HOST MICROBE INTERACTIONS

Getting under the birds’ skin: tissue tropism of *Borrelia burgdorferi* s.l. in naturally and experimentally infected avian hosts

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**Abstract**

Wild birds are frequently exposed to the zoonotic tick-borne bacteria *Borrelia burgdorferi* sensu lato (s.l.), and some bird species act as reservoirs for some *Borrelia* genospecies. Studying the tropism of *Borrelia* in the host, how it is sequestered in different organs, and whether it is maintained in circulation and/or in the host’s skin is important to understand pathogenicity, infectivity to vector ticks and reservoir competency.

We evaluated tissue dissemination of *Borrelia* in blackbirds (*Turdus merula*) and great tits (*Parus major*), naturally and experimentally infected with *Borrelia* genospecies from enzootic foci. We collected both minimally invasive biological samples (feathers, skin biopsies and blood) and skin, joint, brain and visceral tissues from necropsied birds. Infectiousness of the host was evaluated through xenodiagnoses and infection rates in fed and moulted ticks. Skin biopsies were the most reliable method for assessing avian hosts’ *Borrelia* infectiousness, which was supported by the agreement of infection status results obtained from the analysis of chin and lore skin samples from necropsied birds and of their xenodiagnostic ticks, including a significant correlation between the estimated concentration of *Borrelia* genome copies in the skin and the *Borrelia* infection rate in the xenodiagnostic ticks. This confirms a dermatropism of *Borrelia garinii*, *B. valaisiana* and *B. turdi* in its avian hosts. However, time elapsed from exposure to *Borrelia* and interaction between host species and *Borreli*a genospecies may affect the reliability of skin biopsies. The blood was not useful to assess infectiousness of birds, even during the period of expected maximum spirochetaemia. From the tissues sampled (foot joint, liver, spleen, heart, kidney, gut and brain), *Borrelia* was detected only in the gut, which could be related with infection mode, genospecies competition, genospecies-specific seasonality and/or excretion processes.

**Keywords** Microorganism tropism · Avian reservoir hosts · Tick-borne pathogens · Lyme borreliosis · Skin biopsies

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**Introduction**

Microorganism tropism, which is the ability to infect a specific organ or tissue of the host, is the most causal factor for disease pathology and clinical disease manifestations in the hosts. Survival of a microorganism within a host depends on its ability to remain in latent states in immunoprivileged tissues (e.g. hair follicles, skin, extracellular matrix of interstitial tissues) evading the host immune system [1–3]. As microorganism transmission modes and efficiency are strongly linked to its physical location, tropism is very important for microorganism fitness, affecting its survival, proliferation and dissemination. This is because microorganism tropism (a) affects the probability for direct transmission between hosts (e.g. influenza virus infecting the upper respiratory tract of hosts [4]), (b) influences their availability to vectors (e.g. infection relapses in which microorganisms peak in the bloodstream becoming...
available to blood-sucking vectors—e.g. avian malaria [5]) and (c) may affect the host’s behaviour promoting the completion of its life cycle (e.g. multi-host parasites infecting the brain of the intermediate host altering its behaviour to facilitate predation by the definite host [6]). Therefore, microorganism tropism plays a pivotal role in disease ecology. In addition, the study of pathogen tropism is valuable in disease diagnosis because the simultaneous assessment of infection in different organs reduces the rate of false negatives during latent phases of infection [7]. This study will examine the tropism of some members in the *Borrelia burgdorferi* sensu lato (s.l.) bacterial complex, which comprises over 20 genospecies [8–10]. Those include pathogenic and non-pathogenic genospecies to humans, vectored by *Ixodes* sp. ticks, and presenting different associations with vertebrate reservoir hosts.

*Borrelia burgdorferi* s.l. (from here onwards ‘*Borrelia*’) present different abilities to invade host tissues. The skin of the vertebrate host is the first physical and immune barrier encountered by the spirochetes after the bite of the vector tick, and it constitutes an interface and filter which is permeable to only some strains. Those will disseminate from the feeding lesions and infiltrate other host tissues, whereas others remain localised in the area of the tick bite [11, 12]. As there is different tissue tropisms among *Borrelia* genospecies due to differential binding patterns to host cells and allelic variations in chemotaxis genes, the symptoms of infection depend on the specific *Borrelia* genospecies involved [11, 13]. Pathogenicity manifestations in humans include erythema migrans at the tick bite site (skin infection often associated with *B. afzelii* infection [9]) and tertiary symptoms by *Borrelia*’s local actions at corresponding tissues, including degenerative arthritis (joints—*B. burgdorferi* sensu stricto [14]), lesions in the central and peripheral nervous system (*B. garinii* [9]) and myocarditis (heart—*B. burgdorferi* sensu stricto [15]).

