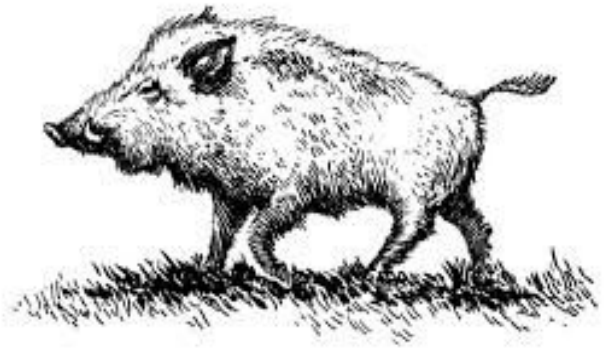
# A Survey of ixodid species parasitizing wild boar (*Sus scrofa*) in Collserola Natural Park and molecular detection of selected tick-borne pathogens



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# A Survey of ixodid species parasitizing wild boar (*Sus scrofa*) in Collserola Natural Park and molecular detection of selected tick-borne pathogens

**Luis Magen Moreno**

## Abstract

Collserola Natural Park (CNP) is a 8,000 ha peri-urban park surrounded by the highly populated Barcelona metropolitan area. Wild boar (*Sus scrofa*) displays an abundant and increasing population in CNP and could play an important role as a reservoir of zoonotic diseases. Furthermore, wild boar can be infested by hard ticks (Ixodidae) which can act as vector of some of these diseases (tick-borne diseases). Tick-borne diseases also represent a cause of health concern and tick abundance may be prompted by both global warming and hosts abundance. A survey of ticks feeding on wild boar was performed between years 2013 and 2016 in CNP. A total of 2,235 ticks were removed from 261 hunter harvested or captured wild boars. Furthermore, 180 tick pools and 167 spleen samples were screened by RT-PCR for *Rickettsia* spp. and *Coxiella burnetti.*

Ticks were morphologically classified as *H. lusitanicum* (1,143), *R. sanguineus* (s.l.) (558), *D. marginatus* (533) and *R. bursa* (1). *H. lusitanicum* and *R. sanguineus* (s.l.) presented more activity during warm months in the south of the park and *D. marginatus* in cold months in the north. Infestation prevalence was 53.7% with an abundance of 8.48 ticks per boar. One-tick species infestation predominated (67.05%) and *H. lusitanicum* was the most represented species parasitizing the 55.93% of infested wild boar. According to the model selected (GLM), sex, age and body condition of wild boar as well as season explained the 25% of variability of tick abundance on wild boar from CNP.

*Rickettsia* DNA was amplified in 96 of 180 tick pools showing differences among species. *D. marginatus* (71.62% positives) and *R. sanguineus* (67.44% positives) were the most infected species whereas *H. lusitanicum* presented lower number of positives (3.22% positives). All spleen samples were negative for *Rickettsia* spp. Regarding *C. burnetii*, all tick pools and spleen samples resulted negative.

Our study demonstrates rickettsial infection in ticks parasitizing wild boar, but there is no evidence of the pathogen circulation between ticks and boars. Hence, despite wild boar does not seem to have

a role in the epidemiology of *Rickettsia* sp. in the study area, could be indirectly contributing to increase its vector abundance.


## Keywords

Wild boar – Ixodids – Tick-borne pathogens – *Rickettsia* – *Coxiella burnetii* - Collserola Natural Park


## Background

Collserola Natural Park (CNP) is a peri-urban park located in Catalonia (northeastern Spain). It is a relatively small area (8,000 ha) completely surrounded by the Barcelona metropolitan area, where more than three million people inhabit. Wild boar (*Sus scrofa*) is the biggest wild species inhabiting in CNP with an abundant and increasing population (6-9 animals /Km2) [1]. CNP supports a high anthropic pressure and some wild boar penetrate into urban areas mainly searching for anthropogenic food resources (including garbage, pet food and even direct food supply by certain inhabitants). This urban habituation behavior is more frequent during the warm months, when food sources are poor and the soil is too dry to foraging [2]. Altogether leads to an increasing direct and indirect wild boar-pets-human interactions in the last few years, which represents an increased risk of disease transmission to humans/emergence given that wild boar can play an important role as a reservoir of zoonotic diseases [3].

