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Ticks climb the mountains: ixodid tick infestation and infection by tick-borne pathogens in the Western Alps.

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ABSTRACT

In mountain areas of northwestern Italy, ticks were rarely collected in the past. In recent years, a marked increase in tick abundance has been observed in several Alpine valleys, together with more frequent reports of Lyme borreliosis. We then carried out a four-year study to assess the distribution and abundance of ticks and transmitted pathogens and determine their altitudinal limit in a natural park area in Piedmont region.

Ixodes ricinus and *Dermacentor marginatus* were collected from both the vegetation and hunted wild ungulates. Tick abundance was significantly associated with altitude, habitat type and signs of animal presence, roe deer's in particular. *Ixodes ricinus* prevailed in distribution and abundance and, although their numbers decreased with increasing altitude, we recorded the presence of all active life stages of up to around 1700 m a.s.l., with conifers as the second most infested habitat after deciduous woods.

Molecular analyses demonstrated the infection of questing *I. ricinus* nymphs with *B. burgdorferi* sensu lato (15.5%), *Rickettsia helvetica* and *R. monacensis* (20.7%), *Anaplasma phagocytophilum* (1.9%), *Borrelia miyamotoi* (0.5%) and *Neoehrlichia mikurensis* (0.5%). One third of the questing *D. marginatus* were infected with *R. slovaca*. We observed a spatial aggregation of study sites infested by *B. burgdorferi* s.l. infected ticks below 1400 m. *Borrelia*-infected nymphs prevailed in open areas, while SFG rickettsiae prevalence was higher in coniferous and deciduous woods. Interestingly, prevalence of SFG rickettsiae in ticks doubled above 1400 m, and *R. helvetica* was the only pathogen detected above 1800 m a.s.l.

Tick infestation on hunted wild ungulates indicated the persistence of tick activity during winter months and, when compared to past studies, confirmed the recent spread of *I. ricinus* in the area.

Our study provides new insights into the population dynamics of ticks in the Alps and confirms a further expansion of ticks to higher altitudes in Europe. We underline the importance of adopting a multidisciplinary approach in order to develop effective strategies for the surveillance of tick-borne diseases, and inform the public about the hazard posed by ticks, especially in recently invaded areas.

Keywords: Ixodidae; *Borrelia* spp.; SFG rickettsiae; altitude; Alps

1. INTRODUCTION

Alpine regions, and more generally mountain areas, are susceptible to environmental changes (Rogora et al., 2018). In the Italian Alps, climate change, along with land abandonment and habitat fragmentation, has strongly contributed to changes in vegetation cover and wildlife distribution at high altitudes (Chemini and Rizzoli, 2003). These shifts have favoured the geographic expansion of ixodid ticks, which are well-known vectors of disease agents to humans and animals. In particular, *Ixodes ricinus* - the main vector of Lyme borreliosis (LB) spirochaetes and tick-borne encephalitis (TBE) virus, is emerging in new areas in Europe (Medlock et al., 2013). This tick species was reported above 1000 m above the sea level (a.s.l.) in Western Alpine regions (Burri et al., 2007; Ceballos et al., 2014; Pintore et al., 2014), and up to 1650 m a.s.l. in the Northern Apennines in Central Italy (Martello et al., 2014). Geographic range expansion has also been notable for *Dermacentor marginatus* and *D. reticulatus* in central Europe (Földvári et al., 2016; Walter et al., 2016; Sprong et al., 2019), while their distribution in Alpine areas is not well-defined (Rubel et al., 2016). In the Alpine valleys of Piedmont region, northwestern Italy, ticks were absent or rare in the late 1980's (Rossi and Meneguz, 1989). Accordingly, tick-borne disease cases were uncommon in the region. Indeed, although LB is a notifiable disease in Italy, the Piedmont Regional Service for the Epidemiology of Infectious Diseases (SEREMI) only registered eight cases from 1990 to 2008. From 2008 to 2011, 11 LB cases were reported in Verbano-Cusio-Ossola province, northern Piedmont (Pintore et al., 2014). More recently, the number of LB cases notified to SEREMI increased in the whole region, summing up to 68 from 2012 to 2018 (<https://www.seremi.it/>). The rising number of LB cases suggests an increase in *I. ricinus* distribution and abundance.

This study was prompted by three cases of LB unexpectedly diagnosed in the spring of 2016, in forest rangers operating in a mountain regional park in Piedmont. Ticks were historically absent in this park, which extends in altitude from around 1000 m to 2600 m a.s.l. and is often visited by tourists because of its great natural value. Our aim was to investigate the spatial distribution, abundance and diversity of ticks and transmitted pathogens, considering habitat characteristics and animal presence. In particular, we wanted to assess the current altitudinal limit of *I. ricinus* in the Alps, in an area where it has only recently been

found. The knowledge about the spatio-temporal distribution of ticks and tick-borne pathogens (TBP) in recently invaded regions may indeed be helpful in raising awareness among the population at risk, while providing relevant information for vector-borne disease surveillance.

2. MATERIALS AND METHODS

2.1. Study area

The study was carried out in high Susa Valley, in a natural protected area belonging to Alpi Cozie regional park (45°03'N, 6°54'E), and nearby areas belonging to high Susa Valley hunting district.

Susa Valley has a west-east direction from the city of Turin to the French border and is located between the Graian (north) and the Cottian Alps (south). In view of its geographic location and climatic characteristics, it is considered as a xeric inner Alpine valley. An increase in mean temperatures was observed in the period 1961-2010, in particular during the cold seasons at elevations above 1600 m, with a rise in mean maximum temperature of 0.072°C per year (Acquaotta et al., 2015). Rain and snow are well distributed annually: the first is concentrated mainly during spring and autumn (618 – 950 mm), while snow cover prevails from November to April, reaching an average thickness of 17 – 68 cm, depending on altitude (Regional Agency for the Protection of the Environment, ARPA – Piedmont: www.arpa.piemonte.it). However, a reduced snow cover is accompanying the positive trend in winter temperatures (Acquaotta et al., 2015).

This Alpine valley has a high biodiversity. Coniferous forests cover the area above 900 m, namely European spruce (*Picea abies*), silver fir (*Abies alba*), larch (*Larix decidua*) and stone pine (*Pinus cembra*). Mainly on slopes exposed to the south, conifers are combined with species of temperate and Mediterranean climate such as European beech (*Fagus sylvatica*), ash (*Fraxinus excelsior*), birch (*Betula* spp.) and Scots pine (*Pinus sylvestris*). Summer grasslands prevail at high altitudes, enabling the existence of pastoral-extensive livestock systems, which coexist with a large variety of wildlife, including five species of wild ungulates: Alpine ibex (*Capra ibex*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), Northern chamois (*Rupicapra rupicapra*) and wild boar (*Sus scrofa*). Censuses carried out on a yearly basis reported a marked increase in deer population densities since their reintroduction in the area during the 1960s. Nowadays, the

populations of red deer and Northern chamois are the most abundant, regulated by climate constraints, predation by wolf (*Canis lupus italicus*) and hunting (Scialabba, 2016).

