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Broad diversity of host responses of the white-footed mouse *Peromyscus leucopus* to *Borrelia* infection and antigens

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Abstract

Peromyscus leucopus , the white-footed mouse, is one of the more abundant mammals of North America and is a major reservoir host for at least five tickborne diseases of humans, including Lyme disease and a newly-recognized form of relapsing fever. In comparison to *Mus musculus*, which is not a natural reservoir for any of these infections, there has been little research on experimental infections in *P. leucopus*. With the aim of further characterizing the diversity of phenotypes of host responses, we studied a selection of quantitative traits in colony-bred and –reared outbred *P. leucopus* adults that were uninfected, infected with the relapsing fever agent *Borrelia hermsii* alone, or infected after immunization with Lyme disease vaccine antigen OspA and keyhole limpet hemocyanin (KLH). The methods included measurements of organ weights, hematocrits, and bleeding times, quantitative PCR for bacterial burdens, and enzyme immunoassays for serum antibodies against both the immunization proteins and cellular antigens of the infecting organism. The results included the following: (i) Uninfected animals displayed wide variation in relative sizes of their spleens and in their bleeding times. (ii) In an experiment with matched littermates, no differences were observed between females and males at 7 days of infection in bacterial burdens in blood and spleen, relative spleen size, or antibody responses to the *B. hermsii* specific-antigen, FbpC. (iii) In studies of larger groups of males or females, the wide variations between bacterial burdens and in relative spleen sizes between individuals was confirmed. (iv) In these separate groups of males and females, all animals showed moderate-to-high levels of antibodies to KLH but wide variation in antibody levels to OspA and to FbpC. The study demonstrated the diversity of host responses to infection and immunization in this species and identified quantitative traits that may be suitable for forward genetics approaches to reservoir-pathogen interactions.

Keywords

tickborne; Lyme disease; relapsing fever; reservoir; vaccine; wildlife

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Introduction

The genus *Peromsyscus* is one of the most widely distributed, ecologically variable, and speciose among rodent taxa of North America (Baker, 1968; Kirkland and Layne, 1989). As E.R. Hall describes peromyscines in *Mammals of North America*, "In most places within the geographic range of the genus, these mice are the most abundant mammal" (Hall, 1979). The deer mouse *P. maniculatus* and the white-footed mouse *P. leucopus* have broad and overlapping distributions and are particularly important as major reservoirs for several human pathogens. In the case of *P. maniculatus*, which exists in variety of habitats mainly in central and western United States, these pathogens include hantaviruses (Amman et al., 2013). *P. leucopus*, which is distributed across much of the central and eastern United States, serves as a reservoir host for at least five tick-borne human pathogens: the Lyme disease agent *Borrelia burgdorferi* (Levine et al., 1985), the cause of human granulocytic anaplasmosis *Anaplasma phagocytophilum* (Telford et al., 1996), the babesiosis agent *Babesia microti* (Telford and Spielman, 1993), the flavirus that causes deer tick virus encephalitis (Ebel et al., 2000), and the newly-recognized disease agent *Borrelia miyamotoi* (Barbour et al., 2009; Scoles et al., 2001). For all these infections the disease vector for humans in the northeastern and north-central United States and adjoining areas of Canada is the northern form of the blacklegged tick *Ixodes scapularis*. The larval and nymphal stages of this tick heavily exploit *P. leucopus* as a source of their blood meals in many if not all of the areas where the aforementioned pathogens are endemic (Piesman and Schwan, 2010). *P. leucopus* is the first candidate for transmission-blocking vaccines targeting wildlife with the aim of reducing the prevalence of *B. burgdorferi* in ticks (Bhattacharya et al., 2011; Meirelles Richer et al., 2011; Tsao et al., 2004).

Given the key role of *P. leucopus* in the life cycle of several different human pathogens, including the cause of Lyme disease, the most frequent arthropod-borne disease in the United States (Centers for Disease Control, 2014), one might expect that it would be the subject of much attention as a laboratory model for these infections. But that has not been the case. The number of reports of experimental infections of the laboratory mouse *Mus musculus* greatly outnumber those of *P. leucopus*, even though the house mouse is not a reservoir. Among the possible explanations for this neglect of *P. leucopus* is the assumption that because rodent is familiarly called a "mouse", it is not only expedient but also sufficient to substitute the laboratory mouse.

Doubtless much has been learned from experimental infections with *B. burgdorferi* of *M. musculus*, especially in its highly defined inbred varieties (Barthold et al., 2010). But it is unclear to what extent the findings from this model animal can be extrapolated to *P. leucopus*, let alone other natural reservoirs, like shrews (Brisson et al., 2008). A presumption of close-relatedness between these two "mice", which superficially resemble each other, does not match current understanding of the evolution of rodents. The genus *Peromyscus* is in the family *Cricetidae*, along with hamsters and voles, while *M. musculus*, as well as that other laboratory standby, *Rattus rattus*, is in *Muridae*, a different family, (Steppan et al., 2004). The *Peromsycus* lineage is estimated to have diverged from the common ancestor of *Mus* and *Rattus* 25 million years ago (Dewey and Dawson, 2001). One *Cricetidae* member with a long history of utility for studies of infectious diseases in the laboratory is the golden

hamster, *Mesocricetus auratus*. And, indeed, the first published report of infection of a rodent with *B. burgdorferi* in the laboratory was on hamsters (Johnson et al., 1984). But citations to experimental *B. burgdorferi* infections of *M. auratus* are dwarfed by those to *M. musculus*.

Cognizant of the lengthening list of infectious agents carried by *Peromyscus* species and the evidence of increasing distribution and incidence of these zoonoses in North America (Centers for Disease Control, 2014), we set out to further develop *P. leucopus* as an experimental model for those tick-borne pathogens for which it serves as a major reservoir. A *P. leucopus* experimental system is feasible in part because there are at least two institutional sources for colony-bred and –reared outbred *P. leucopus* in the United States: in South Carolina (Crossland et al., 2014) and in Massachusetts (Bhattacharya et al., 2011). Advances in the physical mapping and sequencing of the genomes of different *Peromyscus* species (Kenney-Hunt et al., 2014; Worley, 2015), including *P. leucopus*, also mean that investigators likely will not be dependent on using inbred strains for the identification of loci for either Mendelian or quantitative traits. Such forward genetics approaches as high-density SNP microarrays and genome-wide association studies are most informative when there are well-characterized phenotypes of biological relevance and with no or limited co-variation. To this end we examined several distinct parameters of host response of *P. leucopus* in experiments with either *Borrelia hermsii* infection alone or infection in combination with immunization with antigens that were anticipated to be irrelevant to the course of the infection. The soft tick-borne relapsing fever agent *B. hermsii* was chosen for the experiment because of the expectation of higher pathogen densities in the blood with this organism than with *B. burgdorferi* (Barbour et al., 2009). We had found that *P. leucopus* could be infected with *B. hermsii* by needle inoculation and for there to be a detectable and specific antibody response to the infection (Baum et al., 2012).

