



Published in final edited form as:

*Ticks Tick Borne Dis.* 2019 February ; 10(2): 352–359. doi:10.1016/j.ttbdis.2018.11.017.

## An immunocompromised mouse model to infect *Ixodes scapularis* ticks with the relapsing fever spirochete, *Borrelia miyamotoi*

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### Abstract

The hard tick-borne relapsing fever spirochete, *Borrelia miyamotoi*, has recently gained attention as a cause of human illness, but fundamental aspects of its enzootic maintenance are still poorly understood. Challenges to experimental studies with *B. miyamotoi*-infected vector ticks include low prevalence of infection in field-collected ticks and seemingly inefficient horizontal transmission from infected immunocompetent rodents to feeding ticks. To reliably produce large numbers of *B. miyamotoi*-infected ticks in support of experimental studies, we developed an animal model where immunocompromised *Mus musculus* SCID mice were used as a source of *B. miyamotoi*-infection for larval and nymphal *Ixodes scapularis* ticks. Following needle inoculation with  $1 \times 10^5$  spirochetes, the SCID mice developed a high spirochetemia (greater than  $1 \times 10^7$  copies of *B. miyamotoi purB* per mL of blood) that persisted for at least 30 d after inoculation. In comparison, immunocompetent *M. musculus* CD-1 mice developed transient infections, detectable for only 2–8 d within the first 16 d after needle inoculation, with a brief, lower peak spirochetemia ( $8.5 \times 10^4 - 5.6 \times 10^5$  *purB* copies per mL of blood). All larval or nymphal ticks fed on infected SCID mice acquired *B. miyamotoi*, but frequent loss of infection during the molt led to the proportion infected ticks of the resulting nymphal or adult stages declining to 22–29%. The ticks that remained infected after the molt had well-disseminated infections which then persisted through successive life stages, including transmission to larval offspring.

### Keywords

*Borrelia miyamotoi* ; *Ixodes scapularis* ; Mouse model; Transmission; Transovarial transmission

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.ttbdis.2018.11.017>.

## 1. Introduction

The relapsing fever spirochete, *Borrelia miyamotoi*, is increasingly recognized as a cause of human illness (named hard tick-borne relapsing fever or *Borrelia miyamotoi* disease) in North America, Europe, and Asia (Platonov et al., 2011; Krause et al., 2013, 2014; Sato et al., 2014; Molloy et al., 2015; Wagemakers et al., 2015; Fiorito et al., 2017). The primary vector of *B. miyamotoi* to humans in the eastern United States is the blacklegged tick, *Ixodes scapularis*, which also transmits the Lyme disease spirochetes, *Borrelia burgdorferi* sensu stricto (s.s.) and *Borrelia mayonii* (Krause et al., 2015a; Eisen, 2018; Eisen and Eisen, 2018). In nature, infection of nymphal and adult *I. scapularis* ticks with *B. miyamotoi* is relatively infrequent, often with a 10-fold lower infection prevalence compared with *B. burgdorferi* s.s. (Tsao et al., 2004; Barbour et al., 2009; Krause et al., 2015b; Nelder et al., 2016; Johnson et al., 2018). This discrepancy raises the question of whether natural maintenance relies on the same modes of transmission for both spirochetes. Elucidating how *B. miyamotoi* is maintained in enzootic transmission cycles also will clarify whether or not control strategies specifically targeting enzootic transmission of *B. burgdorferi* s.s. among tick vectors and rodent reservoirs can be assumed to also impact *B. miyamotoi*.

Horizontal transmission, where infected ticks transmit spirochetes to vertebrate hosts, and naïve ticks then acquire spirochetes while feeding on the infectious hosts, is the predominant mode of natural transmission for *B. burgdorferi* s.s. (Anderson et al., 1985; Donahue et al., 1987; Rollend et al., 2013). Natural maintenance of *B. miyamotoi* is still poorly understood, but in contrast to *B. burgdorferi* s.s., it is perpetuated not only via horizontal transmission among tick vectors and vertebrate reservoirs but also by transovarial (vertical) spirochete passage from infected females to their offspring (Scoles et al., 2001; Richter et al., 2012; Rollend et al., 2013; Breuner et al., 2018). If transovarial transmission proves to be of primary importance in maintaining *B. miyamotoi* in tick populations, control efforts focused on reducing abundance of host-seeking ticks will be more effective than strategies attempting to disrupt horizontal transmission of this spirochete. In addition, if both horizontal and transovarial transmission contribute substantially to the natural maintenance of *B. miyamotoi*, it will require further investigation as to why this spirochete nevertheless consistently is less prevalent in nymphal and adult *I. scapularis* ticks compared with *B. burgdorferi* s.s. (Scoles et al., 2001; Barbour et al., 2009; Hoen et al., 2009; Nelder et al., 2016).

Previous studies assessed the efficiency of transmission of *B. miyamotoi* from transovarially-infected *I. scapularis* larvae or nymphs to experimental hosts (Breuner et al., 2017, 2018). However, neither included an evaluation of the efficiency of acquisition of *B. miyamotoi* by larval ticks from infected hosts and subsequent transstadial passage to the nymphal stage, nor did they assess the efficiency of transmission from horizontally *B. miyamotoi*-infected *I. scapularis* ticks to experimental hosts. Though field-collected *Ixodes* ticks have been used for previous studies of this nature (Scoles et al., 2001; van Duijvendjik et al., 2016), the low prevalence of *B. miyamotoi* in ticks collected from the wild presents an added challenge for experimental work, and limits the ability to control for factors including pathogen strain and co-infections with other pathogens. Here we report on the use of a mouse model to infect immature *I. scapularis* ticks with a North

American isolate of *B. miyamotoi*. Experiments were conducted to describe 1) kinetics of spirochetemia in immunocompetent and immunocompromised *Mus musculus* mice, and 2) infection dynamics in ticks fed on *B. miyamotoi*-infected immunocompromised mice, including spirochete acquisition by larvae and nymphs, and transstadial passage of infections to successive life stages.

