Co-infections and transmission dynamics in a tick-borne bacterium community exposed to songbirds

Reference:
Heylen Dieter, Fonville Manoj, van Leeuwen Arieke Docters, Sprong Hein.- Co-infections and transmission dynamics in a tick-borne bacterium community exposed to songbirds
Environmental microbiology - ISSN 1462-2912 - 18:3(2016), p. 988-996
Full text (Publishers DOI): http://dx.doi.org/doi:10.1111/1462-2920.13164
To cite this reference: http://hdl.handle.net/10067/1311710151162165141

Co-infections and transmission dynamics in a tick-borne bacterium community exposed to songbirds

Dieter Heylen[1†], Manoj Fonville[2], Arieke Docters van Leeuwen[2], Hein Sprong[2]

[1] Evolutionary Ecology Group, Department of Biology, University of Antwerp, Belgium

[2] Laboratory for Zoonoses and Environmental Microbiology, National Institute for Public Health and Environment (RIVM), Bilthoven, the Netherlands

[†] **Author to whom correspondence should be addressed**

E-mail: Dieter.Heylen@uantwerpen.be

Summary

We investigated the transmission dynamics of a community of tick-borne pathogenic bacteria in a common European songbird (*Parus major*). Tick-naïve birds were recurrently exposed to field-collected *Ixodes ricinus* nymphs, carrying *Ricketssia helvetica* (16.9 %), *Borrelia garinii* (1.9 %), *Borrelia miyamotoi* (1.6 %), *Anaplasma phagocytophylum* (1.2 %), and *Candidatus* Neoehrlichia *mikurensis* (0.4 %), bacteria. Fed ticks were screened for the pathogens after moulting to the next developmental phase. We found evidence for an early stage transmission (within 2.75 days after exposure) of *R. helvetica* and *B. garinii*, and to a lesser extent of *A. phagocytophylum* based on the increased infection rates during the first exposure.

Throughout the course of infestations, *R. helvetica* infection rate remained constant. On the other hand, the infection rate of *B. garinii* further increased, indicating a more gradual development of host tissue infection. No interactions were found among the different bacterium species during transmission. Birds did not act as transmitter, nor amplifier for the other bacterial species. We experimentally show that one bird can simultaneously transmit several pathogenic bacterium species using different mechanisms, and that the transmission facilitation by the birds results in a strong increase of co-infections in ticks.

Key words: songbird, *Ricketssia helvetica*, *Anaplasma phagocytophylum*, *Candidatus* Neoehrlichia *mikurensis*, *Borrelia miyamotoi*, *Borrelia burgdorferi*, *Ixodes ricinus*, reservoir, Lyme borreliosis, transmission, co-infections

Introduction

Simultaneous infections of multiple pathogen species (co-infection) can lead to increased pathogenicity, affect the pathogen's proliferation dynamics, distribution in the body, and influence the host's immune responses, but also can complicate the diagnosis and treatment of disease (Thomas et al., 2001; Moro et al., 2002; Regev-Yochay et al., 2004; Holden et al., 2005; Civitello et al., 2010; Duncan et al., 2015; Susi et al., 2015). Transmission dynamics of multiple vector-borne micro-organisms in wildlife hosts determine the pathogen communities within vector individuals to which susceptible hosts will be exposed. Understanding the mechanisms underlying co-infections in ticks is important, as co-infections in hosts in which tick bites are relatively low (e.g. humans) can result from the attachment of a single co-infected tick individual, usually rather than sequential bites of multiple singly-infected ticks

(Ginsberg, 2008). Not only mammals, but also birds are recognized to make a significant contribution to the distribution of tick-borne pathogenic bacteria. However, only limited information is available on the competence of birds to sustain tick-borne zoonoses other than Lyme borreliosis (caused by *Borrelia burgdorferi* s.l.) (Richter et al., 2000; Humair, 2002; Heylen et al., 2014).

In the Holarctic region, every year many questing ticks are exposed to high bird densities, largely consisting of juvenile songbirds (Gray, 1991; Marsot et al., 2012; Heylen et al., 2014). Juvenile birds are particularly important for the establishment of foci of tick-borne pathogens in endemic areas, as they have a low acquired resistance and hence facilitate the proliferation and transmission of ticks and their pathogens (Richter et al., 2000; Heylen et al., 2010; Dubska et al., 2011; Heylen et al., 2014). Although many observational studies have reported infections in bird-derived ticks at the time of migration (Comstedt et al., 2006; Elfving et al., 2010; Hornok et al., 2014), when birds introduce infected ticks along their passage routes, little information is available on the extent to which juvenile songbirds are able to maintain endemic pathogen transmission cycles.

The main vector of the zoonotic tick-borne bacteria in Europe is the ixodid tick *Ixodes ricinus*, which infests a broad range of terrestrial vertebrates, including birds. This tick has a three-stage life cycle (larva, nymph, and adult), with each stage feeding only once (except for the adult male which seldom feed). In addition to *Borrelia burgdorferi* sensu lato, other zoonotic pathogens, like *B. miyamotoi*, *Rickettsia helvetica*, *Anaplasma phagocytophilum* and *Candidatus* Neoehrlichia *mikurensis* (*N. mikurensis*) have been reported in *I. ricinus* (Sprong et al., 2009; Jahfari et al., 2012; Tijsse-Klasen et al., 2013; Cochez et al., 2014; Jahfari et al., 2014), but only in small mammals has effort been paid in identifying the reservoir hosts (Burri et al., 2014). The dominating *Borrelia burgdorferi* sensu lato genospecies in ticks collected from European songbirds are *Borrelia garinii* and *B. valaisiana*, both causing Lyme borreliosis (Humair and Gern, 2000). *Borrelia miyamotoi* is a member belonging to the relapsing fever group of *Borrelia* spirochaetes, and can be hosted by rodents (Burri et al., 2014). *Rickettsia helvetica* belongs to the spotted fever group, and is - in contrast to the spirochaetes - an obligate intra-cellular bacterium. Neurological and cardiac problems in humans have been ascribed to this *Rickettsia* species (Nilsson et al., 1999; Nilsson et al., 2010). *Anaplasma phagocytophilum* is a rickettsial bacterium obligate of neutrophils, causing granulocytic anaplasmosis in humans, livestock and companion animals (Dumler et al., 2005). The rickettsial bacterium *N. mikurensis* is associated with febrile patients (Maurer et al., 2013), and has been detected in tissues of wild rodents (Burri et al., 2014; Szekeres et al., 2015). Further details on the status of the bacteria above, are reviewed in the work of Burri *et al.* (2014).