In Europe, wild birds are often infested with the tick vectors, especially ground-foraging birds, which are frequently exposed to infected *Ixodes ricinus* ticks in undergrowth vegetation [16, 17]. *Borrelia* infection rates in ticks feeding on wild birds may be as high as 37% (Norte, A.C. unpublished; [18]); however, variation among songbird species in tick exposure and infectiousness is large [19]. In Europe, there are three genospecies associated with songbirds: *B. garinii*, *B. valaisiana* and *B. turdi* [19]. *Borrelia garinii* is the most virulent for humans ( Lyme neuroborreliosis), while the pathogenicity and tropism of the other two genospecies are currently unknown. The study of *Borrelia* tissue tropism in avian hosts has so far been restricted to very few captive-bred domesticated bird species, with limited relevance for the *Borrelia* natural enzootic cycle. In those artificially *Borrelia*-inoculated birds, no detectable clinical signs of disease and gross or histopathological lesions were detected, although viable spirochetes were cultured or detected from visceral organs [20–22]. In mammal laboratory models, symptoms could be linked to the location of the spirochetes that have been artificially administered, but only with variable success [11, 23]. In contrast, natural reservoir hosts usually show little or no symptoms of *Borrelia* infection [24–26].

It has been suggested that, after an infectious period in the skin, *Borrelia* can persist in other immune-privileged sites (mainly extracellular matrix of interstitial tissues, basement membranes, collagen [3]) escaping effective clearance by the immune system [27]. Similar patterns are observed in some ubiquitous avian parasites such as *Plasmodium* and several other Haemosporidia (e.g. *Haemoproteus* and *Leucocytozoon*), which may be hidden in visceral organs during certain periods of their life cycles inside the vertebrate host, and present in cells of the peripheral bloodstream before transmission towards an arthropod vector [7]. Spirochetes in the skin may persist at low densities, undetectable by molecular techniques, but which are still infective to feeding ticks [28]. During periods of stress-induced immunosuppression, the infections may reactivate, thus increasing the chances of isolation or detection in peripheral tissues as was the case in redwings (*Turdus iliacus*) under migratory stress [29]. The study of *Borrelia* tropism in the vertebrate host is important because the occurrence and duration of spirochetaemia, i.e. presence of spirochetes in the circulating blood, and how the infection persists in the host’s skin and other tissues is key to understanding their pathogenicity and transmission efficiency. It will also be useful to assess the utility of biological samples collectable from wild birds as indicators of their infectious status. Although *Borrelia* has been detected or isolated from the blood of wild birds, the success of detection from skin biopsies has been generally higher [30, 31]. Different combinations of hosts and *Borrelia* strains likely yield different tropism patterns and transmission outcomes, which so far have not been investigated in wild songbirds.

Among the European birds that are known to facilitate *Borrelia* transmission, blackbirds (*Turdus merula*) and great tits (*Parus major*) are the most common [32]. Both species have been experimentally tested for their reservoir competence via detections in the ticks that fed on them [30, 33–35]. Great tits and blackbirds were shown to amplify *B. garinii* and *B. valaisiana*, and blackbirds also *B. turdi*. In this study, we evaluate tissue dissemination of these three genospecies in birds naturally and experimentally infected through exposure to vector nymphs in previously reported experiments ([34, 35]; see Fig. 1 for an overview of the study designs). In addition to the *Borrelia* screening of vital organs (liver, spleen, heart, kidney, gut and brain) and foot joint, we assessed whether minimally invasive collectable samples (blood, skin biopsies, head feather pulp) that can be easily collected from wild birds in the field, reflect the infection and infectiousness of the bird and hence provide a monitoring technique usable in the field.
Materials and Methods

Blackbirds and great tits were either captured in the wild or bred in captivity and were either naturally infected (blackbirds) or experimentally infected via infected nymphs (blackbirds and great tits; Fig. 1). Infection was assessed through the molecular analysis of tissues and fed ticks (larva and nymphs). Details of these procedures are given below.

Wild Blackbirds’ *Borrelia* Infection

Thirteen wild blackbirds were captured using mist nets in a peri-urban *Borrelia* enzootic foci from 10 to 27 February 2015 in Belgium (51° 19’ N, 4° 58’ E). The birds were weighed (in g), their tarsus length (in mm) was measured, they were inspected for attached ticks (which were removed) and then, they were taken into captive conditions. They were

Grey ticks represent *I. frontalis* and black ticks represent *I. ricinus*; larvae are represented by tick icons with three pairs of legs and nymph and adults with four pairs of legs. Large ticks belonging to the same developmental stage represent engorged ticks.

Figure 1 Graphical overview of the transmission experiments performed on wild blackbirds (a), experimental blackbirds (b) and great tits (c). Infection status is represented within circles by (−) negative, (+?) likely positive and (?) unknown. Birds in black represent wild birds of unknown infection status; birds in grey represent naïve birds raised in captivity.
maintained indoors in individual cages with unlimited access to food and water. Individuals’ infection status was assessed through xenodiagnoses: a first batch of 10 naïve I. ricinus larvae from a laboratory colony (IS Insect Services GmbH, Berlin, Germany) was placed on each bird within 7 days after capture, and a second batch of a mix of 50 I. ricinus and I. frontalis larvae 5 days later (for xenodiagnoses and also to obtain challenge nymphs of these two tick species—see below). Birds with larvae placed underneath their crown feathers were immobilised inside an air-permeable cotton bag for 2 h (size 20 cm × 15 cm) inside a darkened cage which kept them inactive and facilitated tick attachment. After tick exposure, the birds were placed back in their individual cages with a wire mesh floor (40 cm × 80 cm) and a plastic tray underneath containing damp filter paper and edges streaked with Vaseline, all to prevent detached larvae from escaping. The engorged larvae that dropped through the mesh were collected for Borrelia screening (methods detailed below). Part of the ticks of the second batch were maintained in a climate room (16-h:8-h light:dark photoperiod; 25 °C:15 °C temperature cycle; > 90% humidity) until moult (‘challenge nymphs’, see below) [34]. Sixteen to 25 days after capture, a blood sample was collected from the brachial vein into EDTA tubes and a skin biopsy (< 2 mm²) was collected from the chin area. This area was chosen because almost all ixodid ticks in the Holarctic prefer the heads of songbirds above any other body part (Fracasso et al. 2019, in press) and it should increase the probability of detecting an infection. Blood and skin biopsies were maintained at − 80 °C until molecular analysis for Borrelia infection. All birds were released after experimental procedures.