## 2. Materials and methods

### 2.1. *Borrelia miyamotoi* isolate, experimental vertebrate hosts, and *I. scapularis* ticks

The North American CT13–2396 *B. miyamotoi* isolate (Kingry et al., 2017) was used to needle-inoculate *Mus musculus* mice (Sections 2.2 and 2.3). Experimental mouse hosts included 6 wk old female Fox Chase SCID mice (CB17/Icr-Prkdc<sup>scid</sup>/IcrIcoCrI) and 8 wk old female CD-1 mice (Charles River Laboratories, Wilmington, MA, USA). Larval and nymphal *I. scapularis* ticks used in transmission experiments with infected SCID mice (Section 2.3) were sourced from the Oklahoma State University Tick Rearing Facility (Stillwater, OK, USA) and the Medical Entomology Laboratory at the Centers for Disease Control and Prevention (Atlanta, GA, USA) and a subset (a pool of 50 larvae per clutch, or a pool of 10 nymphs for every 100 used) were tested at CDC using the assay described below, and determined to be *B. miyamotoi*-free (*purB* negative). *Ixodes scapularis* nymphs with transovarially acquired infections, descended from field-collected females (see supplemental materials), were included in the study solely for comparison of spirochete load with nymphs harboring laboratory-acquired infections.

### 2.2. Duration of infection with *B. miyamotoi* in needle-inoculated mice

Ten CD-1 mice and 10 SCID mice were inoculated via needle with low passage (P3) *B. miyamotoi* spirochetes grown at 34 °C in modified Barbour-Stoenner-Kelly (BSK) medium (in-house BSK-R medium) without antibiotics. The mice were inoculated intraperitoneally with 100 µl suspended culture at a concentration of approximately  $1 \times 10^6$  spirochetes/mL ( $1 \times 10^5$  spirochetes inoculated per mouse). Starting at 24 h after spirochete inoculation, a small amount of blood (15 µl) was extracted from each mouse by lateral tail vein nick and collected using MICROSAFE capillary tubes (Medicore, Nashville, TN, USA). To comply with Institutional Animal Care and Use guidelines for weekly limit of blood volume extracted, one of two groups of each mouse strain were sampled on alternating days, so that samples were collected from each individual mouse at 48 h intervals, and five mice of each strain were sampled daily. Blood collection was concluded for CD-1 mice at 15 or 16 days post-inoculation (d.p.i.), when three consecutive samples had yielded negative results for 9 of 10 mice. We collected blood from SCID mice over a period of 30 d.p.i., with frequency of collection reduced from 48 h intervals to biweekly intervals after 16 d.p.i. The methods for detection and quantification of *B. miyamotoi* spirochetes in mouse blood are described in Section 2.4. All animal procedures were approved by the Centers for Disease Control and Prevention Division of Vector-Borne Diseases Animal Care and Use Committee, in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

### 2.3. Acquisition of *B. miyamotoi* from infected SCID mice by feeding larval or nymphal ticks and transstadial passage

After first assessing the kinetics of infection in mice, 2 additional sets of 10 SCID mice were inoculated with *B. miyamotoi* via needle (as described in Section 2.2) and then exposed to feeding ticks. Ticks were introduced following an incubation period of at least 7 d.p.i. and not exceeding 21 d.p.i., a time period during which mouse spirochetemia was expected to be high and stable in these immunodeficient mice based on the first experiment outlined in Section 2.2. Mice were exposed to either naïve larval ticks (50–100 larvae for each of 10 mice) or naïve nymphal ticks (25 nymphs for each of 10 mice). Mouse blood was collected (as described in Section 2.2) on the final day of tick feeding and then tested by qPCR (see Section 2.5) to confirm *B. miyamotoi* infection. Within 24 h of tick drop off, a subset of freshly fed larval ticks (15 per infected mouse) were set aside for detection and quantification of *B. miyamotoi* spirochetes, as described in Section 2.4. The remaining fed larvae were placed within glass desiccators (90 to 95% relative humidity) in a growth chamber maintained at 21 to 23 °C with a 16:8 h light:dark cycle. Detection and quantification of *B. miyamotoi* spirochetes was also done for a subset of larvae harvested 3 wk post-drop off (9 per infected mouse) and for nymphs harvested shortly after the molt (10–15 per infected mouse) and 2 mo post-molt (up to 10 per infected mouse). The remaining nymphs were fed on non-infected CD-1 mice, and approximately 2 mo after adults had molted, females and males from this tick cohort were placed on a New Zealand White rabbit (Charles River Laboratories) and allowed to feed. Males and unfed females were collected and stored in 70% ethanol at 4 °C until qPCR was performed. Engorged females were tested after oviposition was completed or after being removed during unsuccessful feeds. A similar process was followed for ticks first fed on infectious mice as nymphs, with detection and quantification of *B. miyamotoi* spirochetes performed both on freshly molted adults, as well as on adults following exposure to a rabbit. Engorged females were included in our estimate of proportion infected with *B. miyamotoi*, but were excluded from median copy number calculation.

### 2.4. Detection and quantification of *B. miyamotoi* spirochetes from mouse blood and ticks

Collected mouse blood (15 µl) was immediately transferred into microcentrifuge tubes containing 200 µl lysis buffer (180 µl ATL - Qiagen, Valencia, CA, USA; 20 µl Proteinase K - Qiagen; 1 µl Carrier RNA - Applied Biosystems, ThermoFisher Scientific, Houston, TX, USA; and 1.5 µl DX Reagent - Qiagen) before clotting could occur. Samples were then incubated at 56 °C for 60 min and the lysates were stored at 4 °C until DNA extraction was performed. Ticks were surface cleaned with sodium hypochlorite diluted to 0.01% in water and rinsed in Milli-Q water (Millipore Corporation, Billerica, MA, USA). Dry ticks were stored at –80 °C until DNA extraction was performed. Whole ticks were ground using a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, OK, USA) and 2.0 mm Very High Density Ytria stabilized zirconium oxide beads (GlenMills, Clifton, NJ, USA) immersed in 350 µl tissue lysis buffer (327.25 µl ATL, 20 µl Proteinase K, 1 µl Carrier RNA, and 1.5 µl DX Reagent). DNA was extracted from blood lysates or whole tick triturates using the KingFisher Flex system with MagMAX kit (ThermoFisher Scientific) and eluted into 90 µl elution buffer.

