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Transmission dynamics of *Borrelia burgdorferi* s.l. in a bird tick community

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Summary

We examined the *Borrelia burgdorferi* sensu lato circulation in a tick community consisting of three species (*Ixodes ricinus*, *I. frontalis*, *I. arboricola*) with contrasting ecologies, but sharing two European songbird hosts (*Parus major* and *Cyanistes caeruleus*). *Parus major* had the highest infestation rates, primarily due to larger numbers of *I. ricinus*, and probably because of their greater low-level foraging. The prevalence of *Borrelia* in feeding ticks did not significantly differ between the two bird species; however, *P. major* in particular hosted large numbers of *Borrelia*-infected *I. frontalis* and *I. ricinus* larvae, suggesting that the species facilitates *Borrelia* transmission. The low but significant numbers of *Borrelia* in questing *I. arboricola* ticks also provides the first field data to suggest that it is competent in maintaining *Borrelia*. Aside from *Borrelia garinii*, a high number of less dominant genospecies was observed, including several mammalian genospecies and the first record of *Borrelia turdi* for North-Western Europe. *Borrelia burgdorferi* sensu lato IGS genotypes were shared between *I. arboricola* and *I. ricinus* and between *I. frontalis* and *I. ricinus,* but not between *I. arboricola* and *I. frontalis*. This suggests that the *Borrelia* spp. transmission cycles can be maintained by bird-specific ticks, and bridged by *I. ricinus* to other hosts outside bird-tick cycles.

Introduction

Research on parasite communities and the evolution of host–parasite interactions is becoming an important focus in the fields of parasitology and epidemiology. In addition to the consequences of co-occurring parasites on both the hosts' and parasites' fitness (Frank, 1996; West and Buckling, 2003; Krasnov *et al*., 2005; Bell *et al*., 2006), there is great interest in understanding of the transmission of diseases that are vectored by macro-parasites. In many of these pathogens, multiple vectors can participate in transmission but may contribute differently to disease ecology and evolution (Bown *et al*., 2008; Bataille *et al*., 2009; Gomez-Diaz *et al*., 2010; Farajollahi *et al*., 2011).

In the present study we investigate to what extent a community of ixodid ticks in European resident songbirds contributes to the transmission dynamics of tick-borne bacteria belonging to the *Borrelia burgdorferi* sensu lato complex. At least seven genospecies of this complex cause Lyme borreliosis in humans (Franke *et al*., 2010 and references herein), the leading vector-borne disease in Europe and North America. The role of songbirds as reservoir hosts for *Borrelia* spirochaetes has been recognized for many years (Humair, 2002). Birds can have a significant role in the dispersal and sustainment of populations of *Borrelia* bacteria (Comstedt *et al*., 2006), and they are considered to sustain some specialist ornithophilic tick populations (McCoy *et al*., 2001; Heylen and Matthysen, 2010) and diseases that can be found in close proximity to humans (Gray *et al*., 1999). It is also well documented that numerous species of passerine birds are infested with more than one tick species (Hillyard, 1996; Spitalska *et al*., 2011); however, little is known about the implications of multiple tick infestations on the flow of tick-borne pathogens.

The vast majority of human Lyme borreliosis cases in Europe are transmitted by the nymphal stage of *Ixodes ricinus*. *Ixodes ricinus* is a widespread forest-dwelling, exophilic tick, and has been recognized as a key vector of numerous human and/or animal pathogens, including bacteria (e.g. *B. burgdorferi* sensu lato, *Rickettsia* spp.), viruses (e.g. tick-borne encephalitis virus) and protozoans (e.g. *Babesia* sp.) (Gray, 1991; Jongejan and Uilenberg, 2004). This generalist tick infests a broad range of vertebrate hosts, including birds and humans. The enzootic maintenance of the pathogen *B. burgdorferi* s.l. by *I. ricinus* and mammals is well documented, whereas its more cryptic maintenance by specialist ticks (i.e. with

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a narrow host range) and wildlife hosts remains largely unexplored (cf. *Ixodes scapularis* and *I. dentatus*, Hamer *et al*., 2011). Here, we examine the enzootic cycles of *B. burgdorferi* s.l. in a tick community which consists of three species: *Ixodes arboricola* (Schulze and Schlottke), *I. frontalis* (Panzer) and *I. ricinus*. These three species have contrasting ecologies and off-host habitats, but share two common songbird hosts: the great tit (*Parus major*) and the blue tit (*Cyanistes caeruleus*). *Parus major* and *C. caeruleus* are two of the most common resident birds of European gardens and woodlands (Gosler, 1993; Liebisch, 1996) and are highly infested with ixodid ticks (Heylen *et al*., 2010; 2012; Heylen and Matthysen, 2010). Both *P. major* and *C. caeruleus* breed and roost in natural tree-holes, but readily accept nest boxes as surrogates as well. The entire life cycle of *I. arboricola* is restricted to these natural tree-holes and nest boxes, where it infests roosting or breeding birds (Arthur, 1963; Hudde and Walter, 1988; Hillyard, 1996). *Ixodes frontalis* is considered as an ornithophilic tick of terrestrial ecosystems, which has been found inside bird nests (Hillyard, 1996).

