Distribution of *Borrelia burgdorferi* s.l. and *Borrelia miyamotoi* in *Ixodes* tick populations in Northern Germany, co-infections with Rickettsiales and assessment of potential influencing factors

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Abstract. To determine *Borrelia* spp. (Spirochaetales: Spirochaetaceae) prevalence and species distribution in Northern Germany, *Ixodes* ticks were sampled from April to October in 2018 and 2019 by the flagging method at three locations each in five regions. Analysis by quantitative real-time PCR of 3150 individual ticks revealed an overall prevalence of 30.6%, without significant differences between tick stages (31.7% positive adults, 28.6% positive nymphs). Significant differences were observed in seasonal infection rates, but not between regions, landscape types or sampling years. Analysis of co-infections with Rickettsiales indicated a negative association between *Borrelia* and *Anaplasma phagocytophilum* infection. The most frequent *Borrelia* species differentiated by Reverse Line Blot were *B. afzelii* and *B. garinii/B. bavariensis*, followed by *B. valaisiana, B. burgdorferi* sensu stricto, *B. spielmanii* and *B. lusitaniae*. Furthermore, *B. miyamotoi* was identified in 12.9% of differentiable samples. No effect of region nor landscape type on species composition was found, but significant variations in the distribution at the different sampling sites within a region were observed. The detected monthly fluctuations in prevalence and the differences in intra-regional *Borrelia* species distribution underline the importance of long-term and multi-location monitoring of *Borrelia* spp. in ticks as an essential part of public health assessment.

Key words. *Borrelia, Ixodes inopinatus, Ixodes ricinus, Lyme borreliosis, tick-borne diseases.*

Introduction

Lyme borreliosis (LB), caused by spirochaetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex, is the most common tick-borne disease within the Northern Hemisphere. For Europe, an LB incidence of more than 200 000 annual cases has been estimated, with considerable regional differences (Sykes & Makiello, 2017). For Germany, the resulting annual costs for the national health care system have been estimated at more than 30 million Euros for hospitalization of LB patients and additional 51 million Euros for outpatient care (Müller et al., 2012; Lohr et al., 2015). The most common clinical manifestation of LB is erythema migrans, followed by neuroborreliosis and arthritis, whereas carditis, borrelial lymphocytoma, acrodermatitis chronica atrophicans and eye involvement are rare clinical variants of LB (Strle & Stanek, 2009).

Clinical symptoms of LB may vary according to the different species of the *Borrelia burgdorferi* s.l. complex. To date,
12 different species are known to occur in Europe, whereby *Borrelia afzelii* and *B. garinii* occur most frequently in ticks and as causative agents in human clinical cases (Rudenko *et al.*, 2011; Strnad *et al.*, 2017). An infection with *B. garinii* often causes Lyme neuroborreliosis, whereas *B. afzelii* is the main agent of acrodermatitis chronica atrophicans, but usually induces less specific symptoms (Strle & Stanek, 2009). By contrast with North America, *B. burgdorferi* sensu stricto (s.s.), which is mainly associated with Lyme neuroborreliosis and Lyme arthritis, is less frequently involved in clinical cases in Europe (Rudenko *et al.*, 2011). Additionally, *B. bavariensis*, *B. lusitaniae*, *B. spielmanii*, *B. bissettiae* and *B. valaisiana* also possess pathogenic potential (Rudenko *et al.*, 2011), and *B. kurtzenbachii* has been detected in humans in Europe (Margos *et al.*, 2010). By contrast, *B. carolinensis*, *B. turdi* and *B. finlandensis* have not yet been isolated from humans (Casjens *et al.*, 2011; Rudenko *et al.*, 2011).

Furthermore, the relapsing fever spirochaete *B. miyamotoi* occurs at low prevalences between 0.4 and 2.8% in nymphs and 3.0 and 4.3% in adults of European *I. ricinus* populations (Kubiak *et al.*, 2021). Relapsing fever due to *B. miyamotoi* is associated with a non-specific, flu-like illness in the majority of cases, but in immunocompromised patients, *B. miyamotoi* may cause meningoencephalitis (Kubiak *et al.*, 2021). The average prevalence of *Borrelia in I. ricinus* is estimated at 12.3% in Europe (Strnad *et al.*, 2017). Regarding Germany, *Borrelia* prevalences in questing ticks between 7.2 and 34.1% have been determined in northern parts of the country (May *et al.*, 2015; Blazekaj *et al.*, 2018; Raileanu *et al.*, 2020) and between 12.7 and 27.0% in the southern half (Hildebrandt *et al.*, 2010; Zubrikova *et al.*, 2020). Thus, despite differences in examination methods between the studies, regional differences in *Borrelia* prevalence in Germany are discernible.

Knowledge about the current, regional distribution of *Borrelia* spp. and influencing factors is crucial for public health risk assessment. The present study evaluated the *Borrelia* prevalence and species composition in *Ixodes* ticks in five different regions in northern Germany during two consecutive years. Potential regional, seasonal, biennial and landscape associated influences on *Borrelia* prevalence were investigated. Furthermore, a detailed analysis of co-infection patterns with *Rickettsia* spp. as well as *Anaplasma phagocytophilum* is presented, as the same ticks were investigated with regard to Rickettsiales infections in a previous study (Knoll *et al.*, 2021).

**Materials and methods**

**Tick material**

In 2018 and 2019, questing ticks were collected each month from April to October by the flagging method in five regions located in the northern German federal states Lower Saxony, Bremen and Hesse (Fig. 1). At three locations each in the regions of Bremen (B1–B3), Emsland (E1–E3), Hanover (H1–H3), Kassel (K1–K3) and Uelzen (U1–U3), 15 *Ixodes* ticks were collected per month (five females, males and nymphs each were envisaged). The locations corresponded to different landscape types, comprising four broadleaved (B3, H3, K1, K2), four coniferous (E2, E3, U1, U3) and three mixed forest areas (E1, H1, U2) as well as three urban (B1, H2, K2) and one agricultural area (B2).

