Title: Inefficient co-feeding transmission of *Borrelia afzelii* in two common European songbirds

Dieter J. A. Heylen[1*], Hein Sprong[2], Aleksandra Krawczyk[2], Natalie Van Houtte[1], Dolores Genné[4], Andrea Gomez-Chamorro[4], Kees van Oers[3], Maarten J. Voordouw[4]

[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.

[3] Department of Animal Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands.

[4] Laboratory of Ecology and Evolution of Parasites, Institute of Biology, University of Neuchâtel, Switzerland.

[*] Corresponding author email: Dieter.Heylen@uantwerpen.be

Abstract

The spirochete bacterium *Borrelia afzelii* is the most common cause of Lyme borreliosis in Europe. This tick-borne pathogen can establish systemic infections in rodents but not in birds. However, several field studies have recovered larval *Ixodes ricinus* ticks infected with *B. afzelii* from songbirds suggesting successful transmission of *B. afzelii*. We reviewed the literature to determine which songbird species were the most frequent carriers of *B. afzelii*-infected *I. ricinus* larvae and nymphs. We tested experimentally whether *B. afzelii* is capable of co-feeding transmission on two common European bird species, the blackbird (*Turdus merula*) and the great tit (*Parus major*). For each bird species, four naïve individuals were infested with *B. afzelii*-infected *I. ricinus* nymphal ticks and pathogen-free larval ticks. None of the co-feeding larvae tested positive for *B. afzelii* in blackbirds, but a low percentage of infected larvae (3.33%) was observed in great tits. Transstadial transmission of *B. afzelii* DNA from the engorged nymphs to the adult ticks was observed in both bird species. However, BSK culture found that these spirochetes were not viable. Our study suggests that co-feeding transmission of *B. afzelii* is not efficient in these two songbird species.

Key words: *Borrelia afzelii*, *Borrelia burgdorferi*, co-feeding transmission, trans-stadial transmission, *Ixodes ricinus*, Lyme borreliosis, reservoir host, songbird, tick-borne pathogen

Introduction

The tick-borne spirochete bacterium *Borrelia afzelii* is the most common etiological agent of Lyme borreliosis (LB) in Europe [1-3]. This pathogen is transmitted by *Ixodes ricinus* ticks and is adapted to infect rodent reservoir hosts [3-7]. In these hosts, *B. afzelii* establishes a long-term, systemic infection that facilitates high rates of host-to-tick transmission [6,8-10]. In contrast to bird-adapted *Borrelia* species such as *B. garinii* and *B. valaisiana*, experimental infection studies with blackbirds, pheasants, and great tits have shown that *B. afzelii* is not able to establish a systemic infection in these bird species [11-13]. The ability of *B. afzelii* to infect rodent but not avian hosts (and vice versa for the bird-adapted *Borrelia* species) appears to be mediated by the vertebrate complement system [14,15]. Thus, the general consensus is that *B. afzelii* is unable to use avian hosts to infect new ticks [1-3,16].

Recent field studies on birds have questioned this consensus of whether *B. afzelii* is strictly incompatible with avian hosts. Many species of birds are frequently exposed to *B. afzelii*-infected *I. ricinus* nymphs [17-23]. More importantly, *B. afzelii*-infected larval ticks have been recovered from a number of bird species including *Fringilla coelebs* L., *Troglodytes troglodytes* L., *Parus major* L., *Turdus merula* L., and *Turdus iliacus* L. (see Table 1). Given that vertical transmission of LB pathogens is thought to be rare in *Ixodes* ticks [24-26], these observations suggest that these larval ticks acquired *B. afzelii* spirochetes from avian hosts.

Co-feeding transmission is one strategy by which *B. afzelii* might infect larval ticks feeding on avian hosts. This mode of transmission occurs when infected and uninfected ticks feed in close spatial and temporal proximity on the same host [27-29]. A number of studies have documented co-feeding transmission of *B. afzelii* on competent rodent reservoir hosts [27,30-33]. The observation that this mode of transmission can occur in the absence of a systemic infection raised the hypothesis that co-feeding transmission could allow *Borrelia* pathogens to evade the hostile immune system of otherwise incompetent hosts [28,29,34]. For example, co-

feeding transmission of *B. afzelii* and *B. garinii* has been documented on ungulates, which are believed to be refractory to systemic infection [35,36]. An experimental infection study using a Japanese strain of *B. garinii* demonstrated co-feeding transmission on laboratory mice [37]. However, an alternative explanation for this study is that this strain actually belonged to the closely related but rodent-adapted *B. bavariensis*, as this species was recently shown to be widespread in Asia, including Japan [38].

The purpose of the present study was to test whether *B. afzelii* can use co-feeding transmission to infect *I. ricinus* larval ticks on two different species of songbird: the blackbird (*Turdus merula*) and the great tit (*Parus major*). We chose these two songbird species because they are common in Europe, are often exposed to immature *I. ricinus* ticks in nature, and they are highly competent reservoir hosts for bird-adapted *Borrelia* genospecies. The blackbird can amplify *B. garinii*, *B. valaisiana* and *B. turdi* [23,39,40] and the great tit can amplify *B. garinii* [11]. In addition, we performed a literature review to determine how often songbirds carry *B. afzelii*-infected immature *I. ricinus* ticks in nature.

Results

Blackbird experiment: In the blackbird experiment, each of the four birds was infested with 11–12 nymphs before being infested with 40–50 co-feeding larvae 24 hours later. The challenge nymphs had been randomly selected from a population where the percentage of infected nymphs was 68.1% (47 infected/69 total). For the blackbirds, the nymphal and larval attachment rates (mean ± standard deviation) were 93.7 ± 12.5% per bird and 96.5 ± 4.7% per bird, respectively. A total of 20 engorged challenge nymphs and 128 engorged co-feeding larvae were recovered (mean ± standard deviation: 5.0 ± 0.8 nymphs per bird and 32 ± 12 larvae per bird). The engorged challenge nymphs were allowed to moult into adult ticks, which were tested using qPCR to determine whether the birds had been exposed to