All ixodid ticks have a three-stage life cycle (larva, nymph and adult), with ticks feeding only once during each developmental stage. Transmission of *Borrelia* spirochaetes via the host generally depends on the development of a systemic infection in the host. As the uninfected ticks feed on an infected host, the pathogens are taken up in the bloodmeal. However, transmission may also occur in the absence of a systemic infection, between infected and uninfected ticks co-feeding in close proximity on the same hosts (Randolph *et al*., 1996). *Borrelia* spirochaetes are rarely, if at all, transmitted transovarially (i.e. from mother tick to offspring) (Richter *et al*., 2012). Therefore, one may assume that the presence of infected larvae on a host supports the conclusion that this host transmitted the *Borrelia* to the larvae (Dubska *et al*., 2009) either by systemic infection or by co-feeding transmission.

The main objective of our study is to investigate whether the above tick-songbird system contributes to the local dynamics of *Borrelia* spirochaetes in woodland ecosystems, by quantifying the birds' infestation levels and the *Borrelia* infections in ticks isolated from birds and their nesting sites. These data will provide important information on the capacity of the ticks and songbirds to acquire and maintain *Borrelia* spirochaetes in the wild. Second, by comparing the *B. burgdorferi* sensu lato IGS genotypes diversity among the three tick species, we will evaluate to what extent the *Borrelia* exposure and transmission overlap in the tick community, and hence whether *Borrelia* cycles of the bird-specialized ticks are intruded by *I. ricinus*.

Results

Tick infestations

The prevalence of tick infestation in free living *P. major* was 25.4% (mean infestation: 0.86 ± 0.04; range: 1–56; $n = 5116$ birds), while only 7.9% *C. caeruleus* were infested (mean infestation: 0.20 ± 0.03; range: 1–48; $n = 2702$ birds). Identification of 2631 ticks isolated from 710 birds (Table 1) revealed that in both bird species *I. ricinus* is the commonest (prevalence of infestation in *P. major*: 21.5%; *C. caeruleus*: 3.9%), *I. arboricola* the second commonest (*P. major*: 2.9%; *C. caeruleus*: 3.1%) and *I. frontalis* the least common (*P. major*: 2.1%; *C. caeruleus*: 0.8%). The prevalence of *I. ricinus* infestation (logit: 1.90 ± 0.15; $Z = 170.8$; $P < 0.001$) and *I. frontalis* (logit: 1.00 ± 0.34; $Z = 8.9$; $P = 0.003$) were significantly higher in *P. major* compared with *C. caeruleus*, while the prevalence of *I. arboricola* infestation did not differ between the two bird species (logit: 0.09 ± 0.19; $Z = 0.3$; $P = 0.61$). In *I. ricinus*, only immature stages (larvae and nymphs) were detected. In contrast, in the two bird-specialized ticks also adult females parasitized the birds (Tables 1 and 2). *Parus major* had a significantly higher relative proportion of *I. ricinus* larvae that co-fed with *I. ricinus* nymphs (25.8%; logit: 2.50 ± 0.73; $Z = 11.8$; $P < 0.001$; Table 1). Neither in *I. arboricola* ($\chi^2 = 0.009$; d.f. = 1; $P = 0.9$) nor in *I. frontalis* (exact Fisher, $P = 1$) there was a significant host difference in the relative proportion of co-feeding ticks of different developmental stages. The relative proportion of mixed infestations with different tick species did not significantly differ between *P. major* (5.5%) and *C. caeruleus* (1.8%; exact Fisher; $P = 0.15$).

Table 1. Prevalence of infestation by *Ixodes* species in *Parus major* ($n = 598$ captured birds) and *Cyanistes caeruleus* ($n = 112$ captured birds) and the per cent distribution of infestations with either a single tick developmental stage (larva, nymph, adult female) or a mix of different developmental stages.

	Parus major								*Cyanistes caeruleus*							
	Prev.	L	N	A	L&N	L&A	N&A	L&N&A	Prev.	L	N	A	L&N	L&A	N&A	L&N&A
Ixodes ricinus	21.5	49.2	25.0	0	25.8	0	0	0	3.9	31.1	68.1	0	0.8	0	0	0
Ixodes arboricola	2.9	47.8	37.3	4.5	10.4	0	1.5	0	3.1	50	38.6	0	4.5	4.5	0	2.3
Ixodes frontalis	2.1	53.1	34.7	6.1	6.1	2.0	0	0	0.8	72.7	27.3	0	0	0	9.1	0

Prev. = prevalence; developmental stages: L = larva, N = nymph, A = adult female; &: co-feeding ticks of different developmental stages.

Table 2. *Borrelia* prevalence in screened ticks collected from *Parus major* and *Cyanistes caeruleus*.

