Diversifying forest communities may change Lyme disease risk: extra dimension to the dilution effect in Europe

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SUMMARY

Lyme disease is caused by bacteria of the *Borrelia burgdorferi* genospecies complex and transmitted by Ixodid ticks. In North America only one pathogenic genospecies occurs, in Europe there are several. According to the dilution effect hypothesis (DEH), formulated in North America, nymphal infection prevalence (NIP) decreases with increasing host diversity since host species differ in transmission potential. We analysed *Borrelia* infection in nymphs from 94 forest stands in Belgium, which are part of a diversification gradient with a supposedly related increasing host diversity: from pine stands without to oak stands with a shrub layer. We expected changing tree species and forest structure to increase host diversity and decrease NIP. In contrast with the DEH, NIP did not differ between different forest types. Genospecies diversity however, and presumably also host diversity, was higher in oak than in pine stands. Infected nymphs tended to harbour *Borrelia afzelii* infection more often in pine stands while *Borrelia garinii* and *Borrelia burgdorferi* ss. infection appeared to be more prevalent in oak stands. This has important health consequences, since the latter two cause more severe disease manifestations. We show that the DEH must be nuanced for Europe and should consider the response of multiple pathogenic genospecies.

Key words: Biodiversity, *Borrelia burgdorferi* genospecies, dilution effect, disease ecology, forest diversification, public health, *Ixodes ricinus*, Lyme disease.

INTRODUCTION

In the temperate regions of the northern hemisphere, Lyme disease is the most prevalent vector-borne disease, with important medical and economic consequences (World Health Organization, 2004; Wormser *et al*. 2006). When left untreated, infection can lead to chronic manifestations affecting skin, joints, heart or the nervous system, causing permanent disabilities (Stanek *et al*. 2012). This multisystemic disorder is caused by some bacteria of the *Borrelia burgdorferi sensu lato* (*sl*.) ('*Borrelia*') species group, a complex of more than 18 distinct genospecies of which at least nine occur in Europe: *Borrelia afzelii, Borrelia garinii, B. burgdorferi sensu stricto* (*ss*.), *Borrelia valaisiana, Borrelia lusitaniae, Borrelia spielmanii, Borrelia bavariensis, Borrelia turdii* and *Borrelia bissettii* (Margos *et al*. 2010, 2011; Rudenko *et al*. 2011; Pérez *et al*. 2012; Coipan *et al*. 2013; Heylen *et al*. 2013). At least five of these genospecies are known to cause disease in humans (see Stanek *et al*. 2012 and the references therein). Humans become infected with *Borrelia* via *Ixodes* ticks, and in the Western Europe, the castor bean tick *Ixodes ricinus* L. is the tick vector that most frequently transmits *Borrelia* to humans (Piesman and Gern, 2004). The genospecies of the *B. burgdorferi sl*. complex are all associated with a specific host species, or a range of hosts, and, if pathogenic, cause different disease symptoms in humans, when humans are selected as a host by the tick. *Borrelia afzelii*, for example, is associated with small rodents such as mice and voles and usually causes skin manifestations in humans, while *B. garinii* is associated with birds and can lead to neuroborreliosis (Balmelli and Piffaretti, 1995; Humair *et al*. 1995, 1998; Comstedt *et al*. 2006). Each *Borrelia* genospecies can be considered as a different pathogen with its own transmission dynamics in wildlife hosts, and different clinical manifestations and pathogenicity in humans.

Transmission potential varies between hosts, with some hosts transmitting *Borrelia* efficiently to ticks (i.e. efficient or competent reservoir species) and others rarely or not (i.e. poor reservoirs) (Matuschka *et al*. 1992; LoGiudice *et al*. 2003; Comstedt *et al*. 2006; Heylen *et al*. 2014). This

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variation in reservoir competence has led to the formulation of the dilution effect hypothesis (DEH) (Ostfeld and Keesing, 2000*b*): a high diversity of host species can lower the prevalence of *Borrelia* in ticks because ticks then have a higher likelihood to feed on a host species that is a relatively poor reservoir. The hypothesis assumes that species-poor host communities consist mostly of competent reservoir species, such as mice, and that with increasing biodiversity, the proportion of poor reservoir species in the host community increases and more tick bites will then be wasted – from the point of view of *Borrelia* transmission – on poor reservoirs (Norman *et al*. 1999; Ostfeld and Keesing, 2000*a*, *b*). While the DEH makes predictions about the proportion of nymphs infected with *Borrelia* (i.e. the nymphal infection prevalence), the actual disease risk for humans also depends on the contact rate between ticks and humans, and the density of host-seeking infected nymphs (Begon, 2008; Ogden and Tsao, 2009; Wood and Lafferty, 2013). A reduction in nymphal infection prevalence (NIP) alone does not necessarily provide a decreased Lyme disease risk, because adding more species to the host community can increase tick abundance and hence tick–human contact rate and the associated disease risk (Ogden and Tsao, 2009; Randolph and Dobson, 2012). To counter the effect of increased tick abundances, the reduction in NIP should be substantial to be of public health significance (Schmidt and Ostfeld, 2001).

