

# Abundance of questing ticks and molecular evidence for pathogens in ticks in three parks of Emilia-Romagna region of Northern Italy

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## Abstract

**Introduction and objective.** Infectious and parasitic diseases transmitted by ticks, such as Lyme diseases, granulocytic anaplasmosis and piroplasmosis, have been frequently reported in Europe, with increasing attention to them as an emerging zoonotic problem. The presented study was performed to assess the distribution and the density of questing ticks in three regional parks of Emilia-Romagna region of Northern Italy, and to seek molecular evidence of potential human pathogens in tick populations.

**Materials and Methods.** In the period April–October 2010, 8,139 questing ticks were collected: 6,734 larvae, 1,344 nymphs and only a few adults – 28 females and 33 males. The abundance of *Ixodes ricinus* questing ticks was compared among different sampling sites and related to microclimate parameters. 1,544 out of 8,139 ticks were examined for the presence of pathogens: PCR was used to detect piroplasm DNA and Real time Taqman PCR for *Anaplasma phagocytophilum* and *Borrelia burgdorferi* s.l.

**Results.** The predominant species was *I. ricinus* (overall abundance 1,075.9/100 m<sup>2</sup>); more rarely, *Dermacentor marginatus* (n = 37 – 0.45%), *Scaphixodes frontalis* (n = 13 – 0.16%), *Hyalomma* spp. (n = 6 – 0.07%) and *Ixodes acuminatus* (n = 3 – 0.04%) were also found. 28 out of 324 (8.6%) samples of ticks were PCR-positive for piroplasm DNA. 11 amplicons of 18S rRNA gene were identical to each other and had 100% identity with *Babesia* EU1 (*Babesia venatorum*) using BLAST analysis. Real time Taqman PCR gave positive results for *A. phagocytophilum* in 23 out of 292 samples (7.9%), and for *B. burgdorferi* s.l. in 78 out of 292 samples (26.7%). *I. ricinus* was the only species found positive for pathogens by molecular analysis; 16 tick samples were co-infected with at least 2 pathogens.

**Discussion.** The peak of nymph presence was in May, and the higher prevalence of pathogens occurred in April–June, most often in nymphs; therefore, spring season could represent the higher risk period for the transmission of pathogens. These data could provide guidelines for the preventions of tick-transmitted diseases in this region.

## Key words

questing ticks, *Ixodes ricinus*, *Babesia* EU1, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* s.l.; Emilia Romagna region (Italy)

## INTRODUCTION

Ixodid ticks (hard ticks) are parasites of great importance for both animal and human health. They are able to harm the host, directly as bloodsucking parasites causing anaemia, toxicosis, paralysis and allergies, but they can also transmit pathogens. Infectious and parasitic diseases transmitted by ticks, such as Lyme diseases, granulocytic anaplasmosis and piroplasmosis, have been frequently reported in Europe, with increasing attention on them as an emerging zoonotic problem. In recent decades, in addition to climate change [1], the increase of wild animals in rural and peri-urban areas and the increased interest of people in outside activities, have led to closer contacts between people and the natural cycles in which ticks occur [2]. Monitoring of ticks, and in particular *Ixodes ricinus*, in areas used by people for

recreational activities makes it possible to assess the risk of tick infestation and infection related to pathogens transmitted by these arthropods [3].

The distribution of ixodid ticks in Italy is influenced greatly by the different habitats present on the peninsula, from the Alps region to the far south of Sicily. The presented study was performed to assess the distribution and the density of ticks in 3 regional parks of the Emilia-Romagna Region of Northern Italy, in which no studies have been performed previously, and to seek molecular evidence in these tick populations of potential human pathogens, such as piroplasm, *Anaplasma phagocytophilum* and *Borrelia burgdorferi* s.l.