Our primary question for the present study was, To what extent do these colony-bred animals differ in selected phenotypes of responses to infection and immunization? In other words, how much would they individually vary if placed under the same experimental conditions?

Materials and Methods

Animals

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine. Severe-combined immunodeficient mice (SCID) C.B-17/Icr-*Prkdc*^{scid}/IcrIcoCrl were obtained from Charles River Laboratories and were housed in sterile barrier cages (Tecniplast USA Inc., Exton, PA). Adult male and female colony-bred and –reared *Peromyscus leucopus* (LL stock) were obtained from the Peromyscus Genetic Stock Center (PGSC) colony at the University of South Carolina, Columbia, SC (Peromyscus Genetic Stock Center, 2015). The colony was derived from 38 wild ancestors captured at a single site near Linville, NC between 1982 and 1985. Pedigrees are kept on all mice, and siblings are excluded from breeding pairs (Crossland et al., 2014). The mice were routinely monitored for common rodent viruses, intestinal parasites, and mites (Wiedmeyer et al., 2014). At U.C. Irvine, animals were housed under BSL2 conditions

in groups of 5 in Tecniplast cages (Tecniplast USA Inc., Exton, PA). They were accomodated to the new environment for at least 3 days before experiments. Rodent feed (Teklad 8604 Rodent Diet, Harlan Laboratories, Indianapolis, IN) and water were supplied ad libitum. Animals were on a 12 hour light and 12 hour dark schedule and at an ambient temperature of 22 °C. Total body masses and dissected organ masses were determined to 0.01 g with a PL3002 laboratory balance scale (Mettler-Toledo, Columbus, OH) before, during, or at termination of experimental infections. Body lengths (i.e. from nose to base of tail) were measured with Adobe Photoshop v. 7 from digital photographs of the animals on a calibrated grid.

Bacterial strains

B. hermsii strain CC1 (Dai et al., 2006) was propagated in SCID mice from frozen stocks of infected mouse plasma as described (Lewis et al., 2014). The concentrations of *B. hermsii* in the blood were determined by phase-contrast microscopy of wet mounts in a Petroff-Hausser counting chamber as described (Barbour and Bundoc, 2001). The plasma was diluted with Barbour-Stoenner-Kelly II (BSK II) culture medium (Barbour, 1984) to provide the chosen inoculum in a volume of 50 µl.

Infection and immunization

On day 0, *P. leucopus* animals were each injected subcutaneously with *B. burgdorferi* strain B31 recombinant lipidated OspA (lot # 42537 from Aventis Pasteur, Swiftwater, PA) (Pal et al., 2003; Tsao et al., 2004) at a dose of 0.45 µg and keyhole limpet hemocyanin (Sigma–Aldrich, St. Louis, MO) (Martin et al., 2007a) at 1.4 µg per gram of animal mass and in a volume of 100 µl of the following buffer: 50 mM Tris, pH 7.5–10 mM NaCl-0.3% Triton X-100. The purity and the absence of proteolysis of the recombinant OspA was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoreis. Protein concentrations were determined with a Microplate BCA Protein Assay Kit (Pierce, Rockford, IL). On day 21, *P. leucopus* were each intraperitoneally injected with 10^4 cells of *B. hermsii* in infected plasma. Animals were euthanized on day 28 by CO_2 overdose, followed immediately by cardiocentesis and cervical dislocation. Blood was collected into 1.7 ml tubes coated with lithium heparin (Becton-Dickinson, Franklin Lakes, NJ). Plasma was recovered by serial 5 s centrifugations at 3000 × *g* in a microcentrifuge and then pooled. The liver, spleen, and heart were removed intact by dissection and weighed. Whole blood specimens and spleens were kept frozen at −80° C until DNA extraction. The dissected livers were fixed in 10% neutral-buffered formalin solution.

Enzyme immunoassay (EIA)

Antigens were purified recombinant Fibronectin Binding Protein C (FbpC; also known as BHA007) of *B. hermsii* (Lewis et al., 2014), recombinant *Borrelia miyamotoi* GlpQ protein (Krause et al., 2014), and the recombinant OspA and KLH preparations used for the immunization. To the wells of 96-well Microtest™ U-bottomed polystyrene microtiter plates (Becton Dickinson Co., Franklin Lakes, NJ) were added 100 µl protein solution at a concentration of 1 µg per milliliter of carbonate buffer (0.35 M Na_2CO_3- 0.036 M $NaHCO_3$, pH 9.6), and then the plates were incubated for 16 h at 22°C. The wells were washed 3 times

with 300 µl phosphate-buffered saline (PBS) with 0.05% Tween 20 (Bio-Rad, Irvine, CA) (PBS-T) and then blocked for 1 h with 200 µl 10% bovine serum albumin (EMD Milipore, Billerica, MA) in PBS (PBS-A) at 22°C. This was followed by 3 washes with 300 µl of PBS-T each. To each well was then added 100 µl of serum diluted 1:100 in PBS-A. Each sample was assayed in triplicate. When replicates of the assays were carried out on different days the coefficients of determination (R^2) were ≥0.95. Sera from uninfected *P. leucopus* and wells with buffer only were used as negative controls. The plates were incubated for 3 h at 22°C, and then the plates were washed 3 times with 300 µl PBS-T. Bound antibody was detected with a 100 µl volume of alkaline phosphatase-conjugated goat anti-*Peromyscus leucopus* IgG heavy and light chain antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1:2000 in PBS-A. The plates were incubated for 16 h at 22°C. The wells were washed 3 times with 300 µl PBS-T, and the colorimetric reaction was performed with 100 µl per well of *p*-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO). The optical density was determined at 405 nm with a Synergy II plate reader (BioTek, Winooski, VT).

Quantitative polymerase chain reaction (qPCR)

DNA was isolated from blood and spleens using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). In brief, dissected and cut-up tissues or whole blood were lysed 12 to 16 hours at 56°C in 180 µl ATL buffer and 20 µl Proteinase K (20 mg/ml). Sample processing was automated in a QiaCube apparatus (Qiagen, Valencia, CA). Eluted samples were then cleaned and concentrated using ZymoResearch Clean and Concentrator kit (Zymo Research, Irvine, CA) following the manufacturer's protocol. The elution volume was 30 µl. DNA concentrations were determined with a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA) or a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA). qPCR for 16S ribosomal DNA of *B. hermsii* was carried out as described (Barbour and Travinsky, 2010). The probe for *B. burgdorferi* 16S ribosomal DNA in the assay served as a control for the specificity of the *B. hermsii* probe in quantitating genome copies of *B. hermsii* in the extracted DNA. Assays were carried out in duplicate; R^2 values between replicates were ≥ 0.95.