A dilution effect for Lyme disease has already been found in some regions in North America (Allan and Ostfeld, 2003; LoGiudice *et al*. 2003), but the effect may not be generalizable to other regions. In North America, *B. burgdorferi* ss. is the main genospecies and acts as a host generalist in this region (Kurtenbach *et al*. 2006; Stanek *et al*. 2012). Furthermore, the host community of larval ticks in North America consists mainly of one very efficient reservoir species and several alternative host species which are not, or much less, efficient in transmitting *Borrelia* infection. In contrast, in Europe there are multiple pathogenic *Borrelia* genospecies, which are associated with specific ranges of hosts, and most of the host communities probably consist of multiple competent reservoirs (Ostfeld and Keesing, 2000*b*).

In many Western Europe regions, forests are being converted from homogenous conifer plantations to more natural, structure-rich mixed forests, dominated by indigenous broadleaved trees (Olsthoorn *et al*. 1999; Spiecker *et al*. 2004). Tack *et al*. (2012*a*, 2013) found an elevated tick abundance after conversion of pine stands to stands dominated by oak. This is partially due to the higher shrub cover in more structurally diverse, converted stands, which leads to more suitable abiotic conditions for the ticks. Such a forest conversion will also induce changes in the host community (Brockerhoff *et al*. 2008; Du Bus de Warnaffe and Deconchat, 2008; Tack *et al*. 2012*b*). Structure-rich mixed forests, the intended result of forest conversion, are considered to contain a higher host species diversity than homogenous non-indigenous pine plantations (Kennedy and Southwood, 1984; Laiolo, 2002; Alexander *et al*. 2006; Carnus *et al*. 2006; Brockerhoff *et al*. 2008; Du Bus de Warnaffe and Deconchat, 2008). According to the Dilution Effect Hypothesis, this may dilute the prevalence of *B. burgdorferi sl*. in the ticks. Furthermore, an increase in host diversity may lead to an increase in genospecies diversity, which, due to the association between genospecies and clinical manifestations, can change Lyme disease risk. Large-scale conversions of pine forests in Western Europe provide a unique opportunity to test the DEH in Europe in a complex setting of multiple pathogenic genospecies and increasing host diversity.

By using the different *Borrelia* genospecies as distinct pathogens while testing the dilution effect in Europe, this study contributes to the knowledge gap on the interaction between the *Borrelia* genospecies, tick vector and hosts. In addition, most studies testing the dilution effect use indirect measures of human disease risk such as NIP or tick abundance, while we use the density of infected questing nymphs, which better predicts risk (Begon, 2008; Ogden and Tsao, 2009). Here we investigated forest stands that differed in dominant tree species and presence of shrub cover, leading to four different forest types with supposedly increasing host diversification degree: from stands without a shrub layer dominated by pine trees to stands with a substantial shrub layer dominated by oak. Pine stands with a shrub layer and oak stands without a shrub layer are intermediate in diversification degree. We expect that changing dominant tree species and forest structure leads to diversified forest communities, which entails increased host diversity, and changes Lyme disease risk. We hypothesize that forest community diversification changes the density of infected nymphs (DIN) by: (i) increasing the abundance of nymphal ticks and (ii) decreasing the NIP of *B. burgdorferi sl*. through a dilution effect. Furthermore, we expect the *Borrelia* genospecies community composition to change with forest community diversification, with nymphs from diversified forests housing a more diverse genospecies community.

MATERIALS AND METHODS

Study species

The life cycle of *I. ricinus* consists of three parasitic stages: larva, nymph and adult. Each of these stages needs one blood meal (except for adult males, which do not feed) and waits in the vegetation

for a suitable host to pass to which it can attach itself (i.e. questing). *Ixodes ricinus* becomes infected with *Borrelia* by taking up spirochetes from the blood from an infected host, and afterwards passes infection from stage to stage (i.e. transstadial transmission) (Gray, 1998). An infected tick can, in its turn, infect an uninfected host. Larvae usually hatch free of infection (Barbour and Fish, 1993; Parola and Raoult, 2001). The nymphal stage is most responsible for transmitting *Borrelia* to humans (Barbour and Fish, 1993), and thus the most interesting stage to examine in the framework of public health risk. *Ixodes ricinus* is vulnerable to desiccation (Needham and Teel, 1991) and therefore occurs predominantly in forests that supply enough humidity by their dense vegetation cover (Gray *et al*. 1998). Forests moreover harbour a large enough host community for ticks to feed on (Gray *et al*. 1998; Ruiz-Fons *et al*. 2012).