Transmission of tick-borne bacteria via the host generally depends on the development of an infection in a susceptible host. As the uninfected ticks feed on an infected host, the pathogens are taken up in the bloodmeal. This transmission requires a time period between the hosts being bitten by an infected tick and becoming infective ('latent period'), of which the duration depends on the host-pathogen combination. However, transmission may also occur between infected and uninfected ticks co-feeding in close proximity on the same hosts, in the absence of or prior to the establishment of a systemic infection. For this pathway, the latent period is generally very short (1-3 days) (Gern and Rais, 1996; Randolph et al., 1996).

The main objective of our experimental study was to investigate to what extent songbirds facilitate the transmission and amplification of the bacterium community (*R. helvetica*, *A. phagocytophylum*, *N. mikurensis*, *B. miyamotoi*, *B. garinii*) of *Ixodes ricinus* ticks, by quantifying the infections in fed ticks from repeatedly exposed juvenile birds. We used the great tit (*Parus major* L.) as a host, since this species is an abundant resident bird of woodlands and gardens in Europe, and is frequently infested by *Ixodes ricinus* ticks that are themselves infected by a variety of bacterium species. We used juvenile birds that had not been exposed to ixodid ticks before. Birds were exposed to ticks in three sessions, each lasting 4-5 days (the normal time for ticks to engorge) and separated by 5-6 days without ticks (Fig. 1). We hypothesized that when the birds facilitate transmission via a gradual infection development of host tissue, the infection rate in the fed ticks should significantly increase over successive infestation sessions (Richter et al., 2000; Massung et al., 2004; Heylen et al., 2014). If the birds facilitate transmission via co-feeding between ticks alone, we hypothesize that infection rates should increase in the fed ticks from the first infestation onwards, but do not increase over the course of the experiment (Zemtsova et al., 2010; Heylen et al., 2014). Furthermore, we expect that facilitation of pathogen transmission in the birds will change the bacterium community in the fed ticks, and will increase the occurrence of co-infected ticks.

Results

A schematic overview of the study design is summarized in Fig. 1. The infection rates in the questing ticks and the mean infection rates per bird over the three infestation sessions are presented for each bacterium species in Fig. 2.

Overall, infection rates strongly varied among the bacterium species in the 1225 questing ticks (*Ricketssia helvetica* (16.9 %), *Anaplasma phagocytophylum* (1.2 %), *Neoehrlichia mikurensis* (0.4 %), *Borrelia garinii* (1.9 %), *Borrelia miyamotoi* (1.6 %)), as well as in the 854 experimentally fed ticks after moulting (*R. helvetica* (50 %), *A. phagocytophylum* (2.3 %), *N. mikurensis* (0.0 %), *B. garinii* (31.9 %), *B. miyamotoi* (1.4 %)). Note that *N. mikurensis* (5 questing ticks and 0 fed ticks) is not included in the statistical models reported below, because of the small sample size.

Infection profiles

The infection rate in the fed ticks obtained from the first infestation was significantly higher compared to the questing ticks for: *B. garinii* (Odds Ratio (O.R.) $_{(inf.1 - questing)}$ [95% confidence limits]: 4.80 [2.68 – 8.54], t = 5.62, df = 21.56, P < 0.0001), *R. helvetica* (3.98 [1.75 – 9.05], t = 3.31, df = 228, P = 0.0011) and *A. phagocytophylum* (3.03 [1.61 – 5.70], t = 3.66, df = 19.3, P = 0.002), but not for *B. miyamotoi* (0.89 [0.59 – 1.33], t = -0.58, df = 133.3, P = 0.57). The *B. garinii*'s increase did not differ from that of *R. helvetica* (t = -0.77, df = 38.3, P = 0.45), but was higher than the *A. phagocytophylum*'s increase (t = -1.96, df = 136.7, P = 0.052). During the first infestation, for each of the latter pathogens we found significantly higher infection rates in the fed ticks compared to the questing ticks, starting from 2.75 days after exposure (Table 1).

The change in infection rate over the course of successive infestations differed significantly among the bacterium species (interaction change x bacterium species: $F_{4,196.6} = 18.78$, P < 0.0001). For *B. garinii* (O.R.: 3.46 [2.59 – 4.63]/session; t = 8.56, df = 57.02.4, P < 0.0001) the infection rate increased, while for all other pathogens, including *R. helvetica* (1.12 [0.92 – 1.36]/session, t = 1.15, df = 270.4, P = 0.25), the infection rate in the fed ticks remained unchanged.

Overall, in twenty-four birds in which the *R. helvetica* infection rates increased, also these of *B. garinii* went up (Fisher's P = 0.042). Based on the empirical Bayes estimates for the bird individual's infection rate profiles, there were no correlations between the different bacteria, neither in the changes over the three sessions, nor in the difference with the questing ticks.

Co-infections after exposure to the experimental birds

As a result of the differential transmissions, the bacterium community in the fed ticks increasingly deviated from that found in the questing ticks (Fig. 3). The proportion of the ticks with co-infections was higher in the first infestation session (7 %) compared to the questing ticks (0.97 %) (O.R.: 7.63 [4.02 – 14.49]; t = 6.50, df = 28, P < 0.0001), and further increased during the course of infestations (2.49 [1.81 – 3.42]/session; t = 5.69, df = 77, P < 0.0001). Of the fed ticks with co-infections, by far the most dominant combination was *B. garinii* x *R. helvetica* (17.1 %). In 0.1 % of the co-infected fed ticks, we found three different bacterium species (*B. garinii* x *R. helvetica* x *B. miyamotoi*).

Discussion

This is the first study that experimentally demonstrates differential transmission in a community of endemic tick-borne bacterium genera exposed to a songbird. With the exception of *N. mikurensis*, the presence of all bacterium species (*R. helvetica*, *A. phagocytophylum*, *B. garinii*, *B. miyamotoi*) after the ticks have moulted gives proof for successful trans-stadial transmission after being exposed to the bird's skin and blood. Furthermore, this study shows for the first time that songbirds effectively facilitate the transmission of *R. helvetica* to ticks, which - in addition to the yet proven development of bacteraemia (Hornok et al., 2014) - gives evidence for their role as amplifying hosts in *R. helvetica*'s epidemiology.

