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Longitudinal Analysis of Tick Densities and *Borrelia*, *Anaplasma*, and *Ehrlichia* Infections of *Ixodes ricinus* Ticks in Different Habitat Areas in The Netherlands ▽

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ABSTRACT

From 2000 to 2004, ticks were collected by dragging a blanket in four habitat areas in The Netherlands: dunes, heather, forest, and a city park. Tick densities were calculated, and infection with *Borrelia burgdorferi* and *Anaplasma* and *Ehrlichia* species was investigated by reverse line blot analysis. The lowest tick density was observed in the heather area (1 to 8/100 m^2). In the oak forest and city park, the tick densities ranged from 26 to 45/100 m^2. The highest tick density was found in the dune area (139 to 551/100 m^2). The infection rates varied significantly for the four study areas and years, ranging from 0.8 to 11.5% for *Borrelia* spp. and 1 to 16% for *Ehrlichia* or *Anaplasma* (*Ehrlichia/Anaplasma*) spp. *Borrelia* infection rates were highest in the dunes, followed by the forest, the city park, and heather area. In contrast, *Ehrlichia/Anaplasma* was found most often in the forest and less often in the city park. The following *Borrelia* species were found: *Borrelia* sensu lato strains not identified to the species level (2.5%), *B. afzelii* (2.5%), *B. valaisiana* (0.9%), *B. burgdorferi* sensu stricto (0.13%), and *B. garinii* (0.13%). For *Ehrlichia/Anaplasma* species, *Ehrlichia* and *Anaplasma* spp. not identified to the species level (2.5%), *Anaplasma schotti* variant (3.5%), *Anaplasma phagocytophilum* variant (0.3%), and *Ehrlichia canis* (0.19%) were found. *E. canis* is reported for the first time in ticks in The Netherlands in this study. *Borrelia lusitaniae*, *Ehrlichia chaffeensis*, and the human granylocytic anaplasmosis agent were not detected. About 1.6% of the ticks were infected with both *Borrelia* and *Ehrlichia/Anaplasma*, which was higher than the frequency predicted from the individual infection rates, suggesting hosts with multiple infections or a possible selective advantage of coinfection.

INTRODUCTION

Blood-sucking ticks parasitizing animals and humans are found worldwide. Their involvement in zoonotic disease transmission, transmission of microorganisms (viruses, bacteria, and parasites) from animal reservoirs to humans, is well-known. Over 800 tick species have been described, but only a few of the *Ixodes*, *Rhipicephalus*, *Dermacentor*, *Hyalomma*, and *Haemaphysalis* tick species are known to transfer diseases to humans (10, 17). In The Netherlands and in Europe, the most common tick is *Ixodes ricinus*. *I. ricinus* ticks may transmit the spirochete *Borrelia* spp. causing Lyme borreliosis, as well as other diseases (33). Other well-known tick-transmitted pathogenic microorganisms are the intracellular bacteria *Anaplasma* and *Ehrlichia* (9), *Rickettsia* (25), the intracellular eukaryotic protozoan parasites *Babesia* and *Theileria* (9, 12) and tick-borne encephalitis virus. Several species or genomospecies of these organisms have been associated with distinct diseases. *Borrelia garinii* has been associated with neuroborreliosis, *Borrelia burgdorferi* senso stricto has been

associated with arthritis, and *Borrelia afzelii* has been associated with acrodermatis chronica atropicans (3, 24, 34, 35, 37). *Ehrlichia chaffeensis* (2) may cause human monocytic anaplasmosis, and the human granylocytic anaplasmosis agent (HGA), which has been found to be *Anaplasma phagocytophilum* (8), affects neutrophils (5).

Environmental factors, such as climate, vegetation type, and abundance of suitable hosts, limit the geographic distribution of the ticks and the pathogens they may carry. A comparison of the *Borrelia* species in Europe and the United States shows that there are some clear differences: *B. burgdorferi* sensu stricto is the sole *B. burgdorferi* genomospecies in the United States, while in Europe, *B. afzelii* and *B. garinii* are the predominant species and *B. burgdorferi* sensu stricto is found only in a minority of the cases. *Borrelia valaisiana* (or VS116) and *Borrelia lusitaniae* (or PotiB2) are two other subspecies that are found in European ticks and may be associated with human disease. In the United States, *Ixodes scapularis* is the most common disease-transmitting tick, while in Europe, it is *I. ricinus* (26, 27). Environmental factors, such as climate (changes), (de)forestation, increases in the roe deer population, or introduction of new animal reservoirs, may lead to changing numbers of ticks and dispersal of the tick population and the pathogens they carry. Such changes may lead to a new status quo of the risk of tick bites for human and animal health (16, 23, 31). Monitoring tick distribution and the prevalence of tick-transmitted pathogens is therefore essential to describe and understand the risk of tick-borne disease of the predominant tick species and probably the sole vector for Lyme disease. Earlier studies in The Netherlands have shown that *I. ricinus* may carry different *Borrelia*, *Anaplasma*, and *Ehrlichia* species and sporadically, some *Babesia* species (13).

