



Original article

Effects of cattle grazing on *Ixodes ricinus*-borne disease risk in forest areas of the NetherlandsHein Sprong^{a,*}, Sander Moonen^{b,1}, Sipke E. van Wieren^b, Tim R. Hofmeester^{b,2}^a Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, 3720 MA, Bilthoven, The Netherlands^b Resource Ecology Group, Wageningen University, Wageningen, The Netherlands

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ABSTRACT

Cattle grazing has been suggested to reduce the risk for Lyme borreliosis by decreasing the density of questing *Ixodes ricinus* infected with *Borrelia burgdorferi* sensu lato. We tested the hypotheses that cattle grazing used in woodland management decreases the density of questing *I. ricinus*, and that it decreases the nymphal infection prevalence of *B. burgdorferi* sensu lato. We further expected the nymphal infection prevalence of tick-borne pathogens that utilize cattle as amplifying hosts, namely *Anaplasma phagocytophilum*, and *Babesia* sensu stricto, to increase. To test these hypotheses, we compared the densities of questing *I. ricinus* between twenty pairs of plots in grazed and ungrazed forest areas. The density of *I. ricinus* adults, but not nymphs, was lower in areas grazed by cattle than in ungrazed areas. Nymphs were tested for the presence of *Borrelia burgdorferi* s.l., *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, and *Babesia* s.s. DNA from twelve paired areas. *Anaplasma phagocytophilum* and *Babesia* s.s. from qPCR-positive tick lysates were identified further to the ecotype and species level, respectively, by DNA sequencing. The infection prevalence of *A. phagocytophilum* was lower, and infection prevalence of *Babesia* s.s., identified as *Babesia venatorum*, was higher in grazed areas. In contrast, infection prevalence with *B. burgdorferi* s.l. or *B. miyamotoi* did not differ between grazed and ungrazed areas. As a consequence, conventional cattle grazing in forested areas had no effect on the densities of questing nymphs infected with *B. burgdorferi* s.l. and *B. miyamotoi*. Similarly, we found no effect of cattle grazing on the density of infected nymphs with *B. venatorum*. The marked difference in the densities of questing nymphs infected with *A. phagocytophilum* could potentially be explained by the presence of a higher density of roe deer (*Capreolus capreolus*) in ungrazed areas, as the majority of typed *A. phagocytophilum* from ungrazed areas were the non-zoonotic ecotype II, which is associated with roe deer.

1. Introduction

Ixodes ricinus transmits pathogens of medical and veterinary importance (Sprong et al., 2018). Particularly, infections with spirochetes of the *Borrelia* genus cause a serious health concern in humans (Steere et al., 2016; Wagemakers et al., 2015), whereas *Anaplasma phagocytophilum* and *Babesia* sensu stricto cause considerable economic loss in domestic animals (Beugnet and Moreau, 2015; Jalovecka et al., 2019; Stuenkel et al., 2013). Control of *I. ricinus*-borne diseases in humans primarily consists of the promotion of personal preventive actions for the public and for risk groups, such as forest workers, by providing information and education (Beaujean et al., 2013; Piesman and Eisen, 2008). Personal protective measures have poor rates of compliance and

their effectiveness has been difficult to demonstrate in terms of reducing disease cases. For example, providing information and education has not resulted in a decline in the incidence of LB in the Netherlands, not even after intensified efforts since 2003 (Hofhuis et al., 2015).

Environmental-based approaches to control tick-borne diseases rely on reduction of tick suitable habitats, the disruption of the tick lifecycle, or interference with pathogen transmission (Braks et al., 2016). An advantage of environmental-based control options is that most of them can readily be applied in various practical situations, as they involve existing nature management options, such as fencing, mowing or intensive grazing (Eisen and Dolan, 2016; Eisen and Eisen, 2018). Several studies have indicated that cattle grazing on pastures or forested areas could be used to reduce the risk for Lyme borreliosis (LB) by decreasing