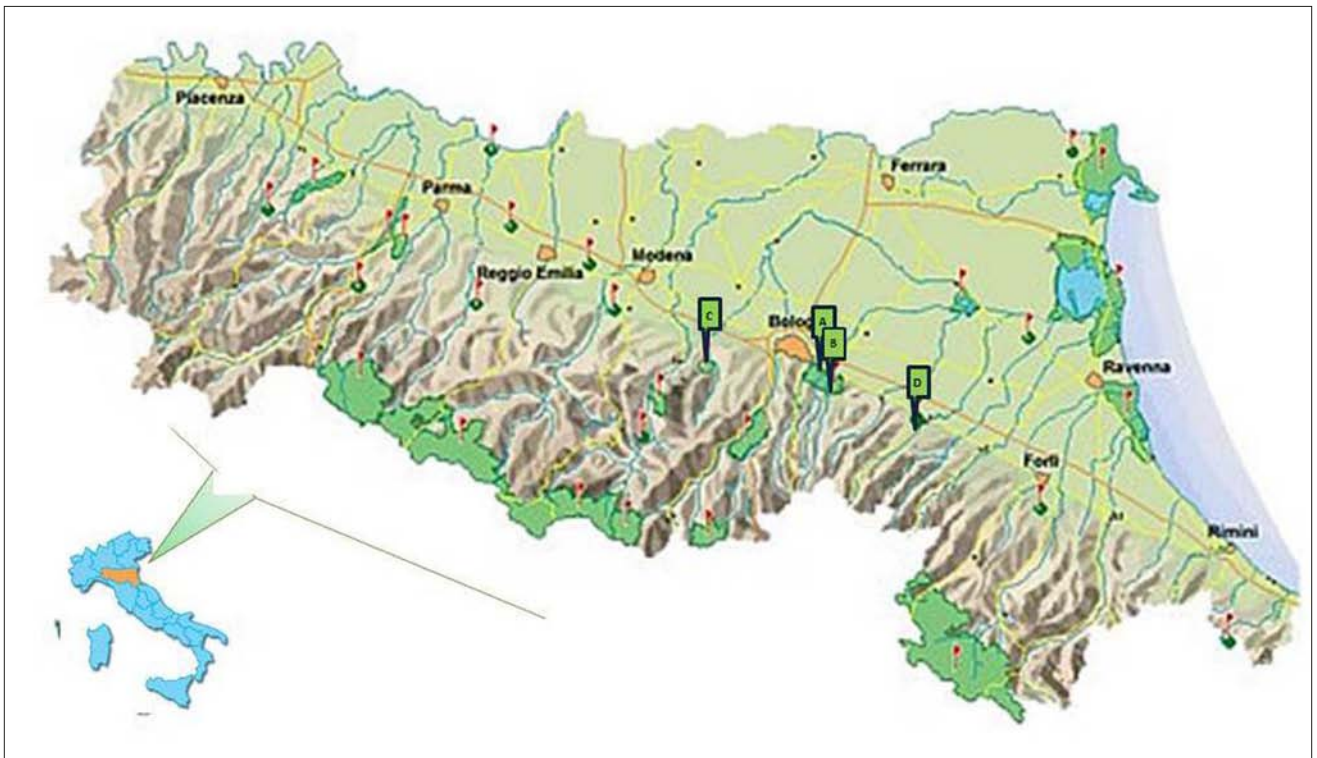
## MATERIALS AND METHOD

**Sampling.** 4 sampling sites in 3 parks of the Emilia-Romagna Region of Northern Italy were selected for the study: the Regional park of ‘Gessi Bolognesi and Calanchi dell’Abbadessa’, the regional park of ‘Monteveglia Abbey’, both in Bologna Province, and ‘Carnè’ Park, within the ‘Vena

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**Figure 1.** Sampling sites: A (locality "Ca' de Mandorli") and B (locality "Ciagnano") in the "Gessi Bolognesi and Calanchi dell'Abbadessa Park"; C – "Monteveglio Abbey park", D – "Carnè Park". Emilia Romagna Region, Italy

del Gesso Romagnola' regional park in Ravenna Province (Fig. 1). These parks are distributed along the hilly part of the Apennines, in a complex territory characterized by a series of gypsum outcrops, karst system with sinkholes, closed valleys, cliffs, wooded mountains, and grey calanques, alternated with agricultural areas, with the traditional hill-type configuration. At these sites there are natural pathways and picnic areas, interspersed with woods and meadows. The sampling was carried out in picnic areas and along footpaths that are usually frequented by people.

In the 'Gessi Bolognesi and Calanchi dell'Abbadessa' park, 2 sampling sites were selected: the first was situated in the locality 'Ca' de Mandorli' (site A – 44° 26' 32" N, 11° 26' 30" E, 66 m asl). At this site, a wooded area of 35 m<sup>2</sup> and 3 transects of 20 m at the border of a wood with grass mantle were selected. The second was located in locality 'Ciagnano' (site B – 44° 24' 57" N, 11° 27' 16" E, 240 m asl); at this site, 6 transects along a path, each of 20 m, were sampled. At site C (44° 28' 01" N, 11° 5' 14" E 252 m asl), located in the 'Monteveglio Abbey' park, 6 transects – each of 20 m, along the pathway, and 2 transects – each of 30 m, at a border of a meadow were examined. At site D (44° 13' 34" N, 11° 44' 22" E 387 m asl), 'Carnè', an area of 236 m<sup>2</sup>, including a playground and a picnic zone, and 6 transects – each of 20 m along a path, were chosen.

The areas selected were indicated by barrier tape and sampled every 15 days for questing ticks, from April – October 2010 (from May – October at site D, because the park was closed in April). Ticks were collected by continuously flagging with a 1 m × 1 m white cotton cloth, from transects of 20 m along the uphill side of the paths, which typically has higher tick densities than the downhill side [4], while the areas were flagged completely. The total surface flagged was 95 m<sup>2</sup> at site A, 120 m<sup>2</sup> at site B, 180 m<sup>2</sup> at site C and 356 m<sup>2</sup>

at site D. The ticks were removed every 2 m to reduce the effect of tick drop-off [5].

Collected ticks were immediately put into 70% ethanol and stored at room temperature until morphological identification, made by using identification keys [6, 7], and subsequent DNA extraction. The number of ticks, species and stage of development were recorded for each transect or area. During each sampling day and in each transect or area examined, environmental factors were recorded, such as the dominant vegetation (presence of grass or dry leaf litter) and the microclimate, assessed by measuring air temperature and percentage of humidity at 5 cm above the soil [8] using a thermometer with hygrometer (Oregon Scientific, USA).

