Forest Diversity Reduces the Prevalence of Pathogens Transmitted by the Tick *Ixodes ricinus*

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Tick-borne diseases represent the majority of vector-borne human diseases in Europe, with *Ixodes ricinus*, mostly present in forests, as the main vector. Studies show that vertebrate hosts diversification would decrease the prevalence of these pathogens. However, it is not well known whether habitat diversity can have similar impact on ticks and their infection rates. We measured the presence and abundance of different stages of *I. ricinus*, and the prevalence of associated pathogens in a large-scale forest experiment in which we manipulated tree diversity and moisture level. We showed that larval abundance was influenced by tree species identity, with larvae being more present in pine plots than in oak plots, while nymph abundance increased with canopy tree density. The proportion of *Borrelia burgdorferi* s.l.-infected nymphs decreased with increasing tree diversity. Our findings suggest that tree overstorey composition, structure and diversity, can affect tick abundance and pathogen prevalence. They support the idea that forest habitats may have "diluting" or "amplifying" effects on tick-borne diseases with direct relevance for human health.

Keywords: biodiversity, forest ecosystems, human health, ORPHEE, ticks, tick-borne disease

INTRODUCTION

A growing body of observational and experimental evidence confirms that more diverse forests provide better support to the provision of ecosystem services and improve nature's contributions to people (Brockerhoff et al., 2017; Messier et al., 2021). Provisioning services such as biomass production (Zhang et al., 2012; Jactel et al., 2018), regulating services such as climate change mitigation through carbon sequestration (Harmon et al., 2013; Lange et al., 2015), pest and fungal disease control (Jactel et al., 2017, 2021), habitat provision for biodiversity (Ampoorter et al., 2020; Stemmelen et al., 2021) are on average often higher in mixed forests than in pure forests. However, it is important to note that forests and other ecosystems can also provide "disservices," i.e., risks

for humans (Dunn, 2010), such as those related to human health. Ecosystem disservices have been much less in the focus of science, although they have recently gained attention with the emergence of epidemics affecting human populations that are increasingly in contact with the wilderness due to deforestation (Jones et al., 2013; Smith et al., 2014; Everard et al., 2020). Even less known is the role of forest diversity in reducing the risk of zoonotic diseases, i.e., whether biodiversity in forests can mitigate ecosystem disservices. The dilution effect hypothesis (Schmidt and Ostfeld, 2001; Halliday et al., 2020) suggests that host species diversity would play a protective role in the spread of pathogens because some host species would not allow the multiplication of viruses or bacteria (low reservoir competence), constituting transmission dead ends, or because the complexity of food webs would allow top down regulation of disease hosts. This theory seems to apply well to the risk of tick-borne diseases (Diuk-Wasser et al., 2021). For example, LoGiudice et al. (2003) predicted a reduction in the prevalence of Lyme disease caused by bacteria of the genus *Borrelia*, with increasing host diversity. Hofmeester et al. (2017) reported that increasing the abundance of predatory mammals reduced the density of disease-carrying tick nymphs in prey rodents. American studies on *Ixodes scapularis*-borne disease showed that the diversity of vertebrate hosts could decrease *Borrelia* prevalence in ticks through the dilution effect of competent hosts by incompetent hosts (Ostfeld and Keesing, 2012; Linske et al., 2018). However, these arguments remain controversial, because the density of competent hosts may not always decrease sufficiently with increasing host diversity to reduce the density of infected nymphs, or because ticks can show a strong preference for competent hosts (Randolph and Dobson, 2012). For example, Ruyts et al. (2018) found no dilution effects in European forests for *Ixodes ricinus,* the most common tick species in western Europe (de la Fuente, 2008). In addition to this host-dilution hypothesis, Ehrmann et al. (2018) suggested that habitats can also be classified into "dilution" and "amplification" habitats that reduce or increase the prevalence of tick-borne disease vectors, respectively, at the local patch or even regional landscape scale. Such "dilution habitats" were characterized by a structurally and functionally diverse tree layer, diverse and abundant understorey vegetation, and increased levels of deadwood in European forests. However, we still lack investigations that are able to disentangle the role of tree diversity from that of other environmental factors in regulating tick abundance and the prevalence of tick-borne diseases. Experiments that manipulate tree diversity independently from other factors offer one promising way into this direction.

The main vector of tick-borne diseases in Europe (de la Fuente, 2008), *I. ricinus* (castor bean tick) is mainly present in forests and woodlands (Estrada-Peña, 2001). It is an obligate hematophagous that requires three blood meals to complete its life cycle and pass from larva to nymph, from nymph to immature adult and from immature adult to egg-laying female, respectively. These different stages depend on different habitat characteristics and microclimates (Ehrmann et al., 2017). *I. ricinus* is responsible for the transmission of several pathogenic bacteria, viruses and protozoa (Rizzoli et al., 2014; Prinz et al., 2017) including the bacterial complex *Borrelia burgdorferi* sensu lato, the tick-borne agent of Lyme borreliosis. But other pathologies are also emerging, related to *Anaplasma*, *Borrelia*, *Neoehrlichia*, *Rickettsia* and *Babesia* species (Kilpatrick and Randolph, 2012; Keesing and Ostfeld, 2021). Because there is no vaccine available against these growing diseases (Steere et al., 2004) with sometimes very serious health consequences, it is very important to search for methods to prevent the risk of transmission.

Ticks can be infected in three ways: (i) by feeding a blood meal on infected hosts (ii) by co-feeding, which is a direct transmission from a tick to another one feeding on the same host at the same time, and (iii) by transovarial (or vertical) transmission. This latter pathway is only possible for a few pathogens (Sprong et al., 2009; Burri et al., 2014) such as some *Rickettsia* or *Babesia* species for unfed *I. ricinus* larvae, and is the main way for larvae to become infected before a first blood meal. Knowing that the probability for one tick to be infected increases with the number of blood meals taken and that nymphs are more abundant in the environment than adults, many studies consider the density of infected nymphs as an indicator of the level of contamination risk in the environment (Mather et al., 1996; Stafford et al., 1998; Diuk-Wasser et al., 2012; Pepin et al., 2012). As a result, the presence and abundance of tick larvae remain less frequently monitored. Although larvae have a lower potential for transmission, their survival in the environment influences directly the abundance of future nymphs, infected or not, and indirectly the epidemiological risk associated (Ehrmann et al., 2017).