In the ticks of the first infestation session, considerably higher infection rates (after moulting to the adult stage) compared to the questing ticks were observed for *R. helvetica*, *B. garinii* and – to a lesser extent - *A. phagocytophylum*, which cannot be due to the presence of the bacteria in the naïve birds prior to feeding. For *Borrelia*, the relatively slow dissemination of the spirochetes through the tissues of the vertebrate host, means that more distantly located tissues will become infectious after the latent period (ca. 7 days, *cf.* American robins (Richter et al., 2000)), thereby strongly increasing the transmission probability. However, the site of the tick bite can become infectious before the end of this latent period, when ticks co-feed, likely explaining the significant increase during the first days after exposure (Table 1). To obtain further evidence for this co-feeding transmission pathway of *B. garinii* in songbirds, we need experiments to test whether naïve ticks get infected when placed in close proximity to an infected nymph, whilst those placed further away remain naïve. Studies in laboratory mammalian hosts have shown this pathway could be relevant for *B. garinii* in the wild (Sato and Nakao, 1997; Hu et al., 2003)). Nevertheless, seen the strong *Borrelia* infection increase over the three infestation sessions, it is more than likely that the spirochetes have disseminated over larger areas of tissue than only the sites of tick bites, which potentially leads to the development of systemic infections (Humair et al., 1998; Kurtenbach et al., 1998; Hanincova et al., 2003; Heylen et al., 2013a; Lommano et al., 2014).

Information on the infection dynamics of rickettsial bacteria (*R. helvetica* and *A. phagocytophylum*) in songbirds is still lacking. Screenings of bird blood have shown that songbirds can become bacteraemic for *R. helvetica* and *A. phagocytophilum* (Hornok et al., 2014). In addition, a number of studies have shown that wild birds often carry infected ticks, including larvae (Santos-Silva et al., 2006; Elfving et al., 2010; Jahfari et al., 2014; Wallmenius et al., 2014) indicating successful transmissions via host tissue. Here we show a a rapid increase in *R. helvetica* infected ticks, from the first infestation session onwards. This observation again suggests co-feeding transmission (*cf.* mammals: Levin and Fish, 2000; Kocan and de la Fuente, 2003; Zemtsova et al., 2010) and/or a very rapid systemic infection as found in mammals experimentally injected with different rickettsial strains (Beati et al., 1999). On the other hand, the low infection rates of *A. phagocytophylum* give evidence that great tits do not act as competent reservoirs for this bacterium species. However, this observation should not preclude the possibility that other songbird species do transmit the pathogen (Hornok et al., 2014; Jahfari et al., 2014).

In the *Borrelia* genospecies, our study showed that the infection susceptibility in the great tit was high for *B. garinii*, while the transmission of *B. miyamotoi* was not facilitated at all. The outcome suggests that this common European songbird is neither a competent reservoir, nor transmitter for latter bacterium. While in the USA and Europe small rodent species have been implicated as reservoirs for latter genospecies (Scoles et al., 2001; Burri et al., 2014), the role of native bird species remain unclear. The transmission dynamics of other *Borrelia* genospecies (*B. afzelii*, *B. spielmanii*, *B. burgdorferi* s.s. and *B. valaisiana*) in the bird species under study, have been discussed in previous work (Heylen et al., 2014).

Despite the slight initial increase of the *A. phagocytophylum* infected ticks, the infection rates remained very low. Also *N. mikurensis*, a bacterium that is considered to be mammal associated (Burri et al., 2014) and so far has not been detected in bird tissues (Hornok et al., 2014), was not detected after the first infestation. The infection progression of both bacteria suggests a lack of transmission and amplification, possibly due to an efficient innate immune response. However, since both bacteria were found to be rare in the questing *I. ricinus*, their absence in fed ticks may simply represent a chance outcome and/or the infection prevalence was too low to provide successful amplification.

As a result of the differential transmission and amplification, the bacterium community of the questing ticks significantly changed after feeding on the birds. However, the changes in each of the bacterium species did not show any interaction: neither the increase over different infestation sessions, nor the initial difference with the unfed questing nymphs showed any correlation among the bacterium species. To gain better insights into whether pathogens within hosts show negative (competition) or positive (facilitation) interactions (Ginsberg, 2008), future experiments are required in which bacterium proliferations in single- and co-infections are compared with each other.

This study is the first that experimentally simulates the transmission dynamics in a situation whereby resident songbirds are recurrently exposed to a natural community of tick-borne bacteria genera that are pathogenic to humans. Birds act as hosts that are permissive for a broad range of bacterium species, but selectively amplify a narrower range. Our data show that in the bird-tick system, simultaneous transmission occurs of different bacterium species, in particular *R. helvetica* and Lyme *B. garinii* spirochaetes, leading to mixed infections in the detached tick. This raises the problem of co-infection in vertebrates, a poorly studied issue but with strong potential implications and relevance for public health (Ginsberg, 2008; Civitello et al., 2010). Further experimental work on the reservoir and transmission competence of other avian hosts, tick species and their pathogenic agents will further improve our understanding of the transmission dynamics of bacteria communities in the wild, and the generalizability of the outcomes in the system we investigated in current study.

Acknowledgments

We are most grateful to Frans Fierens and Joris Elst for their technical assistance. Wild birds were introduced into aviaries with a licence (number: S8/VERG/07-U5R26) of the Agency for Nature and Forests (Flemish Government, Belgium). The infestation procedure is approved by the Ethics Committee for Animal Experiments of the University of Antwerp.
This research was supported by the FWO (grant G0.049.10) and the University of Antwerp (KP BOF UA 2015). Dieter Heylen is a postdoctoral fellow at the Fund for Scientific Research - Flanders Belgium (FWO). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors have declared that no competing interests exist. This work was done under the frame of COST action TD1303 EurNegVec.

Experimental procedures

Birds

The great tit (body mass 15-20 g) commonly breeds in rural and urbanized areas throughout the Palearctic. Nest boxes are often used as surrogates for tree-holes, in which they naturally breed and roost. Great tits are abundant (estimated European population: 31 - 52 million individuals; 5 breeding pairs/ha producing on average 7-8 nestlings per nest in optimal habitat (Hagemeijer and Blair, 1997)), and birds in our Belgian study population frequently host relatively high *I. ricinus* tick burdens (maximum number of larvae: 40, nymphs: 17) (Heylen et al., 2009; Heylen et al., 2013b). Great tits tolerate infestations with *I. ricinus* nymphs

(Heylen et al., 2010). They act as general transmitters for several *Borrelia burgdorferi* s.l. genospecies, allowing trans-stadial transmissions, but develop strong infections in bird tissue only for *B. garinii* (Dubska et al., 2009; Heylen et al., 2013a; Heylen et al., 2014).