87 *B. afzelii*. A total of 17 challenge nymphs and 90 co-feeding larvae were tested for the four
88 blackbirds (Table 2).
89 Two of the four blackbirds produced 2 and 4 infected adult ticks (Table 2) indicating
90 that they were properly challenged. The presence of *B. afzelii* in 6 adult ticks suggests that
91 there was nymph-to-adult transtadial transmission but we do not know whether these
92 spirochetes were dead or alive. The other two birds produced 2 and 4 uninfected adult ticks
93 (Table 2). Given that the estimated proportion of infected challenge nymphs was 0.681, the
94 probability that these two birds would produce 6 uninfected adult ticks is $(1 - 0.681)^6 = 0.001$.
95 Our method of estimating nymphal attachment suggests that 9 and 11 challenge nymphs
96 attached to these two birds. The probability that these two birds were infested with at least one
97 *B. afzelii*-infected nymph is therefore very high (0.9999659 and 0.9999965, respectively).
98 Thus we are confident that all four birds encountered at least one *B. afzelii*-infected nymph.
99 However, none of the 90 xenodiagnostic larval ticks (tested as engorged larvae or as flat
100 nymphs) that had co-fed with the challenge nymphs tested positive for *B. afzelii* (Table 2).
101 All ticks that had fed on the blackbirds and that had tested positive for *B. afzelii* on the
102 qPCR were sequenced with respect to the *ospC* gene and the 5S-23S (rrfA-rrlB) intergenic
103 spacer (IGS) region gene. We obtained 3 *ospC* sequences and 5 IGS sequences and all of
104 them belonged to *B. afzelii*. This sequencing work confirms that the nymphs used to challenge
105 the blackbirds were infected with *B. afzelii*.
106 **Great tit experiment:** In the great tit experiment, each of the four birds was infested
107 with 11–12 nymphs before being infested with 40–50 co-feeding larvae 24 hours later. The
108 challenge nymphs had been randomly selected from a population where the percentage of
109 infected nymphs was 91.5% (130 infected/142 total). For the great tits, the nymphal and larval
110 attachment rates (mean ± standard deviation) were 58.3 ± 21.5% per bird and 85.6 ± 9.8% per
111 bird, respectively. A total of 16 engorged challenge nymphs and 115 engorged co-feeding

112 larvae were recovered (mean ± standard deviation: 4.0 ± 2.7 nymphs per bird and 28.8 ± 6.8
113 larvae per bird). The engorged challenge nymphs were either tested directly or were allowed
114 to moult into adult ticks. A total of 16 challenge nymphs and 90 co-feeding larvae were tested
115 for the four great tits (Table 2).

116 Analysis of the challenge ticks showed that all four great tits had been exposed to *B.*
117 *afzelii* (2, 3, 8, and 2 infected ticks per bird; Table 2). Three of the 76 xenodiagnostic larval
118 ticks (tested as engorged larvae) that had co-fed with the challenge nymphs tested positive for
119 *B. afzelii*, but the pathogen was not detected in any of the 14 nymphs (moulted from the
120 engorged larvae) (Table 2). Four of the five adult ticks obtained from three birds tested
121 positive for *B. afzelii* based on the qPCR (Table 2), but the culture of these ticks in BSK-II
122 medium did not yield any viable spirochetes.

123 **Summary of the infection experiments:** Overall, the *B. afzelii*-infection rates in co-
124 feeding larvae were low in both blackbirds (0.00% = 0/90) and great tits (3.33% = 3/90). In
125 summary, we found limited co-feeding transmission of *B. afzelii* for the two bird species used
126 in this study. We emphasize that our sample size was limited with only 4 individuals for each
127 bird species.

128 **Literature review:** Our review of the literature found 13 of 19 studies in which *B.*
129 *afzelii* has been reported in songbird-derived *I. ricinus* ticks. Seven species of songbird could
130 play a role in the transmission of *B. afzelii* to larval *I. ricinus* ticks (Table 1). The hosts that
131 were most often reported to have *B. afzelii*-infected larvae were the European robin
132 (*Erithacus rubecula*) and the great tit (2 studies). When considering birds that carried *B.*
133 *afzelii*-infected nymphal ticks, we found 20 different bird species, of which the blackbird (7
134 studies), songthrush (*Turdus philomenos*) (6 studies), dunnock (*Prunella modularis*) (5
135 studies), European robin (5 studies), and great tit (4 studies) were most often reported.
136

Discussion

Our study suggests that the rodent-adapted Lyme disease pathogen, *B. afzelii*, cannot use co-feeding transmission as an efficient strategy to infect naive ticks on two species of songbird. There was no co-feeding transmission of *B. afzelii* on the four blackbirds and only three larval ticks acquired *B. afzelii* via co-feeding transmission on the four great tits. The efficiency of co-feeding transmission of *B. afzelii* on the great tit was therefore low (3/90 = 3.33%). In contrast, the isolate of *B. afzelii* used in the great tit experiment (isolate NE4049; also referred to as *ospC* strain A10) has high co-feeding transmission (> 50.00%) on competent rodent reservoir hosts, and in these hosts there is successful trans-stadial transmission [31-33]. We acknowledge that one limitation of the current study is the small sample size (n = 8 birds). However, we point out that studies with similar sample sizes have detected co-feeding transmission of *B. afzelii* on rodents [27,31,32]. Recent theoretical studies have shown that co-feeding transmission makes a modest contribution to the reproductive number (R_0) of *B. burgdorferi* pathogens [41-43]. Specifically, a co-feeding transmission efficiency of 50.0% increases the R_0 value by 2.07–6.68% depending on a variety of ecological factors [41]. These analyses suggest that a co-feeding transmission efficiency of 3.33% would have a negligible effect on the R_0 of *B. afzelii*. In summary, *B. afzelii* is transmitted efficiently via co-feeding transmission on rodent hosts but not on the two bird species investigated. Studies on *B. afzelii* in laboratory rodents have shown that strains differ in the efficacy of co-feeding transmission [31,33]. Studies on *B. burgdorferi* in North American passerines have shown that reservoir competence can vary widely between bird species [44-46]. We therefore emphasize that we cannot generalize these results to other strains of *B. afzelii* and other songbird species.