2.2. Tick collection

Questing ticks were collected in 45 pre-defined transects, which were selected between 959 and 1884 m a.s.l., according to environmental characteristics such as habitat type, sun exposure side and land-use (Appendix A). Habitats were categorized according to dominant vegetation species: 1) open areas: included sun-exposed pastures and hiking areas, characterized by herbaceous and semi-shrubs species and surrounded by woods; 2) coniferous woods: included species such as *A. alba*, *L. decidua*, *P. abis*, *P. sylvestris* and *P. cembra*; and 3) deciduous woods: included *Betula* spp., *F. excelsior*, *Salix* spp., *Populus* spp., *F. sylvatica*.

Monthly tick collections were performed from April to November during 2016-2019 by dragging a 1-square-meter white-cotton cloth on the ground vegetation along 100 m of transect. During the sampling, we measured temperature and humidity at each transect using a HI9564 thermo-hygrometer (HANNA instruments®, Milan, Italy), and recorded signs of animal presence (faeces, footprints and other traces). Snow or frost generally cover the vegetation from December to March, so we did not perform dragging during winter.

Feeding ticks were collected on game presented by hunters at the check station of the local Hunting Management Unit (CA TO2 - <http://www.cato2.it/>), from 2017 to 2019. Hunters bring the hunted animals to the site of inspection as soon as the check station opens, then time may pass after the killing, and they do not adopt any special protocol for the transportation of carcasses. Each animal carcass was visually inspected by the same operator for about 15-20 minutes, including the head, sternum and abdomen regions, and internal parts of front and hind legs. Collected ticks were stored in vials with 70% alcohol for later identification. We recorded data regarding animal species, age, sex and shooting date and location.

2.3. Pathogen detection

Collected ticks were stored in 70% ethanol at room temperature and morphologically identified to stage and species level (Cringoli et al., 2005; Estrada-Peña et al., 2017). *Ixodes* spp. were checked for being *I. inopinatus*, a new species reported in Mediterranean-type habitats (Estrada-Peña et al., 2014). We randomly selected a sample of questing *I. ricinus* nymphs for molecular analyses, considering all dragging sites and sessions. The sample size was determined considering a 25% expected prevalence of *Borrelia burgdorferi*, a 95% confidence level and 5% error, both below and above 1400 m a.s.l. – in order to evaluate possible differences in the infection prevalence among altitudes. Moreover, all questing *D. marginatus* were analysed. The DNA of individual ticks was extracted by using DNAzol reagent® (Life Technologies LTD, Warrington, United Kingdom), according to the manufacturer's instructions. Prior to DNA extraction, ticks were cut apart with sterile scalpel blades and immersed in lysis buffer (NaCl 0.1M, Tris-HCl 0.21M, pH8 EDTA 0.05M, SDS 0.5%) with proteinase-K (20 mg/mL) at 56°C overnight. *Ixodes ricinus* nymphs were screened by multiplex quantitative polymerase chain reaction (qPCR) assays for *B. burgdorferi* sensu lato (s.l.) (*OspA* and *flagellin B* genes) and *B. miyamotoi* (*flagellin* gene) (van Duijvendijk et al., 2016), *Neoehrlichia mikurensis* (*groEL* gene) and *Anaplasma phagocytophilum* (*msp 2* gene) (Jahfari et al., 2014), and *Rickettsia* spp. (*gltA* gene, with a specific probe for *R. helvetica*) (Heylen et al., 2016). Positive rickettsia-*gltA* samples, negative for *R. helvetica* specific probe, were further tested by conventional PCR targeting a fragment of the *OmpA* gene (Regnery et al., 1991). *Dermacentor marginatus* ticks were screened for *Rickettsia* spp. only, as described in Martello et al. (2019). In order to determine the nucleotide sequence, the DNA from qPCR-positive samples were subjected to end-point PCRs for *B. burgdorferi* s.l. (5S-23S rDNA intergenic spacer; Coipan et al., 2013), *Rickettsia* spp. (*gltA* and *OmpA* genes), and *A. phagocytophilum* (*groEL* gene; Alberti et al., 2005). Positive controls and negative water controls were used on every plate tested. For all qPCRs, plasmids were used as positive controls, which were manufactured by GenScript Biotech (Leiden, Netherlands). The PUC57 plasmid contained an artificial DNA sequence consisting of a primer-probe-primer sequence. Between both primer sequences and the probe six nucleotides, 5'-ATG-TTC-3', were incorporated. All positive controls were therefore different in length and sequence from target sequences, allowing us to check the possibility of contamination of samples with control DNA. To minimize

contamination and avoid false-positive samples, the DNA extraction, PCR mix preparation, sample addition, and (q)PCR analyses were performed in separate air locked dedicated labs.

2.4. Statistical analyses

Data were analysed by RStudio Team (2015) for Windows. Binomial Exact test was performed to calculate tick infestation prevalence of questing and feeding *I. ricinus*, and TBP infection rates in questing nymphs, with 95% confidence intervals (CI). The Pearson's Chi-squared test with Yates' correction for continuity was used to study the association between altitudinal range and habitats with presence of ticks and TBP in dragging sites. Non-parametric tests (Wilcoxon and Kruskal Wallis) were chosen to study the relationship between tick abundance and altitude, habitat and animal signs. We performed Fisher's exact test to evaluate the association between tick infestation in wild ruminants, animal characteristics (sex, age, animal species and altitude of shooting location) and month of collection. We applied Kappa statistic to identify the relationship between concurrent infections by different TBP in the same tick; values not significantly different from zero indicate no agreement beyond chance (Fleiss, 1981).

Nearest neighbour index (NNI) was calculated by using QGIS software (Version 3.4.5) to evaluate the spatial relationship among sampling sites and ticks infected by *B. burgdorferi* s.l. and SFG rickettsiae. Values less than 1 indicate that the observed average distance is closer than in a random situation, and therefore a clustered pattern exists, whereas values $NNI \geq 1$ indicate a randomly dispersed pattern. The significance level is given by the Z-statistic (Pinder and Witherick, 1972).

For all statistical tests, a two-tailed significance level of $\alpha = 0.05$ was adopted.

We performed only descriptive analysis of *D. marginatus* distribution and infection, due to the small sample size.

3. RESULTS

3.1. Abundance and seasonality of questing ticks.

A total of 6510 ticks were collected from the vegetation across the study period, namely 6492 *I. ricinus* and 18 *D. marginatus*.

