

Research



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Authors for correspondence:

Yi-Pin Lin

e-mail: yi-pin.lin@health.ny.gov

Maria A. Diuk-Wasser

e-mail: mad2256@columbia.edu

†Contributed equally to this work.

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Cellular and immunological mechanisms influence host-adapted phenotypes in a vector-borne microparasite

Yi-Pin Lin^{1,2,†}, Danielle M. Tufts^{3,4,†}, Matthew Combs^{3,†}, Alan P. Dupuis II¹, Ashley L. Marcinkiewicz¹, Andrew D. Hirsbrunner¹, Alexander J. Diaz¹, Jessica L. Stout¹, Anna M. Blom⁵, Klemen Strle^{1,2}, April D. Davis¹, Laura D. Kramer^{1,2}, Sergios-Orestis Kolokotronis^{6,7,8} and Maria A. Diuk-Wasser³

¹Division of Infectious Diseases, Wadsworth Center, NYSDOH, Albany, NY, USA

²Department of Biomedical Sciences, SUNY Albany, Albany, NY, USA

³Department of Ecology, Evolution, and Environmental Biology, Columbia University, New York, NY, USA

⁴Infectious Diseases and Microbiology Department, University of Pittsburgh, Pittsburgh, PA, USA

⁵Division of Medical Protein Chemistry, Department of Translational Medicine, Lund University, Malmö, Sweden

⁶Department of Epidemiology and Biostatistics, School of Public Health,⁷Institute for Genomic Health, and⁸Division of Infectious Diseases, Department of Medicine, College of Medicine, SUNY Downstate Health Sciences University, Brooklyn, NY, USA

Y-PL, 0000-0002-5240-2031; DMT, 0000-0002-8084-7556; MAD-W, 0000-0001-8809-3050

Predicting pathogen emergence and spillover risk requires understanding the determinants of a pathogen's host range and the traits involved in host competence. While host competence is often considered a fixed species-specific trait, it may be variable if pathogens diversify across hosts. Balancing selection can lead to maintenance of pathogen polymorphisms (multiple-niche-polymorphism; MNP). The causative agent of Lyme disease, *Borrelia burgdorferi* (*Bb*), provides a model to study the evolution of host adaptation, as some *Bb* strains defined by their outer surface protein C (*ospC*) genotype, are widespread in white-footed mice and others are associated with non-rodent vertebrates (e.g. birds). To identify the mechanisms underlying potential strain × host adaptation, we infected American robins and white-footed mice, with three *Bb* strains of different *ospC* genotypes. *Bb* burdens varied by strain in a host-dependent fashion, and strain persistence in hosts largely corresponded to *Bb* survival at early infection stages and with transmission to larvae (i.e. fitness). Early survival phenotypes are associated with cell adhesion, complement evasion and/or inflammatory and antibody-mediated removal of *Bb*, suggesting directional selective pressure for host adaptation and the potential role of MNP in maintaining *OspC* diversity. Our findings will guide future investigations to inform eco-evolutionary models of host adaptation for microparasites.

1. Introduction

Predicting the potential for pathogen spillover events to human and non-human hosts has become crucial with increasing frequency and magnitude of emergence events [1]. Critical traits involved in the potential for pathogen emergence are the range of hosts that a pathogen can infect (host range) and the competence of potential host species [2,3]. Host competence traits include the capacity to support initial infection establishment, development of infection over time, and pathogen transmission, while the pathogen's host range trait is defined as the breadth of species for which a pathogen can achieve these three criteria [4]. Given the importance to human health, recent reviews and meta-analyses have sought to identify host- and pathogen-derived traits contributing to potential zoonotic emergence. Host-associated traits included life-history traits [5–7], relationships among hosts [8], species interaction dynamics

(e.g. domestication) [9–11] and phylogenetic relationships [12,13]. Analyses of pathogen traits suggest that host range, adaptive potential, and mechanisms of infection are key predictors of zoonoses [2,14,15]. However, with a focus on observed patterns of host–pathogen association, these studies offer limited insights into the underlying molecular mechanisms that determine host competence and potentially shape pathogen adaptation.

A challenge in characterizing host competence is that it is a composite trait resulting from multiple processes encompassing host–pathogen interactions, any of which exhibits a broad range of variation within species [1,16,17]. Intraspecific variability in host competence has often been linked to host-specific traits such as local adaptation [16], phenotypic plasticity [1], development [18], sex [19] or environmentally dependent physiological condition (e.g. resource availability, stressors) [20,21]. However, there is limited understanding of how competence varies depending on the host genotype–pathogen genotype interaction [16]. In fact, pathogen polymorphisms within host populations (or ‘niches’), have been proposed to maintain multiple phenotypes within a pathogen and drive variable outcomes in host competence through a balancing selection mechanism, known as ‘multiple niche polymorphism (MNP)’ [22,23]. MNP is predicted when the environment (hosts) is heterogeneous, and no single pathogen genotype reaches the highest fitness in all hosts [24,25]. Thus, MNP assumes selective advantages for pathogen strains to adapt to certain hosts, reducing their host range compared to more generalist strains [26]. These selective advantages may be manifested as increased probability of initial establishment, faster development of infection (i.e. growth rates), or increased transmissibility [3,27].

Borrelia burgdorferi sensu stricto (Bb), the Lyme disease agent in North America, is an ideal system to study the evolution of host–pathogen interactions because it is one of the few zoonotic pathogens for which molecular mechanisms for host adaptation have been proposed [22,28,29]. *Bb* is transmitted by generalist tick vector, *Ixodes* spp., driving unique selective pressures across many host species, which exhibit strong variability in overall competence [28,30]. MNP is proposed as a mechanism maintaining extensive *Bb* strain diversity, particularly driving the polymorphism in a *Bb* gene, *ospC* [22]. Evidence of MNP is based on laboratory studies documenting fitness variation among *Bb* strains across hosts, as well as observations of the associations between hosts and genetic markers in nature [22,31–36]. Determining the extent of intraspecific variation in pathogen fitness and evaluating molecular mechanisms is pivotal for characterizing the potential selective pressures mediating host–pathogen interactions and influencing human disease risk.

