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Year-to-year variation in the density of *Ixodes ricinus* ticks and the prevalence of the rodent-associated human pathogens *Borrelia afzelii* and *B. miyamotoi* in different forest types.

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ABSTRACT

The human pathogens *Borrelia afzelii*, which causes Lyme borreliosis and *B. miyamotoi*, which causes relapsing fever, both circulate between *Ixodes ricinus* ticks and rodents. The spatiotemporal dynamics in the prevalence of these pathogens have not yet been fully elucidated, but probably depend on the spatiotemporal population dynamics of small rodents. We aimed to evaluate the effect of different forest types on the density of infected nymphs in different years and to obtain more knowledge about the spatial and temporal patterns of ticks and tick-borne pathogens. We analysed unfed nymphal ticks from 22 stands of four different forest types in Belgium in 2009, 2010, 2013 and 2014 and found that the density of nymphs in general and the density of nymphs infected with *B. afzelii* and *B. miyamotoi* varied yearly, but without temporal variation in the infection prevalence. The yearly variation in density of infected nymphs in our study thus seems to be caused most by the variation in the density of nymphs, which makes it a good predictor of disease risk. The risk for rodent-associated tick-borne diseases also varied between forest types. We stress the need to elucidate the contribution of the host community composition to tick-borne disease risk.

KEYWORDS

Host community; Lyme borreliosis, mast year; spatiotemporal dynamics; tick-borne disease risk
Tick-borne diseases are a growing public health concern (Dantas-Torres et al., 2012). The most common tick-borne disease in the northern hemisphere is Lyme borreliosis, which is caused by some genospecies of the *Borrelia burgdorferi* sensu lato (s.l.) complex (Stanek et al., 2012), of which *B. afzelii* is the most prevalent in many Western European regions (Bingsohn et al., 2013; Gassner et al., 2011; Rauter and Hartung, 2005; Ruyts et al., 2016). Both *B. afzelii* and *B. miyamotoi*, the latter causing relapsing fever, circulate in the same tick species and the same vertebrate hosts (Cosson et al., 2014; Hanincová et al., 2003). In Europe, *Ixodes ricinus* is the main vector for *B. afzelii* transmission to humans (Piesman and Gern, 2004) and especially the host-seeking nymphs contribute most to the Lyme borreliosis risk (Barbour and Fish, 1993). In addition, this tick species is an important carrier for *B. miyamotoi*, and as with other tick-borne pathogens, *B. afzelii* regularly co-occurs with *B. miyamotoi* in the same tick individuals (Cosson et al., 2014; Gern et al., 2010; Kjelland et al., 2015).

The different genospecies of *B. burgdorferi* s.l. and *B. miyamotoi* each appear to be associated with a particular host species, or a range of hosts. *Borrelia afzelii* is commonly transmitted to ticks by small rodents, such as the wood mouse (*Apodemus sylvaticus* Linnaeus, 1758) and the bank vole (*Myodes glareolus* Schreber, 1780) (Hanincová et al., 2003; Humair et al., 1995). Also Eurasian red squirrels (*Sciurus vulgaris* Linnaeus, 1758) and European hedgehogs (*Erinaceus europaeus* Linnaeus, 1758) have been suggested to transmit *B. afzelii* to ticks (Jahfari et al., 2017; Pisanu et al., 2014; Ruyts et al., 2017; Skuballa et al., 2012). Like *B. afzelii*, *B. miyamotoi* appears to be associated with rodents (Barbour et al., 2009; Cosson et al., 2014; Taylor et al., 2013).

The preferential habitat of *I. ricinus* is forest, due to the sheltered microclimate and availability of vertebrate hosts for their blood meals (Gray et al., 1998; Lindstrom and Jaenson, 2003). Juvenile ticks (larvae and nymphs) generally feed most often on small to medium sized hosts, while adults tend to feed on medium sized to large hosts. A recent European meta-analysis including 44 hosts, however, showed that only a few host species (small rodents, thrushes and roe deer) feed the majority of *I. ricinus* individuals (Hofmeester et al., 2016). Roe deer are generally the most important feeding host for female ticks in Europe and are important in the maintenance and reproduction of *I. ricinus* populations (Gray, 1998; Hofmeester et al., 2016; Ruiz-Fons and Gilbert, 2010). In most regions, larvae mainly feed on small rodents, and rodents are
generally responsible for the majority of *B. burgdorferi* s.l. infections in *I. ricinus* larvae (Hofmeester et al., 2016). The densities of small rodents such as wood mouse and bank vole in our study region, but also of other important host species such as roe deer, are positively correlated with the presence of a shrub layer and are higher in deciduous forests than in coniferous forests (Tack, 2013; Tack et al., 2012a). Furthermore, infection prevalence of nymphs with *B. afzelii* tends to be higher in pine than in oak forests, which suggests that small rodents feed more larvae in pine than in oak forests, relative to other host species (Ruyts et al., 2016). The densities of nymphs are also highest in structure rich deciduous forests (Gray et al., 1998; Ruyts et al., 2016; Tack et al., 2012b). Besides the type of forest, also the availability of seeds influences the occurrence and population dynamics of rodents, which is shown to affect the density of nymphs (Ostfeld et al., 2006, 2001; Tack, 2013; van Duijvendijk, 2016). Therefore, it is expected that the spatial and temporal differences in population dynamics of small mammals are important in explaining the density of infected nymphs, which is a commonly used tick-borne disease risk measure (Ostfeld et al., 2006).