Dermacentor marginatus ticks (10 larvae, 2 nymphs and 6 adults) were sporadically collected in six dragging transects in 2017 and 2018. Infested locations corresponded to exposed-open areas characterized by medium height grasses and shrubs, up to 1340 m a.s.l. *Dermacentor marginatus* was collected across the sampling season, however immatures were observed only in summer, in particular in July and September. *Ixodes ricinus* ticks (3940 larvae, 2388 nymphs and 164 adults) were present during the entire sampling period (April to November; Fig. 1, A-C). Their questing activity was synchronous across most of the sampling sites; however, some transects above 1450 m were not sampled in the first dragging session (April) because of persistence of snow cover. We recorded the maximum abundance of nymphs and adults from April to June, with 4.5 and 0.33 specimens per 100 m², respectively. By contrast, in late autumn (November) we recorded the lowest tick activity (0.09 larvae, 0.16 nymphs and 0 adults per 100 m²), although ticks were still recovered in around 25% of transects. Nymphs were the most prevalent life stage collected, in particular during spring (April-June), when we recorded the highest apparent density (Fig. 1B). Nymph abundance varied significantly among sampling locations (Kruskal – Wallis test, $p < 0.001$), reaching a maximum mean number of 19.3 nymphs/100 m² in the most infested sites. These locations corresponded to moist woods between 1201 to 1400 m a.s.l., where transects were infested in nearly 85% of samplings (Table 1). The percentage of sites infested by larvae slightly prevailed during summer (July-August), when they overlapped with nymph activity. On the other hand, adults were the least abundant life stage and were mainly collected during spring (April – May) with a maximum average number of 0.4/100 m² (Fig. 1, C and I).

Altitude and habitat type strongly influenced the presence and abundance of *I. ricinus*. It was recorded up to 1884 m a.s.l., although its abundance declined with altitude (Wilcoxon rank sum test, $p < 0.001$) (Table 1); indeed, only three nymphs and one larva were collected over 1800 m a.s.l. during the entire study, while no

adult was found at these altitudes. Immature and adult ticks were instead collected simultaneously in 51% (n= 23) of the study sites up to 1697 m a.s.l. When considering altitude, nymphs and adults did not show great variations in their seasonality (number of infested sites) above and below 1400 m a.s.l. (Fig. 1, E and F). Their abundance, however, considerably decreased in time above 1400 m, with nymphs ranging from 5.8 specimens per 100 m² in April to 0.1 in November (Fig. 1H). Conversely, larvae gradually declined in time below 1400 m a.s.l., with a maximum of 42.4% infested sites in July to a minimum of 16.7% in November; at higher altitudes they showed an initial increase in activity from April, peaking in June (with 40.6% sites infested) and then dropped dramatically over time (Fig. 1D).

Ixodes ricinus nymphs were more abundant in deciduous and coniferous woods compared to open areas (Pearson's chi squared test, $p < 0.01$) (Fig. 2A). All active life stages were observed across all habitats investigated; deciduous woods represented the most frequently infested habitat (91.2%; 95% CI= 84.5 – 95.7) and presented the highest tick abundance (Table 2). Tick abundance in coniferous woods and open areas was significantly greater at lower altitudes (Wilcoxon rank sum test, $p < 0.001$; Table 2). No statistically significant differences in questing nymphal densities, on the other hand, were observed across deciduous woods according to altitude (Wilcoxon rank sum test, $p = 0.16$), with 7.7 nymphs/100m² below 1400 m a.s.l., and 8.1 above.

During the sampling period, we recorded signs of presence of domestic animals (dogs), livestock (cattle, horses) and wildlife in almost all dragging sites (n= 42). In general, tick occurrence was not associated with signs of animal presence (Pearson's chi squared test, $p = 0.25$); however, ticks were more abundant in sites where wild animal signs were registered, in particular roe deer's (Kruskal – Wallis test, $p < 0.05$).

3.2. Tick-borne pathogens infection in questing ticks

We tested a total of 786 *I. ricinus* nymphs and 34.0% (95% CI= 30.6 – 37.4) were infected by at least one pathogen, with *B. burgdorferi* s.l. and spotted fever group (SFG) rickettsiae being the most prevalent bacteria (Table 3). We identified four *B. burgdorferi* s.l. genospecies: *B. afzelii* [GenBank: MT038899], *B. garinii* [MT038900], *B. valaisiana* [MT038902] and *B. burgdorferi* sensu stricto [MT038901]. *Borrelia afzelii*

was predominant, infecting 68 specimens (55.7% of positives; 95%CI = 46.5 – 64.7), followed by *B. garinii* (n= 13; 10.7%, 95%CI= 5.8 – 17.5), *B. valaisiana* (n= 6; 4.9%, 95%CI= 1.8 – 10.4) and *B. burgdorferi* sensu stricto (n= 6; 4.9%, 95%CI= 1.8 – 10.4). *Rickettsia helvetica* was detected in 16.0% nymphs (95%CI= 13.5 – 18.8). Other rickettsiae infected 4.7% *I. ricinus* nymphs (95%CI= 3.3 – 6.4), and we could identify *R. monacensis* in nine samples by DNA sequencing [MT025711]. *Anaplasma phagocytophilum* belonged to ecotype I [MT025713]. The emerging *Neoehrlichia mikurensis* and the relapsing fever spirochete *Borrelia miyamotoi* (MG451835; Ravagnan et al., 2018) were less frequently detected (Table 3). *Rickettsia slovaca* [MT025712] infected 33.3% of *D. marginatus* (95%CI= 13.3 – 59.0), namely the two nymphs and four females.

Among *I. ricinus* infected nymphs, 37 (13.9%; 95% CI= 10.0 – 18.6) were coinfecting by up to three different pathogens. The most frequent coinfection was *B. burgdorferi* s.l. with SFG rickettsiae (n= 32), in particular *B. afzelii* and *B. garinii* with *R. helvetica*. Kappa, as a measure of concurrent infection by bacterial groups in the same tick, indicated no agreement beyond chance between pathogens (K= 0.06, 95%CI= -0.01 – 0.2). Coinfections by more than two bacteria were observed in only four nymphs; in particular two nymphs were infected by *B. afzelii*, *A. phagocytophilum* and *R. monacensis*, one by *B. afzelii*, *A. phagocytophilum* and *R. helvetica* and one by *B. afzelii*, *B. miyamotoi* and *R. helvetica*.

As regards pathogens spatial distribution, we observed an aggregation of dragging sites with infected ticks, being more evident for *B. burgdorferi* s.l. (NNI = 0.53; p<0.0001) compared to SFG rickettsiae (NNI = 0.75; p<0.01) (Fig. 3).

Altitude affected pathogens distribution in the area. *Borrelia burgdorferi* s.l. prevalence was significantly higher below 1400 m a.s.l. (p < 0.05), whereas SFG rickettsiae were more frequently detected in ticks sampled above 1400 m (p < 0.001), where their prevalence doubled (Table 3). Interestingly, *R. helvetica* was the only pathogen detected above 1800 m a.s.l., in two nymphs.

Considering habitat type, deciduous and coniferous woods showed the highest rates of ticks infected by *B. burgdorferi* s.l. and SFG rickettsiae (Fig. 2B). The contrary was observed for open areas, where *Borrelia*-infected nymphs prevailed over those infected by *Rickettsia* spp.