To detect and quantify spirochetes in mouse blood, a duplex assay targeting the *B. miyamotoi* adenylosuccinate lyase (*purB*) gene (forward primer - TCC TCA ATG ATG AAA GCT TTA, reverse primer - GGA TCA ACT GTC TCT TTA ATA AAG, probe – CalRd610-TCG ACT TGC AAT GAT GCA AAA CCT-BHQ2) (Graham et al., 2016) and rodent GAPDH (Applied Biosystems® TaqMan® Rodent GAPDH ControlReagents kit; ThermoFisher Scientific) was used. For tick samples, a duplex assay targeting *purB* and the *I. scapularis* actin gene (forward primer - GCC CTG GAC TCC GAG CAG, reverse primer - CCG TCG GGA AGC TCG TAG G, probe – Quas705-CCA CCG CCG CCT CCT CTT CTT CC-BHQ3) (Hojgaard et al., 2014) was used. Actin and GAPDH served as controls for DNA purification and qPCR. The Biotechnology Core Facility Branch at the Centers for Disease Control and Prevention (Atlanta, GA, USA) synthesized all oligonucleotides.

A recombinant plasmid containing the *purB* sequence (Graham et al., 2016) was used to create standard curves for each plate of samples, allowing quantitation cycle (Cq) values to be converted to gene copy numbers. Reactions were performed in 96 well plates using 1X iQ Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA, USA) containing 300 nM of each primer and 200 nM of each probe per 10 µl reaction. Between 4.8 to 5.3% of DNA extracted from a tick was used per reaction as template. Real-time cycling conditions followed a previously described protocol (Graham et al., 2016), and qPCR samples were analyzed using CFX Manager 3.1 software (Bio-Rad) with Cq determination set to regression.

We used a dilution series of the recombinant plasmid, which included six replicates per dilution, to determine the limit of detection for *purB* copies. Though as few as a single copy per reaction could be detected in some samples, the assay used for blood and ticks was consistently able to detect 6 copies per reaction, resulting in a limit of detection of 8.2 copies per microliter of blood ( $8.2 \times 10^3$  copies per mL of blood) and 113 copies per tick. A single *purB* copy was considered equivalent to one spirochete, and whole tick spirochete loads were estimated by correcting *purB* copy number for the fraction of tick DNA included in the qPCR assay.

## 2.5. Studies on dissemination of *B. miyamotoi* spirochetes in infected nymphs

Dissemination of *B. miyamotoi* spirochetes in horizontally infected, unfed nymphs (see Section 2.3) was assessed by qPCR and direct fluorescence assay (DFA). Individual tick tissues, including midguts and salivary glands were dissected out on microscope slides using an Olympus SZX12 stereo microscope (Olympus, Tokyo, Japan). Sterile PBS was used to facilitate tissue extraction. For qPCR, individual tick tissues were then transferred to tubes containing 200 µl lysis buffer. Tick tissue lysates were incubated and stored as described for mouse blood lysates, and DNA extraction and detection and quantification of *B. miyamotoi* spirochetes was performed as described for whole ticks in Section 2.4. For DFA, tick tissues were smeared on slides, air dried, fixed in acetone, and stored at  $-20^{\circ}\text{C}$  until further use. Prior to labelling, slides were washed twice in sterile PBS and blocked with SuperBlock Blocking Buffer (Thermo Scientific) for 30 min, followed by three washes with PBS. Tissues were then treated with murine monoclonal anti-flagellin antibody H9724 (Barbour et al., 1986; Gugliotta et al., 2013) (1:200) for 1 h, washed 3 times in PBS, and

stained with anti-mouse IgG H + L Alexa Fluor 594 (1:500) (Molecular Probes by Life Technologies, Carlsbad, CA, USA) for 1 h. Finally, slides were washed 3 times with PBS and rinsed with dd H<sub>2</sub>O, and coverslips were mounted using ProLong DIAMOND anti-fade (Molecular Probes by Life Technologies) and allowed to dry overnight at 4 °C. Samples were viewed using a Zeiss LSM 800 confocal laser scanning microscope (Carl Zeiss, Inc. Oberkochen, Germany) and images produced using the LSM Software Zen 2 (Blue Edition).

## 2.6. Statistical analysis

Data were analyzed and graphed using JMP 13 software (SAS Institute, Cary, NC, USA). Kruskal-Wallis or Mann-Whitney U tests were used for comparisons of median *purB* copy numbers among or between groups, respectively, including for mouse strain, ticks originating from different mice, and tick groups tested at different life stages. H and U statistics were approximated as the  $\chi^2$  values produced using JMP.