**DNA analyses.** Ticks were processed individually when adults, or in pools of 5 nymphs or 10 larvae [9]. Each pool consisted of ticks from the same site and time of sampling. Overall, 1,544 ticks (324 samples) were examined as follows: 320 larvae of *I. ricinus* (32 samples) 1,165 nymphs (232 samples of *I. ricinus* and 1 *Scaphixodes frontalis*) and 59 adults (52 *I. ricinus*, 3 *Ixodes acuminatus*, 1 *Dermacentor marginatus* 3 *Hyalomma m. marginatum*). Ticks were washed twice with PBS and centrifuged at 10,000 x g for one minute to remove the alcohol, then cut with a steril scalpel. DNA was extracted with Nucleo Spin Extract II (Macherey Nagel), according to manufacturer's instructions, except for an increased period of lysis of ticks from 10 min to 30 min.

All the tick samples (n = 324) were examined for piroplasms DNA. PCR assays were performed amplifying ~400 bp of 18S rRNA gene of piroplasms [10]. All the PCR products were purified using NucleoSpin Extract II (Macherey Nagel GmbH, Düren, Germany), and both strands were sequenced by BMR genomics (Padua, Italy) in an ABI-3730XL analyzer.

The sequences were corrected by visual analysis in BioEdit 7.0.9 and subjected to database search by BLAST.

292 samples (nymphs and adults; no larvae were examined) were tested for the presence of *A. phagocytophilum* DNA with highly sensitive real-time Taqman PCR (StepOnePlus, Applied Biosystem, Life Technologies Corporation, Carlsbad, California, USA) assay targeting the multiple-copy *msp2/p44* gene [11]. Samples with a cycle threshold (Ct) value of <40 and a characteristic amplification curve were considered positive.

The same 292 samples were also screened for the presence of *B. burgdorferi* s.l. DNA with highly-sensitive real-time Taqman PCR (StepOnePlus, Applied Biosystem, Life Technologies Corporation, Carlsbad, California, USA) assay targeting 16S rDNA region, according to Barbour et al. [12], modified by increasing the number of cycles to 50, rather than 45, and decreasing the extension temperature to 60 °C instead of 63 °C. Samples with a cycle threshold (Ct) value of <40 and a characteristic amplification curve were considered positive.

**Statistical analysis.** To compare between sampling sites, data on *I. ricinus* – the species mainly represented – were converted to abundance indices (AI), which express the number of ticks collected in 100 m<sup>2</sup>. This parameter was calculated for the total of ticks (AI), and larvae (AIL), nymphs (AIN) and adults (AIA), according to the formula  $AI = TR \times 100/a$ , where ‘TR’ is the number of *I. ricinus* ticks recorded and ‘a’ is the sampled area in square meters [13]. The non-parametric Kruskal-Wallis test was used to compare tick abundance among the different sampling sites. Non-parametric correlation (Spearman Rho) was used to assess the relationship between microclimate parameters (temperature, humidity) and ticks abundance. A  $\chi^2$  test was performed to evaluate different positive rates of pathogens in ticks among sampling sites, month of sampling, temperature (T°) and humidity (RH) at the time of sampling, and among nymphs and adults of *I. ricinus*. Values of  $p < 0.05$  were considered statistically significant. Statistical analysis was performed by SPSS software (IBM SPSS Statistics ver. 21).

## RESULTS

**Tick abundance.** During the period April-October 2010, 8,139 ticks were collected at the 3 sites. The highest number of ticks was found at site B (4,187), followed by site A (2,123), then site D (967), and finally site C (862). The majority were larvae (6,734) and to a lesser extent nymphs (1,344); only a few specimens collected were adults (28 females and 33 males). The predominant species was *I. ricinus* (n=8080 – 99.28%); rarely, *Ixodes acuminatus* (n=3 – 0.04%), *Dermacentor marginatus* (n=37 – 0.45%), *Scaphixodes frontalis* (n=13 – 0.16%) and *Hyalomma* spp. (n=6 – 0.07%) were also found (Tab. 1).

The greatest richness of tick species were detected at site B. Since species other than *I. ricinus* were found in smaller numbers and only occasionally, only this latter species was taken in account for the following considerations. The overall abundance of *I. ricinus* was 1,075.9/100 m<sup>2</sup>, with a greater abundance of larvae (891.3/100 m<sup>2</sup>) than nymphs (150.9/100 m<sup>2</sup>) and adults (7.1/100 m<sup>2</sup>). AI, AIL and AIN were significantly higher at site B than the other sites ( $p < 0.01$  for AI and AIL and  $p < 0.05$  for AIN) (Tab. 2).