3.3. Feeding ticks

During 2017, 2018 and 2019 hunting periods (September to December), we inspected the skin of 373 carcasses, belonging to 207 red deer, 23 roe deer and 143 chamois. *Ixodes ricinus* (n= 285; 51 nymphs and 234 adults) and *D. marginatus* (n= 8; 1 nymph and 7 adults) were again the only tick species collected. Adults were the most prevalent stage and were mainly located in the axillary and inguinal regions, while nymphs were exclusively found on the ears. *Dermacentor marginatus* was found on red deer and chamois, not on roe deer. Ixodid ticks infested 27.1% (95%CI= 22.6 – 31.9) of the animals, especially red deer (73/207) and roe deer (9/23), with an average of 3.2 (min./max.= 1 – 13). and 6.1 (min./max.= 2 – 14) ticks/individual, respectively. Chamois had a significantly lower infestation rate (n = 19; 13.3%; 95%CI = 8.2 – 20.0) ($p < 0.05$), although the tick load was similar to red deer, 3.1 (min./max.= 1 – 14) ticks/individual. No significant differences were observed regarding sex, age groups and month of collection ($p > 0.05$). We registered the maximum infestation rate in October (37.8% animals infested; 95%CI= 24.8 – 52.1), followed by November (29.2%) and December (23.1%).

Shooting location altitude ranged between 750 m and 2800 m a.s.l. Hunted animals from areas located below 1400 m were significantly more parasitized (47.5%; 95%CI= 39.1 – 56.1) than those from high altitudes (20.0%; 95%CI= 14.2 – 26.7) ($p < 0.001$). We observed, however, 52 adult ticks infesting 11 ungulates from higher altitudes (1800 – 2000 m a.s.l.).

4. DISCUSSION

Our study provides new insights into the population dynamics of ticks in the Alps, focusing on their association with altitude and habitats. In particular, it shows the presence of stable *I. ricinus* populations in a mountain area where this tick species was recently introduced. Indeed, while *D. marginatus* had occasionally been collected on wildlife in the past (Rossi and Meneguz, 1989), *I. ricinus* was not reported; park rangers and local hunters state that they started noticing the presence of *I. ricinus* in the early 2000s. Nowadays, *I. ricinus* largely prevails in abundance and it has reached a wide geographical distribution. We collected it during the entire sampling period and it infested almost 90% of our study sites, with greater

abundance in wooded areas rich in undergrowth and leaf litter cover. These findings are in accordance with the usual habitat distribution of *I. ricinus* (Estrada-Peña, 2001; Tack et al., 2012; Ceballos et al., 2014). We, however, recorded its presence up to 1890 m a.s.l., also in coniferous forests, the second most infested habitat after deciduous woods. These results are remarkable since such altitudes and habitats have been usually considered unsuitable for *I. ricinus* survival and development, due to extreme temperatures, the low content of secondary vegetation and dry soils (Estrada-Peña, 2001).

In Italy, a similar geographical spread of *I. ricinus* was observed in the northern Apennine, where it was also absent until the 2000s (Martello et al., 2014). Previous studies carried out in Alpine areas had reported the presence of *I. ricinus* at altitudes up to 1450 m in north Piedmont region (Pintore et al., 2014). Interestingly, our study shows the presence of all tick life stages in sites up to around 1700 m a.s.l., suggesting the maintenance of the tick life cycle at such altitudes and a further increase in the altitudinal limit for ticks in the Italian Alps.

As observed by other authors (Danielová et al., 2010; Pintore et al., 2014), *I. ricinus* abundance was negatively affected by the increase in altitude. Environmental factors are the underlying key mechanisms, in particular, temperature and relative humidity have a major impact on tick survival and activity (Daniel, 1993; Randolph et al., 2004; Ehrmann et al., 2017). In fact, climate in mountainous areas is characterized by great temperature fluctuations, which are more pronounced as altitude increases (Acquaotta et al., 2015). These conditions may limit the questing behaviour and developmental rate of ticks. The persistence of snow cover during the coldest seasons, nevertheless, may protect ticks from temperature fluctuation and freezing temperatures (Medlock et al., 2013). High ground humidity after snow melting and the increasing temperature may explain the relatively high numbers of ticks and prevalence of infested sites we observed above 1400 m in April-May, then decreasing with drier climate in the following months.

Other factors such as habitat characteristics and vertebrate host abundance may limit tick establishment at high altitudes. In fact, dry open pastures are predominant as altitude increases, and suitable areas for *I. ricinus* are reduced to patches of woods and ecotones that serve as source of food and refuge for wildlife (Mazurkiewicz and Rajska-Jurgiel, 1987).

The synchronous activity between nymphs and larvae during summer may be relevant for TBP, favouring co-feeding transmission on the same host and increasing the infection prevalence among the ticks. For instance, the co-occurrence of nymphs and larvae feeding on the same rodent-host may favour the horizontal transmission of TBE virus from infected nymphs to uninfected larvae (Cagnacci et al., 2012). In this study, we did not investigate TBEV presence in ticks, since the virus is endemic in northeastern Italy only. However, future studies should target TBEV, since it might appear in the Western Italian Alps following its recent establishment in bordering areas of Switzerland (Casati Pagani et al., 2019).

We observed the presence of several TBP. We detected for the first time *B. miyamotoi*, that was simultaneously identified in the Eastern Alps (Ravagnan et al., 2018), and *Neoehrlichia mikurensis*. These bacteria were found at low prevalence, like *A. phagocytophilum* ecotype I, and their importance for public health has to be assessed. On the other hand, *B. burgdorferi* s.l. and SFG rickettsiae were widespread in our study area. We did not detect either positive or negative association between the pathogens detected in *I. ricinus*, as it has been reported in previous studies (Moutailler et al., 2016; Raulf et al., 2018).

The infection by different *B. burgdorferi* genospecies denotes the relevance of vertebrate communities as reservoir hosts across different habitats (Takumi et al., 2019). The finding of *B. afzelii* as the dominant genospecies suggests that small rodents play an important role as hosts for immature *I. ricinus* in the study area (Gern et al., 1994). Other vertebrates, such as birds, may also act as hosts for tick immature stages. We detected a low prevalence of bird-associated *B. burgdorferi* s.l. genospecies in *I. ricinus*, such as *B. garinii* and *B. valaisiana*. Birds can transmit other tick-borne pathogens, like *R. helvetica* (Heylen et al., 2016), which might explain the concurrent infections by *B. garinii* and *R. helvetica* observed in nymphs. On the other hand, the role of rodents in the transmission cycle of *R. helvetica* is still uncertain, although the bacterium was detected in their blood and in feeding ticks (Sprong et al., 2009; Obiegala et al., 2016). Thus, coinfections by *B. afzelii* and *R. helvetica* could be due to different mechanisms of transmission: while *B. afzelii* infection probably took place from rodents, *R. helvetica* infection may be due to transovarial transmission or co-feeding.