The risk of tick bites and disease transmission in forests is directly influenced by the density of infected ticks in the environment (Fischhoff et al., 2019), which in turn depends on interactions between biotic (e.g., host community) and abiotic (e.g., microclimate) conditions. Moreover, understorey vegetation plays a central role in the population dynamics of ticks. First of all, the structure of the understorey vegetation directly conditions the ability to reach the hosts for the questing ticks that are hanging to plant parts (McCoy and Boulanger, 2015). Then the understorey may provide more or less favorable feeding, breeding or resting sites for the vertebrate hosts that ensure the survival, infection and transport of ticks (Allan et al., 2010). Finally, the structure and composition of the understorey condition the microclimate that affects the survival and questing activity of ticks (McCoy and Boulanger, 2015; Ehrmann et al., 2017) and more particularly the humidity (Boyard et al., 2007; Estrada-Peña, 2008). Forest diversity may therefore indirectly influence tick abundance through its effect on understorey vegetation. Several studies have indeed established a causal link between tree diversity and understorey diversity or composition (Gazol and Ibáñez, 2009; Ampoorter et al., 2014; Corcket et al., 2020). Tree species diversity and composition have also been shown to mitigate understorey microclimate (De Frenne et al., 2019). Mixed forests are characterized by a high degree of canopy closure, made possible by a greater complementarity in crown shape, a process known as canopy packing (Jucker et al., 2015; Williams et al., 2017). A denser canopy then produces a shading effect that can increase the humidity in the understory, which favors the survival of ticks in case of drought. Other studies have demonstrated that forest species diversity can alter the abundance

and foraging behavior of herbivorous vertebrates (i.e., potential tick hosts), often with contrasting effects depending on the size of these animals, reducing the browsing activity of small animals (e.g., mice and voles) and increasing that of large ones (e.g., moose and deer) (Milligan and Koricheva, 2013; Jactel et al., 2017). However, very few studies have attempted to measure the effects of forest species diversity on tick abundance and tick-borne pathogen prevalence (but see Ruyts et al., 2016; Ehrmann et al., 2017, 2018) and, to our knowledge, none has isolated the effect of tree species richness from other environmental drivers in an experimental setting.

In this study, we used a long-term experiment where both tree diversity and water supply are manipulated to test their interactive effects on *I. ricinus* larvae and nymphs abundance and on associated pathogens. We sampled ticks in forest plots ranging from one (monoculture) to three tree species mixtures, in both irrigated and non-irrigated conditions. More particularly, we tested the following hypotheses:

(1) Tick abundance and proportion of infected tick nymphs are lower in mixed than in pure forest plots, due to stand characteristics associated with "habitat-dilution" effects.
(2) Effects of tree species diversity on ticks are principally mediated by changes in understorey density and composition, being the main habitat for larvae and nymphs.
(3) The negative effects of drought (in non-irrigated plots) on ticks are mitigated by forest diversity, due to higher crown space filling and shading of the understorey.

Although we were unable to sample vertebrate hosts, our goal was to quantify the direct and indirect (microclimate, understorey) drivers of the effects of overstorey tree diversity on the risk of disease transmission by ticks (**Figure 1**).

MATERIALS AND METHODS

Study Site

The study was conducted in the tree diversity experiment ORPHEE, located in southwestern France, in the Landes de Gascogne forest. The region is characterized by a mild oceanic climate, with mean annual temperature of 12.5 °C and mean total precipitations of ca. 950 mm. The ORPHEE experiment, which belongs to the global network of tree diversity experiments TreeDivNet (Verheyen et al., 2016; Paquette et al., 2018), was established in 2008 on a 12 ha clearcut of maritime pine stands, on a sandy podzolic soil. It is composed of eight blocks of 32 randomly distributed plots corresponding to all the possible combinations of one to five tree species. Five native tree species were planted in monocultures (European birch: *Betula pendula*, ROTH; Pedunculate oak: *Quercus robur*, L.; Pyrenean oak: *Quercus pyrenaica*, WILLD.; Holm oak: *Quercus ilex*, L.; and Maritime pine: *Pinus pinaster*, AIT.) as well as all combinations with two, three, four and five tree species. Each plot is composed of 10×10 trees, planted 2 meters apart, resulting in 100 trees per plot, with a plot area of 400 m^2. Tree species are planted according to a systematic alternate pattern (see details in the

study by Castagneyrol et al., 2013; Damien et al., 2016). Four out of eight blocks are irrigated since 2015. Blocks are sprinkled at night with 42 m^3 of water (i.e., 3 mm precipitation, block area of 1.28 ha), daily from May to October, which significantly increases water availability as compared to non-irrigated blocks (Maxwell et al., 2020). The site has been fenced, which may have reduced the presence or passage of large mammal in the plots.