The collected ticks were identified by light microscopy (Zeiss SteREO Discovery.V8, Oberkochen, Germany) on the basis of morphological keys described by Estrada-Peña *et al.* (2014, 2018). As *I. ricinus* and *I. inopinatus* are morphologically very similar (Younsi *et al.*, 2020), ticks collected in 2018 were additionally differentiated molecularly by amplifying a part of the 16S rRNA gene and subsequent Sanger sequencing as described by Hauck *et al.* (2019). The genomic DNA was extracted from each individual tick as described elsewhere (May & Strube, 2014; Knoll *et al.*, 2021) and already analysed for infections with *Rickettsia* spp. and *Anaplasma phagocytophilum* in a previous study (Knoll *et al.*, 2021).

**Detection of Borrelia spp.**

Until further investigation, isolated DNA was stored at –20°C. For detection of *Borrelia* spp. DNA, each tick sample was analysed individually by a probe-based quantitative real-time PCR (qPCR) targeting the 5S-23S rRNA intergenic spacer (IGS) as published by Strube *et al.* (2010) with previously described modifications (May *et al.*, 2015). To verify successful DNA isolation, the *Ixodes* ITS 2 region was co-amplified (Strube *et al.*, 2010).

**Borrelia species identification**

* Borrelia qPCR positive samples were subjected to a conventional PCR amplifying a part of the 5S-23S IGS by use
of biotin-linked B5S-Bor forward, BMiya-For forward and 23S-Bor reverse primers (Alekseev et al., 2001; Blazjak et al., 2018). To identify the *Borrelia* species, a Reverse Line Blot (RLB) was conducted as described by Blazjak et al. (2018) and modified by Springer et al. (2020). In addition to the relapsing fever spirochaete *B. miyamotoi*, the employed RLB detects the different species of the *B. burgdorferi* s.l. complex occurring in Europe with exception of the very rare *B. turdi* and *B. finlandensis*. However, *B. bavariensis* is recognized by co-hybridization of the *B. garinii* probe (GA), so that a differentiation between these two species is not possible with RLB. Likewise, no differentiation between *B. burgdorferi* s.s. and *B. carolinensis* can be achieved, as the *B. burgdorferi* s.s. probe (SS) binds to both species. Therefore, samples showing a positive signal with either of these probes were re-amplified and the 5S-23S fragment was custom Sanger sequenced (Microsynth Seqlab, Göttingen, Germany).

**Statistical analyses**

Statistical analyses were carried out in R v. 3.6.1. The effect of different factors on *Borrelia* prevalence in ticks was tested by a generalized linear mixed effects model (GLMM) with binomial error structure and logit-link function (R function ‘glmer’, package ‘lme4’) (Bates et al., 2015). The predictor variables ‘year’, ‘sampling month’, ‘tick developmental stage’, ‘landscape type’ and ‘region’ as well as infection status with *Rickettsia* spp. and *A. phagocytophilum* were defined as fixed factors, while ‘sampling site’ was included as a random factor. In a second model, the developmental stage level ‘adults’ was split into ‘females’ and ‘males’. For factors with more than two levels, multiple comparisons were conducted based on Tukey contrasts using the function ‘glht’ from the package ‘multcomp’ (Hothorn et al., 2008) with single-step-*P*-value adjustment. To evaluate the differences in the *Borrelia* infection rate between the two tick species *I. ricinus* and *I. inopinatus*, a similar GLMM with the added predictor variable ‘tick species’ was calculated based only on the dataset from 2018, containing molecularly identified ticks. Full models were compared with null models containing the random factor in a likelihood ratio test (R function ‘anova’, method = ‘chisq’). 

For each region as well as for each month, differences in *Borrelia* prevalence between 2018 and 2019 were assessed by χ² tests with subsequent Bonferroni correction. The *Borrelia* prevalences of the three sampling sites within each region were compared using χ² tests followed by Bonferroni–Holm correction.

To evaluate the impact of different regions as well as landscape types on the *Borrelia* species composition, a permutational multivariate analysis of variance (PERMANOVA) was conducted based on Bray–Curtis distances of species proportions among the successfully differentiated samples from each site (R function ‘adonis’, package ‘vegan’, 10,000 permutations) (Oksanen et al., 2019). Additionally, differences in the relative species abundance between the three sampling sites in each region were compared using Fisher’s exact test with subsequent Bonferroni–Holm correction. To illustrate possible effects of region and landscape type on the *Borrelia* species composition of each site, a principal component plot based on Bray–Curtis distances was created.

Regarding co-infections with *Rickettsiales*, the relationship between co-infection rates and pathogen prevalences was examined using Spearman rank correlation. Mathematically expected and determined values of co-infections were compared using χ² test.

**Results**

**Tick material and species identification**

A total of 3150 ticks, including 1052 females, 1048 males and 1050 nymphs, were collected. All ticks were morphologically identified as belonging to the *I. ricinus*/*I. inopinatus* complex. Among the molecularly identified ticks from 2018, 94.9% (1495 of 1575) were *I. ricinus* and 5.1% (80 of 1575) were *I. inopinatus* (Knoll et al., 2021). The ratio between *I. ricinus* and *I. inopinatus* at different developmental stages was 96.0% (506 of 527) vs. 4.0% (21 of 527) in females, 94.6% (495 of 523) vs. 5.4% (28 of 523) in males and 94.1% (494 of 525) vs. 5.9% (31 of 525) in nymphs.