Our study also found evidence that avian blood is borreliacidal for *B. afzelii*. For the blackbirds, the probability that two birds would produce six uninfected adult ticks was highly unlikely (p = 0.001), given that an independent sample suggested that 68.1% (47 infected/69

total) of the challenge nymphs were infected with *B. afzelii* before feeding on these birds. Our results are similar to a previous study where *B. afzelii* was cleared from *I. ricinus* challenge nymphs after they had fed on pheasants, whereas bird-adapted *Borrelia* species were not cleared from the challenge nymphs [12]. Additional evidence for the borreliacidal effects of avian blood on *B. afzelii* was our demonstration using BSK-II cultures that none of the qPCR-positive adult ticks that had fed as nymphal ticks on the great tits contained viable spirochetes. Previous work has shown that the ability to detect *Borrelia* infections by culturing ticks in BSK media is similar to PCR-based methods [47]. This result suggests that our qPCR assay is detecting dead spirochetes in the adult ticks and shows the limitations of using DNA-based methods to infer the reservoir competence of a particular host species. Further studies using other combinations of pathogen strains and songbird species should investigate the generality of whether avian blood kills *B. afzelii* in *I. ricinus* during tick blood feeding.

Numerous field studies have shown the association of *B. afzelii* with rodent reservoir hosts [4-6,48,49] and of *B. garinii* and *B. valaisiana* with avian reservoir hosts [7,11,12,20,23,39,40,50-52]. The cycling of *B. afzelii* and *B. garinii* in different classes of vertebrate hosts is also supported by studies on wild *I. ricinus* nymphs, which have shown that these two sympatric *Borrelia* species rarely co-occur in the same nymphal tick [53-55]. The host-specificity of *B. afzelii* for rodents and *B. garinii* for birds is believed to be mediated by the complement system of the vertebrate host [14,15,54,55]. *In vitro* assays have shown that *B. afzelii* is tolerant to rodent complement but is lysed by bird complement, and vice versa for bird-adapted *Borrelia* species like *B. garinii* and *B. valaisiana* [14,15]. However, as mentioned previously, there are very few in vivo studies showing that *B. afzelii* spirochetes are killed in nymphs that feed on avian hosts [12]. Two recent studies that quantified the abundance of rodent- and bird-adapted *Borrelia* species in wild questing *I. ricinus* nymphs provided indirect evidence for the complement hypothesis [53,56]. In the first study, the spirochete load of nymphs co-infected with rodent- and

187 bird-adapted *Borrelia* species was significantly lower than the additive expectation of when
188 the species occurred alone [53]. In the second study, co-infections between *B. afzelii* and *B.*
189 *garinii* were surprisingly common in wild nymphs, however, the spirochete load of the
190 dominant *Borrelia* species was always an order of magnitude higher than the sub-dominant
191 species [56]. Taken together, these two studies provide indirect evidence that some component
192 of the vertebrate blood meal (e.g. complement) was reducing the spirochete load of the mal-
193 adapted *Borrelia* species [53,56]. Thus co-infections between rodent- and bird-adapted *Borrelia*
194 species in *I. ricinus* nymphs may be much more common than previously thought but the
195 spirochete population of one of the two species is probably dead.
196 Migratory songbirds have a great capacity to disperse ticks and tick-borne pathogens
197 to new geographic locations [57]. Interestingly, phylogenetic studies have shown that *B. afzelii*
198 has much more spatial genetic structure than *B. garinii*, which may reflect the migratory
199 potential of their rodent and bird hosts [58,59]. Our literature review found that ground-dwelling
200 birds such as the blackbird, song thrush, European robin and dunnock were common carriers
201 of *B. afzelii*-infected immature *I. ricinus* ticks. These studies have led to speculation that *B.*
202 *afzelii* can use bird hosts to achieve transmission and is not as restricted to rodent hosts as
203 previously thought [60]. However, all of these studies used PCR-based methods to determine
204 *Borrelia* infection and none of these studies used culture-based methods to show that the
205 spirochetes are actually alive. The present study shows that nymph-to-adult transtadial
206 transmission of *B. afzelii* DNA can occur on birds but that the spirochetes are not necessarily
207 viable. We suggest that PCR-based studies demonstrating that birds can amplify *B. afzelii* or
208 that rodents can amplify *B. garinii* should be interpreted with great caution.
209 We propose three alternative explanations for the observation that *B. afzelii*-positive
210 larval ticks are regularly collected from wild birds (Table 1). First, the larval ticks could have
211 acquired *B. afzelii* via vertical transmission. There is a general consensus that vertical

212 transmission in *Ixodes* ticks is rare for *B. burgdorferi* s. l. pathogens but common for the
213 relapsing fever spirochete *B. miyamotoi* [24,25]. A second explanation is partial blood feeding
214 where larval ticks take multiple meals from different vertebrate hosts. Host blood meal
215 analysis of wild *I. ricinus* ticks in Switzerland suggests that 9.5–19.5% of larval ticks feed on
216 multiple hosts [61,62]. An early study on *B. burgdorferi* s. s. in *I. scapularis* showed that partially
217 fed larval ticks could acquire spirochetes [63]. Thus larval ticks could acquire *B. afzelii* from a
218 partial blood meal on a rodent and then attach to a bird to feed to repletion. A recent study in
219 the Netherlands reported that wild *I. ricinus* larvae carried *B. afzelii* (prevalence was 0.62%),
220 and these larvae were able to infect pathogen-free rodents [26]. The authors suggested that their
221 data were consistent with both vertical transmission and partial blood meals [26]. A third
222 explanation involves variation in the efficiency of co-feeding transmission between strains of
223 *B. afzelii*. Like many vector-borne pathogens, populations of *B. afzelii* consist of multiple
224 strains [56,64-67]. Two recent studies found that some *B. afzelii* strains are much more efficient at
225 co-feeding transmission than other strains [31,33]. The *B. afzelii* strains in the blackbird
226 experiment were derived from naturally infected *Apodemus* mice, and their genetic identity
227 and co-feeding transmission efficiency on rodent hosts are currently unknown. For this
228 reason, we used *B. afzelii* isolate NE4049 in the great tit experiment because it has a high
229 efficiency of co-feeding transmission (>50%) on lab mice [31,33].
230 We conclude that blackbirds and great tits do not allow efficient co-feeding
231 transmission of viable *B. afzelii* spirochetes. The present study supports the hypothesis that
232 the bird complement system inhibits the rodent-adapted *B. afzelii* from exploiting avian hosts
233 for spirochete transmission. The generality of our results for other combinations of *B. afzelii*
234 strains and bird species remains to be investigated.
235
236 **Methods**

Birds: Eurasian blackbirds and great tits are two abundant bird species in Europe. The Eurasian blackbird is frequently infested with tens of immature *I. ricinus* ticks [23,68,69]. The great tits in our Belgian study population frequently carry high burdens of immature *I. ricinus* ticks (maximum number of larvae = 40; nymphs = 17) [70,71]. Both bird species are competent reservoir hosts for bird-adapted *B. burgdorferi* s. l. pathogens. Blackbirds transmit *B. garinii*, *B. valaisiana*, and *B. turdi* [23,39,40], whereas great tits transmit *B. garinii* [11,72-74].