Tick-naïve birds were obtained by collecting nestlings from parasite-free boxes in the wild and introducing them with their parents into tick-free aviaries (for details see Heylen et al., 2010). During the month preceding the breeding season (March), selected nest boxes were sprayed twice with permethrin (2.2 g/L). Permethrin has a low toxicity to birds and mammals (Elliott, 1977). In addition, after hatching of the chicks, nest material was microwaved three times at 4-day intervals. A few days before fledging, nestlings and parents of five nests were transferred into outdoor aviaries located on the University of Antwerp (Belgium), where parents continued to feed the nestlings until independence. In total 29 naïve *P. major* individuals participated in the experiment. During the experiment, five individuals died. Birds received food and water *ad libitum,* and birds had the opportunity to take a bath in fresh water. Ringing procedures and experiments were carried out in accordance with national environmental legislation and university regulations.

Study design

When birds were 9 weeks old, individuals were infested with *I. ricinus* nymphs three times in succession (Infestation 1-3; Fig. 1; for details see also Heylen et al. 2010). Each infestation lasted 4 - 5 days, and birds were kept free of ticks for a duration of 5 - 6 days between the consecutive infestations. In an area suspected to be endemic for tick-borne bacteria, approximately 3000 *I. ricinus* nymphs were caught by dragging a white flannel flag over suitable vegetation. After capture, the ticks were kept under sterile conditions in a climate room at > 90% relative humidity and 16h:8h (light:dark photoperiod; 25°C:15°C temperature cycle) until infestation. We infested the birds with tick loads around the maximum level found under natural conditions in our study population (Heylen et al., 2013b). Using moistened tweezers 17 nymphs for *P. major* were put underneath the feathers on the head of each bird in each infestation session. Immediately afterwards birds were kept for 2 h in an air-permeable cotton bag (sized: 20 cm x 15 cm) inside a darkened cage which kept them inactive (Heylen and Matthysen, 2008). After tick exposure, birds were placed in individual cages with a wire-mesh floor (40 cm x 80 cm). Below the wire-mesh was a plastic tray containing damp filter paper and edges were streaked with vaseline to prevent nymphs from escaping. The engorged nymphs that dropped through the mesh cage were collected at 7 a.m. and 7 p.m. each day with minimal disturbance to the host. The proportion (%) of ticks that successfully engorged was high over all infestation sessions($\geq 67 \pm 4$ %/session) (for details see Heylen et al., 2010). Engorged nymphs were kept in individual tubes at 25°C and > 90 % relative humidity until moulting to the adult stage was completed. The moulting success (%) was high over all infestation sessions($\geq 82 \pm 4$%/session). As a consequence of the lack of resistance against the *I. ricinus* nymphs, birds were unable to prevent the direct harm (acute blood depletions) caused by tick feeding. However, birds compensated the erythrocyte loss, without reduction in general body condition (Heylen et al., 2010).

DNA extraction and PCR-based detection of bacterium species

1225 questing nymphs and 854 nymphs that had fed and moulted to the adult stage, were immersed in 70% ethanol and stored at -20°C before testing. All the ticks were screened for the presence of *Ricketssia helvetica*, *Anaplasma phagocytophylum*, *Neoehrlichia mikurensis*, *Borrelia garinii*, *Borrelia miyamotoi*. DNA was extracted by alkaline lysis (Schouls et al., 1999). Details of the qPCR protocols for genospecies determinations by PCR amplification and the detection of *B. burgdorferi* s.l. (Heylen et al., 2013a), *Borrelia miyamotoi* (Cochez et al., 2014), *Candidatus* Neoehrlichia *mikurensis* (Jahfari et al., 2012), and *Anaplasma phagocytophylum* (Jahfari et al., 2014) are given in the respective references. For the detection of *Rickettsia helvetica*, a duplex qPCR was designed consisting of a specific qPCR for *Rickettsia helvetica* and an already existing qPCR for spotted fever and typhus group rickettsia (Stenos et al., 2005; PMID: 16354816). Briefly, we used 5'-tcgcaaatgttcacggtacttt-'3 and 5'-atgatccgtttaggttaataggcttcggtc-3' as forward primers, 5'-tcgtgcatttctttccattgtg-'3 and 5'-ttgtaagagcggattgttttctagctgtc-3' as reverse primers and 5'-Atto520-tgc aat agc aag aac cgt agg ctg gat g-BHQ1-3' and 5'-Atto425-cgatccacgtgccgcagt-BHQ1-3' were used as probes. For the qPCR-protocol, sequences of primers and probes were based on the DNA sequences of these gene fragments available in Genbank and were evaluated using Bionumerics (Applied Maths NV, Sint-Martens-Latem, Belgium). The specificity of primers and probes was tested with tick lysates containing the DNA from the following microorganisms: *R. africae, R. conorii*, *R. helvetica*, *R. rickettsii*, *R. monacensis, R. raoultii*. Correct sizes of DNA fragments of qPCR-amplicons were regularly confirmed on a bioanalyzer (Agilent Technologies, Palo Alto, CA). qPCR was performed using the iQ Multiplex Powermix PCR reagent kit, which contains iTaq DNA polymerase (Bio-Rad Laboratories, Hercules, USA), in a LightCycler 480 Real-Time PCR System (F. Hoffmann-La Roche, Basel, Switzerland). Optimal reaction conditions in a final volume of 20ul were iQ multiplex Powermix, primers 200 nM each, probes at 100 nM each, and 3 µl of template DNA. Cycling conditions included an initial activation of the iTaq DNA polymerase at 95°C for 5 min, followed by 60 cycles of a 5 s denaturation at 95°C followed by a 35 s annealing-extension step at 60°C (Ramp rate 2.2 °C/s and a single point measurement at 60 °C) and a cooling-cycle of 37 °C for 20 s. Analysis was performed using the second derivative calculations for cp (crossing point) values. Amplification curves were assessed visually. Samples with positive scores for both targets and only positive for the specific *R. helvetica* qPCR were assumed to be positive for *R. helvetica*. Specificity of the positive signals was confirmed using a conventional PCR and sequencing as described (Sprong et al., 2009). For each PCR and multiplex qPCR, positive, negative controls and blank samples were included. In order to minimize contamination, the PCR proceedings were performed in three separate rooms, of which the reagent setup and sample addition rooms were kept at positive pressure, whereas the DNA extraction room was kept at negative pressure. All rooms had airlocks.