To understand the role of intraspecific *Bb* variation in host competence and assess the plausibility of MNP as a driver of *Bb* diversification, we experimentally evaluated the interaction between two reservoir vertebrate hosts and three genotypically distinct *Bb* strains. Hosts were rodents (white-footed mice, *Peromyscus leucopus*) and birds (American robins, *Turdus migratorius*) that are competent for *Bb* and frequently infected in nature [5]. We aimed to address the following questions: (i) do *Bb* strains with distinct *ospC* genotypes exhibit variation in fitness (transmission to ticks) across two representative host species? (ii) are strains with higher fitness in one host (‘host adapted’), less fit in the other host compared to strains with similar fitness across hosts

(‘generalists’)? That is, are there trade-offs involved in host adaptation? and (iii) which cellular and immunological mechanisms are involved in initial strain survival and persistence and are these consistent with differences in fitness, resulting in divergent strain × host phenotypes?

2. Results

(a) *Bb* strains vary in their fitness across hosts

We compared fitness between *Bb* genotypes commonly found in wild-caught rodents (*ospC* type A, K) [14,27,28] or rarely found in rodents but infectious in birds (*ospC* type E) [29]. These strains included tick-isolated B31-5A4 (*ospC* type A) and cN40 (*ospC* type E), or human patient-isolated 297 (*ospC* type K) (electronic supplementary material, table S1) and displayed similar growth rates in culture *in vitro* (electronic supplementary material, figure S1). We generated *Ixodes scapularis* nymphs carrying each of these strains and permitted these nymphs to feed on robins and white-footed mice. We found indistinguishable bacterial burdens between the three strains in flat and engorged nymphs (electronic supplementary material, figure S2 and table S2). We then compared the ability of B31-5A4, 297 and cN40 to be transmitted from these *Bb*-infected robins and mice to naive ticks by determining the *Bb* burdens in xenodiagnostic *I. scapularis* larvae at various time points. We found significantly higher proportions of robin-fed cN40-positive larvae than 297-positive larvae, when all time points were combined (electronic supplementary material, table S3–S4). Additionally, the proportion of cN40-positive larvae was significantly lower at 14 days post feeding (dpf), but significantly higher at 28 dpf, than B31-5A4-positive larvae (electronic supplementary material, table S3–S4). We also found significantly greater *Bb* loads in the larvae that fed on cN40-infected robins relative to 297-infected robins throughout the experiments. The bacterial burdens in larvae from cN40-infected robins were significantly lower at 14 dpf but significantly higher at 28 and 56 dpf than larvae from B31-5A4-infected robins (figure 1*a–d*; electronic supplementary material, table S2). Although the percent positivity of larvae varied over time and among different strains (figure 1*e*), the percentage of positive larvae at 56 dpf (45, 27, 25% for larvae that fed on cN40-, B31-5A4-, 297-infected robins, respectively) suggest that cN40 is more fit in robins than B31-5A4 and 297.

In white-footed mice, the proportion of cN40-positive larvae was significantly lower than B31-5A4- or 297-positive larvae when the results from all time points were combined (electronic supplementary material, tables S3–S4). At 28 and 35 dpf, there were significantly lower bacterial burdens in 297-positive than B31-5A4-positive larvae (figure 1*f–i*; electronic supplementary material, table S2). We also found *Bb* burdens in cN40-positive larvae were significantly lower than those from B31-5A4- or 297-positive larvae throughout the experiments (figure 1*f–i*; electronic supplementary material, tables S2–S4). While the percent positivity differed in the larvae from B31-5A4-, 297- or cN40-infected mice at different time points and among different strains (figure 1*j*), the percentage of positive larvae at 56 dpf (7, 69, 65% for larvae that fed cN40-, B31-5A4-, 297-infected mice, respectively) suggest cN40 is less fit in mice than B31-5A4 or 297.

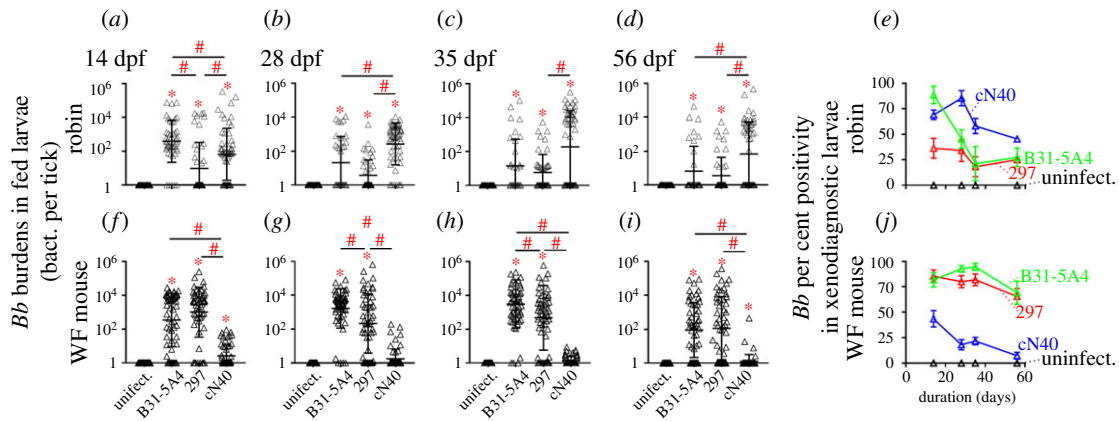


Figure 1. *Bb* displayed strain-specific, robin- or white-footed (WF) mouse-dependent spirochete transmission to larvae. Nymphs carrying *Bb* strains B31-5A4, 297, cN40 or naive nymphs (uninfect.) fed to repletion on robins or WF mice. (a–d, f–i) Data represent the geometric mean \pm geometric s.d. of *Bb* burdens in xenodiagnostic larvae from indicated animals. Significance ($p \leq 0.05$) from the uninfected group (*) or between groups (#) is indicated. (e and j) Data represent the geometric mean \pm s.e.m. of *Bb* per cent positivity in the xenodiagnostic larvae derived from each animal. (Online version in colour.)

(b) The fitness of *Bb* strains corresponds with their ability of initial survival and persistence

Because tick-transmitted bacterial loads can vary [37], we confirmed the above data and also evaluated early injection site survival of *Bb* by intradermally injecting robins and white-footed mice with each strain (10^4 spirochetes per individual). At 1 day post injection (dpi), B31-5A4- or cN40-infected robins showed significantly greater burdens at the injection sites than uninfected robins, whereas B31-5A4- or 297-infected mice had significantly higher burdens at these sites than uninfected mice (figure 2a,b; electronic supplementary material, table S2). These findings indicate that B31-5A4, 297 and cN40 differ in their initial survival in robins and white-footed mice, corresponding with their fitness.