Erythema migrans (EM) is a clear clinical manifestation of Lyme disease and serves as an indicator for transmission of *Borrelia* sensu lato. EM is found in about 90% of the human cases of Lyme borreliosis (22). A study using questionnaires filled out by a large group of Dutch general practitioners in the period from 1994 to 2001 showed a doubling of the reports of tick-biting incidence and the diagnosis of EM (7). Recently, this study has been repeated and again showed an increase in these incidences for 2006 (11). This suggests that the number of ticks is increasing or that people are coming into contact with ticks more often. Here we report results of tick densities in the period from 2000 to 2004 in four different areas in The Netherlands that are open for recreation: a dune area with rich vegetation near the North Sea (Duin and Kruidberg), a city park near Amsterdam (Bijlmerweide), and two areas in the Koninklijke Houtvesterijen region, an oak forest with blueberries and a heather area. Using PCR and subsequent reverse line blot (RLB) hybridizations, we determined which proportion of the collected ticks was infected with various *Borrelia* sensu lato species and *Ehrlichia* or *Anaplasma* (*Ehrlichia/Anaplasma*) species. In our RLB assay, we included the species that have been found earlier in our country and some other species found elsewhere in Europe that might have been newly introduced here, such as *B. lusitaniae*, *E. chaffeensis*, and *Ehrlichia canis* (19, 28, 29, 32).

MATERIALS AND METHODS

Origin of the samples.

Ticks were collected by dragging a blanket in four different areas in The Netherlands open to the public: Duin and Kruidberg, a dune area rich in vegetation (2000 to 2004); Bijlmerweide, a city park near Amsterdam (2000 to 2002); and two sites in the Koninklijke Houtvesterijen separated from each other by 200 m, an oak forest rich in blueberries (2000 to 2002) and a heather area (2001 to 2002) (Fig. 1). In the dune area, several species of deciduous trees and shrubs were present, and 60% of the soil was covered with vegetation litter. Ninety percent of the forest area in the Koninklijke Houtvesterijen was covered with blueberries, while the heather area consisted of heather only, with a single pine tree and very little vegetation litter. Many deciduous trees and a few shrubs with a rich secondary vegetation were seen in the city park. Eighty percent of the soil in this park was covered with vegetation litter. Every month from April to October, a maximum of 50 questing ticks were collected from each habitat. The density was calculated by multiplying the number of ticks with the number of dragged m^2. After the ticks were collected, they were immersed in 70% ethanol and stored at –20°C. Preparation of DNA extracts from ticks was done as described previously (32). Briefly, the ticks were taken from the 70% ethanol solution, air dried, and boiled for 20 min in 200 μl of 0.7

M ammonium hydroxide. After the vial was allowed to cool, it was left open for 10 min at 80°C to allow the ammonia to evaporate, and the lysate was stored at –20°C until further use.

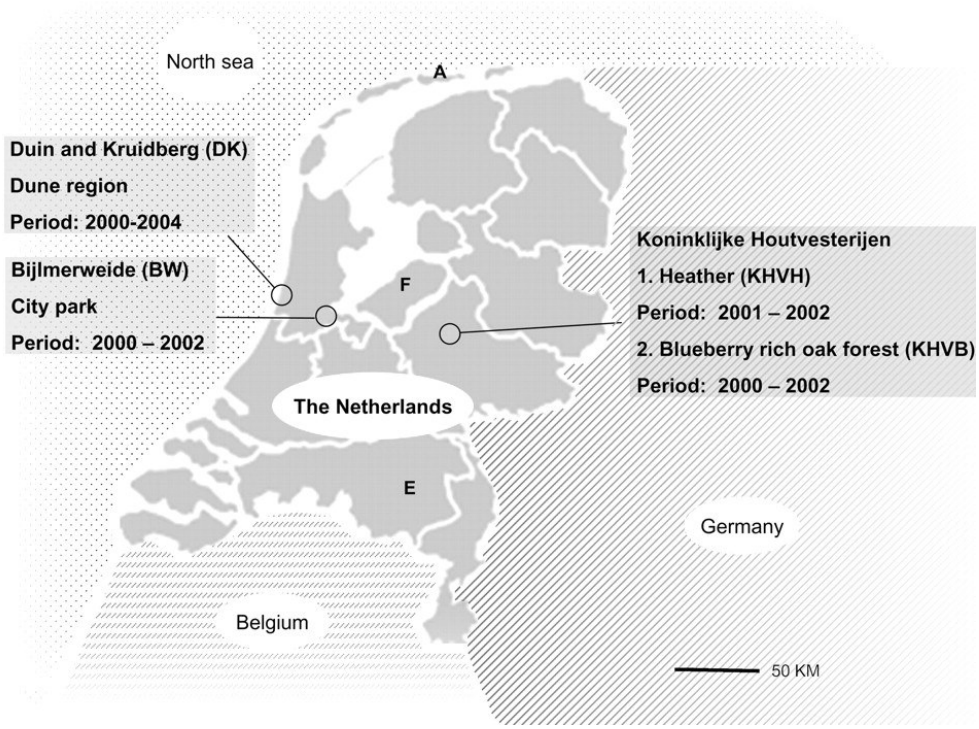

FIG. 1. Locations of the four different habitat areas that were studied. The Netherlands is positioned between the North Sea to the north and west, Belgium to the south, and Germany to the east, and the map shows the four areas that were studied: (i) a dune area, Duin and Kruidberg (DK); (ii) a city park, Bijlmerweide (BW); and the Koninklijke Houtvesterijen, (iii) a blueberry-rich oak forest (KHVB) and (iv) a heather area (KHVH). The years that the ticks were collected in the different areas are indicate. The letters A (Ameland), F (Flevopolder), and E (Eindhoven) indicate the areas previously studied by others (13, 30, 32).

