across the 22 combinations of *Borrelia burgdorferi* strain and mouse sex. The necropsy tissue spirochete load is based on the seven mouse tissues tested at necropsy (day 97 PI) using qPCR. The ear biopsy spirochete load is based on the last biopsy of the right ear (day 89 PI) using qPCR. Both values are expressed as the log$_{10}$-transformed ratio of the B. burgdorferi 23S rRNA copies/10$^6$ mouse Beta-actin copies. The correlation between the two variables is positive and highly significant ($r = .923, p = 9.551 \times 10^{-10}$) [Colour figure can be viewed at wileyonlinelibrary.com]
FIGURE 5 Relationship between the strain-specific estimates of the mean spirochete load in the mouse tissues and the strain-specific frequency in *Ixodes scapularis* ticks in North America. The MLST was used to determine how many times each strain appeared in *I. scapularis* ticks based on two published studies (Ogden et al., 2011; Travinsky et al., 2010). The strain-specific frequencies were calculated by dividing the counts for each strain by the sum of the counts for all our strains (*n* = 251). The mean spirochete load in the mouse tissues is based on the subset of infected mouse tissues tested at necropsy (day 97 PI) and is averaged over the two sexes and the seven necropsy tissue types. Spirochete loads are expressed as the log_{10}-transformed ratio of the *B. burgdorferi* 23S rRNA copies/10^6 mouse Beta-actin copies. A GLM with quasibinomial errors found a significant relationship between the two variables (*p* = .045). Horizontal and vertical error bars represent the 95% confidence intervals for each variable.

other strains. Strains with higher infection prevalence and higher abundance in mouse tissue are expected to have higher transmission to feeding *I. scapularis* ticks and to have higher frequencies in nature. Interestingly, we found a significant positive relationship between our strain-specific estimates of the mean spirochete load in mouse tissues and our estimates of the strain frequencies in nature (Figure 5; see Section S5 in Appendix S1). Thus, strains of *B. burgdorferi* that established a higher spirochete load in the tissues of the C3H/HeJ mice were more commonly found in the PubMLST database and in field-collected *I. scapularis* ticks. This result agrees with another study, which found that *B. burgdorferi* strains with high versus low prevalence in *I. scapularis* nymphs established high versus low abundance in the tissues of the white-footed mouse (*Peromyscus leucopus*), an important reservoir host of *B. burgdorferi* in nature (Baum et al., 2012). Studies on the European Lyme disease spirochete *B. afzelii* found a positive relationship between spirochete abundance in the host tissues and spirochete transmission to feeding *I. ricinus* nympha (Genné et al., 2021; Råberg, 2012). We have previously shown that *B. afzelii* strains with higher transmission from infected hosts to *I. ricinus* ticks are more common in nature (Durand et al., 2017; Tonetti et al., 2015). We therefore hypothesize that *B. burgdorferi* strains with high abundance in mouse tissues have higher host-to-tick transmission, which causes these strains to have higher frequencies in nature.

The MLST was used to match our strains to the PubMLST database and the published literature and to estimate their frequencies in nature. The *Borrelia* MLST is based on eight housekeeping genes that are located on the bacterial chromosome (Hoen et al., 2009; Margos et al., 2008, 2012), but this strain typing system does not provide any information on the many linear and circular plasmids that carry the genes necessary for infection in the vertebrate host and the tick vector (Casjens et al., 2012, 2017; Grimm et al., 2005; Stewart et al., 2005). However, numerous studies have found strong linkage disequilibrium between the chromosomal MLST and plasmid-encoded genes such as ospC (Bunikis, Garpmo, et al., 2004; Hellgren et al., 2011; Qiu et al., 2004; Travinsky et al., 2010), suggesting that MLST might be a reasonable guide to link variation in strain-specific life history traits measured in the laboratory (e.g., abundance in host tissues and host-to-tick transmission) to variation in strain-specific frequencies in nature.

4.2 Most *B. burgdorferi* strains establish persistent infections in C3H/HeJ mice

Of the 12 strains of *B. burgdorferi* used in this study, 11 established persistent infections in C3H/HeJ mice. The strain that failed to infect mice (strain 111) was missing a plasmid shared by the other 11 strains (see Section S1 in Appendix S1). Each infected mouse showed consistent detection of *B. burgdorferi* in ear biopsies and in the necropsy tissues at 97 days PI. This result was similar to another study demonstrating that six strains of *B. burgdorferi* were able to establish persistent infection in the white-footed mouse (*P. leucopus*) up to 35 days PI following needle inoculation (Baum et al., 2012). For
most strains, the spirochete loads in the right ear tissue biopsies of infected mice remained stable over time but for strains 66, 57 and 54 there was a significant decrease in abundance in the right ear tissue over time. Interestingly, these three strains also had the highest abundance in the right ear on day 29 PI. This observation suggests that there is a trade-off between high abundance early in the infection versus the ability to maintain high abundance over the duration of the infection. Others have suggested that B. burgdorferi strains can evolve different life history strategies, such as persistent strains versus rapidly cleared strains (Haven et al., 2012).

