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Summary

The principal European vector for *Borrelia burgdorferi* s.l., the causative agents of Lyme disease, is the host-generalist tick *Ixodes ricinus*. Almost all terrestrial host-specialist ticks have been supposed not to contribute to the terrestrial *Borrelia* transmission cycles. Through an experiment with blackbirds, we show successful transmission by the widespread *I. frontalis*, an abundant bird-specialized tick that infests a broad range of songbirds. In the first phase of the experiment, we obtained *Borrelia*-infected *I. frontalis* (infection rate: 19 %) and *I. ricinus* (17 %) nymphs by exposing larvae to wild blackbirds that carried several genospecies (*Borrelia turdi*, *B. valaisiana*, *B. burgdorferi* s.s.). In the second phase, pathogen-free blackbirds were exposed to these infected nymphs. Both tick species were able to infect the birds, as indicated by the analysis of xenodiagnostic *I. ricinus* larvae which provided evidence for both co-feeding and systemic transmission (infection rates: 10-60%). *Ixodes frontalis* was shown to transmit *B. turdi* spirochetes, while *I. ricinus* transmitted both *B. turdi* and *B. valaisiana*. Neither species transmitted *B. burgdorferi* s.s.. European enzootic cycles of *Borrelia* between songbirds and their ornithophilic ticks do exist, with *I. ricinus* potentially acting as a bridging vector towards mammals, including man.

Key words: songbird, vector-competence, *Borrelia burgdorferi* sensu lato, Lyme borreliosis, enzootic cycle, *Ixodes ricinus*, *Ixodes frontalis*, *Borrelia turdi*

Introduction

The capability of an organism to transmit pathogens is fundamental in understanding the epidemiology of infectious diseases. Several organisms can participate to the pathogen transmission dynamics, but each of them may contribute differently. In addition, pathogens, vectors and reservoir hosts may interact, making outcomes of cycles of diseases complex. The detection of a pathogen in an organism does not necessarily mean that it is truly involved in the transmission and spread of diseases (Leirs et al., 1999; Keele et al., 2006; Soares et al., 2006; Bown et al., 2008; Bataille et al., 2009; Farajollahi et al., 2011; Lindeborg et al., 2012). Therefore, experimental evaluations of the competence of an organism to act as vector and/or reservoir are essential to unravel the transmission dynamics and eco-epidemiology of infectious pathogenic agents.

In Europe, the ticks *Ixodes ricinus* and *I. persulcatus* are considered the main vectors for *Borrelia burgdorferi* sensu lato bacterial spirochetes (hereinafter '*Borrelia*' refers to '*B. burgdorferi* s.l.') (Gray, 1998; Jongejan and Uilenberg, 2004), the causative agents of Lyme borreliosis in humans and domestic animals. This tick-borne bacterial species complex comprises at least 20 genospecies, some of which are known to cause disease (Margos et al., 2011; Margos et al., 2014; Margos et al., 2015). Both tick species are extreme host generalists that infest a very broad range of vertebrates, including mammals, reptiles and birds (Hillyard, 1996). *Borrelia* has also been detected in a several host-specialized tick species that solely act as a carrier, acquiring the spirochetes after feeding on an infected host, but being incapable of maintaining them through moult to the next developmental stage, and transmitting them to the next host (Gray, 1998; Piesman and Gern, 2004; Heylen et al., 2014c).

After decades of research on *Borrelia* in wildlife, there are still numerous research questions that remain about the importance of the songbird-tick-*Borrelia* system in the maintenance and spread of *Borrelia* spirochetes. Our study aims to test whether the most abundant and widely spread songbird-specialized tick of Europe, *I. frontalis* (Panzer), acts as a competent vector in the *Borrelia* transmission cycles. *Ixodes frontalis* shares all of its host species with *I. ricinus*, including some very common songbird species such as the blackbird (*Turdus merula*). The blackbird is probably the most abundant resident bird of European gardens and woodlands (Hagemeijer and Blair, 1997), is very often infested with ixodid ticks, including cases of co-feeding between different tick species (Norte et al., 2013a), and is known to be an important reservoir host for several *Borrelia* genospecies (Humair et al., 1998; Taragel'ova et al., 2008; Norte et al., 2013a). The ecology of *I. frontalis* is not well known, but it is considered as an abundant ornithophilic tick of terrestrial ecosystems. The tick has been found inside bird nests, but also in the understorey-vegetation and the ground layer of forests (Doby, 1998; Schorn et al., 2011; Heylen et al., 2013). Screenings of bird-derived *I. frontalis* ticks indicate that this species is able to carry *Borrelia* spirochetes (Estrada-Peña et al., 1995; Heylen et al., 2013; Norte et al., 2013b); however, a first test of its competence in the hole-breeding great tit (*Parus major*) indicates it is not competent in transmitting two avian *Borrelia* spirochetes (*B. valaisiana* and *B. garinii*) to a subsequent host (Heylen et al., 2014c). However, the transmission and amplification outcomes depend upon an interaction with factors that are unique to the particular species of host, *Borrelia* and tick (Kurtenbach et al., 2002b; Brisson and Dykhuizen, 2004; Gomez-Diaz et al., 2010a; Gomez-Diaz et al., 2010b; Hamer et al., 2011; Heylen et al., 2014b), and therefore generalizations on vector and/or host competences can only be made based on data of experimental multi-species comparison that simulate the parasite-vector-host interactions in the wild.

In the light of the this, we tested whether the bird-specialized tick *I. frontalis* acquires *Borrelia* from wild naturally-infected blackbirds (host-to-tick transmission) and whether *Borrelia*-infected ticks were capable of transmitting the spirochetes to naive blackbirds (tick-to-host transmission). In the experiment, we explicitly tried to investigate the interactions between the tick and the complete European spectrum of avian genospecies (*B. garinii*, *B. valaisiana*, and the recently discovered *B. turdi* (Hasle et al., 2011; Norte et al., 2013b; Heylen et al., 2013), by focusing on *Borrelia* foci where these genospecies naturally occur. Additionally, we tested whether the generalist tick *I. ricinus* is able to vector all these genospecies (including *B. turdi*, which has not been proven to date) when using the blackbird as a host, and thus could act as bridging vector towards other, non-avian hosts. Results will be compared with those obtained in the great tit in a previous study (Heylen et al., 2014c). Successful transmission of *Borrelia* spirochetes was tested by xenodiagnoses, i.e. the molecular screening of *I. ricinus* and *I. frontalis* larvae for *Borrelia* DNA (Humair et al., 1998; Richter et al., 2000).

Results

A graphical overview and a Gantt chart of the various phases of the study design are provided in Fig. 1 and Fig. 3, respectively. Arithmetic means (± standard error) have been used to calculate the average infection rates over the bird individuals.