The temporal dynamics in the prevalence of many important tick-borne pathogens, such as the Lyme borreliosis bacteria, remain largely unclear. In the light of the reported rise in incidence of tick-borne diseases in recent years, the study of the ecology and the spatial and temporal patterns of ticks, hosts and tick-borne pathogens is becoming increasingly important (Estrada-Peña et al., 2011; Gray et al., 2009; Randolph, 2010). With our temporal survey, we provide data on the annual variability of the impact of forest characteristics on the density of ticks and the infection prevalence of the rodent-associated pathogens *B. afzelii* and *B. miyamotoi*.

**MATERIALS AND METHODS**

**Study area**

This study was performed in two forest sites in the Campine region in northern Belgium; one in the municipality Postel (51°17’26.35” N, 5°11’40.11” E), the other between the municipalities Herselt and Averbode (51°02’42.91” N, 4°57’17.19” E). The climate is temperate with warm summers (Peel et al., 2007). Forests in this region mainly consist of even-aged homogenous stands of Scots pine (*Pinus sylvestris* L), and to a smaller extent Corsican pine (*P. nigra* Arnold subsp. *laricio* Poiret). Maire] interspersed with
more diverse, structure-rich deciduous forests composed of pedunculate oak (*Quercus robur* L.), Northern red oak (*Q. rubra* L.), common beech (*Fagus sylvatica* L.), silver birch (*Betula pendula* Roth.) and downy birch (*B. pubescens* Ehrh.). The forests in the Campine region are frequently visited for recreational purposes and Lyme borreliosis incidence in this region is relatively high compared to other regions in Belgium (Linard et al., 2007; Vanthomme et al., 2012).

**Forest stand selection**

The 22 forest stands we used in this study were selected in the framework of the study of Tack et al. (2012b) and were also studied in Ruyts et al. (2016). The forest stands lie in a larger matrix of forest stands of different forest types. We investigated stands of four different forest types, i.e. stands dominated either by pines (‘pine stands’) or oaks (‘oak stands’), with (> 50% of the forest floor covered by shrubs) or without (< 25%) a well-developed shrub layer. The 22 studied stands included five pine stands without a shrub layer, six pine stands with a shrub layer, six oak stands without a shrub layer and five oak stands with a shrub layer. The forest stands were on average 1 ha in size, ranging from 0.5 to 4 ha. In our study region, the years 2006, 2007 and 2011 were mast years of pedunculate oak and 2011 was a mast year of beech (Nussbaumer et al., 2016). Corsican pine experienced a high seed crop in 2012 and 2013 and Scots pine in 2013 (Verstraeten A., personal communication). No data for these pine species are available for our region before 2009.

**Data collection**

Questing nymphs were sampled three to four times per year in a fixed representative part of each forest stand between June and October in 2009, 2010, 2013 and 2014. All stands were sampled with comparable intensity and in the same period each year. For the exact procedure of tick sampling we refer to Ruyts et al. (2016). The differences in structure and composition of the herbaceous community between the different stands were negligible so that the sampling could be performed in a standardized way (Tack et al., 2012a). The stands were sampled in a random order each time, to account for daily fluctuations in temperature and humidity during the sampling sessions. Nymphs were removed from the blanket after sampling each transect and transferred to vials containing 70% ethanol and afterwards stored at -22 °C. We counted and pooled nymphs from all sampling occasions from each year per forest stand. From each pool, 35 individual nymphs were randomly selected to examine for infection with *B. burgdorferi* s.l. genospecies and *B. miyamotoi*. For the procedure of DNA extraction of the individual nymphs and the simultaneous detection of *B. burgdorferi* s.l.
and *B. miyamotoi* by multiplex qPCR, and for the identification of *B. burgdorferi* s.l. genospecies, we refer to the methods described in Hansford et al. (2014). As the conventional *Borrelia*-PCR followed by Sanger sequencing is less sensitive than our duplex *Borrelia*-qPCR, we could not assign a genospecies to all ticks that were *B. burgdorferi* s.l.-positive by qPCR. To correct for this shortcoming, we approximated the infection prevalence of nymphs with each *B. burgdorferi* s.l. genospecies for each plot following the procedure described in Jahfari et al. (2017).

