Received: 23 December 2020 | Accepted: 28 July 2021

DOI: 10.1111/tbed.14268

Transboundary and Emerging Diseases WILEY

ORIGINAL ARTICLE

Ticks on wild boar in the metropolitan area of Barcelona (Spain) are infected with spotted fever group rickettsiae

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Abstract

Tick-borne pathogens (TBPs) constitute an emerging public health concern favoured by multidimensional global changes. Amongst these, increase and spread of wild boar (*Sus scrofa*) populations are of special concern since this species can act as a reservoir of zoonotic pathogens and promote tick abundance. Thus, we aimed to make a first assessment of the risk by TBPs resulting from wild boar and ticks in the vicinity of a highly populated area. Between 2014 and 2016, we collected spleen samples and 2256 ticks from 261 wild boars (out of 438 inspected) in the metropolitan area of Barcelona (MAB; northeast Spain). We morphologically identified four tick species: *Hyalomma lusitanicum* (infestation prevalence: 33.6%), *Dermacentor marginatus* (26.9%), *Rhipicephalus sanguineus* sensu lato (18.9%) and *R. bursa* (0.2%). Ticks were pooled according to species and individual host. A total of 180 tick pools and 167 spleen samples were screened by real-time PCR and/or reverse line blot hybridization assay for *Ehrlichia* sp., *Anaplasma* sp., *Babesia* sp., *Rickettsia* sp., *Borrelia burgdorferi* sensu lato and *Coxiella burnetii*. Seventy-two out of the 180 tick pools were positive to *Rickettsia* spp. (minimum prevalence of 8.7%), including *Rickettsia massiliae*, *R. slovaca* and *R. raoultii*. We did not detect *Rickettsia* spp. in wild boar spleens nor other TBPs in ticks or wild boars. Since the ticks identified can bite humans, and the recorded spotted fever group (SFG) rickettsiae are zoonotic pathogens, there is a risk of SFG rickettsiae transmission for MAB inhabitants. Our results suggest a broader distribution of *H. lusitanicum*, competent vector for the Crimean-Congo haemorrhagic fever virus than previously known. Wild boar is not a *Rickettsia* spp. reservoir according to the spleen negative results. However, its abundance could favour tick life cycle and abundance, and its proximity to humans could promote the infection risk by *Rickettsia* spp.

KEYWORDS

Hyalomma lusitanicum, *Rhipicephalus sanguineus*, *Rickettsia* sp, *Sus scrofa*, urban area

1 | INTRODUCTION

Ticks are the most important vectors of disease transmission to livestock, pets and humans (Jongejan & Uilenberg, 2004), and both the number of tick-borne pathogens (TBPs) and the incidence of tick-borne diseases are increasing globally as a result of multidimensional global changes (Colwell et al., 2011; Dantas-Torres et al., 2012). Accordingly, human tick-borne diseases are emerging and constitute a major public health concern (Doudier et al., 2010; Mansfield et al., 2009; Parola & Raoult, 2001).

Tick ecology and TBPs epidemiology are driven by environmental factors including host composition and abundance (James et al., 2013; Randolph, 2004; Ruiz-Fons et al., 2012). The greater the host density, the higher the probability of ticks finding a suitable host, completing their life cycle and multiplying (Estrada-Peña & de la Fuente, 2014; Randolph, 2004). Hence, wildlife can display a significant role in TBPs epidemiology, as they can act as reservoirs of human pathogens and increase the tick range and abundance (Dantas-Torres et al., 2012; Varela-Castro et al., 2018). Moreover, with the increasing number of human-wildlife interactions in densely populated areas, we face new epidemiological scenarios where zoonotic pathogens can spread (Bradley & Altizer, 2007; Fernández-Aguilar et al., 2018).

The risk of transmission of TBPs to humans can be assessed through the study of ticks carried by sympatric species, and the Eurasian wild boar (*Sus scrofa*) can be a good sentinel. The wild boar is commonly infested by hard ticks (Ortuño et al., 2007; Ruiz-Fons et al., 2006), its populations have increased across Europe since 1965 (Massei et al., 2015; Sáez-Royuela & Tellería, 1986) and it is in proximity to humans, as it is occupying or using urbanized areas (Castillo-Contreras et al., 2018; Licoppe et al., 2013). This is the case in the metropolitan area of Barcelona (MAB), in northeast Spain, where wild boars have grown in numbers for the last 20 years (González-Crespo et al., 2018), and they are often seen in urban areas including the city of Barcelona (Cahill et al., 2012; Castillo-Contreras et al., 2018).

Tick species commonly reported on wild boars in Spain are *Hyalomma marginatum marginatum*, *Rhipicephalus bursa* and *Dermacentor marginatus* (Ortuño et al., 2007; Ruiz-Fons et al., 2006). However, *D. reticulatus*, *R. sanguineus* sensu lato and *Ixodes ricinus* can also parasitize wild boars in northern Spain (Astobiza et al., 2011; Estrada-Peña et al., 1992). Moreover, several zoonotic TBPs such as *Ehrlichia* sp., *Anaplasma* sp., *Rickettsia* sp., *Babesia* sp., and *Borrelia burgdorferi* sensu lato have been previously detected in ticks collected from wild boar (de la Fuente et al., 2004; Estrada-Peña et al., 2005; Iori et al., 2010). Most of these and other TBPs have been also identified in wild boar tissues or sera (Astobiza et al., 2011; Faria et al., 2015; Petrovec et al., 2003; Selmi et al., 2009; Tampieri et al., 2008).

All the above raise concern regarding the risk of TBPs infection for MAB inhabitants owing to direct and indirect effects of wild boar expansion and proximity to humans. Our aim is to make a first assessment of TBPs risk and its determining factors in the MAB through two specific objectives: (1) assessing the tick diversity and abundance in wild boars from the MAB and the drivers of their spatiotemporal distribution and (2) identifying and determining the frequency of zoonotic TBPs infecting wild boars from the MAB and their ticks.

2 | MATERIAL AND METHODS

2.1 | Study area

The study area includes different locations within the MAB (Figure 1), located in Catalonia (northeastern Spain). The MAB encompasses 36 municipalities, has more than three million inhabitants and occupies 63,600 ha (Statistical Institute of Catalonia, 2019). Most wild boars come from three main locations: the Collserola Natural Park (Collserola, hereafter), the municipality of Barcelona and the campus of the Autonomous University of Barcelona (UAB). Collserola is located in the centre of the MAB, is 11,100 ha in size and has its highest point at 510 m above sea level (Parc de Collserola, 2020a). Its landscape is composed of a mixture of Mediterranean forests, scrublands, grasslands, croplands and built-up areas (Parc de Collserola, 2020b), and its wild boar population has been estimated to increase almost 10-fold (from 165 to 1500 individuals) from 2000 to 2015 (González-Crespo et al., 2018). Collserola is used by MAB inhabitants and visitors for leisure activities and receives approximately 3,000,000 visitors every year (Parc de Collserola, 2020c). The municipality of Barcelona is located southeast of Collserola, with a population of 1,600,000 inhabitants in 10,100 ha (Statistical Institute of Catalonia, 2019). Barcelona is mostly urbanized, although it comprises 2900 ha of green and forested areas (Ajuntament de Barcelona, 2018). The UAB campus is located north of Collserola, is roughly 260 ha in size and is regularly used by more than 45,000 people (Universitat Autònoma de Barcelona, 2018). It is urbanized but contains gardens, forestry and agricultural patches that cover approximately 60% of its surface (Universitat Autònoma de Barcelona, 2019a, 2019b).