Statistical analysis

Generalized linear mixed effects models (GLMM) were fitted to test hypotheses on bacterium infection rates (logit-link, binomial-distributed residuals), taking into account the correlation structure of the repeated measurements from the same individual (Verbeke and Molenberghs, 2001; Molenberghs and Verbeke, 2005). Infection rates in the fed ticks of the first session (Inf. 1) were compared with these of the questing ticks, to test whether co-feeding transmission and/or an early systemic infection took place. To test whether the linear trend (change) with infestation order differed among bacterium species, we modeled the interaction terms change x bacterium and added a random intercept and slope on the level of the bird individual and nest of origin. The bird's nest of origin did not significantly explain the variation in the bacterium infection profiles (all P-values > 0.05) and therefore its random effects were excluded from the statistical analyses. For the inference of the maximum likelihood estimates of the fixed effects, Kenward Roger approximation was used to estimate the denominator degrees of freedom of the F-distributed test statistics, which takes into account the correlation of observations within the same cluster (Verbeke and Molenberghs, 2001; Molenberghs and Verbeke, 2005). Odds ratio estimates are reported with their 95%

confidence limits. Odds ratio estimates of continuous variables are assessed as one unit offsets from the mean. All data manipulations and statistical analyses were performed using SAS v9.2 (SAS Institute, Cary, North Carolina, USA).

References

Beati, L., Kelly, P.J., Mason, P.R., and Raoult, D. (1999) Experimental infections of vervet monkeys (*Cercopithecus pygerythrus*) with three spotted fever group rickettsiae. *S Afr J Sci* **95**: 448-449.

Burri, C., Schumann, O., Schumann, C., and Gern, L. (2014) Are *Apodemus* spp. mice and *Myodes glareolus* reservoirs for *Borrelia miyamotoi*, *Candidatus* Neoehrlichia mikurensis, *Rickettsia helvetica*, *R. monacensis* and *Anaplasma phagocytophylum*? *Ticks Tick-Borne Dis* **5**: 245-251.

Civitello, D.J., Rynkiewicz, E., and Clay, K. (2010) Meta-analysis of co-infections in ticks. *Isr J Ecol Evol* **56**: 417-431.

Cochez, C., Heyman, P., Heylen, D., Fonville, M., Hengeveld, P., Takken, W. et al. (2014) The presence of *Borrelia miyamotoi*, a relapsing fever spirochaete, in questing *Ixodes ricinus* in Belgium and in The Netherlands. *Zoonoses Public Health* **62**: 331-333.

Comstedt, P., Bergstrom, S., Olsen, B., Garpmo, U., Marjavaara, L., Mejlon, H. et al. (2006) Migratory passerine birds as reservoirs of lyme borreliosis in Europe. *Emerg Infect Dis* **12**: 1087-1095.

Dubska, L., Literak, I., Kocianova, E., Taragelova, V., and Sychra, O. (2009) Differential role of passerine birds in distribution of *Borrelia* spirochetes, based on data from ticks collected from birds during the postbreeding migration period in Central Europe. *Appl Environ Microbiol* **75**: 596-602.

Dubska, L., Literak, I., Kocianova, E., Taragelova, V., Sverakova, V., Sychra, O., and Hromadko, M. (2011) Synanthropic birds influence the distribution of *Borrelia* species: Analysis of *Ixodes ricinus* ticks feeding on Passerine Birds. *Appl Environ Microbiol* **77**: 1115-1117.

Dumler, J.S., Choi, K.S., Garcia-Garcia, J.C., Barat, N.S., Scorpio, D.G., Garyu, J.W. et al. (2005) Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*. *Emerg Infect Dis* **11**: 1828-1834.

Duncan, A.B., Agnew, P., Noel, V., and Michalakis, Y. (2015) The consequences of co-infections for parasite transmission in the mosquito *Aedes aegypti*. *J Anim Ecol* **84**: 498-508.

Elfving, K., Olsen, B., Bergstrom, S., Waldenstrom, J., Lundkvist, A., Sjostedt, A. et al. (2010) Dissemination of spotted fever Rickettsia agents in Europe by migrating Birds. *Plos One* **5**.

Elliott, M. (1977) Synthetic pyrethroids. *Am Chem Soc Symp Ser* **42**: 1-28

Gern, L., and Rais, O. (1996) Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). *J Med Entomol* **33**: 189-192.

Ginsberg, H.S. (2008) Potential effects of mixed infections in ticks on transmission dynamics of pathogens: comparative analysis of published records. *Exp Appl Acarol* **46**: 29-41.

Gray, J.S. (1991) The development and seasonal activity of the tick *Ixodes ricinus*: a vector of Lyme borreliosis. *Rev Med Vet Entomol* **79**: 323-333.

Hagemeijer, E.J.M., and Blair, M.J. (1997) *The EBCC Atlas of European Breeding Birds: their distribution and abundance.* London: T & A D Poyser.

Hanincova, K., Taragelova, V., Koci, J., Schafer, S.M., Hails, R., Ullmann, A.J. et al. (2003) Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. *Appl Environ Microbiol* **69**: 2825-2830.

Heylen, D., Matthysen, E., Fonville, M., and Sprong, H. (2014) Songbirds as general transmittors but selective amplifiers of *Borrelia burgdorferi* sensu lato genotypes. *Environ Microbiol* **16**: 2859-2868.

Heylen, D., Adriaensen, F., Dauwe, T., Eens, M., and Matthysen, E. (2009) Offspring quality and tick infestation load in brood rearing great tits *Parus major*. *Oikos* **118**: 1499-1506.

Heylen, D., Tijsse, E., Fonville, M., Matthysen, E., and Sprong, H. (2013a) Transmission dynamics of *Borrelia burgdorferi* s.l. in a bird tick community. *Environ Microbiol* **15**: 663-673.

Heylen, D., Adriaensen, F., Van Dongen, S., Sprong, H., and Matthysen, E. (2013b) Ecological factors that determine *Ixodes ricinus* tick burdens in the great tit (*Parus major*), an important avian reservoir of *Borrelia burgdorferi* s.l.. *Int J Parasitol.* **43**: 603-611.