Other blood studies

For the hematocrit measurements, cardiac blood was collected with heparinized capillary tubes (Fisher Scientific, Waltham, MA), and these were centrifuged in a ZIPocrit Microhematocrit Centrifuge (LW Scientific, Lawrenceville, GA) for 5 minutes. The bleeding time was the time in 30 second intervals for bleeding to cease after 1 mm of the tip of the tail was snipped with sterile scissors and blood intermittently drawn off with a gauze.

Histopathology

Livers from *P. leucopus* were fixed in 10% neutral-buffered formalin and then processed at University of California Davis' Comparative Pathology Laboratory, where they were paraffin-embedded, sectioned, and stained with hematoxylin and eosin. The sections were examined blindly as to infection state. The histology of the liver was evaluated and graded with respect to five pathologic features: hemophagocytosis by Kupfer cells, portal and

periportal infiltration by mononuclear cells, lymphofollicular hyperplasia, centrilobular lipoidosis, and presence and extent of ground-glass appearance. The absence of a feature was assigned a score of 0, and assessments of mild, moderate, or severe were assigned scores of 1, 2, or 3, respectively. The composite pathology score was the sum of the individual feature scores with a maximum score of 13. Livers from two uninfected mice had scores of 0 by blind reading.

Statistics

Descriptive statistics of means, medians, standard deviations (SD), coefficients of variation (CV), 95% confidence intervals (CI), coefficients of determination (R^2), linear and power regressions, and general linear models were calculated by Stata/MP v. 10.1 (Stata Corp., College Station, TX) or SYSTAT v. 13 (Systat Software, Chicago, IL). Parametric hypothesis testing was by 2-tailed paired or unpaired *t* test or ANOVA. The Shapiro-Wilk W test for normal data was used.

Results

Overview of the design and conditions

The subjects for this study were colony-bred and –reared *P. leucopus* of both sexes and aged 13 to 81 weeks, with the majority in the range of 20 to 50 weeks. *P. leucopus* have life spans of up to 8 years (416 weeks) in captivity (Joyner et al., 1998), so these were mature but not aged animals. Four different experiments were carried out: 1, a group of 28 uninfected males; 2, a group with littermate-matched males and females; 3, a group of 30 males; and 4, a group of 30 females, for an overall total of 102 animals. In experiment 2 the mixed sex set of animals were infected with *Borrelia hermsii* and examined with respect to the differences in pathogen burden and host responses. The two antigens, GlpQ and FbpC, used in the immunoassays were known to be immunogenic during *B. hermsii* infections of *M. musculus* (Lewis et al., 2014; Schwan et al., 1996).

In experiments 3 and 4, the two larger sets with single sex, the animals were also infected with *B. hermsii* but after first being immunized 3 weeks before with single unadjuvanted doses of keyhole limpet hemocyanin (KLH), a commonly used antigen in experimental studies, including of *P. leucopus* (Martin et al., 2007a), and *B. burgdorferi*'s OspA lipoprotein, which is the basis of a recombinant dog vaccine against Lyme disease (Conlon et al., 2000) and the principal candidate antigen for a wildlife vaccine targeting *P. leucopus* and possibly other reservoirs in the field (Bhattacharya et al., 2011; Meirelles Richer et al., 2011; Tsao et al., 2004).

Diversity of selected traits in uninfected animals

In experiment 1, the 28 males were the offspring of 25 different mating pairs and were aged 119 to 525 days (mean 258 d; median 175 d). Their body masses ranged from 14.0 to 24.9 g (mean 18.4 g; median 18.7 g), and their lengths from nose to the base of tail were from 8.0 to 9.3 cm (8.7 cm mean; 8.8 cm median). Neither body mass (R^2 = 0.008) nor length (R^2 = 0.015) correlated with age among these animals. The mean (CI) change in body mass between two weighings 21 d apart was −0.19 (−0.40 to +0.02) g.

Body mass (*M*) scaled to body length (*L*) from nose to the base of the tail with an exponent of 2.8 (i.e., $M \propto L^{2.8}$), close to the theoretical expectation of 3 (Thompson, 1961) (Figure 1). The allometric scaling of liver mass to body mass with an exponent of 1.25 was similar to the value of 1.23 that one obtains with the combined data from 6 different strains of *M. musculus* of Konarzewski and Diamond (Konarzewski and Diamond, 1995). For 16 week old males of 7 strains of *M. musculus* (B6D2F1, BALB/c, C3H/HeJ, C56BL/6, CBA, DBA/2, and FVB/N) the exponent for the power trend line of liver mass on body mass was 1.21 (The Jackson Laboratory, 2015). Within each of the 6 inbred strains in Konarzewski and Diamond study, the CV ranged from 0.07 to 0.10 for wet body mass and 0.08 to 0.16 for liver mass. In contrast, the corresponding CV values for the 28 uninfected *P. leucopus* were 0.17 for body mass and 0.23 for liver mass, evidence of broader variation in these traits among the outbred *P. leucopus*.

The ratio of spleen mass to whole body mass was more variable than that observed for the liver, but the allometric exponent at 1.39 was similar (Figure 1). The ratio of heart to whole body also was more variable between animals than the liver to body ratio. Unlike the liver and spleen, which tended to be proportionately greater as body mass increased, the heart mass (*Ht*) of larger animals was proportionately smaller ($Ht \propto M^{0.57}$). (Another quantitative trait, bleeding time, that varied between uninfected animals is described below.)

Comparison of infected male and female animals

In experiment 2, fourteen animals (7 females and 7 males) were injected with 10^4 *B. hermsii* on day 0 and then euthanized on day 7. Male and female littermates were paired, and, accordingly, the mean (CI) ages in days were 211 (192–229) were the same for the two sub-groups. Although males were heavier than matched female littermates, by a mean (CI) of 18% (7–25%), before the infection, there was no discernible difference between sexes in the amount of change in mass between days 0 and 21 (Figure 2 and Table 1). Females and males were comparable in their relative spleen, liver, and heart sizes at the peak of infection. For the combined group of 14 animals, the heart mass scaled to body mass similarly to uninfected animals ($Ht \propto M^{0.54}$), but the liver masses (*Lv*) were proportionately smaller in the infected animals ($Lv \propto M^{0.92}$) and more variable (Figures 1 and 2). There was no association between age in days and relative spleen size ($R^2 = 0.03$) for the 14 animals in the sample.

Littermate-matched females and males were also similar in their spirochete burdens in the blood and spleen and in their antibody responses to the FbpC protein after 7 days of infection (Table 1). By linear regression there was no discernible association at the 0.05 level of confidence of age with spirochete burdens in blood or spleen or with anti-FbpC antibody levels in this sample. The variation in spleen masses in the 14 infected animals (Figure 2) was not accounted for by spirochete burdens in the spleen, as estimated by qPCR ($R^2 = 0.08$).