**Experimental Blackbirds’ Borrelia Infection**

Nineteen naïve juvenile blackbirds (40–50 days old) were obtained from a local breeder (Houthalen, Belgium) in mid-September 2015. These experimental birds were maintained in individual cages with unlimited access to food and water and a 12-h:12-h light:dark photoperiod. The experimental birds were randomly divided in two groups in a scheme stratified by sex (females:males): ‘control’ (n = 2; 1:1) and ‘exposed to challenge nymphs’ (n = 17; 9:8). The control birds were infested with I. ricinus nymphs from a laboratory colony. The exposed birds were infested randomly with either I. ricinus or I. frontalis nymphs that were presumably infected (see previous paragraph) as part of a study on vectorial capacity of I. ricinus and I. frontalis for avian-associated Borrelia genospecies [34]. Birds were exposed twice with 20 challenge nymphs, by placing the ticks underneath the bird’s head feathers. The two consecutive infestation sessions were 12 days apart. Tick loads were within the natural infestation range for this bird species [36]. Subsequently, 60–70 xenodiagnostic I. ricinus larvae were placed on the birds to verify whether they acquired the infection via the ‘challenge nymphs’ (6 days after exposure to the second nymphal exposure).

Of the 17 blackbirds exposed to challenge nymphs, 4 individuals (3 females and 1 male) did not produce infected xenodiagnostic ticks and were considered uninfected. None of the control birds yielded infected xenodiagnostic ticks, as was expected. Seven days after the exposure to the xenodiagnostic larvae, we took a blood sample (into an EDTA-coated tube) and collected a skin biopsy from the chin area. Birds naturally died (n = 7) or were euthanised (n = 12) 56–63 days after Borrelia exposure to challenge nymphs. They were preserved at − 80 °C until necropsy.

**Experimental Great Tits’ Borrelia Infection**

Great tits were obtained by collecting nestlings from parasite-free nest boxes in the wild and transferring them with their parents (10 broods) into tick-free aviaries (see [35, 37] for details). Young birds, 9 weeks of age, were infested with field-collected I. ricinus nymphs from an enzootic area (Borrelia infection rate in questing ticks = 12.2%), 3 times in succession (17 ticks per batch) separated by 5–6 days. Birds were kept in similar cages as in the blackbird experiments. Fed nymphs were collected from the trays and kept at 25 °C and > 90% humidity until moult, after which they were analysed for Borrelia infection. Three birds died during the experimental infestation (one after the second infestation and two after the third infestation), and four died for unknown reasons after the experimental procedures were completed. Overall, the 7 birds died 17–96 days after the first exposure to ticks.

**Ethical Approval**

All bird captures were performed under licences of the Agency for Nature and Forests (Flemish Government, Belgium) and experimental setups were approved by the Ethics Committee for Animal Experiments of the University of Antwerp (2009-32 and 2014-49).

**Laboratory Procedures**

A small aliquot of blood (ca. 30 μL) was collected from the EDTA tube containing the blood sample of experimental blackbirds into a capillary tube with no anticoagulant. This was centrifuged at 14,000g for 10 min and theuffy coat fraction plus the plasma were used for DNA extraction because Borrelia concentrate in theuffy coat layer during centrifugation [38]. Whole blood,uffy coat fractions and skin biopsies from blackbirds were maintained at − 80 °C until further analysis.

During necropsies, we collected samples of feathers from the head and skin (pooled sample from chin and lore areas),
the foot joint, heart, liver, lungs, spleen, gut, kidneys and brain. Due to the birds’ emaciation state and friable state of the organs of some individuals, spleen could only be collected from 8 birds.