Borrelia burgdorferi s.l. infection prevalence is in line with other studies in the Western Alps, and was higher in sites with higher tick densities as previously observed (Jouda et al., 2004; Burri et al., 2007; Pintore et al., 2014). Accordingly, we detected an aggregation of infected study sites in the altitudinal belt 950-1400 m (Fig. 3A), mainly deciduous woods characterized by milder temperatures.

A lower tick burden characterized coniferous woods and open areas. Nevertheless, the latter are more frequented by the public, since they include sites equipped with tables and seats and walking paths, which are often visited by tourists. Interestingly, the infection rate by SFG rickettsiae was higher than that of *B. burgdorferi* s.l. in deciduous and coniferous woods, but it was the opposite in open areas. This could be due to the fact that open areas are characterized by increased ecotone density, which favours the presence and abundance of small vertebrates, acting as reservoir hosts of *B. burgdorferi* s.l. This hypothesis, however, must be further investigated, as well as the probability of exposure of people to ticks in different habitats, in order to assess disease risk (Millet et al., 2019).

We observed a lower aggregation of SFG rickettsiae infected sites (Fig. 3B), and these pathogens were significantly more abundant over 1400 m. This finding is surprising; previous studies, indeed, indicated no difference in SFG rickettsiae infection prevalence among different habitats (Rosà et al., 2018). Transovarial transmission of *Rickettsia* spp. should indeed ensure its maintenance in tick populations, regardless the presence of vertebrate hosts (Tomassone et al., 2018). Therefore, we would have expected SFG rickettsiae prevalence not to vary across altitudes. Alternatively, we would have expected a higher prevalence at lower altitudes as in the case of *B. burgdorferi* s.l., considering the higher tick abundance and the possible involvement of vertebrates as reservoir hosts in rickettsiae cycle. For example, Rosà et al. (2018) detected a higher *Rickettsia* spp. prevalence in areas with higher tick densities, as for *B. burgdorferi* s.l. Future studies should investigate more in depth the relative frequency of rickettsiae according to altitude, vegetation and vertebrate community composition.

The other tick species collected, *D. marginatus*, was found in localized open-exposed areas, distant from one another and differing in altitude. The more focal distribution of *Dermacentor* spp. compared with *I. ricinus* (Rubel et al., 2016), and the low suitability of dragging for the collection of adult ticks, may explain

the low number of *D. marginatus* collected. However, one third of the specimens was infected by *R. slovaca*, the main causative agent of tick-borne lymphadenopathy/*Dermacentor*-borne necrosis-erythema-lymphadenopathy (TIBOLA/DEBONEL) in humans (Ibarra et al., 2006). Although the encounter of *D. marginatus* is less likely compared to *I. ricinus*, this tick species was associated with outbreaks of TIBOLA/DEBONEL in areas highly populated by wildlife, wild boar in particular (Selmi et al., 2008).

As regards feeding ticks, red deer and roe deer were the species most frequently and intensively infested. These results confirm the great contribution of deer to the maintenance of tick populations (Mihalca and Sándor, 2013), also in our study area. Roe deer seemed to particularly affect questing nymph abundance, even though it shares the same habitat with red deer and chamois. This may rely on interspecific differences regarding land use and dietary habits; roe deer, indeed, preferentially browse in dense woodlands, while red deer and chamois also graze in open pastures (Hoffmann, 1989; Bertolino et al., 2008). We did not record high tick burdens on ungulates. This may be due to the visual inspection technique, that was performed in a limited amount of time and has limits in the detection of immature stages, due to their small size. This may explain the higher number of adult ticks collected compared to immatures. Moreover, carcasses were often brought to the hunting station a few hours after death, which might have entailed tick falling during the transportation; carcasses of large animals were generally not put in plastic bags so it was not possible to collect detached ticks. Thus, we have probably lost many feeding ticks. Previous studies in the Alps indeed reported higher tick burdens on deer; for example, Carpi et al. (2008), by inspecting the lower part of roe deer forelegs – that were immediately collected and stored after animal death, reported an average of about 36.0 larvae and 4.8 nymphs per individual. Accordingly, Vor et al. (2010) reported high nymph infestation rates in Germany, through visual inspection of the head and neck regions of roe deer. However, also these studies may have underestimated tick numbers, since selected body parts of the animals were inspected. In other researches, where ticks were allowed to fall down from hides and legs of carcasses suspended over water, up to around 500 larvae, 100 nymphs and 60 adults were collected from a single roe deer (Jaenson and Tälleklint, 1992). Notwithstanding, our results (35.3% of red deer and 39.1% of roe deer infested) highlight the spread of ticks in the area in the past thirty

years. In fact, in 1989 Rossi and Meneguz visually inspected 243 red deer and 54 roe deer in our same hunting district and season, and reported just one red deer infested, by a single tick (*D. marginatus*). As observed with dragging, a higher infestation was registered in ungulates sampled at lower altitudes (below 1400 m). Data on wildlife also indicate the persistence of tick activity during winter months. Passive surveillance on wildlife was thus a valuable source of data, providing information on tick presence in periods and years in which dragging was not performed.

5. CONCLUSIONS

Ticks are nowadays widespread across the Alpine arch, also in the Western Italian side that was deemed unsuitable for *Ixodes* ticks. The abandonment of traditional land-use practices, reforestation and the increase of wild ungulate populations may have driven their expansion, together with increased temperatures. Mountain areas are markedly susceptible to climatic changes, hence global warming may be particularly relevant for tick expansion at higher altitudes.

Our research indeed demonstrates ticks and TBP spread above 1800 m a.s.l., and across different habitats. Future studies will need to investigate the risk posed by ticks according to human exposure, and their impact on public health in the area. In fact, although several pathogens infect ticks in Piedmont region, relatively few cases of disease in humans have been notified in the last decades. We believe human cases are underdiagnosed and/or underreported, due to the lack of a specific surveillance programme and the low awareness about tick-borne diseases, among health professionals too. Multidisciplinary collaboration among professionals working in environmental, human and animal health is essential for the development of effective actions and strategies for the surveillance of tick-borne diseases, and inform the public about the hazard posed by ticks, especially in recently invaded areas.

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Declaration of Competing Interest

All authors declare the absence of any financial or personal interests that could inappropriately influence the current work. The final article has been approved by all authors.