Heylen, D.J.A., and Matthysen, E. (2008) Effect of tick parasitism on the health status of a passerine bird. *Funct Ecol* **22**: 1099-1107.

Heylen, D.J.A., Madder, M., and Matthysen, E. (2010) Lack of resistance against the tick *Ixodes ricinus* in two related passerine bird species. *Int J Parasitol* **40**: 183-191.

Holden, K., Hodzic, E., Feng, S., Freet, K.J., Lefebvre, R.B., and Barthold, S.W. (2005) Coinfection with *Anaplasma phagocytophilum* alters *Borrelia burgdorferi* population distribution in C3H/HeN mice. *Infect Immun* **73**: 3440-3444.

Hornok, S., Kovats, D., Csorgo, T., Meli, M.L., Gonczi, E., Hadnagy, Z. et al. (2014) Birds as potential reservoirs of tick-borne pathogens: first evidence of bacteraemia with *Rickettsia helvetica*. *Parasit Vectors* **7**.

Hu, C.M., Cheminade, Y., Perret, J.L., Weynants, V., Lobet, Y., and Gern, L. (2003) Early detection of *Borrelia burgdorferi* sensu lato infection in Balb/c mice by co-feeding *Ixodes ricinus* ticks. *Int J Med Microbiol* **293**: 421-426.

Humair, P.F. (2002) Birds and *Borrelia*. *Int J Med Microbiol* **291**: 70-74.

Humair, P.F., and Gern, L. (2000) The wild hidden face of Lyme borreliosis in Europe. *Microbes Infect* **2**: 915−922.

Humair, P.F., Postic, D., Wallich, R., and Gern, L. (1998) An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes. *J Med Microbiol Virol Parasitol Infect Dis* **287**: 521-538.

Jahfari, S., Fonville, M., Hengeveld, P., Reusken, C., Scholte, E.J., Takken, W. et al. (2012) Prevalence of *Neoehrlichia mikurensis* in ticks and rodents from North-west Europe. *Parasit Vectors* **5**.

Jahfari, S., Coipan, E.C., Fonville, M., Van Leeuwen, A.D., Hengeveld, P., Heylen, D. et al. (2014) Circulation of four *Anaplasma phagocytophilum* ecotypes in Europe. *Parasit Vectors* **7**.

Kocan, K.A., and de la Fuente, J. (2003) Co-feeding studies of ticks infected with *Anaplasma marginale*. *Vet Parasitol* **112**: 295-305.

Kurtenbach, K., Peacey, M., Rijpkema, S.G.T., Hoodless, A.N., Nuttall, P.A., and Randolph, S.E. (1998) Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. *Appl Environ Microbiol* **64**: 1169-1174.

Levin, M.L., and Fish, D. (2000) Immunity reduces reservoir host competence of *Peromyscus leucopus* for *Ehrlichia phagocytophila*. *Infect Immun* **68**: 1514-1518.

Lommano, E., Dvorak, C., Vallotton, L., Jenni, L., and Gern, L. (2014) Tick-borne pathogens in ticks collected from breeding and migratory birds in Switzerland. *Ticks Tick-Borne Dis* **5**: 871-882.

Marsot, M., Henry, P.-Y., Vourc'h, G., Gasqui, P., Ferquel, E., Laignel, J. et al. (2012) Which forest bird species are the main hosts of the tick, *Ixodes ricinus*, the vector of *Borrelia burgdorferi* sensu lato, during the breeding season? *Int J Parasitol* **42**: 781-788.

Massung, R.F., Priestley, R.A., and Levin, M.L. (2004) Transmission route efficacy and kinetics of *Anaplasma phagocytophilum* infection in the white-footed mouse, *Peromyscus leucopus*. *Vector-Borne Zoonotic Dis* **4**: 310-318.

Maurer, F.P., Keller, P.M., Beuret, C., Joha, C., Achermann, Y., Gubler, J. et al. (2013) Close geographic association of human Neoehrlichiosis and tick populations carrying "*Candidatus* Neoehrlichia mikurensis" in Eastern Switzerland. *J Clin Microbiol* **51**: 169-176.

Molenberghs, G., and Verbeke, G. (2005) *Models for Discrete Longitudinal Data*. Berlin-Heidelberg-New York Springer-Verlag.

Moro, M.H., Zegarra-Moro, O.L., Bjornsson, J., Hofmeister, E.K., Bruinsma, E., Germer, J.J., and Persing, D.H. (2002) Increased arthritis severity in mice coinfected with *Borrelia burgdorferi* and *Babesia microti*. *J Infect Dis* **186**: 428-431.

Nilsson, K., Lindquist, O., and Pahlson, C. (1999) Association of *Rickettsia helvetica* with chronic perimyocarditis in sudden cardiac death. *Lancet* **354**: 1169-1173.

Nilsson, K., Elfving, K., and Pahlson, C. (2010) *Rickettsia helvetica* in patient with meningitis, Sweden, 2006. *Emerg Infect Dis* **16**: 490-492.

Randolph, S.E., Gern, L., and Nuttall, P.A. (1996) Co-feeding ticks: Epidemiological significance for tick-borne pathogen transmission. *Parasitol Today* **12**: 472-479.

Regev-Yochay, G., Dagan, R., Raz, M., Carmeli, Y., Shainberg, B., Derazne, E. et al. (2004) Association between carriage of *Streptococcus pneumoniae* and *Staphylococcus aureus* in children. *J Am Med Assoc* **292**: 716-720.

Richter, D., Spielman, A., Komar, N., and Matuschka, F.R. (2000) Competence of American robins as reservoir hosts for Lyme disease spirochetes. *Emerg Infect Dis* **6**: 133-138.

Santos-Silva, M.M., Sousa, R., Santos, A.S., Melo, P., Encarnacao, V., and Bacellar, F. (2006) Ticks parasitizing wild birds in Portugal: Detection of *Rickettsia aeschlimannii*, *R. helvetica* and *R. massiliae*. *Exp Appl Acarol* **39**: 331-338.

Sato, Y., and Nakao, M. (1997) Transmission of the Lyme disease spirochete, *Borrelia garinii*, between infected and uninfected immature *Ixodes persulcatus* during cofeeding on mice. *J Parasitol* **83**: 547-550.