		Parus major				*Cyanistes caeruleus*			
		Total	L	N	A	Total	L	N	A
Ixodes sp.	No. of screened ticks (infested birds)	563 (178)	173 (88)	383 (137)	7 (7)	77 (49)	17 (13)	60 (37)	0 (0)
	Prevalence *Borrelia*-infected ticks (infected birds) %	19.9 (30.3)	16.7 (26.1)	21.4 (23.7)	14.3 (14.3)	16.9 (16.3)	5.9 (7.7)	20 (21.6)	0 (0)
Ixodes ricinus	No. of screened ticks (infested birds)	450 (147)	131 (70)	319 (125)	0 (0)	40 (34)	9 (7)	31 (28)	0 (0)
	Prevalence *Borrelia*-infected ticks (infected birds) %	20.9 (29.9)	17.6 (27.1)	22.3 (28.0)	0 (0)	15.0 (14.7)	11.1 (14.3)	16.1 (17.9)	0 (0)
Ixodes arboricola	No. of screened ticks (infested birds)	80 (22)	18 (8)	57 (12)	5 (5)	36 (15)	7 (5)	29 (10)	0 (0)
	Prevalence *Borrelia*-infected ticks (infected birds) %	10.0 (18.2)	0 (0)	14.0 (33.3)	0 (0)	19.4 (20)	0 (0)	13.8 (10.0)	0 (0)
Ixodes frontalis	No. of screened ticks (infested birds)	33 (22)	24 (14)	7 (7)	2 (2)	1 (1)	1 (1)	0 (0)	0 (0)
	Prevalence *Borrelia*-infected ticks (infected birds) %	30.3 (36.4)	25.0 (28.6)	42.9 (42.9)	50.0 (50.0)	0 (0)	0 (0)	0 (0)	0 (0)

Developmental stages: L = larva; N = nymph; A = adult female.

In 5% of the 450 inspected nest boxes we found *I. arboricola* (range: 1–450 ticks per nest box). Only in 1% of nest boxes we observed *I. frontalis* (one to two adult females per nest box). No off-host *I. ricinus* ticks were found inside these nest boxes.

Borrelia *infections in ticks isolated from birds*

In total, 640 ticks collected from birds were screened for *Borrelia*. The overall proportion of infested birds hosting *Borrelia*-infected ticks was higher in *P. major* (30.3%, $n = 178$ birds) compared with *C. caeruleus* (16.3%, $n = 49$ birds; logit: 0.78 ± 0.41; $Z = 3.9$; $P = 0.048$). The *Borrelia* spp. infection in ticks did not statistically differ between *P. major* (19.9%, $n = 563$; Table 2) and *C. caeruleus* (16.9%, $n = 77$) when controlling for tick species and tick instar (logit: -0.11 ± 0.40; $Z = -0.3$; $P = 0.8$). *Borrelia* was detected in all tick species (Table 2). *Borrelia* prevalence in *I. arboricola* (18.2%, $n = 80$) was significantly lower than in *I. frontalis* (36.4%; $n = 33$; logit: -1.78 ± 0.63; $Z = -2.8$; $P = 0.004$) and tended to be lower than in *I. ricinus* (29.9%, $n = 450$; logit: -0.97 ± 0.54; $Z = -1.8$; $P = 0.07$) when controlling for tick instar and bird species. The *Borrelia* prevalence in *I. frontalis* tended to be higher than in *I. ricinus* (logit: 0.81 ± 0.45; $Z = 1.8$; $P = 0.07$).

In *P. major*, 29 out of 173 larvae (16.8%) were found to be *Borrelia*-infected, all belonging to *I. frontalis* (infection rate: 25.0%) and *I. ricinus* (17.6%). In *C. caeruleus* we observed one *Borrelia*-infected *I. ricinus* larva out of 9 larvae (11.1%). No *Borrelia*-infected *I. arboricola* larvae were isolated from the birds.

Borrelia *infections in ticks isolated from nest boxes*

In total, a sample of 142 *I. arboricola* ticks collected from nest boxes was screened for *Borrelia*. We found a significant number of *Borrelia*-infected *I. arboricola* ticks among the unfed nymphs (17.7%) and adults (10.7%; Table 3). This contrasts with the unfed *I. arboricola* larvae, in which no *Borrelia*-infected ticks were observed (40 individuals

Table 3. *Borrelia* prevalence in *Ixodes arboricola* ticks found inside nest boxes.

	Engorged				Questing			
	Total	L	N	A	Total	L	N	A
No. of screened ticks (infested boxes)	97 (47)	61 (30)	31 (17)	5 (3)	85 (18)	40 (5)	17 (6)	28 (13)
Prevalence *Borrelia*-infected ticks (infected boxes) %	4.1 (8.5)	1.6 (3.3)	9.7 (17.6)	0 (0)	7.1 (27.8)	0 (0)	17.7 (33.3)	10.7 (23.1)

Developmental stages: L = larva; N = nymph; A = adult female.

Table 4. Distribution of *Borrelia* genospecies in *Ixodes ricinus*, *I. arboricola* and *I. frontalis*.

	Ixodes ricinus				*Ixodes arboricola*				*Ixodes frontalis*			
	Total	L	N	A	Total	L	N	A	Total	L	N	A
Borrelia turdi-like	0 (0%)	0	0	–	0 (0%)	0	0	0	5 (55.6%)	1	3	1
Borrelia afzelii	5 (10%)	1	4	–	2 (18.2%)	0	1	1	1 (11.1%)	1	0	0
Borrelia garinii	36 (72%)	7	29	–	7 (63.6%)	0	6	1	3 (33.3%)	3	0	0
Borrelia gariniibavariensis	1 (2%)	0	1	–	0 (0%)	0	0	0	0 (0%)	0	0	0
Borrelia sensu stricto	3 (6%)	3	0	–	0 (0%)	0	0	0	0 (0%)	0	0	0
Borrelia spielmanii	2 (4%)	1	1	–	1 (9.1%)	0	1	0	0 (0%)	0	0	0
Borrelia valaisiana	3 (6%)	0	3	–	1 (9.1%)	1	0	0	0 (0%)	0	0	0

Developmental stages: L = larva; N = nymph; A = adult female.

from five different boxes). Of the 97 ticks that had recently engorged and detached inside the nest box, four tested positive (one larva and three nymphs).