**Prevalence of *Borrelia* spp.**

The total *Borrelia* prevalence was 30.6%, with 31.7% infected adults (32.4% females, 30.9% males) and 28.6% infected nymphs (Table 1). The GLMM revealed no significant differences regarding the developmental stage (Table 2, Table S1). Regarding the different regions, the highest prevalence was detected in Hanover with 33.2% and the lowest in Bremen (26.8%). Within the five regions, statistical comparisons revealed local prevalence differences in Emsland as well as in Kasel (Table 1), whereas no statistically significant inter-regional differences were observed (Table 2). Likewise, no significant effect of landscape type was found (Table 2).

Regarding sampling years, the overall *Borrelia* prevalence was 31.6% (497/1575) in 2018, with no significant difference to 2019 (29.7% [468 of 1575]; *P* = 0.203; Table 2). On a regional level, a significant biannual difference was only detected in Hanover (41.0% in 2018 vs. 25.4% in 2019; *χ²* = 16.5, df = 1, *P* < 0.001; Fig. 2). Analyses of the annual differences for each sampling month revealed no statistically significant differences (Fig. 3). Combining data from both years, the overall prevalence was significantly lower in May (27.1% in 2018 and 20.0% in 2019) than in April (35.6%, 32.4%), June (36.9%, 32.0%) and October (30.2%, 42.7%) (*P* < 0.05; Table 2, Fig. 3).

Analysing the data from 2018 regarding tick species, the *Borrelia* infection rate in *I. ricinus* was 31.7% (474/1495) compared with 28.8% (23/80) in *I. inopinatus*. No significant effect of tick species on infection rate was found (*P* = 0.706; Table S2).

**Borrelia species differentiation in ticks**

*Borrelia* species differentiation by RLB was successful in 58.0% (560 of 965) of qPCR positive samples. Differentiation
success was dependent on the number of IGS copies, whereby identification success was 89.9% (98 of 109) among samples containing $\geq 10^4$ SS-23S IGS copies, 81.9% (271 of 331) in samples with $\geq 10^3$ to $< 10^4$ copies, 70.7% (135 of 191) in samples with $\geq 10^2$ to $< 10^3$ copies, 48.3% (42 of 87) in samples with $\geq 10$ to $< 10^2$, and 5.7% (14 of 247) in samples with $< 10$ copies. The most frequently detected species was *B. afzelii* (34.1%), followed by *B. garinii/B. bavariensis* (30.4%) and *B. valaisiana* (22.1%). Additionally, *B. burgdorferi s.s./B. carolinensis* (7.9%), *B. spielmani* (2.7%) and *B. lusitanae* (0.7%) were detected, whereby the latter was only determined at one location in Kassel (Fig. 4). In 12.9% of positive samples, the relapsing fever spirochaete *B. miyamotoi* was detected. Most of the positive ticks were infected by one *Borrelia* species (90.4%), whereas 8.6% of ticks were double and 1.1% triple infected (Table 3).

A high degree of heterogeneity was observed in the species distribution between different sampling sites and regions. Significant intra-regional differences in the *Borrelia* species composition were found in each region with the exception of Bremen (Fig. 4). *Borrelia afzelii* was the most frequently detected species at all three sampling sites in Bremen as well as at two sites in Emsland and one site each in Hanover and Uelzen, whereas *B. garinii/B. bavariensis* were detected more frequently than *B. afzelii* in Kassel as well as at two sites in Uelzen and one site each in Hanover and Emsland (Fig. 4). However, no significant influence of region nor landscape type on the *Borrelia* species composition was detected by PERMANOVA analysis (Table 4, Fig. 5).

Sanger sequencing of the 170 *B. garinii/B. bavariensis*-positive tick samples resulted in *B. garinii* in 84.1% (143 of 170) and *B. bavariensis* in 0.6% (1 of 170) of cases. The remaining samples were not successfully differentiated due to co-infections with other species (Table 3) or inferior sequence quality. Out of the 44 *B. burgdorferi s.s./B. carolinensis* positive samples, 77.3% (34 of 44) were identified as *B. burgdorferi s.s.*, whereas Sanger sequencing failed or resulted in identification of a co-infecting species in the remaining 10 samples.

**Co-infections with Borrelia spp. and Rickettsiales**

In a previous study, an infection rate of 29.6% (931 of 3150) for *Rickettsia* spp. and 6.4% (202 of 3150) for *A. phagocytophilum* was determined in the ticks used in this study (Knoll et al., 2021). The total rate of co-infections with *Borrelia* spp. and Rickettsiales was 9.9% (Table 5), which did not deviate significantly from the mathematically expected value of 11.0% ($\chi^2 = 8.1, df = 1, P = 0.004$). The co-infection rate of *Borrelia* spp. with *Rickettsia* spp. met the mathematically expected value of 9.1% with a detected rate of 9.2% ($\chi^2 = 0.175$) in ticks. The *Borrelia–Rickettsia* co-infection rates determined at each site correlated significantly with the local *Borrelia* prevalence as well as the local *Rickettsia* prevalence (*Borrelia*: rho = 0.69, $P = 0.004$; *Rickettsia*: rho = 0.84, $P < 0.001$). No significant relationship between a *Rickettsia* infection and a *Borrelia* infection was found in the GLMM (Table 2) and a calculated odds ratio (OR) of 1.0 confirmed the independence between these infections. Co-infections with *Borrelia* spp.