DNA was extracted from whole blood, buffy coat fraction, skin biopsies and tissue samples collected during necropsies using Qiagen DNAeasy blood and tissue kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions with the following details: starting material for DNA extraction was 10 μL of blood and all available material of buffy coat layer. Elution was performed in 50 μL (buffy coat layer), 100 μL (whole blood) and 80 μL (remainder tissues) of AE. DNA was extracted from the calamus of a pool of five feathers from the head of each bird with 5% Chelex 100 (Bio-Rad, Hercules, USA). Briefly, the calamus was incubated at 56 °C for 2 h in a solution of 5% Chelex and proteinase K, boiled for 8 min, vortexed and centrifuged for 5 min at 15000 g to separate the supernatant. After extraction, DNA concentration was measured in NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). DNA concentration in the samples (assumed as mostly hosts’ DNA) was adjusted with ultrapure DNase-free water to a final concentration below 100 ng/μL to avoid PCR inhibition by excess DNA. DNA from xenodiagnostic larvae was extracted using Qiagen DNAeasy blood and tissue kit (Qiagen, Hilden, Germany). Moulted ticks DNA (from experimental great tits; adults) was extracted by alkaline lysis extraction [39].

Borrelia DNA was detected and quantified in bird tissues by quantitative polymerase chain reaction (qPCR) targeting the flaB gene following the protocol of [40] for Borrelia and using a serial dilution quantification standard of B. turdi (isolate T2084A) [41] in a LightCycler 480 (Roche Molecular Systems, Pleasanton, CA, USA). DNA was extracted from the B. turdi isolate T2084A (3rd passage) using the Qiagen DNAeasy blood and tissue kit. DNA was eluted in 100 μL of ultrapure DNase-free water and its concentration estimated with Qubit DNA quantification kit (Invitrogen, Carlsbad, CA, EUA). The DNA concentration measured was assumed to be mainly from Borrelia and assuming a genome size of approximately 1.3 × 10^6 bps [42], we created a serial dilution of standards ranging from an estimation of 10 to 10^6 genome copies per microlitre. We used the estimation of the number of Borrelia genome copies in each tested sample given by the cycler standard curve to extrapolate the number of Borrelia genome copies per host’s tissues DNA weight (in ng). We recognise that primer and probe ability to bind to the DNA of different Borrelia genospecies present in our study may differ, and this could affect our estimations of Borrelia genome copies in samples containing DNA of other Borrelia genospecies than B. turdi (the quantification standard used in our study). But the primers’ and probe’s sequences are very conserved among genospecies, with minimal differences among them (maximum of 1 nt per annealing sequence [43] and our own data), suggesting similar sensitivities of this protocol among genospecies. All quantitative PCR runs included seven negative controls throughout the plate and had efficiencies (the fraction of target molecules that are copied in one PCR cycle) above 1.8.

Borrelia infection in ticks was detected by a duplex quantitative polymerase chain reaction (qPCR) targeting ospA and flaB genes, as described in [44] and a nested PCR targeting the flaB gene using the primers of [45]. Borrelia genospecies were identified in positive samples by sequencing the amplicons of the flaB gene (obtained by qPCR in tissue samples or nested PCR in xenodiagnostic ticks from wild blackbirds) and of the variable 5S–23S (rrfA-rrlB) intergenic spacer region [46]—remainder xenodiagnostic ticks from blackbirds and fed and moulted ticks from great tits.

**Statistical Analyses**

Infection rates in birds obtained using different approaches (xenodiagnoses, analysis of skin biopsies and skin samples) was compared with a chi-square test (experimental blackbirds) or, because of the low sample size, with Fisher’s exact test (wild blackbirds). Infection rates in xenodiagnostic ticks from experimental blackbirds were correlated with the estimated Borrelia concentration in their skin samples with a Spearman correlation. Results are presented as mean ± standard error (SE).

**Results**

**Borrelia Infection in Minimally Invasive Biological Samples (Skin Biopsy and Blood)**

All 13 blackbirds captured in the wild were found to be infectious to xenodiagnostic ticks. Their skin biopsies revealed an infection rate of 76.9% (10/13), which is not statistically different from the rate derived from the xenodiagnoses 4–24 days later (Fisher exact test, *P* = 0.22). The molecular analysis of the blood failed to detect any Borrelia positive individual (0/13; Table 1).

Both control birds in the blackbird experiment showed to be free of *Borrelia*, based on the screening outcomes of ticks, blood (total blood and buffy coat fraction), and skin. Of the 17 experimentally exposed birds to challenge nymphs, 13 (76.5%) individuals tested positive by xenodiagnoses but only 3 (17.6%) had positive skin biopsies collected 7 days later (Table 1; Fig. 2). This represents a significantly different detection probability between xenodiagnoses and skin biopsy analyses (*χ^2^ 1,12 = 11.79, *P* < 0.001; Fig. 2). None of the birds presented positive feather pulpa, blood or buffy coat fractions.

Of the 13 infectious wild blackbirds, 7 (53.8%) transmitted *B. valaisiana* to xenodiagnostic ticks, 5 (38.5%) transmitted...
Table 1  Overview of *Borrelia* tropism patterns in infectious (i.e. that produced positive *Borrelia*-infected ticks) blackbirds (*Turdus merula*) and great tits (*Parus major*) in minimally invasive collectable tissues and tissues collected during necropsies. Values are means ± SE