2.2 | Sampling

Between 2014 and 2016, we examined 438 wild boars, either hunted or captured and euthanized, from the above-mentioned areas: Collserola ($n = 117$), Barcelona ($n = 230$), UAB ($n = 79$) and other locations within the MAB ($n = 12$). Wild boars were culled for population control or conflict management purposes. Hunted wild boars were shot by authorized local hunters during the regular hunting season, whereas euthanized wild boars were previously anaesthetized with a blowpipe by a veterinarian within the framework of the contracts 13/051, 15/0174, 16/0243 and 16/0243-00-PR/01 with the Barcelona City Council (*Ajuntament de Barcelona*).

We performed a post-mortem external and internal examination of wild boar carcasses, manually removed all the ticks feeding on each wild boar and collected spleen samples. Both ticks and spleen samples were stored in sterile 5-ml tubes (one tube per wild boar and sample type) at −20°C until further processing. We recorded wild boar age

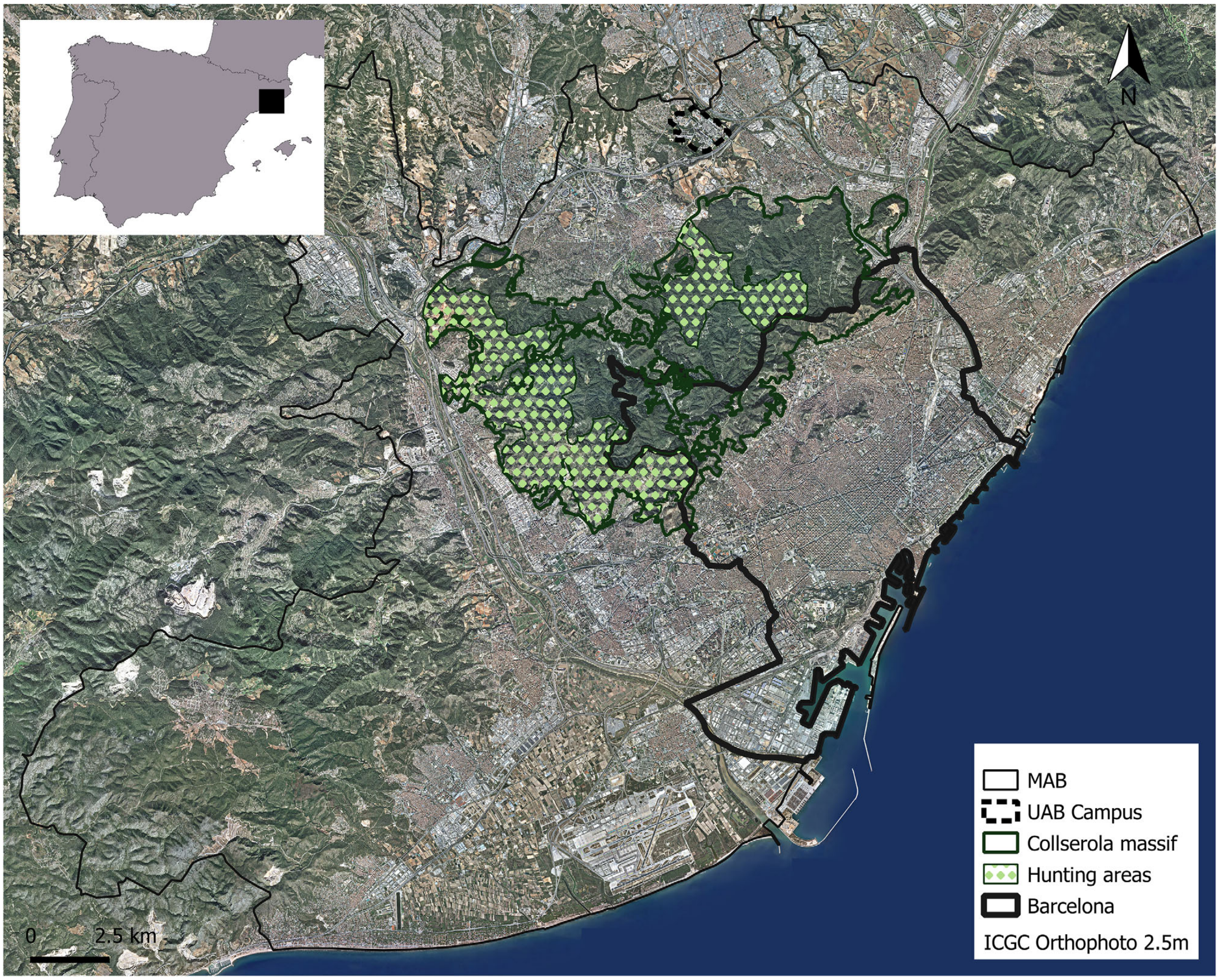

FIGURE 1 Metropolitan area of Barcelona (MAB). Top left: Location of the MAB (black square) in the Iberian Peninsula. Orthophoto from Institut Cartogràfic i Geològic de Catalunya

class, date and sampling area. We determined wild boar age using dentition patterns and wear (Boitani & Mattei, 1992) and assigned the corresponding age class: piglet (up to 6 months), juvenile (6 to 12 months), yearling (12 to 24 months) and adult (over 2 years). Northern hemisphere seasons were considered.

2.3 | Tick identification and pooling

We identified tick specimens, determined the tick life stage (adult, nymph or larva) and sex using a stereo microscope and morphological keys (Estrada-Peña et al., 2004; 2017). Ticks collected from every wild boar were sorted into smaller pools (n = 380) and stored into sterile 1.5-ml microcentrifuge tubes at −20°C until further processing. Each pool contained between 1 and 49 ticks of the same tick species and life stage.