In our study, we focused on 14 different plots with the following four tree species: *Q. robur*, *Q. ilex*, *P. pinaster*, and *B. pendula*, planted in monocultures ($n = 4$), two-species mixtures ($n = 6$), and three-species tree mixtures ($n = 4$). All plot compositions were replicated eight times, four in irrigated blocks and four in non-irrigated blocks, for a total of 112 plots.
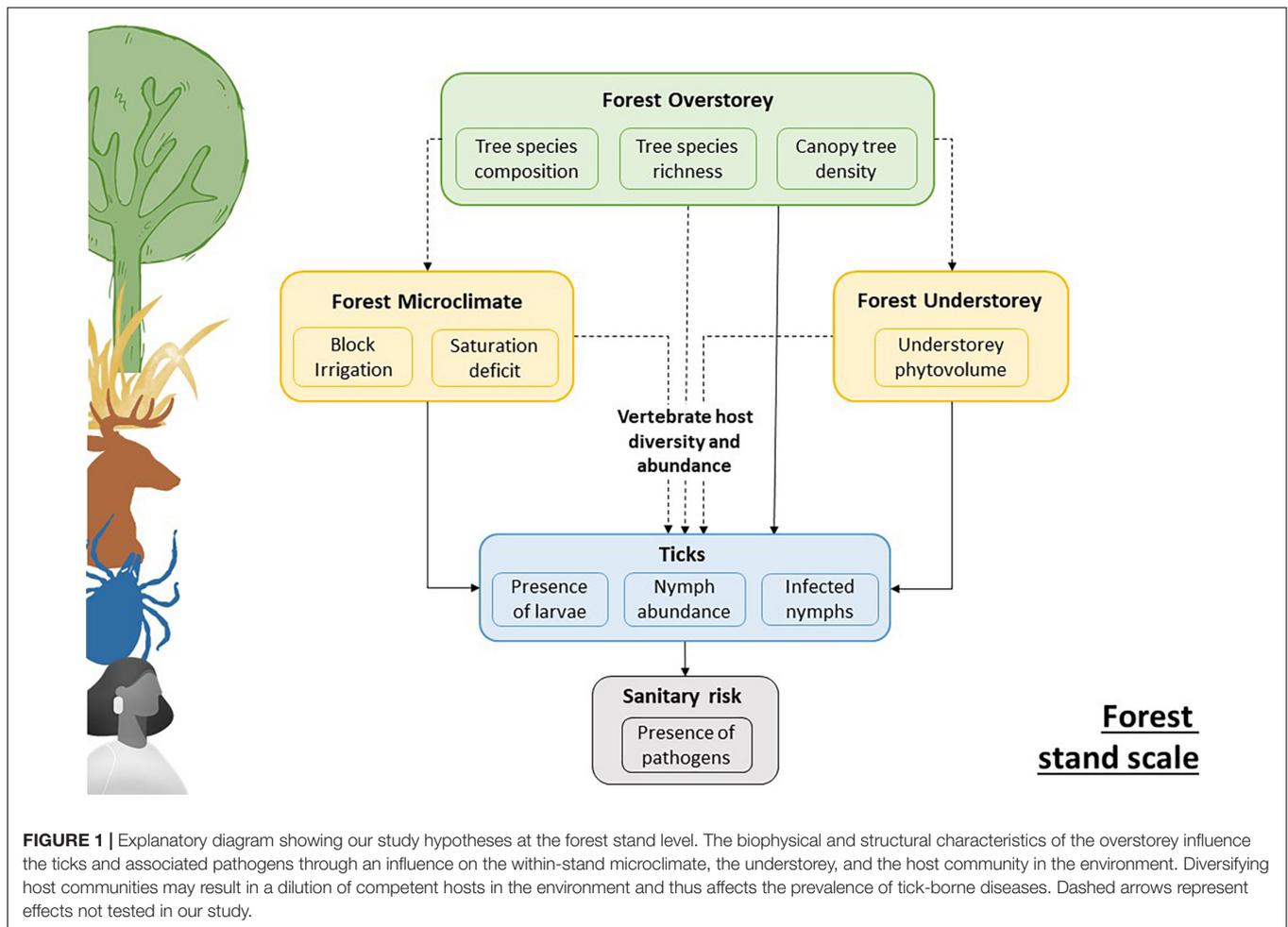
Tick Sampling and Identification

Free-living ticks, being larvae, nymphs or adults, were collected in June and July 2020 on days without rain during the peak of tick activity season, using the dragflag technique (Macleod, 1932; Vassallo et al., 2000). The total number of ticks collected along four parallel 20 meter long transects in each plot (80 m^2 sampled area) were denoted $N_{\{i,j,k\}}$ where $i \in [1; 14]$ represents the plot composition, $j \in [1; 8]$ represents the replication of plot composition and $k \in [1; 4]$ represents the kth parallel long transect. To take into account the effect of different understorey diversity or composition on the sampling rate (Bord et al., 2014), i.e., the proportion of ticks collected by the drag in the questing tick population, each transect was passed four times, corresponding to two return trips, with a 1 m × 1 m white flannel cloth of dragged on the ground at a constant speed (walking pace). Ticks were counted after each successive sampling, stage determined and kept in a 70% ethanol tube at the end of each pass by transect. Abundance of adults, nymphs and larvae was confirmed after binocular examination in the laboratory.

Three plots could not be sampled due to the presence of dense gorse (*Ulex europaeus*) that would have torn the flannel. A total of 109 plots were therefore sampled, corresponding to 436 transects and 1744 sampling passes. Tick species were determined after binocular examination using the systematic key from Estrada-Peña (2017). Too few adults were collected (n = 35 including 29 *I. ricinus*). We therefore decided to focus only on *I. ricinus* nymphs and larvae.

Identification of Tick-Borne Pathogens in *I. ricinus* Nymphs

All *I. ricinus* collected nymphs ($n = 173$) were isolated for pathogen identification. We used a microfluidic PCR technique that provides information on the presence of 35 different pathogens of the following genius: *Borrelia, Anaplasma, Rickettsia, Francisella, Bartonella, Babesia* and *Coxiella* potentially present in tick nymphs (developed by Michelet et al., 2014).

Tick nymphs were rinsed twice with distilled water. Prior to extraction, ticks were crushed and processed in a homogeniser at 5,500 rpm for 20 s with 100 µl of elution buffer BE, 40 µl of lysis buffer MG and 10 µl of liquid proteinase K and six steel balls. DNA was extracted using a Nucleospin DNA insect kit

FIGURE 1 | Explanatory diagram showing our study hypotheses at the forest stand level. The biophysical and structural characteristics of the overstorey influence the ticks and associated pathogens through an influence on the within-stand microclimate, the understorey, and the host community in the environment. Diversifying host communities may result in a dilution of competent hosts in the environment and thus affects the prevalence of tick-borne diseases. Dashed arrows represent effects not tested in our study.

(Macherey-Nagel, Germany), and DNA from each sample was stored at −20°C in 50 µl of rehydration solution until next step.

DNA pre-amplification reactions was performed with 2.5 µl of PreAmp Master Mix according to the manufacturer's instructions (Fluidigm, CA, United States). Prior to experiments, primers targeting all pathogens were pooled in equal volumes (200 nM each). The experiment was performed with 1 µL of PreAmp Master Mix, 1.25 µL of pooled primers mix, 1.5 µL distilled water, and 1.25 µL DNA for 5 µL final volume. The pre-amplifications were performed with one cycle at 95°C for two minutes, 14 cycles at 95°C for 15 s and 60°C for four minutes. Pre-amplified DNA was diluted 1:10 and stored at −20°C. Finally, a high-throughput real-time PCR was performed with the BioMark system (Fluidigm, United States) using the method explained in Michelet et al. (2014).