**Table 1.** Number of ticks collected from four sampling sites\*, divided into stages of development and sampling site

SPECIES	STAGES	SITE A	SITE B	SITE C	SITE D	TOTAL
<i>I. ricinus</i>	Larvae	1876	3614	520	684	6694
	Nymphs	242	493	330	268	1333
	Females	2	11	6	3	22
	Males	2	16	6	7	31
<i>I. acuminatus</i>	Females		2			2
	Males				1	1
<i>S. frontalis</i>	Larvae				1	1
	Nymphs		9		2	11
	Females				1	1
<i>D. marginatus</i>	Larvae		36			36
	Females	1				1
<i>H. marginatum marginatum</i> ( <i>Hyalomma</i> sp.)	Larvae		3			3
	Females		2			2
	Males		1			1
		2123	4187	862	967	8139

\* Sampling sites: A - ‘Ca’ de Mandorli; B - ‘Ciagnano’, both in the ‘Gessi Bolognesi and Calanchi dell’Abbadessa Park; C - ‘Montevoglio Abbey Park; D - ‘Carnè Park; Emilia Romagna Region, Italy.

**Table 2.** Mean abundance of larvae, nymphs and adult of *I. ricinus* per sampling site\*\*

Site of sampling	Abundance: mean (standard deviation)			
	total ticks/100 m <sup>2</sup>	larvae/100 m <sup>2</sup>	nymphs/100 m <sup>2</sup>	adults/100 m <sup>2</sup>
Site A	147.23 (271.48)	124.12 (27881)	22.78 (37.12)	0.32 (0.84)
Site B	246.07 (333.88)*a	215.11(345.43)*a	29.34 (38.35)*b	1.60 (2.70)
Site C	34.21 (35.91)	20.63 (28.84)	13.09 (21.09)	0.48 (0.92)
Site D	44.63 (59.38)	33.34 (57.37)	10.90 (25.69)	0.39 (1.23)
Total	106.27 (216.68)	87.52 (219.06)	18.08 (30.78)	0.66 (1.60)

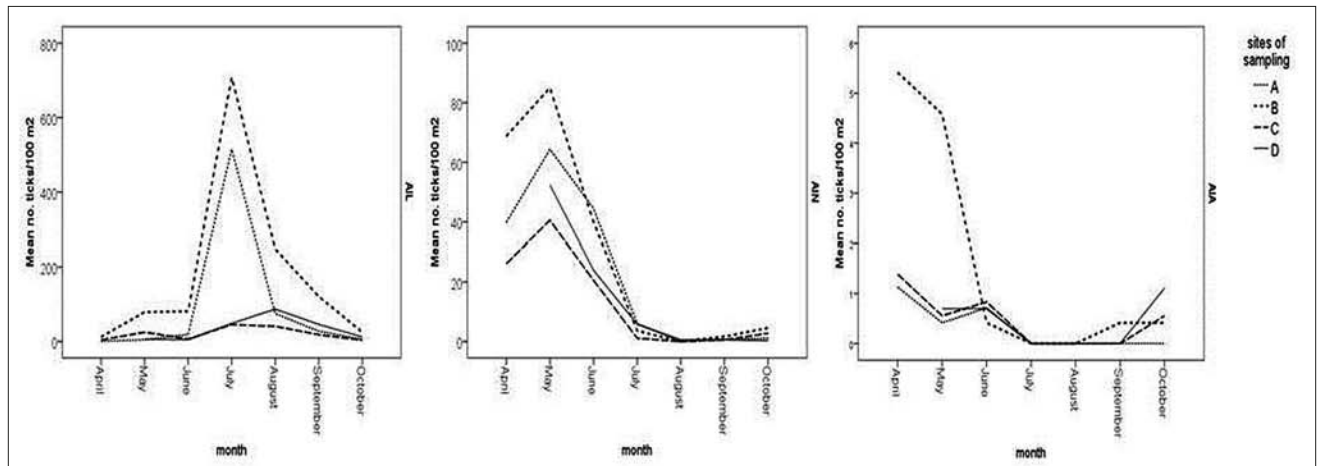
\*significantly higher than other sites; a =  $p < 0.01$ ; b =  $p < 0.05$ .

\*\* Sampling sites: A - ‘Ca’ de Mandorli; B - ‘Ciagnano’ both in the ‘Gessi Bolognesi and Calanchi dell’Abbadessa Park; C - ‘Montevoglio Abbey park; D - ‘Carnè Park; Emilia Romagna Region, Italy