Study area

In Belgium, converting structure-less pine forests to structure-rich, natural forests dominated by broad-leaved tree species occurs on a large scale in the Campine region, a region with a high risk of Lyme disease (Linard *et al*. 2007; Verheyen *et al*. 2007; Vanthomme *et al*. 2012). The Campine region is an area with nutrient-poor and acid sandy soils located in the northeastern part of Belgium and spanning the provinces Limburg and Antwerp. The region has a temperate climate with warm summers (Peel *et al*. 2007) with a mean annual precipitation and temperature of 800 mm and 9·0 °C, respectively (Royal Meteorological Institute of Belgium, http://www.kmi.be, accessed December 19, 2014). In the 19th and the beginning of the 20th century, the original oak–birch forests on former heathland were mainly replaced by pine plantations of Scots pine (*Pinus sylvestris* L) and, to a lesser extent, Corsican pine [*Pinus nigra* Arnold subsp. *laricio* (Poiret) Maire]. The current characteristic forest structures of this region are pine plantations, interspersed with deciduous forests composed of pedunculate oak (*Quercus robur* L.), red oak (*Q. rubra* L.), common beech (*Fagus sylvatica* L.), silver birch (*Betula pendula* Roth.) and downy birch (*Betula pubescens* Ehrh.). The shrub layer mainly consists of rowan (*Sorbus aucuparia* L.), black cherry (*Prunus serotine* Ehrh.), alder buckthorn (*Frangula alnus* Miller), pedunculate oak, red oak and silver birch. The most common species in the herbaceous vegetation are wavy hair-grass [*Deschampsia flexuosa* (L.) Trin.], purple moor-grass [*Molinia caerulea* (L.) Moench], broad buckler-fern [*Dryopteris dilatata* (Hoffmann) A. Gray], narrow buckler-fern [*D. carthusiana* (Vill.) H.P. Fuchs], blackberry (*Rubus fruticosus* L. agg.), European blueberry (*Vaccinium myrtillus* L.), heather [*Calluna vulgaris* (L.) Hull] and honeysuckle (*Lonicera periclymenum* L.) (Waterinckx and Roelandt, 2001).

For this study, we primarily used the same stands as Tack *et al*. (2012*a*). These stands are dominated by either oak (pedunculate or red oak) or pine (Scots or Corsican pine), hereafter called oak and pine stands, respectively. The stands belong to one of four forest types, namely oak or pine stands with (>50% of the forest ground covered with shrub layer) or without (<25%) a well-established shrub layer, and represent a gradient of forest community diversification from low diversified pine stands without a shrub layer to highly diversified oak stands with a well-established shrub layer. The pine stands with a shrub layer and the oak stands without a shrub layer are less straightforward to rank mutually according to forest community diversity, but we can reasonably assume they are both intermediate between the high and low diversity forest types. A total of 93 stands were selected in 20 different forest sites: 20 pine stands without shrub layer, 32 pine stands with shrub layer, 19 oak stands without shrub layer and 22 oak stands with shrub layer. We attempted to select at least one stand of each type in every forest site. Due to the strong association between host species and tree species, the host community may differ between the two oak species. In our study, we selected pedunculate oak stands, as well as stands of non-indigenous red oak, but investigated their (indirect) effect on the ticks and *Borrelia* infection later during analysis. Forest stands were on average 1 ha large, ranging from 0·5 to 4 ha, and were distributed between 51° 16′N, 4°29′E in the northwestern part of the Campine region and 50°54′N, 5°39′E in the southeast.

Data collection

Each forest stand was visited once when the vegetation was dry between June and September 2013. Questing ticks were sampled by sweeping a white flannel blanket (1×1 m^2) attached to a wooden pole over the herbaceous vegetation ('flagging'). Six 25 m parallel transects were performed in the centre in a representative part of each stand. The sampled area was calculated as the product of distance flagged (6×25 m $= 150$ m^2) and width of blanket (1 m); nymphs per 100 m^2 was used to as our measure of relative nymphal population density. The composition of the herbaceous layer was comparable between the different stands so we believe that it did not influence the density estimates by impeding the sampling technique (Tack *et al*. 2012*b*). For that reason, also, sampling in dense shrubbery and bracken fern [*Pteridium aquilinum* (L.) Kuhn] was avoided. After each transect, nymphs were removed from the blanket with forceps and stored in vials containing 70% ethanol.