Phase 1: Ability of the ticks to acquire *Borrelia burgdorferi* s.l. spirochetes

Obtaining challenge nymphs

From the 23 birds that were captured in the wild, we obtained one or more *Borrelia*-infected ticks from 17 individuals (76 %). *Borrelia*-infected *I. frontalis* ticks were obtained from 10 birds , whereas 13 birds yielded *Borrelia*-infected *I. ricinus* ticks. For six of those birds, we obtained *Borrelia*-infected individuals of both tick species. The average tick infection rate per bird for the moulted *I. frontalis* larvae was 19 ± 6 % (N = 133 screened ticks), while this was 17 ± 9 % for *I. ricinus* (N = 49 screened ticks). When considering only those birds in which successful *Borrelia* transmission had occurred, i.e. birds with at least one infected tick, the average infection rate per bird was 28 ± 8 % (N = 74 ticks) and 31 ± 16 % (N = 18 ticks) for *I. frontalis* and *I. ricinus*, respectively.

Genospecies distribution

In those infected *I. frontalis* and *I. ricinus* nymphs (i.e. moulted larvae) in which the genospecies was successfully identified, exclusively the bird-associated *B. turdi* (2 *I. frontalis* and 7 *I. ricinus* ticks) and *B. valaisiana* (2 *I. frontalis* and 4 *I. ricinus* ticks) were detected, except for the three *I. frontalis* nymphs derived from three birds that carried *B. burgdorferi*

s.s. (Table 2). In one bird individual, we identified two genospecies (*B. turdi* in 1 tick, and *B. burgdorferi* s.s. in another tick).

Phase 2: Testing the vector competence of the *Borrelia*-infected ticks

Xenodiagnosis via* Ixodes ricinus *larvae

Overall, we detected *Borrelia* DNA in the *I. ricinus* xenodiagnostic larvae that fed on 13 out of the 18 experimental birds (72 %), that were either exposed to infected nymphs of *I. frontalis* (10 infected out of 13 birds; N = 465 larvae tested) or *I. ricinus* (3 infected out of 5 birds; N = 427 larvae tested; Table 1 and Fig. 2).

The infection rates increased over the course of successive infestations [OR: 36.8 (18.3–74.1) session $^{-1}$, t = 10.1, d.f. = 598.6, P < 0.0001] (Fig. 2), but the change in infection rate did not significantly differ between the *I. frontalis*- and *I. ricinus*-exposed birds (interaction change × tick species: $F_{1,551.1}$ = 0.21, P = 0.65). Final infection rates after the second nymphal infestation ('Inf. 2') did not differ between the two exposure groups ($F_{1,112.4}$ = 0.02, P = 0.89). In six of the *I. frontalis*-exposed birds, some fed xenodiagnostic larvae ('Xen. 1') that co-fed with infected nymphs of the first nymphal exposure ('Inf. 1') became infected (10 $\pm$ 4 %; N = 147 fed 'larvae), while there was no evidence for co-feeding transmission in the *I. ricinus*-exposed birds (N = 60 fed larvae).

Several of the infections in the *I. ricinus* xenodiagnostic larvae ('Xen. 2') that fed after the final nymphal exposure ('Inf. 2') persisted after they moulted to the nymphal stage, both in the *I. ricinus*-exposed birds (infection rate: 16 $\pm$ 16 %; N = 18 nymphs) and *I. frontalis*-exposed birds (16 $\pm$ 7 %; N = 80 nymphs), but the infection rates after moulting were significantly lower [$OR_{fed - moulted}$: 3.6 (2.0-6.4), t = 4.4, d.f. = 705, P < 0.0001]. Also in the engorged infected nymphs that moulted to the adult developmental phase, successful trans-

stadial persistence occurred in the two infestation sessions (Inf. 1 and Inf. 2) for *I. ricinus*-exposed birds (Inf. 1: 50 ± 20 %, N = 58 adults; Inf. 2: 29 ± 24 %, N = 23 adults) and *I. frontalis*-exposed birds (Inf. 1: 22 ± 8 %, N = 110 adults; Inf. 2: 38 ± 11 %, N = 51 adults).

Xenodiagnosis via* Ixodes frontalis *larvae

In the *Borrelia*-naïve *I. frontalis* larvae that fed 14 days after the second batch of *I. ricinus* xenodiagnostic larvae ('Xen. 3'), *Borrelia* DNA was detected in 15 birds (83.3%) that had been exposed either to *I. ricinus* nymphs (3 infected out of 5 birds; N = 78 larvae tested) or *I. frontalis* nymphs (12 infected out of 13 birds; N = 252 larvae tested). Thus, in addition to the previous 13 birds that were shown to be positive by *I. ricinus* xenodiagnoses, two extra birds were shown to be infected by the screening of *I. frontalis* larvae. The *I. frontalis* larvae were infected in both the *I. ricinus*-exposed and *I. frontalis*-exposed birds. Here, final infection rates tended to be higher for the birds exposed to *I. frontalis*-nymphs (77 ± 10 %, N = 156 fed larvae) than for those exposed to *I. ricinus* (35 ± 18 %, N = 48 fed larvae; [$OR_{I.frontalis\text{-}I.ricinus}$: 27.3 (0.82–909.81), t = 2.04, d.f. = 12.53, P = 0.06]). Both in the *I. frontalis*-and *I. ricinus*- exposed birds, the *I. frontalis* larval infection rates were higher [range OR's: 3.05 (1.20 –7.80) - 8.05 (3.64–17.79), range P-values: < 0.0001 - 0.02] than the last batch of *I. ricinus* larvae that had fed two weeks before.

Several of the *Borrelia* infections in the larvae persisted after the moulting to the nymphal stage (*I. ricinus*-exposed: 21 ± 13 %, N = 30 nymphs; *I. frontalis*-exposed: 34 ± 12 %, N = 96 nymphs), but the infection rates were significantly lower after moulting [$OR_{fed\text{ - }moulted}$: 3.6 (2.2-5.7), t = 5.3, d.f. = 328, P < 0.0001].

Genospecies distribution

In the xenodiagnostic larvae (engorged and moulted) in which genospecies could be determined based on the sequence data, only the bird-associated *B. turdi* (in four *I. ricinus*-exposed birds, and eleven *I. frontalis*-exposed birds) and *B. valaisiana* (in two *I. ricinus*-exposed birds) were identified (Table 2 and Fig. 2). The latter two *B. valaisiana*-infected birds also yielded xenodiagnostic larvae infected with *B. turdi*, indicating mixed infections in these birds. Co-feeding transmission of *B. turdi*-DNA between *I. frontalis* nymphs ('Inf. 1') and fed *I. ricinus* larvae ('Xen. 1') was found in five birds. Notably, in the two experimental birds with mixed infections (*B. valaisiana* and *B. turdi*), only one of the tested *I. frontalis* xenodiagnostic larvae was infected with *B. valaisiana* (7 fed larvae tested), while a significant proportion of the *I. ricinus* xenodiagnostic larvae was (23 fed and 2 moulted larvae out of 32 larvae tested). Transstadial persistence of *B. turdi*- and *B. valaisiana*-DNA was registered in both tick species (larva-to-nymph-transmission: 'larvae moulted' in Table 2; nymph-to-adult-transmission: 'nymphs moulted' in Table 2). Sample sizes of the sequence data were too low for further statistical analysis.