PCR amplification.

PCR amplifications and reverse line blotting were performed as described before (30) with some modifications (32). Briefly, PCRs were performed in 50-μl volumes using the HotStarTaq master mix kit (QIAGEN, Westburg, The Netherlands) using the primers (Invitrogen) displayed in Table 1. PCR amplification of *Ehrlichia/Anaplasma* DNA was done using 80 pmol of each primer and the following program: (i) 15 min at 94°C; (ii) 20 s at 94°C, 30 s at 67°C, and 30 s at 72°C, lowering the annealing temperature by 1°C each cycle until it reaches 55°C; (iii) 20 cycles of 20 s at 94°C, 30 s at 55°C, and 20 s at 72°C; (iv) 20 cycles of 20 s at 94°C, 30 s at 63°C, and 20 s at 72°C; and (v) a final step of 10 min at 72°C. For *Borrelia* sensu lato, 40 pmol of each primer was used with the following program: (i) 15 min at 94°C; (ii) 20 s at 94°C, 30 s at 70°C, and 30 s at 72°C, lowering the annealing temperature by 1°C each cycle until it reaches 60°C; (iii) 40 cycles of 20 s at 94°C, 30 s at 60°C, and 20 s at 72°C; and (iv) a final step of 10 min at 72°C.

TABLE 1. Primers and RLB probes used in this study[a]

Oligonucleotide	Sequence (5'-3')	Type	Species	Target	Reference
23S borSeq	TCAGGGTACTTAGATGGTTCACTTCC	Primer	*B. burgdorferi* sensu lato	23S-5S spacer	1
B-5SBor	Biotin-GAGTTCGCGGGAGAGTAGGTTATT	Primer	*B. burgdorferi* sensu lato	23S-5S spacer	1
BB-A sensu lato	CTTTGACCATATTTTTATCTTCCA	Probe	*B. burgdorferi* sensu lato	23S-5S spacer	29
A-borsl2	CTTCCATCTCTATTTAGCCAATTT	Probe	*B. burgdorferi* sensu lato	23S-5S spacer	This study
A-borsl3	TATTTTTATCTTCCATCTCTATTTT	Probe	*B. burgdorferi* sensu lato	23S-5S spacer	This study
B31-A sensu stricto	AACACCAATATTTAAAAAACATAA	Probe	*B. burgdorferi* sensu stricto	23S-5S spacer	29
Ga2-garinii	AACATGAACATCTAAAAACATAAA	Probe	*B. garinii*	23S-5S spacer	29
Vs461N2afzelii	AACATTTAAAAAATAAATTCAAGG	Probe	*B. afzelii*	23S-5S spacer	29
A-Ruskii	GAATAAAACATTCAAATAATATAAAC	Probe	*B. ruski* (*B. afzelii* like)	23S-5S spacer	1
VsII62 val	CATTAAAAAAATATAAAAAATAAATTTAAGG	Probe	*B. valaisiana* (VS116)	23S-5S spacer	29
A-LusiP	CAAAAAAATGAACATTTAAAAAAC	Probe	*B. lusitaniae* (PotiB2)	23S-5S spacer	6
B-GA1B	Biotin-CGGGATCCCGAGTTTGCCGGGACTTCTTCT	Primer	*Ehrlichia/Anaplasma* genus	16S rRNA gene	32
16S8FE	GGAATTCAGAGTTGGATCMTGGYTCAG	Primer	*Ehrlichia/Anaplasma* genus	16S rRNA gene	4
A-EhrAll	TTATCGCTATTAGATGAGCC	Probe	*Anaplasma* genus	16S rRNA gene	32
A-Phago	TTGCTATAAAGAATAATTAGTGG	Probe	*A. phagocytophilum*	16S rRNA gene	32
A-DPhago	TTGCTATGAAGAATAATTAGTG	Probe	*A. phagocytophilum* (variant)	16S rRNA gene	32
A-HGE	GCTATAAAGAATAGTTAGTGG	Probe	HGA agent	16S rRNA gene	32
A-D-HGE	GCTATGAAGAATAGTTAGTG	Probe	HGA agent (variant)	16S rRNA gene	32
A-EChaf	ACCTTTTGGTTATAAATAATTGTTA	Probe	*E. chaffeensis*	16S rRNA gene	32
A-Eschot	GCTGTAGTTTACTATGGGTA	Probe	*A. schotti* (variant)	16S rRNA gene	32
A-ECan	TCTGGCTATAGGAAATTGTTA	Probe	*E. canis*	16S rRNA gene	32
A-EmurisT	AGCTATAGGTTTGCTATTAGT	Probe	*E. muris* T variant	16S rRNA gene	1
A-Wolbach	CTACCAAGGCAATGATCTA	Probe	*Wolbachia*	16S rRNA gene	This study

[a] Probes were 5' amino labeled.