Nymphs were pooled per forest stand and 35 individuals were selected randomly from each pool. For one stand, only 34 individuals were analysed due to a low number of nymphs caught. DNA extraction from each of these ticks was done by alkaline lysis in ammonium hydroxide, as described previously (Schouls *et al*. 1999). For the detection of *B. burgdorferi* sl., a duplex qPCR, targeting ospA and flaB genes, was used. For the sequences of primers and probes we refer to Appendix A. The qPCR was performed using the iQ Multiplex Powermix PCR reagent kit (Bio-Rad Laboratories, Hercules, USA), in a LightCycler 480 Real-Time PCR System (F. Hoffmann-La Roche, Basel, Switzerland). The reaction mix consisted of iQ multiplex Powermix, 100 nM of the B-FlaB-Rc and B-FlaB-Rt primers, 200 nM of the B-FlaB-F, 400 nM of the B-OspA_modF and B-OspA_borAS primers, 100 nM of the B-OspAmodPatto probe, 200 nM of the B-FlaB-Patto probe, and 3 μL of template DNA in a final volume of 20 μL. Cycling conditions included an initial activation of the iTaq DNA polymerase at 95 °C for 5 min, followed by 60 cycles of a 5 s denaturation at 95 °C, followed by a 35 s annealing-extension step at 60 °C (ramp rate 2·2 °C s^{-1} and a single point measurement at 60 °C) and a cooling cycle of 37 °C for 20 s. Analysis was performed using the second derivative calculations for crossing point values. For each run positive and negative controls and blank samples were included. For confirmation of presence of *B. burgdorferi* sl. DNA, the positive samples of the qPCR were further submitted to PCR targeting the variable 5S–23S intergenic spacer region (IGS). The PCR was performed according to the protocol described in Coipan *et al*. (2013). Identification of *Borrelia* genospecies was done based on the DNA sequence of IGS. PCR products were sequenced using an ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems). Sequences were confirmed by sequencing both strands (Sanger *et al*. 1977). Storage and analysis of the IGS sequences were performed in BioNumerics version 7.0 (Applied Math, Belgium). *Borrelia* genospecies were assigned based on sequence identity with reference DNA sequences from GenBank (http://www.ncbi.nlm.nih.gov).

Data analysis

All analyses were conducted in R version 3.2.0 (R Core Team, 2015). Graphs were made with the package *ggplot2* (Wickham, 2009). We defined NIP as the proportion of nymphs infected with *B. burgdorferi sl*. and the density of nymphs (DON) as the number of nymphs caught per 100 m^2. The DIN is the product of NIP and DON. First, we fitted effects for tree species ('pine' or 'oak'), the presence of a shrub layer ('yes' or 'no') and the interaction between tree species and the presence of a shrub layer on DON, NIP and DIN using generalized linear models (*glm*). DON and DIN resembled overdispersed count data, so we applied a negative binomial error distribution using the package *MASS* (Venables and Ripley, 2002). For NIP we used a *glm* with binomial error distribution for proportional data. Significances in all model fits were assessed through analysis of deviance with Chi-square (χ^2) test. We checked for heterogeneity of the residuals following the approach described in Zuur *et al*. (2009).

Second, to examine the impact of the different types of forest composition and structure on the *Borrelia* genospecies community compositions, we fitted a multivariate generalized linear model (*manyglm*) to our data (Warton *et al*. 2012) as implemented in the R package *mvabund* (Wang *et al*. 2012). The fitted model assumed a binomial distribution of the prevalence (proportion) data. Predictor variables included tree species, the presence of a shrub layer and the interaction between tree species and presence of a shrub layer. An analysis of deviance for multivariate *glm* fits with likelihood ratio test was employed to determine which variables contributed significantly to the differences between the community compositions. Afterwards, we considered the *Borrelia* genospecies as distinct pathogens, and analysed the effect of the predictor variables on the prevalence of the individual genospecies the same way as described above for the response variable NIP with a binomial *glm*.

Finally, we calculated the diversity of each genospecies community (exponent of Shannon index or equivalent species numbers) with the function *diversity* in the R package *vegan* (Oksanen *et al*. 2015) and tested for the effect of the forest type variables with a *glm*.

To test the impact of the ecologically relevant difference between the stands of indigenous pedunculate oak and non-indigenous red oak, we conducted all above-mentioned analyses on the whole dataset of 93 stands and afterwards on a limited dataset of the oak stands. The outcomes of the analysis on the dataset of the red oak stands were then compared with those of the pedunculate oak stands. No difference was found for any response variable, at the level of *B. burgdorferi sl*. or the genospecies, between the indigenous pedunculate oak stands and the non-indigenous red oak stands, so we considered the category 'oak' as robust.