**Statistical analysis**

All analyses were conducted in R version 3.3.1 (R Core Team, 2017). DON is the average yearly density of nymphs per plot. The nymphal infection prevalence (NIP) is the proportion of infected nymphs per year, averaged over all sampling occasions per year per plot, and the density of infected nymphs (DIN) is the product of DON and NIP. We calculated NIP and DIN for the *B. burgdorferi* s.l. complex, for each *B. burgdorferi* s.l. genospecies and for *B. miyamotoi*. Due to low numbers for *B. burgdorferi* s.l. genospecies other than *B. afzelii*, only NIP<sub>afzelii</sub>, DIN<sub>afzelii</sub>, NIP<sub>miyamotoi</sub> and DIN<sub>miyamotoi</sub> were included in the statistical analyses.

We used linear mixed-effect models (*lme*) from the package *nlme* (Pinheiro et al., 2015) to explore the effect of sampling year and forest characteristics on the response variables DON, NIP<sub>afzelii</sub>, DIN<sub>afzelii</sub>, NIP<sub>miyamotoi</sub> and DIN<sub>miyamotoi</sub>. As fixed effects, we used sampling year (levels ‘2009’, ‘2010’, ‘2013’, ‘2014’), the dominant tree species (‘pine’ or ‘oak’), the presence of a shrub layer (‘yes’ or ‘no’) and all two-way interactions. We added forest stand as a random effect to take into account the repeated measures in each stand. Significance of the predictor variables in all model fits were assessed using analysis of variance (ANOVA) with Chi-square ($\chi^2$) test and we checked for heterogeneity of the residuals following the approach described in Zuur et al. (2009). Finally, to estimate if changes in DON correlate to changes in NIP, we performed a Spearman Rank Correlation using the package *Hmisc* (Harrell et al., 2016) on DON and NIP<sub>afzelii</sub> and NIP<sub>miyamotoi</sub>.

We did not statistically test the effect of weather variables such as precipitation and temperature on the tick-borne disease risk, since our sample size of four years and 22 stands was too low.
RESULTS

In the 22 forest stands, a total of 21,376 questing *I. ricinus* nymphs were collected. We used 3,080 nymphs for further analysis. Overall, 17.63% of the analysed nymphs was infected with at least one pathogen. We identified six different *B. burgdorferi* s.l. genospecies in 341 of the 471 (72.4%) infected nymphs, namely *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana*, *B. spielmanii* and *B. bavariensis* (Supplementary Table), but we were unable to identify the genospecies in 130 *B. burgdorferi* s.l.-positive nymphs. Thirteen nymphs were co-infected with *B. burgdorferi* s.l. and *B. miyamotoi*. For eight of these co-infected nymphs, *B. miyamotoi* occurred together with *B. afzelii*. The *B. burgdorferi* s.l. genospecies in the remaining five cases of co-infection could not be identified.

Figure 1 visualizes DON, *NIP*<sub>afzelii</sub> and *DIN*<sub>afzelii</sub> in each year. DON (p < 0.01) significantly differed among years, with highest values in 2010 and lowest in 2014 (Table 1 and Fig. 1). DON was consistently higher in oak forests than in pine forests (Fig. 1, Table 1). DON was significantly higher in stands with a shrub layer than in stands without a shrub layer in 2009 and 2010, but no difference was found in 2013 and 2014.

The variables *NIP*<sub>sl</sub>, *NIP*<sub>afzelii</sub> and *NIP*<sub>miyamotoi</sub> did not show significant temporal variation (Table 1). *NIP*<sub>afzelii</sub> was significantly higher in pine forests, consistently throughout the years (Table 1 and Fig. 1). We found no correlation between DON and *NIP*<sub>sl</sub> (p = 0.17, ρ = -0.15), between DON and *NIP*<sub>afzelii</sub> (p = 0.24, ρ = -0.13) or between DON and *NIP*<sub>miyamotoi</sub> (p = 0.32, ρ = -0.11).