Pathogens detected with BioMark system were confirmed by conventional PCR or nested PCR on several samples presenting low Ct values. Primers targeting different genes or regions than those of the BioMark system were selected from the literature (listed **Supplementary Table 1**) and PCR were performed with Phusion High fidelity kit (Thermo scientific, MA, United States). The positive samples after gel migration were sent to sequencing at Eurofins (Germany) and assembled using

BioEdit software (Ibis Biosciences). An online BLAST (NCBI; National Center for Biotechnology Information) was used to identify the sequenced organisms.

Response Variables

We analyzed four response variables for *I. ricinus*: *larvae presence, nymph abundance* and *proportion of infected nymphs by Borrelia burgdorferi s.l.* and *proportion of infected nymphs by Rickettsia. Nymph abundance* indicator was the cumulative number of nymphs collected after the four successive samplings $\sum_{k=1}^{4} N_{\{i,j,\,k\}_l}$ where $i \in [1;\,14]$, $j \in [1;\,8]$, $k \in [1;\,4]$ and $l \in [1;\,4]$ corresponding to the plot composition, the repetition number, the parallel long transect and the successive samplings, respectively. *Larvae presence* variable is binomial: it is set to 1 when at least one larva was found in the plot, and 0 if none was found. As we performed individual PCR's, the proportion of nymphs infected by each pathogen was tested in the model as a vector including the number of pathogen-positive nymphs and pathogen-negative nymphs in the plot.

Explanatory Variables

We selected three tree layer variables. First, *canopy tree density,* corresponding to the percentage of canopy individuals in

the plot (here pine and/or birch, since oak trees are much smaller in ORPHEE, still being part of the understorey layer; Damien et al., 2016).

Second, *tree composition* corresponds to different types of tree mixtures (a total of nine type of mixtures). In order to obtain sufficient statistical power to test the effect of plot composition on ticks variables, individual plot compositions were combined into four composition types: (1) Pure pine (including monocultures of pines and mixtures of pines and oaks, which are still small enough to be considered part of the understorey); (2) Pure birch (including monocultures of birches and mixtures of birches and oaks); (3) Mixtures of pine and birch (including mixtures of pines and birches, and mixtures of pines, birches and oaks); (4) Oaks (including monocultures of oaks and mixtures of only oaks) (**Table 1**).

Third, *tree species richness* corresponds to the number of different tree species in the plot (including the oak species).

Further, we included understorey (*understorey phytovolume*) and microclimatic variables (presence/absence of *irrigation* and the *saturation deficit* of the plot) into the models.

To describe the understorey vegetation, we sampled one 1 m × 1 m quadrat in each transect passed for the ticks (i.e., four quadrats per plot). In each quadrat, we estimated the percentage of bare soil, litter and moss cover, the percentage cover of the main plant types found in ORPHEE (Corcket et al., 2020), i.e., grasses, heath, ferns and shrubs (mainly gorses), and the maximum height of each plant type. We considered not only plants directly rooted in the quadrat but also those whose aerial parts partially or completely covered the surface of the quadrat. The percentage cover of each type was transformed in eight categorical classes: O: 0%; A: 1–5%; B: 6–15%; C: 16–25%; D:26–50%, E:51–75%; F:75–99%; G: 100% [inspired by the Braun-Blanquet approach (Braun-Blanquet, 1964)]. The maximal height

of each plant type was measured on the highest individual in the quadrat using a measuring pole. Finally, we aggregated the data at the plot level by averaging across the four quadrats to calculate the *understorey phytovolume* (cm³) according to equation (1):

$$Understorey\ phytovolume =$$
$$\sum_{types} mean \left[\frac{cover\ types}{100} \right] \max height\ types \qquad (1)$$

Finally, temperature (T) and relative atmospheric humidity (RH) were measured in each plot $N_{i,j}$ before each sampling (with Kestrel 3500 NV), in order to calculate the *Saturation Deficit* in millimeters of mercury, according to equation (2) (Vourc'h et al., 2016):

$$Saturation\ Deficit = \left(1 - \frac{RH}{100} \right) 4.9463 \exp\ (0.0621\ T) \quad (2)$$

Saturation Deficit, a proxy for tick desiccation potential, is considered a relevant predictor of nymph foraging activity (Gray, 2008).

Statistical Analysis

According to our hypotheses we were interested in testing the effects of tree layer variables, understorey vegetation variables and microclimatic variables on tick response variables. A first sorting of the explanatory variables was done with univariate tests. Variables with a *p* value < 0.1 were selected as candidates for the full models.

Tick nymphs, larvae and associated pathogens were analyzed separately. For *nymph abundance*, we built one negative-binomial generalized linear model (glmer.nb) suitable for overdispersed data (Breslow, 1990). We modeled the *presence of larvae* and *proportion of infected nymphs* with binomial generalized linear models (glm, binomial family).

Nymph abundance, *presence of larvae*, and *proportion of infected nymphs* were modeled as a function of *tree composition* or *canopy tree density*, *irrigation*, *tree species richness*, *understorey phytovolume* and their interactions, and *saturation deficit* as fixed effects. Spatial autocorrelation of plots in the block was accounted for by including *Block* as random factor.

All continuous predictor variables were scaled and centered prior to modeling to make their coefficients comparable (Schielzeth, 2010). We first built full models fitting the most complex model consistent with the experimental design including all fixed effect factors, interactions and random factors. Then we applied model simplification by sequentially removing one-by-one, the less significant terms to allow a non-singular fit (Barr, 2013). Once optimally simplified, we selected the best fitted model with the lowest AIC. Statistical analyses were performed using the R software version 4.0.5 (R Core Team, 2021).

RESULTS

We collected three tick species in ORPHEE, namely *I. ricinus* (L., 1758), *Ixodes frontalis* (PANZER 1795), and *Dermacentor*

TABLE 1 | Table representing the combination of the different tree compositions chosen for the analyses.