RESULTS

In the 93 forest stands, a total of 9554 *I. ricinus* nymphs were caught, with a mean density (±S.D.) of 40·5 nymphs (±34·8) per 100 m^2. 3254 nymphs were examined for *Borrelia* spirochetes, and 508 were infected. This corresponds with a mean (±S.D.) NIP of 15·6% (±8·1) over all forest stands. Average DIN was 5·9 nymphs (±6·1) per 100 m^2. *Borrelia*-positive nymphs were found in all forest stands

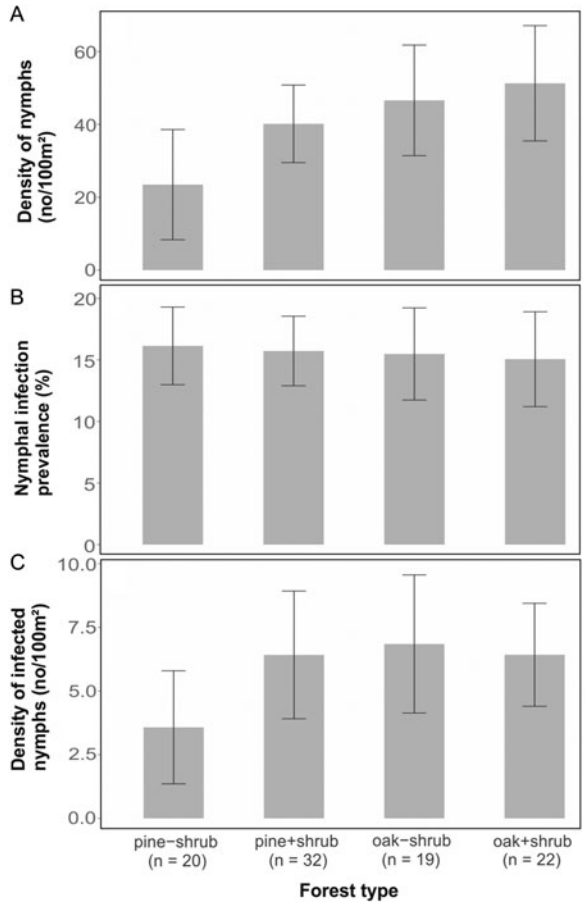

Fig. 1. Density of nymphs (A), NIP (B) and DIN (C) in the different forest types (mean ± S.D.). *n*, number of stands used per forest type. Note the difference in scaling of the *y*-axis, and the units used.

examined. We identified six different genospecies in 462 (91%) infected nymphs, namely *B. afzelii*, *B. garinii*, *B. burgdorferi ss.*, *B. valaisiana*, *B. spielmanii* and *B. bavariensis*. For the other 46 infected nymphs, genospecies could not be defined because of the lower sensitivity of the conventional PCR in comparison with the qPCR. Only one genospecies was identified in each infected nymph. From the six detected genospecies, *B. afzelii* was the most prevalent genospecies, occurring in 10·6% of the nymphs (±7·0) or 73·8% (±27·4) of the infected nymphs. The mean prevalence of *B. garinii* and *B. burgdorferi ss.* in nymphs was 1·5% (±2·5) and 1·2% (±2·1), respectively. The mean prevalences of the other genospecies were lower than 1%.

The *glm* showed a significant effect of tree species on DON ($P = 0{\cdot}02$) and a marginally significant effect of the presence of a shrub layer ($P = 0{\cdot}05$) with lowest densities in pine stands without a shrub layer (Fig. 1). The interaction between tree species and the presence of a shrub layer had a marginally significant effect on DIN ($P = 0{\cdot}09$). We found no significant effect of any predictor variable on NIP (Fig. 1).

The community analysis of *Borrelia* genospecies revealed a significant effect of tree species on the diversity of *Borrelia* genospecies communities (Fig. 2, Appendix B), with more diverse communities in nymphs from oak stands ($t = -2{\cdot}21$, $P = 0{\cdot}03$). No significant effect of tree species or the presence of a shrub layer was found on the genospecies community composition, although the effect of tree species was marginally significant (Dev = 4·44, $P = 0{\cdot}07$). When we considered the *Borrelia* genospecies as separate pathogens in the *glm*, we could not detect a significant effect of the forest type variables on the NIP of the genospecies. Nevertheless we can clearly see in the genospecies community composition (Fig. 2) a trend towards a higher prevalence of *B. afzelii* in nymphs from pine compared with oak stands, and for *B. garinii* and *B. burgdorferi ss.* a trend towards a higher prevalence in oak compared with pine stands. The genospecies community in the infected nymphs appeared to be dominated by *B. afzelii* in all forest types, but it was more pronounced in the pine stands [83·9% (95% confidence interval [71·4, 91·6]] of the infected nymphs) compared with the oak stands (62·4% [46·9, 75·8]) (Fig 2, Appendix B). *B. garinii* and *B. burgdorferi ss.* occurred in 6·7% [2·4, 17·6] and 4·9% [1·4, 15·3] of the infected nymphs from the pine stands compared with 12·5% [5·4, 26·5] and 16% [7·6, 30·5] in the oak stands, respectively. The share of *B. valaisiana*, *B. spielmanii* and *B. bavariensis* in the genospecies community in nymphs was relatively low, namely 1% [0·1, 13·3], 3% [0·6, 13·3] and 0·4% [$4{\cdot}8\times10^{-5}$, 2·4] in pine, and 5·7% [1·6, 18·5], 2·9% [0·5, 15·6] and 0·3% [$1{\cdot}9\times10^{-5}$, 3·9] in oak stands, respectively.

