Presence of host-seeking Ixodes ricinus and their infection with Borrelia burgdorferi sensu lato in the Northern Apennines, Italy

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Abstract

Host-seeking ticks were collected in the Northern Apennines, Italy, by dragging at 35 sites, at altitudes ranging from 680 and 1670 m above sea level (asl), from April through November, in 2010 and 2011. *Ixodes ricinus* (4431 larvae, 597 nymphs and 12 adults)and *Haemaphysalis punctata* (11209 larvae, 313 nymphs, and 25 adults) were the most abundant species, followed by *Haemaphysalis sulcata* (20 larvae, five nymphs, and 13 adults), *Dermacentor marginatus* (42 larvae and two adults) and *Ixodes hexagonus* (one nymph). Greatest numbers of ticks were collected at locations characterised by southern exposure and limestone substratum, at altitudes < 1400 m asl; *I. ricinus* was most abundant in Turkey oak (*Quercus cerris*) wood, whereas *H. punctata* was mostly collected in hop hornbeam (*Ostrya carpinifolia*) wood and on exposed rocks. *I. ricinus* was also found up to 1670 m asl, in high stand beech tree (*Fagus sylvatica*) wood. The overall prevalence of *Borrelia burgdorferi* sensu lato (sl) in 294 host-seeking *I. ricinus* nymphs was 8.5%. *B. garinii* was the most frequently identified genospecies (64.0% of positive nymphs), followed by *B. valaisiana*, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. lusitaniae*. Based upon the comparison with the results of previous studies at the same location, these research findings suggest the recent invasion of the study area by the tick vector and the agents of Lyme borreliosis.

Keywords

Haemaphysalis spp., *Dermacentor marginatus*, Ixodidae, Northern Apennines, Italy.

Introduction

In the last decades, latitudinal and altitudinal expansions of the geographic range of *Ixodid* ticks, as well as increasing incidence of tick-borne infections, have been reported in Europe (Danielová et al., 2010; Daniel et al., 2009; Lindgren and Gustafson 2001; Jore et al., 2011; Jore et al., 2014; Hartelt et al., 2008; Gilbert, 2010; Sprong et al., 2012; Pintore et al., 2015). In addition to *Ixodes ricinus*, the main vector of Lyme borreliosis and tick-borne encephalitis, other tick species, such as *Dermacentor spp.,* became more widespread and abundant (Karbowiak, 2014; Kiewra and Czulowska, 2013). Data collections carried out at different times, at the same locations can be useful to confirm changes in distribution and frequency of ticks and tick-borne agents (Gern et al., 2008; Jaenson et al., 2012; Schwarz et al., 2012).

In a study carried out in 1994 and 1995, *D. marginatus* was the dominant tick species collected from wild rodents in the Northern Apennines, in Tuscany, Italy, whereas *Haemaphysalis punctata* was found by dragging in locations characterised by southern exposure and limestone rocks as the geological substrate (Mannelli et al., 1997). No host-seeking *I. ricinus* was found by dragging a total sampled area of approximately 50000 m^2, whereas only one out of 128 small rodents was infested by *I. ricinus* larvae. Furthermore, no *B. burgdorferi* sensu lato (sl) could be revealed by cultivation of skin biopsies from rodents. By capturing wild rodents in the same area in 2009-2012, Martello et al. (2014) demonstrated the geographic range expansion of *I. ricinus*, up to beech tree (*Fagus sylvatica*) wood habitat at 1670 m above the sea level (asl). In the present work, we collected host-seeking ticks by dragging the vegetation in the Northern Apennines, and carried out subsequent laboratory analysis for the detection of *B. burgdorferi* sl in *I. ricinus*. Our objectives were: to integrate the data which were collected on small rodents by Martello et al. (2014); to further support the range expansion of *I. ricinus* by including different habitat types; to investigate the occurrence of the agents of Lyme borreliosis in the newly-colonised

area; to study changes in habitat distribution and abundance of *D. marginatus* and *H. punctata*, in comparison with the study of 1994-1995 (Mannelli et al., 1997).

Materials and methods

Study area

The study was carried out on the southern, Tuscan side of the Tuscan-Emilian Apennine National Park, Italy (44°12′ N, 10°22′ E), at altitudes ranging from 680 and 1670 m asl. Climate is characterized by relatively cold winters and cool summers. In the period 1996–2010, the monthly mean temperature was 1.9°C in January (minimum), and 17.7°C in July and August (maximum) (Regione Toscana, 2015a,b). Annual rain precipitations are among the greatest in Italy, and showed much variation in the considered period (1350 - 2150 mm). Snow cover normally lasts from December through March–April. Temperature > 10 °C, which is considered as compatible for the development of *I. ricinus* (Gray 1991; Daniel et al., 2015), have been recorded at ~ 1200 m asl from April through October, during the years 2010 and 2011 (Regione Toscana, 2015a).