Initial tree compositions		Combined tree compositions	
Monocultures	Bp	Birch	Bp
	Qr		BpQr
	Qi		BpQi
	Pp		BpQrQi
		Pine	Pp
2 species mixtures	BpQr		PpQr
	BpQi		PpQi
	BpPp		PpQrQi
	PpQr		
	PpQi	Pine and Birch	PpBp
			PpBpQr
			PpBpQi
3 species mixtures	BPQrQi		
	BpQrPp	Oaks	QiQr
	BpQiPp		Qr
	PpQrQi		Qi

Bp, Betula pendula; Qr: Quercus robur; Qi, Quercus ilex; Pp, Pinus pinaster.
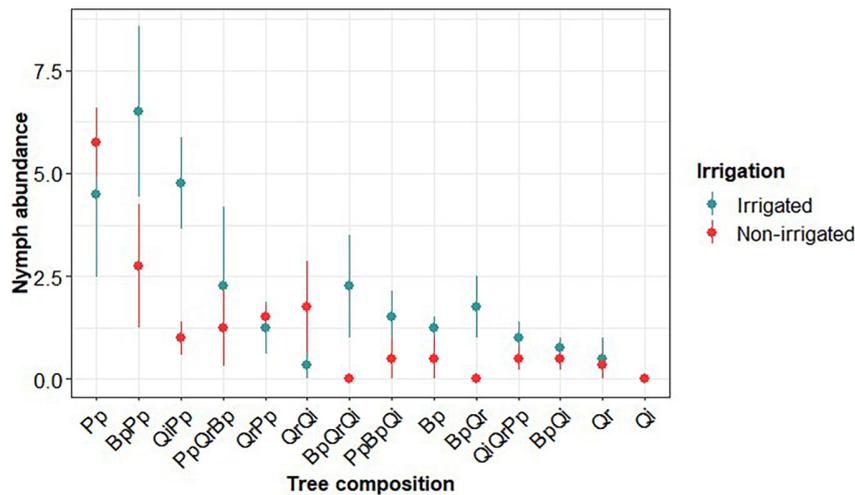
FIGURE 2 | *Ixodes ricinus* nymph abundance as a function of tree composition in all sampled plots, in irrigated plots (blue) and non-irrigated plots (red). Pp: *Pinus pinaster,* Bp*: Betula pendula,* Qr*: Quercus robur,* Qi*: Quercus ilex.* Dots and solid lines represents mean ± SE calculated on raw data.

TABLE 2 | Summary of results of final negative binomial GLM models for Ixodes ricinus nymph abundance, binomial GLMMs for presence of larva and binomial logit model for proportion of infected nymph by Borrelia and Rickettsia pathogens.

	Presence of larvae		Nymph Abundance		Nymph w *Borrelia* s.l.		Nymph w *Rickettsia* spp.	
Predictors	*df*	χ^2-value (*p*)	*df*	χ^2-value (*p*)	*df*	χ^2-value (*p*)	*df*	χ^2-value (*p*)
(Intercept)		(0.706)		(0.798)		**(0.001**)		**(0.03)**
Tree composition	3	**9.00** (0.03)						
Canopy tree density			1	**5.04** (0.02)				
Tree species richness	2	2.05 (0.36)			2	**6.72** (0.003)	2	2.24 (0.33)
Irrigation	1	**4.12** (0.04)	1	**4.13** (0.04)	1	**9.34** (0.02)	1	0.65 (0.41)
Understorey phytovolume			1	**10.16** (0.001)			1	0.98 (0.32)
Saturation deficit					1	2.30 (0.12)		
Random Effect								
σ^2	3.29		0.75		3.29		3.29	
τ_{00}	0.19 Block		0.00 Block		0.00 Block		0.00 Block	
ICC	0.05		0.00		0.00		0.00	
N	8 blocks		8 blocks		8 blocks		8 blocks	
Observations	109		109		65		65	
R^2m / R^2c	0.204 / 0.247		0.483 / 0.483		0.198 / 0.198		0.059 / 0.059	
AIC	148.2		348.7		88.5		105.5	
θ (Dispersion parameter)			2.11					

$\chi2$ and P-values are reported for each model, values in bold are those that were significant. Marginal (R^2m) and conditional (R^2c) regression coefficients, and AIC are given for the minimal model obtained by model simplification.
The random effect corresponds to the "block" effect.
The dispersion parameter is indicated for the model with over-dispersed data.

reticulatus (FABRICIUS, 1794). However, *I. ricinus* was by far the most abundant with 1458/1535 larvae and 173/179 nymphs. We therefore focused our analyses on this species. The abundance of *I. ricinus* nymphs greatly varied between tree composition and the water supply of forest plots (**Figure 2**).

Presence of *I. ricinus* Larvae Along the Tree Diversity Gradient

Larvae were present in 56 out of the 109 sampled plots and their abundance was on average (±sd, n = 1458)

13.38 ± 0.37 per plot and 26.03 ± 0.46 in the plots where they were present. However, because larval distribution was highly aggregated due to clustering in larval nests, we analyzed only presence/absence data.

The probability of presence of the larvae significantly differed between tree compositions (**Figure 3**). Presence of larvae was highest in pine plots (pure and mixed with birch), intermediate in birch plots and lowest in oak plots. We also found a significantly positive effect of irrigation, with higher probability of presence in irrigated plots, irrespective of plot composition (i.e., lack of significant interaction) (**Table 2** and **Figure 3**). The probability
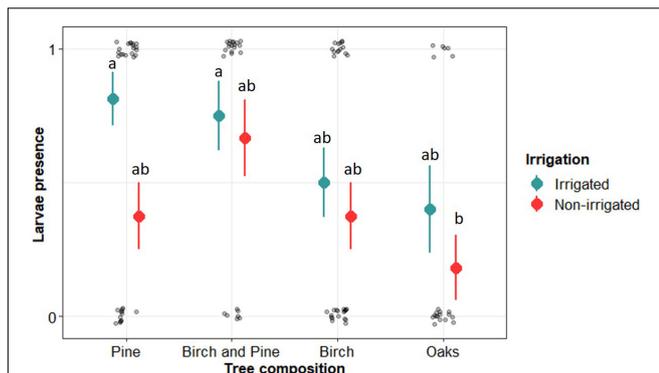
FIGURE 3 | Presence of Ixodes ricinus larva as a function of tree composition and irrigation in the plot. Graph made on raw data. Pine include Pine with or without oaks, Birch includes Birch with or without oaks, Birch and Pine include Birch with pine with or without oaks, and Oaks includes oaks only. Mean values with common letters were not significantly different (Tukey's *post hoc* test). Dots and solid lines represents mean ± SE calculated on raw data.

of larvae presence was significantly higher in irrigated pure and mixed pine plots than in non-irrigated oak plots (**Figure 3**).