## 3. Results

### 3.1. Duration and magnitude of spirochetemia in mice infected with *B. miyamotoi* via needle inoculation

Throughout the sampling period, spirochetemia (estimated as *purB* copy number per mL blood) was variable among immunocompetent CD-1 mice, fluctuating between peaks as high as  $6 \times 10^5$  copies/mL and concentrations below the limit of detection of our assay (Fig. 1). All mice had detectable infections on both sampling dates between 5–8 d.p.i., yet, by 16 d.p.i., three consecutive blood samples had tested negative for 9 of 10 mice. In the single exception, only one (13 d.p.i.) of the last three blood samples taken had detectable *purB*. In each of the immunocompromised SCID mice, *purB* copy numbers increased rapidly, reaching  $> 1 \times 10^7$  copies/mL of blood by 11 d.p.i. (Fig. 1), after which all 10 mice maintained between  $1 \times 10^7$  and  $7 \times 10^7$  copies/mL of blood through the conclusion of the sampling period at 30 d.p.i. The median peak spirochetemia for SCID mice ( $4.6 \times 10^7$  *purB* copies/mL) was 2 logs higher than for CD-1 mice ( $2.7 \times 10^5$  *purB* copies/mL) (Table 1; U = 14.3; d.f. = 1;  $P = 0.0002$ ). The median number of d.p.i. until the first detection of *purB* did not differ significantly between the mouse strains (2 d in both cases). However, median peak spirochetemia was reached later for SCID mice (14 d; range = 12–30 d) than for CD-1 mice (6.5 d, 1–10 d) (U = 14.4; d.f. = 1;  $P = 0.0001$ ).

### 3.2. Acquisition of *B. miyamotoi* from infected SCID mice by feeding ticks and transstadial passage

*PurB* copies were quantified in blood from the SCID mice used to infect larval *I. scapularis* directly following cessation of tick drop off, confirming that elevated spirochetemia ( $4.3 \times 10^6$ – $4.1 \times 10^7$  copies/mL blood) was attained in all 10 tick-exposed mice. All 150 freshly fed larvae examined contained *B. miyamotoi* DNA (median: 12,500 copies per tick, range: 3500–35,200) (Fig. 2). The highest median *purB* copy number for ticks collected from an individual mouse was 17,300 (range of 5600–28,400), whereas the lowest was 8300 (range of 4600–20,100). While statistically significant differences in median values for tick *purB* copy number were detected among individual mice ( $\chi^2 = 33.6$ ; d.f. = 9;  $P = 0.0001$ ), all ticks tested contained at least 3500 copies and no more than 35,000 copies.

At three weeks post-drop off, 9 larvae from each mouse were tested and the percentage positive had declined to 46%, while the median *purB* copy number for positive ticks had decreased slightly to 9900 copies (Fig. 3). Infection prevalence then declined further to 24.6% in molted, flat nymphs, with a median *purB* copy number of 89,800 per tick (Fig. 3). Copy numbers for infected molted nymphs (median 89,800; range 42,300–258,000) were significantly higher than for replete larvae both at drop off (12,500; 3500–21,100;  $\chi^2 = 74.6$ ; d.f. = 1;  $P = 0.0001$ ) and at 21 d after drop off (9900; 300–44,400;  $\chi^2 = 45.0$ ; d.f. = 1;  $P = 0.0001$ ). There was no statistically significant difference in median copy number between the two groups of fed larvae. Prevalence of infection between groups of nymphs tested shortly after molting, and at 60 d post-molt was similar (Table 2) and median copy number did not differ significantly ( $\chi^2 = 3.12$ ; d.f. = 1;  $P = 0.077$ ). Molted nymphs retained high *purB* copy numbers well beyond ecdysis; three individuals among those dissected 6 mo post-molt had combined tissue totals exceeding 130,000 copies each. Finally, we observed high success of transstadial retention of infection between the nymphal and adult stages (Table 2).

### 3.3. Dissemination of *B. miyamotoi* spirochetes in infected nymphs

Salivary glands and midguts were extracted from 50 additional nymphs at time points between 1 wk and 6 mo post-molt. Sixteen (32%) of these ticks tested positive for *B. miyamotoi* and both tissue types were positive in each of the 16 infected ticks. Median *purB* copy number was estimated at 53,400 (8900–225,900) in midguts and 10,500 (2,000–24,200) in salivary glands. Moreover, midguts and salivary glands from four nymphs testing positive by qPCR were processed for DFA and each of the tissues contained large numbers of distinguishable spirochetes (Fig. 4).

## 4. Discussion

As expected, the dynamics of *B. miyamotoi* infection differed markedly between immunocompetent and immunocompromised mouse strains. In immunocompetent CD-1 mice, which had previously been shown susceptible to *B. miyamotoi* infection via tick bite (Breuner et al., 2017, 2018), spirochetemia fluctuated between peak and nondetectable levels for a period of 10–14 d after needle inoculation, with *purB* blood concentrations peaking at 2–3 orders of magnitude below those of immunocompromised SCID mice. For the SCID mice, *purB* copy numbers increased steadily, until peak blood concentration was attained at 10–12 d following inoculation, and remained at, or near peak concentration through the 30-day sampling period in all individuals. Despite differences in strain and inoculums, the kinetics of spirochetemia in our SCID mice were consistent with previous descriptions of infection in immunocompromised mice where the LB-2001 *B. miyamotoi* isolate was used (Krause et al., 2015b; Wagemakers et al., 2016) and we attribute the delay in peak spirochetemia in SCID mice (relative to CD-1 mice) to the additional period of spirochete replication required to reach higher blood concentrations.

Feeding *I. scapularis* on infected SCID mice proved a highly effective method of experimentally exposing ticks to *B. miyamotoi*. All larvae and nymphs contained *B. miyamotoi* DNA when tested shortly after completion of blood feeding on an infected

mouse, and even at the low end of the range, copy numbers were substantial. However, despite universal exposure to spirochetes by feeding larvae, overall transstadial survival was inefficient, as evidenced by the decline in infection prevalence from drop off through ecdysis, at which point only one in four nymphs retained spirochete loads that were detectable by our assay. Interestingly, while a majority of ticks exposed to a large quantity of *B. miyamotoi* via SCID mouse cleared spirochetes (at least to numbers below detectable levels) during the period between exposure and completion of molt, qPCR results indicate that robust spirochete replication occurred in a subset of these ticks during the molting process. Inclusion of direct culture of tick tissue or a molecular assay targeting spirochete mRNA should be used to confirm these findings in future experimental work. While we were not able to investigate the mechanism(s) leading to clearance of high spirochete loads from our engorged ticks, we speculate that serotype may play a role in the likelihood of successful colonization of ticks. Wagemakers et al. (2016) previously demonstrated that *B. miyamotoi*, like two closely related relapsing fever spirochetes, *Borrelia hermsii* and *Borrelia turicatae*, expresses alternating surface proteins known as variable membrane proteins (VMPs) which contribute to evasion of host immunity. In SCID mice, both *B. hermsii* and *B. turicatae* are known to express multiple serotypes simultaneously, with distinctive tissue tropisms (Cadavid et al., 1994, 2001; Mehra et al., 2009). Though the relationship between serotype and arthropod host has not been described for relapsing fever spirochetes, it has been shown that variants within an isogenic population of *B. burgdorferi* circulating in a host differ in their ability to colonize ticks fed on that host (Rego et al., 2014). We therefore suggest that further consideration should be given to the potential relationship between serotype and vector susceptibility.