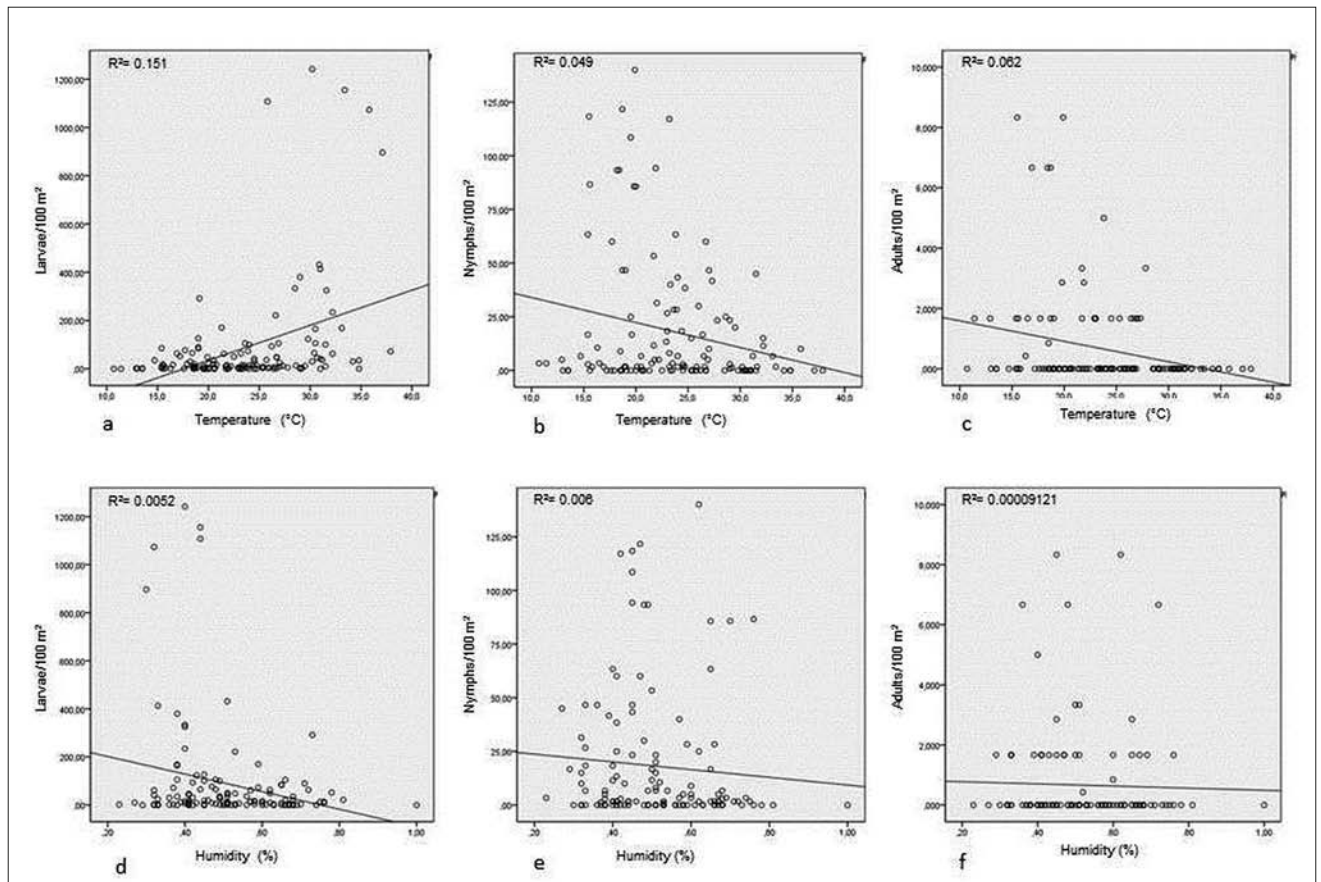
The peak of AIL was recorded in July at sites A, B and C, while at site D the peak occurred in August. At site C, where the AIL was lower, another peak was present in May. The peak of AIN (at a lower level than AIL) occurred in May at all the sites. The occurrence of adult ticks was rather sporadic and fairly distributed throughout the sampling, with a peak in April-May at site B (Fig. 2).

The temperature registered at 5 cm above the soil during the sampling period ranged from 10.7 – 37.9 °C. At all the sites, a higher AIL was observed at temperature > 30 °C, while the main AIN was observed at temperature less than 22 °C. No adults were found at temperature >30 °C. Overall, in the range examined, there was a significant correlation between abundance of collected questing ticks and temperature: AIL tends to increase with the increase in temperature, versus the AIN and AIA which tended to decrease (Fig. 3). The RH ranged from 23% – 81%. No significant differences in IAL, IAN and IAA were found at the different RH, although the AIL was higher at low RH (Fig. 3).

Finally, considering the dominant vegetation (presence of grass or dry leaf litter), it was observed that AIL and AIN were higher on dry leaf litter than on grass (Fig. 4).



**Figure 2.** Tick abundance during the time in the different sampling sites. A (locality “Ca’ de Mandorli”) and B (locality “Ciagnano”) in the “Gessi Bolognesi and Calanchi dell’Abbadessa Park”; C – “Montevoglio Abbey park”, D – “Carnè Park”; ALL larvae abundance, AIN nymph abundance, AIA adult abundance



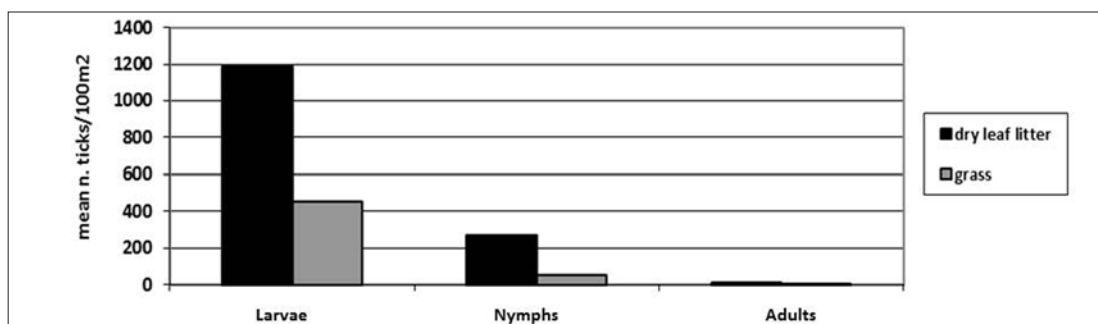
**Figure 3.** Abundance of larvae, nymphs and adults at different temperature (a,b,c) and percentage of humidity (d, e, f) registered at 5 cm above the soil