Wild ungulates were reintroduced during the 1960-1970s. In 2011, the red deer (*Cervus elaphus*) and mouflon (*Ovis orientalis musimon*) populations were estimated at 9.2 and 12.3 head/100 ha. The roe deer (*Capreolus capreolus*) was once rare (Ciucci, 1994), but it underwent a remarkable population increase in the last two decades; in 2011, population density was estimated at 13.2 head/100 ha (Bongi P., unpublished report). The wild boar (*Sus scrofa*) is abundant, although census data are not available.

Tick collection

Dragging transects were chosen based upon the distribution of hiking trails within the Park, to cover the main habitat types of the study area. This can

be divided into two major sections: 1) the southern slopes of Mount Pania di Corfino (Mt. Pania), ranging from 680 to 1400 m asl; it is characterised by a limestone geological substratum and by a cover of exposed rocks and mixed deciduous woods dominated by hop hornbeam (*Ostrya carpinifolia*), which is typical of areas with poor soil, and Turkey oak (*Quercus cerris*); xerophilic, evergreen oaks (*Q. ilex*) can also be found; 2) the upper part of the study area (1200 - 1670 m asl), characterised by sandstone as geological substratum, and dominated by high stand, beech tree wood (*Fagus sylvatica*), with deep leaf litter (Ciancio et al. 2006). Above the limit of the tree vegetation (approximately 1700-2054 m asl), the Apennine ridge is characterised by pastures which were not included in the collection of host-seeking tick.

In 2010 and 2011, dragging was monthly performed in 35 sites, from spring to autumn (April-May to October-November). At each site, questing ticks were collected by one operator by dragging a 1 m^2 cotton cloth on the ground vegetation, along a 100 m^2 transect. The operator stopped approximately every 20 m^2 to check the cloth for the presence of ticks. The Cartesian coordinates (UTM, Zone 32T, datum WGS84) were taken with a GPS device for each dragging location.

Ticks were preserved in 70% ethanol for later identification using keys by Manilla (1998). *Haemaphysalis* spp. larvae were encountered in very high number at certain locations, thus, a maximum number of 60 specimens per collection tube were individually identified to species level, based on the shape of the *basis capituli* and the presence of a ventral spur on the palp III article. When more numerous, the species of the remaining larvae was attributed to *Haemaphysalis spp.* on the basis of the fraction of each species which was identified in the first 60 specimens, in the same tube.

Laboratory analyses

A sample of *I. ricinus* nymphs, which was randomly chosen from all dragging

sites and sessions, was screened by PCR assays to detect *B. burgdorferi* sl. Tick DNA was extracted by using the QIAGEN DNeasy tissue kit (Qiagen GmbH, Hilden, Germany). For each extraction set of five samples, a negative control (distilled water) was added. The infection by *B. burgdorferi* sl was investigated by a PCR protocol targeting a 337-bp fragment of the intergenic spacer (IGS) region included between genes coding for the 5S and 23S subunits of ribosomal RNA (Rijpkema et al. 1995). The 25µl PCR reactions contained 2.5ul of DNA extracts in RNase and DNase free water, 10X PCR Buffer, 1.5 mM $MgCl_2$, 1U Platinum Taq DNA Polymerase (Invitrogen, Milan, Italy), 0.2 mM dNTP mix (Qiagen, Milan, Italy), 0.4uM of each primer. The reaction conditions were 94°C for 3 min, 10 cycles (94°C for 20 s, annealing for 30s with temperatures ranging from 60°C to 52°C, with a decrease of 2°C every second cycle, 72°C for 30s), 40 cycles (94°C for 20 s, 50°C for 30 s, 72°C for 30 s), and 72°C for 7 min. In each PCR run, one negative control (distilled water) was added. DNA from *B. afzelii* (Nancy strain) and *B. garinii* (BL3 strain) were used as positive controls. Amplicons were purified using ExoSAP-IT PCR Clean-up Kit (GE Healthcare Limited, Chalfont, UK) and sent to an external service (Macrogen Inc., Amsterdam, The Netherlands) for automatic sequencing. The sequences were analyzed by Chromas 2.0 software (Technelysium, Helensvale, Australia) and submitted to BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi) to identify similarities to known sequences and therefore to classify pathogens at species level. Moreover, to confirm genospecies identification, a restriction fragment length polymorphism (RFLP) analysis was done *in silico* and a "virtual hybridization" was performed, as described in Rudenko et al. (2009).

Statistical analyses

Numbers of ticks per 100 m^2 were represented by boxplots, by ticks species, stage, and vegetation cover which was divided into: mixed deciduous wood dominated by Turkey oak, mixed deciduous wood dominated by hop hornbeam, and exposed rocks (on the southern slopes of Mt. Pania), and beech tree wood (on the upper part of the study area). Data from 2010 and 2011 were combined.