Combined infection and immunization

Two groups of 30 animals of the same sex were studied separately, but the protocol was the same: immunization with KLH and OspA on day 0, inoculation with *B. hermsii* on day 21,

and euthanasia with blood and tissue collection on day 28. The most comprehensive studies were done on a group of 30 males that were offspring of 29 different mating pairs (experiment 3). They ranged in age from 90 to 566 d with a median of 200 and a mean of 270 d. Table 2 summarizes the values of several of the parameters representing the animals' bodies, the burdens of spirochetes in the blood and spleen, pathology of the liver, and antibodies to proteins in response to a single immunization 28 d previously (KLH and OspA) and to the infecting pathogen after 7 d (GlpQ and FbpC). The CV of the preinfection body masses was 0.19, similar to the 0.17 observed with 28 males in the uninfected group. After 7 d of the infection there was little change in body mass for the animals, but there were greater variances in relative spleen size. The R^2 for spleen mass regressed on body mass was only 0.11, similar to what was observed with 14 females and males (Figure 2).

The spirochete burdens in the blood and spleen 7 days into the infection varied over several orders of magnitude between individual animals. The log-transformed values for both blood and spleen approximated normal distributions (Shapiro-Wilk W test $p > 0.3$). In this experiment with a larger sample size (n = 30) we noted a positive association between the $\log_{10}$ of the genome copies per μg of extracted spleen DNA and the relative spleen size ($R^2 = 0.21$; $p = 0.01$). The burden of spirochetes in the spleen in turn plausibly was accounted for by the density of spirochetes in the blood at the time of euthanasia, according to log-log regression ($p < 0.0001$) (Figure 3). Although older males tended to have lower spirochete burdens than younger animals in the blood (n = 27; $R^2 = 0.18$; $p = 0.03$), the variances for untransformed values were similar for the 19 animals aged 90–246 days (CV = 2.2) and the 8 animals aged 379–566 days (CV = 1.8) ($F_{1,25} = 0.84$; $p = 0.36$). There was no discernible association of spirochete burdens in the blood or spleen with composite liver pathology scores ($R^2 = 0.01$), which ranged from 1 to 7, or with the most commonly noted feature, hemophagocytosis by Kupfer cells, which ranged from 1 to 3 in score.

There was also variation between individual animals in their antibody responses to the infection and the immunization (Table 2 and Figure 4). While all 30 animals responded to the immunization with KLH with EIA values of 0.724 or higher and little variation between individuals, only a minority of the animals had more than moderately elevated antibodies to FbpC, GlpQ, and/or OspA. Those animals that responded to one of these three borrelial antigens tended to respond to the others as well (Figure 4). There was no discernible association between age and antibody levels to KLH, FbpC, GlpQ, or OspA ($R^2 \leq 0.03$). The distinctions between animals in their antibodies to FbpC and GlpQ may be accountable by differences in the dynamics of antibody formation rather than the specificity; this was only 7 days into the infection. But that would not explain a similarly wide spread of values among the mice immunized with OspA along with KLH four weeks prior.

In experiment 4, the group of 30 females were the offspring of 21 different mating pairs. They ranged in age from 143 to 378 d with a median of 273 and a mean of 265 d The 0.08 value for the R^2 for spleen mass regressed on body mass was again lower in the infected animals than observed in the uninfected rodents (Figure 1). We noted once more a weak positive association between the relative spleen size and the number of *B. hermsii* genomes in the spleen after 7 d of infection ($R^2 = 0.11$; $p = 0.09$), as well as a weak negative

association between age and spirochete burdens in the blood (R^2 = 0.15; p = 0.07) in the female group.

Figure 5 summarizes for the infection of 30 females (experiment 4) the findings of relative spleen size, *B. hermsii* genomes in spleen and blood, and EIA values for antibodies to KLH, OspA, and FbpC, and compares the values with those of the group of males in experiment 3. Overall, the females were similar to the males in the diversity of the infection phenotypes, in particular, the log normal distributions of *B. hermsii* in the blood and spleen. Like the males, all the females produced antibodies to KLH within a comparatively narrow range but were more varied in their antibody responses to OspA and FbpC. The distributions of antibody concentrations for these two borrelial antigens among the individuals in this group were skewed, if not to the extent of the males' values (Figure 5).

Bleeding time

In the course of taking low-volume blood samples from the animals, we noted variation between the animals in the length of time for bleeding from the clipped tail to stop. This was investigated in more depth with a standardized bleeding time assay that was repeated 4 d later for 30 male mice before infection (experiment 3). The results were reproducible (Figure 6). The averaged replicates for each of the 30 animals ranged from 1.5 to 12 min, and the CV was 0.71 (Table 2). There was little or no association (R^2) between mean bleeding time and age (0.00), pre-infection body mass (0.01), hematocrit (0.00), relative spleen size (0.04), the log-transformed number of *B. hermsii* genomes in the blood (0.06), or the antibody response to OspA immunization (0.00).

Discussion

The experimental animals in this study were outbred rather than inbred, and presumably the sample more broadly represented the phenotypic diversity of species populations undergoing infections in nature. Though outbred rodents have long taken a back seat to inbred varieties of a species in biomedical research, the increasing application of forward genetics to non-model organisms means that experimental systems need not be limited to species that are second or third choice for an investigation. The laboratory mouse, *M. musculus*, in either inbred or outbred versions, has been elucidative for pathogenesis studies of *Borrelia* infections but is less relevant for the ecology of these infectious diseases, for modeling pathogen-reservoir-vector interactions and dynamics, and for measures to control these diseases at their sources, such as by targeting vaccines or other interventions to the reservoirs themselves. Needless to say, the outputs from forward genetics or mathematical modeling are only as good as the informativeness and replicability of the phenotypes to be scored in screens or incorporated for simulations.

Further assessment of the diversity of responses to infection and immunity of *P. leucopus* was the study's principal aim. There have been previous studies of experimental infections of *Peromyscus* species, including hantaviruses in *P. maniculatus* (Botten et al., 2000) and several of *B. burgdorferi* in *P. leucopus* (reviewed in Barthold et al., 2010). However, in these studies either the data on individual animals were largely qualitative or the sample sizes were small. One exception was our earlier *P. leucopus* study, in which we noted a wide

range of host responses among individuals animals infected with *B. burgdorferi*, but the emphasis in that study was on differences between strains of *B. burgdorferi* in the infections they caused in the animals (Baum et al., 2012). For the descriptive study presented here, the primary end-points instead were quantitative assessments of diversity within groups of up to 30 animals under the same experimental conditions, and not a comparison of treated with untreated animals per se.