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the density of questing *I. ricinus* infected with *B. burgdorferi* sensu lato (Gassner et al., 2008; Richter and Matuschka, 2006). Cattle can act as feeding and propagation host for *I. ricinus*, thus supporting local tick populations on one hand (Mierzejewska et al., 2015). On the other hand, cattle can negatively affect the survival of ticks by modifying the vegetation structure and litter layer (Jorritsma et al., 1999) as well as the rodent distribution (Smit et al., 2001). Furthermore, cattle do not support *B. burgdorferi* s.l. transmission, and can therefore act as dilution hosts (Perkins et al., 2006). Since cattle can be systematically infected with *A. phagocytophilum* and *Babesia* s.s., they might act as amplifying hosts for these two tick-borne pathogens (Stuenkel et al., 2013; Suarez et al., 2019). Woodland management using cattle grazing, a practise that is commonly applied in the Netherlands (Piek, 1998), could thus potentially influence tick-borne disease risk in forested areas.

Since the number of studies where the effect of cattle grazing in forest areas on *I. ricinus*-borne disease risk is rather limited, we investigated the following three hypotheses: Grazing by cattle in forested areas I) decreases the density of questing *I. ricinus*, II) decreases the *B. burgdorferi* s.l. prevalence in questing ticks, and III) increases the *A. phagocytophilum* and *Babesia* s.s. prevalence in questing ticks. We therefore predict that forested areas with cattle grazing will have a reduced risk for Lyme borreliosis, and have a neutral or increased risk for anaplasmosis and babesiosis. We tested our hypotheses in a cross-sectional study, where we compared the densities of questing *I. ricinus* infected with tick-borne pathogens between twenty grazed and ungrazed forest areas. Cattle grazing in these areas was ongoing for more than five years prior to our study for woodland management purposes.

2. Materials and methods

2.1. Study sites

We selected twenty locations where we could compare paired grazed and ungrazed plots in forested areas (Supplementary Table I). In order to ensure independence between areas, we used the rule that each grazing area, a fenced area where cattle are present for grazing management, was sampled once. However, as the same nature reserve could have multiple grazing areas, we regularly sampled multiple areas within the same larger management unit, where we kept a minimum distance of 4 km between areas to ensure independence of the tick populations. The paired plots within each area were separated by a fence, but were situated in the same forest area, with the same forest type and similar understory vegetation to ensure a similar abiotic and biotic environment within pairs. Between the two paired plots, there was a distance between 100 and 500 m, which ensured that there was no overlap in rodent home ranges between plots (Benhamou, 2001). The ungrazed plots were selected by signs for the presence of wild herbivores (deer, wild boar). In the Netherlands, cattle grazing for woodland management is sometimes combined with other large grazers, such as horses, goats and sheep (Piek, 1998). For this reason within some of the grazed plots, groups of one or more of these species were present.

2.2. Density of questing *I. ricinus*

Each area was visited once in June or July 2015, and ticks were collected from paired plots on the same day with optimal conditions: on dry days, with air temperature > 10 °C, and in dry vegetation < 60 cm high. Ticks were collected using blanket-dragging covering a surface of 200 m² in each plot as described previously (Hofmeester et al., 2017). We counted all the nymphs and adults after every 10 m of dragging to avoid the risk of losing collected ticks. All nymphs were collected in microcentrifuge tubes and stored at -18 °C until pathogen analysis. In areas with relatively low nymphal densities, we continued dragging after the initial 200 m to increase the sample size for pathogen prevalence. We did not consider these extra nymphs in the density

estimate. Upon arrival in the laboratory, the ticks were identified by an experienced technician using morphological keys as described (Arthur, 1963; Hillyard, 1996). Only *I. ricinus* nymphs were used for further analysis.