The percents of tick-positive transects, where at least one host seeking tick was collected, were calculated by month, tick species and stage, and were represented by barplots (Bisanzio et al., 2008). Prevalence of infection with *B. burgdorferi* sl, and exact binomial 95% confidence interval (CI) were calculated for *I. ricinus* nymphs which were collected in each type of vegetation cover.

Mapping of the distribution of host-seeking *I. ricinus* nymphs, and of the detection of *B. burgdorferi* sl by PCR, at each dragging site, was carried out using the R software (R Core Team, 2013). Altitude data of the study area were downloaded from the NASA's Shuttle Radar Topography Mission (SRTM) (http://www2.jpl.nasa.gov/srtm/) and were imported in R (*raster* package) to produce a map of the study area, where elevation was represented by contour lines (*contour* function) with an interval of 100 m asl.

Results

In 2010-2011, we collected host seeking ticks by dragging in a total of 414 x 100 m^2 transects. In certain months, the frequency of dragging was reduced due to adverse weather conditions, and only 15 and 22 transects were sampled in October and in November, respectively. We collected a total of 16670 ticks. *I. ricinus* (4431 larvae, 597 nymphs, and 12 adults) and *H. punctata* (11209 larvae, 313 nymphs, and 25 adults) were the most abundant species, followed by *H. sulcata* (20 larvae, 5 nymphs, and 13 adults), *D. marginatus* (42 larvae, and 2 adults) and one *I. hexagonus* nymph.

Immature *I. ricinus* were found in all dragging sites. They were most abundant at altitudes below 1400 m asl on the southern slopes of Mt. Pania, and greatest counts were found in Turkey oak wood at 1000-1200 m asl (Figure 1). Immature *I. ricinus*, especially nymphs, were also relatively abundant at greater altitudes in beech tree wood, and they were found up to 1670 m asl, near the altitudinal limit of the tree vegetation on the Apennine ridge (Figure 1; Figure 5). The percent of sites which were positive for *I.*

ricinus larvae was greatest in June (73.9%), it gradually decreased until October and was lowest in November (Figure 3). The percent of positive sites for *I. ricinus* nymphs was greatest in spring and early summer (maximum = 75.0% in May), although nymphs were found until November. Adult *I. ricinus*, including three females and nine males, were collected from April through September, in all habitat types; a maximum of five specimens was found in beech tree wood.

In comparison with *I. ricinus*, *H. punctata* was more markedly associated with habitats such as exposed rocks and hop hornbeam wood, on the southern slopes of the Mt. Pania (Figure 2). Here, *H. punctata* larvae were particularly abundant (up to 1050 larvae per 100 m^2), whereas nymphs were much less numerous. Rare immatures were also found in beech tree wood, at ~ 1400 m asl. The percent of positive sites for *H. punctata* larvae was greatest from August through October, whereas nymphs peaked in June (Figure 4). Adults (13 females and 12 males) were most abundant in April and May, in hornbeam wood, where 14 individuals were collected.

D. marginatus larvae were collected from June through August. They showed a preference for Turkey oak and hop hornbeam woods, accounting for 69% of specimens; only eight larvae were found in beech tree wood, at ~ 1200 m asl. Two females were also found in September, in Turkey oak wood and on exposed rocks. *H. sulcata* larvae were found from July through September, whereas nymphs were collected in June, July and September, and adults from April through June. Hop hornbeam wood accounted for 82% of ticks of this species. The abundance of *H. sulcata* larvae might have been underestimated in 46 cases where more than 60 *Haemaphysalis* spp. larvae were collected, and no *H. sulcata* was identified; in fact, in these cases, all of the remaining larvae were classified as *H. punctata*. One *I. hexagonus* nymph was collected in hop hornbeam wood in September.

Overall, 294 *I. ricinus* nymphs were tested for the presence of *B.*

burgdorferi sl by PCR. The overall prevalence of infection was 8.5% (95% CI = 5.6; 12.3). Infected ticks were collected in different habitat types, with a greater frequency of positives in sites facing south (Table 1; Figure 5). Indeed, prevalence of *B. burgdorferi* sl was greatest in nymphs collected on exposed rocks, followed by hop hornbeam wood, whereas it was lower in beech tree and Turkey oak woods.

We identified five genospecies of *B. burgdorferi* sl. *B. garinii* was the most frequent, being detected in 16 nymphs and accounting for 64% of PCR-positive specimens (Table 1). It was found in all habitat types, except Turkey oak wood, including beech wood site up to ~ 1400 m asl, and it showed 98% similarity with the PBi German strain (GenBank accession no. CP000013). *B. valaisiana* was found in five nymphs from all habitat types; it had a 99% similarity to the Tom4006 strain from Siberia (CP009117). *B. burgdorferi* sensu stricto (ss) was detected in one nymph from hop hornbeam wood and in one nymph from beech tree wood, showing 100% similarity to the reference strain B31 (CP009656). *B. afzelii* and *B. lusitaniae* were detected in one nymph each, from hop hornbeam wood. *B. afzelii* was 97% similar to the German PKo strain (CP000395), *B. lusitaniae* 99% similar to the PoTiB6 strain from Portugal (EU078961). One sequence per each genospecies was submitted to GenBank, with the accession no. KU291351-5.