**Piroplasms.** Overall, 28 out of 324 (8.6%) samples of ticks examined for piroplasms DNA were positive by PCR. From all sampling sites, only *I. ricinus* ticks were found positive: 2 male, one female and 25 pools of nymphs (Tab. 3); no larval samples were tested positive. Prevalence was significantly higher in site C ( $p < 0.05$ ). Independently from the sampling site, PCR-positive ticks were collected only in April, May and June, with a higher prevalence in May (14.8%). Prevalence was higher in specimens collected at temperature  $> 35^{\circ}\text{C}$  (28.6%), and/or at HR  $< 30^{\circ}\text{C}$  (18.2%); however these data were not statistically significant.

11 amplicons from 9 pools of nymph and 2 adults from all sites were identical to each other, and the sequences showed 100% identity with *Babesia* EU1 (*B. venatorum*) (AY046575 and other strains) by BLAST analysis.

The amplicons from 2 pools of nymphs collected at site D had 99% identity with *B. divergens* (AY572456) and *B. capreoli* (FJ944828). 15 isolates from sites A, B and C, identity varying from 85% – to 100% with different strains of *Theileria buffeli*, *T. sergenti*, and *T. orientalis*, and were therefore included in the *T. buffeli/sergenti/orientalis* complex.





**Figure 4.** Mean abundance of larvae, nymphs and adult ticks collected in different dominant vegetation (presence of grass or dry leaf litter)

**Table 3.** *I. ricinus* samples positive for piroplasms, *A. phagocytophilum* and *B. burgdorferi* s.l. by PCR (none of the other tick species tested positive and are not included in the Table). Note: each samples consist of individual adults, or 5 nymphs or 10 larvae

	n. positive to PCR/n. examined (%)				
	Site A	Site B	Site C	Site D	Total
<b>Piroplasms</b>					
Adult male	0/2	1/15 (6.7) 1 (EU1)	1/7 (14.3) 1 (th)	0/7	<b>2/31 (6.4)</b>
Adult female	0/2	1/12 (8.3) 1 (EU1)	0/4	0/3	<b>1/21 (4.8)</b>
Nymph	2/43 (4.6) 1 (EU1) + 1 (Th)	9/89 (10.1) 6 (EU1) + 3 (Th)	11/56 (19.6) 1 (EU1) + 10 (th)	3/44 (6.8) 1 (EU1) + 2 (Bc/d)	<b>25/232 (9.9)</b>
Larvae	0/11	0/21	–	–	<b>0/32 (0%)</b>
<b>Total</b>	<b>2 /58 (3.4)</b>	<b>11 /137 (8.0)</b>	<b>12 /67 (17.9)</b>	<b>3 /54 (5.6)</b>	<b>28/316 (8.9)</b>
<b>Anaplasma phagocytophilum</b>					
Adult male	0/2 (0)	2/15 (13.3)	2/7 (28.6)	0/7 (0)	<b>4/31 (8.9)</b>
Adult female	0/2 (0)	0/12 (0)	0/4 (0)	0/3 (0)	<b>0/21 (0)</b>
Nymph	5/43 (11.6)	6/89 (6.7)	8/56 (14.3)	0/44 (0)	<b>19/232 (8.2)</b>
<b>Total</b>	<b>5/47 (10.6)</b>	<b>8/116 (6.9%)</b>	<b>10/67 (14.9)</b>	<b>0/54 (0)</b>	<b>23/284 (8.1)</b>
<b>Borrelia burgdorferi</b> s.l.					
Adult male	0/2	1/15 (6.7)	2/7 (28.6)	1/7 (14.3)	<b>4/31 (12.9)</b>
Adult female	1/2 (50)	1/12 (8.3)	1/4 (25)	1/3 (33.3)	<b>4/21 (19)</b>
Nymph	17/43 (39.5)	25/89 (28)	14/56 (25)	14/44 (31.8)	<b>70/232 (30.2)</b>
<b>Total</b>	<b>18/47 (38.3)</b>	<b>27/116 (23.3)</b>	<b>17/67 (25.4)</b>	<b>16/44 (36.4)</b>	<b>78/284 (27.5)</b>

EU1 – isolates with 100% identity with *Babesia* EU1 AY046575; Bc/d – isolates with 99% identity with *B. capreoli* FJ944828 and *B. divergens* AY572456; Th – isolates with 85%–100% identity with *Theileria buffeli/sergenti/orientalis*

**Anaplasma phagocytophilum.** A total of 23 samples out of 292 (7.9%) tested positive for *A. phagocytophilum* infection by real-time PCR. *I. ricinus* was the only species found positive, including 8.9% of males and 8.2% of pools of nymphs. No female tick was found positive and no positive samples were found at site D (Tab. 3). The prevalence of *A. phagocytophilum* was significantly higher in April (15.8%) than in May (5.7%;  $p=0.03$ ) and June (4.2%;  $p=0.037$ ); no positive samples were found in ticks collected in the other months. Furthermore, the prevalence was significantly higher ( $p<0.05$ ) in ticks collected at 20–26 °C (18.8%), compared to ticks collected at higher or lower temperatures (4.6% – 4.9% or negative). There were not significant differences between ticks collected at different RH.