Reverse line blot.
The RLB technique has been described before (18, 30, 32), and the probes to detect the different species and subspecies are displayed in Table 1 (1, 4, 6, 29, 32). Briefly, solutions with 5'-amino-linked oligonucleotide probes ranging from 100 to 1000 pmol (in 0.5 mM $NaHCO_3$, pH 8.4) were coupled covalently to an activated Biodyne C membrane in a line pattern by using a miniblotter (Immunetics, Cambridge, MA). After binding of the oligonucleotide probes, the membrane was taken from the miniblotter, washed in 2x SSPE (1x SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) and 0.1% sodium dodecyl sulfate (SDS) (2x SSPE-0.1% SDS) at 60°C, and again placed in the miniblotter with the oligonucleotide lines perpendicular to the slots. Ten microliters of the biotin-labeled PCR product was diluted in 150 ml of 2x SSPE-0.1% SDS, denatured for 10 min at 99°C, and cooled rapidly on ice. The slots of the miniblotter were filled with the denatured PCR product, and hybridized for 1 h at 42°C. The samples were removed from the slots by aspiration, and then the membrane was removed from the miniblotter and washed twice for 10 min with 2x SSPE-0.1% SDS at 52°C. To visualize hybridization, the membrane was incubated for 30 min at 42°C with streptavidin-peroxidase (Boehringer Mannheim GmbH, Mannheim, Germany) in 2x SSPE-0.5% SDS, washed twice for 10 min with 2x SSPE-0.5% SDS, and then incubated with enhanced chemiluminescence detection liquid (Pharmacia Biotech). Luminescence was recorded using a LAS-300 charge-coupled device camera system from Fuji film (Rotterdam, The Netherlands). To minimize cross contamination and false-positive results, positive and negative controls were included in each batch tested by the PCR and RLB assays, and DNA extraction, PCR mix preparation, sample addition, and PCR analysis were performed in specialized and separate labs.

RESULTS

Tick densities and developmental stage of the ticks.
The highest number of ticks was found in the dune area, and for this area, ticks were collected each year of this study (2000 to 2004). Table 2 shows for each different area the number of ticks caught each year and month (April to September). The dune area had the highest tick density, followed by the forest and the city park; the heather area had a very low tick density. A comparison of the tick densities in the dune area in five consecutive years showed a slight increase over time. However, the increase is very moderate compared to the large variations between years. The average density of the ticks caught in each area is shown in Table 2. A comparison of tick densities over time shows that the highest tick densities were in the months June, July, and August. Table 3 shows the developmental stage of the ticks collected in each area. Overall, most ticks were nymphs (55%), followed by larvae (38%) and a small number of adult males and females (both 3%). Notably, relatively large numbers of larvae were found in the heather area, and relatively large numbers of nymphs were found in the forest and dune areas.

TABLE 2. Yearly and monthly densities of ticks collected in four different habitats in The Netherlands

Year or month	Average density (no. of ticks/100 m^2) ± SD in:				Density in all areas (%)[d]
	Dune (*n* = 1,500)	City park (*n* = 693)	Forest (*n* = 783)	Heather (*n* = 153)	
Years[a]					
2000	139 ± 107	29 ± 19	31 ± 14	ND	
2001	258 ± 207	26 ± 26	45 ± 16	8 ± 9	
2002	220 ± 121	38 ± 24	43 ± 26	1 ± 1	
2003	342 ± 245	ND[b]	ND	ND	
2004	551 ± 557[c]	ND	ND	ND	
Months					
April	169	14	15	1	9
May	249	23	35	1	14
June	282	38	61	1	17
July	296	40	47	8	17
August	628	37	36	1	31
September	188	28	34	7	11

[a] The average was determined for the period from April to September each year.
[b] ND, no data.
[c] This relatively high density is due to a single high density of 1,600 ticks per m^2 found in August (note the large standard deviation); leaving out this value gives a density of 328 ± 205 ticks per m^2.
[d] Percentage calculated as the average number of ticks collected in the four areas per 100 m^2 and per month divided by the total number collected.

TABLE 3. Percentages of the different developmental stages of the ticks collected in four different habitat areas

Development stage	% of development stage of ticks collected from:				
	Dune (*n* = 1,500)	City park (*n* = 693)	Forest (*n* = 783)	Heather (*n* = 153)	All four sites (overall) (avg ± SD)
Larval	23	49	16	67	38 ± 21
Nymph	67	46	80	28	55 ± 21
Adult male	6	2	3	1	3 ± 1
Adult female	4	2	2	4	3 ± 1

Prevalence of *Borrelia* sensu lato and *Ehrlichia/Anaplasma* in ticks in four Dutch habitats.