Like DON, *DIN*<sub>sl</sub> (p = 0.02) significantly differed among years, with highest values in 2010 and lowest in 2014 (Table 1 and Fig. 1). *DIN*<sub>miyamotoi</sub> and *DIN*<sub>afzelii</sub> did not show significant temporal variation (Table 1). Like DON, *DIN*<sub>sl</sub> and *DIN*<sub>miyamotoi</sub> were higher in oak forests than in pine forests, consistently throughout the years (Table 1). *DIN*<sub>sl</sub> was significantly higher in stands with a shrub layer compared to stands without a shrub layer in 2010, while in the other years, no significant effect could be detected of the presence of a shrub layer.

DISCUSSION

In this temporal survey, we looked at the inter-annual dynamics in tick densities and the infection prevalence of tick-borne bacteria, with special attention to the rodent-associated human pathogens *B. afzelii* and *B.
miyamotoi, in relation to forest types in Belgium. Our results indicate that the risk of rodent-associated tick-borne disease varies both between different types of forest and between years. This spatiotemporal variation can be related to the response of both ticks and hosts to the biotic and abiotic conditions influenced by the dominant tree species, and can be predicted by the density of nymphs.

In our study, the rodent-associated pathogens B. afzelii and B. miyamotoi were the most common bacteria in the investigated nymphs. The bird-associated B. burgdorferi s.l. genospecies B. garinii and B. valaisiana occurred at low infection prevalence in our study sites. Together, this suggests that rodents are most likely the most important feeding hosts for larvae in our study area, as stated by Hofmeester et al. (2016). In our study, B. miyamotoi displayed co-infection with B. afzelii, which supports the assumption that they share the same hosts (Barbour et al., 2009; Cosson et al., 2014; Taylor et al., 2013).

Our results show that DON, but not NIP, displays inter-annual fluctuations. Some European studies have reported that an increased supply of acorns can increase the population density of wood mouse and bank vole the next year (Tack, 2013; van Duijvendijk, 2016). Moreover, they show that this increased rodent density leads to more feeding opportunities for larvae, and a high DON one year later, while NIP remains stable. Also densities of other host species, such as roe deer, red squirrel and wild boar, may increase after a high seed crop of oak, beech or pine (Cutini et al., 2013; Tixier and Duncan, 1996; Wauters et al., 2004; Wauters and Lens, 1995). Oak experienced a high seed crop in 2006, 2007 and 2011, beech in 2011 and pine in 2012 and 2013. Based on this, we would expect DON to be highest in the years 2009, 2013 and 2014. However, DON is highest in 2010 and 2013. Yearly variation in weather conditions such as temperature and the amount of precipitation can also influence DON. Since ticks are sensitive to desiccation (Needham and Teel, 1991), they will be more prone to death in dry conditions, or will seek shelter in the litter layer or lower vegetation which makes it more difficult to collect them with the standard sampling methods and thereby biasing the results. In our study, it is not possible to conclude if mast years or weather conditions affect DON, as these and other possible influencing factors are not accounted for.

Lyme borreliosis incidence has increased significantly the last decades in many European countries (Ducoffre, 2010; Hofhuis et al., 2006; Sprong et al., 2012). We found no clear pattern in DON, NIP\textsubscript{d} or DIN\textsubscript{d} but rather DON and DIN\textsubscript{d} varied from one year to the other. Similar to our results, Estrada-Peña et al. (2011) detected no specific temporal trend at the European level in the prevalence of B. burgdorferi s.l. genospecies
and relate the prevalence of genospecies across Europe to temperature and vegetation stress, which are important drivers of both tick and host populations. Like in other studies (James et al., 2013; Jouda et al., 2004; Vourc’h et al., 2016), but contrary to Tälleklint and Jaenson (1996), we found no correlation between DON and NIP. As NIP in our study did not vary from year to year, the temporal variation in DIN resembles the temporal variation in DON. This confirms that DON can be a good predictor of disease risk, as already suggested by e.g. Jaenson et al. (2009). The relationship between DON and NIP, however, can depend on the specific host community composition (Kurtenbach et al., 2006; Tälleklint and Jaenson, 1996; van Buskirk and Ostfeld, 1995).