The separation of *B. valaisiana* and *B. turdi* according to the QPCR-results based on the flaB- and ospA-markers (for details see 'Study design' section 'DNA extraction and PCR-based detection of *Borrelia* in ticks') enlarged the sample sizes, but did not change the conclusions above. We found one extra *I. frontalis*-exposed bird in which co-feeding transmission of potentially *B. turdi* had occurred, suggested by one *I. ricinus* larva (i.e. positive signal for the ospA-marker). Furthermore, we found one *I. ricinus* larva in which a FlaB-signal was detected in an *I. frontalis*-exposed bird (i.e. suggesting *B. valaisiana*-DNA). However, all *Borrelia*-sequences of the 10 other infected larvae were from *B. turdi*, making it unlikely that *B. valaisiana*-transmission had occurred via *I. frontalis* nymphs.

Discussion

Here we experimentally proved the carrier capacity and vector-competence of an ornithophilic tick (*I. frontalis*) for a genospecies (*B. turdi*) belonging to the *Borrelia burgdorferi* s.l. complex. Furthermore, we showed that the most important *Borrelia*-vector in Europe, *I. ricinus*, is also able to transmit *B. turdi* spirochetes that were acquired via *I. frontalis*-exposed birds, increasing the chance that non-avian hosts can be exposed to this *Borrelia*-genospecies via *I. ricinus* and become infected ('bridging').

After exposure to *I. ricinus* and *I. frontalis* nymphs carrying an endemic *Borrelia*-community obtained from local blackbirds (*B. turdi*, *B. valaisiana*, *B. burgdorferi* s.s.), the pathogen-free naïve birds started to selectively amplify only the two avian genospecies (*B. turdi* and *B. valaisiana*). Thus, the unexpectedly obtained DNA from some phase 1-birds of *B. burgdorferi* s.s., a genospecies known to be maintained in Europe by mammals as is *B. afzelii* and *B. spielmanii* (Kurtenbach et al., 2002a; Kurtenbach et al., 2002b; Richter et al., 2004), was not detected again in any of the ticks in further steps of the experiment (Fig. 2 and Table 2). Some *B. burgdorferi* s.s. may possibly circulate in blackbirds and their ticks in Europe, as is observed in American songbirds, but this could not be confirmed from the experiment described here. The selective amplification of avian genospecies is in agreement with previous experiments that have tested the reservoir competence of European wild birds for endemic *Borrelia* (Humair et al., 1998; Norte et al., 2013a; Heylen et al., 2014b; Heylen et al., 2016).

In the two phases in the experiment, naïve larvae of both *I. ricinus* and *I. frontalis* were able to acquire spirochetes from the infected birds and transstadial persistence occurred, as indicated by the detection of *Borrelia* several weeks post-moult. Transstadial persistence was also proven in the nymphs of the two ixodid tick species. For both stages, the infection rates

were significantly lower after moulting, indicating that the development of fed ticks to the next stage challenges the spirochetes to persist and to stay involved in the transmission cycles, as is known from other *Borrelia* studies (Piesman et al., 1990; Burkot et al., 1994).

Throughout phase 2, *I. ricinus* transmitted both avian genospecies, while *I. frontalis* only transmitted *B. turdi*, despite this tick species was infected with *B. valaisiana*-DNA in phase 1 (only in two tick individuals) (Fig. 2 and Table 2). Some engorged *I. ricinus* xenodiagnostic larvae that co-fed with infected *I. frontalis* nymphs contained *B. turdi* DNA, suggesting co-feeding transmission. This mode of transmission occurs when infected and uninfected ticks feed in close spatial and temporal proximity on the same host, and can occur in the absence of a systemic infection (Gern and Rais, 1996; Voordouw, 2015). Further investigations are needed to test whether the detected DNA represents viable spirochetes that survive moulting and are able to infect new hosts. We did not find evidence for co-feeding transmission in *B. valaisiana*. Despite this genospecies has the capacity to use this mode of transmission in laboratory mice (Hu et al., 2003), as far as we know, there is no evidence for it in natural hosts (i.e. songbirds).

The transmission outcomes in the blackbirds of the present study, in combination with those of a previous vector-competence experiment in great tits (*Parus major*) (Heylen et al., 2014c) provide evidence that *Borrelia* transmission and amplification strongly depend upon an interaction with factors that are not only unique to the *Borrelia* genospecies and host species, but also to the tick species that are involved (Soares et al., 2006). In the previous study of Heylen et al. (2014c) it was shown that the presence of *Borrelia* in ornithophilic ticks (*I. frontalis* and *I. arboricola*) collected from great tits in the wild (Heylen et al., 2013) was due to infection only of the ticks and not to their ability to act as biological vectors. These conclusions are in line with a previous study on bird-derived ticks from a Spanish population

(Estrada-Peña et al., 1995). In that study, a high *Borrelia* prevalence was found in *I. frontalis*, but only in those locations where *I. ricinus* was present. This was interpreted as evidence for the hypothesis that *I. frontalis* is not a vector of *B. burgdorferi* s.l., and that in the absence of *I. ricinus*, the bird - *I. frontalis* - *Borrelia* cycle is broken (Estrada-Peña et al., 1995). In contrast, here we convincingly show by using the blackbird as a reservoir host - instead of the great tit - that *I. frontalis* is a highly competent vector for at least *Borrelia turdi.* As an explanation for the difference in results between our study and Heylen et al. (2014c) we suggest that some genospecies are more capable of establishing an infection in some bird and tick species than in others. However, in the study of Heylen et al. (2014c), *B. turdi* was probably absent, while in this study, *B. garinii* was probably absent, making the comparison of both studies incomplete. Either great tits and blackbirds were not acting as competent reservoirs for *B. turdi* and *B. garinii*, respectively, or these genospecies were not present in the nymphs by which the birds were challenged. In neither study was there an indication of successful *B. valaisiana* transmission by *I. frontalis*, while *I. ricinus* was clearly confirmed to be vector-competent for this genospecies. A possible explanation for these findings, is that some component of the avian immune system selectively clears *B. valaisiana* spirochetes - but not *B. turdi* spirochetes - inside the *I. frontalis* nymphs during feeding. In vector-competent ticks, surface proteins of the *Borrelia* spirochete are known to interact with proteins of the vector, which could help the spirochete in evading the immune response of the host during transmission (Soares et al., 2006; Hovius et al., 2007). An absence of these protective protein-protein interactions with *B. valaisiana* in *I. frontalis* could have led to its clearance during the feeding. Although we found *B. valaisiana* DNA in a very low number of *I. frontalis* nymphs (N = 2) (Table 2), they were not necessarily viable spirochetes. Another explanation could be that there is competition between *Borrelia* genospecies and that this competition could differ between the tick species, in that *B. turdi* outcompetes *B. valaisiana*

in *I. frontalis*, but not in *I. ricinus*. Limited resources and bottle necks linked to pathogen transmission and survival, increase competition among pathogen strains inside arthropod vectors, including ticks (Schmid-Hempel, 2011; Rego et al., 2014; Reif et al., 2014). A stronger proliferation of *B. turdi* than the other genospecies in bird skin (e.g. because of a shorter latent period) can increase the chances of *Borrelia* transmission (Råberg, 2012), and this may well explain the overall dominance of *B. turdi* throughout the experiment.