We also noted an increase in infection prevalence between nymphs and adults of the same cohort. While it is possible that uninfected ticks acquired infection while co-feeding with infected ticks as nymphs, a previous study suggests that little, if any amplification in infection prevalence of *B. miyamotoi* occurs during co-feeding of *I. ricinus* larvae (van Duijvendijk et al., 2016). It is therefore more likely that some nymphs in our experiment were infected at levels below detection, and subsequent spirochete replication that occurred as a result of the nymphal blood meal may have increased copy numbers above the threshold of detection in some adult ticks. The stable infection prevalence of the cohort we exposed to infection as larvae presents in contrast to a similar study of *B. burgdorferi* s.l., where infection prevalence and spirochete load were observed to decline between the nymphal and adult stages (Jacquet et al., 2017). This difference may reflect two distinctive biological strategies of *Borrelia* species, where a peak in infection prevalence at the nymphal stage would maximize the likelihood of horizontal transmission of infection, whereas a continued increase in spirochete load and stable infection prevalence in a tick population through the adult stage would be more beneficial to vertical transmission of infection.

Though infection dynamics in SCID mice may offer insights into pathogenesis of tick-borne infections such as *B. miyamotoi* disease, it is important to acknowledge that they do not reflect natural exposure of a tick to an infected immunocompetent host, and are described here for the purpose of production of infected ticks for use in experimental studies. However, along with the unfed nymphs infected with *B. miyamotoi* as larvae via SCID mice in the laboratory, we also quantified infection in transovarially-infected unfed



nymphs originating from field-collected females collected in Connecticut or Minnesota (Breuner et al., 2017, 2018). In support of the biological relevance of our SCID mouse model, the transovarially-infected nymphs from field-collected females had similarly high to higher spirochete loads (CT nymphs, median *purB* copy number of 147,700 per tick; MN nymphs, median *purB* copy number of 301,400 per tick, listed in Supplemental material) compared with the laboratory-infected nymphs (see Table 2; median *purB* copy number of 110,026 for recently molted ticks). The lone prior study reporting quantitative figures from field-collected *I. scapularis* nymphs infected with *B. miyamotoi* listed spirochete loads of 1–2 orders of magnitude lower than those described here (Barbour et al., 2009). Notably, the quantitative assay we used measured genetic copies, which do not equate to single spirochetes. So while we were not able to make a direct comparison between our lab-infected ticks and previously described field-collected specimens, Kitten and Barbour (1992) reported that *B. hermsii* obtained both from mice and culture contained multiple chromosomal copies per cell, which suggests that *purB* copy number may overestimate the quantity of *B. miyamotoi* spirochetes in mouse blood and ticks.

Additionally, we determined that *I. scapularis* feeding as nymphs were similarly susceptible to infection as feeding larvae. In a natural setting, though phenology and host association for this tick species are likely to limit the significance of infected adults for horizontal transmission relative to infected nymphs, acquisition of infection during the bloodmeal of either immature stage would facilitate vertical transmission of spirochetes. As it concerns the further study of transovarial transmission, experimental introduction of infection at the nymphal stage includes the potential benefits of reduction in tick developmental period and animal resources associated with an additional life stage. Based on our results, infestation of an infected SCID mouse with 100 naïve larvae would result in the production of approximately 20–25 molted infected nymphs, should all larvae feed and molt successfully. In addition, 100% of tested larvae produced by one infected female were *purB*-positive, indicating that transovarially-infected tick lines can be established using our mouse model.

Examination of individual tick tissues added further insight into the biology of *B. miyamotoi* infection within the vector. Salivary glands and midguts dissected from flat nymphs contained numerous visible spirochetes, and *purB* copy numbers were detected at high quantities in these tissues as long as 6 months after the molt. The disseminated infections observed in these nymphs correspond with our previous observation that nymphs can transmit *B. miyamotoi* to mice within 24 h of attachment (Breuner et al., 2017). Other relapsing fever spirochetes, including *B. hermsii* and *B. turicatae* are known to establish persistent infections in salivary glands (Schwan and Hinnebusch, 1998; Takano et al., 2012; Boyle et al., 2014). While this is evidently advantageous for spirochete transmission by rapidly feeding soft ticks, the extent of any benefit conveyed to spirochete transmission by hard ticks, which are attached to their hosts for days, remains to be elucidated.