Because of the correspondence of data trends during both needle and tick infections, we used a more physiologically relevant route of tick infections to evaluate the ability of these strains to survive in the bloodstream of American robins and white-footed mice. At 7 dpf, B31-5A4- and cN40-, but not 297-infected, robins developed significantly greater levels of bacteremia than uninfected robins, whereas robins infected with all strains displayed significantly greater bacteremia than uninfected robins at 14 dpf (figure 2c,d; electronic supplementary material, table S2). At 21 dpf, cN40-infected robins yielded significantly greater levels of bacteremia than uninfected robins, and bacteremia in cN40-infected robins was significantly greater than B31-5A4-infected robins (figure 2e; electronic supplementary material, table S2). At 28 dpf, robins infected with all strains displayed levels of bacteremia indistinguishable from uninfected robins (figure 2f; electronic supplementary material, table S2). Thus, cN40 induces longer-lasting bacteremia in robins. By contrast, the bacteremia levels from B31-5A4- or 297-, but not cN40-, infected white-footed mice were significantly greater than uninfected mice at 7 and 14 dpf, and B31-5A4- or 297-infected mice had significantly higher bacteremia levels than cN40-infected mice at 14 dpf (figure 2g,h; electronic supplementary material, table S2). None of these strains were detected in mouse blood at 21 and 28 dpf (figure 2i,j). Thus, cN40 is less capable than B31-5A4 and 297 at surviving in the white-footed mouse bloodstream.

We also evaluated the ability of B31-5A4, 297 and cN40 to colonize robin and white-footed mouse tissues at 64 dpf. Robins infected with all strains had significantly greater spirochete burdens in skin than uninfected robins (figure 2k; electronic supplementary material, table S2). Additionally, *Bb* burdens were significantly greater in the heart and brain of cN40-infected, and the heart of B31-5A4-infected, than uninfected robins (figure 2l,m; electronic supplementary material, table S2). The burdens in livers from robins infected with each strain were indistinguishable from uninfected robins (figure 2n; electronic supplementary material, table S2). These data showed persistent robin skin colonization of all tested strains, but the ability of each strain to colonize other tissues varied. In mice, the bacterial burdens in the outer ears (skin), joints, heart and bladder from B31-5A4 or 297 infections were significantly greater than uninfected or cN40-infected mice (figure 2o–r; electronic supplementary material, table S2). Thus, B31-5A4 and 297, but not cN40, persistently colonized these mice. Taken together, these results showed a positive correspondence of these *Bb* strains' initial survival and persistence in robins and white-footed mice.

(c) Multiple cellular and immunological phenotypes correspond with *Bb* strain-specific fitness, providing mechanistic insights for *Bb* survival and persistence within a host

(i) Cell adhesion: *Bb* strains differed in adhesion to robin and white-footed mouse fibroblasts

We incubated B31-5A4, 297 and cN40 with robin or white-footed mouse fibroblasts and compared the levels of bacterial attachment microscopically with a non-adherent *Bb* strain, B314 [38] (electronic supplementary material, figure S3). Compared to B314, we detected significantly greater attachment of B31-5A4 and cN40 (figure 3a; electronic supplementary material, table S2). We also observed significantly greater numbers of B31-5A4 and 297 attaching to white-footed mouse fibroblasts relative to B314, and 297 had greater attachment than cN40 (figure 3b; electronic supplementary material, table S2). These results showed differential cell adhesion of these *Bb* strains as a cellular