| Tissue                        | n infected/tested | Estimated *Borrelia* genome copies/ng hosts’ DNA in infected tissues | Genospecies in tissue (%) | *Borrelia* infection rate in fed/xenodiagnostic ticks (%; stage; number of days elapsed between tick feeding and tissue collection) | Genospecies in fed/xenodiagnostic ticks |
|-------------------------------|-------------------|---------------------------------------------------------------|---------------------------|----------------------------------------------------------------|----------------------------------------|
| Blackbirds Wild              |                   |                                                               |                           |                                                                |                                        |
| Blood                         | 0/13              | na                                                           | na                        | 67.17 ± 7.2 (L; 4–24 d)                                          | 61.54 ± 14.0 *B. valaisiana*            |
| Buffy coat                    | na                | na                                                           | na                        |                                                                |                                        |
| Skin biopsy                   | 10/13             | 16.8 ± 6.02                                                  | *B. turdi* (40); *B. valaisiana* (60)                           |                                                                |                                        |
| Blackbirds Experimental       |                   |                                                               |                           |                                                                |                                        |
| Blood                         | 0/13              | na                                                           | na                        | 68.4 ± 8.2 (N; 7 d)                                              | 81.1 ± 7.7 *B. turdi*                   |
| Buffy coat                    | 0/13              | na                                                           | na                        |                                                                | 38.46 ± 18.1 *B. turdi*                 |
| Skin biopsy                   | 3/13              | 2.36 ± 1.71                                                  | *B. turdi* (66.7); undefined (33.3)                            |                                                                | 61.54 ± 21.3 undefined                 |
| Feather pulpa                 | 0/13              | na                                                           | *B. turdi* (75); *B. valaisiana* (25)                           |                                                                |                                        |
| Skin chin and lore            | 8/13              | 4.58 ± 2.07                                                  |                           |                                                                |                                        |
| Gut                           | 0/13              | na                                                           | na                        | 68.4 ± 8.2 (N; 49–55 d)                                          |                                        |
| Other                         | 0/82              | na                                                           | na                        |                                                                |                                        |
| Great tits                    |                   |                                                               |                           |                                                                |                                        |
| Feather pulpa                 | 0/7               | na                                                           | na                        | 66.4 ± 4.2 (A; 17–96 d)                                          | 51.4 ± 11.0 *B. garinii*                |
| Skin chin and lore            | 3/7               | 21.5 ± 102.7                                                 | *B. garinii* (100)                                               |                                                                | 2.9 ± 2.2 *B. valaisiana*              |
| Gut                           | 1/7               | 2.84                                                         | *B. turdi* (100)                                                |                                                                | 5.2 ± 1.5 *B. afzelii*                 |
| Other                         | 0/45              | na                                                           | na                        |                                                                | 40.5 ± 9.6 undefined                   |

L, larvae; A, adults; d, days; na, non-applicable

Others include (n blackbird/ n great tit): foot joint (13/7), liver (13/7), spleen (4/3), heart (13/7), kidney (13/7), lungs (13/7) and brain (13/7)
B. turdi and in one bird, the genospecies could not be identified. Genospecies distribution in the 10 positive skin biopsies was 50% B. valaisiana and 50% B. turdi. However, in 3 out of the 10 positive individuals, in which both screening methods were performed (and in which the genospecies could be identified), the genospecies in the xenodiagnostic ticks did not match those in the skin biopsies. In experimentally infected blackbirds (n = 13), the most prevalent genospecies in positive xenodiagnostic ticks (n = 327 larvae) was B. turdi (mean B. turdi infection rate per individual ± SE = 81.1 ± 7.7 %), but B. valaisiana was also detected in 2 birds with mixed infections (B. valaisiana = 8.3 ± 6.8 %; Table 1 [34]). In two out of the three positive skin biopsies from these birds, the genospecies could be identified as B. turdi, in agreement with data from xenodiagnoses.

Borrelia Infection in Tissue and Organ Samples

The negative control blackbirds did not contain any Borrelia positive tissue sample. In one out of the four experimentally exposed blackbirds that were presumed to be negative (i.e. all xenodiagnostic larvae tested negative), we did find Borrelia (B. garinii) in tissue (gut tissue; estimation of 0.42 Borrelia genome copies per ng hosts’ DNA). From the 13 experimental blackbirds that tested positive by xenodiagnoses, Borrelia was only detected in skin samples collected from the chin and lore areas (n = 8), but not from any other tissue collected during necropsy (foot joint, heart, liver, lungs, spleen, gut, kidneys and brain; Table 1). There was no significant difference in detection probability between xenodiagnostic ticks and analyses of skin from the chin and lore areas (χ² 1,12 = 3.1, P = 0.78, Fig. 2) and there was a significant correlation between Borrelia infection rate in xenodiagnostic ticks from infected experimental blackbirds (68.4 ± 8.2%) and the estimated concentration of Borrelia genome copies in their chin and lore skin samples (4.58 ± 2.1 Borrelia genome copies/ng hosts’ tissue DNA; r_s = 0.81, P = 0.0008, n = 13).

In the 8 positive skin samples from the chin and lore, B. turdi dominated (77.8%), in accordance with data from xenodiagnostic ticks (see above). Borrelia valaisiana was detected in skin samples from two birds (22.2%; Fig. 3), of which one yielded xenodiagnostic ticks infected with B. valaisiana and B. turdi, while the other bird only originated B. turdi-infected ticks.