Borrelia *genospecies and IGS genotype distribution*

Overall, six known *Borrelia* genospecies were detected (Table 4) using IGS-based genotyping. In *I. ricinus* ($n = 55$) all genospecies were found and four of them were also detected in *I. arboricola* ($n = 16$). In the nine infected *I. frontalis* ticks *Borrelia afzelii, B. garinii* and a potentially new genospecies for North-Western Europe were detected. Further phylogenetic comparison based on concatenated sequences of the MLST housekeeping genes, showed that the discovered *Borrelia* (IF 412, 413 and 414) was similar but not identical to the previously described *Borrelia turdi* (Ya501) (Fig. 1) (Fukunaga *et al*., 1996). Despite several attempts, the *clpA* sequences of the *B. turdi*-like isolates (IF 412, 413 and 414) could not be amplified.

Detailed analyses of the 5S-23S IGS revealed that the six genospecies could be subdivided in 24 IGS genotypes. *Ixodes ricinus* shared six IGS genotypes with *I. arboricola* (belonging to *B. garinii*, *B. valaisiana* and *B. afzelii*), and only two IGS genotypes with *I. frontalis* (belonging to *B. garinii*) (Fig. 2). Remarkably, *I. frontalis* did not share any IGS genotype with *I. arboricola*. In ticks of *C. caeruleus* we detected two IGS genotypes (belonging to *B. garinii*) while all except one IGS genotype (belonging to *B. valaisiana*) were found in ticks isolated from *P. major.* This last IGS genotype was found in an *I. arboricola* collected from a nest box.

In co-feeding ticks with identified genospecies (38 ticks isolated from 12 birds) we found one group with mixed *Ixodes* species, which had fed on *P. major*. In this group, one *I. arboricola* nymph and a mix of immature *I. ricinus*

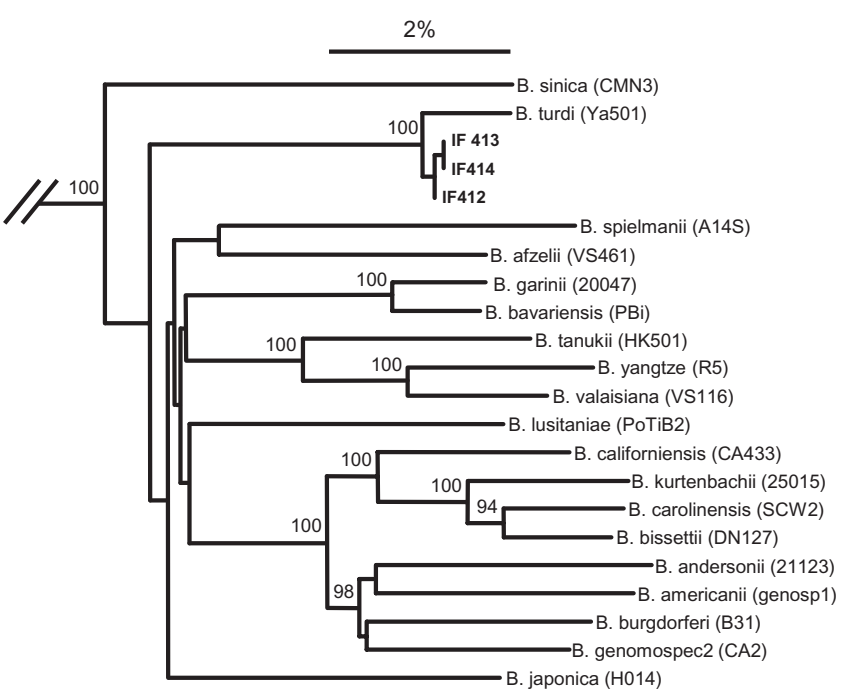

Fig. 1. A neighbour joining tree with Jukes & Cantor correction was generated using the concatenated sequences of MLST housekeeping genes from references strains and from the three *Borrelia* strains (IF 412–413) isolated from *I. frontalis*. Bar shows 2% divergence. *Borrelia hermsii* and *B. turicatae* were used as outgroup. Branch confidence values were calculated using a bootstrap procedure with 500 replicates. Values > 90 are indicated.