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**Table 1.** Local and regional distribution of *Borrelia* spp. prevalence in questing ticks in northern Germany (positives/total ticks).

| Location | Landscape type | % of total ticks | % of nymphs | % of females | % of males |
|----------|----------------|-----------------|-------------|-------------|------------|
| Bremen   | Urban area     | 26.8 (169/630)  | 27.6 (58/210)| 26.7 (56/210)| 26.2 (55/210) |
| B1       | Urban area     | 26.7 (56/210)  | 24.3 (17/70) | 27.1 (19/70) | 28.6 (20/70)  |
| B2       | Agricultural area | 25.7 (54/210) | 32.9 (23/70) | 24.3 (17/70) | 20.0 (14/70)  |
| B3       | Broadleaved forest | 28.1 (59/210) | 25.7 (18/70) | 28.6 (20/70) | 30.0 (21/70)  |
| Emsland  | Mixed forest   | 33.0 (208/630) | 32.9 (69/210)| 31.0 (65/210)| 35.2 (74/210) |
| E1       | Mixed forest   | 37.1 (78/210)  | 31.4 (22/70) | 30.0 (21/70) | 50.0 (35/70)  |
| E2       | Coniferous forest | 37.6 (79/210)| 45.7 (32/70) | 40.0 (28/70) | 27.1 (19/70)  |
| E3       | Coniferous forest | 24.3 (51/210) | 21.4 (15/70) | 22.9 (16/70) | 28.6 (20/70)  |
| Hanover  | Mixed forest   | 33.2 (209/630) | 29.0 (61/210)| 38.6 (81/210)| 28.6 (20/70)  |
| H1       | Mixed forest   | 31.1 (66/212)  | 30.0 (21/70) | 32.9 (23/70) | 30.6 (22/72)  |
| H2       | Urban area     | 32.9 (68/207)  | 20.0 (14/70) | 45.7 (32/70) | 32.8 (22/67)  |
| H3       | Broadleaved forest | 35.5 (75/211) | 37.1 (26/70) | 37.1 (26/70) | 32.4 (23/71)  |
| Kassel   | Broadleaved forest | 29.0 (183/630)| 23.3 (49/210)| 31.9 (67/210)| 31.9 (67/210)|
| K1       | Broadleaved forest | 24.2 (51/211) | 20.0 (14/70) | 26.8 (19/71) | 25.7 (18/70) |
| K2       | Broadleaved forest | 22.9 (48/210) | 18.6 (13/70) | 28.6 (20/70) | 21.4 (15/70) |
| K3       | Urban area     | 40.2 (84/209)  | 31.4 (22/70) | 40.6 (28/69) | 48.6 (34/70)  |
| Uelzen   | Coniferous forest | 31.1 (196/630)| 30.0 (63/210)| 34.0 (72/210)| 29.3 (61/210)|
| U1       | Coniferous forest | 30.0 (63/210)| 22.9 (16/70) | 31.0 (22/71) | 36.2 (25/69) |
| U2       | Mixed forest   | 30.5 (64/210)  | 34.3 (24/70) | 33.8 (24/71) | 23.2 (16/69) |
| U3       | Coniferous forest | 32.9 (69/210) | 32.9 (23/70) | 37.1 (26/70) | 28.6 (20/70) |
| Total    |                | 30.6 (965/3150)| 28.6 (300/1050)| 32.4 (341/1052)| 30.9 (324/1048)|

*Significantly lower prevalence in E3 than in K1 ($\chi^2$ test, $P < 0.001$) and K2 ($\chi^2$ test, $P < 0.001$).

†Significantly higher prevalence in K3 than in K1 ($\chi^2$ test, $P < 0.001$) and K2 ($\chi^2$ test, $P < 0.001$).
Table 2. Results of GLMM testing the influence of different predictor variables on the probability of infection with *Borrelia* spp.