Four pathogen-free blackbirds and four pathogen-free great tits were obtained, respectively, from a certified Belgian breeder and a laboratory colony at the Netherlands Institute of Ecology (NIOO-KNAW) [75]. Environmental conditions consisted of a 12 h light: 12 h dark cycle (7:00 to 19:00) and ambient temperature varied with outdoor conditions. Birds were given food and water *ad libitum*, and had access to a fresh water bath. Birds were kept in individual cages and were allowed to habituate to the lab environment for at least four days before the start of the experiment. Experiments were carried out in accordance with national environmental legislation and university regulations. The Ethics Committee for Animal Experiments of the University of Antwerp approved the tick infestation procedure (Dossier 2009-32) and the transmission experiment (Dossier 2014-49).

***Ixodes ricinus* ticks:** Pathogen-free *I. ricinus* larval ticks from the laboratory colony at the University of Neuchâtel were fed on *B. afzelii*-infected rodents and were allowed to moult into *B. afzelii*-infected nymphs (hereafter referred to as the challenge nymphs). The creation of the challenge nymphs was different for the blackbirds and great tits (see below). The pathogen-free *I. ricinus* larvae that were used for co-feeding with the infected challenge nymphs were obtained from a German laboratory colony (IS Insect Services GmbH, Berlin).

For the blackbirds, the challenge nymphs had been fed as larval ticks on 7 field-captured and naturally infected wood mice (*Apodemus sylvaticus* L.). Infection with *B. burgdorferi* s. l. of each wood mouse was confirmed with a commercial Lyme borreliosis

ELISA assay and qPCR on an ear tissue sample, using protocols described elsewhere [76]. All challenge nymphs were kept in individual Eppendorf tubes to facilitate random sampling. We randomly selected 9–10 nymphs from each of the 7 *Apodemus* mice and tested them for *B. afzelii* infection using qPCR. The infection prevalence of the challenge nymphs used in the black bird experiment was 68.1% (47 infected/69 total).

For the great tits, the challenge nymphs had been fed as larval ticks on 15 *Mus musculus* BALB/c mice that had been experimentally co-infected via tick bite with *B. afzelii* isolates NE4049 and Fin-A3. Infection with *B. afzelii* of each mouse was confirmed with a commercial Lyme borreliosis ELISA assay and qPCR on an ear tissue sample, using protocols described elsewhere [76]. Isolates NE4049 and Fin-A3 were obtained from an *I. ricinus* nymph in Switzerland and a bank vole (*Myodes glareolus*) in Finland. 454-sequencincing of the *ospC* gene found that isolates NE4049 and Fin-A3 were monogenic for the *ospC* major group alleles A10 and A3, respectively. We used isolate NE4049 (also referred to as *ospC* strain A10) because it has very efficient co-feeding transmission in lab mice [31,33]. All challenge nymphs were kept in individual Eppendorf tubes to facilitate random sampling. We randomly selected 7-10 nymphs from each of the 15 mice and tested them for *B. afzelii* infection using qPCR. The infection prevalence of the challenge nymphs used in the great tit experiment was 91.5% (130 infected/142 total), of which 75.4% (107/142) and 59.9% (85/142) were infected with isolates NE4049 and Fin-A3, respectively.

Study design: The infestation experiments for the blackbirds and great tits were conducted in November 2015 and February 2016, respectively. For each bird species, four individuals were infested with 11–12 *B. afzelii*-infected *I. ricinus* nymphs that had been randomly selected from the pool of available nymphs. These tick loads are within the range observed in field-captured birds [23,68-71]. Nymphs were placed underneath the crown feathers on the right side of the head above the eye using moistened tweezers, as described in [71] (Fig.

1). After each infestation, birds were kept for 1 h in an air-permeable cotton bag (size: 25 cm x 20 cm for blackbirds; 20 cm x 15 cm for great tits) inside a darkened cage to keep them inactive and to facilitate tick attachment [71]. Twenty-four hours after nymphal exposure, the blackbirds and great tits were additionally infested with 40–50 xenodiagnostic larvae, following the same protocol as for the challenge nymphs. The larvae were placed near the nymphs to facilitate co-feeding transmission [31-33]. After each infestation, the cotton bags were checked for ticks to determine the number of nymphs and larvae that had attached to each bird. Birds were not checked for the number of attached nymphs to avoid disturbing these ticks. Following infestation, birds were returned to their individual cages (40 cm x 80 cm) that had a wire mesh floor to facilitate the daily collection of engorged ticks. Most of the engorged ticks were placed in 80% ethanol and stored at –20°C. The remaining engorged ticks were allowed to moult to the next stage to study transstadial transmission of *B. afzelii* DNA. These ticks were kept in individual tubes under summer conditions (16 h light at 25°C, 8 h at dark at 16°C) and with a relative humidity >90%. For the great tit experiment, we further tested whether the *B. afzelii* spirochetes in the adult ticks were actually viable. Each of five adult ticks that had fed as challenge nymph on three great tits, were cut into two halves using sterile scissors. One tick half was screened for *B. afzelii* infection using qPCR, the other tick half was cultured in tubes containing BSK-II medium [77], incubated at 34°C, and examined by dark-field microscopy every 10 days for 40 days.