The analyses of the fed and moulted nymphs from experimentally infested great tits (n = 7) with field-collected nymphs

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Fig 2 Borrelia infection rate in experimental blackbirds Turdus merula (exposed to challenge Ixodes sp. nymphs) assessed through xenodiagnoses and analyses of skin biopsies (13–15 days after exposure; 7 days after xenodiagnoses) and skin samples collected during necropsy (41–66 days after exposure; 49–55 days after xenodiagnoses). ns, not significantly different; *P < 0.001
revealed that all the birds produced infected adult ticks (*Borrelia* infection rate = 66.4 ± 4.2%, *n* = 205), and these high infection rates were obtained in an increasing way in successive infestations [31] suggesting transmission via gradual dissemination in bird tissue, rather than transmission via co-feeding between ticks alone [46]. Furthermore, the high *Borrelia* infection in ticks compared with their initial infection rate when collected unfed from the field (12.2%, 173/1414) indicates that the nymphal exposed birds amplified and facilitated the transmission of *Borrelia*, and thus, that these birds were infectious (see [35, 47] for further details and discussion). From the 7 infectious great tits, only skin samples from the chin and lore (*n* = 3, 42.8%; estimated concentration of *Borrelia* genome copies/ng hosts DNA = 215.3 ± 102.7) and gut samples (*n* = 1, 14.3%; estimated concentration of *Borrelia* s.l. genome copies/ ng hosts DNA = 2.84) were found to be infected by *Borrelia*. No other organs were infected (Table 1).

While a variety of genospecies was detected in the great tit fed ticks (mean infection rate ± SE *B. garinii* = 34.2 ± 6.4%; *B. valaisiana* = 2.0 ± 1.4%; *B. afzelii* = 3.5 ± 1.0%; see [35] for further details; Fig. 4), only the most prevalent genospecies, *B. garinii*, was found in their skin samples. Although *B. turdi* was not present in any of the ticks, we found this genospecies in the gut sample of one of the birds.

**Discussion**

Tissue tropism of the bacterial complex *B. burgdorferi* s.l. was studied using two infected model bird species by screening skin samples and tissues from several internal organs. Here, by using hosts that contribute to the European *Borrelia* enzootic cycle in nature (blackbird and great tit), in comparison with previous studies on domesticated bird species, we added new substantial insights on *Borrelia* pathogenicity, amplification and transmission potential in common avian European reservoirs.

We evaluated the utility of minimally invasive collectable biological samples (head feather pulpa, skin biopsies and blood) to assess the infectiousness of wild songbirds without taking them into captivity for xenodiagnosis. From all tissues assessed in our study, head skin was the best proxy for the infectiousness of the birds. Although no statistically significant difference was found in the proportion of infected birds when comparing the screening outcomes of the xenodiagnoses and skin biopsies (evaluated 4–24 days apart) in wild

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**Fig 3** *Borrelia* genospecies distribution in a xenodiagnostic ticks from infectious experimental blackbirds *Turdus merula* (*n* = 13) and b their positive chin and lore skin biopsies (*n* = 8) collected during necropsy. Mixed = *B. turdi* and *B. valaisiana*

**Fig 4** *Borrelia* genospecies distribution in a ticks fed on infectious experimental great tits *Parus major* (*n* = 7) and b their chin and lore skin biopsies (*n* = 3) collected during necropsy. Mixed = *B. garinii* and *B. afzelii* (*n* = 3), or *B. garinii*, *B. afzelii* and *B. valaisiana* (*n* = 2)
blackbirds, head skin screenings slightly underestimated the birds’ infectiousness. Skin biopsies were successfully used to assess *Borrelia* infection status in previous studies on birds [30, 33, 48], small mammals [49–51], lizards [52, 53] and several *Borrelia* genospecies were reported from skin biopsies collected from wildlife reservoir hosts [31, 51, 54–56]. This may be a useful methodology to assess hosts’ relative contribution to *Borrelia* maintenance in nature, as viable spirochetes should persist in the skin as it forms the interface where transmission with the ticks occurs [21] and, therefore, the skin is likely the most important source of infection for feeding ticks. Ticks prefer the birds’ head skin, and, as shown in our study, tick infection rates significantly correlate with the head skin’s infection status. Also, the bird’s skin temperature seems to be in the optimal range for *Borrelia* growth, being a bit lower than internal organs [57] as suggested by other studies in which *Borrelia* showed preference for colonising bird’s skin [20, 21]. *Borrelia* persisting for long periods and in relatively high numbers in the skin of their reservoir hosts was also documented from rodents infected with *B. afzelii* [28]. Despite the promising outcome of our results, the use of skin samples to detect *Borrelia* is subject to some criticism. First of all, *Borrelia* was suggested to show a patchy distribution in the vertebrate hosts’ skin [50] and, therefore, it is possible to miss an infection when sampling occurs at a skin area that was not infected. This could be particularly important in early stages of infection before spirochetes disseminate from tick-feeding site and could explain why infection rate based in skin was lower than on xenodiagnoses [33, 49, 50, 58]. Our experimental data corroborated that the sensitivity and reliability of skin biopsies may be affected by the time since exposure, as well as the *Borrelia* genospecies involved (see below). Our data show that skin tissue collected later in the blackbird experiment (during necropsies; i.e. 53–66 days after exposure to *Borrelia*) was more reliable for infection status assessment than that collected early in the experiment (i.e. 13–25 days after exposure). We suggest that the infections by the *Borrelia* genospecies present in birds in our study were localised and restricted to the tick-feeding sites in the initial phase of infection, before spreading over the skin and other organs. Also, *Borrelia* in skin may persist in low, undetectable levels by PCR, but still high enough to infect feeding ticks [28]. Occurrence (and timing) of infection relapses and *Borrelia* redistribution in the organism, possibly related to stress-induced immunosuppression by long-term maintenance in captivity, could also affect detectability in skin tissue. The predominant genospecies detected in the skin from experimental blackbirds was *B. turdi*, and this was generally in agreement with data obtained by xenodiagnoses. In the naturally infected wild blackbirds, both *B. turdi* and *B. valaisiana* infections were detected in skin biopsies, but they were not always reflected in the xenodiagnostic ticks of the respective birds. Potential explanations for the differences in detectability between skin and ticks are unidentified co-infections (only the most dominant genospecies was detected), differential tropism according to genospecies or different competition outcomes among genospecies depending on environment (tick [59] vs. host skin [60]).