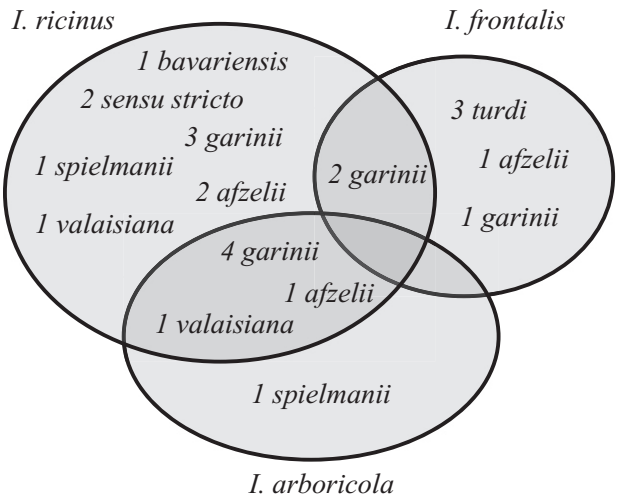

Fig. 2. Venn diagram of *Borrelia* IGS genotypes found in the three ixodid species (number of screened ticks: *I. ricinus* = 490, *I. arboricola* = 258 and *I. frontalis* = 34). IGS genotypes in the overlap areas are shared, whereas IGS genotypes outside the overlap areas are exclusive of a single tick species.

ticks ($n = 10$) shared the same IGS genotype (*B. garinii*). Of all the ticks that were infected with mammalian genospecies (*B. afzelii*, *B. sensu stricto*, *B. spielmanii* and *bavariensis*) none was observed that co-fed with other ticks sharing these genospecies.

Discussion

The purpose of our study was to investigate enzootic cycles of *Borrelia* in a system involving two resident songbirds (*P. major* and *C. caeruleus*) and a tick community consisting of three species (*I. arboricola*, *I. frontalis* and *I. ricinus*). Although these tick species have different off-host habitats and ecologies, during their parasitic phase they share the same hole-breeding songbirds. We found that *P. major* had the highest *Ixodes* infestations. Although the infection rate of ticks did not differ between the two bird species, the proportion of *P. major* with infected ticks was higher compared with *C. caeruleus*. Furthermore, *P. major* hosted more *Borrelia*-infected *Ixodes* larvae. The *Borrelia* prevalence was significantly lower in *I. arboricola* compared with the other tick species. A high diversity of *Borrelia* IGS genotypes was observed, including a unique *Borrelia* type similar to *B. turdi*.

The comparison of tick infestation levels between the two bird species confirms to some degree our expectations, but also adds new insights to our current knowledge on tick ecology. Infestation levels of the tree-hole inhabiting *I. arboricola* did not differ among the bird species, which makes sense because both use cavities for roosting and breeding to the same degree (Perrins, 1979). The strongest infestation differences were found in *I. ricinus*, the field tick that is associated with the humid understorey vegetation and the decaying ground layer of forests (Kahl and Knülle, 1988; Gray, 1991; Mejlon and Jaenson, 1997). We found that *P. major* was more often infested with *I. ricinus* than was *C. caeruleus*. This pattern has been observed in other European populations (Humair *et al*., 1993; Olsen *et al*., 1995; Hubalek *et al*., 1996; Comstedt *et al*., 2006; Kipp *et al*., 2006), and is likely because *P. major* is more inclined than *C. caeruleus* to forage at low heights inside the habitat of *I. ricinus* (Hartley, 1953; Betts, 1955; Gosler, 1987). Indeed, since neither *P. major* nor *C. caeruleus* acquire detectable immunological resistance against *I. ricinus* (Heylen *et al*., 2009), differences in *Ixodes* infestation levels are, as in other bird species, most likely driven by tick contact rates (Comstedt *et al*., 2006). Furthermore, the *I. ricinus* larvae/nymph ratio was significantly higher in *P. major* than in *C. caeruleus* (Table 1); larvae quest closer to the ground than nymphs (Mejlon and Jaenson, 1997). Interestingly, the infestation levels of *I. frontalis* were also higher in *P. major*, although this difference was less prominent than for *I. ricinus*. This result, in combination with the very low incidence of *I. frontalis* in nest boxes, suggests that *I. frontalis* is not closely associated with bird nests, but, similar to *I. ricinus,* occurs in the under-storey vegetation and ground layer. This conclusion is supported by anecdotal reports on captures of *I. frontalis* ticks by flagging under-storey vegetation (Gilot *et al*., 1997; Doby, 1998; Schorn *et al*., 2011) (personal communications of Maxime Madder and Fedor Gassner on the collection of adult female ticks during winter and spring in Belgium and the Netherlands; Estrada-Peña on the collection of immature developmental stages in Spain).

Overall, the *Borrelia* prevalence was high, emphasizing the role of birds in the *Borrelia* spp. transmission cycles in public woodlands situated within densely populated areas (Robertson *et al*., 2000; Pichon *et al*., 2005). The *Borrelia* prevalence in ticks from *P. major* was much higher than in questing *I. ricinus* ticks from the same study area (nymphs: 11.8%, larvae: 1.5%; D. Heylen, unpubl. data), suggesting that this bird species facilitates transmission of *Borrelia* spirochaetes and acts as a competent reservoir host. Because *Borrelia* spirochaetes are not, or inefficiently, transmitted transovarially, their presence in several feeding larvae indicates that they were likely acquired via the host, either by systemic infections or by co-feeding transmission (Dubska *et al*., 2009). The larval xenodiagnoses in other European *P. major* populations (Humair *et al*., 1993; Comstedt *et al*., 2006; Dubska *et al*., 2009) suggest this finding as well. Since infestation burdens in woodland populations of *P. major* can be high (Heylen *et al*., 2010) and this host species is very abundant, it may significantly contribute to the avian *I. ricinus*-borne *Borrelia* cycles. In contrast, *C. caeruleus* has never

been considered as a competent reservoir host based on larval xenodiagnoses in other studies (Humair *et al*., 1993; Comstedt *et al*., 2006; Dubska *et al*., 2009). Therefore, we suggest that the single infected *I. ricinus* larva in this study likely resulted from co-feeding transmission with an infected nymph that shared the same *Borrelia* IGS genotype (*B. garinii*). As shown in other non-competent reservoir host species (Kimura *et al*., 1995; Ogden *et al*., 1997), co-feeding could be an important mode to transmit *Borrelia* in certain songbirds as well (Randolph *et al*., 1996).