|                        | Estimate | Std. error | z-value | P value |
|------------------------|----------|------------|---------|---------|
| Intercept              | −0.30    | 0.39       | −0.76   | 0.447   |
| Developmental stage    |          |            |         |         |
| Adults vs. nymphs      | 0.16     | 0.08       | 1.88    | 0.060   |
| Sampling region        |          |            |         |         |
| Bremen vs. Emsland     | −0.49    | 0.32       | −1.53   | 0.504   |
| Bremen vs. Hanover     | −0.33    | 0.20       | −1.67   | 0.116   |
| Bremen vs. Kassel      | −0.12    | 0.18       | −0.64   | 0.532   |
| Bremen vs. Uelzen      | −0.43    | 0.32       | −1.34   | 0.395   |
| Emsland vs. Hanover    | 0.16     | 0.25       | 0.64    | 0.524   |
| Emsland vs. Kassel     | 0.37     | 0.31       | 1.20    | 0.221   |
| Emsland vs. Uelzen     | 0.06     | 0.16       | 0.37    | 0.695   |
| Hanover vs. Kassel     | 0.21     | 0.18       | 1.18    | 0.240   |
| Hanover vs. Uelzen     | −0.10    | 0.25       | −0.40   | 0.684   |
| Kassel vs. Uelzen      | −0.31    | 0.31       | −1.01   | 0.832   |
| Year                   |          |            |         |         |
| 2018 vs. 2019          | 0.10     | 0.08       | 1.27    | 0.203   |
| Sampling month         |          |            |         |         |
| April vs. May          | 0.52     | 0.15       | 3.49    | 0.009   |
| April vs. June         | −0.02    | 0.14       | −0.17   | 1.000   |
| April vs. July         | 0.30     | 0.15       | 2.05    | 0.038   |
| April vs. August       | 0.19     | 0.14       | 1.35    | 0.428   |
| April vs. September    | 0.30     | 0.15       | 2.06    | 0.037   |
| April vs. October      | −0.10    | 0.14       | −0.70   | 0.938   |
| May vs. June           | −0.55    | 0.15       | −3.65   | 0.005   |
| May vs. July           | −0.22    | 0.15       | −1.46   | 0.170   |
| May vs. August         | −0.33    | 0.15       | −2.15   | 0.032   |
| May vs. September      | −0.22    | 0.15       | −1.45   | 0.156   |
| May vs. October        | −0.62    | 0.15       | −4.16   | <0.001  |
| June vs. July          | 0.32     | 0.15       | 2.22    | 0.283   |
| June vs. August        | 0.22     | 0.14       | 1.52    | 0.133   |
| June vs. September     | 0.32     | 0.15       | 2.23    | 0.278   |
| July vs. August        | −0.10    | 0.15       | −0.70   | 0.922   |
| July vs. September     | −0.00    | 0.15       | 0.01    | 1.000   |
| July vs. October       | −0.40    | 0.14       | −2.75   | 0.087   |
| August vs. September   | 0.11     | 0.15       | 0.71    | 0.992   |
| August vs. October     | −0.29    | 0.14       | −2.04   | 0.391   |
| September vs. October  | −0.40    | 0.14       | −2.76   | 0.085   |
| Landscape type         |          |            |         |         |
| Agricultural area vs. broadleaved forest | 0.05 | 0.26 | 0.19 | 1.000 |
| Agricultural area vs. coniferous forest | 0.05 | 0.34 | 0.14 | 1.000 |
| Agricultural area vs. mixed forest | 0.18 | 0.38 | 0.47 | 0.988 |
| Agricultural area vs. urban area | −0.22 | 0.26 | −0.84 | 0.904 |
| Broadleaved forest vs. coniferous forest | 0.13 | 0.30 | 0.44 | 0.991 |
| Broadleaved forest vs. mixed forest | −0.00 | 0.25 | −0.00 | 1.000 |
| Broadleaved forest vs. urban area | −0.26 | 0.15 | −1.75 | 0.367 |
| Coniferous forest vs. mixed forest | −0.13 | 0.17 | −0.78 | 0.625 |
| Coniferous forest vs. urban area | −0.39 | 0.30 | −1.32 | 0.644 |
| Mixed forest vs. urban area | −0.26 | 0.25 | −1.06 | 0.801 |
| Co-infections          |          |            |         |         |
| *Rickettsia* spp. co-infected | −0.00 | 0.09 | −0.04 | 0.968 |
| *Anaplasma phagocytophilum* co-infected | −0.36 | 0.17 | −2.09 | 0.037 |

The full model was significantly different from a null model containing only the random factor ‘sampling location’ (χ² = 43.1, df = 18, P < 0.001). Significant P values (≤0.05) are printed in bold.

Std. Error, standard error.

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Fig. 2. Prevalence of *Borrelia* spp. in the five different sampling regions in 2018 and 2019. No significant differences in prevalence were observed between the regions, but a significantly higher prevalence in 2018 than in 2019 was detected in Hanover ($\chi^2$ test, $P < 0.001$). [Colour figure can be viewed at wileyonlinelibrary.com]

Fig. 3. Seasonal distribution of *Borrelia*-positive ticks in 2018 and 2019. Overall, the number of *Borrelia*-positive ticks was significantly lower in May vs. April, June and October ($P < 0.05$). No significant annual differences were found. [Colour figure can be viewed at wileyonlinelibrary.com]

and *A. phagocytophilum* were present in 1.6% of samples, which did not differ significantly from the expected value of 2.0% ($\chi^2 = 1.1$, df = 1, $P = 0.299$, Table 5). However, a significant negative association between *A. phagocytophilum* and *Borrelia* spp. infection status in ticks was determined by GLMM, controlling for further influencing factors on *Borrelia* prevalence (Table 2). *Anaplasma*-infected ticks had lower odds of *Borrelia* infection than *Anaplasma*-negative ticks, with an OR of 0.7. If the model was calculated for adult ticks and for nymphal ticks separately, this negative association was only found in adult ticks (adults: Estimate = −0.48, SE = 0.20, $z = −2.38$, $P = 0.017$; nymphs: Estimate = −0.02, SE = 0.32, $z = −0.07$, $P = 0.94$). In addition, the GLMM for nymphs was not significantly different from a null model containing only the random factor ($\chi^2 = 23.9$, df = 17, $P = 0.122$). The local *Borrelia*–*Anaplasma* co-infection rates correlated significantly with the local *A. phagocytophilum* prevalences but not with *Borrelia* prevalences (*Borrelia*: $\rho = 0.27$, $P = 0.332$; *A. phagocytophilum*: $\rho = 0.56$, $P = 0.031$).

Triple infections were detected in 0.9% of the samples, without any significant correlation to pathogen prevalence (*Borrelia*: $\rho = 0.43$, $P = 0.113$; *Rickettsia*: $\rho = 0.46$, $P = 0.086$; *A. phagocytophilum*: $\rho = 0.45$, $P = 0.093$).
Fig. 4. *Borrelia* species distribution in the five investigated regions as determined by RLB. Differences between the intra-regional sampling sites were analysed by Fisher’s exact test. Abbreviations: Baf, *B. afzelii*; Bga/Bba, *B. garinii/B. bavariensis*; Bva, *B. valaisiana*; Bss/Bca, *B. burgdorferi* s.s./*B. carolinensis*; Bsp, *B. spielmanii*; Blu, *B. lusitaniae*; Bmi, *B. miyamotoi*; n.d., not differentiated. [Colour figure can be viewed at wileyonlinelibrary.com]
region and landscape type on the distribution of Table 4. Results of PERMANOVA analysis to test the influence of species and prevalences in questing Lyme borreliosis constitutes the most important tick-borne disease in Europe. In the present study, the regional Borrelia prevalences in questing Ixodes ticks in northern Germany of 26.8–33.2% were considerably higher than the estimated average prevalence of 19.3% in Central Europe (Strnad et al., 2017) and in the upper range of values previously reported from Germany. In the northern half of Germany, regional Borrelia prevalences between 7.2 and 34.1% were determined in previous studies (May et al., 2015; Blazejak et al., 2018; Raileanu et al., 2020), whereas in the southern part they ranged from 12.7 to 27.0% (Hildebrandt et al., 2010; Zubrikova et al., 2020). However, differences in pathogen detection methods, i.e. different sensitivities, need to be considered when comparing different prevalence studies (Strnad et al., 2017).