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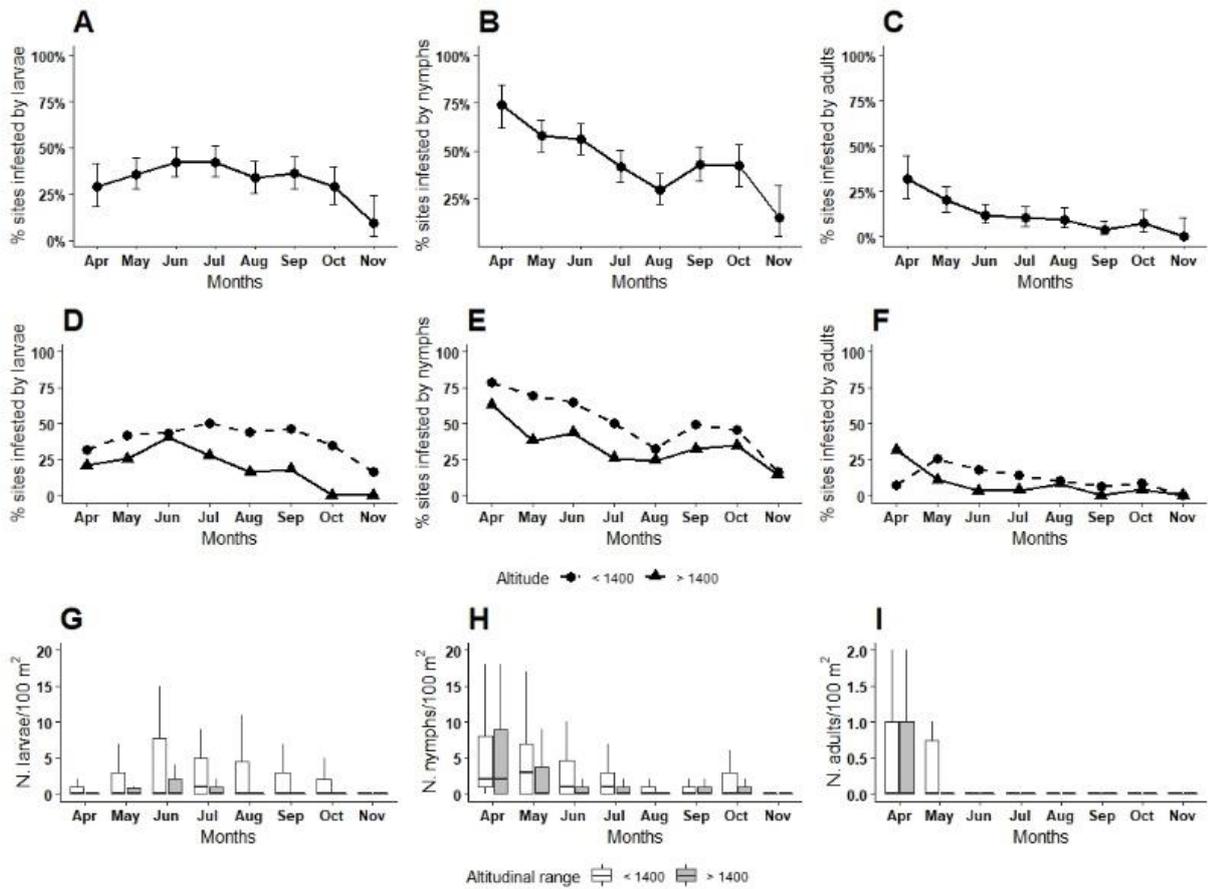


Fig. 1. Seasonal activity of *I. ricinus* life stages by month (A-C) and altitudinal range (below and above 1400 m a.s.l.; D-I) in high Susa valley, April – November 2016-2019. A-C: percentages (95%CI) of dragging sites with at least one questing specimen of *I. ricinus* life stage, by month; D-F: percentage of sampling sites infested by at least one specimen according to altitude (below and above 1400 m a.s.l.); G-I: boxplot representing the number of ticks per 100 m², by month.

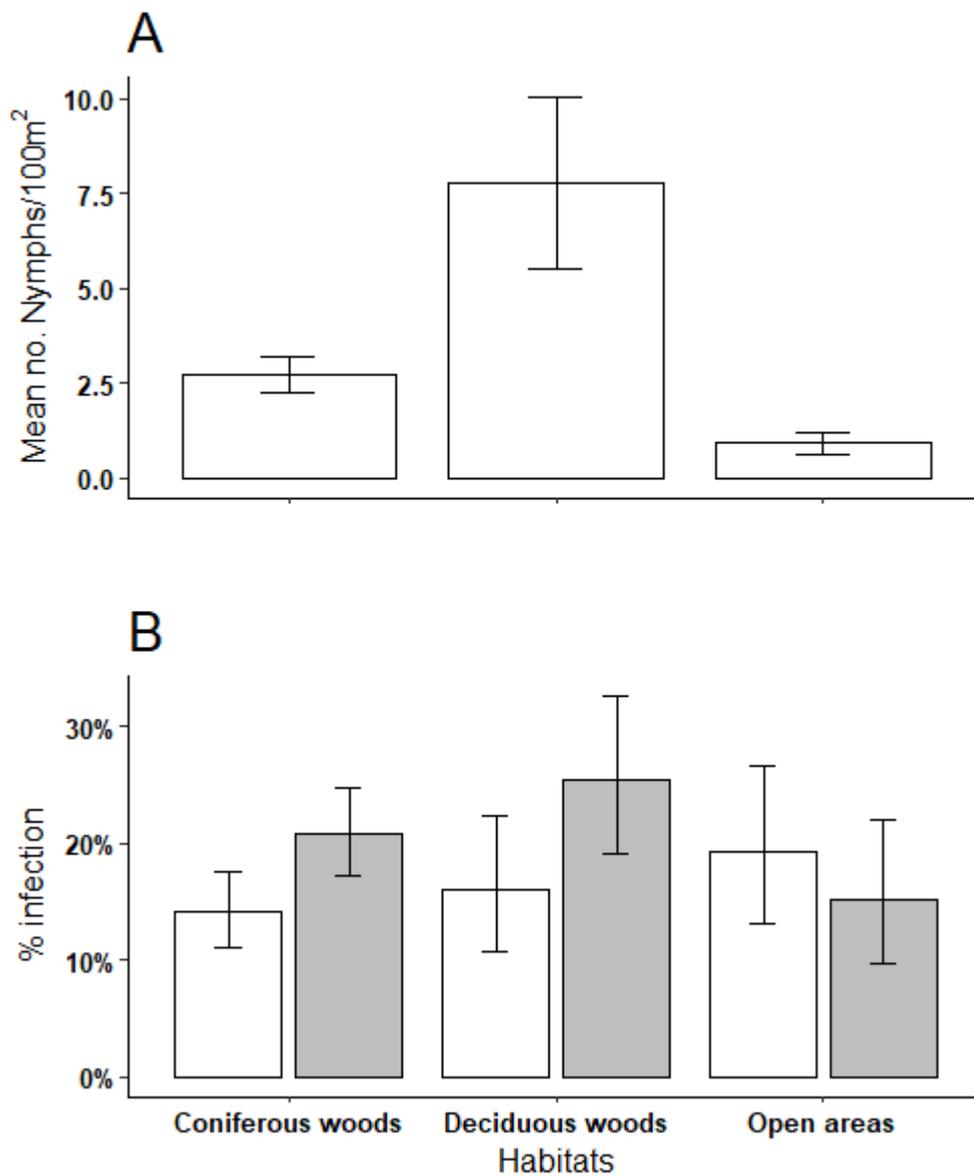


Fig.2. *Ixodes ricinus* nymphs abundance and infection prevalence per habitats: A) abundance of nymphs (mean number/ 100 m² dragging, with 95% CI); B) infection prevalence by *B. burgdorferi* s.l. (white) and *Rickettsia* spp. (grey), with 95% CI; high Susa valley, April – November 2016-2019.