The high prevalence of *B. burgdorferi* s.l. in *I. ricinus* ticks, including important genospecies (*B. garinii*, *afzelii* and *sensu stricto*) that are pathogenic for humans (Anthonissen *et al*., 1994; Franke *et al*., 2010), shows that the sampled area comprises a certain risk for Lyme borreliosis in humans. In *I. arboricola*, the significant numbers of *Borrelia* infections provide the first field data that strongly suggest that this tick species is competent. The infection levels are not consistent with the very low numbers in a previous field study in a Czech endemic area (Spitalska *et al*., 2011). On the other hand, our finding supports the conclusions of an experimental study (Thorud, 1999), which demonstrated the ability of several *Borrelia* genospecies (*B. valaisiana*, *afzelii*, *garinii* and *sensu stricto*) to infect *I. arboricola* by artificial feeding, and the ability to persist in the ticks for many weeks. In addition, we presented data showing that transstadial transmission successfully occurs, since a noteworthy proportion of questing ticks collected in nest boxes were *Borrelia* positive. Unfed larvae were *Borrelia* negative, suggesting that transovarial transmission does not take place (Thorud, 1999; Richter *et al*., 2012). In *I. frontalis*, a remarkably high proportion of ticks were infected, and all *Borrelia*-infected ticks originated from *P. major*. The high prevalence confirms the findings of a Spanish study which suggested that *I. frontalis* is an important carrier of *Borrelia* spirochaetes (Estrada-Peña *et al*., 1995). One should also mention that the presence of the bacteria in the ticks after feeding does not necessarily mean that the tick will effectively transmit the pathogens through salivation when feeding on the next host. Many correlative studies have shown that ticks are *Borrelia* carriers, but proving the vector competence of the ticks requires experimental investigations (Gern *et al*., 1998).

Based on the distributions of IGS genotypes (Fig. 2) we may construct a preliminary hypothetical *Borrelia* transmission cycle within the tick community found on the birds. A larger sample of successfully sequenced genotypes would allow a detailed statistical analysis, taking into account tick instar, bird species and engorgement status. The present data show the sharing of IGS types between *I. ricinus* and each of the bird-specific ticks, but provide no evidence of the sharing of IGS types between the bird-specific ticks. These findings suggest a scenario in which the generalist *I. ricinus* intrudes on the enzootic *Borrelia* cycles supported by specialist ticks, and hence acting as a bridging vector towards other hosts outside these enzootic cycles. In Europe, the only known *Borrelia* example, is the enzootic circulation of *Borrelia* between the hedgehog (*Erinaceus europaeus* L.) and hedgehog specialized *Ixodes hexagonus*, with *I. ricinus* intruding upon this cycle when feeding on the hedgehog (Piesman and Gern, 2004). Analogously, cycles of *Anaplasma phagocytophilum* and *Babesia microti* can be maintained by the nest-dwelling *Ixodes trianguliceps*, which is specialized in infesting small mammals (Bown *et al*., 2008) . These pathogens can then be bridged to the exophilic *I. ricinus*. A relatively high proportion of *Borrelia* IGS genotypes that were found in *I. arboricola*, were also found in *I. ricinus* (Fig. 2). Interestingly, however, there was no overlap at all between *I. arboricola* and *I. frontalis,* although both ticks are considered to have a nidicolous existence (Hillyard, 1996). Possible explanations are the higher diversity of hosts on which *I. frontalis* feed, and differences in host exposures due to dissimilar off-host habitat. *Ixodes frontalis* seldom infests the tree-hole nests that are inhabited by *I. arboricola* (D. Heylen, pers. obs.), and most of the *I. frontalis* records origin from populations of open-nesting birds (Hillyard, 1996) (see also discussion above). A large proportion of the *Borrelia* IGS genotypes in *I. frontalis* was not shared with *I. ricinus*, suggesting enzootic bird – *I. frontalis* cycles, of which the bacteria are neither carried nor vectored by *I. ricinus* to other hosts outside the cycles. This finding is not in line with a previous study (Estrada-Peña *et al*., 1995) in which the authors concluded that in the absence of *I. ricinus*, the bird – *I. frontalis* cycle may be broken, as *Borrelia*-infected *I. frontalis* ticks were only found in those locations where *I. ricinus* was present.