2.4 | DNA extraction

For TBPs analyses, we selected 180 out of the 380 tick pools, which comprised 1 to 6 adult ticks (mean: 4.6 ticks/tick pool, median: 5, total sum: 827 ticks) of the same species, with no sex discrimination and belonging to 180 different wild boar hosts. The selection was made in order to obtain the representation of the four tick species found, the different locations, seasons and wild boar age classes. In the case of wild boars co-infested with more than one tick species, we selected only one tick species per host. We also analysed the 167 spleen samples available belonging to the wild boar hosts from which the selected tick pools were collected. Before DNA extraction, we processed the selected tick pools individually and washed each pool three times with sterile water and once with 70% ethanol. We air-dried the tick specimens and collected them in sterile tubes. For DNA extraction, we used the QIAamp cador Pathogen Mini Kit (Qiagen) to extract DNA from

TABLE 1 Tick-borne pathogens (TBPs) targeted

TBPs (type of assay)	Target gene	Oligonucleotide sequence of primers and probes (5′–3′)	PCR product length (bp)	Reference
Rickettsia spp. (1)	gtlA	RKND03F: GTGAATGAAAGATTACACTATTTAT RKND03R: GTATCTTAGCAATCATTCTAATAGC RKND03: 6FAM-CTATTATGCTTGCGGCTGTCGGTTC-TAMRA	165	Rolain et al. (2009)
Rickettsia (2)	16S rDNA	Rick-F1: GAACGCTATCGGTATGCTTAACACA Rick-R2: Biotin-CATCACTCACTCGGTATTGCTGGA	350–400	Lorusso et al. (2016)
Rickettsia sp. (3)	gltA	CS409d: CCTATGGCTATTATGCTTGC Rp1258n: ATTGCAAAAAGTACAGTGAACA	750	Roux et al. (1997)
Coxiella burnetii (1)	IS1111	IS1111F: GCGTCATAATGCGCCAACATA IS1111R: CGCAGCCCACCTTAAGACTG IS1111: 6FAM-TGCTCAGTATGTATCCACCG-TAMRA	200	Brouqui et al. (2005)
C. burnetii (1)	IS30a	Cbis30aF: AATGTCTGCGGGAAATAGGC Cbis30aR: GAGGCCTTTTACCGGAATTC IS30a: 6FAM-TCGAGATCATAGCGTCATT-TAMRA	120	Brouqui et al. (2005)
Borrelia burgdorferi sensu lato (1)	23S rRNA	Bb23Sf: CGAGTCTTAAAAGGGCGATTTAGT Bb23Sr: GCTTCAGCCTGGCCATAAATAG Bb23Sp: 6FAM-AGATGTGGTAGACCCGAAGCCGAGTG-TAMRA	75	Courtney et al. (2004)
Ehrlichia/Anaplasma spp. (2)	16S rDNA	16S8FE: GGAATTCAGAGTTGGATC(A/C)TGG(C/T)TCAG BGA1B-new: Biotin-CGGGATCCCGAGTTTGCCGGGACTT(C/T)TTCT	460–520	Lorusso et al. (2016)
Babesia spp. (2)	18S rDNA	RLB-F2: GACACAGGGAGGTAGTGACAAG RLB-R2: Biotin-CTAAGAATTTCACCTCTGACAGT	460–540	Lorusso et al. (2016)

Note: 1: real-time PCR; 2: reverse line blot hybridization assay; 3: conventional PCR; bp: base pairs.

ticks and spleen samples. We followed the manufacturer's instructions for tissue samples, with the pre-treatment T2. In summary, we physically disrupted the ticks using sterilized scissors and conical tissue grinders in 200 ml of sterile phosphate-buffered saline (PBS). We also mechanically disrupted and homogenized 10 mg of each of the 167 wild boar spleen samples in 200 ml of PBS. We stored the resulting DNA extracts at −20°C until further analysis.

2.5 | PCR protocols

We screened the extracted 180 tick pools and 167 wild boar spleen samples by real-time polimerase chain reaction (PCR) for *Coxiella burnetii* and *B. burgdorferi* s.l. by reverse line blot hybridization assay (RLB) for *Ehrlichia* spp., *Anaplasma* spp., and *Babesia* spp. and by both real-time PCR and RLB for *Rickettsia* spp. In the case of *Rickettsia* spp., only samples yielding a positive result in both assays (with different molecular targets, see Table 1) were considered positive. Target regions, expected length of the PCR products and oligonucleotide sequences of primers and probes are detailed in Table 1. The concentration of extracted DNA was not assessed prior to amplification.

For the molecular detection of *Rickettsia* spp. DNA by real-time PCR, we followed a protocol modified from Mediannikov et al. (2014) and used a total PCR volume of 20 μl (5 μl of extracted DNA and 15 μl of PCR mixture). The PCR mixture included 10 μl of MyTaq™ Mix (Bioline), 0.5 μl (20 pmol/μl) of forward primer RKND03F, 0.5 μl (20 pmol/μl) of reverse primer RKND03R, 2 μl (2 pmol/μl) of FAM and TAMRA-labelled probe RKND03R (Rolain et al., 2009) and 2 μl of distilled water. Amplification conditions started with a first step at 95°C for 3 min, followed by 40 cycles of denaturation at 92°C for 1 s and annealing and extension at 60°C for 35 s and one last cycle at 42°C for 30 s.

Regarding the molecular detection of *C. burnetii* through real-time PCR, we followed the protocol described in Brouqui et al. (2005). As for the molecular detection of *B. burgdorferi* s.l. through real-time PCR, we followed a protocol modified from Courtney et al. (2004).

For the three real-time PCR assays (targeting *Rickettsia* spp., *C. burnetii* and *B. burgdorferi* s.l.), we used distilled water as negative control and a laboratory-cultured *Rickettsia conorii* strain, a known *C. burnetii* strain and a known *B. burgdorferi* strain, respectively, as positive controls. We considered positive those samples with cycle threshold values lower than 35. We used a DNA Engine Opticon 2 Continuous Fluorescence Detector CFD-3220 (MJ Research).

The molecular amplification of *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp. and *Babesia* spp. DNA through RLB consisted of three different amplifications, one for *Rickettsia* spp., one for *Ehrlichia/Anaplasma* spp. and one for *Babesia* spp. The total volume of all three PCRs was 25 μl and comprised 2.5 μl of extracted DNA and 22.5 μl of PCR mix. The mix included 10 μl of MyTaq™ Red Mix (Bioline), 1 μl (20 pmol/μl) of forward primer, 1 μl (20 pmol/μl) of reverse primer and 10.5 μl of distilled water. We followed the amplification conditions described in Lorusso et al. (2016). We used distilled water as the negative control, and a laboratory-cultured *R. conorii* strain, a known *Ehrlichia ruminantium* and a known *Babesia bigemina* served as positive controls for *Rickettsia* spp., *Ehrlichia/Anaplasma* spp. and *Babesia* spp. assays, respectively. We used a Prime Elite Thermal

Cycler (Techne). A detailed RLB protocol for membrane preparation, hybridization and detection can be found in O'Sullivan et al. (2011), and further details on the specific membrane used and the oligonucleotide probes included are available in Lorusso et al. (2016). See Table 1 for target regions, expected length of the PCR products and oligonucleotide sequences of primers.