Discussion

The collection of host-seeking ticks in 2010 and 2011, and subsequent laboratory analysis, demonstrated the recent *I. ricinus* and *B. burgdorferi* sl invasion of the Tuscan side of the Tuscan-Emilian Apennine National park, including beech tree woods near the limit of the tree vegetation. These results confirm and integrate the finding of *I. ricinus* on small rodents, starting in 2009, in the same area (Martello et al., 2014). In particular, the collection of host-seeking *I. ricinus* nymphs provided useful information. In fact, this stage, which was rarely found on rodents, is the most important for the transmission of *B. burgdorferi* sl to people.

Increasing population densities of wild ungulates, and increasing summer temperatures might have favored the range expansion of *I. ricinus* to higher altitude in the Northern Apennines (Léger et al., 2012; Medlock et al., 2013; Martello et al., 2014).

Host-seeking ticks, including immature *I. ricinus*, were most abundant in wooded habitats, as well as on exposed rocks on the southern slopes of the Mt. Pania. Short duration of snow cover at these locations, in comparison with areas at higher altitude, favour a relatively high densities of wild ungulates and other vertebrate hosts for ticks, especially during the colder months of the year (Ciucci, 1994). Although dry habitat conditions limit the geographic range of *I. ricinus* in southern Europe (Estrada-Peña et al., 2004; Bisanzio et al., 2008; Tagliapietra et al., 2011), abundant rainfall in the Northern Apennines probably ensures enough moisture for the development and activity of *I. ricinus*, even in south-facing sites, characterised by poor soil and xerophilic vegetation. The collection of relatively abundant *I. ricinus* in oak (*Quercus* spp.) wood harbouring a diverse fauna is in agreement with results of previous European studies (Tack et al., 2012). However, within this area, *I. ricinus* was the dominant tick species at greater altitudes, in high stand beech tree wood characterised by deep leaf litter, which is considered as favourable to the tick (Rizzoli et al. 2009). Accordingly, immature *I. ricinus* were found on small rodents in the same habitat type by Martello et al. (2014).

The possibility of finding host seeking ticks in pastures above the limits of the tree vegetation should also be investigated, even though no tick was previously found in this habitat (Mannelli et al., 1997). Very few, host seeking *I. ricinus* were found in grassland surrounded by deciduous wood, in the Monti Sibillini National Park, in the Central Apennines (Curioni et al., 2004). Factors underlaying the distribution and the abundance of *I. ricinus* in different habitats, and in different study areas in the Apennines should be the object of further studies.

The activity of host seeking *I. ricinus* larvae started from May in the majority of our dragging sites (Figure 3), whereas the prevalence of this tick stage on small rodents was greatest in July and August (Martello et al., 2014). Activity of *I. ricinus* nymphs was slightly delayed in comparison with the seasonality that was previously observed at another location in Tuscany (Le Cerbaie Hills; Bisanzio et al., 2008); this was probably due to colder spring climate in the Apennines.

The limited number of adult ticks collected, in comparison with immature stages, might be attributable to the relatively low efficiency of dragging in collecting adult ticks (Dantas-Torres et al 2013). Furthermore, checking drag clothes at 20 m^2 intervals might not be frequent enough to collect adults, which might be dislodged by the substrate during dragging (Estrada-Peña et al., 2013b). On the other hand, by using the same dragging method, Bisanzio et al. (2008) collected relatively greater proportions of *I. ricinus* adults and nymphs than in this study, indicating that, in the Apennines, larvae were disproportionately more abundant than the other stages. More in depth studies would be advisable on *I. ricinus* phenology and population dynamics in the Park.

H. punctata was previously shown to prefer relatively dry habitats in Italy (Cringoli et al., 2005; Ceballos et al., 2014); in the Monti Sibillini National Park it accounted for almost the totality (98.9%) of collected host seeking ticks, and it was not found above 1300 m asl (Curioni et al., 2004). In our study, *H. punctata* was found in beech tree wood habitat, showing a recent expansion of its range to higher altitudes in comparison with 1994-1995 (Mannelli et al. 1997). The marked seasonality of *H. punctata* larvae, together with the more extended activity of nymphs that we recorded, are in agreement with results of previous studies in central Italy and in northern Spain (Estrada-Peña et al., 1990; Curioni et al., 2004; Barandika et al., 2006).