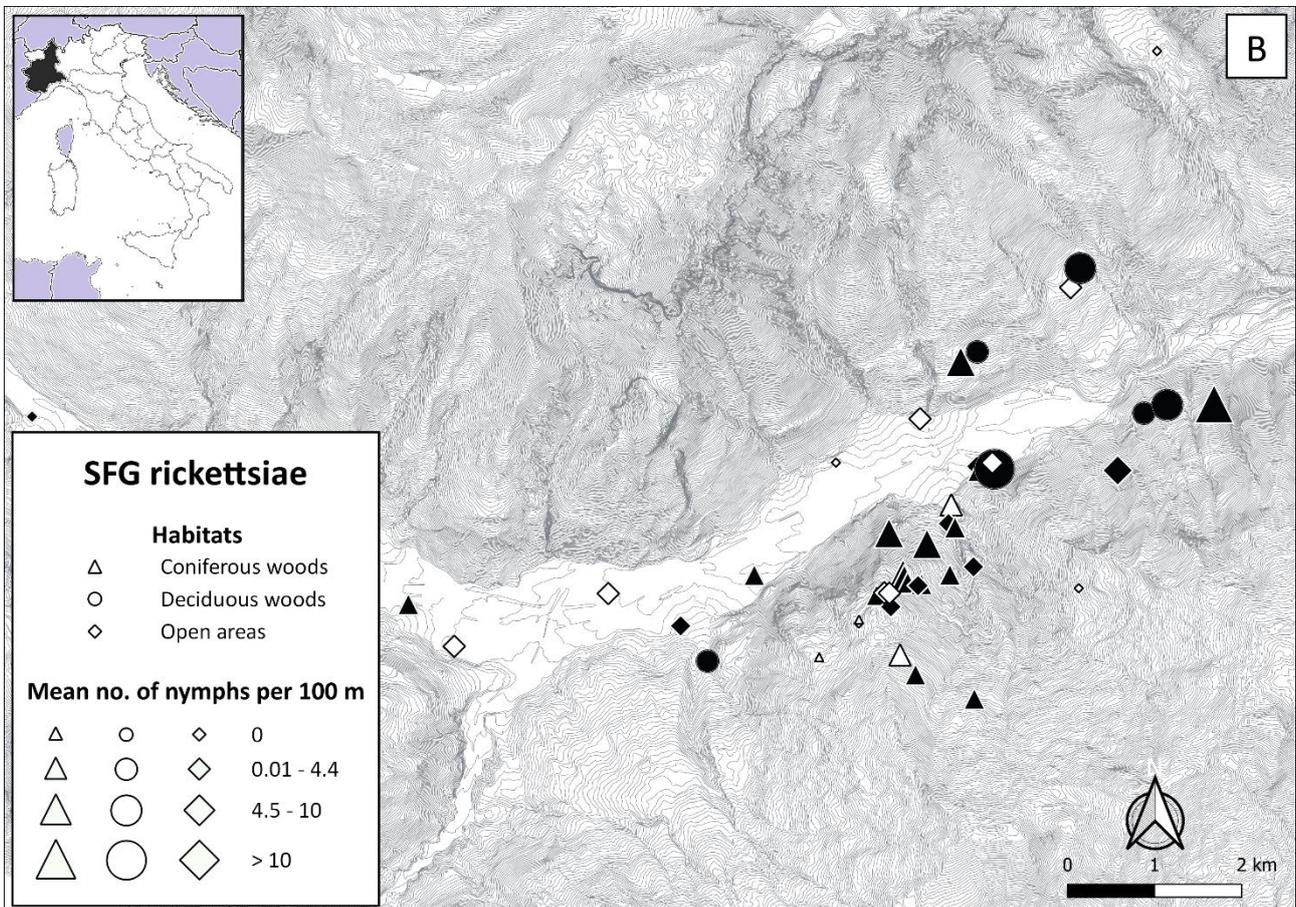
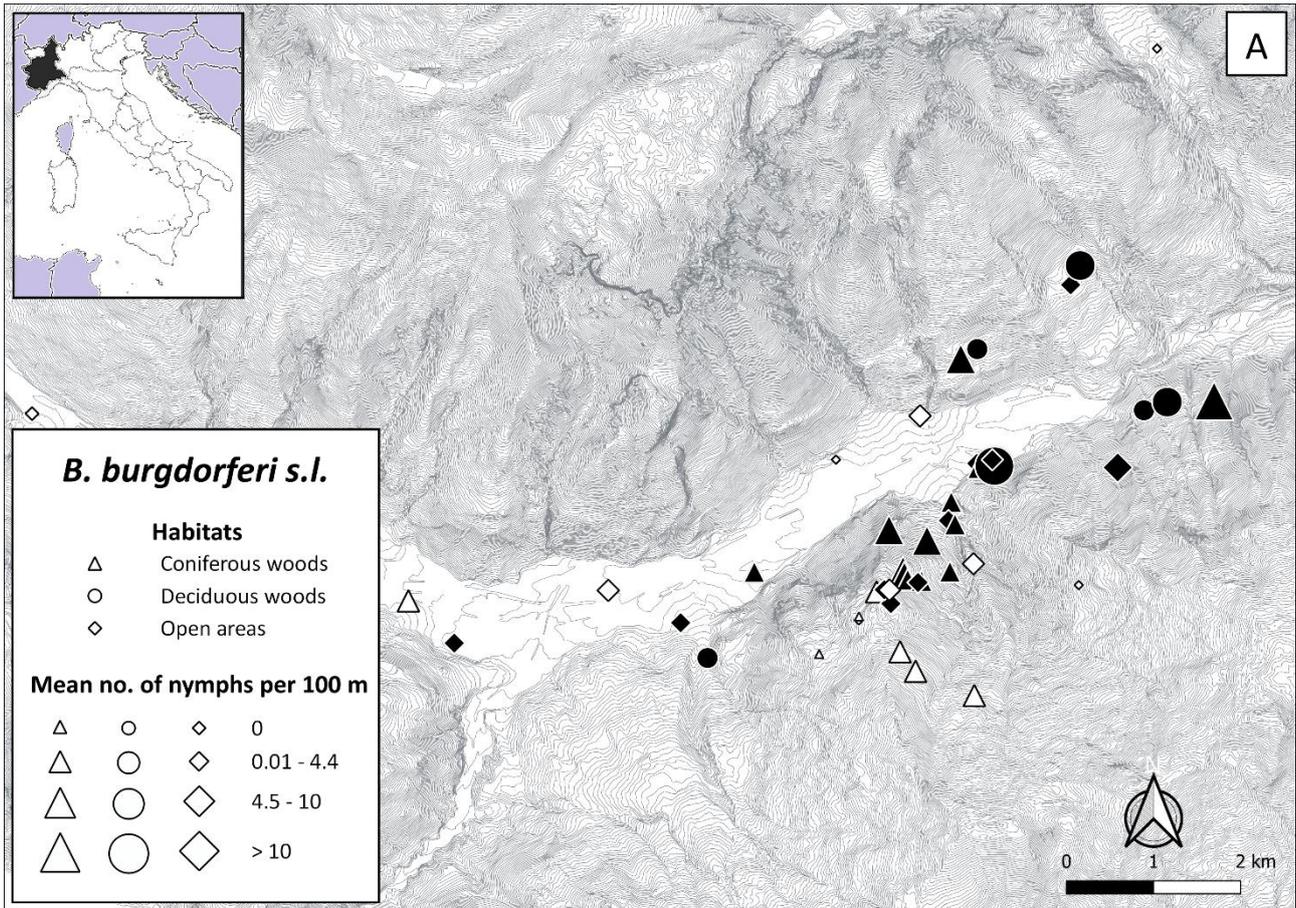