2.6 | *Rickettsia* spp. sequencing

For sequencing, we used the protocol described in Tijsse-Klasen et al. (2011) to amplify a 750-bp fragment of the *Rickettsia* spp. gltA gene, which encodes for a citrate synthase protein. We used a total volume of 20 µl including 2 µl of extracted DNA and 18 µl of PCR mix. The PCR mix included: 10 µl of MyTaq™ Red Mix (Bioline), 1 µl (10 pmol/µl) of forward primer CS409d, 1 µl (10 pmol/µl) of reverse primer Rp1258n and 6 µl of distilled water. We used distilled water as negative control and a laboratory-cultured *R. conorii* strain served as positive control. We used a Prime Elite Thermal Cycler (Techne). We purified the amplicons using the ISOLATE II PCR and Gel kit (Bioline) and measured the DNA concentration with a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Sanger sequencing was performed on the purified amplicons, in both directions, at the Servei de Genòmica i Bioinformàtica (Bellaterra, Spain), using an ABI 3130XL sequencer (Applied Biosystems) and the same primers at a concentration of 10 pmol/µl. We aligned the sequenced data in MEGA version X (Kumar et al., 2018) and identified the species by comparison with the nucleotide collection (GenBank, EMBL, DDBJ, PDB and RefSeq sequences) through NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast). We accepted a result when both the BLAST query cover and identity were equal to or above 99%. These sequence data have been submitted to the GenBank database under accession numbers MW835759 to MW835820.

2.7 | Statistical analyses

We used the R software (version 3.5.0, R Development Core Team, 2018) to perform the statistical analyses. For 95% confidence intervals, we used the binconf function from the Hmisc package (Harrel Jr., 2018).

We looked for patterns in the spatio-temporal distribution of the different tick species identified, as well as for wild boar age-related patterns, in the infested wild boar from Collserola ($n = 82$) and Barcelona ($n = 128$). We did not include in this analysis wild boars from UAB or other locations due to insufficient representation of certain seasons and wild boar age classes. The response variable was the presence or absence of each tick species on a specific wild boar, and the predictors were area (Collserola or Barcelona), sampling year (2014 to 2016), season (winter, spring, summer or autumn) and wild boar age class (piglet, juvenile, yearling or adult), and we also included interactions among them. We used generalized linear models (GLMs; McCullagh & Nelder, 1989) and model selection by means of the function dredge from the package MuMIn (Bartoń, 2018) to choose the best GLMs according to their Akaike Information Criterion value (Burnham & Anderson, 2002). We fitted the GLMs using the glm function within the stats package in R (R Core Team, 2019), with binomial family and logit link function. Regarding TBPs, we applied another GLM (binomial family, logit link function) to explore the presence of *Rickettsia* sp. in 148 tick pools (both positive and negative for *Rickettsia* sp.) from 148 wild boars; the response variable was the positive or negative result obtained from each tick pool, and the predictors were tick species, area, sampling season and wild boar age class. We did not consider tick pools from wild boars from UAB or other locations due to insufficient representation. For all GLMs, we checked that the model assumptions of binary logistic regression were met.

Moreover, to test whether there was a relationship between the tick species and the *Rickettsia* species identified, we applied a Fisher's exact test for count data with the function fisher.test (stats package; R Core Team, 2019).

3 | RESULTS

3.1 | Ticks

We collected 2256 ticks feeding on 261 out of 438 wild boars examined (59.6%). We identified four different tick species, namely *Hyalomma lusitanicum* (1156/2256, 51.2%), *R. sanguineus* s.l. (557/2256, 24.7%), *D. marginatus* (542/2256, 24%) and *R. bursa* (1/2256, 0.04%). Details on the life stage and sex of these ticks are provided in Table 2. At the host level, each infested wild boar carried on average 8.6 ticks, with a median of 5, ranging from 1 to 70 ticks per wild boar. The species parasitizing most wild boars was *H. lusitanicum* (infestation prevalence of 33.6%, 95% confidence interval (CI): 29.3%–38.1%), followed by *D. marginatus* (26.9%, 95% CI: 23%–31.3%) and *R. sanguineus* s.l. (18.9%, 95% CI: 16.4%–23.9%), while *R. bursa* was found on one wild boar (0.2%, 95% CI: 0.01%–1.3%). Tick prevalence per area can be found in Table 3. Regarding co-infestation, most of the infested wild boars carried one tick species only (173 out of 261 infested wild boar; 56.3%). Two tick-species infestations (84/261, 32.18%) mainly involved *H. lusitanicum* and *D. marginatus* ticks (38/261, 14,6%) or *H. lusitanicum* and *R. sanguineus* s.l. ticks (37/261, 14.2%). Only four wild boars carried three tick species (*H. lusitanicum*, *D. marginatus*, *R. sanguineus* s.l.) at the same time (4/261, 1.5%).

With regards to the spatio-temporal distribution of ticks collected from wild boars, two GLMs (GLM-h1 and h2) were selected to explain the presence of *H. lusitanicum* on the infested wild boars. This tick species were found all year round, but primarily from April to October, showing a seasonal pattern with a maximum in summer and a minimum in winter (GLM-h1: spring versus autumn, $Z = 2.64$, $p < .05$; summer versus autumn, $Z = 3.80$, $p < .001$; winter versus autumn: $Z = -2.08$, $p < .05$; GLM-h2: spring versus autumn, $Z = 2.92$, $p < .05$; summer versus autumn, $Z = 3.98$, $p < .001$). As for the age-related patterns, the presence of *H. lusitanicum* significantly increased with wild boar age (GLM-h1: piglets versus adults: $Z = -2.74$, $p < .05$; GLM-h2: piglets versus adults: $Z = -2.80$, $p < .05$; juveniles versus adults:

TABLE 2 Ticks collected from wild boar of the Metropolitan Area of Barcelona (MAB): Distribution of the specimens collected by species, life stage and sex (the latter only for adult ticks), and the percentage in relation to the total amount of collected ticks

	Adults				
Tick species	Females	Males	Total	Nymphs	Total (adults + nymphs)
Hyalomma lusitanicum	265 (32.40%)	797 (59.61%)	1062 (49.28%)	94 (93.07%)	1156 (51.24%)
Rhipicephalus sanguineus sensu lato	305 (37.29%)	245 (18.32%)	550 (25.52%)	7 (6.93%)	557 (24.69%)
Dermacentor marginatus	248 (30.32%)	294 (21.99%)	542 (25.15%)	0 (0.00%)	542 (24.02%)
R. bursa	0 (0.00%)	1 (0.07%)	1 (0.05%)	0 (0.00%)	1 (0.04%)
Total	818 (36.26%)	1337 (59.26%)	2155 (95.52%)	101 (4.48%)	2256 (100%)