Interestingly, *H. punctata* larvae were frequently collected by dragging, but were very rare on rodents in our study area (Martello et al., 2014). Conversely, no host-seeking *D. marginatus* larva was collected in beech wood above 1200 m asl, where these ticks were relatively abundant on rodents (Mannelli et al., 1997; Martello et al., 2013; Martello et al, 2014). This might be attributable to the nidiculous habits of immature *D. marginatus* (Cringoli et al., 2005), which might be more marked at increasing altitudes. Moreover, in the presence of deep beech tree leaf litter, *D. marginatus* larvae might quest very low, near the ground, reducing the likelihood of collection by dragging. Indeed, host-seeking *D. marginatus* were also not found at dragging sites in beech tree woods, above 800 m asl, in Liguria (Ceballos et al., 2014).

Based on the above observations, the collection of host seeking and on host ticks are complementary techniques for the detection of *H. punctata* - which was most frequently collected by dragging - and *D. marginatus* – which was most frequent on rodents. Both tick species might serve as vectors for agents of zoonotic diseases. *D. marginatus* was found infected with *Rickettsia slovaca*—the agent of tick-borne lymphadenopathy (TIBOLA), in our study area (Selmi et al., 2008; Selmi et al., 2009; Martello et al., 2013). *H. punctata* may transmit human pathogens such as rickettsiae, *Francisella tularensis*, and *Babesia* spp. (Curioni et al., 2004; Cringoli et al. 2005).

The spatial distribution of *H. sulcata* was similar to the distribution of *H. punctata*. *H. sulcata* is a characteristic tick of the Mediterranean area (Estrada-Peña et al., 2013a) and it could also have invaded the mountain park area, along with changing environmental conditions. The coexistence of several tick species in the Northern Apennines is in agreement with the tick diversity which was observed in southern Italy (Dantas-Torres and Otranto, 2013). This is probably attributable to habitat and vertebrate host diversity within a limited geographic range.

Several genospecies of *B. burgdorferi* sl were detected in host-seeking *I. ricinus* nymphs. This suggests that, following the recent invasion of the area by the tick vector, multiple species of vertebrate reservoir hosts contributed to the establishment of *B. burgdorferi* sl transmission cycles (Mannelli et al., 2012). The prevalence of *B. burgdorferi* sl, and relative frequency of different genospecies, may vary with altitude and vegetation cover, due to mechanisms involving both microclimate and host abundance (Morán Cadenas et al., 2007). Indeed, Jouda et al. (2004) observed a decreasing tick density and *B. burgdorferi* sl prevalence with increasing altitude in the Swiss Alps.

In our study, the greatest frequency of *B. garinii* and *B. valaisiana* suggests a major role of birds as reservoir hosts, in agreement with observations in other European areas, including North western Italy (Mannelli et al., 2003; Pichon et al., 2006). *B. lusitaniae* was previously associated with lizards in Tuscany (Amore et al, 2007), and these hosts might find favourable conditions especially in the warmer parts of our mountainous study area (Morán Cadenas et al., 2007). *B. afzelii* and *B. burgdorferi* ss might be associated with small rodents and other species of small mammals (such as sciurids). Further studies would be necessary to investigate the association between hosts and genospecies in the newly invaded area, given a non-absolute association between reservoir hosts and genospecies.

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Table title

Table 1 Results of PCR for the detection of *B. burgdorferi* sl in *I. ricinus* nymphs collected by dragging in different habitat types in the Tuscan-Emilian National Park, Italy, in 2010 and 2011

Habitat type	Number of tested nymphs	% prevalence of *B. burgdorferi* sl in nymphs (95% CI)	Identified genospecies of *B. burgdorferi* sl
Hop hornbeam wood	70	11.4 (5.1; 21.3)	*B. garinii*; *B. valaisiana*; *B. burgdorferi* ss; *B. afzelii*; *B. lusitaniae*
Exposed rocks	42	19.0 (8.6; 34.1)	*B. garinii*; *B. valaisiana*
Turkey oak wood	57	1.8 (0.04; 9.4)	*B. valaisiana*
Beech tree wood	125	6.4 (2.8; 12.2)	*B. garinii*; *B. valaisiana*; *B. burgdorferi* ss