First recorded in Japan, the spatial distribution of *B. turdi* was supposed to be confined to Asia (Fukunaga et al., 1996; Kurtenbach et al., 2006). However, the recent European observations prove that this *Borrelia* genospecies is widespread over the Eurasian continent (Fukunaga et al., 1996; Hasle et al., 2011; Palomar et al., 2012; Heylen et al., 2013; Norte et al., 2013b). In Europe, *B. turdi* is almost exclusively found in *I. frontalis* ticks, which made us believe *B. turdi* is only successfully transmitted by *I. frontalis* that is maintaining a cryptic cycle in songbirds. However, in the present study we show that *I. ricinus* is also capable of transmitting *B. turdi* to naïve hosts, potentially bridging this presumed cryptic *B.turdi*-*I.frontalis*-bird cycle towards other host types. We therefore can expect to find *B.turdi* in questing *I. ricinus*, potentially leading to cases of Lyme borreliosis in humans and livestock, although, at present this genospecies is not implicated as a human pathogen. Explanations for the current lack of cases are (1) that *B. turdi* has only recently emerged in Europe and is still too rare to be of epidemiological importance, (2) that the host-finding activity between the spring and autumn active *I. ricinus* individuals (Dautel et al., 2008; Gray, 2008) shows too little temporal overlap with the winter-active *I. frontalis* (Doby, 1998) lowering the chances of successful transmission events, (3) that *B. turdi* has been overlooked (Heylen et al., 2013; Norte et al., 2015), or (4) that *B. turdi* shows a low persistence in *I. ricinus* over the long-term, reducing the detection chances in field-derived ticks.

In conclusion, our study supports the occurrence of terrestrial enzootic cycles of *Borrelia* that are maintained by ornithophilic ticks, and are transmitted by the generalist species, *I. ricinus*, to other hosts, acting as a bridge vector. These findings are unique to the global ecology of *Borrelia* transmission cycles. As the widely spread songbird-specialized tick species under study (*I. frontalis*) is strongly related to the Asiatic *I. turdus* and the American *I. brunneus* (Heylen et al., 2014a), the contribution of ornithophilic species to the terrestrial *Borrelia* enzootic cycles may even occur on a larger scale than just in Europe, whereby the local competent generalist ticks act as bridging vectors to other hosts, including humans and livestock.

Experimental procedures

In summary, the experiment was subdivided in two phases (Fig. 1 and Fig. 3): In the first phase ('phase 1'), we tested whether wild birds naturally infected with *Borrelia* transmit spirochetes to *I. ricinus* and *I. frontalis* larvae. Infected blackbirds were captured from the wild (Belgium and Portugal) (Table 1). In the second phase ('phase 2'), we tested whether the potentially infected *I. frontalis* and *I. ricinus* moulted nymphs from phase 1 were able to infect *Borrelia*-naïve blackbirds.

Phase 1: Ability of ticks to acquire *Borrelia burgdorferi* s.l. spirochetes

For phase 1, in the early Spring of 2015 (February-March), 23 blackbirds were captured with mist nets at a site near Antwerp (N = 13), Belgium, and a site near Coimbra (N = 10), Portugal, and were placed individually in local cages with a wire-mesh floor (40 cm x 80 cm)

housed in a lab in which temperature varied with ambient outdoor conditions. Birds were released back into the wild, immediately after the last experimental ticks had detached.

The wild blackbirds were captured (Feb.- Mar. 2015) from areas known to be endemic for *B. burgdorferi* s.l.. Birds were exposed to a mix of 50 *I. frontalis* larvae (attachment success: 95.8 $\pm$ 1.5 %) and 50 *I. ricinus* larvae (attachment success: 98.4 $\pm$ 0.5 %) that were not infected with *Borrelia* spirochetes (infection rate = 0.0 %; N = 158 randomly sampled larvae from the pool of *I. frontalis*). Larvae were placed underneath the feathers on the head of the bird using a brush. Immediately afterwards, birds were kept for 1.5 hours in an air-permeable cotton bag (sized: 25 cm by 20 cm) inside a darkened cage which kept them inactive (Heylen and Matthysen, 2008). Attachment success is defined as a ratio: (total number of nymphs placed on the bird - the number of nymphs left in the bag) / total number of nymphs placed on the bird. The engorged ticks dropped through the mesh cage into a removable plastic tray that contained damp filter paper, and edges streaked with vaseline to prevent ticks from escaping. Ticks that had dropped were collected daily by separating them from the dirt (food and bird excreta), and were rinsed with distilled water before they were put in ethanol or in climate rooms. The infestation lasted five to six days, and birds were additionally checked for undetached larvae before releasing them back into the wild. *I. frontalis* larvae were obtained from engorged adult female ticks collected in late winter 2015 from experimentally infested song thrushes (*Turdus philomelos*) with ticks from a laboratory colony, and from wild redwings (*Turdus iliacus*) captured near Antwerp, Belgium. Engorged *I. frontalis* females were kept at 25 °C and 90 % R.H. until the emergence of larvae from their eggs. *I. ricinus* larvae that were used for xenodiagnosis in phase 2 were obtained from a German laboratory colony (IS Insect Services GmbH, Berlin). Unfed larvae of the two ixodid tick species were kept under sterile conditions in a climate room at 85 % R.H. and 12h:12h light:dark photophase (20°C:10°C temperature cycle) until infestation. After feeding on the

experimental birds of phase 1, engorged larvae of either species were kept in a climate room until the moult to the nymphal stage was complete (16h:8h light:dark photoperiod; 25°C:15°C temperature cycle; 85% humidity). On average 8.3 ± 1.3 *I. ricinus* larvae, and 28.6 ± 2.1 *I. frontalis* larvae per bird moulted to the nymphal stage. For each tick species, individuals were kept individually in plastic tubes (height: 1 cm; diameter: 0.4 cm) until sampling prior to challenging the birds of phase 2.