Abundance of Tick Nymphs

We collect *I. ricinus* nymphs in 65 out of 109 plots. Nymph abundance was on average (±sd, n = 173) 1.59 ± 0.02, with a median of 1 nymph per plot and 2.66 ± 0.02 in the plots with at least one nymph was present. According to the AIC of compared models, three explanatory variables were retained as significantly influencing the abundance of tick nymphs (**Table 2**). Nymph abundance increased with the density of canopy trees (**Figure 4**) and decreased with the phytovolume of understorey vegetation (**Figure 5**), being significantly higher in irrigated plots, irrespective of their composition (no interaction effect). We did not find any effect of the saturation deficit or tree species richness.

Presence of Infected Nymphs

The control probe was positive for 172 of 173 collected nymphs, so one DNA extraction failed. Infected nymphs were found in 40 of the 65 plots with nymphs, 72 nymphs were infected with at least one pathogen and 6 nymphs presented a co-infection with several pathogens. Among the 72 infected nymphs, the majority of associated pathogens were from the *B. burgdorferi* s.l. complex (n = 35), and *Rickettsia* spp. (n = 32, with 69% of *Rickettsia helvetica* and 31% of *Rickettsia monacensis*), the other pathogens were not found frequently enough to test for a significant relationship with covariates.

The *Borrelia* complex included five different species: 80% of *B. lusitaniae*, 11% *B. afzelii*, 3% *B. valaisiana*, 3% *B. garinii* and 3% *B. burgdorferi s.s*. No *B. spielmanii, B. bissetti,* and no *B. miyamotoi* (relapsing fever *Borrelia*) were found. *B. lusitaniae*, which was the more frequently detected species, is known to be vectored by ticks with reptiles as hosts. Two other reptile-borne pathogens were found: 10 nymphs carried *Rickettsia monacensis* and one carried *Candidatus cryptoplasma*. The proportion of nymphs infected by *B. burgdorferi* s.l. was significantly influenced

by the richness of tree species and also by irrigation, with no interaction between these factors (**Table 2**). The occurrence of nymphs infected by *B. burgdorferi* s.l. pathogen was lower in irrigated plots and decreased with increasing tree species richness, being highest in non-irrigated pure plots and lowest in irrigated three-species mixtures (**Figure 6**).

None of our variables were significantly related to the proportion of ticks infected with a *Rickettsia* pathogen (data not shown).

DISCUSSION

Using a dedicated experiment where both tree diversity and water supply were manipulated, our study demonstrated that the abundance and pathogen load of the most common tick species in Central Europe, *Ixodes ricinus*, were significantly influenced by tree species composition, canopy density, tree species richness, volume of understorey vegetation, and by irrigation. The main trend was a decrease in presence of larvae, abundance of nymphs and proportion of nymphs carrying *Borrelia* pathogens in mixed forests where pine trees were substituted with broadleaved trees, irrespective of plot humidity (be it driven by the weather or controlled *via* irrigation).

Effects of Forest Diversity, Composition and Structure

Our results show that forest diversity and composition can influence tick populations across different stages and their level of infection by pathogens. The probability of presence of larvae was lowest in oak plots, intermediate in birch plots and greatest in pure and mixed pine plots, suggesting a strong tree species identity effect. The abundance of nymphs was significantly affected by canopy tree density, being greater in denser forest plots (e.g., 100% pines, 100% birches or 50% pine–50% birch mixtures), suggesting a stronger effect of forest structure than tree identity. For a tick to be present in an environment, it must (i) have been deposited by a host for nymphal and adult stages or have been laid by a female for the larval stages. (ii) survived in a favorable environment. Overstorey trees can thus have an impact on young tick population by controlling local microclimate (Renaud et al., 2011) and host presence. For example, dense, shaded forests as a result of canopy packing (Jucker et al., 2015; Williams et al., 2017) can be optimal habitats because they provide host animals with food resources while maintaining sufficient moisture for tick survival. A 19-year study in a mature oak-maple mixt forest showed a bottom-up, cascading effect of acorns yield on increasing mouse population levels, resulting in increased abundance of *Ixodes scapularis* (Say, 1821) nymph and associated Lyme disease risks (Ostfeld et al., 2018), and similar results were recently found with *I. ricinus* (Bregnard et al., 2020, 2021). High moisture is important for survival of tick larvae and nymphs (Knülle and Devine, 1972; Tukahirwa, 1976; Davey et al., 1991) and Gray (1998) explains that *I. ricinus* are usually found in environments with 80% atmospheric humidity. The questing activity of tick nymphs occurs higher in the understorey vegetation than that of larvae, so that they can attached to a
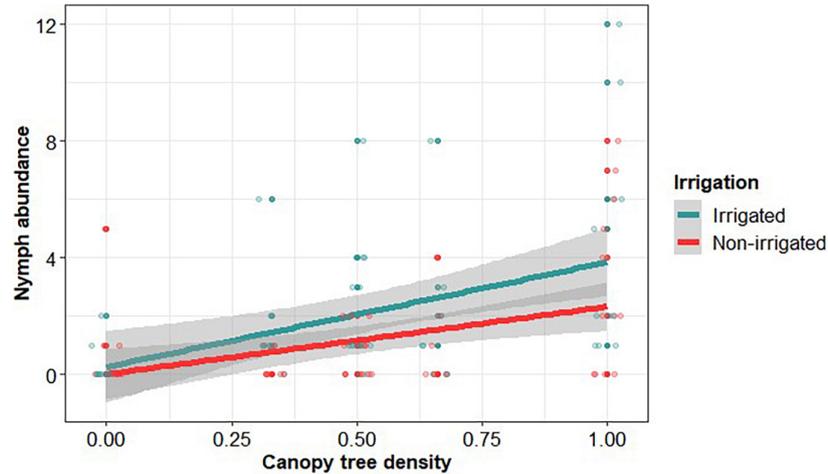
FIGURE 4 | *Ixodes ricinus* nymph abundance as a function of canopy tree density and irrigation in the plot. Canopy tree density is the ratio of the number of birch and/or pine trees to 100 total trees in the plot. Solid lines and gray shaded area correspond to the regression line and its 95% confidence interval, calculated on raw data.
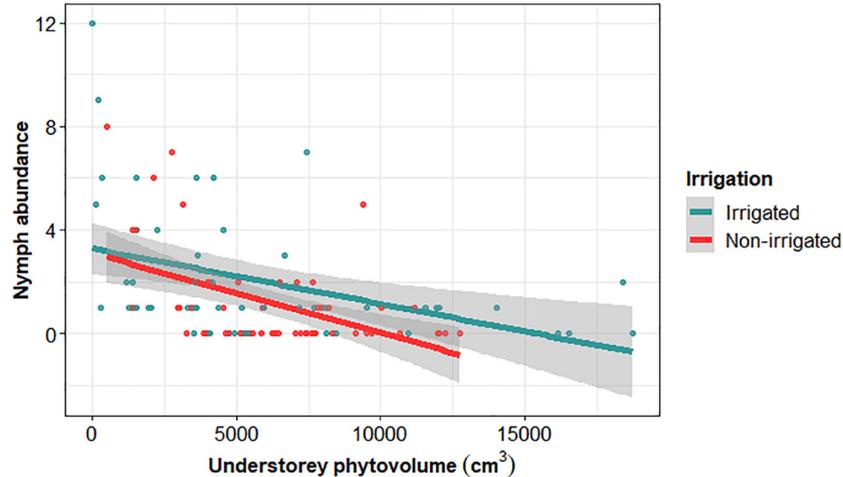