DISCUSSION

This study tests the DEH for Lyme disease in Europe while focusing on the different *Borrelia* genospecies as distinct pathogens, which, to our knowledge, has hardly been performed. Even though we did not test the dilution effect directly, since we did not empirically quantify the host communities, the association between planted forests and biodiversity has been covered sufficiently in literature to make reasonable assessments about the host diversity in the examined forest types (Laiolo, 2002; Carnus *et al*. 2006; Brockerhoff *et al*. 2008; Du Bus de Warnaffe and Deconchat, 2008). We assumed the host diversity to be higher in the oak stands with a substantial shrub layer than in the pine stands without a shrub layer. Further research which empirically quantifies the host communities, however, is needed to confirm this statement. We show that the dilution effect must be nuanced for Europe and should consider the response of multiple pathogenic genospecies. The DIN and associated human health risk will not necessarily decline with increasing biodiversity or host diversity, but will

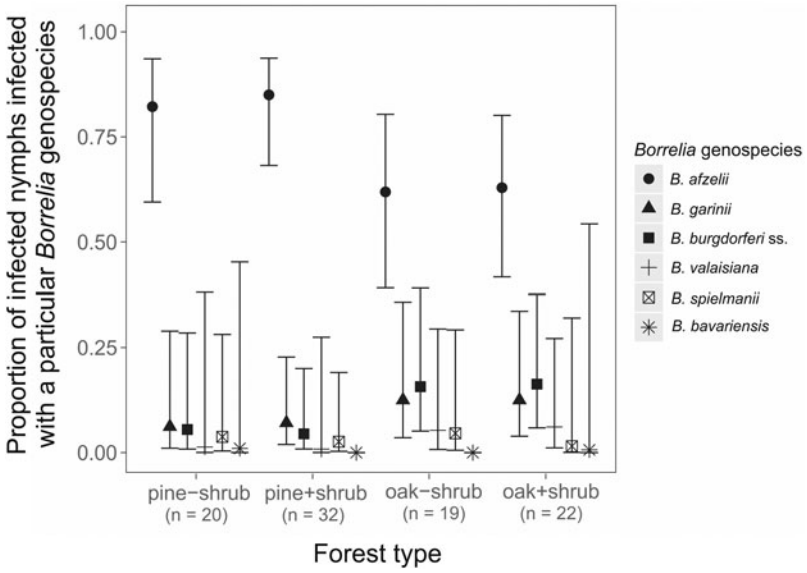

Fig. 2. The community composition of *Borrelia* genospecies in field collected nymphs (mean proportion + 95% confidence interval) in pine and oak stands, with or without shrub layer. *n*, number of stands used per forest type.

instead largely depend on the species assemblages of the host community. The different *Borrelia* genospecies can thus best be considered as separate pathogens, each with a separate possible dilution effect.

Highest densities of nymphs were found in oak stands and lowest in pine stands. Hence, our results show that changing forest composition could lead to increased tick abundances, which confirms the findings of former studies (Tack *et al*. 2012*a*, 2013). This could be due to the fact that more host species are present in oak forests, as suggested by Laiolo *et al*. (2004) and Tack *et al*. (2012*b*). We found an average NIP of 15·6% which is higher than the 10·1% mean NIP usually found in Europe (Rauter and Hartung, 2005). This finding, together with the fact that the forests in the Campine region are readily visited for outdoor activities, suggests that it is an area of potentially high Lyme disease risk, as already proposed by Linard *et al*. (2007) and Vanthomme *et al*. (2012). The NIP did not differ between forest types. The lack of a decline in NIP does not allow us to confirm the DEH. This might indicate that even though host diversity is likely higher in oak forests, the relative proportion of non-competent hosts is probably not substantially large enough to reduce infection prevalence, e.g. because more competent than non-competent species are added to the host community with increasing host diversity (a possible scenario recognized by Ostfeld and Keesing, 2000*b*; Wood and Lafferty, 2013). We could also not detect a significant effect of tree species or the presence of a shrub layer on the DIN. According to Begon (2008) and Schmidt and Ostfeld (2001), a dilution effect only applies to situations where tick bites are wasted on less competent reservoir hosts and when the compensatory increase in vector abundance, caused by the increase in host diversity, is limited. Regardless of whether the host species in question are competent reservoirs or not, an increase in host abundance, and thus feeding opportunities for the ticks, will increase tick abundance (Ogden and Tsao, 2009; Randolph and Dobson, 2012). This indeed appears to be the case in our study, with higher densities of nymphs in oak forests.