A higher Borrelia prevalence in questing ticks could lead to a higher LB incidence in northern Germany compared with other parts of the country. However, LB is not notifiable in all parts of Germany. Incidence data exist for eastern Germany, where acute LB is notifiable, and has fluctuated between 19.5 and 34.9 annual cases per 100 000 inhabitants in recent years (Wilking & Stark, 2014). Published Borrelia prevalence rates in questing ticks from eastern Germany range from 7.2 to 27.0% (Hildebrandt et al., 2010; Szekeres et al., 2017; Raileanu et al., 2020) and are thus frequently lower than in the present study. However, disease incidence does not only depend on pathogen prevalence in ticks, but is also influenced by other factors, e.g. human behaviour.

No significant differences in Borrelia prevalence were detected between the different regions in the present study, but between different locations within the sampled regions of Emsland and Kassel. Similar intra-regional differences in Borrelia prevalence were also found in previous studies (May et al., 2015; Blazejak et al., 2018). A large-scale study on 250 forest patches throughout Europe identified certain habitat properties, which may impact the availability of hosts, as important drivers of Borrelia prevalence in ticks (Ehrmann et al., 2018). However, landscape type had no significant effect in the present study on Borrelia infections, nor on Rickettsiales infections in the same ticks from these sampling sites (Knoll et al., 2021). Likewise, no significant effect of forest type on Borrelia prevalence was observed in a Belgian study (Ruyts et al., 2016), although the grade of urbanization was positively correlated to Borrelia prevalence in Luxembourg (Reye et al., 2010). The number of sampled locations per landscape type was rather low in the present study, ranging from 1 to 4 sites, and a larger sample size may be required to assess landscape effects. Furthermore, information on wildlife density and diversity at the different sampling locations was unfortunately not available.

The two tick species identified via molecular methods, I. ricinus and I. inopinatus, showed similar Borrelia prevalences. Conversely, Hauck et al. (2019) described a significantly lower Borrelia prevalence in I. ricinus (24.8%) than in I. inopinatus (33.6%). In both studies, the number of investigated I. inopinatus was relatively low compared with I. ricinus [present study: I. ricinus: n = 1495, I. inopinatus: n = 80, Hauck et al., 2019; n = 3708 and n = 137]. Due to the close phylogenetic relationship of these species (Estrada-Peña et al., 2014), similar infection rates were expected, although further investigations are required to determine the vector competence of I. inopinatus for Borrelia spirochaetes and other pathogens.

Noteworthy, a significantly higher prevalence in adult ticks compared with nymphs, which was expected due to transstadial transmission, was not observed in the present study. By contrast, a higher or nearly equal infection rate in nymphs compared with adult ticks was detected at a few sampling sites in the present study. Similar findings were described in other European Borrelia prevalence studies (Strnad et al., 2017; Ehrmann et al., 2018).

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Table 3. Borrelia species distribution among the 560 successfully differentiated samples and rate of co-infections with different B. burgdorferi s.l. species and B. miyamotoi.

| Total infections | No. of infected ticks (%) | Mono-infection | No. of infected ticks (%) | Double-infection | No. of infected ticks (%) | Triple-infection | No. of infected ticks (%) |
|------------------|---------------------------|----------------|--------------------------|------------------|--------------------------|------------------|--------------------------|
| Baf              | 191 (34.1)                | Baf            | 167 (29.8)               | Baf + Bga/Bba    | 2 (0.4)                  | Baf + Bga/Bba + Bss/Bca | 1 (0.2)                 |
| Bga/Bba          | 170 (30.4)                | Bga/Bba        | 135 (24.1)               | Bga + Bva        | 3 (0.5)                  | Bga + Bga/Bba + Bsp   | 1 (0.2)                  |
| Bva              | 124 (22.1)                | Bva            | 95 (17.0)                | Bva + Bss/Bca    | 9 (1.6)                  | Bva + Bss/Bca + Bmi  | 2 (0.4)                  |
| Bss/Bca          | 44 (7.9)                  | Bss/Bca        | 32 (5.7)                 | Baf + Bsp        | 3 (0.5)                  | Baf + Bss/Bca + Bsp   | 1 (0.2)                  |
| Bsp              | 15 (2.7)                  | Bsp            | 10 (1.8)                 | Baf + Bmi        | 1 (0.2)                  | Baf + Bss/Bca + Bmi  | 1 (0.2)                  |
| Blu              | 4 (0.7)                   | Blu            | 4 (0.7)                  | Bga/Bba + Bva    | 25 (4.5)                 | Bga/Bba + Bvi        | 25 (4.5)                 |
| Bbi              | 0 (0.0)                   | Bbi            | 63 (11.3)                | Bga/Bba + Bmi    | 4 (0.7)                  | Bga/Bba + Bmi        | 4 (0.7)                  |
| Bka              | 0 (0.0)                   | Bka            | 63 (11.3)                | Bga/Bba + Bvi    | 2 (0.4)                  | Bga/Bba + Bmi        | 2 (0.4)                  |
| Bmi              | 72 (12.9)                 |                |                          |                  |                          |                  |                          |
| Total            | 560 (100)                 |                | 506 (90.4)               |                  | 48 (8.6)                 |                  | 6 (1.1)                  |

Baf, B. afzelii; Bga/Bba, B. garinii/B. bavariensis; Bva, B. valaisiana; Bss/Bca, B. burgdorferi s.s./B. carolinensis; Bsp, B. spielmanii; Blu, B. lusitaniae; Bbi, B. bissettiae; Bka, B. kurtenbachii; Bmi, B. miyamotoi.