An intriguing finding was the presence of a *B. turdi*-like genospecies in five *I. frontalis* individuals, originating from five different birds, captured in three different woodlots. The cluster of sequenceable samples (IF 412, 413 and 414) branched off from *B. turdi* with high certainty, indicating genetic divergence (Fig. 1). One straightforward explanation for the genetic divergence between the original *B. turdi* (Japan) (Fukunaga *et al*., 1996) and our *B. turdi*-like isolates (Belgium) is differentiation by distance. In Europe, *B. turdi* has been recorded only twice very recently, in Norway (Hasle *et al*., 2011) and in Portugal (Norte *et al*., 2012). This genospecies may be more common in Eurasia than previously thought. Despite the screenings of a large number of sympatric *I. ricinus* ticks in our study, the genospecies has only been detected in *I. frontalis*, as was also the case in a Japanese study (Fukunaga *et al*., 1996). These observations contrast with the Norwegian study of Hasle and colleagues (2011) and

the Portugese study of Norte and colleagues (2012), reporting its presence in small numbers of *I. ricinus* ticks. Therefore, we cannot exclude the possibility that *B. turdi* could be relevant for public health, when humans are exposed to *B. turdi*-infected *I. ricinus* nymphs.

A noteworthy prevalence of presumed mammalian genospecies (*B. afzelii*, *B. spielmanii*, *B. bavariensis*) (Richter *et al*., 2004; Franke *et al*., 2010) was present in the ticks, including the larval developmental stages (Table 4). In the case of the bird-specific ticks and *I. ricinus* larvae we suggest that these types of infection likely occurred during co-feeding with ticks that vector mammalian genospecies from a previous host, because mammalian *Borrelia* is not considered to persist as systemic infections in bird tissue (Kurtenbach *et al*., 2002). However, in the case of *I. ricinus,* unusual trans-ovarial transmissions, and the potential reservoir competence of certain bird species for presumed mammal genospecies (Franke *et al*., 2010), may also explain our findings. Another explanation for the presence in the bird-specific ticks, is that they successfully infest infected mammals. This explanation is less likely for *I. arboricola*, since previous experimental exposures of small mammals to this species show very low infestation successes (Lichard and Kozuch, 1967). To the best of our knowledge, there is no evidence that *I. frontalis* infests mammals, other than a single report of an adult female tick attached on man (Gilot *et al*., 1997).

In conclusion, this study shows that significant numbers of the two bird-specific ticks carry *Borrelia* spirochaetes and share several *Borrelia* genospecies with the main European *Borrelia* vector, *I. ricinus*. These results support the hypothesis that the flow of *Borrelia* spirochaetes is partly maintained by bird-specific ticks, and bridged by *I. ricinus* to other host types. Our results highlight the need for experiments to better assess to what extent *I. frontalis* and *I. arboricola* are able to effectively transmit *Borrelia* spirochaetes to susceptible hosts, and hence may contribute to the local dynamics and large-scale dispersal of *Borrelia* spirochaetes. Furthermore, more extensive research is needed to clarify the role of the reservoir competence of birds, the vector competence of bird-specific ticks and the role of spatial isolation in generating and maintaining the genetic diversity of *B. burgdorferi* genospecies.

Experimental procedures

Tick collection

Fieldwork was carried out from 2007 to 2011 in woodland areas situated within a radius of 25 km from the centre of the city of Antwerp (northern Belgium). Free-living birds (*P. major* and *C. caeruleus*) were captured during the post-breeding season with mist nets, and during the breeding season with nest traps inside nest boxes while feeding their nestlings. Most of the nest boxes were from two larger woodland areas (51°08′N, 4°31′E and 51°16′N, 4°29′E) where long-term population studies on *P. major* and *C. caeruleus* have been carried out for many years (Matthysen *et al*., 2001; 2011). We systematically searched the head region of 5116 *P. major* and 2702 *C. caeruleus* individuals for ixodid ticks while holding the beak of the bird between thumb and forefinger and blowing and brushing the birds' feathers apart (Heylen *et al*., 2009). No nestlings were screened for ticks. For a random subset of 710 birds, any ticks ($n = 2631$) found were removed using tweezers. After investigation, the birds were released back into the wild as quickly as possible. In addition, nest boxes were screened for both unfed and engorged ticks. The removable nest box lids allowed easy access to *I. arboricola* ticks that typically move towards the top of the nest box after feeding (D. Heylen, unpubl. obs.). To reduce the disturbance of the *I. arboricola* population, only half of the ticks were collected per nest box. In the laboratory, the species and developmental stages were identified using a stereomicroscope and identification keys (Arthur, 1963; Hillyard, 1996).