TABLE 3 Infested wild boars per tick species and sampling area in the MAB

	Infested wild boars/examined wild boars; infestation prevalence (95% confidence interval)			
	Barcelona	Collserola	University of Barcelona (UAB)	Total*
H. lusitanicum	98/230; 42.61% (36.39%–49.07%)	38/117; 32.48% (24.67%–41.40%)	5/79; 6.33% (2.73%–13.97%)	147/438; 33.6% (29.3%–38.1%)
D. marginatus	28/230; 12.17% (8.56%–17.03%)	74/117; 63.25% (54.22%–71.43%)	13/79; 16.46% (9.88%–26.15%)	118/438; 26.9% (23%–31.3%)
R. sanguineus sensu lato	48/230; 20.87% (16.12%–26.58%)	6/117; 5.13% (2.37%–10.74%)	29/79; 36.71 (26.93%–47.72%)	87/438; 18.9% (16.4%–23.9%)
R. bursa	1/230; 0.43% (0.00%–2.42%)	0/117; 0.00% (0.00%–3.18%)	0/79; 0.00% (0.00%–4.64%)	1/438; 0.2% (0.01 – 1.3%)
Total*	128/230; 55.65% (49.19%–61.93%)	87/117; 74.36% (65.76%–81.41%)	38/79; 48.10% (37.43%–58.95%)	261/438; 59.39% (54.66%–63.95%)

*12 wild boars from locations within the MAB but other than Barcelona, Collserola or UAB are included in the total count.

$Z = -2.01$, $p < .05$). No significant differences were found between areas ($Z = -1.59$, $p > .05$). Year and sex variables were not retained in the selected models or had non-significant effects on the response variable. These models explained 29.9% (GLM-h1) and 30.3% (GLM-h2) of the data variance.

Four GLMs (GLM-d1 to d4) were selected to explain the spatio-temporal distribution of *D. marginatus*. This tick was more frequently found on the infested wild boars from Collserola than from Barcelona (GLM-d3: $Z = 2.83$, $p < .05$; GLM-d4: $Z = 3.20$, $p < .05$), considering the shared sampling period in both areas (autumn and winter). Regarding seasonality, *D. marginatus* was significantly more frequent during autumn-winter than during spring-summer (GLM-d1: spring versus autumn: $Z = -4.79$, $p < .001$; summer versus autumn: $Z = -4.82$, $p < .001$; winter versus autumn: $Z = -2.74$, $p < .05$; Z and p statistics from GLM-d2, GLM-d3 and GLM-d4 are not shown, but their results agree with those from GLM-d1). Year and sex variables were not retained in the selected models or had non-significant effects on the response variable. These models explained between 57.8% and 58.9% of the data variance.

As for *R. sanguineus* s.l., two GLMs (GLM-r1 and r2) were selected to explain its distribution in the infested wild boars. *R. sanguineus* s.l. presence decreased with wild boar age (GLM-r2: juveniles versus adults: $Z = 1.96$, $p < .05$). In spite of a lower number of *R. sanguineus* s.l. ticks collected on wild boars from Collserola, compared to those from Barcelona, no significant differences were found among areas (GLM-r1 and GLM-r2: $Z = 0.01$, $p > .05$). *Rhipicephalus sanguineus* s.l. ticks were collected primarily from February to June, but no seasonal pattern was statistically demonstrated (GLM-r1 and GLM-r2: spring versus autumn: $Z = 0.01$, $p > 0.05$; summer versus autumn: $Z = 0.01$, $p > .05$; winter versus autumn: $Z = 0.01$, $p > .05$). Year and sex variables were not retained in the selected models or had non-significant effects on the response variable. These models explained 57.9% (GLM-r1) and 57.6% (GLM-r2) of the data variance.

3.2 | TBPs

We found 72 out of the 180 tick pools (40%) to be positive for *Rickettsia* spp., which yields an overall minimum prevalence of 8.7% (95% CI: 7–10.8). The minimum prevalence per tick species was 14.7% (95% CI: 10.5–20.2) for *R. sanguineus*, 12.2% (95% CI: 9.1–16.1) for *D. marginatus* and 0.7% (95% CI: 0.2–2.5) for *H. lusitanicum* (Table 4). Since we selected one tick pool per host, the number of wild boars with positive tick pools was 72 (72/180; 40%; 95% CI: 33.1–47.3). There were significant differences in the *Rickettsia* spp. detection among tick species (Figure 2). *Rickettsia* spp. was detected significantly more often in

TABLE 4 *Rickettsia*-positive tick pools, minimum prevalence and *Rickettsia* species identified

Tick species	Positive tick pools (%)	Minimum number of positive ticks (minimum prevalence; 95% CI)	*Rickettsia* species identified (number of positive tick pools)
D. marginatus	40/74 (54.1)	40/329 (12.2; 9.1–16.1)	*Rickettsia slovaca* (24); *R. raoultii* (9); *Rickettsia* sp. (7)
R. sanguineus sensu lato	30/43 (69.8)	30/204 (14.7; 10.5–20.2)	*R. massiliae* (28); *Rickettsia* sp. (2)
H. lusitanicum	2/62 (3.2)	2/293 (0.7; 0.2–2.5)	*R. slovaca* (1); *Rickettsia* sp. (1)
R. bursa	0/1 (0)	–	–
Total	72/180 (40)	72/827 (8.7; 6.97–10.82)	*R. massiliae* (28); *R. slovaca* (25); *R. raoultii* (9); *Rickettsia* sp. (10)

R. sanguineus s.l. tick pools (*D. marginatus* versus *R. sanguineus* s.l.: $Z = 2.44$, $p < .05$; *R. sanguineus* s.l. versus *H. lusitanicum*: $Z = -3.30$, $p < .001$) and less often in *H. lusitanicum* tick pools (*D. marginatus* versus *H. lusitanicum:* $Z = -2.23$, $p < .05$), according to the selected GLM. The explained data variance was 38.2%, and we did not find differences in the overall *Rickettsia* sp. positivity between areas ($Z = -0.36$, $p > .05$). This GLM included the variables tick species, area and a non-significant interaction between both but did not retain sampling season or wild boar age class.

Sixty-two of the 72 *Rickettsia*-positive pools could be sequenced, revealing three different *Rickettsia* species: *R. massiliae* (28 out of 62 sequenced pools, 45.2%), *R. slovaca* (25/62, 40.3%) and *R. raoultii* (9/62, 14.5%; Table 4). Tick species was significantly associated with the *Rickettsia* species identified (Fisher's test, $p < .001$). *Rickettsia massiliae* was only detected in *R. sanguineus* s.l. tick pools; *R. slovaca* was detected in *D. marginatus* and the only *H. lusitanicum* pool that could be sequenced; and *R. raoultii* was only identified in *D. marginatus* (Table 4).