4.3 | Sex-specific differences of B. burgdorferi prevalence and abundance in C3H/HeJ mice

Our study found that male mice had a higher infection prevalence and abundance of B. burgdorferi in their tissues than female mice. This was true for 10 of the 11 successful strains, whereas strain 126 had equal tissue infection prevalence between the sexes. We screened 27 experimental studies of rodent hosts infected with different B. burgdorferi s.l. genospecies but found no reports of such differences between the sexes (see Section S12 in Appendix S1). A study of a relapsing fever spirochete, Borrelia hermsii, found a non-significant trend of male mice having a higher bacteraemia than female mice (Benoit et al., 2010). We found four field studies in which the infection prevalence of B. burgdorferi s.l. was higher in male rodents compared to female rodents (Hamer et al., 2012; Tschirren et al., 2013; Voorduw et al., 2015; Zawada et al., 2020). One limitation with field studies is that they do not control for sex-specific differences in exposure rate, and previous studies have shown that male rodents are more likely to be parasitized by ticks than female rodents (Devey & Brisson, 2012; Ostfeld et al., 2018; Perkins et al., 2003). In our study, both sexes were equally susceptible to infection with B. burgdorferi, but the abundance of B. burgdorferi in the host tissues was 1.45x higher in male mice compared to female mice. One potential explanation for this sex-specific difference is the immunosuppressive effect of testosterone in male vertebrate hosts (Klein, 2004; Klein & Flanagan, 2016; Trigunaite et al., 2015). In mice, testosterone has been directly linked to a greater host impact of infection with males having higher pathogen burden or prolonged infection compared to females (Arroyo-Mendoza et al., 2020; Benten et al., 1997; Hughes & Randolph, 2001; Sasaki et al., 2013). In summary, our study shows that with equal exposure, both male and female mice are susceptible to B. burgdorferi, but male mice have a higher tissue infection prevalence and higher spirochete abundance compared to female mice.

4.4 | Tissue tropism of B. burgdorferi in the vertebrate host

Borrelia burgdorferi s.l. is an extracellular pathogen that targets the extracellular matrix of the host tissues (Cabello et al., 2007), and it is found in a variety of host tissues including skin, joints, bladder and heart. While many vector-borne pathogens are found in the blood of their vertebrate hosts, for B. burgdorferi s.l. it is the host skin that is the most important organ for transmission to feeding ticks (Genné et al., 2021; Grillon et al., 2017; Råberg, 2012; Tsao, 2009). In contrast, the internal organs (joints, bladder, heart), which are distant from the sites of tick attachment, are believed to be a dead-end for spirochete transmission (Tsao, 2009). Thus, natural selection should favour those strains that can persist in the skin of their reservoir hosts and in this study, all strains established persistent infections in the skin as shown by the ear biopsies. The presence and abundance of B. burgdorferi in the host tissues is also influenced by the host immune response, which consists of both humoral and cellular components (Bockenstedt et al., 2021). This surveillance by the cells and molecules of the host immune system is reduced in immune privileged tissues, such as articular cartilage and heart valves, which might explain why the joints and the heart have a high abundance of B. burgdorferi despite being dead-end tissues for transmission to feeding ticks (Embers et al., 2004; Hill et al., 2021).

In our study, the infection status and spirochete load of B. burgdorferi differed significantly among the seven necropsy tissues on day 97 PI. The internal tissues (heart and bladder, but not the kidney) had higher infection prevalence and higher spirochete abundance compared to the external tissues (ventral skin, left ear, right ear). In studies on B. burgdorferi in C3H/HeJ mice taking place over a shorter infection time (7-21 days), the heart and ears were the tissues with the higher spirochete abundance compared to the bladder (Wang et al., 2001, 2002). In a study on B. burgdorferi in P. leucopus over a shorter infection time (5 weeks), the spirochete abundance in the ear and tail was much higher compared to the heart (Baum et al., 2012). One explanation is that the spirochete load in the host tissues changes with the age of the infection in the host. We therefore observed a different pattern of spirochete loads in the host tissues because we killed our mice substantially later (97 days PI) compared to these other experimental infection studies. A second explanation is that our mice were infected via tick bite whereas the other studies infected mice via needle inoculation. A third explanation is that we killed our mice immediately after the third larval infestation and we suspect that larval feeding might reduce the spirochete load in the internal tissues but not the internal tissues (see below). In summary, tissue infection prevalence and tissue spirochete load are influenced by many factors including the B. burgdorferi s.l. genospecies or strain, the species of vertebrate host, the time of euthanasia and the mode of inoculation (i.e., needle vs. tick).