FIGURE 5 | *Ixodes ricinus* nymph abundance as a function of understorey phytovolume in irrigated (blue) and non-irrigated plots (red). Solid lines and gray shaded area correspond to the regression line and its 95% confidence interval, calculated on raw data.

passing animal, but this presents a greater risk of desiccation during this questing period (Schulze and Jordan, 2005). Denser forests with more trees and with a higher tree canopy packing could therefore limit this risk of desiccation and thus favoring the survival of tick nymphs. This explanation for the positive correlation between nymph abundance and proportion of canopy trees in our experimental plots is also consistent with our result of a positive effect of irrigation.

The presence of larvae and their questing activity are related to the microclimate closer to the ground (Yoder et al., 2006; Ehrmann et al., 2017). Leal et al. (2020) suggest that forest types where the most larvae are found differ between tick species. Indeed, several studies have found greater abundance of *I. ricinus* larvae in deciduous forests (Walker et al., 2001; Tack et al., 2012; Ruyts et al., 2018). Due to the much slower

growth of these species, the plots with only oaks in ORPHEE are currently characterized by small trees of about 2 m height and thus an open canopy. Our results in deciduous plots are therefore to be distinguished from the scientific literature on mature deciduous forests. These conditions on ORPHEE were probably less favorable for the maintenance of suitable humidity in the understorey and for the visit of host animals. In contrast, pine plots characterized by dense and tall trees, may have favored larval survival by providing thick needle litter as a protective barrier against desiccation. Birch plots are in an intermediate position in terms of likelihood of larvae presence, probably because they are composed of high trees but with lower canopy closure and perhaps lower quality litter for ticks.

The proportion of nymphs vectoring *Borrelia* pathogens significantly decreased with increasing tree species richness,
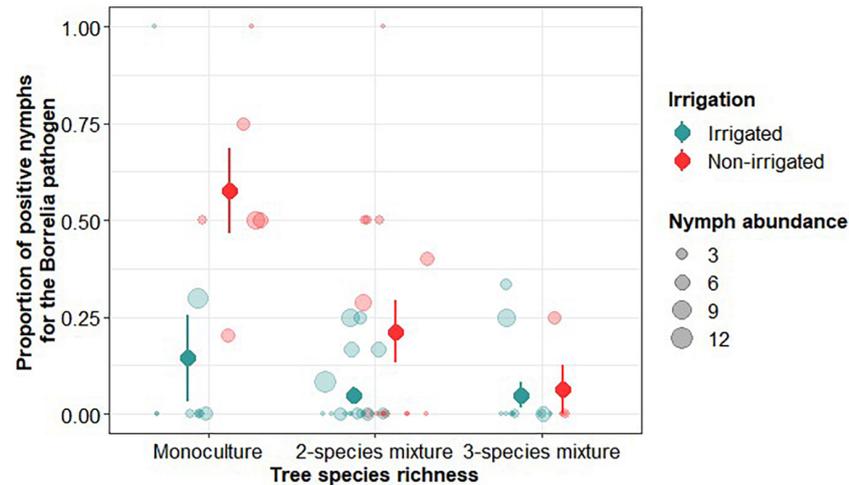
FIGURE 6 | Proportion of positive *Ixodes ricinus* nymphs for *Borrelia burgdorferi* s.l. pathogen as a function of tree species richness and irrigation. The size of the dots is proportional to the total number of nymphs on which the proportion of positive nymphs was calculated. Dots and solid lines represents mean ± SE calculated on raw data.

with the highest frequency in pure pine plots and the lowest in mixtures associating pine, birch and oaks. Our results thus support the finding by Ehrmann et al. (2018) that a reduction in the prevalence of tick-borne diseases is associated with greater forest diversity, confirming the beneficial role of tree species mixing for natural risk reduction (Jactel et al., 2017). Ruyts et al. (2016) did not find a tree species diversity effect on the prevalence of tick-borne pathogens but they found that the risk of Lyme borreliosis differed between different forest compositions. They showed that *B. afzelii* was more often found on ticks in pine plots while *B. garinii* and *B. burgdorferi* s.s. were more prevalent in oak plots. A negative effect of tree species richness on infected nymphs could be explained by an increase in ecological niches for host species, resulting in a change in the ratio of competent to incompetent reservoir hosts, with diluting effects on *Borrelia* (Levi et al., 2016; Ehrmann et al., 2018). Empirical multitaxonomic studies (e.g., Ampoorter et al., 2020) and meta-analyses (e.g., Stemmelen et al., 2021) have indeed shown greater faunal diversity in mixed forests than in pure forests. Additional research is now needed to inventory potential tick host animals in pure and mixed forests and better assess their level of competence for tick-borne pathogens.