We did not find *Rickettsia* spp. DNA in wild boar spleens (0/167; 0%). As for the other TBPs analysed, we did not detect *C. burnetii*, *B. burgdorferi* s.l., *Ehrlichia* sp., *Anaplasma* sp., or *Babesia* sp. either in the tick pools (0/180; 0%) or the wild boar spleen samples (0/167; 0%) analysed.

4 | DISCUSSION

4.1 | Ticks

The prevalence of tick infestation on wild boars in this study, close to 60%, is among the highest previously found on Spanish wild boars, which vary from 9% to 70% depending on the region (Ortuño et al., 2007; Ruiz-Fons et al., 2006). The tick species identified here are commonly found in areas with a Mediterranean climate, and there are several domestic animals among their hosts (Estrada-Peña et al., 2004). Nevertheless, *H. lusitanicum* had never been described in northeastern Spain, which suggests a broader distribution than previously known (Barandika et al., 2011; ECDC & EFSA, 2021). The four tick species have been previously collected from wild boars in Spain (de la Fuente et al., 2004; Márquez, 2009; Ruiz-Fons et al., 2006) but, to our knowledge, only *D. marginatus* had been reported on wild boars from northeastern Spain (Ortuño et al., 2006, 2007). The anecdotal observation of one *R. bursa*, which is common in livestock from Mediterranean areas (Estrada-Peña et al., 2004), could be related to the marginal presence of free-ranging livestock in our study area (Parc de Collserola, 2020d).

Regarding the spatio-temporal distribution of the different ticks collected from wild boars, the intra-annual variation of *H. lusitanicum* infestation is probably due to its questing behaviour, as adults reach a peak in their questing activity in May-July and again in October-November (Estrada-Peña et al., 2004; Requena-García et al., 2017; Valcárcel et al., 2015). The preferred host size of this tick, for example, large and medium-sized domestic and wild ungulates (Apanaskevich et al., 2008), is possibly the reason of the increasing presence as wild boar grow older. It is necessary to stress the large spread of *H. lusitanicum* in Spain in the last decades, its distribution was thought to be restricted to central and south-western Spain and Portugal (Barandika et al., 2011; Estrada-Peña et al., 1992; Ruiz-Fons et al., 2006) and has now colonized an area more than 1000-km away. Since this species is not transported by birds, we can only ascribe its spread to terrestrial vertebrates. The finding in this tick of the Crimean-Congo haemorrhagic fever virus (CCHFV; Estrada-Peña et al., 2012; Moraga-Fernández et al., 2020), an often-fatal zoonotic TBP, makes this increase in its distribution range more concerning. *Dermacentor marginatus* ticks usually prefer areas with dense bushes and tree cover (Estrada-Peña et al., 2004), which could explain why the wild boars from Collserola were more parasitized by this tick than those from Barcelona. Moreover, the observed seasonal pattern for *D. marginatus* agrees with the period of activity of this tick, as adults are active at the end of autumn and throughout winter (Estrada-Peña et al., 2004; Rubel et al., 2016). The presence of *R. sanguineus* s.l., a species with specificity for dogs, was related to wild boar age class, apparently selecting younger wild boars. Occasional hosts of *R. sanguineus* s.l. can develop an efficient protective response against this tick (Ferreira et al., 2003), and thus older wild boars might be able to develop an immune response upon repeated infestations. *Rhipicephalus* ticks also attach more superficially than other ticks due to their short hypostome (Dantas-Torres et al., 2012), so the thinner and softer skin of younger wild boar may make them a better target; conversely, adult wild boar may result more unapproachable to this tick due to their thicker skin and/or more efficient grooming behaviour (Mooring et al., 2004; Welch et al., 1991). Lacking area- and seasonal-related differences could be due to sampling limitations such as scarce sampling in Collserola outside the