In accordance with other studies (Ruyts et al., 2016; Tack et al., 2012b), we found a higher DON in oak forests and a higher NIP_{afzelii} in pine forests. The higher DON in oak stands can be explained by the more favourable biotic and abiotic conditions for ticks in oak forests than in pine forests, such as a better microclimate or a higher abundance of hosts (Gray et al., 1998). Previous research has shown that oak forests in our study region harbour higher densities of small rodents and roe deer compared to pine forests, and thus more feeding opportunities for ticks (Tack, 2013; Tack et al., 2012a). Although the densities of small rodents are higher in oak than in pine forests, it is possible that, due to their wide ecological tolerance (Douglass et al., 1992), wood mouse and bank vole contribute more to the host community in pine than in oak forests, relative to other host species. This way they feed relatively more larvae in pine forests, as already suggested by Ruyts et al. (2016). Squirrels are also generally more abundant in pine than in oak forests (Wauters and Lens, 1995). Since squirrels are, like mice and voles, believed to be associated with B. afzelii (Hanincová et al., 2003; Humair et al., 1995; Pisanu et al., 2014; Ruyts et al., 2017), this might explain the higher NIP_{afzelii} in pine than in oak stands.

From our results, we may conclude that the density of nymphs can be used to predict yearly variation in tick-borne disease risk. We found that the effect of the dominant tree species on the density of nymphs, which reflects changes in biotic and abiotic conditions, is consistent through time. In this study, we did not directly examine the host community of the ticks. Further research should therefore try to determine the exact contribution of the different host species and of the whole host community to the enzootic cycle of human pathogens, and to test the effect of weather conditions and different host community compositions to the tick-borne disease risk.
CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Fig. 1. The density of nymphs (DON), nymphal infection prevalence of the rodent associated pathogen Borrelia afzelii (NIP\textsubscript{afzelii}) and density of nymphs infected with B. afzelii (DIN\textsubscript{afzelii}) in the different sampling years averaged over pine and oak stands (mean ± S.D.).

Table 1. The effect of sampling year, dominant tree species and presence of a shrub layer and their two-way interactions on density of nymphs (DON), nymphal infection prevalence of Borrelia burgdorferi s.l. (NIP\textsubscript{sl}), B. afzelii (NIP\textsubscript{afzelii}) and B. miyamotoi (NIP\textsubscript{miyamotoi}), and density of nymphs infected with B. burgdorferi s.l. (DIN\textsubscript{sl}), B. afzelii (DIN\textsubscript{afzelii}) and B. miyamotoi (DIN\textsubscript{miyamotoi}). Values represent F-values obtained by ANOVA (* p < 0.05). Higher F-values indicate higher variation in the response variable.

<table>
<thead>
<tr>
<th></th>
<th>year</th>
<th>tree</th>
<th>shrub</th>
<th>tree:shrub</th>
<th>tree:year</th>
<th>shrub:year</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
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<td>20.29*</td>
<td>8.32*</td>
<td>0.2</td>
<td>2.02</td>
<td>5.90*</td>
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<tr>
<td>NIP\textsubscript{sl}</td>
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<td>4.17</td>
<td>&lt;0.01</td>
<td>0.38</td>
<td>0.03</td>
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<tr>
<td>DIN\textsubscript{sl}</td>
<td>3.82*</td>
<td>6.54*</td>
<td>6.36*</td>
<td>1.04</td>
<td>0.29</td>
<td>2.49</td>
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<td>NIP\textsubscript{afzelii}</td>
<td>0.63</td>
<td>9.43*</td>
<td>0.9</td>
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<tr>
<td>DIN\textsubscript{afzelii}</td>
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<td>1.59</td>
<td>1.49</td>
<td>1.12</td>
<td>0.19</td>
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<td>&lt;0.01</td>
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<td>0.6</td>
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<tr>
<td>DIN\textsubscript{miyamotoi}</td>
<td>2.38*</td>
<td>6.85*</td>
<td>0.2</td>
<td>0.5</td>
<td>0.36</td>
<td>1.56</td>
</tr>
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</table>
Supplementary table 1. The infection prevalence (%) of *Ixodes ricinus* nymphs with *Borrelia miyamotoi* or a distinct *B. burgdorferi* s.l. genospecies in 2009, 2010, 2013 and 2014 in each studied forest type, averaged over all forest stands from that forest type. We approximated the nymphal infection prevalence of the *B. burgdorferi* s.l. genospecies to correct for the samples that were positive in RT-PCR but could not be identified to genospecies level, as written in the text.

<table>
<thead>
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<th>Bacteria</th>
<th>Year</th>
<th>Pine without shrub</th>
<th>Pine with shrub</th>
<th>Oak without shrub</th>
<th>Oak with shrub</th>
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<td></td>
<td></td>
<td>2009</td>
<td>2010</td>
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<td></td>
<td>2009</td>
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<tr>
<td><em>B. afzelii</em></td>
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<td></td>
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<td>17.9</td>
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