**Borrelia burgdorferi** s.l. Real time taqman PCR for *B. burgdorferi* s.l. was positive in 78 out of 292 samples (26.7%). Only *I. ricinus* was found to be positive (Tab. 3). No statistically significant differences were found in prevalence between different sites of sampling, while the prevalence was higher in nymphs than adults (30.2% vs. 12.9%;  $p<0.01$ ). The prevalence of *B. burgdorferi* s.l. in ticks was higher in April, May and

June, but the difference compared to the other months was not statistically significant. No differences were found between ticks collected at different temperatures and RH.

16 tick samples were co-infected with at least 2 of the pathogens mentioned above (Tab. 4). The association of

**Table 4.** Co-infections in *I. ricinus* ticks samples from the different sampling sites\*

Concomitant infection	SITE A	SITE B	SITE C	SITE C
<i>A. phagocytophilum</i> / <i>B. burgdorferi</i> s.l.	2 nymphs	1 nymph	1 nymph + 1 male	1 male
<i>A. phagocytophilum</i> / <i>Babesia</i> EU1	1 nymph	1 male	-	-
<i>B. burgdorferi</i> s.l./ <i>Babesia</i> EU1.	-	3 nymphs	-	-
<i>B. burgdorferi</i> s.l./ <i>B. divergens-capreoli</i>	-	-	-	1 nymph
<i>B. burgdorferi</i> s.l./ <i>Theileria</i> spp.	-	-	3 nymphs + 1 male	-

\* A (locality 'Ca' de Mandorli'; B – 'Ciagnano', both in the 'Gessi Bolognesi and Calanchi dell'Abbadessa' Park; C – 'Montevoglio Abbey' Park; D – 'Carnè' Park; Emilia Romagna Region, Italy)

*A. phagocytophilum*/*B. burgdorferi* s.l. was more frequent (6 samples) and was found in ticks from all the sampling sites

## DISCUSSION

The diversity of tick species found in this study was higher than in other studies conducted in other parks of the Emilia Romagna region [14], which found only *I. ricinus* by dragging; in contrast, a higher richness of species diversity was recently found in an oak forest of South Italy [15]. The recovery of *S. frontalis* (one larva, 11 nymphs and one female) at sites A and D is interesting. Males were not detected and are rarely described in the literature. This tick, parasitizing primarily avian species, especially Passeriformes, has been reported in most regions of Italy, but to-date, there have been no reports of its presence in the Emilia-Romagna Region [6, 7], although it is not surprising that it should be in that region as it is easily transported by birds during migration.

*Ixodes ricinus* is by far the most common species in Europe and also in the current study, it appears to be the predominant species, with 8,080 specimens collected. This species was reported at all the sampling sites, although some differences in AI were found that related to the developmental stage, sampling site, collection period, microclimate (temperature and humidity) and vegetation at the site of sampling. AIL was higher than AIN and AIA according to Barandika et al. [13] in Northern Spain and by Dantas Torres and Otranto [15] in Southern Italy. A decreased presence of mature stages, with respect to juveniles, is not surprising, because only 0.1% of the eggs produced by the female can reach the adult stage [16]. In the presented study, the detection of adults was rather sporadic and distributed throughout the sampling period. The overall AIA (7.1/100 m<sup>2</sup>) was similar to the finding reported by Barandika et al. [13]. The highest AI of *I. ricinus* was observed at site B, where the ixodid tick species richness was highest overall; this was probably related to the presence of a rich and varied local fauna, as the sampling was performed along a narrow path that winds through the hilly terrain in a forest at the edge of the calanques. Randolph et al. [17] in England showed that, despite annual differences, the larvae show a higher peak around the month of July, while the nymphs show a greater activity in May, which is in agreement with the presented results and counter to those of Barandika et al. [13] in Northern Spain.

During sampling, an increase of AIL with increasing temperature was observed, but the data obtained are not completely in accord with other authors [3] who reported reduced activities of ticks when the temperature is higher. The larval stages are usually considered more sensitive to high temperatures, and various authors indicate the temperatures of 32 °C as critical for the survival of *I. ricinus*, because they cause rapid dehydration [18]. Usually, it is presumed that moisture is a crucial factor for the survival of *I. ricinus*; larvae and nymphs choose micro-habitats in which the relative humidity is above 80% to avoid desiccation [19]. The active, seeking ticks, in order to avoid dehydration, tend to penetrate into the upper layers of soil, or beneath the foliage at the base of vegetation, where high humidity creates a microclimate that allows rehydration. Once rehydrated, the young and hungry ticks tend to climb on the top layers of turf or leaves on the forest floor where they have a high host-seeking activity [20]. In the presented work, it was observed that

the abundance of larvae tends to decrease with increasing humidity, with the highest AIL when the humidity was less than 35%; this can be explained by the fact that the ticks collected by flagging were those in active search of a host, and increased their activity precisely when the humidity was low [20]. There was a greater abundance of *I. ricinus*, especially larvae and nymphs, in woodland areas where grass was not present and there was a small shrub vegetation and ground covered with dry leaves. The data obtained can be explained by the fact that the areas presenting turf were also more exposed to the sun, which probably limited the active presence of ticks in the upper layers, reducing the attack of the flag.