Probability that each bird was challenged by at least one *B. afzelii*-infected nymph: If avian blood clears spirochetes from feeding nymphs, the post-hoc analysis of such ticks is not be a reliable indicator as to whether the bird was challenged or not. For example, after feeding *B. afzelii*-infected *I. ricinus* nymphs on pheasants, 0 of the 56 engorged nymphs tested positive for *B. afzelii* [12]. In this case, it is critical to know the prevalence of *B. afzelii* infection in the flat nymphs (q) before they are placed on the birds, and the number of nymphs

312 that attached to the bird (n). With this information one can calculate the probability (P) that
313 each bird was bitten by at least one *B. afzelii*-infected challenge nymph as follows: $P = 1 - (1$
314 $- q)^n$. The exact value of n is often unknown: the maximum is the number of nymphs that
315 attached to the bird (n_{max}) and the minimum is the number of blood-engorged nymphs that
316 were recovered (n_{min}). For example, for a bird that was infested with 12 challenge nymphs
317 with an expected prevalence of infection of 0.681 and for which 4 engorged challenge
318 nymphs were recovered, the probability that at least one of the challenge nymphs was infected
319 with *B. afzelii* ranges from $P_{max} = 0.9999989$ to $P_{min} = 0.9896447$.

320 **PCR-based detection of *B. afzelii*:** Total tick DNA was purified using the DNeasy
321 Blood & Tissue Kit following the protocol for the purification of total DNA from ticks. All
322 ticks were screened for the presence of *B. burgdorferi* s. l. using a duplex qPCR that was
323 designed based on existing qPCR protocols that target fragments of the *ospA* gene [78] and the
324 *flagellin* gene [79]. A detailed description of primers, probes and the qPCR protocol is given in
325 an earlier study [74]. For the subsample of qPCR-positive ticks that had fed on the blackbirds,
326 the *B. burgdorferi* s. l. genospecies was determined by PCR amplification and sequencing of
327 the *ospC* gene [80] and the variable 5S-23S (rrfA-rrlB) intergenic spacer (IGS) region gene [74].
328 For each PCR and multiplex qPCR, positive controls, negative controls, and blank samples
329 were included. To minimize contamination, the three steps of the PCR protocol were
330 performed in separate rooms. The DNA extraction room was kept at negative pressure,
331 whereas the reagent setup and sample addition rooms were kept at positive pressure. All
332 rooms had airlocks.

333 **Literature review:** We used an extensive systematic literature search that is described
334 in Hofmeester *et al.* (2016) [81]. The search strings and selection procedure as well as the
335 dataset are provided in the supplementary material of that study (URL:
336 http://iopscience.iop.org/article/10.1088/1748-9326/11/4/043001/meta). The search was done

using PubMed, Web of Science and Scopus to review the occurrence of *B. burgdorferi* s. l. pathogens in Europe, in songbird hosts and their *I. ricinus* ticks. The last literature search was carried out in January 2015 and used the years 1945-2014. We added one more study to that dataset [21]. Only studies that identified the *Borrelia* genospecies in infected larvae and nymphs derived from songbirds were included, which resulted in 19 usable studies.

References

1 Piesman, J. & Gern, L. Lyme borreliosis in Europe and North America. *Parasitology* **129**, S191-S220 (2004).

2 Kurtenbach, K. *et al.* Fundamental processes in the evolutionary ecology of Lyme borreliosis. *Nat Rev Microbiol* **4** (2006).

3 van Duijvendijk, G., Sprong, H. & Takken, W. Multi-trophic interactions driving the transmission cycle of *Borrelia afzelii* between *Ixodes ricinus* and rodents: a review. *Parasites & Vectors* **8**, 1-11 (2015).

4 Hanincova, K. *et al.* Association of *Borrelia afzelii* with rodents in Europe. *Parasitology* **126**, 11-20 (2003).

5 Hu, C. M., Humair, P. F., Wallich, R. & Gern, L. *Apodemus sp*. rodents, reservoir hosts for *Borrelia afzelii* in an endemic area in Switzerland. *Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases* **285**, 558-564 (1997).

6 Humair, P. F., Rais, O. & Gern, L. Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance. *Parasitology* **118**, 33-42 (1999).

7 Kurtenbach, K. *et al.* Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. *Appl Environ Microbiol* **64**, 1169-1174 (1998).

8 Gern, L. *et al. Borrelia burgdorferi* in rodents (*Apodemus flavicollis* and *A. sylvaticus*): Duration and enhancement of infectivity for *Ixodes ricinus* ticks. *Eur J Epidemiol* **10**, 75-80 (1994).

9 Talleklint, L. & Jaenson, T. G. T. Is the small mammal (Clet*hrionomys glareolus*) or the tick vector (*Ixodes ricinus*) the primary overwintering reservoir for the Lyme borreliosis spirochete in Sweden. *J Wildl Dis* **31**, 537-540 (1995).

10 Richter, D., Klug, B., Spielman, A. & Matuschka, F. R. Adaptation of diverse Lyme disease spirochetes in a natural rodent reservoir host. *Infection and Immunity* **72**, 2442-2444 (2004).

11 Heylen, D., Matthysen, E., Fonville, M. & Sprong, H. Songbirds as general transmitters but selective amplifiers of *Borrelia burgdorferi* sensu lato genotypes in *Ixodes rinicus* ticks. *Environmental Microbiology* **16**, 2859-2868 (2014).

12 Kurtenbach, K. *et al.* Differential survival of Lyme borreliosis spirochetes in ticks that feed on birds. *Infection and Immunity* **70**, 5893-5895 (2002).

13 Matuschka, F. R. & Spielman, A. Loss of Lyme disease spirochetes from *Ixodes ricinus* ticks feeding on European blackbirds. *Exp Parasitol* **74**, 151-158 (1992).

14 Kurtenbach, K. *et al.* Host association of *Borrelia burgdorferi* sensu lato - the key role of host complement. *Trends Microbiol* **10**, 74-79 (2002).

15 Kurtenbach, K., Sewell, H. S., Ogden, N. H., Randolph, S. E. & Nuttall, P. A. Serum complement sensitivity as a key factor in Lyme disease ecology. *Infection and Immunity* **66**, 1248-1251 (1998).

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

17 Comstedt, P. *et al.* Migratory passerine birds as reservoirs of Lyme borreliosis in Europe. *Emerg Infect Dis* **12**, 1087-1095 (2006).