PCR and reverse line blot analyses of total DNA extracted from the ticks showed that the ticks from all four areas studied carried *Borrelia* and *Ehrlichia/Anaplasma*. Figure 2A and B show the percentage of infected ticks found in the four areas over time, showing that the infection rates per area and per year varied substantially. The overall infection rates determined for all ticks analyzed (all years and areas) was 7.6% for *Borrelia* and 6.8% for *Anaplasma/Ehrlichia*. Table 4 shows the mean percentage of infected ticks that were collected at the different areas. Figure 2 and Table 4 show that the lowest *Borrelia* sensu lato infection rates were found in the heather area, twofold-higher infection rates were found in the forest area and city park, and the highest rates were found in the dune area. For *Ehrlichia/Anaplasma* spp. not identified to the species level, the lowest infection rates were found in the city park, about fourfold-higher levels were found in the dune area, and the highest levels were found in the heather and forest areas. The percentage of ticks that was found positive for *Borrelia* sensu lato (Fig. 2A) and for *Ehrlichia/Anaplasma* spp. (Fig. 2B) clearly decreased in all areas in the year 2001 and increased again the following year. In the dune area, this decrease and increase in *Borrelia* sensu lato prevalence was seen again in 2003 and 2004 (Fig. 2A). The *Ehrlichia/Anaplasma* infection rate for the dune area showed similar dips in 2001 and 2003 and peaks in 2002 and 2004. For the other areas, there was no clear dip in 2001. However,

compared to 2001, there was a strong increase in the prevalence in 2002 in the forest and heather areas.

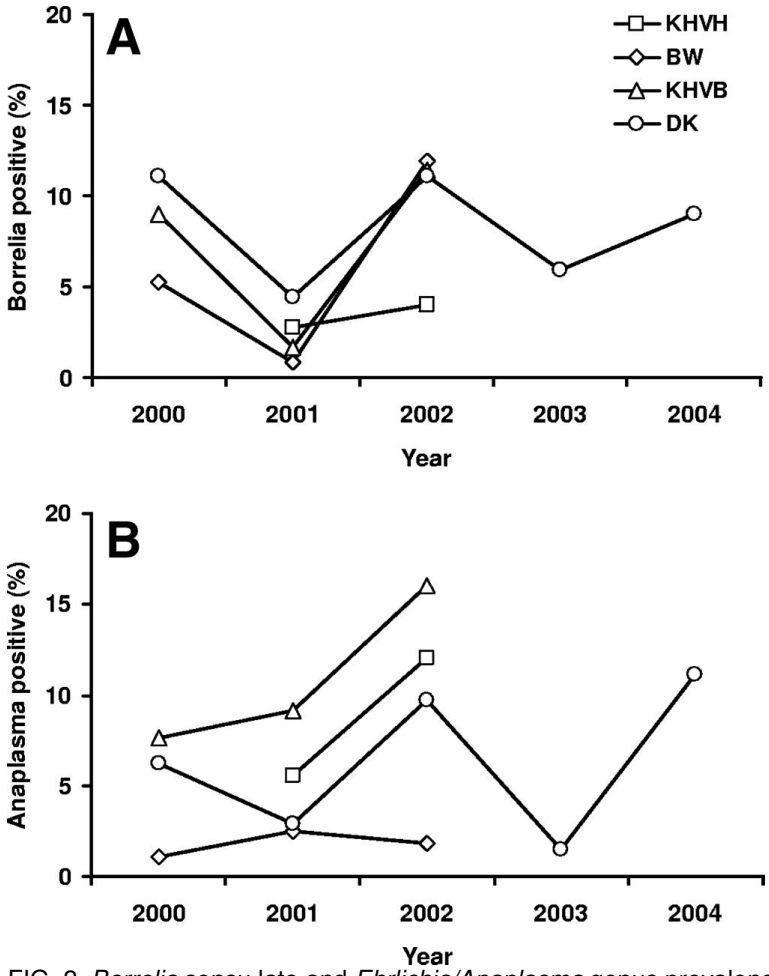

FIG. 2. *Borrelia* sensu lato and *Ehrlichia/Anaplasma* genus prevalences over time. Percentage of ticks carrying (A) *Borrelia* sensu lato and (B) *Ehrlichia/Anaplasma* spp. for the four different areas, the dune area (Duin and Kruidberg [DK]), city park (Bijlmerweide [BW]), forest (Koninklijke Houtvesterijen [KHVB]), and heather area (Koninklijke Houtvesterijen [KHVH]). Note that data for all 5 years in the period from 2000 to 2004 were analyzed only for the dune area.

TABLE 4. Comparison of the *Borrelia*, *Anaplasma*, and *Ehrlichia* spp. found in ticks from the four different areas