CI = exact binomial confidence interval

Figures

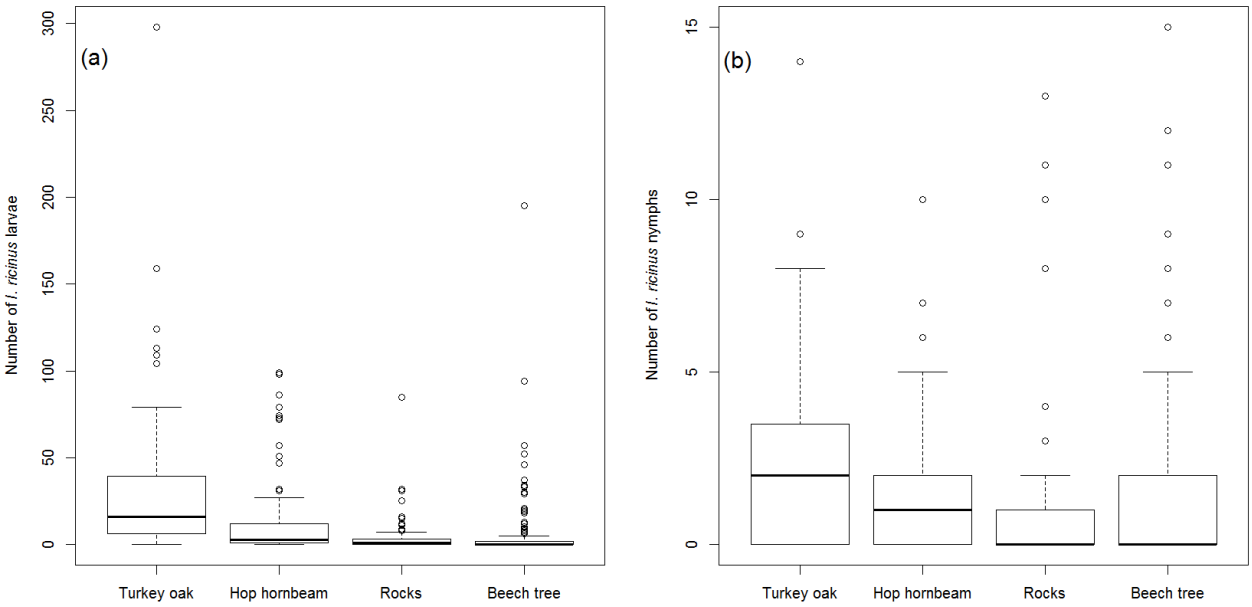

Fig. 1 Numbers of host-seeking *I. ricinus* collected by dragging in 100 m^2 transects, in different habitat types in the Tuscan-Emilian National Park, Italy, in 2010 and 2011, (a) larvae; (b) nymphs. In the boxplot, the bottom of the box represents the first quartile of the distribution and the top the third quartile. The bar inside the box represents the median. Small circular symbols indicate values differing from the box more than 1.5 times the interquartile range

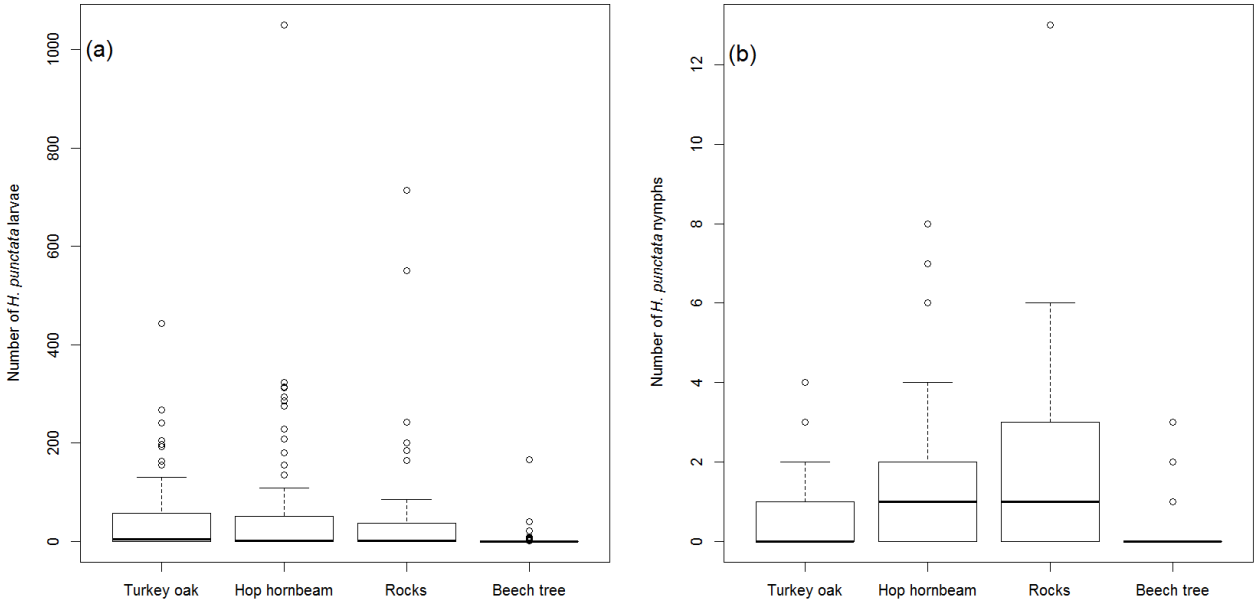

Fig. 2 Numbers of host-seeking *H. punctata* collected by dragging in 100 m^2 transects,in different habitat types in the Tuscan-Emilian National Park, Italy, in 2010 and 2011, (a) larvae; (b) nymphs. Symbols as in Fig. 1