2.3. Pathogen identification and prevalence

We tested for the presence of microorganisms in nymphs from a subset of twelve areas (Supplementary Table I), and excluded ticks from paired locations where very low number of ticks were collected. DNA extraction from the individual questing ticks was done by alkaline lysis in ammonium hydroxide (Schouls et al., 1999). For the detection of *B. burgdorferi* s.l. DNA, a duplex qPCR was used, based on the detection of fragments of the outer surface protein A (ospA) and flagellin genes (Heylen et al., 2013). For detection of *B. miyamotoi*, a qPCR assay was used that targets a region of the flagellin gene, specific for *B. miyamotoi* (Hovius et al., 2013). The detection and typing of *A. phagocytophilum* DNA was performed as described previously (Jaarsma et al., 2019; Jahfari et al., 2014). The detection and typing of *Babesia* species from the *Babesia* s.s. clade was performed as described previously (Øines et al., 2012; Wielinga et al., 2009). These qPCRs were carried out on a LightCycler 480 (Roche Diagnostics Nederland B.V, Almere, the Netherlands) in a final volume of 20 µl with iQ multiplex Powermix, 3 µl of sample and 0.2 µM for all primers and different concentrations for probes (Kazimírová et al., 2018). Positive controls were based on plasmids containing the primer-probe-primer sequences for the target qPCR plus unique nucleotide codes between the primer and the probes. These constructs enable us to distinguish potential contaminations of samples with positive controls. Negative processing controls (50 µl distilled water) were used during the whole DNA extraction and qPCR procedure, and negative qPCR controls (distilled water) were also used with the positive controls on every plate tested. To minimize contamination, and false-positive samples, the DNA extraction, PCR mix preparation, sample addition, and (q)PCR analyses were performed in separated air locked dedicated labs. DNA sequences of *A. phagocytophilum* obtained in this study are in GenBank (accession numbers MN398177-MN398185). DNA sequences of *B. venatorum* are identical to the sequence with GenBank accession number KF447532.

2.4. Statistical analyses

We tested for differences in *I. ricinus* density, infection prevalence of the different tick-borne pathogens (NIP) and differences in the density of infected nymphs (DIN) for the different pathogens with generalized linear mixed models (GLMMs). For tick densities and DIN, we used a negative binomial distribution and log link function, which accommodates the overdispersion in the count data (Kim et al., 2014). For NIP, we used a binomial distribution with logit link function, which accommodates the limitation to 0 and 1 of presence/absence data. All models had a random intercept per area to account for the paired sampling of grazed and ungrazed plots. All analyses were performed in R 3.6.0 (R Core Team (2019), 2019) using the glmmTMB package (Brooks et al., 2017)

3. Results

In total, 2633 *I. ricinus* nymphs and 124 *I. ricinus* adults were collected in the 40 plots (Supplementary Table I). The mean density of questing nymphs (66/200 m²; range: 0–197) was 21 times higher than the density of questing adults (3,1/200 m²; range: 0–14). There was no difference in densities of questing *I. ricinus* nymphs between grazed and ungrazed plots ($\beta = -0.07$, $p = 0.56$). In contrast, we found a negative association between grazing and *I. ricinus* adult densities ($\beta = -0.49$, $p = 0.08$; Fig. 1A).

We tested 2240 *I. ricinus* nymphs from 24 plots (Supplementary Table I) for the presence of tick-borne pathogens. The mean infection

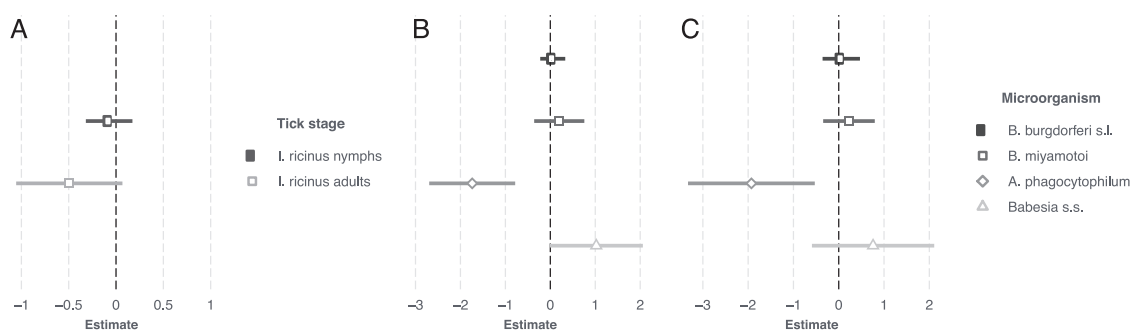


Fig. 1. Model estimates from generalized linear mixed models for the difference between grazed and ungrazed plots. A: Estimated differences in *Ixodes ricinus* nymphal and adult densities, B: Estimated differences in nymphal infection prevalence for the different pathogens, C: Estimated differences in density of infected nymphs for the different pathogens. Horizontal bars represent the 95 % confidence interval of the estimates. Positive values show a higher density or infection prevalence in grazed plots.