18 Dubska, L. *et al.* Synanthropic birds influence the distribution of *Borrelia* species: analysis of *Ixodes ricinus* ticks feeding on passerine birds. *Appl Environ Microbiol* **77**, 1115-1117 (2011).
19 Geller, J. *et al.* Tick-borne pathogens in ticks feeding on migratory passerines in western part of Estonia. *Vector-Borne and Zoonotic Diseases* **13**, 443-448 (2013).
20 Hanincova, K. *et al.* Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. *Appl Environ Microbiol* **69**, 2825-2830 (2003).
21 Hasle, G., Bjune, G. A., Midthjell, L., Roed, K. H. & Leinaas, H. P. Transport of *Ixodes ricinus* infected with *Borrelia* species to Norway by northward-migrating passerine birds. *Ticks and Tick-Borne Diseases* **2**, 37-43 (2011).
22 Kipp, S., Goedecke, A., Dorn, W., Wilske, B. & Fingerle, V. Role of birds in Thuringia, Germany, in the natural cycle of *Borrelia burgdorferi* sensu lato, the Lyme disease spirochaete. *International Journal of Medical Microbiology* **296**, 125-128 (2006).
23 Taragel'ová, V. *et al.* Blackbirds and song thrushes constitute a key reservoir of *Borrelia garinii*, the causative agent of borreliosis in Central Europe. *Appl Environ Microbiol* **74**, 1289-1293 (2008).
24 Richter, D., Debski, A., Hubalek, Z. & Matuschka, F. R. Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks. *Vector-Borne and Zoonotic Diseases* **12**, 21-27 (2012).
25 Rollend, L., Fish, D. & Childs, J. E. Transovarial transmission of *Borrelia* spirochetes by *Ixodes scapularis*: A summary of the literature and recent observations. *Ticks and Tick-borne Diseases* **4**, 46-51 (2013).
26 van Duijvendijk, G. *et al.* Larvae of *Ixodes ricinus* transmit *Borrelia afzelii* and *B. miyamotoi* to vertebrate hosts. *Parasites & Vectors* **9** (2016).
27 Gern, L. & Rais, O. Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). *J Med Entomol* **33**, 189-192 (1996).
28 Randolph, S. E., Gern, L. & Nuttall, P. A. Co-feeding ticks: epidemiological significance for tick-borne pathogen transmission. *Parasitol Today* **12**, 472-479 (1996).
29 Voordouw, M. J. Co-feeding transmission in Lyme disease pathogens. *Parasitology* **142**, 290-302 (2015).
30 Hu, C. M. *et al.* Early detection of *Borrelia burgdorferi* sensu lato infection in Balb/c mice by co-feeding *Ixodes ricinus* ticks. *International Journal of Medical Microbiology* **293**, 421-426 (2003).
31 Jacquet, M., Durand, J., Rais, O. & Voordouw, M. J. Strain-specific antibodies reduce co-feeding transmission of the Lyme disease pathogen, *Borrelia afzelii*. *Environmental Microbiology* **18**, 833-845 (2016).
32 Richter, D., Allgower, R. & Matuschka, F. R. Co-feeding transmission and its contribution to the perpetuation of the Lyme disease spirochete *Borrelia afzelii*. *Emerg Infect Dis* **8**, 1421-1425 (2002).
33 Tonetti, N., Voordouw, M. J., Durand, J., Monnier, S. & Gern, L. Genetic variation in transmission success of the Lyme borreliosis pathogen *Borrelia afzelii*. *Ticks and Tick-borne Diseases* **6**, 334-343 (2015).
34 Gern, L. *et al.* European reservoir hosts of *Borrelia burgdorferi* sensu lato. *Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases* **287**, 196-204 (1998).
35 Kimura, K. *et al.* Detection of Lyme disease spirochetes in the skin of naturally infected wild sika deer (*Cervus nippon yesoensis*) by PCR. *Appl Environ Microbiol* **61**, 1641-1642 (1995).

36 Ogden, N. H., Nuttall, P. A. & Randolph, S. E. Natural Lyme disease cycles maintained via sheep by cofeeding ticks. *Parasitology* **115**, 591-599 (1997).

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

38 Margos, G. *et al. Borrelia bavariensis* sp nov is widely distributed in Europe and Asia. *Int J Syst Evol Microbiol* **63**, 4284-4288 (2013).

39 Humair, P. F., Postic, D., Wallich, R. & Gern, L. An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes. *Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases* **287**, 521-538 (1998).

40 Norte, A. C., de Carvalho, I. L., Nuncio, M. S., Ramos, J. A. & Gern, L. Blackbirds *Turdus merula* as competent reservoirs for *Borrelia turdi* and *Borrelia valaisiana* in Portugal: evidence from a xenodiagnostic experiment. *Environmental Microbiology Reports* **5**, 604-607 (2013).

41 Harrison, A. & Bennett, N. The importance of the aggregation of ticks on small mammal hosts for the establishment and persistence of tick-borne pathogens: an investigation using the R(0) model. *Parasitology* **139**, 1605-1613 (2012).

42 Harrison, A., Montgomery, W. I. & Bown, K. J. Investigating the persistence of tick-borne pathogens via the R-0 model. *Parasitology* **138**, 896-905 (2011).

43 Hartemink, N. A., Randolph, S. E., Davis, S. A. & Heesterbeek, J. A. P. The basic reproduction number for complex disease systems: Defining R-0 for tick-borne infections. *Am Nat* **171**, 743-754 (2008).

44 Ginsberg, H. S. *et al.* Reservoir competence of native north American birds for the Lyme disease spirochete, *Borrelia burgdorferi*. *J Med Entomol* **42**, 445-449 (2005).

45 Mather, T. N., Telford, S. R., Maclachlan, A. B. & Spielman, A. Incompetence of catbirds as reservoirs for the Lyme disease spirochete (*Borrelia burgdorferi*). *J Parasitol* **75**, 66-69 (1989).

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

47 Morán Cadenas, F. *et al.* A comparison of two DNA extraction approaches in the detection of *Borrelia burgdorferi* sensu lato from live *Ixodes ricinus* ticks by PCR and reverse line blotting. *Vector-Borne and Zoonotic Diseases* **7**, 555-561 (2007).