Table 4. Results of PERMANOVA analysis to test the influence of region and landscape type on the distribution of Borrelia species.

|                      | Df | Sums of square | Mean square | Pseudo-F | P value |
|----------------------|----|----------------|-------------|----------|---------|
| Region               | 4  | 0.42           | 0.10        | 1.70     | 0.164   |
| Landscape type       | 4  | 0.15           | 0.04        | 0.63     | 0.776   |
| Residuals            | 6  | 0.37           | 0.06        |          |         |
| Total                | 14 | 0.94           |             |          |         |
A different host spectrum is relevant for the different developmental stages of *Ixodes* ticks. Larvae preferentially parasitize rodents and birds, which constitute the main *Borrelia* reservoirs, whereas nymphs are frequently present on larger mammals like hares and deer (Tälleklint & Jaenson, 1997). Especially roe deer, which are abundant in Germany, may have an important impact on *Borrelia* prevalence in adult ticks, as roe deer do not act as competent reservoir hosts for *B. burgdorferi* s.l. despite being infested by high numbers of nymphal ticks (Kurtenbach et al., 2002). Furthermore, wild ruminants may even cause elimination of the *B. burgdorferi* s.l. burden in feeding ticks, similar to domestic ruminants (Richter & Matuschka, 2010), resulting in a reduced prevalence in the moulted adults. Therefore, nearly equal infection rates in nymphs as in adult ticks might be driven by roe deer abundance. Another indication of the influence of roe deer can possibly be seen in the negative association between *A. phagocytophilum* and *Borrelia* infections in adult ticks. By contrast with *Borrelia*, roe deer and red deer are one of the main reservoirs for *A. phagocytophilum* (Dugat et al., 2015). Therefore, a blood meal on deer could result in *Borrelia* elimination, while simultaneously leading to *A. phagocytophilum* infection. This was also corroborated by a significantly higher *A. phagocytophilum* prevalence in adult ticks than in nymphs, as nymphs are more likely to feed on larger mammals like deer than larvae (Knoll et al., 2021).

A long-term study in the northern German city of Hanover showed a relatively constant *Borrelia* prevalence over a period of 10 years (Blazejak et al., 2018). Accordingly, no significant difference in the overall *Borrelia* prevalence was observed between 2018 and 2019 in the present study. Conversely, when analysing annual differences for each region separately, a significant decline in prevalence from 2018 to 2019 was detected only in the region of Hanover. Interestingly, significantly less *A. phagocytophilum* and *Rickettsia* infections in 2019 than in 2018 were detected in the same tick material, with the largest prevalence difference observed in the region of Hanover (Knoll et al., 2021). The years 2018 and 2019 were characterized by a rather hot and dry climate in Europe [German Meteorological Service (http://www.dwd.de/)]. Drought conditions have a negative impact on vegetation, rodent populations, tick survival and also incidence of tick-borne diseases (Brown et al., 2014). The effect of this climatic extreme may have been more pronounced in Hanover than in the other regions.

Monthly variations in *Borrelia* prevalence were observed, without a general seasonal pattern, similar to other studies in Central Europe (e.g. Reye et al., 2010; May et al., 2015; Szekerés et al., 2017; Blazejak et al., 2018). Pathogen prevalence in questing ticks reflects infections, which were acquired in earlier developmental stages, and monthly fluctuations in prevalence may result from differences in activity of infected and uninfected
ticks (Lefcort & Durden, 1996). For example, it has been shown that *B. burgdorferi* s.l. infection confers survival advantages to *I. ricinus* under hot and dry conditions (Herrmann & Gern, 2010). In addition, ticks active in spring usually molt in summer, followed by a diapause during the autumn and winter months with early spring activity. By contrast, ticks active in autumn overwinter in a diapause and molt during the next spring, becoming active again in early summer (Gray et al., 2016). In consequence, different ticks contribute to the questing tick populations in spring and autumn. Importantly, the wide range between the lowest rate of 20.0% in May 2019 and the highest rate of 42.7% in October 2019 shows the necessity of long-term studies to determine *Borrelia* prevalence. Similar monthly fluctuations in prevalence were also observed regarding Rickettsiales, and again no general trend could be identified when comparing the results to other studies (Knoll et al., 2021). Furthermore, no clear pattern was discernible when comparing monthly prevalence fluctuations of the three investigated pathogens in the same tick material.

The success in *Borrelia* species identification by RLB is comparable to other studies using the same protocol [52.7% by Blazejak et al., 2018, 57.1% by Springer et al., 2020] and is positively correlated to the number of 55-23S IGS copies detected by qPCR (Blazejak et al., 2018). The higher sensitivity of qPCR compared with conventional PCR is the main reason for the fact that not all samples could be differentiated (Strube et al., 2010). In addition, the RLB technique is not able to detect *B. finlandensis* or *B. turdi*. So far, *B. finlandensis* was only detected in one *I. ricinus* in Finland and *B. turdi* was only found with low prevalence in *I. ricinus* collected from birds in Europe (Casjens et al., 2011; Palomar et al., 2017), so that it seems unlikely that non-differentiated samples contained these species.