Further progress toward the prevention of human *B. miyamotoi* disease cases should include a more robust comparison of the relative contributions of horizontal and vertical transmission in the natural maintenance of this spirochete. For the latter, this should include determination of both the frequency of transovarial transmission among a population of females, as well as quantifying successful acquisition of infection within filial clutch. The

SCID mouse model described here allows for reliable production of *I. scapularis* ticks infected with specific, well-characterized *B. miyamotoi* isolates, which is a prerequisite for downstream experimental studies focusing on the biology of this emerging human pathogen.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

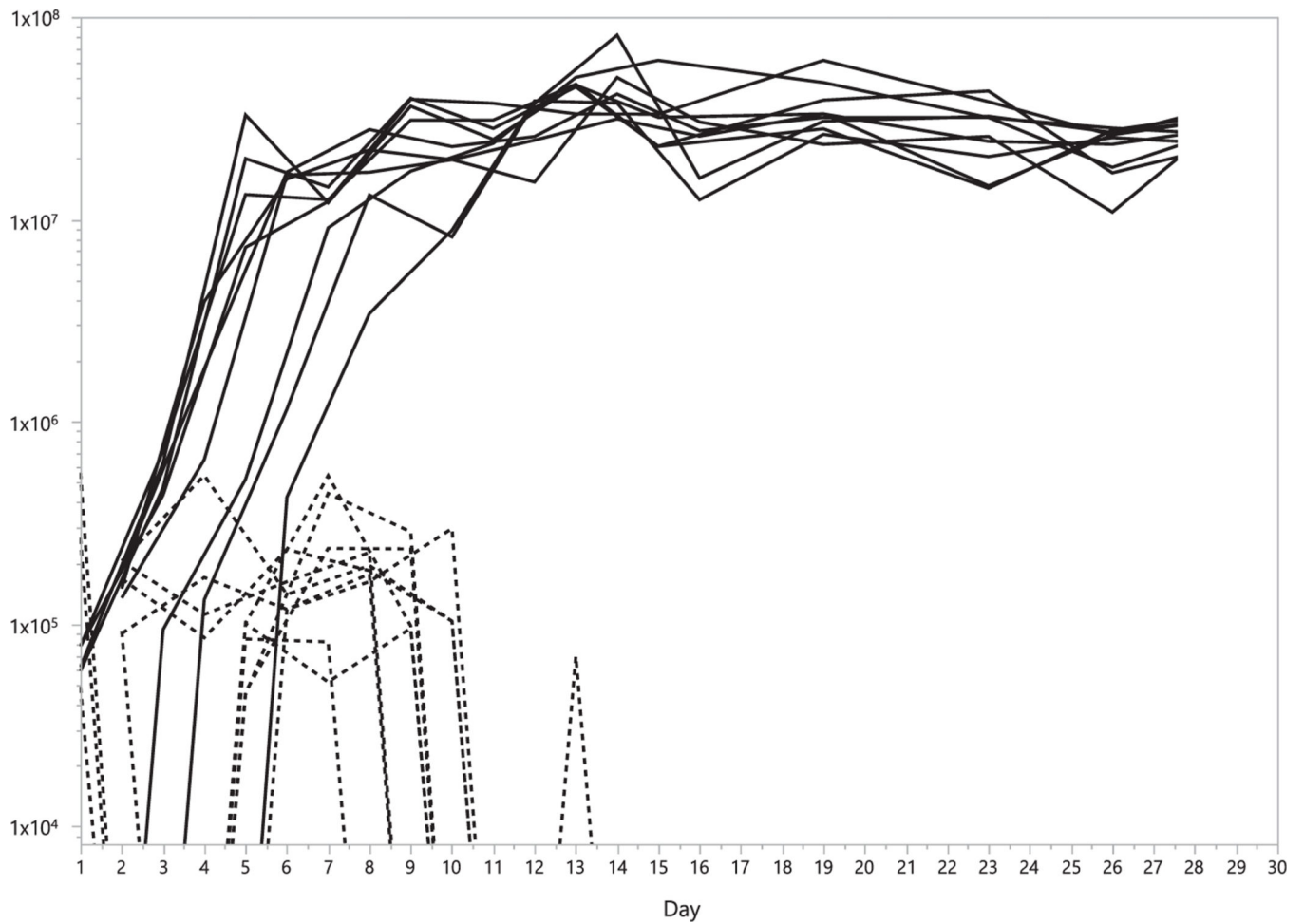
We thank Alan Barbour of U.C. Irvine for sharing the H9724 antibody; we also thank Karen Boegler, Christine Graham, Christopher Sexton, and Shanna Williams, all of the Centers for Disease Control and Prevention for technical assistance and/or advice with experimental procedures.