To test whether the birds transmitted *Borrelia* spirochetes to the ticks, approximately four months before the start of the infestations in phase 2, we took a random sample of 3.1 ± 0.6 *I. ricinus* and 4.3 ± 0.5 *I. frontalis* nymphs from the vials containing the phase 1 nymphs that co-fed on the same bird, that were subsequently screened for *Borrelia* DNA. If nymphs tested positive, the remaining nymphs that co-fed with the *Borrelia*-positive nymphs on the same infected bird were used for phase 2.

Phase 2: Testing the vector competence of Borrelia-infected ticks

For phase 2, in the Autumn of 2015 (September-November), 18 pathogen-free birds were obtained from a breeder (Houthalen, Belgium) that kept birds in inside aviaries with paved floors, that are free of ticks. All birds were confirmed to be pathogen-free via the xenodiagnosis, taking place before the first nymphal exposure (9.7 ± 0.3 *I. ricinus* larvae per bird tested; N = 175 larvae). Xenodiagnosis is a pathogen detection technique by which vector-competent, pathogen-free ticks (usually larvae) are allowed to feed on a potentially infected host, and are screened after detachment for the presence of the pathogen. Over the duration of phase 2 in the experiment, birds were maintained with a photophase beginning at 07.30 a.m. and a scotophase beginning at 7.30 p.m. Temperature varied with ambient outdoor

conditions. Birds received food and water *ad libitum* and were habituated to the laboratory environment for at least four days before the start of the experiments.

The *Borrelia*-naïve birds were exposed twice in succession ('Inf. 1': 24 Sept.;'Inf. 2': 5 Oct.) to a random sample of 20 nymphs that were obtained in phase 1 (Fig. 2). Five experimental birds were infested with *I. ricinus* (attachment success Inf. 1: 95.0 ± 3.2 %; Inf. 2: 98.0 ± 1.2%), the other 13 with *I. frontalis* (attachment success Inf. 1: 96.2 ± 1.2 %; Inf. 2: 90.8 ± 2.3 %). In contrast to the experimental larvae exposures (see above), nymphs were placed underneath the feathers on the head of the bird using moistened tweezers. Two days after the nymphs had been placed on the birds' skins, 30-40 *I. ricinus* larvae were added (attachment success: 90.0 ± 2.6 %) in order to test for co-feeding transmission to the larvae ('Xen. 1'), thus using the *I. ricinus* larvae in a xenodiagnostic approach. In addition, 18 days after the first nymphal exposure (12 Oct.), 60-70 *I. ricinus* xenodiagnostic larvae were placed on each bird's head (attachment success: 94.8 ± 1.6 %), in order to test for *Borrelia* transmission via an infection of the host ('Xen. 2'). This time period is assumed to be sufficiently long, since it exceeds the latent period between the hosts being bitten by an infected tick and becoming infective (6 - 12 days in American robins *Turdus migratorius* (Richter et al., 2000) and great tit *Parus major* (Heylen et al., 2014b; Heylen et al., 2016), 14 days in juvenile chickens *Gallus gallus* (Piesman et al., 1996)). An additional set of 80-100 *I. frontalis* ('Xen. 3') larvae were placed 31 days after the first nymphal exposure (25 Oct., attachment success: 97.9 ± 0.7 %).

All engorged nymphs, and random subsamples of engorged *I. ricinus* larvae (after nymphal infestation) were kept in climate rooms until moulting to the next developmental stage was complete, and subsequently screened for *Borrelia*. At the end of the experiment, birds were euthanized by a veterinarian who injected intravenously a dose of 200 µL embutramide (T-61®; INTERVET Belgium N.V.; Brussels) (Baert and De Backer, 1999). Euthanization took

place approximately three months after the first exposure with potentially infected nymphs in phase 2.

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

Ticks (both engorged and moulted) were immersed in 70% ethanol and stored at -20°C before screening for *B. burgdorferi* s.l. using PCR. DNA was purified using the DNeasy Blood & Tissue Kit according to the manufacturer's manual (Qiagen, 2006, Hilden; Germany) following the protocol for the purification of total DNA from ticks. For the detection, a duplex Q-PCR was designed based on existing Q-PCR protocols on fragments of the OspA (Gooskens et al., 2006) and flagellin genes ('FlaB' (Schwaiger et al., 2001)). A detailed description of primers, probes and the Q-PCR protocol is given in an earlier study (Heylen et al., 2013). From a proportion of the Q-PCR-positive samples (388 out of 564 positive samples; 68.8 %), the genospecies were determined by PCR amplification and sequencing of the variable 5S-23S (rrfA-rrlB) intergenic spacer region. We could successfully genotype 80.2 % of these infected ticks (see below). For each PCR and multiplex Q-PCR, positive controls, negative controls, and blank samples were included. A 10^{-3} to 10^{-5} dilution of DNA from a *B. burgdorferi* culture (*B. burgdorferi* s.s. B31 strain) was used as a positive control. To minimize contamination, the three steps of the PCR protocol were performed in separate rooms; 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.

As virtually all *B. turdi*-positive ticks (269 out of 274 ; 98.2 %) showed to be positive only for the OspA-marker, while the vast majority of the *B. valaisiana*-positive ticks tested positive for the FlaB-marker (34 out of 36 ; 94.4 %) we could assign the QPCR-positive samples over

the two genospecies with high certainty. To verify whether the only three *B. turdi*-positive xenodiagnostic ticks that also tested positive for FlaB-marker were co-infected with *B. valaisiana*, we developed a non-*B. turdi* specific marker, targeting the RecG gene. The primer pair (RecG-F; 5'-gtttcagtgactcttttgactggc-3', RecG-R; 5'-aagtagtaataggtaaacgacccttg-3') amplified *B. valaisiana,* but not *B.turdi*. Cycling conditions included an initial activation of the Taq DNA polymerase at 95°C for 15 min, followed by seven cycles of a 30 s 94°C, 30 s 56°C, 30 s 72°C lowering the annealing temperature 1°C each cycle till reaching 49°C, then 20 cycles at this annealing temperature and ending by five min 72°C. PCR products were sequenced and typed using reference sequences (PMID: 27125686). We concluded that one of those three ticks was co-infected with both *Borrelia* genospecies, while the two others were only infected with *B. turdi*, proving very rare interaction of *B. turdi* with FlaB in duplex Q-PCR, hence very low error rates in further analyses.

Statistical analysis

Generalized linear mixed effects model (binomially distributed residuals) were fitted for comparisons between the two tick species, taking into account the correlation of the repeated measurements from the same bird individual (Molenberghs and Verbeke, 2005). The odds ratios obtained from the analyses are reported with their 95% confidence limits. In the 'Results' and 'Experimental procedures' section, the arithmetic mean (± standard error) was used to calculate the average infection rate and infestation successes over the bird individuals. Data management and statistical analyses were performed using SAS v 9.2 (SAS Institute, Cary, North Carolina, USA).