To a large degree the study succeeded, as discussed below. Nevertheless, there were limitations to the study. The subjects differed from animals captured in the field in being detectably free of other infectious diseases and parasites, in the predictability and adequateness of their nutrition (Martin et al., 2007a), and their habitat's controlled, unvarying conditions (Martin et al., 2008). The animals were infected by needle inoculation and not by tick bite, although this may not be an important distinction for experimental *B. hermsii* infections (Policastro et al., 2013). Though more than a hundred animals were studied in total, these were divided for the experiments in groups of 30 or less. Consequently, the study may have been underpowered for detecting some true differences between uninfected and infected or between males and females. Though we preferred to exclude siblings in an experimental group, the exigencies of breeding schedules and availability contingencies meant that a small number of siblings (but not clones) were represented in some experiments (and were specifically requested for the mixed sex experiment). Finally, although *P. leucopus* can be infected with *B. hermsii* by needle inoculation (Baum et al., 2012), it is not the usual reservoir for this species. *B. burgdorferi* would be suitable, but we reasoned that for a first investigation a pathogen that more predictably produced higher blood and organ burdens in this species could provide a greater potential range of host responses for assessments of phenotype diversity. *P. leucopus* can be infected with *B. miyamotoi* (Scoles et al., 2001), and thus this species would be another choice.

The principal findings of the four experiments were the following:

1. Values for burdens of spirochetes in the blood or spleen, as measured by qPCR with normalization for total DNA in reactions, fit a log-normal distribution with a wide divergence among individual animals (Figure 5), similarly to what was observed in infected wild animals that were captured (Barbour et al., 2009). Spirochete DNA in the spleen closely tracked burdens in the blood at the same time. Residual blood accounts for some or much of the genome target that was measured in the spleen, while additional DNA may represent live or dead sequestered bacterial cells or their breakdown in the spleen. This was a narrow time window for sampling an infection, and delaying the collection a day or two later may have yielded different results, especially if immune responses are building. Notwithstanding this limitation, the employed protocol yielded similar results upon replication. The broad range of burdens in blood and spleen among individuals, first observed in the group of males, was also noted in the study of the female group. While the apparent effect of age on spirochete burdens was modest, it is a parameter to be controlled for in future studies. Among the several testable hypotheses to account for the variances of pathogen burdens are individual

differences in a rate-limiting step in the adaptive immune response or innate immunity signaling pathways.

2. Among uninfected animals, spleen mass scaled allometrically to body mass and similarly to liver mass scaling, but there was comparatively greater variation in relative size of spleen than of the liver. The variation of relative spleen size was greater than what had been observed with *M. musculus* across a collection of inbred strains. After one week of infection there was further divergence in this characteristic between individual *P. leucopus* to the point of little or no discernible association of spleen mass with terminal body mass. Pre-infection spleen mass plausibly was an independent determinant of mid-infection spleen mass. But the remaining variance appears to be only partially attributable to the burden of live (or dead) spirochetes in the spleen. Change in relative spleen size during infection may then be an informative quantitative trait that is not solely dependent on pathogen burden. Given the important role of the spleen in local and systemic immunity (Bronte and Pittet, 2013), including for *Borrelia* infections (Meleney, 1928; Smith et al., 1985), this phenomenon may not be trivial.

3. All animals in two different experiments of 30 subjects had moderate to high levels of antibodies to the reference antigen KLH four weeks after single dose immunization. The antibody responses were age-independent and were similar in magnitude to what Martin et al. (2007b) observed in *P. leucopus*. In contrast, only a minority of animals in either of the two groups had comparable EIA values for antibodies to the OspA protein, which had been administered with the KLH. The doses of OspA were equivalent on weight basis to what had elicited strong antibody responses in two inbred strains of *M. musculus* injected with the same preparation of OspA (Pal et al., 2003). Higher variances for anti-OspA antibody levels than for anti-KLH antibodies across a panel were also noted among 30 females in a separate experiment. The same animals that had high antibody levels to OspA also had higher than the mean levels of antibodies to two other borrelial proteins, FbpC and, to a lesser extent, GlpQ. These antibodies developed in response to an acute infection and not immunization. Like OspA, FbpC is a lipoprotein, so an explanation for co-variation in antibody levels to these proteins might lie in signalling pathways for innate responses to bacterial lipoproteins in general (Salazar et al., 2009). Whatever the explanation, this heterogeneity of response in *P. leucopus*, the main candidate for OspA-based, transmission-blocking vaccines (Meirelles Richer et al., 2011; Tsao et al., 2004), may have implications for further development and implementation of this reservoir-targeting immunization project.

4. A serendipitous finding among uninfected animals was reproducible differences over a 10-fold range in the time for hemostasis after the tail was clipped (Figure 6 and Table 2). The technique that was used corresponds to the Duke method for the bleeding time test for defects in hemostasis of humans (Harker and Slichter, 1972). The test is mainly used for detecting evidence of platelet dysfunction, but the time can also be prolonged when platelet concentrations in the blood are abnormally low. Platelet counts in 40 *P. leucopus* from the PGSC had a mean of 394,000 per μl with a SD of 139,000 and CV of 0.35 (Wiedmeyer et al., 2014). While this is

within the range of normal platelet counts for humans, it is about half of what is reported for *M. musculus* (Sun et al., 2014). *P. leucopus* and *P. maniculatus* have also been noted to have unusual timing of the coagulation of their blood, which is a phenomenon that is distinguished from the bleeding time assay used here (Folk et al., 2010). The cause of the varied bleeding times in *P. leucopus* remains to be determined. But the observed variation conceivably has consequences for tick feeding and the duration of blood meals, given the importance of hemostasis for the embedded tick (Sonenshine and Anderson, 2014).

Conclusions

P. leucopus, as represented by these samplings of an outbred but colony-bred population, manifested considerable diversity in phenotype among uninfected animals and to the same if not greater extent among animals in their responses to infection and immunization. The natural variation observed in animals in a stable and protected environment would be the foundation upon which several different environmental effects, such as other infectious diseases, predators, and climate, would build under natural conditions. Attempts to model the ecology of tick-borne zoonoses should incorporate estimates of variance among these important reservoir hosts for inferences to hold true under field validation. Reservoir-targeted vaccination programs in their planning cannot assume that individual animals will respond in an “average” way. Finally, the findings encourage further development of *Peromyscus* as a model organism that is particularly relevant for ecological and population genetics studies in North America. Of particular interest as quantitative traits of this reservoir host are relative spleen size, spirochete burdens, and antibody responses to certain lipoproteins.