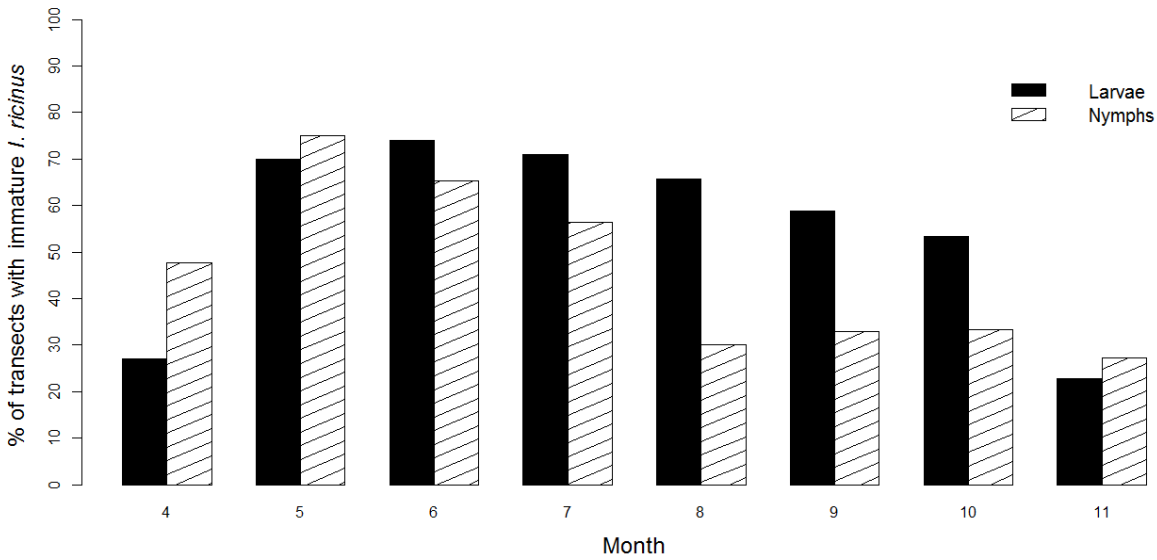

Fig. 3 Percent of 100 m^2 transects where at least one host-seeking immature *I. ricinus* was collected by dragging, from April through November, in 2010 and 2011, in the Tuscan-Emilian National Park, Italy.

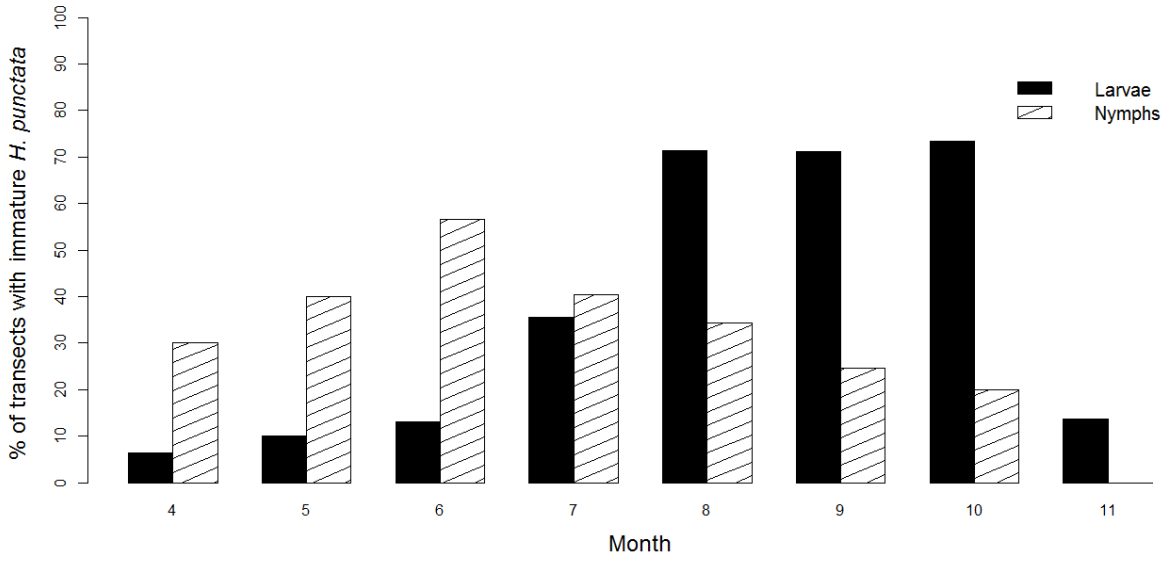

Fig. 4 Percent of 100 m^2 transects where at least one host-seeking immature *H. punctata* was collected by dragging, from April through November, in 2010 and 2011, in the Tuscan-Emilian National Park, Italy.