RLB-identified genotype	% of infected ticks (no. of infected ticks) in:			
	Dune (*n* = 704)	City park (*n* = 384)	Forest (*n* = 395)	Heather (*n* = 97)
Total *Borrelia* sensu lato	8.4 (59)	6.8 (26)	8.1 (32)	3.1 (3)
Total *Ehrlichia/Anaplasma*	6.8 (48)	1.8 (7)	11.4 (45)	7.2 (7)
Doubly infected ticks, determined[b]	1 (7)	0.8 (3)	3.3 (13)	2.1 (2)
Doubly infected ticks, predicted	0.6	0.1	0.9	0.2
Borrelia spp. sensu lato[c]	2.8 (20)	2.9 (11)	2.3 (9)	–
B. burgdorferi sensu stricto	0.1 (1)	0.3 (1)	–	–
B. garinii	0.1 (1)	–	0.3 (1)	–
B. afzelii	2.1 (15)	2.1 (8)	3.8 (15)	2.1 (2)
B. ruski (*B. afzelii* like)	0.6 (4)	0.5 (2)	–	–
B. valaisiana	1 (7)	1 (4)	0.8 (3)	1 (1)
B. lusitaniae	–[a]	–	–	–
B. garinii/B. afzelii	0.1 (1)	–	–	–
B. afzelii/B. ruski	1.4 (10)	–	1 (4)	–
Ehrlichia/Anaplasma spp.[c]	2.1 (15)	1.8 (7)	3.8 (15)	3.1 (3)
A. phagocytophilum	–	–	–	–
A. phagocytophilum variant	0.6 (4)	–	–	–
HGA	–	–	–	–
HGA variant	–	–	–	–
A. schotti variant	3.4 (24)	–	6.8 (27)	4.1 (4)
E. chaffeensis	–	–	–	–
E. canis	0.3 (2)	–	0.3 (1)	–
E. muris T variant	–	–	–	–
Wolbachia	0.4 (3)	–	0.5 (2)	–

[a] The minus symbol indicates that this genotype was not found.
[b] The predicted percentage of doubly infected ticks was calculated as the percentage of *Borrelia* times the percentage of *Ehrlichia/Anaplasma*-infected ticks.
[c] *Ehrlichia/Anaplasma* spp. not identified to the species level that reacted only with the catchall probes.

Identification of *Borrelia*, *Ehrlichia*, and *Anaplasma* spp. using RLB.
The infection rate of the ticks for different *Borrelia*, *Ehrlichia*, and *Anaplasma* species was determined by RLB analysis. The infection rates of ticks from the four areas and ticks from the dune area each year are displayed in Table 4 and Table 5, respectively. The predominant *Borrelia* species in all four areas were *B. afzelii* (overall frequency of 2.5%), *B. valaisiana* (overall frequency of 0.9%), and *Borrelia* spp. sensu lato not identified to the species level (overall frequency of 2.5%). *Borrelia burgdorferi* sensu stricto was detected in ticks from the dune area and city park (Table 4). Ticks from these two areas also contained a *B. afzelii*-like species designated *Borrelia ruski. B. garinii* was found only in the dune and heather areas. In the latter areas, about 1% of the ticks appeared to contain both *B. afzelii* and *B. garinii*, showing that double infection with two distinct *Borrelia* genomospecies does occur. One tick from the dune area carried both *B. garinii* and *B. ruski. B. lusitaniae* was not detected in any of the ticks analyzed.

TABLE 5. Comparison of the *Borrelia*, *Anaplasma*, and *Ehrlichia* spp. found in ticks collected in the dune area in the period from 2000 to 2004

RLB-identified genotype	% of RLB-identified genotype (no. of RLB-identified genotypes) in:				
	2004 (*n* = 144)[a]	2003 (*n* = 136)	2002 (*n* = 144)	2001 (*n* = 136)	2000 (*n* = 144)
Total *Borrelia* sensu lato	9 (13)	5.9 (8)	11.1 (16)	4.4 (6)	11.1 (16)
Total *Ehrlichia/Anaplasma*	11.1 (16)	1.5 (2)	9.7 (14)	2.9 (4)	6.3 (9)
Borrelia spp. sensu lato[c]	4.2 (6)	2.9 (4)	1.4 (2)	2.2 (3)	3.5 (5)
B. burgdorferi sensu stricto	–[b]	–	0.7 (1)	–	–
B. garinii	–	0.7 (1)	–	–	–
B. afzelii	2.1 (3)	0.7 (1)	3.5 (5)	0.7 (1)	3.5 (5)
B. valaisiana	0.75 (1)	–	4.2 (6)	–	–
B. lusitaniae	–	–	–	–	–
B. ruski (*B. afzelii* like)	1.4 (2)	–	1.4 (2)	–	–
B. afzelii/B. ruski	0.7 (1)	1.5 (2)	–	0.7 (1)	4.2 (6)
B. garinii/B. afzelii	–	–	–	0.7 (1)	–
Ehrlichia/Anaplasma spp.[c]	2.8 (4)	–	4.2 (6)	2.2 (3)	2.8 (4)
A. phagocytophilum	–	–	–	–	–
A. phagocytophilum variant	2.8 (4)	–	–	–	–
HGA agent	–	–	–	–	–
HGA agent variant	–	–	–	–	–
A. schotti variant	5.6 (8)	1.5 (2)	4.9 (7)	2.2 (3)	2.8 (4)
E. chaffeensis	–	–	–	–	–
E. canis	0.7 (1)	–	0.7 (1)	–	–
E. muris T variant	–	–	–	–	–
Wolbachia	0.7 (1)	0.7 (1)	–	–	0.7 (1)

[a] Total number of ticks analyzed by RLB for each year.
[b] The minus symbol indicates that this genotype was not found.
[c] *Ehrlichia/Anaplasma* spp. not identified to the species level that reacted only with the catchall probes.