## References

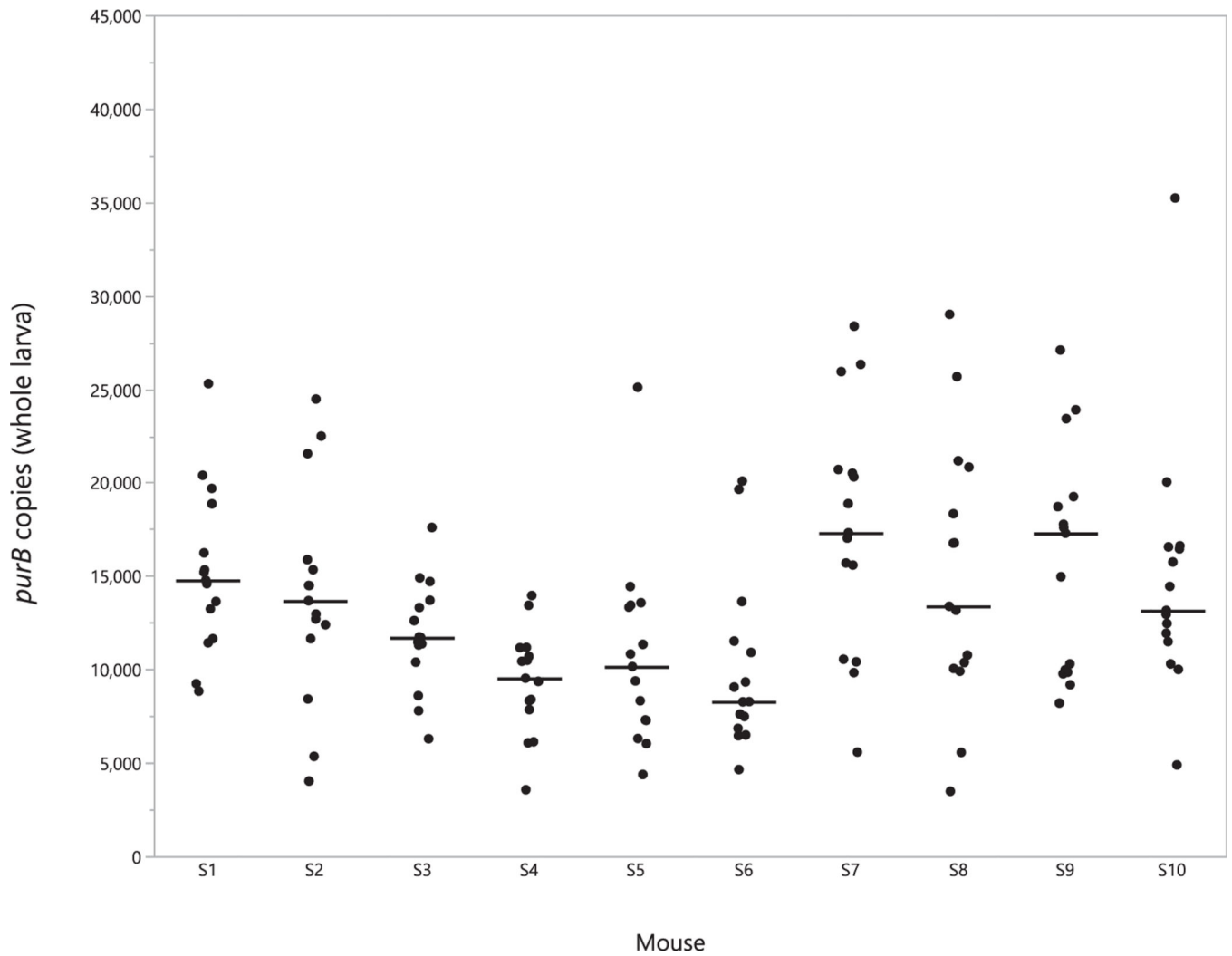
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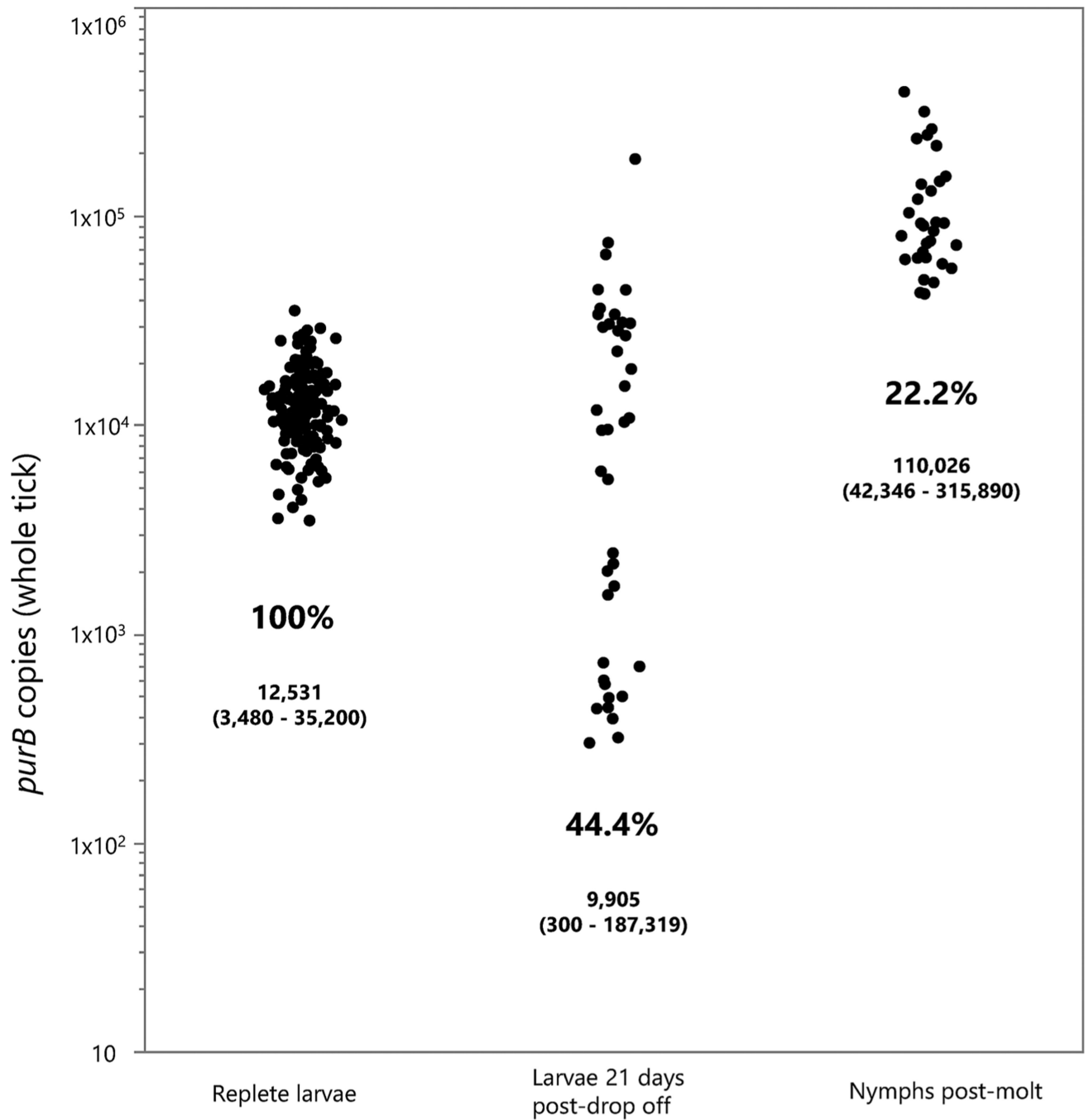
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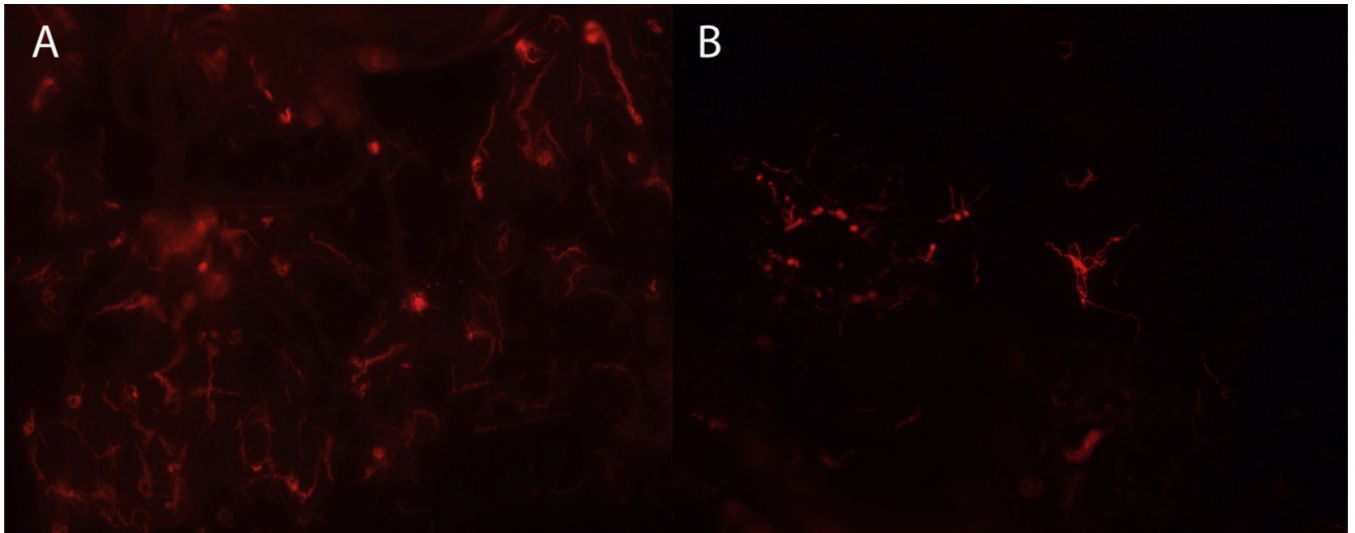
**Fig. 1.** Spirochetemia in mice. Changes in blood concentration of *purB* (copies/mL) over time for individual mice following inoculation of *B. miyamotoi* CT13–2396 culture. Copy numbers are represented by dashed lines for immunocompetent CD-1 mice and solid lines for immunocompromised SCID mice. Blood collection was discontinued after 16 d for CD-1 mice.