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Author contributions

D.H. and A.C.N. conceived and designed the study. D.H. and A.C.N. performed the experiments. H.S. and A.K. designed primers and performed the molecular analyses. H.S. and D.H. provided funding. All authors contributed to writing the manuscript.

Additional information

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Table legends

Table 1. Phase 1: *Borrelia* infection in the generalist tick *Ixodes ricinus* (‘*I.r.*’) and the ornithophilic tick *I. frontalis* (‘*I.f.*’) that had fed as larvae on 23 blackbirds (*Turdus merula*) from *Borrelia*-endemic areas in Belgium and Portugal. Phase 2: *Borrelia* infection in the xenodiagnostic (‘xeno.’) *I. ricinus* and *I. frontalis* larvae that fed on naïve blackbirds exposed to *I. ricinus* nymphs (5 blackbirds) and *I. frontalis* nymphs (13 blackbirds) obtained from phase 1. See Gantt chart of the study design in Fig. 2 and the main text for further details.

Table 2. Distribution of *Borrelia* genospecies that were identified in *I. ricinus* (‘*I.r.*’) and *I. frontalis* (‘*I.f.*’) ticks that fed on 23 blackbirds (*Turdus merula*) from the wild as a larvae (*‘Phase 1 - larvae - moulted’*) and subsequently fed as nymphs on 18 naïve birds and moulted (‘*Phase 2 – nymphs – moulted’*). In addition, the genospecies’ distribution is shown for the *I. ricinus* and *I. frontalis* xenodiagnostic larvae that co-fed (*‘Phase 2 – Xen. 1 – fed/moulted’*) with infected nymphs of Phase 1, and larvae that fed on the birds after the two consecutive exposures with the infected nymphs of Phase 1 (*‘Phase 2 – Xen. 2 & 3 – fed/moulted’*).

Figure captions

Fig. 1. Graphical overview of the study set-up and results. Dashed lines indicate organisms that are infected with *B. burgdorferi* s.l., solid lines indicate organisms that are *Borrelia*-free. Grey ticks represent *I. ricinus*, white ticks represent the ornithophilic tick *I. frontalis*. Large ticks and small ticks are nymphal and larval stages, respectively. Phase 1: Ability of ticks to acquire *B. burgdorferi* s.l. spirochetes. Wild blackbirds were captured from known endemic areas for *B. burgdorferi* s.l.. and exposed to naïve *I. ricinus* and *I. frontalis* larvae. These fed larvae moulted to the nymphal stage, and were screened for *Borrelia* infections.

Phase 2: Testing the vector competence of *Borrelia*-infected nymphs. (a) *Borrelia*-naïve birds were exposed to *Borrelia*-infected nymphs from Phase 1. (b) *Borrelia*-naïve *I. ricinus* and *I. frontalis* larvae were allowed to feed on the birds of Phase 2 (a) (xenodiagnoses) and were screened for *Borrelia* infections when moulted to nymphs.

Fig. 2. Mean infection prevalence (+ 1 standard error) of *Borrelia burgdorferi* s.l. in *Ixodes ricinus* (small circles) and *I. frontalis* (small triangles) xenodiagnostic larvae ('*Xen.0*', '*Xen.1*', '*Xen.2*', '*Xen.3*') that fed on naïve blackbirds that were exposed twice with *I. ricinus* (large grey circles) and *I. frontalis* (large grey triangles) *Borrelia* infected nymphs ('*Inf. 1*' and '*Inf. 2*'). Arrows indicate the transstadial persistence from engorged to moulted xenodiagnostic larva. Proportions of *Borrelia* genospecies found in positive ticks are represented by different colours in the legend of the pie charts. Number of positive ticks of which the *Borrelia* genospecies was successfully identified is indicated next to each of the pie charts. Pie charts for *Xen.2* and *Xen.3* combine the information of both engorged and moulted larvae. We refer to Tables 1 and Table 2 for the specific details on values, and Fig. 3 for the Gannt chart for the dates of the procedures.

Fig. 3. Gantt chart of the study design. See main text for further details.

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	Average n° of infected samples /tested samples per bird (%)		Overall n° of infected samples /tested samples (%)	
	Fed	**Moulted**	**Fed**	**Moulted**
Phase 1: Ability to acquire *Borrelia* spirochetes:				
I. ricinus xeno. larvae (Belgium only; N = 13 birds) – 8 Mar.	0.88/1.29 (79 ± 9)	-	15/ 22 (68)	-
I. ricinus larvae – Portugal: 19 Feb. and 6 Mar.; Belgium: 16 Mar.	-	0.20/3.13 (17 ± 9)		3/49 (6)
I. frontalis larvae – Portugal: 19 Feb. and 6 Mar.; Belgium: 16 Mar.	-	0.70/4.30 (19 ± 6)		21/ 133 (16)
Phase 2: Testing the vector competence:				
I. ricinus infectious nymphs:				
Infestation 1 (Inf. 1) – 24 Sept.	-	5.00/11.60 (50 ± 20)	-	25/ 58 (43)
Infestation 2 (Inf. 2) – 5 Oct.	-	1.25/5.75 (29 ± 24)	-	5/23 (22)
I. frontalis infectious nymphs:				
Infestation 1 (Inf. 1) – 24 Sept.	-	2.23/8.46 (22 ± 8)	-	29/ 110 (26)
Infestation 2 (Inf. 2) – 5 Oct.	-	1.69/3.92 (38 ± 11)	-	22/51 (43)
I. ricinus xeno. larvae:				
Before exposure to nymphs (Xen. 0) – 14 Sept.	0.00/9.72 (0 ± 0)	-	0/185 (0)	-
Co-feeding with *I. r.* nymphs (Xen. 1) – 27 Sept.	0.00/12.00 (0 ± 0)	-	0/60 (0)	-
Co-feeding with *I. f.* nymphs (Xen. 1) – 27 Sept.	1.23/11.31 (10 ± 4)	-	16/147 (11)	-
Birds double-exposed to *I. r.* nymphs (Xen. 2) – 12 Oct.	6.40/24.80 (39 ± 21)	0.66/6.00 (16 ± 16)	32/124 (26)	2/18 (11)
Birds double-exposed to *I. f.* nymphs (Xen. 2) – 12 Oct.	14.92/23.62 (60 ± 11)	1.18/7.27 (16 ± 7)	194/307 (63)	13/80 (16)
I. frontalis xeno. larvae:				
Birds double-exposed to *I. r.* nymphs (Xen. 3) – 25 Oct.	4.25/12.00 (35 ± 18)	2.00/7.50 (21 ± 13)	17/48 (35)	8/30 (27)
Birds double-exposed to *I. f.* nymphs (Xen. 3) – 25 Oct.	9.23/12.00 (77 ± 10)	3.16/8.00 (34 ± 12)	120/156 (77)	38/96 (40)

Table 2. Distribution of *Borrelia* genospecies that were identified in *I. ricinus* ('*I.r.*') and *I. frontalis* ('*I.f.*') ticks that fed on 23 blackbirds (*Turdus merula*) from the wild as a larvae (*'Phase 1 - larvae - moulted'*) and subsequently fed as nymphs on 18 naïve birds and moulted ('*Phase 2 – nymphs – moulted'*). In addition, the genospecies' distribution is shown for the *I. ricinus* and *I. frontalis* xenodiagnostic larvae that co-fed (*'Phase 2 – Xen. 1 – fed/moulted'*) with infected nymphs of Phase 1, and larvae that fed on the birds after the two consecutive exposures with the infected nymphs of Phase 1 (*'Phase 2 – Xen. 2 & 3 – fed/moulted'*).