From the 7 experimentally infected great tits, *Borrelia* infection was detected in chin and lore skin samples collected during necropsies from only three individuals. Interestingly, these three individuals had naturally died 17–23 days after first exposure to *Borrelia*-infected nymphs. The skin samples collected from birds that died 79–96 days after exposure were not infected. This contrasts with the probability of *Borrelia* detection in skin samples from experimental blackbirds at different time frames after exposure to *Borrelia* and might be attributed to differences in infectivity and reservoir capacity of these two bird species in the long term (i.e. more transient in great tits) and/or differential genospecies tropism. While in blackbirds infections were caused by *B. turdi* and *B. valaisiana, B. garinii* was the dominant genospecies in great tits. *Borrelia garinii* was shown to have the highest temperature for optimal growth when compared with *B. burgdorferi* s.s. and *B. afzelii* [61]. Although the optimal growth temperature for *B. turdi* is not known, it has been originally isolated from cultures growing at 31–32 °C [62]. Because the birds’ skin (and air sacs) are the tissues that present lower temperatures compared with other (internal) organs [57], this could help to explain the differences in detection probability of different genospecies in the bird’s skin with time. However, *B. garinii* has been previously isolated from wild blackbirds’ skin biopsies [30]. Different *Borrelia* genospecies and strains express different surface proteins (e.g. decorin and dermatan sulphate-binding adhesin DlpA), and others that reside in the periplasm [63] that affect the spirochetes’ attachment to cells or to the extracellular matrix of host tissues. These surface proteins, by preferentially binding to host proteins expressed in different target tissues [12, 64, 65] are responsible for the capacity of colonisation of specific tissues. These adhesion properties, balanced by the immune clearance by the host, likely determine the tropism of different *Borrelia* and could explain our different results among genospecies and hosts.

The results of our study on blackbirds (wild and experimentally infected) revealed that blood was not a reliable biological sample to assess avian infection status: none of the blood samples (buffy coat fraction included) was positive in infected birds. Other studies have shown a variable success of *Borrelia* detection from blood samples. Culture of avian blood in growth medium likely facilitates *Borrelia* detection [22, 66–69], but *Borrelia* DNA has also been detected directly from the blood of wild birds [70–73] and experimentally inoculated captive-bred birds [20, 74]. In some cases, a similar infection rate to that assessed by screening ticks collected
feeding on the same birds was reported [68, 69], but it is generally agreed that the sensitivity of blood is lower than that of skin biopsies [22, 30, 31, 33]. Explanations for the variable success of *Borrelia* detection in blood could be either technical or biological. Technical reasons include the detection limit of molecular techniques not being sufficiently low to detect the usual low number of circulating spirochetes in the bloodstream, because, except during periods of spirochaetemia (often transient [25, 75]), *Borrelia* frequently remains in latent states in internal tissues or in the skin [76]. Differences in the timing of sampling during the course of infection can result in variable detection chances of *Borrelia* in the blood, which can only be evaluated experimentally. However, in our study, blood samples collected during periods of expected maximum infectivity to ticks (12–30 days after exposure to *Borrelia* [77]) yielded negative results. Excess hosts’ DNA may potentially inhibit the PCR reaction or preclude the detection of *Borrelia* DNA in, comparatively, lower abundance. However, blood samples with hosts’ DNA concentrations ranging from 50–100 ng/μL (similar to the concentration range of the samples tested in this study), spiked with an estimated number of 10^2 *Borrelia* genome copies, yielded positive results (data not shown). Biological causes may be related to interactions among bird species and *Borrelia* genospecies, resulting in different *Borrelia* tropism patterns. It is also possible that the genospecies in our study show no preference for blood.