The most common genospecies found in questing nymphs in Europe are *B. afzelii* and *B. garinii* but the prevalence of the different genospecies varies between regions (Rauter and Hartung, 2005). In our study region, we found that *B. afzelii* and *B. garinii* constituted 74·5 and 9·3% of the genospecies communities, respectively. Since *B. afzelii* is associated with small rodents, and the majority of infected nymphs were infected with this genospecies, we can assume that rodents such as mice and voles are important feeding hosts for *I. ricinus* larvae and important transmission hosts of *Borrelia* infection. *B. garinii* (and also *B. valaisiana*) is associated with passerine birds (Heylen *et al*. 2013), and birds appear to be less important reservoir hosts in the *Borrelia* transmission cycle of *I. ricinus* ticks than rodents, since the infection prevalence of questing nymphs with *B. garinii* and *B. valaisiana* is much lower than that with *B. afzelii*. This lower prevalence of infection can be due to the fact that passerine birds are less important feeding hosts for the larvae of *I. ricinus* or that they are less efficient at transmitting *Borrelia* infection or both (Brunner *et al*. 2008). A recent meta-analysis demonstrated that birds are generally more important feeding hosts for nymphs than for larvae (unpublished results, although this strongly depends on bird species, see Comstedt *et al*. 2006; Marsot *et al*. 2012), so that questing larvae are less likely to

obtain infection with *B. garinii* than questing nymphs. In the nymphs in our study area, we also found an unusually high prevalence of *B. burgdorferi ss.*, namely 9·8% (Rauter and Hartung, 2005; Burri *et al.* 2007; Bingsohn *et al.* 2013). This is the highest reported prevalence of this genospecies in questing nymphs in Western Europe as far as we know. *B. burgdorferi ss.* is most probably associated with Eurasian red squirrel (*Sciurus vulgaris*) but because this association is not yet strictly determined (Humair and Gern, 1998; Pisanu *et al.* 2014, but see Kurtenbach *et al.* 2002), it is plausible that another host species, or range of hosts, is responsible for the transmission of *B. burgdorferi ss.* to the ticks in our study area. It is however not yet clear which one.

The genospecies community composition in questing nymphs did not significantly differ between forest types, but tree species had a marginally significant effect on the community composition. There was a clear effect of tree species on the diversity of the genospecies communities, with more diverse communities in oak stands compared with pine stands. This increase in *Borrelia* genospecies diversity in the ticks from diversified forest communities can pose serious health risks. *Borrelia burgdorferi ss.* and *B. garinii* are most often associated with Lyme arthritis and neuroborreliosis, respectively, while an infection with *B. afzelii* commonly causes skin manifestations such as erythema migrans or acrodermatitis chronica atrophicans (Strle and Stanek, 2009). These conditions can be regarded as less severe, compared to damage of joints or the nervous system caused by *B. burgdorferi ss.* and *B. garinii*. This indicates that human health risk can increase with increasing host diversity, when the contribution to the genospecies community of the more 'dangerous' *Borrelia* genospecies increases.

Compared with the average home range of some host species, the forest stands we investigated are of relatively small size (Verkem *et al.* 2003) and often imbedded in a mosaic of stands of a different forest type. While ranging, a host species may cross different unsuitable habitats and spend some of its time there. As ticks are disseminated while being attached to their host, it is plausible that nymphs that have fed as a larva on a particular host species are occasionally found in a habitat that is not suitable for that host. Thus ticks, and so also typical host specific *Borrelia* genospecies, can occur in a forest type that is not the favourable habitat of the host. Hosts will, however, spend more of their time in their preferred habitat, so that the chance that a tick drops off in that habitat type instead of in an unfavourable habitat type is higher. Moreover, despite the small size of forest stands, Tack (2013) found that populations of bank vole and wood mouse differed between small neighbouring stands of different forest types, with different populations being composed of different individuals. Most larvae will thus probably be disseminated by their host to a forest stand that is of a favourable forest type for the host, and so the association between *Borrelia* genospecies and forest type remains reasonable. We believe, however, that our estimates of *Borrelia* genospecies prevalence and diversity are only conservative estimates. In a more homogenous landscape, with larger stands of the same forest type, the pattern in *Borrelia* genospecies occurrence we see now would be even clearer and the differences between the forest types even larger.