Europe-wide, *B. afzelii* and *B. garinii* are the predominant *Borrelia* species in *I. ricinus* ticks, followed by *B. valaisiana* and *B. burgdorferi* s.s. (Strnad et al., 2017). The overall species distribution determined in the present study confirms this and is comparable to results of previous studies conducted in Hanover and Hamburg. In most regions, differences in prevalence of individual *Borrelia* species were noted between the different sampling sites. Similar observations have been made in previous studies, which identified *B. afzelii* as the predominant species in Hanover, whereas in Hamburg it was *B. garinii/B. bavariensis* (May & Strube, 2014; Blazejak et al., 2018). The predominance of *B. afzelii* versus *B. garinii/B. bavariensis* in Hanoverian ticks was only confirmed at one site, whereas at the other sites both species occurred at nearly equal frequency. Due to the heterogeneity in species composition between the sampling sites within each region, no significant effect of region on the species composition was found. The *Borrelia* species distribution at a sampling site largely depends on the availability of different host species for ticks, as different *B. burgdorferi* s.l. species are associated with different reservoir hosts. For example, *B. afzelii* is preferentially transmitted by rodents, whereas birds are the main reservoir for *B. garinii* and *B. valaisiana*. Furthermore, *B. burgdorferi* s.s. occurs in birds as well as rodents (Kurtenbach et al., 2002). The reservoir hosts of *B. lusitaniae* are lizards (Richter & Matuschka, 2006), which is an explanation for the low prevalence of this species, which was detected at only one sampling site.

Furthermore, the relapsing fever spirochaete *B. miyamotoi* has been detected in various vertebrate hosts, but rodents constitute the main reservoir (Kubiak et al., 2021). By contrast with other studies (Reye et al., 2010; Ruys et al., 2016), the present study did not find a significant impact of landscape type on *Borrelia* species composition. Probably, the variability in abiotic and biotic factors between the locations within one landscape type was too high to detect an effect with the given sampling size. The proportion of *B. miyamotoi* among *Borrelia*-positive ticks as determined by RLB leads to a calculated prevalence of 3.9% for all examined ticks. In Europe, the reported *B. miyamotoi* prevalences in *I. ricinus* nymphs range from 0.4 to 2.8% and in adults from 3.0 to 4.3% (Kubiak et al., 2021). For each of the northern German federal states Saxony and Mecklenburg-Western Pomerania, a value of 1.2% was published (Szekeres et al., 2017; Raileanu et al., 2020), while a value of 4.1% was determined for the city of Hanover (Blazejak et al., 2018). Regarding human exposure, a *B. miyamotoi* seroprevalence of 2.0% was noted in blood donors in the Netherlands and a seroprevalence of 10.0% in forestry workers as a high-risk group (Jahfari et al., 2014). This shows that *B. miyamotoi* should be considered as an aetiological agent in unspecific feverish illness after a tick bite in Europe.

Considering co-infections with different *B. burgdorferi* s.l. species, the most frequent combination was *B. garinii/B. bavariensis* with *B. valaisiana*, which may result from the fact that both *B. garinii* and *B. valaisiana* are associated with birds as reservoir hosts (Kurtenbach et al., 2002). The low proportion of the rodent-associated *B. bavariensis*, as determined by sequencing of *B. garinii/B. bavariensis*-positive samples, is consistent with an average proportion of 2% among *Borrelia*-positive tick samples throughout Europe (Strnad et al., 2017). In other studies from northern Germany, *B. bavariensis* prevalence was also low (May et al., 2015; Blazejak et al., 2018; Raileanu et al., 2020). Sequencing of the *B. burgdorferi* s.s./*B. carolinensis* positive samples revealed that no tick was infected with *B. carolinensis*, similar to previous studies using this method (Blazejak et al., 2018; Springer et al., 2020).

Concerning co-infection rates of ticks with *Borrelia* spp. and *Rickettsia* s.l., these were mostly close to the mathematically expected values. A positive association between *Borrelia* and *Rickettsia* spp. infections, as described in a meta-analysis by Raulf et al. (2018), was not detected. The *Borrelia*–*Rickettsia* spp. co-infections were driven mainly by the prevalence of each pathogen. The co-infection rate of *Borrelia* spp. and *A. phagocytophilum* depended mainly on the prevalence of *A. phagocytophilum*, which is probably due to the low prevalence of this pathogen compared with *Borrelia*. A negative association of *A. phagocytophilum* and *Borrelia* infection status became apparent when analysing the data by GLMM and controlling for further influencing factors. In mouse models, co-infected mice showed higher bacterial loads of *Borrelia* spp. and more effective pathogen transmission to ticks than mono-infected mice (Thomas et al., 2001). Thus, a positive correlation would be expected if rodents served as the main reservoir of both pathogens. However, roe deer and red deer are regarded as more important *A. phagocytophilum* reservoirs than rodents in Europe.
Conclusions

The overall Borrelia prevalence of 30.6% indicates that Borrelia infection constitutes a health hazard after a tick bite in northern Germany. Monthly variations in prevalence were detected, thus prevalence studies should be carried out over several months to minimize seasonal impact. For public health risk assessment, the Borrelia species distribution is also important. A high variability in the species composition was noted between the different sampling sites, which was not explained by geographic region or landscape type. This demonstrates that multiple locations need to be investigated per region to obtain a comprehensive picture. These factors should be taken into account when designing Borrelia prevalence studies.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1: Results of GLMM testing the influence of different predictor variables on the probability of infection with Borrelia spp.

Table S2: Results of GLMM testing the influence of the tick species Ixodes ricinus and Ixodes inopinatus and other factors on the probability of infection with Borrelia spp.

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Author contribution

CS: conceptualisation, project administration, funding acquisition, supervision; SK, DH: investigation; SK, AS: formal analysis; SK: visualization, writing – original draft; AS, DH, BS, SP, VF, CS: writing – review and editing.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

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