48 Humair, P. F. & Gern, L. Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland. *Acta Trop* **69**, 213-227 (1998).

49 Humair, P. F., Péter, O., Wallich, R. & Gern, L. Strain variation of Lyme disease spirochetes isolated from *Ixodes ricinus* ticks and rodents collected in two endemic areas in Switzerland. *J Med Entomol* **32**, 433-438 (1995).

50 Norte, A. C., Ramos, J. A., Gern, L., Nuncio, M. S. & Lopes de Carvalho, I. Birds as reservoirs for *Borrelia burgdorferi* s.l. in Western Europe: circulation of *B. turdi* and other genospecies in bird-tick cycles in Portugal. *Environmental Microbiology* **15**, 386-397 (2013).

51 Poupon, M. A. *et al.* Prevalence of *Borrelia burgdorferi* sensu lato in ticks collected from migratory birds in Switzerland. *Appl Environ Microbiol* **72**, 976-979 (2006).

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

53 Herrmann, C., Gern, L. & Voordouw, M. Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*. *Appl Environ Microbiol* **79**, 7273-7280 (2013).

54 Kurtenbach, K. *et al.* Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies found in individual questing ticks from Europe. *Appl Environ Microbiol* **67**, 4926-4929 (2001).

55 Rauter, C. & Hartung, T. Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis. *Appl Environ Microbiol* **71**, 7203-7216 (2005).

56 Durand, J. *et al.* Cross-immunity and community structure of a multiple-strain pathogen in the tick vector. *Appl Environ Microbiol* **81**, 7740-7752 (2015).

57 Hasle, G. Transport of ixodid ticks and tick-borne pathogens by migratory birds. *Frontiers in Cellular and Infection Microbiology* **3** (2013).

58 Vollmer, S. A. *et al.* Host migration impacts on the phylogeography of Lyme Borreliosis spirochaete species in Europe. *Environmental Microbiology* **13**, 184-192 (2011).

59 Vollmer, S. A. *et al.* Spatial spread and demographic expansion of Lyme borreliosis spirochaetes in Eurasia. *Infect Genet Evol* **14**, 147-155 (2013).

60 Franke, J., Moldenhauer, A., Hildebrandt, A. & Dorn, W. Are birds reservoir hosts for *Borrelia afzelii*? *Ticks and Tick-borne Diseases* **1**, 109-112 (2010).

61 Humair, P.-F. *et al.* Molecular identification of bloodmeal source in *Ixodes ricinus* ticks using 12S rDNA as a genetic marker. *J Med Entomol* **44**, 869-880 (2007).

62 Morán Cadenas, F. M. *et al.* Identification of host bloodmeal source and *Borrelia burgdorferi* sensu lato in field-collected *Ixodes ricinus* ticks in Chaumont (Switzerland). *J Med Entomol* **44**, 1109-1117 (2007).

63 Piesman, J. Experimental acquisition of the Lyme disease spirochete, *Borrelia burgdorferi*, by larval *Ixodes dammini* (Acari, Ixodidae) during partial blood meals. *J Med Entomol* **28**, 259-262 (1991).

64 Pérez, D., Kneubühler, Y., Rais, O., Jouda, F. & Gern, L. *Borrelia afzelii ospC* genotype diversity in *Ixodes ricinus* questing ticks and ticks from rodents in two Lyme borreliosis endemic areas: Contribution of co-feeding ticks. *Ticks and Tick-borne Diseases* **2**, 137-142 (2011).

65 Andersson, M., Scherman, K. & Raberg, L. Multiple-strain infections of *Borrelia afzelii*: a role for within-host interactions in the maintenance of antigenic diversity? *American Naturalist, The* **181**, 545-554 (2013).

66 Hellgren, O., Andersson, M. & Raberg, L. The genetic structure of *Borrelia afzelii* varies with geographic but not ecological sampling scale. *J Evol Biol* **24**, 159-167 (2011).

67 Strandh, M. & Raberg, L. Within-host competition between *Borrelia afzelii ospC* strains in wild hosts as revealed by massively parallel amplicon sequencing. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **370** (2015).

68 Marsot, M. *et al.* 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 (2012).

69 Norte, A. C. *et al.* Do ticks and *Borrelia burgdorferi* s.l. constitute a burden to birds? *Parasitol Res* **112**, 1903-1912 (2013).

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

71 Heylen, D. J. A. & Matthysen, E. Effect of tick parasitism on the health status of a passerine bird. *Funct Ecol* **22**, 1099-1107 (2008).
72 Dubska, L., Literak, I., Kocianova, E., Taragelova, V. & Sychra, O. 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 (2009).
73 Heylen, D., Fonville, M., van Leeuwen, A. D. & Sprong, H. Co-infections and transmission dynamics in a tick-borne bacterium community exposed to songbirds. *Environmental Microbiology* **18**, 988-996 (2016).
74 Heylen, D., Tijsse, E., Fonville, M., Matthysen, E. & Sprong, H. Transmission dynamics of *Borrelia burgdorferi* s.l. in a bird tick community. *Environmental Microbiology* **15**, 663-673 (2013).
75 Drent, P. J., van Oers, K. & van Noordwijk, A. J. Realized heritability of personalities in the great tit (*Parus major*). *Proceedings of the Royal Society B-Biological Sciences* **270**, 45-51 (2003).
76 Jacquet, M., Durand, J., Rais, O. & Voordouw, M. J. Cross-reactive acquired immunity influences transmission success of the Lyme disease pathogen, *Borrelia afzelii*. *Infection, Genetics and Evolution* **36**, 131-140 (2015).
77 Sinsky, R. J. & Piesman, J. Ear punch biopsy method for detection and isolation of *Borrelia burgdorferi* from rodents. *Journal of Clinical Microbiology* **27**, 1723-1727 (1989).
78 Gooskens, J., Templeton, K. E., Claas, E. C. & van Dam, A. P. Evaluation of an internally controlled real-time PCR targeting the *ospA* gene for detection of *Borrelia burgdorferi* sensu lato DNA in cerebrospinal fluid. *Clinical Microbiology and Infection* **12**, 894-900 (2006).
79 Schwaiger, M., Peter, O. & Cassinotti, P. Routine diagnosis of *Borrelia burgdorferi* (sensu lato) infections using a real-time PCR assay. *Clinical Microbiology and Infection* **7**, 461-469 (2001).
80 Wang, I. N. *et al.* Genetic diversity of *ospC* in a local population of *Borrelia burgdorferi* sensu stricto. *Genetics* **151**, 15-30 (1999).
81 Hofmeester, T. R. *et al.* Few vertebrate species dominate the *Borrelia burgdorferi* s.l. life cycle. *Environmental Research Letters* **11** (2016).
82 Kjelland, V., Stuen, S., Skarpaas, T. & Slettan, A. *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected from migratory birds in Southern Norway. *Acta Vet Scand* **52** (2010).