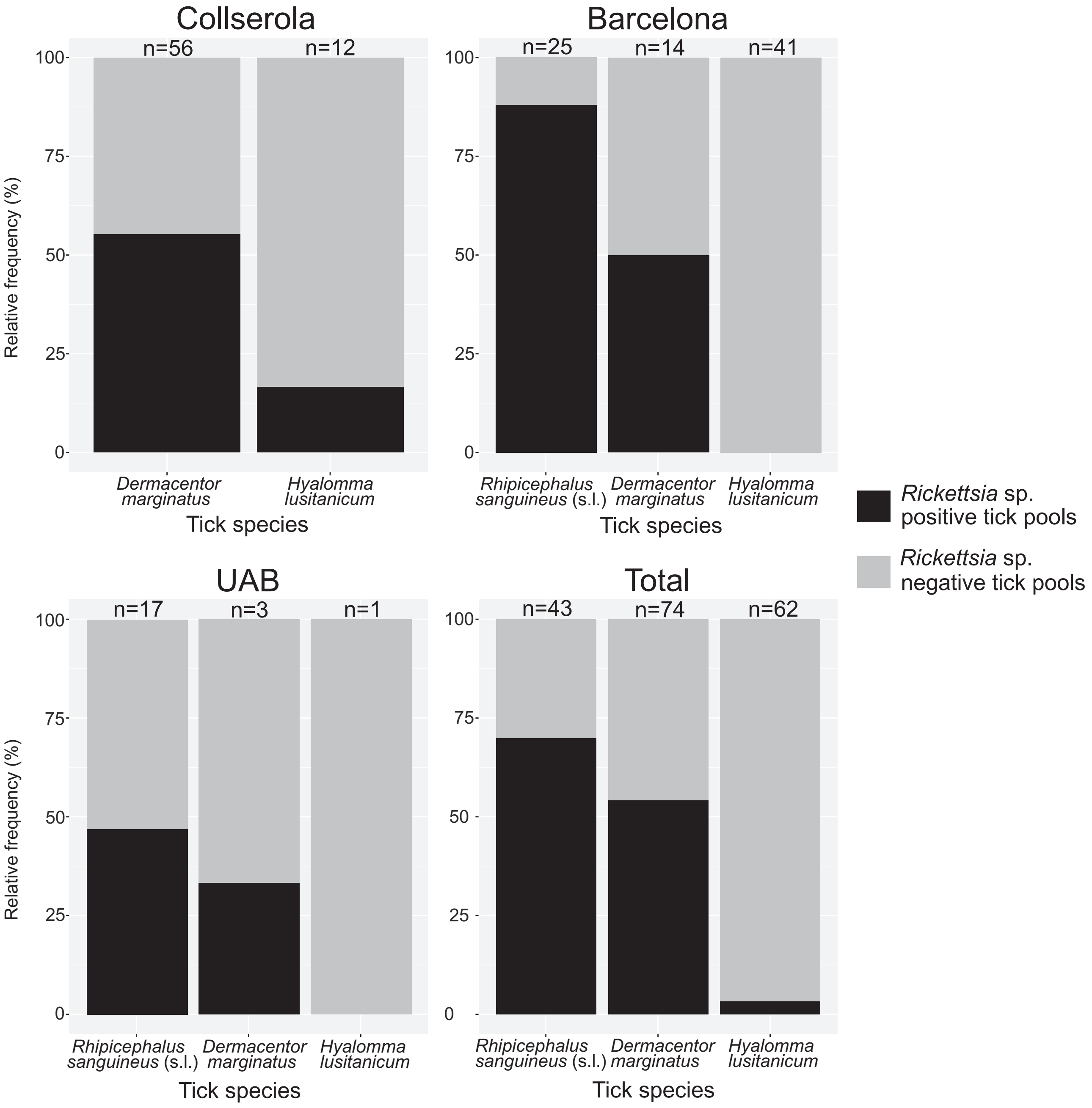

FIGURE 2 *Rickettsia* spp. positive (black) and negative (grey) tick pools per tick species and sampling area. UAB: Campus of the Autonomous University of Barcelona. The "total" count includes 10 additional pools whose wild boar hosts were sampled in areas within the MAB other than Collserola, Barcelona and UAB

hunting period or in Barcelona during the cold seasons. In addition, it would be worth addressing the dog population in both areas.

4.2 | TBPs

The identification of three *Rickettsia* species (*R. massiliae*, *R. slovaca* and *R. raoultii*) belonging to the presumable emerging zoonotic spotted fever group rickettsiae (SFG) represents a public health concern (Brouqui et al., 2007; Oteo & Portillo, 2012). Both *R. slovaca* and *R. raoultii* cause tick-borne lymphadenopathy, also known as *Dermacentor*-borne necrosis erythema and lymphadenopathy (Parola et al., 2009; Raoult et al., 1997), the most prevalent tick-borne rickettsiosis in Europe after *R. conorii*-caused Mediterranean spotted fever (Oteo & Portillo, 2012). *Rickettsia massiliae* infection, although less common, has also been described as a cause of disease in humans since its first description (Eldin et al., 2018; Vitale et al., 2006).

Moreover, the most abundant tick species identified in our study are vectors of several zoonotic pathogens and are known to bite humans. *Hyalomma* ticks, for instance, are vectors of several viruses, including the above-mentioned CCHFV (Estrada-Peña et al., 2012). *Rhipicephalus sanguineus* is the main vector of *R. conorii* and can also transmit other *Rickettsia* species such as *R. raoultii* (Estrada-Peña & Jongejan, 1999; Olivieri et al., 2018). *Dermacentor marginatus* is the main vector of *R. slovaca*, in accordance with our results, and the tick most commonly found feeding on humans in the Palearctic region (Estrada-Peña & Jongejan, 1999). In fact, previous studies show that humans in northern Spain are exposed to this tick, to *R. slovaca* and to *R. raoultii* (Antón et al., 2008; Lledó et al., 2014; Merino et al., 2005). Altogether, it suggests that there is a palpable risk of *Rickettsia* spp. exposure and potential for other TBPs exposure to people living in and visiting the MAB. In fact, 99 people attending a health care centre in the MAB between 2012 and 2017 were diagnosed with rickettsiosis, 13 of which required hospital care (AQuAS, 2018).

Regarding the *Rickettsia* spp. prevalence, the value obtained for *R. sanguineus* s.l. (nearly 15%) falls within the range previously described (2% to 25%; Chisu et al., 2014; Pereira et al., 2018; Toledo et al., 2009). Conversely, our *D. marginatus* ticks (12%) displayed a lower prevalence than the values previously reported (34%—65%; Márquez, 2009; Ortuño et al., 2007; Selmi et al., 2009). As for *H. lusitanicum*, the observed prevalence (less than 1%) agrees with the 0%–2% range previously reported and further indicates that *H. lusitanicum* is a less competent vector of *Rickettsia* spp. (Pereira et al., 2018; Toledo et al., 2009). In any case, our estimations are minimum prevalences, assuming that each of the *Rickettsia*-positive tick pools just contained one positive tick, and hence the actual prevalences might be higher.

The significant association observed between the *Rickettsia* species identified and the hosting tick species agrees with previous studies. *Rickettsia massiliae* has been detected in *R. sanguineus* s.l. ticks collected from wild boar (Chisu et al., 2014; Leulmi et al., 2016), whereas *R. slovaca* and *R. raoultii* have both been identified in *D. marginatus* ticks, also from wild boar (Leulmi et al., 2016; Márquez, 2009; Pereira et al., 2018; Sgroi et al., 2020). To the best of our knowledge, this is the first time that *R. slovaca* is reported in *H. lusitanicum* ticks. Nonetheless, the detection of DNA of a certain pathogen in ticks does not demonstrate their role in pathogen transmission, so the vector competence of *H. lusitanicum* for *R. slovaca* needs to be confirmed.

In contrast to our negative results, *Ehrlichia*, *Anaplasma*, *Babesia* and *B. burgdorferi* s.l. species have been previously detected in ticks collected from wild boar, either in Spain (de la Fuente et al., 2004; Estrada-Peña et al., 2005) or other countries such as the Czech Republic, Italy or Germany (Honig et al., 2017; Iori et al., 2010; Silaghi et al., 2014). Conversely, our negative results for *C. burnetii* agree with previous studies in wild boar ticks (Astobiza et al., 2011; Sgroi et al., 2020). Regarding the detection of TBPs in wild boar tissues, *Anaplasma*, *Rickettsia* or *Babesia* species have not been reported in tissues from wild boars in Spain, and *B. burgdorferi* s.l. and *Ehrlichia* species have not been reported yet in wild boar tissues (Kazimírová et al., 2018; Pereira et al., 2016; Silaghi et al., 2014), which agrees with our results. However, *Anaplasma phagocytophilum* has been reported in wild boars from northeastern European countries (Kazimírová et al., 2018; Petrovec et al., 2003; Silaghi et al., 2014); and different species of *Rickettsia* and *Babesia* have been detected in wild boars from Italy or Algeria (Selmi et al., 2009; Tampieri et al., 2008; Zanet et al., 2014; Zeroual et al., 2018). Last, *C. burnetii* has been previously found in wild boar tissues only in endemic areas of Spain (Astobiza et al., 2011; Jado et al., 2012). The negative results obtained from wild boar tissues prevent us from concluding a reservoir role of this species for *Rickettsia* spp. in our study area, despite the detection of antibodies against SFG *Rickettsia* in wild boars from central and northeastern Spain (Fernández de Mera et al., 2013; Ortuño et al., 2007). Altogether, it might indicate the ability of wild boar to control *Rickettsia* infections, being difficult to molecularly detect the pathogen and systemic infections through cross-sectional studies. This ability has been previously suggested for *A. phagocytophilum* in wild boars (de la Fuente & Gortázar, 2012). However, the negative results obtained in our study should be interpreted with caution since the pathogen DNA integrity was not assessed prior to amplification and thus their prevalence might be higher.