The *Borrelia* spp. sensu lato not identified to the species level in the ticks from the dune area were found at a more or less constant rate. In contrast, infection with *B. afzelii* dipped in 2001 and 2003 (Table 5). *B. valaisiana* and *B. ruski* were found only in the *Borrelia* peak years 2002 and 2004 with large variations in the *B. valaisiana* prevalence. *B. burgdorferi* sensu stricto, *B. garinii*, and the *B. garinii/B. afzelii* combination were found only sporadically.

The *Anaplasma schotti* variant was the most frequently identified species in the ticks collected from three of the four areas, but not in the city park. *Ehrlichia/Anaplasma* species not identified to the species level were found in all areas. Next in prevalence was *E. canis* found in the dune and forest area. *A. phagocytophilum* variant, detected by the A-DPhago probe, was present only in the dune area in the year 2004 but at a relatively high prevalence (2.8%). None of the ticks reacted with the HGA agent, *E. chaffeensis*, and *Anaplasma muris* T probes. Of all the ticks, five (from the dune and forest area) contained *Wolbachia* species, an endosymbiont found in many insects which is also amplified by the *Ehrlichia/Anaplasma* generic PCR and which can clearly distinguished from *Anaplasma* and *Ehrlichia* by RLB. The *A. schotti* and *Ehrlichia/Anaplasma* variants not identified to the species level were found almost every year in the ticks from the dune area at a relatively constant level, but with a strong dip in prevalence in 2001. *E. canis* was found only in the high-prevalence years 2002 and 2004.

***Borrelia* and *Ehrlichia/Anaplasma* double infections.**
Comparison of the rates of *Borrelia* and *Ehrlichia/Anaplasma* double infection in the four areas (Table 4) showed that in the dune area and city park, about 1% of the ticks were doubly infected. For the other areas, this percentage was higher: 2.1% and 3.3% in the heather area and the forest, respectively. The theoretically predicted percentage of double infection can be calculated from the individual *Ehrlichia/Anaplasma* and *Borrelia* prevalence rates. Comparison of the actual and predicted percentages showed that in all areas the actual percentage of double infection was higher than expected (Table 4). The percentage of doubly infected ticks

in the dune area was most in agreement with the predicted value; however, it was still two times higher than predicted.

***Borrelia*, *Ehrlichia*, and *Anaplasma* infections in the different developmental stages.** Table 6 shows the distribution of the development stages in relation to the infection. The lowest rate of infection was found in larvae. For *Ehrlichia/Anaplasma* infection, the prevalence tended to increase with the development stage. For *Borrelia* infection, the prevalence in larvae was twice as low as that in nymphs and male adult ticks. Remarkably, female adult ticks had lower levels of *Borrelia* infection than male ticks did (5.2% versus 8.3%). For the double infections, the prevalence increased from larvae to nymphs and stayed the same in adult males, but in contrast to the single infections, it doubled in females. A comparison of the predicted and determined double infections (Table 6) showed that particularly in the larval and adult females, the rate of double infection was relatively high.

TABLE 6. Comparison of the percentages of *Borrelia* sensu lato and *Anaplasma*-infected ticks for the different development stages of the ticks

Development stage	% of infected ticks		% of double infections	
	Borrelia positive	*Anaplasma* positive	Determined	Predicted
Larval	4.2	1.8	0.6	0.08[a]
Nymph	8.5	7.9	1.8	0.67
Male	8.3	9.7	1.4	0.81
Female	5.2	10.3	3.4	0.54
Overall	7.6	6.8	1.6	0.50

[a] See Table 4, footnote *b*.

DISCUSSION

We investigated the density and infection rate of ticks in four different areas in The Netherlands in the period from 2000 to 2004 and found that these varied substantially for the different areas and years studied. The tick densities peaked between June and August, and the overall tick densities tended to increase slightly over time. The increase over the 5-year period was most obvious in the dune area, which was also the area with the highest tick density. The increasing trend was less clear in areas with lower tick densities. Very low tick densities were found in the heather area, about 100 times lower than the lowest densities found in the dune area. This shows that the heather area is probably the area with the lowest risk of sustaining tick bites, whereas dune areas pose the greatest threat. Morphological examination showed that all collected ticks belonged to *I. ricinus*. Overall, most ticks were nymphs (55%), followed by larvae (38%), and only a minority (6%) were adult ticks. However, the distribution of larvae and nymphs varied considerably in the different areas. The highest nymph levels were found in the dune area (67%) and the blueberry-rich oak forest (80%), and the lowest levels were in the city park (46%) and heather area (28%). Conversely, most larvae were found in the city park (49%) and heather area (67%), and fewer were found in the dunes (23%) and blueberry-rich oak forest (16%). The clear difference between the dune and heather areas, with the latter having relatively high levels of larvae and low levels of nymphs, might indicate that ticks in the heather area have difficulty surviving because of the lack of vegetation litter and difficulty in development, which again might be due to the lack of vegetation litter and suitable hosts for a first blood meal. However, we cannot exclude the possibility that the method of tick collection may play a role in the observed fluctuations, because the height of the vegetation may influence the chance of a tick to come into contact with the blanket.