Schouls, L.M., Van de Pol, I., Rijpkema, S.G.T., and Schot, C.S. (1999) Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. *J Clin Microbiol* **37**: 2215-2222.

Scoles, G.A., Papero, M., Beati, L., and Fish, D. (2001) A relapsing fever group spirochete transmitted by *Ixodes scapularis* ticks. *Vector-Borne Zoonotic Dis* **1**: 21-34.

Sprong, H., Wielinga, P.R., Fonville, M., Reusken, C., Brandenburg, A.H., Borgsteede, F. et al. (2009) *Ixodes ricinus* ticks are reservoir hosts for *Rickettsia helvetica* and potentially carry flea-borne *Rickettsia* species. *Parasit Vectors* **2**.

Stenos, J., Graves, S.R., and Unsworth, N.B. (2005) A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group Rickettsiae. *Am J Trop Med Hyg* **73**: 1083-1085.

Susi, H., Barres, B., Vale, P.F., and Laine, A.L. (2015) Co-infection alters population dynamics of infectious disease. *Nature Commun* **6**.

Szekeres, S., Coipan, E.C., Rigo, K., Majoros, G., Jahfari, S., Sprong, H., and Foldvari, G. (2015) *Candidatus* Neoehrlichia mikurensis and *Anaplasma phagocytophilum* in natural rodent and tick communities in Southern Hungary. *Ticks Tick-Borne Dis* **6**: 111-116.

Thomas, V., Anguita, J., Barthold, S.W., and Fikrig, E. (2001) Coinfection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis alters murine immune responses, pathogen burden, and severity of Lyme arthritis. *Infect Immun* **69**: 3359-3371.

Tijsse-Klasen, E., Sprong, H., and Pandak, N. (2013) Co-infection of *Borrelia burgdorferi* sensu lato and *Rickettsia* species in ticks and in an erythema migrans patient. *Parasit Vectors* **6**.

Verbeke, G., and Molenberghs, G. (2001) *Linear Mixed Models for Longitudinal Data*. Berlin-Heidelberg-New York Springer-Verlag.

Wallmenius, K., Barboutis, C., Fransson, T., Jaenson, T.G.T., Lindgren, P.-E., Nystrom, F. et al. (2014) Spotted fever *Rickettsia* species in *Hyalomma* and *Ixodes* ticks infesting migratory birds in the European Mediterranean area. *Parasit Vectors* **7**.

Zemtsova, G., Killmaster, L.F., Mumcuoglu, K.Y., and Levin, M.L. (2010) Co-feeding as a route for transmission of *Rickettsia conorii israelensis* between *Rhipicephalus sanguineus* ticks. *Exp Appl Acarol* **52**: 383-392.

Table legends

Table 1. The distribution of infected fed ticks over the time after exposure to great tits (Infestation 1). *P*-values of Fisher's exact tests by which the proportions of pathogen-infected fed ticks are compared with these in the set of unfed questing nymphs are shown. When sample sizes were too low (< 5), no statistical conclusions were drawn.

Figure captions

Fig. 1 Schematic overview of the study design. Three times in succession, tick-naïve great tit individuals were exposed to 17 questing *I. ricinus* nymphs carrying a bacterial community consisting of *Ricketssia helvetica*, *Anaplasma phagocytophylum*, *Neoehrlichia mikurensis*, *Borrelia miyamotoi*, *Borrelia burgdorferi* sensu lato. Fed nymphs were kept in individual tubes until moulting to the adult stage was completed, and were subsequently screened for the presence of the bacteria species.

Fig. 2 Mean infection rates (+ 1 standard error) of bacteria in *Ixodes ricinus* ticks that fed as a nymph on naïve great tit individuals ('Inf. 1-3'). Great tit individuals were repeatedly infested with 17 nymphs collected from the vegetation in an a pathogen endemic area ('Questing'). Symbols for the bacterial species are indicated in the figure's legend.

Fig. 3 Proportions of infected unfed ('Questing') ticks and ticks that fed on the juvenile great tits ('Inf.1-3'). Different colors represent bacterium (co-) infections. Plain parts indicate the proportion of co-infected ticks, hatched parts singly-infected. Number of positive ticks and the prevalence of co-infected ticks are presented below each pie chart.

FIGURES

Figure 1

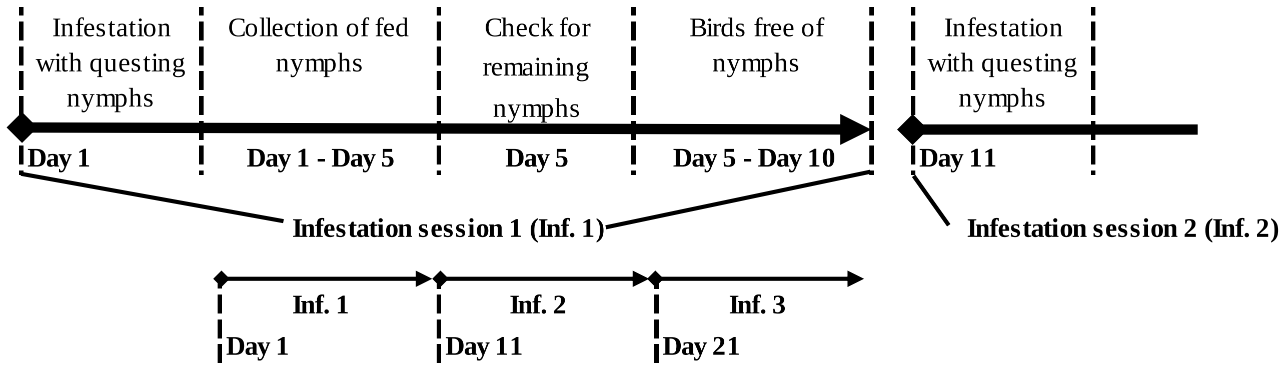

Fig. 1 Schematic overview of the study design. Three times in succession, tick-naïve great tit individuals were exposed to 17 questing *I. ricinus* nymphs carrying a bacterial community consisting of *Ricketssia helvetica*, *Anaplasma phagocytophylum*, *Neoehrlichia mikurensis*, *Borrelia miyamotoi*, *Borrelia burgdorferi* sensu lato. Fed nymphs were kept in individual tubes until moulting to the adult stage was completed, and were subsequently screened for the presence of the bacteria species.