The presented study also investigated molecular evidence of potential zoonotic agents. Out of 324 samples, 8.6% tested positive for piroplasms by PCR. Data in the literature from European countries show extremely variable prevalences ranging from 1% in Germany [21] up to 60% in Poland [22]. Also, in the presented study different infection rates of piroplasms in ticks were detected depending on the sampling site, finding values significantly higher at site C, probably because at this site there is a niche conducive to the maintenance of the biological cycle of the parasites; in particular, in recent years, a clear population growth of roe deer has been documented (data from the Emilia Romagna regional agency). A higher prevalence was detected in ticks collected in May, although the differences between the different periods of the year were not significant; conversely, other authors have shown higher prevalences in June and October [22]. The nymphs were more frequently infected than adults and larvae (all negative), but these differences were not statistically significant; in contrast, in Poland, adults showed higher levels of positivity compared to nymphs [22]; nevertheless, the latter play the main role in the spread of pathogens due to their higher number. Significant differences were not found in relation to the temperature and humidity encountered during the sampling phase of our work. Previous studies [23] had shown that when the the minimum temperature required for the activation of ticks is reached, then the temperature has a minimal effect on the progress of the diffusion of piroplasms.

Sequencing DNA identified *Babesia* EU1, *B. capreoli*/*B. divergens* and the *Theileria buffeli/sergenti/orientalis* complex. *Theileria* spp. belonging to this species complex are not distinguishable on the basis of analysis of the fragment amplified in the presented study, including the 18S rRNA, a highly conserved region of the genome of piroplasms. This complex of species is still considered minimally pathogenic to animals (domestic and wild ruminants) and this, combined with the fact that this organism does not appear to be zoonotic, has decreased interest in this group of parasites. Moreover these species do not represent a significant risk to the health of Italian cattle [24]. Samples collected in site C show highest percentage of positivity. Grazing cattle are not present in this area; therefore, perhaps the reservoirs are wild ruminants even if in these animals, in Italy, this species complex has not yet been reported.

In the presented study, in 2 samples it was not possible to discriminate between *B. capreoli*/*B. divergens*, because analysis of the 18S-rRNA did not allow to distinguish them, as also reported in the literature. *Babesia* EU1, also called *Babesia venatorum*, was present at all sampling site, with higher prevalence at site B. This parasite is of greater



importance because it is a zoonotic pathogen. This species has been isolated for the first time in 2 human cases, one in Austria and the other in Italy [25]. The latter involved a splenectomized human who had supposedly been infected during hunting on the Apennines at few kilometers east of the parks examined. The infected ticks analysed in the presented study were found in parks frequented by people, particularly in the nature trails, picnic areas and children's play areas. The results obtained indicate the presence of the risk of contact with infected ticks and the consequent possibility of transmission to humans of infection by *Babesia* EU1.

In the current study, the overall prevalence of *A. phagocytophilum* in questing ticks was 8.1%, in line with studies performed in other European countries. The presence of this pathogen was recently investigated in questing ticks from other sites of the Emilia Romagna region; the first study [14] found only 2 out of 100 nymphs (2%) positive for *A. phagocytophilum*, while in the second [26], none of the 115 examined were positive. These authors used conventional PCR, which is likely considered less sensitive than real time PCR used in the present study.

The presence of *B. burgdorferi* s.l. in ticks has been extensively studied in Europe; in Italy has been mainly investigated in northern regions, with prevalence ranging from 10% – 20% [26, 27]. The presented results show an overall prevalence of 26.7%, without significant differences between the different sampling sites.

16 samples showed concomitant infections of 2 pathogens. Concomitant infections in ticks have also been described in Europe, and the presence of *A. phagocytophilum*, *B. burgdorferi* s.l. and *B. microti* simultaneously in ticks also reported [28]. In the presented study, however, no more than two infectious agents were ever found in a single tick.

## CONCLUSIONS

The results of this study, performed in areas not previously subjected to epidemiological investigation, contribute to the understanding and mapping of the spread of *I. ricinus* and assessment of the risk of the spread of infections transmitted by this vector species. The public parks in which the sampling was performed are crossed by nature trails and picnic areas and often used by families with children for recreation. Increased outdoor activities and an increase in the number of immunocompromised people increases the risk of diseases transmitted by ticks. There was a peak in the numbers of *I. ricinus* nymphs in May, and higher prevalence of pathogens in April-June, most often in nymphs, suggesting that particular precautions should be taken during the spring season. These data could provide guidelines for the precautions to be observed during periods of increased risk.

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