Since *Rickettsia* spp. can be transmitted trans-stadially and trans-ovarially, infected ticks could have acquired *Rickettsia* spp. while feeding on a previous infected host during immature stages or congenitally (Azad & Beard, 1998). Similarly, *Rhipicephalus* ticks collected from carnivores in a study developed in our study area were infected with *Rickettsia* spp. but their carnivore hosts were not, suggesting that the infection occurred when feeding on other hosts as immature ticks (Millán et al., 2016). Also, some of the *Rickettsia*-positive ticks in our study could have been infected via co-feeding, as this way of transmission has already been proven for some *Rickettsia* species (Moraes-Filho et al., 2018; Zemtsova et al., 2010).

Although wild boar does not seem to be a *Rickettsia* spp. reservoir in our study area, both wild boar abundance and expansion into highly populated areas could be acting as promoting factors of the vector capacity of ticks for *Rickettsia* spp. It has already been suggested that the vector capacity of ticks–the real ability to transmit a pathogen under natural conditions–is determined, either upwards or downwards, by factors other than mere vector competence, such as their abundance (Duron et al., 2015; Varela-Castro et al., 2018). Thus, the increasing trend of wild boar populations during the last years (Massei et al., 2015) is probably facilitating the life cycle of ticks and, therefore, their abundance (Estrada-Peña & de la Fuente, 2014). Moreover, wild boars could be favouring the *Rickettsia* spp. transmission among ticks via co-feeding, even if wild boars are not infected (Moraes-Filho et al., 2018; Zemtsova et al., 2010). On the other hand, human-wildlife coexistence is generating new paradigms of interactions (Conejero et al., 2019; Martínez-Abraín et al., 2019; Soulsbury & White, 2015). This may acquire bigger dimensions in scenarios such as the MAB, where wild species and humans live in sympatry, the human population numbers at risk of zoonotic diseases is high, and where health interactions between wildlife and people have already been reported (Arce et al., 2013; Fernández-Aguilar et al., 2018). MAB inhabitants may be at risk when practising their daily or leisure activities and information to visitors in parks should be provided through informative or warning panels and information campaigns. Nevertheless, the infection risk may spread further since hosts can disperse infected ticks (Palomar et al., 2012). Wild boars can travel distances of several kilometres daily (Podgórski et al., 2013), and some of them are colonising new urban and peri-urban areas (Cahill et al., 2012; Castillo-Contreras et al., 2018; Licoppe et al., 2013). In the particular case of Barcelona, wild boar presence occurs within and around the city such as in urban parks, private and public gardens (Castillo-Contreras et al., 2018), so ticks and TBPs may reach places where the infection risk is supposed to be low or non-existent and hence more difficult to predict. Managers and policy makers must be aware of this risk in order to encourage the design and application of monitoring, prevention and management measures.

To better characterize tick ecology, TBPs epidemiology and improve risk prevention, further studies should be directed at the collection and identification of questing ticks from vegetation and to assess the relationship between wild boar and tick abundances on the one hand, and on the other hand, to screen them for TBPs, especially *Rickettsia* spp. This would allow us to better describe the tick community in our study area and to better understand the ecology of these pathogens in urban and peri-urban environments.

5 | CONCLUSION

Wild boars carry ticks infected with zoonotic *Rickettsia* species in the MAB, an area that is home to three million people that live in sympatry with wild boars. In this study, we describe the presence of four tick species; *H. lusitanicum* had never been reported in northeastern Spain, and only *D. marginatus* had been previously collected from wild boars in our region. Moreover, we identified three emerging zoonotic pathogens belonging to the SFG rickettsiae, namely, *R. massiliae*, *R. slovaca* and *R. raoultii*, in ticks infesting wild boars. However, we did not detect these pathogens in wild boar tissues, suggesting that wild boar do not play a major role as a reservoir host of *Rickettsia* spp. Even so, the increasing trend of wild boar populations could be promoting tick abundance and enhancing *Rickettsia* transmission among ticks via co-feeding or vertically. Also, wild boar presence in urbanized areas could be favouring the dispersion of ticks into these areas. Therefore, a risk of human exposure to *Rickettsia* spp. can be expected, even in urban locations where both the presence of ticks and the TBPs infection risk is supposed to be low or non-existent and hence more difficult to predict and prevent.

ACKNOWLEDGEMENTS

We wish to thank all the people from SEFaS who collaborated in the sample collection, sample processing and TBP analyses, especially Oscar Cabezón. Also, we would like to thank Babagana M. Adam and Isabel G. Fernández de Mera for their support in laboratory analyses, as well as Agustín Estrada-Peña and Pedro Enrique Encinosa Guzmán for their critical review of the manuscript. Our thanks to local hunters and Josep Maria López Martín for providing us access to wild boars from Collserola. Finally, our thanks to all the people involved in the wild boar monitoring and management on the UAB campus, especially Anna Florensa. RCC, XFA and ACC benefited from pre-doctoral grants by *Agència de Gestió d'Ajuts Universitaris i de Recerca* (Government of Catalunya) and the European Social Fund; file numbers 2016FI_B 00425, 2017FI_B1 00040 and 2018FI_B2_00030 for RCC, FI-DGR 2013–2015 for XFA, and FI-DGR 2014–2016 for ACC. JLH was supported by the University of Salford. *Ajuntament de Barcelona* funded this study through the contracts 13/051, 15/0174, 16/0243 and 16/0243-00-PR/01 with Universitat Autònoma de Barcelona but had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Gregorio Mentaberre is a Serra Húnter fellow.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as samples were not obtained for research purposes. Nonetheless, the animals were captured, euthanized and sampled in accordance with national (BOE-A-2013-1337) and international (Directive 2010/63/EU) legislation.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Castillo-Contreras, R., Magen, L., Birtles, R., Varela-Castro, L., Hall, J. L., Conejero, C., Aguilar, X. F., Colom-Cadena, A., Lavín, S., Mentaberre, G., & López-Olvera, J. R. (2021). Ticks on wild boar in the metropolitan area of Barcelona (Spain) are infected with spotted fever group rickettsiae. *Transboundary and Emerging Diseases*, 1–14. https://doi.org/10.1111/tbed.14268