From all the other tissues collected during necropsies, *Borrelia* DNA was detected only in two samples from the gut: one from an experimental blackbird and one from a great tit. *Borrelia* was reported to colonize gastrointestinal tissue’s interstitial sites, often associated with collagen, in laboratory immune-deficient mice [78] but *Borrelia* detections in this type of tissue have not been reported in songbirds [22]. Gut tissue could have been contaminated with *Borrelia* DNA on the way to be excreted from the kidney to the cloaca [22]. Urinary routes likely play a role in *Borrelia* excretion as suggested from positive kidney and excreta samples in artificially *Borrelia*-infected birds [20, 22, 74] and positive kidneys and bladder samples in mammals [23, 79, 80].

Curiously, the captive-bred blackbird in the experiment with a positive gut sample did not show to be infectious based on the screening of its xenodiagnostic ticks. In addition, the genospecies detected from the gut was *B. garinii*, which had not been detected in the challenge nymphs and other blackbirds in the same experimental setup. Although *B. garinii* has often been found in wild blackbirds (ticks and tissue [30, 81, 82]), in the presence of a more dominant genospecies (*B. turdi*, in our study), this genospecies could show tropism away from the skin and/or be outcompeted by other genospecies present in the host [34, 60]. *Borrelia garinii* possibly avoids to end up in an incompetent vector species by moving towards the gut [83], as our experiment took place in early autumn, when the vector of *B. garinii* (*I. ricinus*) shows low host-searching activity in our study region [84–86], while the vector of *B. turdi* (*I. frontalis*) does. Another explanation could be that the bird ingested *B. garinii*-infected ticks, although we are not aware of the presence of *B. garinii* challenge nymphs. Gut infection in the experimentally *Borrelia*-exposed great tit was caused by *B. turdi*. This genospecies had not been detected in the ticks that fed on the birds [35]. Possibly, *B. garinii* is better adapted to great tit hosts and could outcompete *B. turdi* that could only survive in detectable numbers in less privileged tissues in terms of transmission potential, such as the gut. A similar reasoning in terms of *Borrelia* phenology could hold in this case: great tits were exposed during summer, the moment when *I. ricinus* is most active. Accidentally, introduced *B. turdi* spirochetes could latently remain in the gut until autumn/winter, when its vector, *I. frontalis*, is more active [87].

None of the other organs (brain, heart, liver, kidney and spleen) showed evidence of infection. Other studies have detected *Borrelia* (DNA or via spirochete isolation) in the liver of both wild and captive birds [22, 88, 89], and in the spleen of captive birds in an early stage of infection, i.e. within 4 weeks after experimental exposure via inoculation [21, 22]. We have sampled birds much later, 53–96 days after the infection which was performed via infected endemic ticks, thus in a way that mirrors natural infection. We hypothesize that differences in the timing of sampling and mode of infection may result in different tropism patterns. While *Borrelia* bacteria in the mammalian host (laboratory mice and wild or captive-bred small mammal reservoirs) have often been detected in joints, causing inflammation [90–92], in the birds in our study, this does not seem a preferential site for the *Borrelia* genospecies.

If co-feeding (i.e. transmission process that occurs between infected and uninfected ticks co-feeding in time or space in the absence of a systemic infection [93]) was the preferential way of dissemination of avian-associated *Borrelia*, this could explain the lack of infection (or low undetectable infection levels) in the blood and distal tissues in birds in our study. However, although co-feeding infection may be an additional mean of *Borrelia* transmission when ticks feed on vertebrates, including birds, the often detected *Borrelia*-infected larvae in larvae-only-infested wild birds [31, 94], the presence of long-lasting infections in the absence of infected nymphs [29, 77] and data from transmission experiments [33–35] suggest that simultaneous tick feeding is not necessary for successful transmission.

It is possible that the concentration of *Borrelia* in internal organs and tissues may be very low, and its DNA concentration being under the sensitivity limit of our real-time PCR assay. *Borrelia* culture performed in parallel to PCR could potentially increase the detection chance (due to *Borrelia* multiplication in the growth media) and allows the distinction of
viable spirochetes (with implications on infectiousness and potential for pathogenicity assessments [95]). Nonetheless, our study shows that skin biopsies are possibly the most useful and practical sampling method for assessing avian hosts’ Borrelia infectiousness, on condition that the initial infection occurs in sufficient time prior to the sampling. It is the least invasive method and does not imply maintaining the hosts in captivity for xenodiagnoses, which is time-consuming, stressful for the animals and maybe logistically difficult. Skin sampling has also been proved useful for monitoring infectiousness of other vertebrates, and presence of other vector-borne infections where transmission occur via the skin (e.g. Rickettsia sp. [96], Onchocerca volvulus [97], Leishmania [98]). The blood, or its buffy coat fraction, was not a reliable sample for assessing Borrelia infectiousness of birds. Furthermore, from the visceral organ samples analysed from the two Borrelia avian reservoir host models used in our study, Borrelia DNA was only detected from gut tissue, which could be related to the excretion processes.

To fully understand the Borrelia tropism and the sensitivity and reliability of skin samples as proxy of the infectious status of birds, we suggest that future studies should further focus on the influence of the time elapsed since initial infection, the host species-dependent Borrelia genospecies distributions and the interaction between genospecies co-infecting the same host (within-host competition and/or facilitation). Furthermore, genospecies-specific seasonality in tropism with respect to the phenology of different tick vectors is a still unsolved research domain that needs further investigation.

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