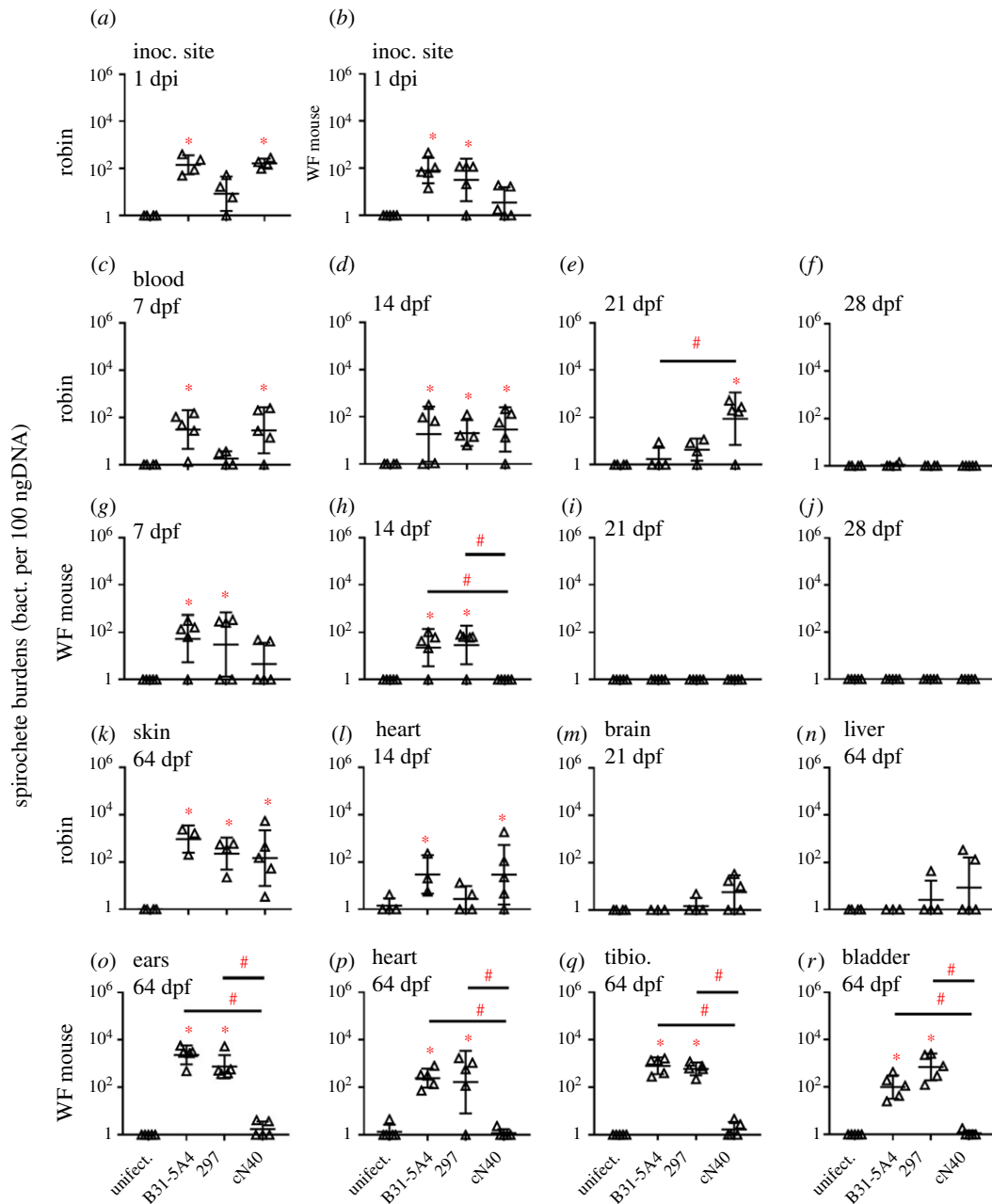


Figure 2. *Bb* strains varied in their ability for early colonization, bacteremia induction and persistence at tissues in robins and white-footed (WF) mice. (a–b) Robins (four per group) and WF mice (five per group) were intradermally inoculated with indicated *Bb* strains or BSK-II medium (uninfect.). The inoculation site of skin (inoc. site) was collected at 1 day post injection (dpi) to determine bacterial burdens by quantitative polymerase chain reaction (qPCR). (c–r) The bacterial loads in blood and tissues at indicated time points post nymph feeding (dpf) determined by qPCR. Data represent geometric mean \pm geometric s.d. Significance ($p \leq 0.05$) from the uninfected group (*) or between groups (#) is indicated.

mechanism that positively corresponds with these strains' fitness in robins and white-footed mice.

(ii) Complement evasion: robin, but not white-footed mouse complement, differentiated between the *Bb* strains serum survivability

We first defined the capability of B31-5A4, 297, and cN40 to evade complement-mediated killing by evaluating the spirochete survival after incubating these strains with naive robin or white-footed mouse sera. More than 88.2% of the spirochetes of these strains survived in heat inactivated robin sera (rendered complement non-functional) [38], as did the high passage, non-infectious and serum sensitive control *Bb* strain, B313 (figure 4a). When incubated in normal robin sera, 8.6% of B313 survived, verifying the bactericidal activity

of robin sera (figure 4a). 95.5% of B31-5A4 and 94.1% of cN40, but 43.6% of 297, survived normal robin sera (figure 4a). All strains survived indistinguishably (at least 80.6% survival) in robin sera treated with *Ornithodoros moubata* complement inhibitor (OmCI), which inactivates avian complement (figure 4b) [37], indicating that 297 is more vulnerable to robin complement-mediated killing than B31-5A4 and cN40. Similarly, at least 97.1% of all strains survived in heat-inactivated, white-footed mouse sera (figure 4c). Whereas only 29.1% of B313 survived normal white-footed mouse sera, at least 97% of all other strains survived in this sera (figure 4c). Additionally, at least 89.1% of all strains survived in white-footed mouse sera treated with cobra venom factor (CVF), which inactivates mammal complement (figure 4d) [37]. These results indicate that these strains evade killing by white-footed mouse complement at indistinguishable levels.

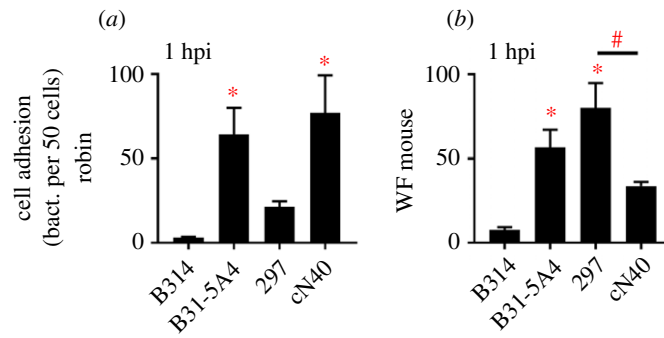


Figure 3. *Bb* strains differed in the levels of cell adhesion in a robin and/or white-footed (WF) mouse-specific manner. Spirochete strains (2×10^6 spirochetes) were incubated with fibroblasts (2×10^5 cells) from robins and WF mice, stained by anti-*Bb* antibodies and 4',6-diamidino-2-phenylindole (DAPI) and counted microscopically from four determinations at 1 h post incubation (hpi). Data represents mean \pm s.d. Statistical significance ($p \leq 0.05$) from B314-treated group (*) or between groups is indicated (#).

(iii) Cytokine induction: *Bb* induced strain-specific varying levels of robin and white-footed mouse pro-inflammatory cytokines during infection

As an indicator of the overall immune response [39,40], we measured the *ex vivo* expression levels of the genes encoding pro-inflammatory cytokines, interferon gamma ($\text{IFN}\gamma$), tumour necrosis factor (TNF) or TNF-induced protein, after incubating robin or white-footed mouse fibroblasts with each *Bb* strain for 24 h. The expression levels of housekeeping genes (18S rRNA and β -actin) were included as controls. We found the expression levels for these genes in cells treated with all *Bb* strains were indistinguishable from mock-treated cells at 1 : 10 (fibroblasts : spirochetes) (electronic supplementary material, figure S4 and table S2). At a ratio of 1 : 100, 297-incubated robin cells had significantly greater expression of $\text{IFN}\gamma$ and TNF-induced protein than mock-treated robin cells (figure 5a–c; electronic supplementary material, table S2). Conversely, cN40-treated mouse cells showed significantly greater expression of $\text{IFN}\gamma$ and TNF than mock-treated cells (figure 5d–f).