	***I. ricinus* vector competence test (5 birds)**					***I. frontalis* vector competence test (13 birds)**				
	Phase 1 ***I.r.* larvae** moulted n (%)	**Phase 2 Inf. 1 + 2** ***I.r.* nymphs** moulted n (%)	**Phase 2 Xen. 1** ***I.r.* larvae** fed/moulted n (%)	**Phase 2 Xen. 2** ***I.r.* larvae** fed/moulted n (%)	**Phase 2 Xen. 3** ***I.f.* larvae** fed/moulted n (%)	**Phase 1** ***I.f.* larvae** moulted n (%)	**Phase 2 Inf. 1 + 2** ***I.f.* nymphs** moulted n (%)	**Phase 2 Xen. 1** ***I.r.* larvae** fed/moulted n (%)	**Phase 2 Xen. 2** ***I.r.* larvae** fed/moulted n (%)	**Phase 2 Xen. 3** ***I.f.* larvae** fed/moulted n (%)
B. turdi	7 (64)	1 (20)	0/ - (0)	5/0 (17)	3/1 (80)	2 (29)	21 (100)	10/ - (100)	184/5 (100)	22/11 (100)
B. valaisiana	4 (36)	4 (80)	0/ - (0)	23/2 (83)	1/0 (20)	2 (29)	0 (0)	0/ - (0)	0/0 (0)	0/0 (0)
B. burgdorferi s.s.	0 (0)	0 (0)	0/ - (0)	0/0 (0)	0/0 (0)	3 (43)	0 (0)	0/ - (0)	0/0 (0)	0/0 (0)
N	18	18	0/ -	32/2	5/4	22	35	15/ -	194/7	24/11

% for Phase 2 larvae is obtained from a pooled set of fed and moulted individuals (fed + moulted).
N: the number of infected ticks for which the *Borrelia* genospecies have been attempted to be identified

Figure 1

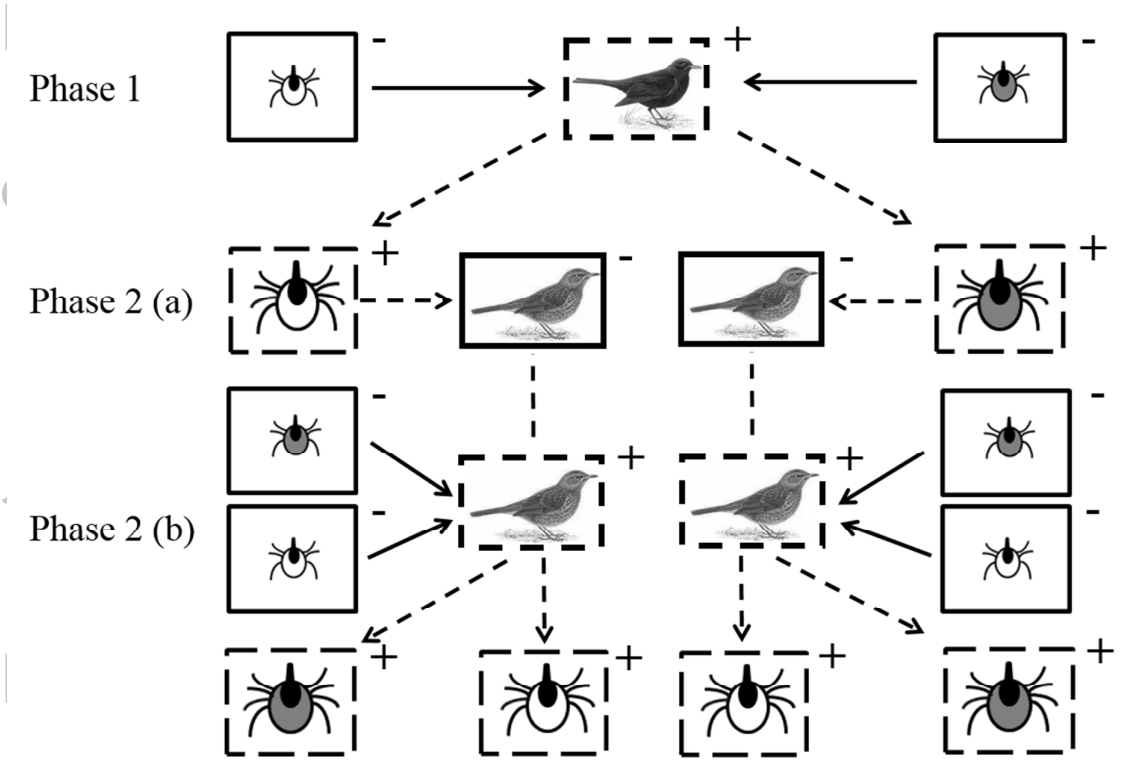

Fig. 1. Graphical overview of the study set-up and results. Dashed lines indicate organisms that are infected with *B. burgdorferi* s.l., solid lines indicate organisms that are *Borrelia*-free. Grey ticks represent *I. ricinus*, white ticks represent the ornithophilic tick *I. frontalis*. Large ticks and small ticks are nymphal and larval stages, respectively. Phase 1: Ability of ticks to acquire *B. burgdorferi* s.l. spirochetes. Wild blackbirds were captured from known endemic areas for *B. burgdorferi* s.l.. and exposed to naïve *I. ricinus* and *I. frontalis* larvae. These fed larvae moulted to the nymphal stage, and were screened for *Borrelia* infections. Phase 2: Testing the vector competence of *Borrelia*-infected nymphs. (a) *Borrelia*-naïve birds were exposed to *Borrelia*-infected nymphs from Phase 1. (b) *Borrelia*-naïve *I. ricinus* and *I. frontalis* larvae were allowed to feed on the birds of Phase 2 (a) (xenodiagnoses) and were screened for *Borrelia* infections when moulted to nymphs.