Ruyts et al. (2016) suggested that the dilution effect would depend on the species of *B. burgdorferi* s.l. In our study, 80% of *B. burgdorferi* s.l. were in fact *B. lusitaniae,* which appears to be carried and multiplied by lizards in several countries around the Mediterranean rim (Michelis et al., 2000; Younsi et al., 2001; Sarih et al., 2003; Tomassone et al., 2017). As ectothermic animals, lizards often prefer environments that allow them significant sun exposure for active thermoregulation (Smith and Ballinger, 2001). This may explain the negative effect of irrigation on the proportion of nymphs infected by *Borrelia burgdorferi* s.l. pathogens, either directly by increasing soil humidity or indirectly by increasing the density and thus shading of understorey vegetation (Corcket et al., 2020). We do not have

yet direct data from the ORPHEE experiment but surveys in the Aquitaine region reported the presence of three lizard species in forests: the common wall lizard [*Podarcis muralis* (Laurenti, 1768)], the viviparous lizard [*Zootoca vivipara* (Lichtentein, 1823)] and the western green lizard (*Lacerta bilineata*, Daudin, 1802) (Berroneau, 2015). All are known as *I. ricinus* potential hosts (Scali et al., 2001; Richter and Matuschka, 2006; Wu et al., 2019; Gwiazdowicz et al., 2020), and two of them (common wall and western green lizards) have already been involved in the transmission of *B. lusitaniae* in *I. ricinus* populations (Richter and Matuschka, 2006; Mendoza-Roldan et al., 2021). To date, no studies on the dilution effect of lizard hosts have been tested on *B. lusitaniae;* it would therefore be interesting to inventory them along a gradient of tree diversity and also to classify them according to their level of competence.

Finally, no variable explained the proportion of *I. ricinus* nymphs infected with *Rickettsia* spp. pathogens. The ecological drivers of distribution and prevalence of *Rickettsia* spp. remain poorly understood in the literature and even less the effect of forest composition or diversity.

Effects of Understorey

Our study showed a distinct negative effect of the phytovolume of understorey vegetation on nymph abundance, independently from canopy diversity or composition. Several studies found opposite results with the abundance of nymphs positively correlated with high density of understorey vegetation, a condition considered favorable for visit by potential tick host animals (Tack et al., 2013; Ostfeld et al., 2018). However, other studies found a negative relationship between high density of understorey vegetation and nymph abundance, suggesting on the contrary that these environments would be less visited by hosts or would not be associated with good tick survival rates (Stein et al., 2008). Similarly, a denser understorey vegetation can also dilute tick infection prevalence (Ehrmann et al., 2018). A dense

understorey can provide a suitable habitat for ticks but also for small mammals; this increases the likelihood of ticks finding a host, but at the same time decreases the likelihood that many ticks can make contact with the same host species. Thus, a lower average tick load and a lower co-feeding probability on hosts could result in dilution of *Borrelia* prevalence.

Bord et al. (2014) also highlighted that the dragflag technique, although very effective for sampling free-living ticks in forest, is less effective when the understorey is too dense, thus resulting in underestimation of tick abundance. In our study, the negative effect of understorey phytovolume on nymph abundance is, however, still present at low levels of understorey phytovolume. In addition, the phytovolume does not take into account plant species identity, an important factor for host attraction. In ORPHEE, the main understorey plant species were *Molinia caerulae, Ulex europaeus, Pteridium aquilinum* and some *Ericaceae* spp. (Corcket et al., 2020), which are not or only little palatable for many herbivorous hosts and whose density or height may limit the passage of large ones.

Medical Relevance and Practical Perspectives

All *Rickettsia* and *Borrelia* pathogens detected in our study are transmissible to humans and of medical importance (Steere et al., 2004; Fournier and Raoult, 2009). *R. helvetica* and *R. monacensis* are belonging to the spotted fever group (Merhej and Raoult, 2011) and *B. afzelii, B. valaisiana, B. garinii* and *B. burgdorferi* s.s. are bacteria involved in the Lyme disease in humans (Cutler et al., 2017). Although the transmission of *B. lusitaniae* to humans has been demonstrated, the link with Lyme disease has yet to be investigated (Stanek and Reiter, 2011). Previous studies found *B. lusitaniae* associated with medical symptoms in humans after a tick bite (Collares-Pereira et al., 2004; Franca et al., 2005; Lopes de Carvalho et al., 2008). In our study, the proportion of infected nymphs did not correlate with nymph abundance, which confirms that it is important to distinguish tick abundance vs. tick-borne pathogen dynamics and to study pathogens at the species level, due to their host and home range specificity.

CONCLUSION

Our results suggest that various habitat characteristics, including tree overstorey composition, structure and diversity, as well as understorey density, can affect tick abundance and pathogen prevalence. They thus support the notion that habitats can have either "diluting" or "amplifying" characteristics for tick-borne diseases with direct relevance for human health (Ehrmann et al., 2018). However, more research is needed to

decipher the underlying mechanisms and to determine the optimal characteristics of heterogeneous forests to inform managers. Still, our results support the generally higher risk to get infected by tick-borne diseases in shady, humid and dense forests during the tick activity period. Nevertheless, evidence is accumulating that "dilution habitats" are characterized by high structural, functional or compositional diversity of the plant community, with propagating effects on host species composition and abundance, and hence on host dilution. If further supported by additional research, this perspective would open possibilities for an active tick-control reducing the survival of ticks and/or decreasing the proportion of infected ticks through forest management that supports the different facets of forest biodiversity.

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.

AUTHOR CONTRIBUTIONS

AB and HJ designed the study, collected the data, and wrote the first draft. SM and CG performed the molecular analysis and formatted the related data. AB computed the data and run the analysis with SB and JD. All authors contributed substantially to subsequent revisions.

FUNDING

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.891908/full#supplementary-material

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