prevalence in nymphs was 0.10 (range: 0.02-0.27) for *B. burgdorferi* s.l., 0.02 (range: 0-0.06) for *B. miyamotoi*, 0.02 (range: 0-0.11) for *A. phagocytophilum*, and 0.01 (range: 0-0.03) for *Babesia* s.s. We found no differences in the prevalence of *B. burgdorferi* s.l. ($\beta = 0.05$, $p = 0.70$) or *B. miyamotoi* ($\beta = 0.20$, $p = 0.49$) in nymphs between grazed and ungrazed plots (Fig. 1B). Remarkably, areas with cattle grazing had a significantly lower *A. phagocytophilum* prevalence in nymphs ($\beta = -1.92$, $p = 0.0004$), and a higher infection prevalence of *Babesia* s.s. ($\beta = 1.02$, $p = 0.05$; Fig. 1B). Consequently, we found no differences in the density of questing nymphs infected with *B. burgdorferi* s.l. ($\beta = 0.05$, $p = 0.79$), *B. miyamotoi* ($\beta = 0.23$, $p = 0.44$), or, surprisingly, *Babesia* s.s. ($\beta = 0.76$, $p = 0.27$) between grazed and ungrazed plots (Fig. 1C). In contrast, the density of questing nymphs infected with *A. phagocytophilum* was lower in grazed plots ($\beta = -1.93$, $p = 0.007$; Fig. 1C).

From the 34 *A. phagocytophilum*-positive ticks, only nine could be typed by conventional PCR and sequencing. These nine ticks were all from ungrazed plots. Three were identified as the zoonotic ecotype I and six as the non-zoonotic ecotype II (Jaarsma et al., 2019; Jahfari et al., 2014). From the 18 *Babesia* s.s.-positive ticks, fourteen could be typed by conventional PCR and sequencing, four from ungrazed and ten from grazed plots. These fourteen ticks were all identified as the zoonotic *B. venatorum* (sequences were identical to GenBank accession number KF447532).

4. Discussion

Grazing by cattle has been suggested as an intervention strategy to reduce tick-borne disease risk, especially for *B. burgdorferi* s.l. (Boyard et al., 2011; Gassner et al., 2008; Richter and Matuschka, 2006; Ruiz-Fons et al., 2012). However, there have been few studies to date that tested the difference between grazed and ungrazed plots over a large spatial scale. Here, we studied 20 pairs of grazed and ungrazed plots to test for an effect of grazing on *I. ricinus* densities, the infection prevalence of *I. ricinus* nymphs (NIP) with four microorganisms, and the density of infected nymphs (DIN) for these four microorganisms. We found that, in contrast to our expectation, grazing reduced the NIP and DIN with *Anaplasma phagocytophilum*. We did not find differences between grazed and ungrazed plots in *I. ricinus* nymphal densities nor in DIN for the other pathogens. NIP of *Babesia* s.s. was higher in grazed plots compared to ungrazed plots, while NIP with *B. burgdorferi* s.l. and *B. miyamotoi* did not differ between grazed and ungrazed plots. The infection prevalence of tick-borne pathogens is generally higher (approximately two times) in questing adult ticks than in nymphs, but their densities are generally much lower than of nymphs (21 times in this study).

A previous study in the Netherlands showed decreased tick densities in grazed plots compared to ungrazed plots (Gassner et al., 2008). That study was performed in one of the areas that was also included in this

study (Telefoonweg). We indeed found slightly higher tick densities in the ungrazed plot in this area, a pattern that was not consistent with the majority of our dataset. This discrepancy shows the difficulty of making general conclusions from single study sites, although studies in single sites are important to generate hypotheses that should then be tested in multiple sites. In this study, we performed that test and found that the general pattern in a cross-sectional study covering multiple areas showed an opposite trend to the original study by Gassner et al. (2008) that generated the hypothesis.