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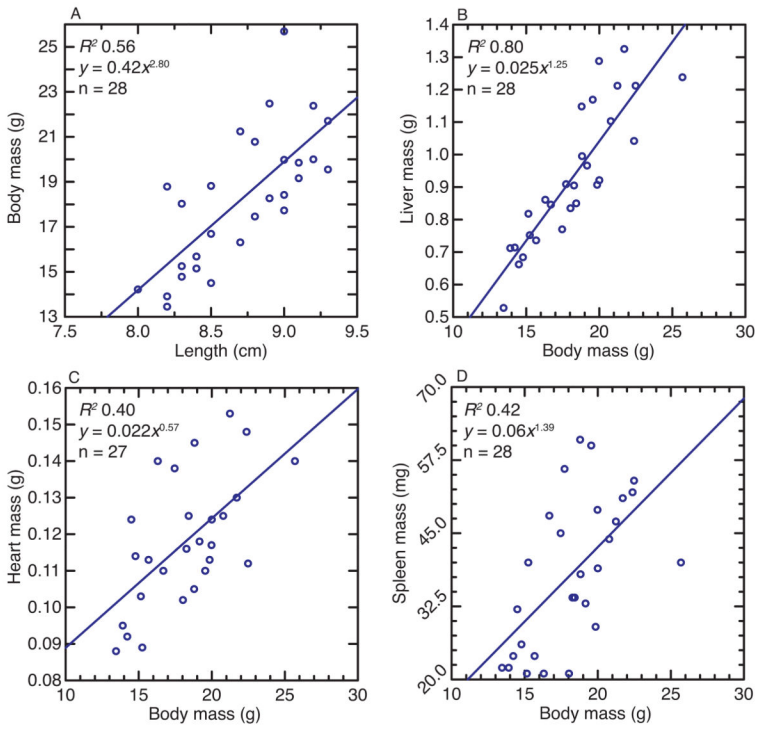

Figure 1.

Four scatter plots of the relationships between body mass and either body length (panel A), liver mass (B), heart mass (C), or spleen mass (D) of 28 uninfected male *P. leucopus* (experiment 1). The least-squares linear regression line, the number (*n*) of animals in the dataset, the coefficient of determination (R^2), and the power equation for the trend line (where *x* is the *x*-axis value and *y* is the *y*-axis value) are shown.

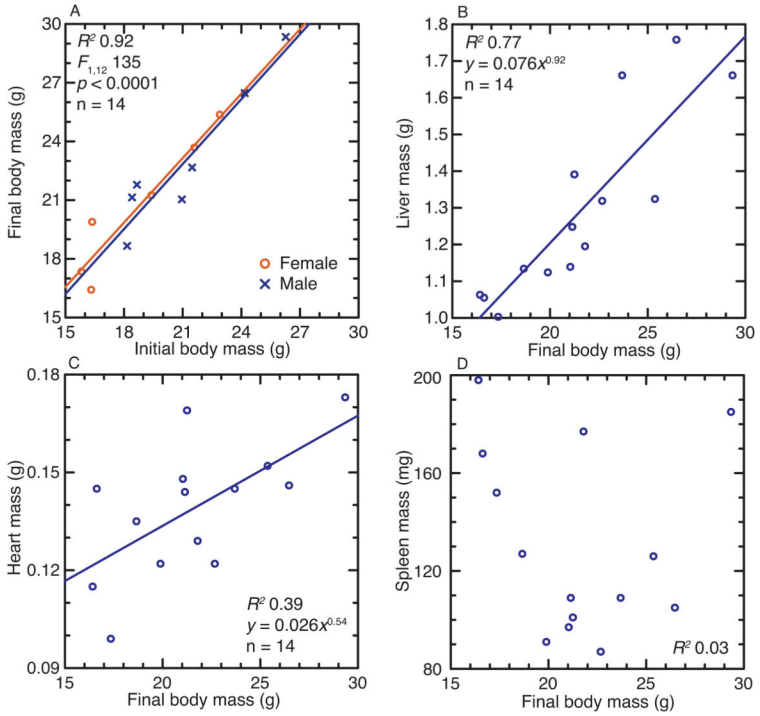

Figure 2.

Four scatter plots of the relationships between final (day 7) body mass and either initial (day 0) body mass (panel A), liver mass (B), heart mass (C), or spleen mass (D) of 7 female and 7 littermate-matched *P. leucopus* infected with *B. hermsii* on day 0 (experiment 2). The least-squares linear regression line, the number (*n*) of animals in the dataset, coefficient of determination (R^2), and the power equation for the trend line (where *x* is the *x*-axis value and *y* is the *y*-axis value) are shown. In panel A the female and male animals are distinguished by symbols and colors, as shown, and by separate regression lines. In the other panels females and males are not distinguished.

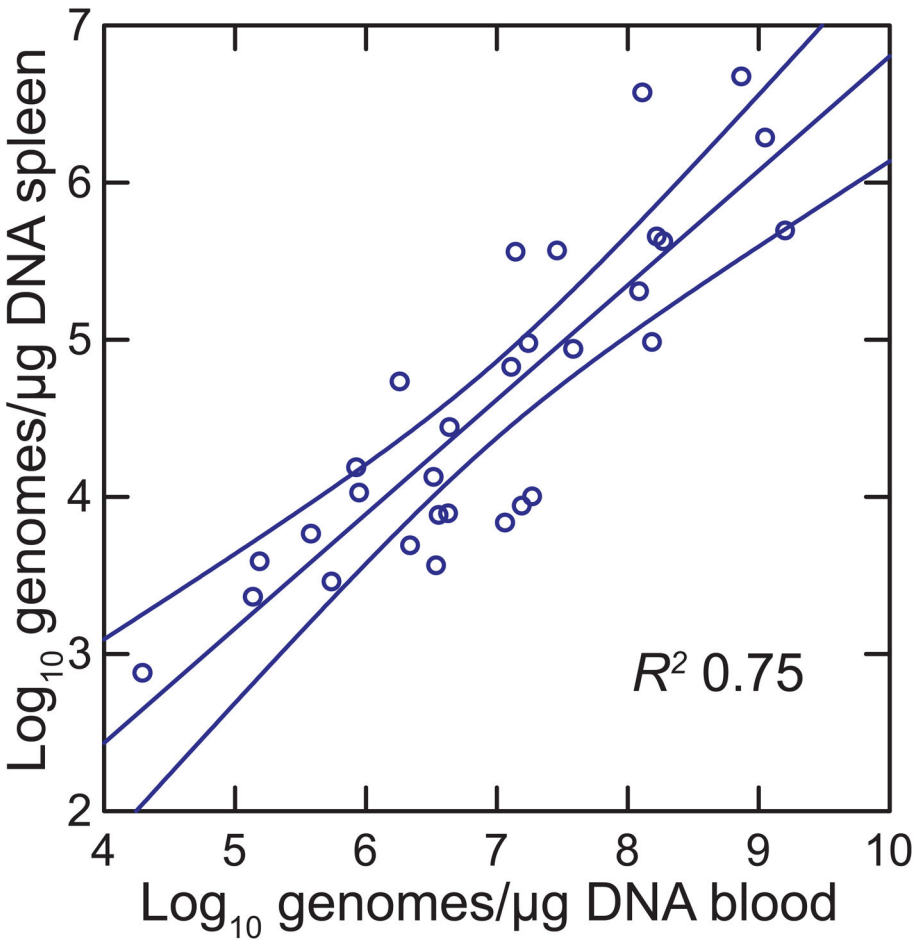

Figure 3.

Scatter plot of log-transformed normalized *B. hermsii* genome copies in spleen on whole blood copies in 30 male *P. leucopus* at day 7 of infection (experiment 3). Spirochete burdens were estimated by species-specific quantitative PCR (qPCR). The least-squares linear regression with 95% confidence limits and coefficient of determination *(R^2)* are shown.