Acknowledgments

Thanks to Tom Fluri, Gaétan Pheulpin, Anouk Sarr, and Olivier Rais for helping to create the *B. afzelii*-infected *I. ricinus* nymphs. This research was supported by the Fund for Scientific Research - Flanders Belgium (FWO) (grant G0.049.10) and the University of Antwerp (KP BOF UA 2015). Dieter Heylen is a postdoctoral fellow at the FWO. This work

was also supported by a Swiss National Science Foundation grant to Maarten J. Voordouw (FN 31003A_141153). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

D.H., M.J.V. and H.S. conceived and designed the study. D.H., A.K. and N.V. performed the experiments. D.G. and A.G.-C. created the *B. afzelii*-infected nymphal ticks. M.J.V., H.S., K.V. and D.H. provided funding. D.H., H.S., and M.J.V. wrote the manuscript. All authors reviewed the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

Supplementary information accompanies this paper at http://www.nature.com/srep

599

Figure legends

601

602 **Figure 1.** Naïve *I. ricinus* larvae co-feed with *B. afzelii*-infected nymphs on the head of a
603 great tit. The larvae (small) and nymphs (large) were placed underneath the crown-feathers on
604 the right side of the head (A: lateral; B: frontal view). By feeding in close spatial and temporal
605 proximity, the *B. afzelii* spirochetes can migrate directly from the infected nymphs to the
606 naïve larvae via co-feeding transmission. Dr. Frank Adriaensen took the photos.

607

608

609

610 **Table 1.** *Borrelia afzelii* infections have been found in *Ixodes ricinus* larvae and nymphs feeding on many different species of birds. Data are

611 from a literature search that included 19 publications that report on *Borrelia* genospecies in bird-derived ticks.

612

	***Ixodes ricinus* larvae**				***Ixodes ricinus* nymphs**			
Bird Species	**# studies reporting *B. afzelii* infections**	**# birds tested**	**# ticks tested**	**# infected ticks**	**# studies reporting *B. afzelii* infections**	**# birds tested**	**# ticks tested**	**# infected ticks**
Anthus trivialis					1 ([52])	120	85	4
Carduelis cabaret					1 ([21])	**	5	1
Carduelis chloris					1 ([18])	1	3	1
Coccothraustes coccothraustes					1 ([52])	2	2	1
Erithacus rubecula	2 ([51,60])	124	38*	8	5 ([18,21,60,72,82])	316	366	11
Fringilla coelebs	1 ([52])	37	42	1	2 ([18,52])	52	50	6
Locustella naevia					1 ([72])	2	5	1
Motacilla cinerea	1 ([72])	3	1	1	1 ([72])	3	9	2
Parus major	2 ([72,74])	187	266	3	4 ([18,19,72,74])	220	403	15
Phoenicurus phoenicurus					1 ([21])	**	38	1
Phylloscopus trochilus					1 ([21])	**	37	2
Prunella modularis					5 ([18,21,23,72,82])	87	430	27
Saxicola rubetra					1 ([21])	**	2	1
Sylvia atricapilla					1 ([23])	16	18	1
Sylvia communis					2 ([52,72])	12	13	4
Sylvia curruca					1 ([21])	**	22	2
Troglodytes troglodytes	1 ([82])	4	5	1				
Turdus iliacus	1 ([60])	19	4	1	2 ([52,60])	28	60	5
Turdus merula	1 ([60])	11	2	1	7 ([18,21-23,52,60,72])	141	1009	35
Turdus philomelos					6 ([18,21,23,51,52,72])	131	436	11
Turdus viscivorus					1 ([52])	2	2	1

* One study did not report on the total number of larvae that were screened, therefore this number is an under-estimation

** Study did not report on the total number of captured birds

613

Table 2. *Borrelia afzelii* infection status is shown for the *Ixodes ricinus* ticks that had co-fed on two species of songbird, the blackbird (*Turdus merula*) and the great tit (*Parus major*). The blood-engorged nymphs and larvae were either placed in ethanol following drop-off or allowed to moult into the next stage (adult and nymph, respectively). All engorged and moulted ticks were screened for *B. afzelii* infection using qPCR. Adult ticks were also cultured in BSKII-medium to test for nymph-to-adult transtadial transmission of viable *B. afzelii* spirochetes.

		Nymphs			***Larvae***	
		Engorged	***Moulted***	***Attached*****	***Engorged***	***Moulted***
Species	Bird N°	*infect./total*	*infect./total*		*infect./total*	*infect./total*
T. merula	1 - ♂	N.A.	0/2	9	0/10	0/7
T. merula	2 - ♀	N.A.	4/5	12	0/10	0/15
T. merula	3 - ♀	N.A.	0/4	11	0/9	0/6
T. merula	4 - ♂	N.A.	2/6	12	0/10	0/23
P. major	1 - ♀	2/2	0/1*	4	0/14	N.A.
P. major	2 - ♀	2/2	1/1*	8	0/24	0/9
P. major	3 - ♂	5/5	3/3*	10	2/22	0/2
P. major	4 - ♂	2/2	N.A.	6	1/16	0/3

* Engorged nymphs were allowed to moult into adult ticks and were cut in half. One half was screened for *B. afzelii* using qPCR and the other half was cultured in BSK II-medium to test for viable spirochetes. None of them yielded spirochete cultures; therefore *B. afzelii* is not capable of transstadial transmission in the presence of bird blood.

** Attached = total number of nymphs placed on the bird minus the number of nymphs left in the bag.