From our results we may conclude that the public health risk associated with Lyme disease in Europe will not only depend on the NIP of *B. burgdorferi sl.* or on DIN, but also on the prevalences of the distinct genospecies. Even if a more diverse host community causes a dilution effect to occur and the DIN declines, disease risk can increase if the prevalence of a more 'dangerous' genospecies increases. As already suggested by Kurtenbach *et al.* (2006), and mentioned above, the interaction between *Borrelia*, ticks and hosts in Europe appears to be much more complex than the situation observed in North America, because of the existence of multiple pathogenic and specialist *Borrelia* genospecies in Europe. We can now confirm the contrast between these two regions and suggest that increasing host diversity, or adding species to the host community, can increase or decrease the prevalence of individual genospecies, depending on the response of the associated host species. Most likely in Europe the prevalence of *B. afzelii* will decline with increasing forest community diversification due to a dilution effect on small rodents, while the prevalence of the more 'dangerous' pathogens *B. garinii* and *B. burgdorferi ss.* will increase. It is then still possible for a dilution effect to occur in Europe on the level of *B. burgdorferi sl.*, when hosts that are added to the host community are less efficient in transmitting *B. burgdorferi sl.* infection to ticks than hosts already occurring in a species poor community (Ostfeld and Keesing, 2000*b*; LoGiudice *et al.* 2003), which decreases the chance that a tick feeds on a competent reservoir species. This scenario is similar to the trend we observed in our study. It is therefore not increasing biodiversity in itself that will decrease Lyme disease risk. The identity of the hosts that are added to the host community and their interactions with each other and the ticks will determine if a dilution effect will occur (Ostfeld and Keesing, 2000*b*; Randolph and Dobson, 2012), both at the level of *B. burgdorferi sl.* and of the *Borrelia* genospecies. These new insights introduce an extra dimension to the DEH for Lyme disease in Europe and pose important implications for the role of biodiversity in disease ecology. This study therefore strongly emphasizes the need to consider the different *Borrelia* genospecies as distinct pathogens and to study the species assemblages of the host community in high risk areas to assess human disease risk.

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APPENDIX

Appendix A. Primers and probes used in the PCR analyses to detect *Borrelia burgdorferi sl*. and *Borrelia* genospecies infections

Target gene/primer & probe	Amplicon length	Sequence
OspA (Outer membrane Protein A, *B. burgdorferi sl.*)	±139 bp	
B-OspA_modF		5′-AAT ATT TAT TGG GAA TAG GTC TAA-3′
B-OspA_borAS		5′-CTT TGT CTT TTT CTT TRC TTA CA-3′
B-OspAmodPatto		5′-Atto520-AAG CAA AAT GTT AGC AGC CTT GA-BHQ1-3′
FlaB (Flagelin B, *B. burgdorferi sl.*)	±89 bp	
B-FlaB-F		5′-CAG AIA GAG GTT CTA TAC AIA TTG AIA TAG A-3′
B-FlaB-Rc		5′-GTG CAT TTG GTT AIA TTG CGC-3′
B-FlaB-Rt		5′-GTG CAT TTG GTT AIA TTG TGC-3′
B-FlaB-Patto		5′-Atto425-CAA CTI ACA GAI GAA AXT AAI AGA ATT GCT GAI CA-Pho-3′

X, BHQ-1-dT; BHQ, Black Hole Quencher.

Appendix B. Proportions of *Borrelia* genospecies detected in the genospecies community in nymphs from the Campine region and the diversity (exponent of Shannon index) of the genospecies communities, per forest type (mean + S.D.)

	Pine		Oak	
Borrelia genospecies	Without shrub ($n = 20$)	With shrub ($n = 32$)	Without shrub ($n = 19$)	With shrub ($n = 22$)
B. afzelii	82·2 (±23·1)	85 (±18·4)	61·9 (±30·7)	62·9 (±31·3)
B. garinii	6·2 (±13·1)	7·1 (±13·2)	12·5 (±20)	12·5 (±15·6)
B. burgdorferi ss.	5·5 (±14·9)	4·5 (±10·1)	15·7 (±25·6)	16·3 (±18·4)
B. valaisiana	1·3 (±4·1)	0·8 (±3·3)	5·3 (±10·2)	6·1 (±9·3)
B. spielmanii	3·8 (±16·8)	2·6 (±8·3)	4·6 (±12·2)	1·5 (±7·1)
B. bavariensis	1 (±4·5)	0 (±0)	0 (±0)	0·6 (±3)
Diversity of *Borrelia* genospecies community	1·4 (±0·5)	1·5 (±0·5)	2 (±0·8)	2·2 (±1)

n, number of stands used per forest type. Mean is given in percentage.