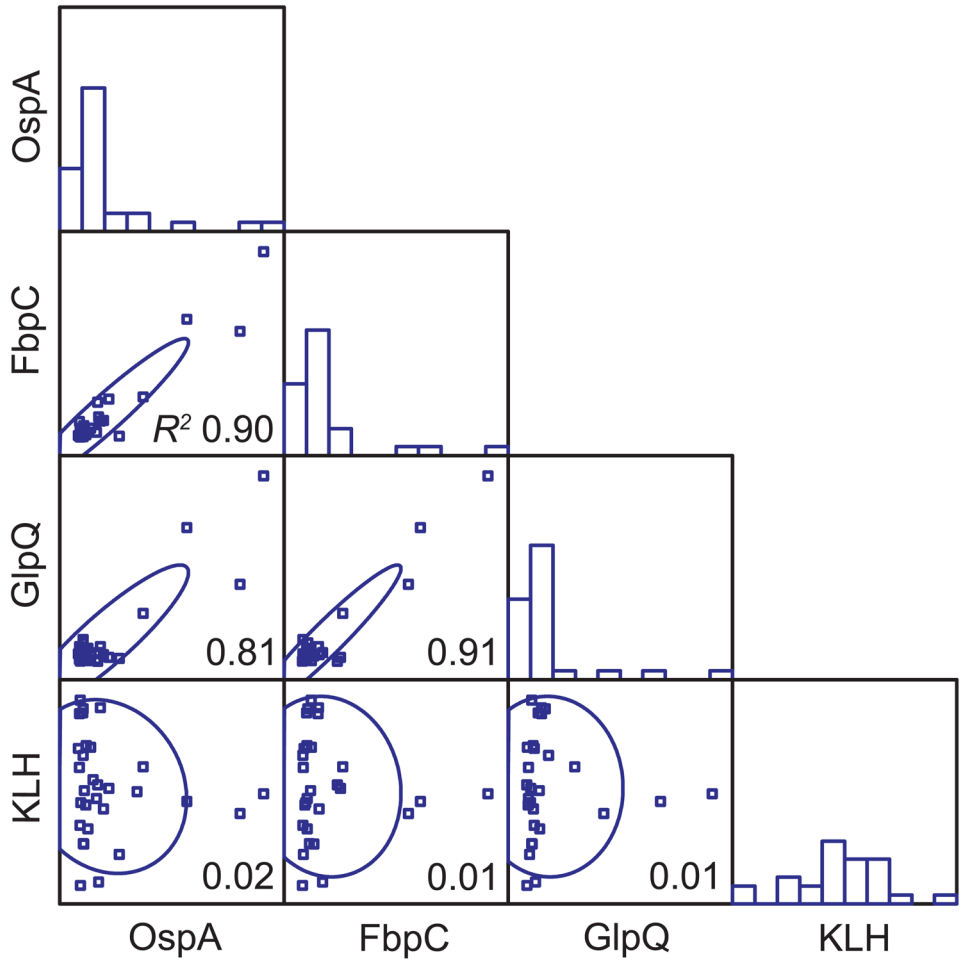

Figure 4.

Combined frequency histograms and scatter plots of the binding of serum antibodies of 30 male *P. leucopus* (experiment 3) to OspA, FbpC, GlpQ, and keyhole limpet hemocyanin (KLH) proteins. The animals were immunized with OspA and KLH on day 0, infected with *B. hermsii* on day 21, and euthanized for blood and tissue collection on day 28. Antibody concentrations of the different specificities were determined by enzyme immunoassay (EIA) as described in text. In the histograms the *x* axes indicate relative EIA values of optical density at 450 nm and the *y* axes indicate relative counts. In the scatter plots both the x and y axes indicate the relative EIA values in a pairwise fashion. For x axes values rise from left to right, and for y axes values rise from bottom to top of the graphs.

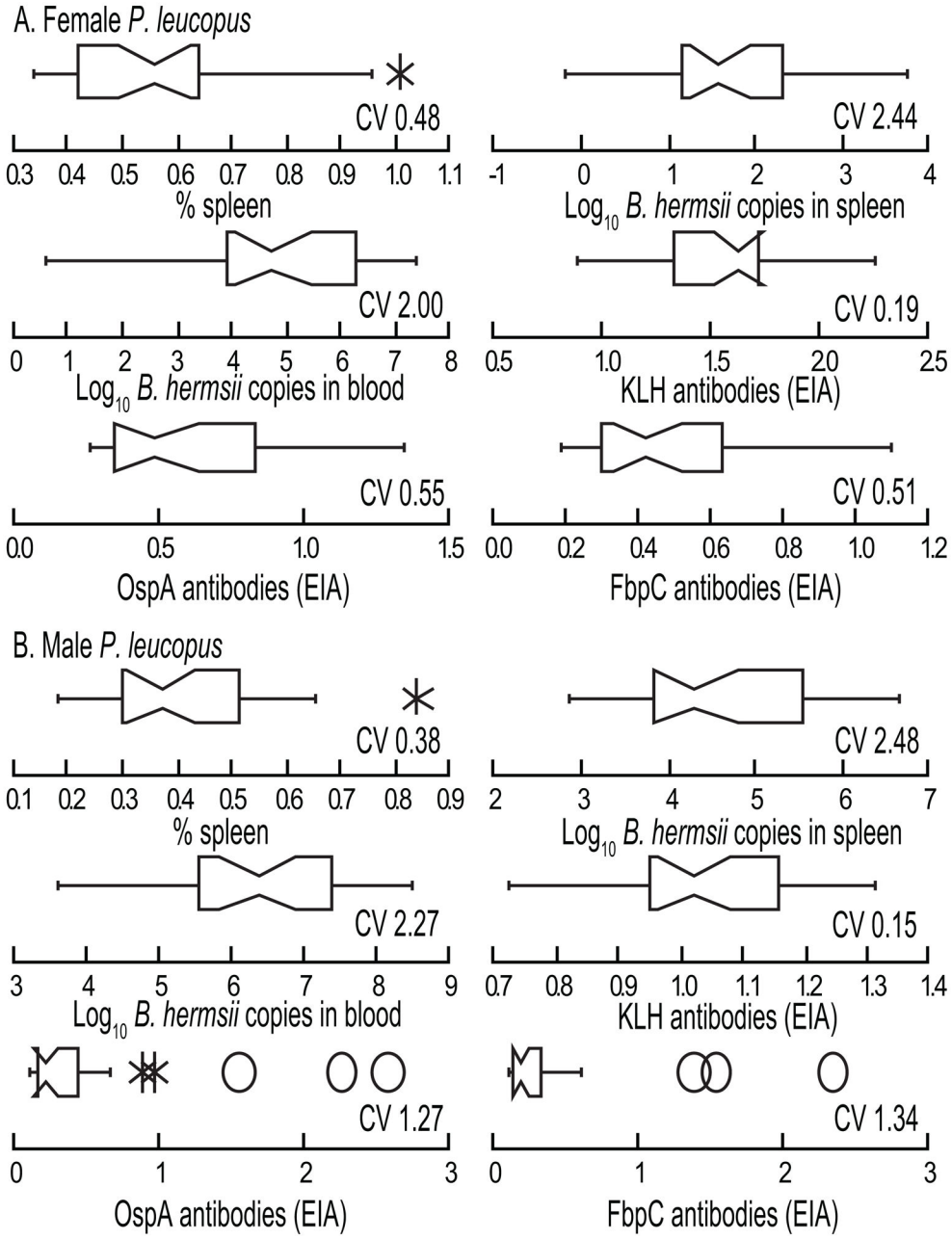

Figure 5.