Fig. 3. Maps illustrating dragging sampling sites in high Susa valley, by habitats. The dimension of symbols indicates *I. ricinus* abundance (expressed as the average number of nymphs per 100 m²), and black colour indicates the infection by tick-borne pathogens: A) *B. burgdorferi* s.l., B) SFG rickettsiae.

Table 1. Questing tick activity (expressed as the percentage of dragging sites where at least one questing tick was collected) and abundance of questing *I. ricinus* larvae, nymphs and adults (mean number/100 m²), by different altitudinal ranges; high Susa valley, April – November 2016-2019.

Altitude range (m a.s.l.)	Dragging sites (n)	% positive sites [95% CI]	Mean no. ticks per 100 m ² [95% CI]		
			Larvae	Nymphs	Adults
950 – 1200	18	61.3 [56.5 – 66.1]	4.2 [3.0 – 5.4]	2.7 [1.9 – 3.5]	0.2 [0.12 – 0.23]
1201 – 1400	8	84.7 [77.5 – 90.2]	9.5 [5.8 – 13.2]	5.2 [3.9 – 6.4]	0.4 [0.2 – 0.6]
1401 – 1600	7	74.6 [66.7 – 81.6]	6.2 [3.8 – 8.5]	3.4 [2.6 – 4.3]	0.2 [0.08 – 0.25]
1601 – 1800	7	22.7 [14.8 – 32.3]	0.3 [0.07 – 0.5]	0.2 [0.1 – 0.3]	0.03 [0 – 0.06]
> 1800	5	5.6 [1.8 – 12.6]	0.01 [0 – 0.3]	0.04 [0.01 – 0.09]	0

Table 2. Prevalence of dragging sites infested by *Ixodes ricinus* and nymph abundance (mean number of nymphs/100 m²), according to habitats and altitudinal range (below and above 1400 m a.s.l.); high Susa valley, April – November 2016-2019.

Habitats (n= 45)	% positive sites [95%CI]	% positive sites [95%CI]		Total nymph abundance (mean per 100 m ²) [95%CI]	Nymph abundance (mean per 100 m ²) [95%CI]	
		< 1400 m a.s.l.	> 1400 m a.s.l.		< 1400 m a.s.l.	> 1400 m a.s.l.
Open areas (n= 19)	38.6 [33.2-44.1]	46.7 [39.8-53.7] *	23.7 [16.2-32.5] *	1.7 [1.2 – 2.1]	1.3 [0.9 – 1.7] ***	0.3 [0.07 – 0.6] ***
Coniferous woods (n= 20)	62.1 [57.4-66.8]	75.2 [69.3-80.5] *	46.7 [39.5-53.9] *	7.6 [6.0 – 9.2]	3.5 [2.7 – 4.3] ***	1.8 [1.3 – 2.3] ***
Deciduous woods (n= 6)	91.2 [84.5-95.7]	90.9 [83.4-95.8]	93.3 [68.0-99.8]	23.1 [17.4 – 28.7]	7.7 [5.2 – 10.3]	8.1 [3.0 – 13.2]

The numbers marked with asterisks within the same line are significantly different (*Pearson's Chi-squared test ($p < 0.05$); *** Wilcoxon rank sum test ($p < 0.001$))

Table 3. Infection prevalence by tick-borne pathogens, with 95% CI, of *I. ricinus* nymphs (n= 786), below and above 1400m a.s.l.; high Susa valley, April – November 2016-2019.

Pathogens	Total prevalence (%) [95%CI] (n= 786)	Prevalence by altitudinal range (%) [95%CI]	
		< 1400 m a.s.l. (n= 475)	>1400 m a.s.l. (n= 311)
<i>Borrelia burgdorferi</i> s.l.	15.5 [13.1 – 18.2]	18.1 [14.7 – 21.9] *	11.6 [8.2 – 15.6] *
<i>Borrelia miyamotoi</i>	0.5 [0.14 – 1.3]	0.4 [0.05 – 1.5]	0.6 [0.07 – 2.3]
<i>Anaplasma phagocytophilum</i>	1.9 [1.1 – 3.1]	2.3 [1.2 – 4.1]	1.3 [0.3 – 3.2]
<i>Neoehrlichia mikurensis</i>	0.5 [0.14 – 1.3]	0.8 [0.2 – 2.1]	0 [0 – 1.2]
<i>Rickettsia</i> spp.	20.7 [17.9 – 23.7]	15.0 [11.8 – 18.5] ***	29.6 [24.5 – 35.0] ***

The numbers marked with asterisks within the same line are significantly different (Pearson's Chi-squared test * ($p < 0.05$); *** ($p < 0.001$))

Appendix A. Supplementary data.

Examples of tick collection sites in high Susa Valley (Piedmont region). Main habitats: 1) Open areas: pastures and hiking areas surrounded by (a) coniferous forest, 959 m a.s.l., (b) mixed woods of European birch and Scots pine, 1078 m a.s.l., (c) European spruce forest, 1112 m a.s.l., and (d) larch wood, 1300 m a.s.l. and (e) 1745 m a.s.l.; 2) Deciduous woods: (f) raspberry shrubs under European ash wood canopy, 1014 m a.s.l., (g) hiking pathways in a dominant ash tree area, 1160 m a.s.l., and (h) in white willow woods, 1324 m a.s.l.; 3) Coniferous woods: (i) recreational area under Scots pines, 1.074m, (j) mixed wood of European spruce and silver fir, 1278; (k) larch wood, 1553 m a.s.l., (l) mixed forest of larch and stone pine, 1850 m a.s.l.