Previous studies also showed a decreased *B. burgdorferi* s.l. infection in grazed plots compared to ungrazed plots (Richter and Matuschka, 2006; Ruiz-Fons et al., 2012). As cattle are non-competent as reservoirs for *B. burgdorferi* s.l. (Richter and Matuschka, 2006), this is to be expected if the cattle feed a sufficient proportion of the *I. ricinus* larvae, either as a function of their tick burden or their density (Hofmeester et al., 2016). Generally, cattle densities as used for management in the Netherlands are very low (Piek, 1998), which could explain why we did not find a diluting effect of cattle on *B. burgdorferi* s.l. infection in questing *I. ricinus* nymphs.

As domestic animals, such as the cattle and other animals that were grazing in the studied areas, can function as a reservoir host for *A. phagocytophilum* (Stuen et al., 2013), we were surprised to find a decreased NIP and DIN with *A. phagocytophilum* in grazed areas. One explanation could be that the majority of infected ticks that we could successfully sequence were infected with the non-zoonotic ecotype II that is most likely maintained by roe deer (Jaarsma et al., 2019; Jahfari et al., 2014). Wild ungulate densities can be lower in grazed areas due to competition over food resources (Kuipers et al., 2005). This could have led to higher roe deer densities in the ungrazed plots, resulting in higher NIP with *A. phagocytophilum* ecotype II. However, the number of *A. phagocytophilum* infected nymphs that we were able to sequence was very low and all ecotype II sequences were from ungrazed areas. As such, we cannot rule out that the lower infection prevalence in grazed areas could in fact be of the zoonotic ecotype I maintained by the domestic grazers rather than the non-zoonotic ecotype II maintained by roe deer (Jaarsma et al., 2019; Jahfari et al., 2014). Furthermore, both densities of domestic grazers and wild ungulates were unknown in our study areas. Future studies should include measures of both wild and domestic ungulate densities and a larger sample size of infections with ecotype identification to disentangle this mechanism.

We found an increased infection prevalence of *Babesia* s.s. in *I. ricinus* nymphs collected from grazed plots. This could be explained by the fact that cattle are reservoir competent hosts for *B. venatorum* and *B. divergens* (Suarez et al., 2019). The differing findings between *B. burgdorferi* s.l., where we did not find a dilution effect, and *Babesia* s.s., where we do find an amplification effect, could be due to the fact that the number of available reservoir competent hosts for *Babesia* s.s. is much lower, resulting in overall lower infection prevalence and therefore a statistically more significant increase as soon as there is a

reservoir competent host present. Another explanation could be that *Babesia* s.s. can be transovarially transmitted, while there this is not known for *A. phagocytophilum* (Severinsson et al., 2010). As cattle are likely to feed mainly adult ticks (Estrada-Peña et al., 1995), this different transmission mechanism might also explain the relationship we found.

5. Conclusion

Overall, we only found minor differences in *I. ricinus* densities, and no differences in the infection prevalence and density of infected nymphs for *B. burgdorferi* s.l. between grazed and ungrazed plots. We thus conclude that conventional grazing as applied in forest systems in the Netherlands will likely not be effective as a management strategy to reduce *I. ricinus* densities or Lyme borreliosis risk. However, we did find reduced infection prevalence and densities of infected nymphs for *Anaplasma phagocytophilum*. As the exact mechanism for this effect is still unknown, we recommend further studies into this effect, and especially the role of deer, before grazing with livestock in low densities can be advised as a management strategy to reduce anaplasmosis risk.

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CRedit authorship contribution statement

Hein Sprong: Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft. **Sander Moonen:** Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Sipke E. van Wieren:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - review & editing. **Tim R. Hofmeester:** Conceptualization, Formal analysis, Investigation, Visualization, Writing - original draft.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.ttbdis.2019.101355>.

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