Figure 2

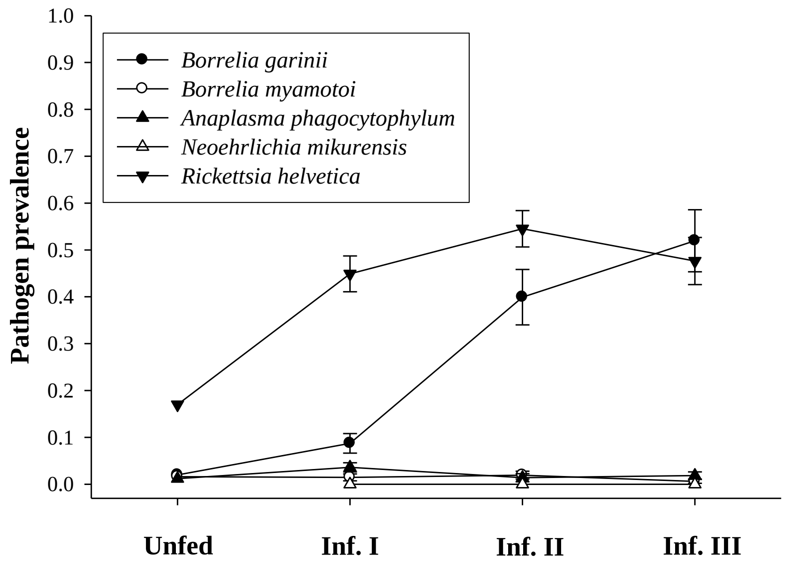

Fig. 2 Mean infection rates (± 1 standard error) of bacteria in *Ixodes ricinus* ticks that fed as a nymph on naïve great tit individuals ('Inf. 1-3'). Great tit individuals were repeatedly infested with 17 nymphs collected from the vegetation in an a pathogen endemic area ('Questing'). Symbols for the bacterial species are indicated in the figure's legend.

Figure 3

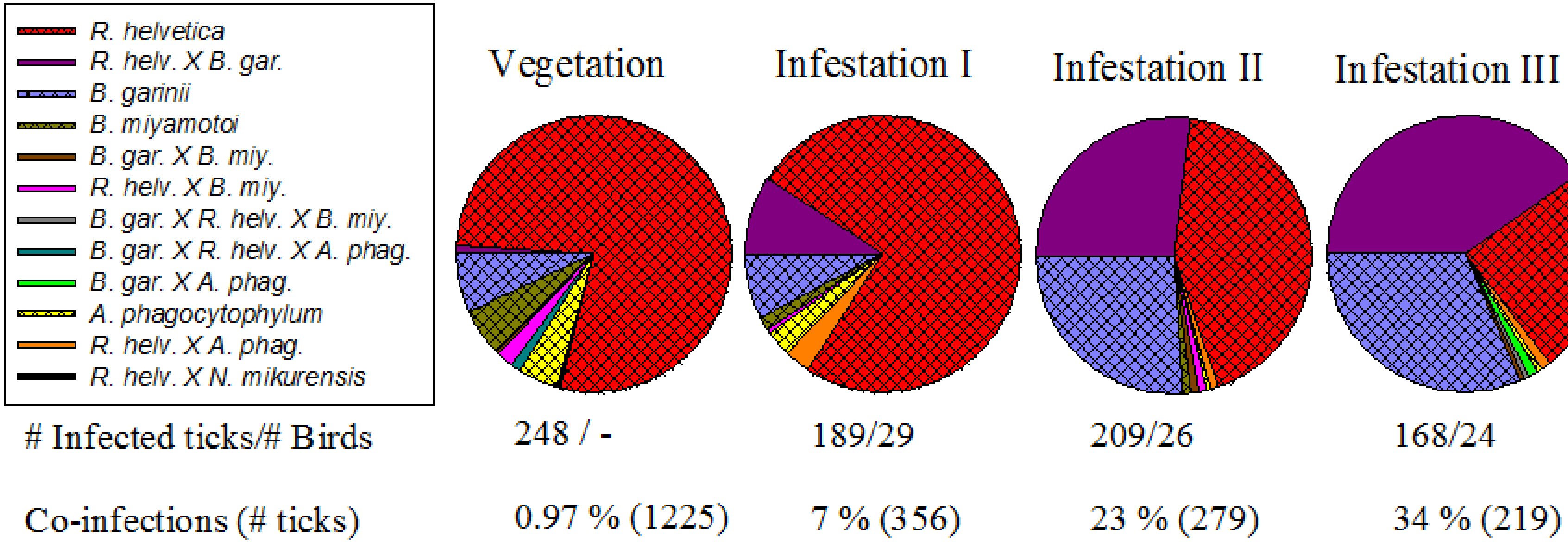

Fig. 3 Proportions of infected unfed ('Questing') ticks and ticks that fed on the juvenile great tits ('Inf.1-3'). Different colors represent bacterium (co-) infections. Plain parts indicate the proportion of co-infected ticks, hatched parts singly-infected. Number of positive ticks and the prevalence of co-infected ticks are presented below each pie chart.

Table 1. The distribution of infected fed ticks over the time after exposure to great tits (Infestation 1). *P*-values of Fisher's exact tests by which the proportions of pathogen-infected fed ticks are compared with these in the set of unfed questing nymphs are shown. When sample sizes were too low (< 5), no statistical conclusions were drawn.

Days after exposure (N [illegible])	*Borrelia garinii* (N positives; P-value)	*Rickettsia helvetica* (N positives; P-value)	*Anaplasma phagocytophylum* (N positives; P-value)
2.75 (164)	0.09 (15; < 0.0001) *	0.42 (69; < 0.0001) *	0.04 (7; 0.10) *
3.25 (26)	0.12 (3; 0.017) *	0.62 (16; < 0.0001) *	0 (0; 1.0)
3.75 (136)	0.10 (13; < 0.0001) *	0.48 (65; < 0.0001) *	0.03 (4; 0.11)
4.25 (28)	0.07 (2; 0.11)	0.43 (12; 0.0014) *	0.04 (1; 0.31)
4.75 (4)	0 (0)	0.5 (2)	0 (0)
5.25 (1)	0 (0)	1 (1)	0 (0)

Infection rate in the 1225 field-collected unfed questing nymphs (N positives):

Borrelia garinii: 0.019 (24)

Rickettsia helvetica: 0.169 (208)

Anaplasma phagocytophylum : 0.012 (15)

* Fisher's exact test *P*-value < 0.05