Selective characteristics of 30 female (panel A; experiment 4) and 30 male (panel B; experiment 3) *P. leucopus* immunized with OspA and keyhole limpet hemocyanin (KLH) and then infected with *B. hermsii*. The box-whisker plots are of the relative spleen size as percentage (%) of total body mass, the burden of spirochetes in the spleen and blood as determined by qPCR of genome copies of *B. hermsii*, and antibody levels to KLH and OspA as determined by enzyme immunoassay with readings in optical density at 450 nm. The coefficient of variation (CV) is shown for each determination. The CV for genome copies was calculated from the original and not the log-transformed values. Each horizontal box

indicates the first and third quartiles, and the indentation inside the box is the median. The 1.5× interquartile range is indicated by the horizontal line (whiskers) bisecting the box, and values outside this range are indicated by asterisks and ovals.

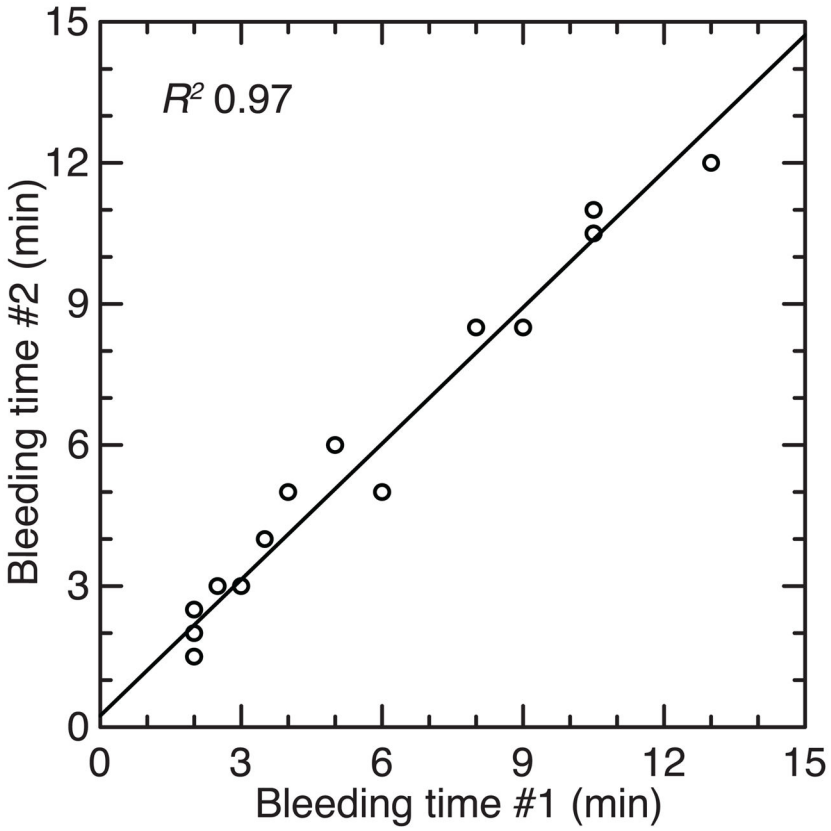

Figure 6.
Paired bleeding times of 30 uninfected male *P. leucopus* (experiment 3) taken 4 days apart. The least-squares linear regression and coefficient of determination *(R^2)* are shown.

Table 1

Characteristics of *Borrelia hermsii* (Bh) infection of female and male *Peromyscus leucopus*

Sex	Change in mass (g) (95% CI) [a]	% spleen mass [b]	% liver mass [b]	% heart mass [b]	Blood Bh genomes × 10^5 [c]	Spleen Bh genomes × 10^3	FbpC EIA (OD) [d]
Female (n=7)	1.91 (1.14–2.69)	0.71 (0.47–0.95)	6.1 (5.7–6.6)	0.68 (0.59–0.77)	1.10 (0.06–18.5)	4.32 (2.26–8.26)	0.72 (0.53–0.91)
Male (n=7)	1.86 (0.91–2.81)	0.55 (0.43–0.67)	5.9 (5.5–6.2)	0.63 (0.57–0.68)	1.52 (0.11–21.0)	7.35 (1.42–38.1)	0.52 (0.27–0.76)
Paired *t* test *p* value	0.91	0.14	0.30	0.39	0.86	0.60	0.10

[a] 95% confidence interval

[b] Percentage (%) of spleen, liver, or heart mass to total body mass

[c] Quantitative PCR for 16S rRNA gene; per µg total DNA of the extract

[d] Enzyme immunoassay optical density (OD) at 450 nm for antibodies to FbpC protein; mean of duplicates

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Table 2

Selected quantitative traits of 30 male *P. leucopus* in combined infection/immunization experiment

Parameter	Median	Mean	SD [a]	CV [b]
Day 21 mass (g)	22.82	23.32	4.46	0.19
Δ mass by day 28 [c]	−0.03	−0.08	1.11	na [d]
Spleen mass (mg)	86.5	93.7	36.0	0.38
Spleen %	0.37	0.41	0.15	0.38
Hematocrit (%)	44.0	43.7	4.3	0.10
Bleeding time (min)	3.0	4.2	3.0	0.71
Pathology score [e]	2.5	2.9	1.7	0.60
Blood genomes [f]	0.91×10^7	13.0×10^7	39.6×10^7	2.27
Spleen genomes [f]	0.22×10^5	4.1×10^5	10.2×10^5	2.48
KLH EIA (OD_{450}) [g]	1.022	1.036	0.158	0.15
FbpC EIA	0.190	0.387	0.517	1.34
GlpQ EIA	0.242	0.283	0.115	0.41
OspA EIA	0.224	0.484	0.616	1.27

[a] SD, standard deviation

[b] CV, coefficient of variation

[c] Change (Δ) in mass in gram (g) between day 21 and day 28

[d] na, not applicable

[e] Composite score of histological findings (see text); maximum score of 13

[f] Number of *B. hermsii* genomes by qPCR per μg of total DNA in extracts of whole blood or spleen

[g] Enzyme immunoassay optical density (OD) at 450 nm; mean of duplicate determinations