We also assessed the levels of the pro-inflammatory cytokine markers $\text{IFN}\gamma$ and $\text{TNF}\alpha$ *in vivo* in robin and white-footed mouse sera at various times after tick infection. For 14 and 21 dpf, we found comparable levels of these cytokines in robins and mice, regardless of *Bb* strain (electronic supplementary material, figure S5 and table S2). At 7 dpf, 297-infected robins had significantly greater levels of $\text{IFN}\gamma$ and $\text{TNF}\alpha$ than uninfected robins and greater levels of $\text{TNF}\alpha$ than cN40-infected robins, whereas cytokine levels in cN40 or B31-5A4-infected robins were indistinguishable from uninfected robins (figure 5g,h; electronic supplementary material, table S2). Conversely, cN40-infected mice displayed significantly greater levels of $\text{IFN}\gamma$ and $\text{TNF}\alpha$ than uninfected mice and $\text{TNF}\alpha$ than 297-infected mice, while 297-infected mice showed indistinguishable cytokine levels from uninfected mice (figure 5i,j; electronic supplementary material, table S2). B31-5A4-infected mice had significantly greater levels of $\text{IFN}\gamma$ at 7 dpf than uninfected mice (figure 5j; electronic supplementary material, table S2). These data indicate differences of pro-inflammatory cytokine induction *ex vivo* and *in vivo* by these *Bb* strains, consistent with the fitness of these strains in robins and white-footed mice.

(iv) Antibodies: *Bb* strains differed in their ability to induce antibodies in a host- and infection stage-dependent manner

We quantified the levels of antibodies induced by infection of each *Bb* strain. To avoid strain-specific antibody recognition

[41,42], we quantified the immunoglobulin G (IgG) titers in sera from infected robins and mice that recognized an equal ratio of mixed lysates of B31-5A4, 297 and cN40 (figure 6a–j). At 7 dpf, 297-infected robins generated higher anti-*Bb* IgG titers than uninfected or other strain-infected robins (figure 6b; electronic supplementary material, table S2). At 14 and 21 dpf, robins infected with any strain developed significantly greater IgG titers than uninfected robins (figure 6c,d; electronic supplementary material, table S2). By 28 dpf, cN40-infected robins generated significantly greater IgG titers than uninfected or other strain-infected robins (figure 6e; electronic supplementary material, table S2). In mice, cN40-infection induced significantly greater anti-*Bb* IgG titers than uninfected or other strain-infected mice at 7 dpf (figure 6g; electronic supplementary material, table S2). At 14 dpf, white-footed mice infected with any strain developed significantly greater anti-*Bb* IgG titers than uninfected mice (figure 6h). B31-5A4- or 297-infected white-footed mice generated significantly greater IgG titers than uninfected or cN40-infected mice at 21 and 28 dpf (figure 6i,j; electronic supplementary material, table S2). Thus, whereas *Bb* strains differed in their ability to induce antibodies in a host- and infection stage-dependent manner, the levels of anti-*Bb* antibodies in robins and white-footed mice at 7 dpf negatively corresponded to spirochete fitness in each of these hosts.

In addition to whole cell lysates, we also compared IgG titers recognizing purified major *Bb* antigens in robins and white-footed mice infected with each *Bb* strain at 7 dpf and found similar negative correspondence between IgG titers and spirochete fitness (electronic supplementary material, figure S6 and table S2). These findings raised the hypothesis that 7 dpf antibodies are bactericidal. To avoid strain-specific bacterial killing, we incubated an equal ratio of B31-5A4, 297 and cN40 with different dilutions of sera from robins or white-footed mice infected with each strain at 7 dpf. We found B31-5A4- and cN40-infected robin sera did not kill spirochetes, but 297-derived sera eliminated 50% of spirochetes at the dilution rate of 1 : 52 (figure 6k). Conversely, cN40-infected white-footed mouse sera eliminated 50% of spirochetes at the dilution rate of 1 : 32 (figure 6l). These results correlate both titers and bactericidal activities of anti-*Bb* antibodies with the fitness of these strains at early stages of infection. Note, the lower IgG titers at late stages of infection (28 dpf) may reflect the lower fitness of the strains in their respective hosts, which warrants further investigation. However, our findings showed early stages-specific

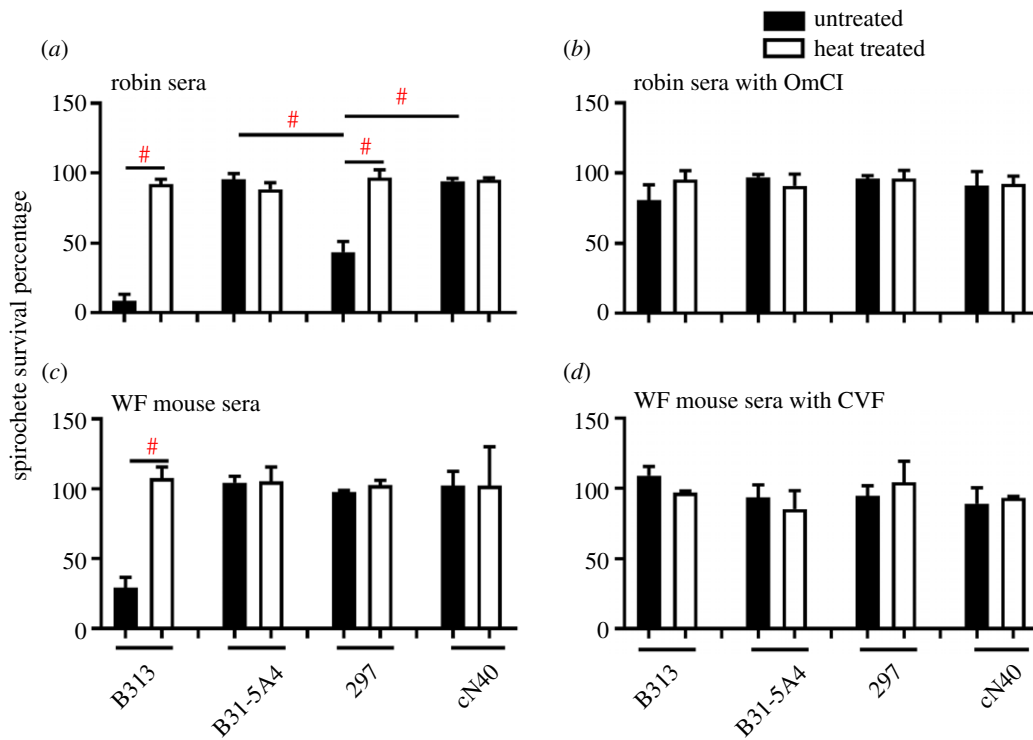


Figure 4. *Bb* strains varied in their ability to survive in robin but not white-footed (WF) mouse sera in a complement-dependent fashion. Percent survival of indicated *Bb* strains is shown in uninfected robin sera in the (a) absence or (b) presence of OmCl, or uninfected WF mouse sera in the (c) absence or (d) presence of CVF. Heat-treated sera were also included. Data represent mean \pm s.d. of per cent survival from three occasions. Significance ($p \leq 0.05$) between groups is indicated (#).

titers and bactericidal activities from the antibodies against B31-5A4-, 297- or cN40, consistent with the fitness of these strains in robins and white-footed mice.