4.5 | Limited evidence for differences in tissue tropism among B. burgdorferi strains

Pathogen species and pathogen strains can differ in their tissue tropism, which influences their transmission and virulence (McCall et al., 2016). For example, the three most common causative agents of LB in humans, B. burgdorferi s.s., B. afzelii and B. garinii, are often
clinically differentiated by their symptoms in the joints, skin and nervous system, respectively (Stanek et al., 2012). In experimental infection studies in which the presence and abundance of pathogen strains are measured across a set of tissues, strain-specific tissue tropisms manifest themselves as a significant strain × tissue interaction. For infection presence, the strain × tissue interaction was not significant, which suggests that the *B. burgdorferi* strains did not differ in their preference for the seven different tissues tested at necropsy (day 97 PI). With respect to the abundance of *B. burgdorferi*, our study found a significant strain × tissue interaction (p = .010), but this interaction was largely driven by the difference between the strain with the highest abundance in the skin, strain 126 (MLST 43, *ospC* N), and two strains with the lowest abundance in the skin, strain 66 (MLST 237 *ospC* type J) and strain 54 (MLST 741 *ospC* type I). Furthermore, the fact that both the presence and abundance of *B. burgdorferi* were strongly and positively correlated among pairs of the seven necropsy tissues further indicates that highly infectious strains (e.g., strain 126) established high presence and abundance in all tissues, whereas moderately infectious strains (e.g., strains 66 and 54) established intermediate presence and abundance in all tissues.

### 4.6 Larval infestation may transiently decrease abundance of *B. burgdorferi* in mouse skin

Our study found that the prevalence of infection in the right ear was 91.7% in the last biopsy (day 89 PI) but dropped to 14.3% at necropsy (day 97 PI). This observation suggests that the spirochete abundance in the right ear decreased by 94.0% over a period of 8 days (see Section S13 in Appendix S1). In this 8-day period, each mouse was infested with ~100 larvae that fed to repletion from day 90 PI to day 95 PI. Thus, the third larval infestation reduced the abundance of *B. burgdorferi* in the mouse tissues below our qPCR detection limit, thereby causing the dramatic drop in infection prevalence in the right ear on day 97 PI. The underlying mechanism involves spirochete loss from the mouse skin during tick feeding followed by a slow recovery of the spirochete population. Spirochetes have periplasmic flagella and are constantly moving within the skin of the mouse (Motalet al., 2015). During tick feeding, spirochetes migrate to the tick bite site whereupon ingestion by the tick they are lost from the spirochete population in the mouse skin (Bockenstedt et al., 2014). *B. burgdorferi* has a slow intrinsic growth rate with a doubling time of 12 h at 34°C (Jutras et al., 2013), and it might take days or even weeks for the spirochete population in the mouse skin to recover following a larval infestation. This transient loss of spirochetes from the mouse skin following larval infestation might explain why the infection prevalence and tissue spirochete abundance were lower in the external organs (skin and ears) compared to the internal organs (bladder, heart, kidney) at necropsy. Importantly, despite the potential impact of the third larval infestation, the abundance of spirochetes in the necropsy tissues was strongly positively correlated with the abundance in the last ear biopsy (Figure 4; Sections S11 and S13 in Appendix S1). This result suggests that the impact of the third larval infestation on the abundance of spirochetes in the necropsy tissues was consistent across strains.