Figure 2

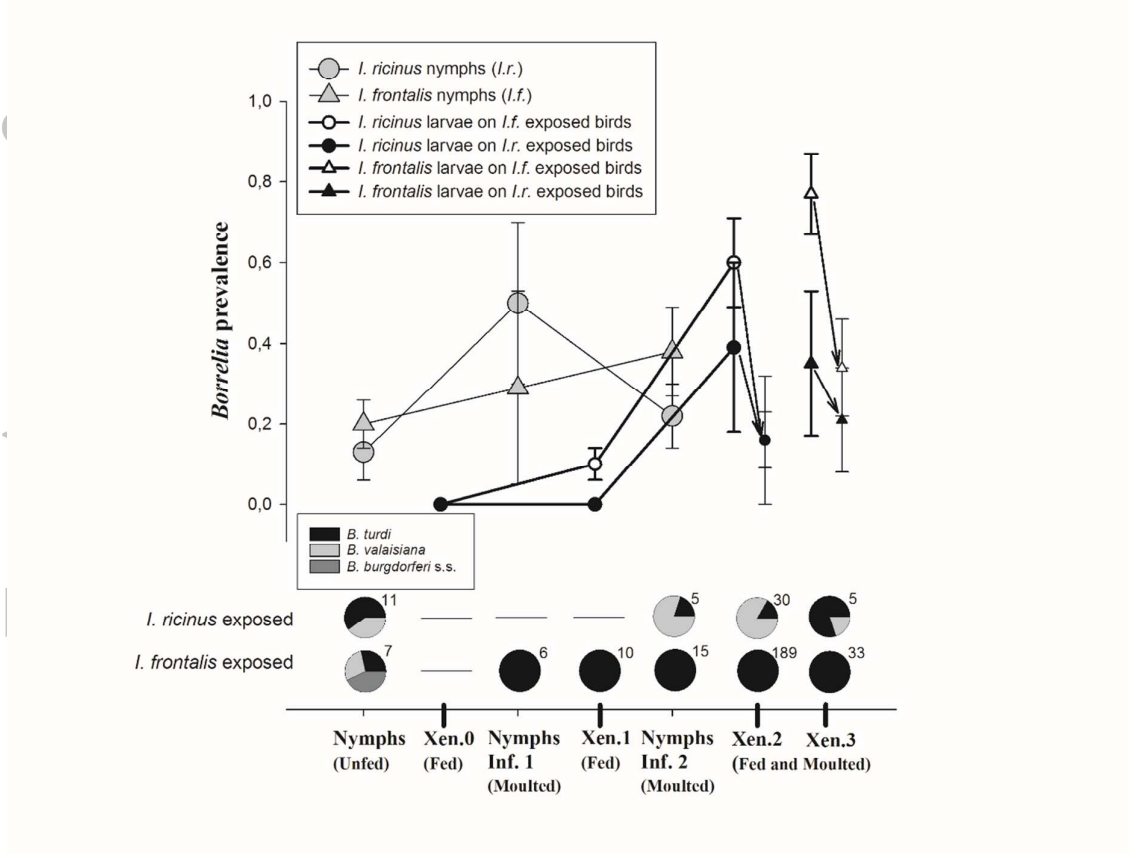

Fig. 2. Mean infection prevalence (+ 1 standard error) of *Borrelia burgdorferi* s.l. in *Ixodes ricinus* (small circles) and *I. frontalis* (small triangles) xenodiagnostic larvae ('*Xen.0*', '*Xen.1*', '*Xen.2*', '*Xen.3*') that fed on naïve blackbirds that were exposed twice with *I. ricinus* (large grey circles) and *I. frontalis* (large grey triangles) *Borrelia* infected nymphs ('*Inf. 1*' and '*Inf. 2*'). Arrows indicate the transstadial persistence from engorged to moulted xenodiagnostic larva. Proportions of *Borrelia* genospecies found in positive ticks are represented by different colours in the legend of the pie charts. Number of positive ticks of which the *Borrelia* genospecies was successfully identified is indicated next to each of the pie charts. Pie charts for *Xen.2* and *Xen.3* combine the information of both engorged and moulted larvae. We refer to Tables 1 and Table 2 for the specific details on values, and Fig. 3 for the Gannt chart for the dates of the procedures.

Figure 3

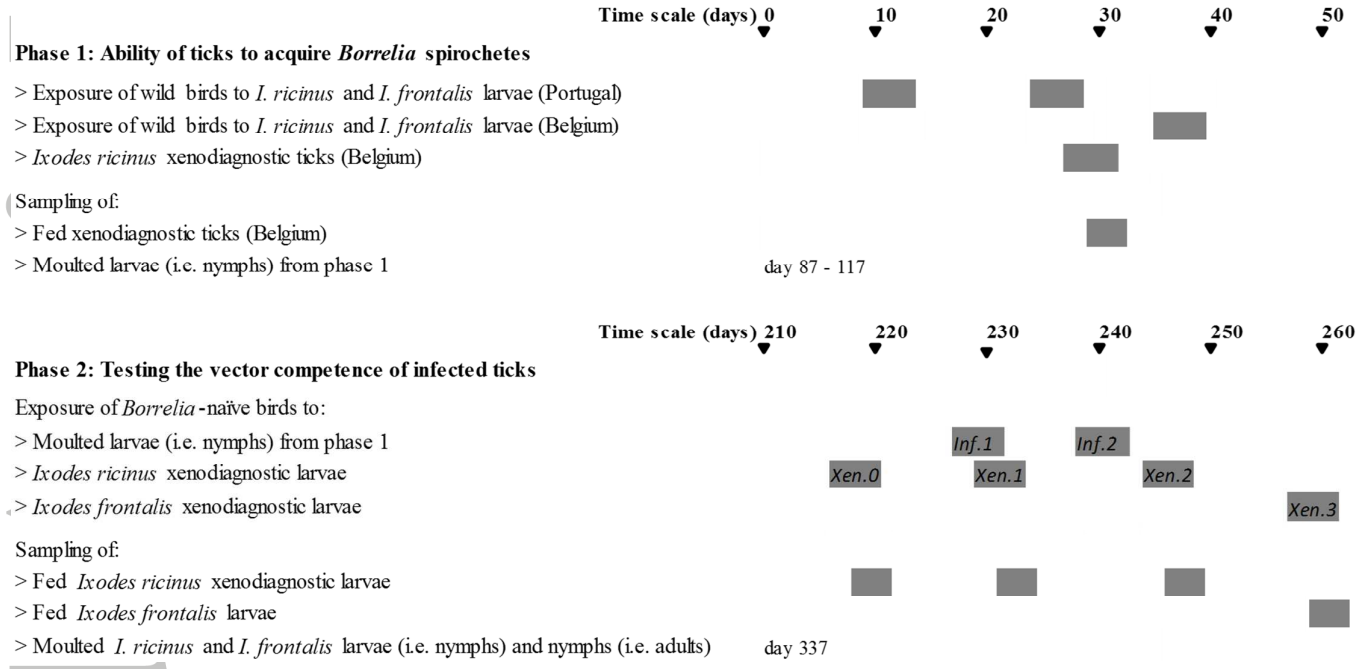

Fig. 3. Gantt chart of the study design. See main text for further details.