3. Discussion

Our results provide evidence for strain-specific fitness variation across American robins and white-footed mice, two host species previously identified as highly competent for *Bb*. Based on significant differences in pathogen acquisition by xenodiagnostic larvae, strains 297 and cN40 appear to be white-footed mouse- and robin-adapted, respectively, and B31-5A4 is more generalist (particularly at early stages of infection). While we observed fitness reduction for host-adapted strains in their non-adapted hosts, our results suggest that *Bb* strains do not occupy strictly specialist or generalist phenotypes, as both strains 297 and cN40 achieved low levels of transmission in their non-adapted hosts.

Competing hypotheses exist for the fundamental drivers of *Bb* diversity, including MNP, suggesting that polymorphisms are maintained through differential fitness experienced by strains across different host environments. An alternative hypothesis, supported by parsimonious models of genome evolution, posits negative frequency-dependent selection (NFDS) to maintain high *Bb* diversity via adaptive immune responses that select against common genotypes in a host-independent manner [43]. Although the evolution and maintenance of *Bb* diversity has been the subject of much theoretical discussion [29,44–46], empirical and mechanistic approaches are uncommon. Previous evidence for host-adapted phenotypes among different *ospC* genotypes revealed weak associations from natural populations [22,47] and fitness variation for multiple *Bb* strains in a single species

[29,41,48]. By demonstrating the presence of divergent host-adapted phenotypes among *Bb* strains across different host species, as well as the cellular and immunological mechanisms that influence host–pathogen interactions, this study provides novel evidence for the potential role of MNP in driving patterns of *Bb* diversity. This does not preclude the potential for NFDS to additionally structure *Bb* strain communities, and indeed synergistic effects of multiple evolutionary drivers have been suggested [46].

Our results indicate that intraspecific pathogen variability is associated with varying competence within and between hosts. While microparasite competence is defined as the capacity for hosts to permit establishment, development and transmission of infection, ‘realized host competence’ for *Bb* has historically been measured as the percent of field-derived hosts, or individuals infected by field-derived nymphs, from which xenodiagnostic larvae can successfully acquire infection [30]. Our findings suggest that measures of competence may be influenced by the relative frequency of different host-adapted genotypes in the environment, as local *Bb* communities (measured by *ospC* types) appear to vary over time and space [33,47,49]. Thus, host overall competence would be an integrated measure of host genotype-specific competence.

Theory suggests that more generalist parasites should experience fitness disadvantages compared to more host-adapted species [3]. However, our results did not indicate the presence of a fitness trade-off during the first two weeks of infection: B31-5A4 exhibited similar levels of *Bb* positive xenodiagnostic larvae and their bacterial burdens in both hosts at 14 dpf. Yet, we did observe a fitness cost for B31-5A4-infected robins during later stages of infection, where acquisition by ticks was significantly reduced