### 4.7 Limitations of using *M. musculus* as a model host for *B. burgdorferi*

One limitation of this study is that we used a laboratory strain of the house mouse (*M. musculus*; strain C3H/HeJ) to compare variation in tissue infection prevalence and abundance among strains of *B. burgdorferi* rather than a common natural reservoir host, such as the white-footed mouse (*P. leucopus*; Barbour, 2017). *Strain C3H/HeJ* possesses a mutation in Toll-like receptor 4 which makes it highly susceptible to Gram-negative bacteria compared to the wild-type C3H/HeN. While this may further impact the performance of *B. burgdorferi* in this host, previous studies have found that C3H/HeJ mice are no different than wild-type in their response to infection with *B. burgdorferi* (Barthold et al., 1991; Ma et al., 1998). Both *M. musculus* and *P. leucopus* have a high probability of becoming infected following exposure to *B. burgdorferi* via needle inoculation or via tick bite (Barbour, 2017; Baum et al., 2012; Derradková et al., 2004; Devevey et al., 2015; Hanincová et al., 2008; Rynkiewicz et al., 2017; Wang et al., 2001, 2002). Experimental infections of *M. musculus* and the European bank vole (*Myodes glareolus*) with the same strains of *B. afzelii* have likewise found no differences in susceptibility (Belli et al., 2017; Genné et al., 2021; Gomez-Chamorro, Battilotti, et al., 2019; Gomez-Chamorro, Heinrich, et al., 2019). Host species can influence the abundance of *B. burgdorferi* s.l. in the tissues of the rodent reservoir host; for example, bank voles have higher abundance of *B. afzelii* in their tissues compared to yellow-necked mice (*Apodemus flavicollis*; Råberg, 2012; Zhong et al., 2019). However, we are not aware of any studies that have compared the tissue abundance of different strains of *B. burgdorferi* among different host species. A study that experimentally infected *P. leucopus* mice with six different *B. burgdorferi* strains found similar levels of variation in tissue spirochete load among strains compared to the present study (Baum et al., 2012). For our study to be relevant to the situation in nature, the variation due to differences among strains averaged across host species should be much larger compared to the variation due to interactions between host species and strains (Råberg et al., 2017). In other words, strains that have high versus low fitness in laboratory mice should have a similar ranking in natural rodent reservoir hosts. One study that compared host-to-tick transmission of two *B. burgdorferi* strains in C3H/HeJ mice and *P. leucopus* mice found that the same *B. burgdorferi* strain had higher transmission in both host species (Hanincová et al., 2008). Future studies should investigate whether the strain-specific mean tissue spirochete loads and strain-specific host-to-tick transmission are correlated between different host species.

In the present study, the mice were sexually mature but relatively young (7–9 weeks of age) at the time of the nymphal infestation, and the spirochete loads in the necropsy tissues were determined...
on day 97 PI when the mice were much older (21–23 weeks of age). Although we are not aware of any studies that have shown that the age at which mice are infected with B. burgdorferi influences the spirochete load in necropsy tissues, we acknowledge that the inferences of this study are restricted to C3H/HeJ mice that are infected and dissected according to the time schedule we used. Field studies have repeatedly shown that the prevalence of B. burgdorferi infection is much higher in adult mice compared to juvenile and subadult mice (Bunikis, Tsao, et al., 2004; Hofmeister et al., 1999; Tschirren et al., 2013; Voordouw et al., 2015), which suggests that many mice become infected during the transition from the subadult stage to the adult stage. In Lyme disease-endemic areas, the monthly probability that a mouse acquires B. burgdorferi can be as high as 60%, which suggests that most mice (>80%) will have become infected at 8 weeks of age (Bunikis, Tsao, et al., 2004; Voordouw et al., 2015). Thus, our decision to infect 7- to 9-week-old mice via tick bite is representative of when naïve rodent reservoir hosts encounter B. burgdorferi in nature.

5 | CONCLUSIONS

Using experimental infections of a laboratory model host with the LB agent B. burgdorferi, we found that estimates of the strain-specific abundance in the host tissues were positively correlated with the frequencies of these strains in wild populations of I. scapularis ticks. Thus, strains that established a higher abundance in the tissues of laboratory mice were more common in nature. While male and female mice were equally susceptible to infection, male mice had consistently higher infection prevalence and abundance of B. burgdorferi in their tissues compared to female mice. In this study, there was no evidence for strain-specific tissue tropism. There were large differences for all strains in the presence and abundance of B. burgdorferi among the seven different necropsy tissues. There was also a 4.8-fold range in the mean spirochete load in the mouse necropsy tissues among strains. In contrast, there was limited evidence for significant strain x tissue interactions indicating that the highly infectious strains were better at establishing themselves in all tissues compared to the moderately infectious strains. An unexpected result was that infestation with I. scapularis larvae dramatically reduced the presence of B. burgdorferi in the mouse ear tissues. Future studies will investigate whether strain-specific variation in tissue presence and abundance influences strain-specific variation in host-to-tick transmission.

AUTHOR CONTRIBUTIONS

MV, CBZ, DB and NHO designed the study. ROMR performed the plasmid content analysis on the strains of B. burgdorferi. CBZ and PRT executed the study. CBZ performed all the molecular work. CBZ conducted all the statistical analyses of the data. CBZ and MV wrote the manuscript. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The qPCR data used in this paper are available on DataDryad (doi: 10.5061).

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