The infection rates in the ticks varied substantially for the four areas and over the 5-year study period; for ticks with *Borrelia* sensu lato, the infection rate was between 0.8 and 11.5%, and for *Ehrlichia/Anaplasma* species, it was between 1 and 16%. Comparison with previous studies in The Netherlands (13, 30, 32) that reported values between 5 and 20% showed that

in this study ticks carry lower levels of pathogens. This is most probably due to regional differences and different methods of tick collection. In previous studies, the ticks were collected in areas different from ours (indicated in Fig. 1), and in two of the studies, ticks were collected from infested roe deer (32) and dogs (13) and not from the vegetation by dragging a blanket as we did in our study. Similar large variations (between 3.5 and 26.7%) have also been reported for questing ticks from different regions in Ireland (20) and elsewhere in Europe with reported *Borrelia* infection rates between 0 and 42% (15). In the current study, the lowest infection rates of *Borrelia* sensu lato were found in the heather area, which was also the area with the lowest tick density and with the highest proportion of larvae. The ticks collected from the dune area had the highest *Borrelia* sensu lato prevalence. The dune area also had the highest tick density. This might suggest a relation between tick density and *Borrelia* sensu lato infection. One hypothesis is that high levels of ticks will cause more animals to be bitten by multiple ticks. This would increase the probability that the host animals become infected and transmit *Borrelia* to other ticks. However, such a correlation between tick density and infection rate was not found for *Ehrlichia/Anaplasma* spp. The levels of *Ehrlichia/Anaplasma* infection also varied substantially between the different areas, with the lowest infection rates for the city park and rates for the other area more than fourfold higher. The latter might be caused by the lack of large host animals, such as roe deer, which are not present in the wild in the city park area and which are present in the wild in the other three areas.

We found that approximately 1.6% of the ticks were doubly infected, which was more than three times higher than the value predicted from the observed number of single infections. Notably, double-infection levels were highest in the blueberry-rich oak forest and heather area (2 to 3%), which was relatively high compared to the predicted levels, and we also detected these double infections in larvae. The relatively high double-infection rate might indicate the relative abundance of hosts carrying multiple infections and/or interaction of the different infections. Also, these doubly infected ticks might impose an increased risk of becoming infected by a tick from these areas, considering the immunosuppressive nature of *Anaplasma* and *Ehrlichia*.

The RLB analysis showed the presence of *B. afzelii*, *Borrelia* sensu stricto, *B. garinii*, *B. valaisiana*, the *B. afzelii*-like species *B. ruski*, and *Borrelia* sensu lato not identified to the species level in the ticks and several ticks with double *B. afzelii/B. ruski* and *B. garinii/B. afzelii* infections. *B. lusitaniae*, a species reported in Portugal, Switzerland, eastern Europe, and northern Africa (14), was not detected in any of the tick analyzed in this study. A very recent study showed that migratory birds in Switzerland appeared to be the reservoir for *B. lusitaniae* (21), and to be able to find this species, one should probably test ticks collected from migratory birds or from migratory bird-rich areas. For the *Ehrlichia* and *Anaplasma* variants studied, the main species were *A. schotti* (overall 3.5%) and *Ehrlichia/Anaplasma* spp. not identified to the species level (overall 2.5%), followed by *E. canis* and *A. phagocytophilum* variant. The *A. phagocytophilum* variant was found only in ticks collected in the year 2004 from the dune area, which was also the area with the highest tick density. *E. canis*, which may cause a fatal disease in dogs (36), was found in the ticks from both the dune and forest areas, the first time it has been found in ticks in The Netherlands. Although the prevalence of *Ehrlichia/Anaplasma* infection is lower than the prevalence in ticks collected from roe deer (32), our study also showed that the *A. schotti* variant and the *A. phagocytophilum* variant were the most abundant. In none of the ticks analyzed was the HGA agent, the HGA agent variant, *A. phagocytophilum* (32), *E. chaffeensis*, or *E. muris* T detected. However, we cannot exclude the possibility that these species might be present at a very low prevalence below our detection limit, which was 0.1% for the area with the highest tick density.

In conclusion, we have shown that tick densities and *Borrelia*, *Ehrlichia*, and *Anaplasma* infection rates in these ticks vary in different areas and even between areas separated by only 200 m, such as the heather area and forest. Our data show a trend of increasing tick densities over the years and increasing infection rates in the peak years (2000, 2002, and 2004). It is not clear what causes these peak years. It may be due to favorable host animal populations or weather conditions, such as warm winters. However, this was not studied here. The peak years, however, suggest that in particular years, the risk of tick-borne diseases for humans and animals may be higher than in other years. The increasing trend in tick numbers over time is in line with the increase in reports of tick-biting incidence in The Netherlands (11).

Comparison of the tick densities and infection rates, particularly of *Borrelia* infections, suggests that increasing infection levels are associated with high tick densities, especially with nymph densities (compare the dune and heather areas). Given the immunosuppressive nature of *Ehrlichia* and *Anaplasma* infections and the relatively high prevalence of doubly infected ticks with these pathogens and *Borrelia*, these infections may be particularly relevant and should be considered in patients with EM bitten in areas where there is a high percentage of doubly infected ticks. To better understand the symptoms of double infections, they should be studied in model systems and/or patients, and the risk to human health should be taken into account in patients with Lyme borreliosis bitten again by infected ticks.

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