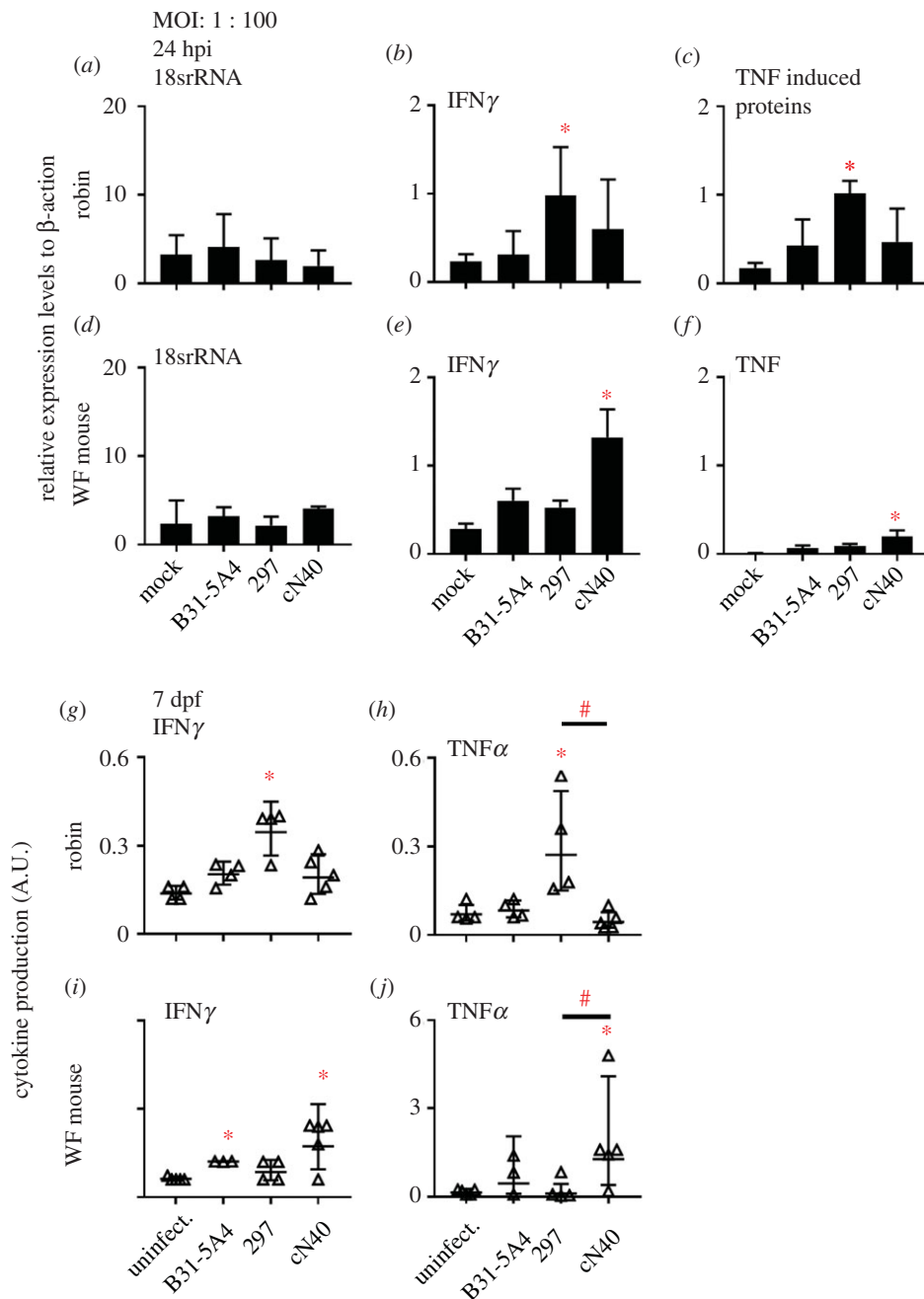


Figure 5. *Bb* strains triggered different levels of cytokine *ex vivo* and at early robin and white-footed (WF) mouse infection. (a–f) Fibroblasts (2×10^5 cells) from robins and WF mice were incubated with *Bb* strains (2×10^7 spirochetes) or cell medium (mock). The expression levels of indicated genes were determined at 24 h post incubation (hpi) by reverse transcription-qPCR (RT-qPCR) and normalized to expression of β -actin genes. (g–j) Sera were obtained 7 days post nymph feeding (dpf) to determine levels of cytokines using the enzyme linked immunosorbent assay (ELISA). Data represent the geometric mean \pm geometric s.d. Significance ($p \leq 0.05$) from the uninfected group (*) or between groups (#) is indicated. MOI, multiplicity of infection.

compared to cN40 at all time points past 14 dpf. This shift in the infectivity profile [45], from ‘persistent’ in white-footed mice to ‘rapidly cleared’ in robins, can influence *Bb* fitness depending on the timing of tick feeding in nature (phenology). ‘Persistent’ genotypes were found to be more frequent in the northeast US than the ‘rapidly cleared’ genotypes, presumably owing to positive selection for the infection to persist from the nymphal abundance peak until they are acquired by the larvae that host-seek nearly two months later [50,51]. Future experiments using combinatorial *Bb* infections across different host species could reveal mechanisms that alter the fitness or infectivity profiles and improve our understanding of the evolutionary drivers of host adaptation and the elevated *Bb* strain diversity observed in nature.

Our results further support cellular and immunological mechanisms mediating the ability to ‘survive’ at early stages and ‘persist’ at late stages. These mechanisms include differential cell adhesion, cytokine induction, complement evasion and triggering of antibodies. During early stages of infection, each strain’s ability to adhere to host fibroblasts positively corresponded to its overall fitness in that host, consistent with a role for *Bb* attachment to cells in permitting infection establishment [52]. Additionally, the tested cytokine markers (IFN, TNF) were selected as the documented strain-specific induction of those markers as representatives of overall immune responses [40]. Although these cytokines impact Lyme disease severity in humans, we did not notice any apparent similar symptoms in those