**Fig. 2.** *purB* copy number in freshly fed larval ticks by source SCID mouse. Black dots indicate *purB* copy number for a single engorged larva (whole), grouped according to the mouse the tick was fed upon. The solid bar indicates the median copy number for ticks sourced from that mouse.



**Fig. 3.** Transstadial kinetics of infection. Black dots represent *purB* copy numbers for individual ticks tested at one of three time points: Replete larvae (at drop off), larvae 21 d post-drop off, and nymphs tested shortly after molting. Percent infected is listed for each group, along with median and range of copy number for each time point.



**Fig. 4.** IFA of *Borrelia miyamotoi* in tick tissues. A. Midgut tissue and B. salivary gland tissue from nymphal *Ixodes scapularis* fed on infected SCID mice as larvae, with spirochetes labelled with anti-flagellin monoclonal antibody (red) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).



Comparison of infection kinetics by mouse strain. For each of the two mouse strains, time of first and last detection of spirochetes are listed as days post-inoculation, along with peak blood concentration of *pwrB* (copies/mL), time of peak blood concentration, and duration of detectable infection in days.

**Table 1**

strain	1 st detected (dpi)		peak concentration (copies/mL)		peak (dpi)		last detected (dpi)		duration (d)	
	median	range	median	range	median	range	median	range	median	range
<i>CD-1</i>	2	(1–5)	$2.66 \times 10^5$	$(8.45 \times 10^4 - 5.60 \times 10^5)$	6.5	(1–10)	9	(7–13)	8	(2–8)
<i>SCID</i>	2	(1–6)	$4.64 \times 10^7$	$(3.42 \times 10^7 - 8.15 \times 10^7)$	14	(12–30)	30	(30)	NA	NA

d.p.i. = days post-inoculation.

d = days.

Interstage infection dynamics in ticks fed on SCID mice. Proportion infected (% prevalence) and *purB* copy numbers are listed (median copies, range) for life stages for *I. scapularis* exposed to infected SCID mice as larvae (top) or nymphs (bottom).

**Table 2**

life stage	stage infection introduced	timepoint	% prevalence (no. + /no. tested)	median copies (range)
larva	larva	drop-off	100 (150/150)	12,531 (3480 – 35,220)
larva	larva	21 d.p.d.	44.4 (40/90)	9905 (300–187,319)
nymph	larva	0 d.p.m.	22.2 (30/135)	110,026 (42,346 – 315,890)
nymph	larva	60 d.p.m.	24.0 (18/75)	74,888 (42,913 – 393,235)
adult male	larva	p.r.f.	37.0 (10/27)	NA
adult female	larva	p.r.f.	55.6 (15/27)	NA
F1 larva	larva	eclosure	100 (15/15)	24,816 (3709– 109,101)
nymph	nymph	drop-off	100 (8/8)	72,148 (3480– 35,220)
adult male	nymph	2–3 m.p.m.	25.0 (9/36)	199,300 (20–3,367,500)
adult female	nymph	2–3 m.p.m.	30.9 (21/68)	916,900 (200–5,191,162) <sup>a</sup>

d.p.d. = days post-drop off; d.p.m. = days post-molt; p.r.f. = post-rabbit feed; m.p.m. = months post-molt; NA = not available.

<sup>a</sup>5 females that were engorged were excluded from calculations.