DNA extraction and PCR-based detection of Borrelia *in ticks*

Ticks were immersed in 70% ethanol and stored at −20°C before testing. In total, a sample of 802 ticks was screened for *Borrelia*, of which 640 were collected from birds and 142 from nest boxes. DNA from engorged ticks was extracted using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol for the purification of genomic DNA from ticks. DNA from unfed ticks was extracted by alkaline lysis (Schouls *et al*., 1999). For the detection of *B. burgdorferi* s.l., a duplex qPCR was redesigned based on existing qPCR protocols on fragments of the OspA (Gooskens *et al*., 2006) and flagellin (Schwaiger *et al*., 2001) genes (Table 5). The oligonucleotides were designed in such way that almost all known genotypes of *B. burgdorferi* s.l. from Europe could be detected with either OspA or FlaB or both. 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) and Visual OMP (Software, Ann Arbor, USA) on the basis of the following criteria: predicted cross-reactivity with closely related organisms, internal primer binding properties for hairpin and primer-dimer potential, length of the desired amplicon, and melting temperatures (T_ms) of probes and primers. The specificity of primers and probes was tested with tick lysates containing the DNA from the following micro-organisms: *Rickettsia africae*, *R. conorii*, *R. helvetica*, *R. rickettsii*, *Anaplasma phagocytophilum*, *Neoehrlichia mikurensis*, *Ehrlichia canis*, *Babesia microtii*, *B. divergens*, *B.* EU1, *Coxiellia burnetii*, *Fransicella tularensis*, *Bartonella henselae* and *Bartonella* species found previously in *I. ricinus*, *Candidatus Midichloria mitochondrii* and tick lysates containing *Wolbachia* species (Sprong *et al*., 2009; Tijsse-Klasen *et al*., 2010; 2011a,b; Wieten *et al*., 2011; Jahfari *et al*., 2012). Correct sizes of DNA fragments of qPCR amplicons were regularly confirmed on a bioanalyser (Agilent Technologies, Palo Alto, CA). Furthermore, the sensitivity of the duplex qPCR turned out to be equal or higher than the OspA qPCR

Table 5. Names and sequences of primers and probes used in the PCR analyses.

Name	Sequence (5′–3′)
B-5S-borseq	GAGTTCGCGGGAGAGTAGGTTATTGCC
B-23S-borseq	TCAGGGTACTTAGATGGTTCACTTCC
B-OspA_modF	AATATTTATTGGGAATAGGTCTAA
B-OspA_borAS	CTTTGTCTTTTTCTTTRCTTACAAG
B-FlaB-F	CAGAIAGAGGTTCTATACAIATTGAIATAGA
B-FlaB-Rc/t	GTGCATTTGGTTAIATTGYGC
B-OspA_mod-probe	FAM-AAGCAAAATGTTAGCAGCCTTGA-BHQ-1™
B-FlaB-probe	JOE-CAACTIACAGAIGAAAXTAAIAGAATTGCTGCTGAICA (X = BHQ-1™-dT)

FAM, 6-carboxyfluorescein; JOE, -carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein.

(Gooskens *et al*., 2006) and the PCR/Reverse Line Blotting (Schouls *et al*., 1999, not shown). qPCR was performed in a multiplex format 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 20 µl were iQ multiplex Powermix, primers B-OspA-modF and B-OspA-borAS at 200 nM each, probeB-OspA_mod-probe-FAM at 100 nM, primers B-FlaB-F and B-FlaB-Rc/t at 200 nM each, probe B-FlaB-probe-JOE at 200 nM, 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^{-1} 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. For overflow of fluorescence from dyes that were used, a colour compensation was conducted. Curves were assessed visually. Samples with a positive score for one or both targets were assumed being positive for *Borrelia*.

From all qPCR-positive tick lysates, *B. burgdorferi* sensu lato genospecies was determined by PCR amplification and sequencing of the variable 5S–23S (rrfA–rrlB) intergenic spacer region (IGS). The PCR was performed with the HotStarTaq master mix (Qiagen, Venlo, the Netherlands) using B-5S-borseq and B-23S-borseq (Table 5) under the following conditions: 15 min 94°C, then cycles of 20 s 94°C, 30 s 70°C, 30 s 72°C lowering the annealing temperature 1°C each cycle till reaching 60°C, then 40 cycles at this annealing temperature and ending by 7 min 72°C. Multilocus sequence typing was performed as described (Margos *et al*., 2008), except that the elongation times were 60 s. For each PCR and multiplex qPCR, positive, negative controls and blank samples were included. A 10^{-3} to 10^{-5} dilution of DNA from a *B. burgdorferi* culture (*B. sensu stricto* B31 strain) was used as a positive control. In order to minimize contamination, the PCR proceedings were performed in three separate rooms, of which the reagent set-up and sample addition rooms were kept at positive pressure, whereas the DNA extraction room was kept at negative pressure. All rooms had airlocks.

DNA sequence analysis

PCR amplicons were sequenced using the described primers (Table 5) and the BigDye Terminator Cycle sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems). All sequences were confirmed by sequencing both strands. The collected sequences were assembled, edited and analysed with BioNumerics version 6.5 (Applied Maths NV, Sint-Martens-Latem, Belgium). *Borrelia* typing was performed by comparing the IGS sequences to reference strains present in GenBank after subtraction of the primer sequences. Multilocus sequence types of reference strains were kindly provided by Gabrielle Margos (University of Bath). DNA sequences are available upon request.

Statistical analysis

Phylogenetic analyses of the sequences and related organisms were conducted using BioNumerics with neighbour-joining algorithm with Kimura's two-parameter model. Bootstrap proportions were calculated by the analysis of 500 replicates for neighbour-joining trees. When testing hypothesizes on prevalence data, marginal models were fitted using generalized estimation equations (logit-link, binomial-distributed residuals) in SAS v 9.1 (SAS Institute, Cary, North Carolina, USA), allowing for dependence of measurements within the same cluster (bird individual or nest box) using exchangeable working correlation matrix (for details see Molenberghs and Verbeke, 2005). When counts were low (< 5), Fisher exact tests were applied (Agresti, 1990). $\alpha = 0.05$ was chosen as the lowest acceptable level of significance.

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