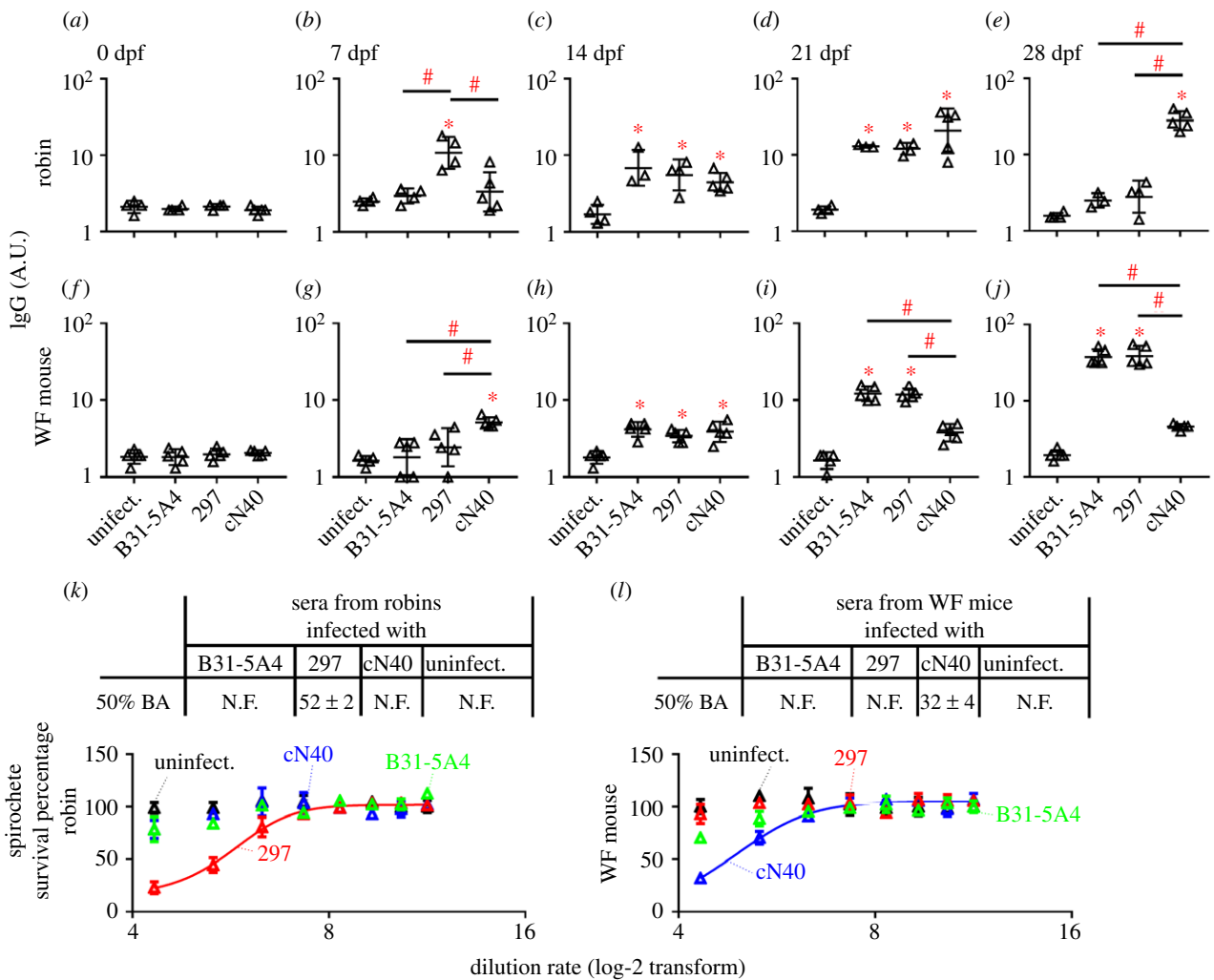


Figure 6. *Bb* strains differed in their ability to induce anti-spirochete antibodies in robin and white-footed (WF) mouse infection. (a–j) Sera were obtained at indicated days post nymph feeding (dpf). The levels of IgG against an equal ratio of B31-5A4, 297 and cN40 lysates were determined by ELISA. Data represents the geometric mean \pm geometric s.d. Significance ($p \leq 0.05$) from the uninfected group (*) or between groups (#) are indicated. (k,l) Sera from infected robins and WF mice at 7 dpf, or naive nymphs (uninfect.). Data represent the mean \pm s.e.m. of the survival percentage. 50% BA, the dilution rate that killed 50% of spirochetes, was obtained from curve-fitting. N.F., not fittable. (Online version in colour.)

reservoir animals (data not shown) [40]. Instead, we found that the extent of cytokine levels *in vitro* and *in vivo* corresponded with *Bb* fitness in reservoir hosts. These findings suggest cytokine marker-mediated signalling promotes *Bb* clearance, and host-adapted *Bb* strains avoid triggering this signalling in their adapted reservoir host. Although it is not clear if this is a result of active immune suppression or lack of stimulation, a lack of early immune responses in adapted reservoir hosts would presumably reduce selective pressures and fitness costs, increasing host permissiveness to *Bb* infection establishment and paving the road for persistence [53]. Moreover, the timing and intensity of antibody response also appeared to mediate variation in strain fitness across hosts, as both 297 and cN40 triggered antibody production earlier in their non-adapted hosts. Given the shift in the infection profile exhibited by B31-5A4 from persistent in mice to rapidly cleared in robins, the increased antibody response at 7 dpf by infected robins to *Bb* lipoproteins relative to mice is also striking. This suggests the capacity to delay host adaptive immune defences may be a key trait in determining infection persistence [53]. Finally, an important difference we observed among hosts is the role for complement-mediated clearance

during early infection, which has been identified as a crucial bottleneck of transmission for Lyme borreliæ [54]. While robin complement effectively reduced 297 survival, mouse complement did not have a similar effect on cN40 survival, consistent with the idea that some mechanisms limiting strain fitness are host-strain specific [54].

We acknowledge limitations of our findings, including that the *in vitro* cultivated fibroblasts do not necessarily reflect *in vivo* results and the chosen *Bb* strains and reservoir hosts do not recapitulate all the bacterial strains and hosts in nature. Thus, future studies should aim to increase both experimental hosts and strain diversity to better characterize these eco-evolutionary dynamics. Additionally, host-independent factors proposed to confer *Bb* adaptation to different hosts (e.g. host–tick species association), should also be considered [55]. Despite these limitations, our findings provide novel insights into the mechanisms structuring *Bb* diversity and provide a model for understanding host adaptation in a complex micro-parasite–host system. The patterns and mechanisms discussed here can guide future efforts to characterize host–pathogen interactions, reservoir competence dynamics, and the potential epidemiological impacts of intraspecific phenotypic variation.

4. Material and methods

(a) Intradermal and tick infection

Animal infections, xenodiagnostic larval placement, and statistical analysis are detailed in the electronic supplementary material.

(b) Quantitative (qPCR) and reverse transcription PCR (RT-qPCR)

Bb genomic equivalents were calculated through amplification of *Bb 16SrRNA* gene using primers detailed in the electronic supplementary material.

(c) Adhesion assays

Fibroblasts were cultivated on 24-well plates and the levels of *Bb* attachment and statistical analysis are detailed in the electronic supplementary material.

(d) Serum resistance, ELISA and borreliacidal assays

The uninfected animal sera or the sera from these animals at 7, 14, 21, or 28 dpf and statistical analysis are detailed in the electronic supplementary material.

Ethics. All experiments involving American robins and white-footed mice were performed in strict accordance with all provisions of the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals and the PHS Policy on Humane Care and Use of Laboratory Animals. Additionally, the protocol was approved by IACUC of Wadsworth Center, NYSDOH (no. 18–412 and 19–451) and

Columbia University (no. AC-AAAY2450) and the City University in New York Advanced Science Research Center (no. 2016–20). To mistnet robins, personnel were approved on scientific collecting permits USFWS Collecting Permit MB035731 and NYSDEC Permit no. 1236.

Data accessibility. This article has no additional data.

Authors' contributions. Y.-P.L.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, writing—original draft, writing—review and editing; D.M.T.: conceptualization, data curation, formal analysis, investigation, methodology, resources, validation, writing—original draft, writing—review and editing; M.C.: data curation, investigation, methodology, writing—original draft, writing—review and editing; A.P.D.: data curation, investigation, methodology, resources, writing—review and editing; A.L.M.: data curation, formal analysis, investigation, methodology, writing—review and editing; A.D.H.: investigation, methodology, resources, writing—review and editing; A.J.D.: investigation, methodology, resources, writing—review and editing; J.L.S.: data curation, investigation, methodology, writing—review and editing; A.M.B.: resources, writing—review and editing; K.S.: conceptualization, writing—review and editing; A.D.D.: conceptualization, investigation, methodology, resources, supervision; L.D.K.: conceptualization, investigation, methodology, project administration, resources, supervision, writing—review and editing; S.-O.K.: writing—review and editing; M.A.D.-W.: conceptualization, funding acquisition, investigation, project administration, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Competing interests. We declare we have no competing interests.

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