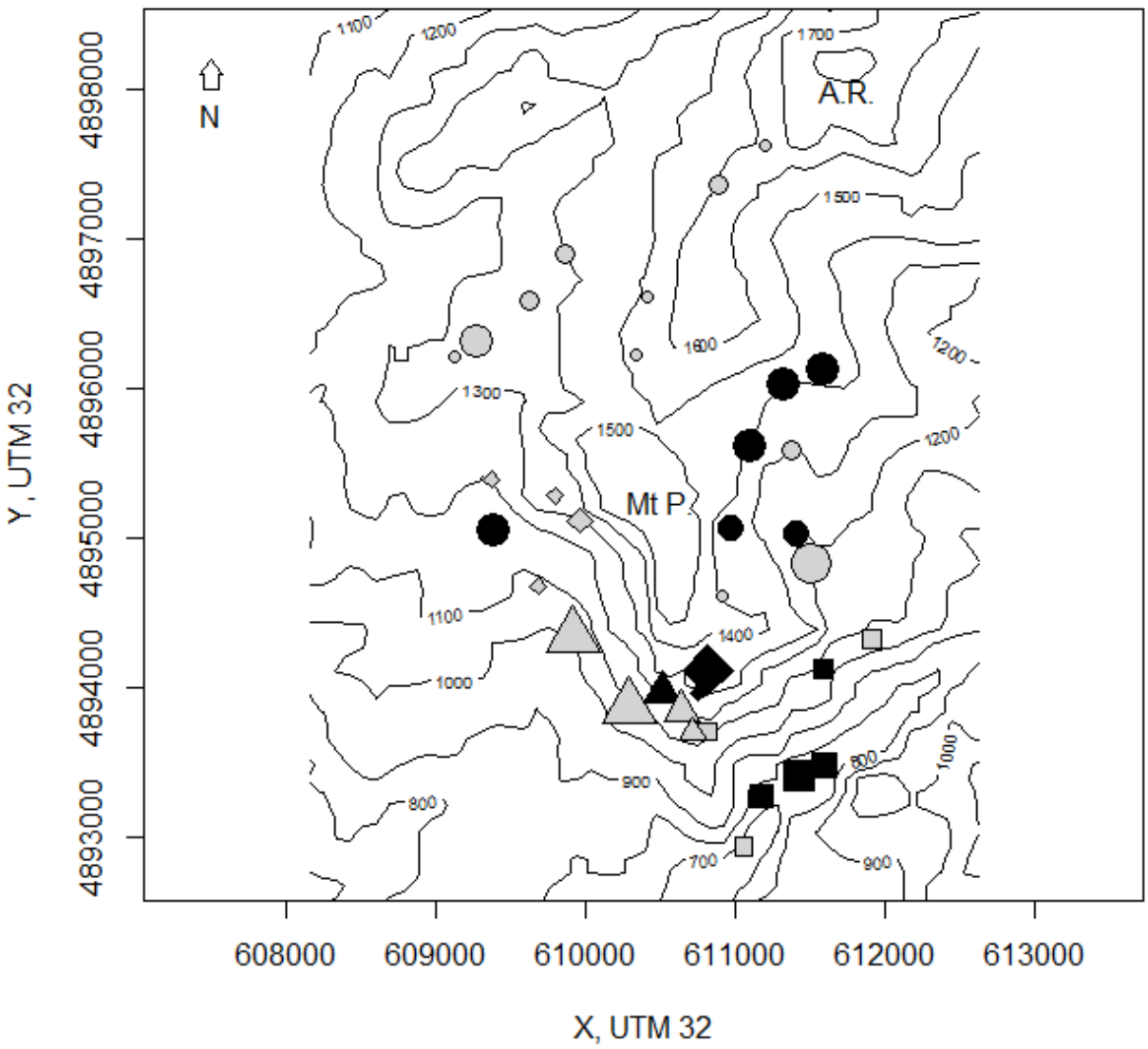

Fig. 5 Contour map of altitude, with an interval of 100 m asl, of the study area, in the Tuscan side of the Tuscan-Emilian National Park, Italy. Symbols represent sites of collection of host-seeking ticks, in 2010 and 2011. Circles: beech tree wood habitat. Triangles: Turkey oak habitat. Diamonds: exposed, limestone rocks. Squares: hop hornbeam habitat. Symbol size is based upon quartile distribution of the average number of *I. ricinus* nymphs per 100 m collected at a dragging site. Min=0.08, first quartile=0.39, median=0.92, third quartile=2.1, max=4.3. Black symbols indicate the detection of *B. burgdorferi* sl in at least one *I. ricinus* nymphs, by PCR. Grey symbols indicate negative PCR results. Mt P.: summit of Mount Pania di

Corfino. A.R.: Apennine ridge