Tick-borne diseases also represent an increasing cause of health concern worldwide and tick abundance may be prompted in some latitudes by both global warming and hosts abundance. Although abiotic conditions such as climate or geographical barriers determine the presence of ticks [4], recent studies have proved the adaptation of exophilic ticks (species that actively seek their hosts out) to different climatic conditions in Spain [5]. In addition, the higher the host density, the greater the probability of ticks attaching and progressing through their life cycle [6]. This is the case of Mediterranean ecosystems, including CNP, where intense ticks parasitization is frequently observed in wild boars, suggesting that the big wild boar population inhabiting CNP is contributing to increased tick abundance. In addition, considering that tick life-cycle is divided in three biological stages (larva, nymph and adult) that feed in different hosts, disease transmission from

wild boar to people via tick bite is also feasible. Wild boar can act as reservoir of livestock and human pathogens such as hepatitis E virus (HEV) [2] and can participate in the transmission or maintenance of several vector-borne zoonotic diseases. Ixodids (family *Ixodidae*), also known as hard ticks, are characterized by the presence of a scutum (hard shield), and comprise a high number of genus that parasitize a wide range of vertebrates including wild boar. Ixodids can be classified according their host-searching behavior as endophilic (passive host-finding in burrows) or exophilic (active host-seeking in the environment) species. An example of endophilic species are *I. ricinus* and *R. sanguineus* while *Hyalomma* and *Dermacentor* genus are described as exophilic. The most common tick species found in wild boar from Mediterranean areas are *Hyalomma marginatum, Rhipicephalus bursa* and *Dermacentor marginatus* [7]. However, *Hyalomma lusitanicum, Ixodes ricinus* and ticks from the *Rhipicephalus sanguineus* complex (*R. sanguineus* sensu lato), comprising *R. sanguineus* sensu stricto and *R. turanicus* species, can also parasitize wild boar in the northeastern of Spain. [8]. Several pathogens can be found in these tick species, including zoonotic agents such as *Rickettsia* sp. and *Coxiella burnetii*.

Rickettsiae refers to a heterogeneus group of microorganisms phylogenetically close to viruses and bacteria. These obligate intracellular Gram-negative coccobacilli are usually transmitted by arthropods and mammals can act as reservoir hosts [9]. Ticks can be reservoirs too [10], but frequently play a vector role [10] [11]. Several species have been described and some can produce important zoonotic diseases with a wide geographic distribution. The spotted fever group (SFG) of rickettsiae comprise several species highly distributed around the world, causing febrile illness episodes in humans. *Rickettsia conorii* is the only SFG rickettsiae prevalent in Europe [10]. It causes a human disease known as Mediterranean Spotted Fever (MSF) characterized by exanthema, fever and ulcer in the inoculation point [12]. MSF is endemic in Catalonia and the brown dog tick, *Rhipicephalus sanguineus* (s.e.), is the main vector. *Rickettsia slovaca* (another SFG rickettsiae) is the aethiological agent of Tick-Borne Lymphadenopathy (TIBOLA), an emergent disease that causes cervical lymphadenopathy with other moderate clinical signs in humans [12]. Recent studies suggest that wild boar can act as reservoir of *R. slovaca* [13]. Recently, *R. raoultii* has also been detected in human TIBOLA cases [14], with some medical reports of the disease in Spain associated to *Dermacentor* tick bites [15] [16]. *Coxiella burnetii* is the aethiological agent of Q fever. It is an obligate *Rickettsia*-related intracellular organism that causes this zoonotic disease endemic of the Mediterranean region. Acute febrile illness is characteristic but sometimes the

infection becomes chronic and asymptomatic. Q fever presents a worldwide distribution and ruminants are the main reservoir [17]. Direct inhalation of excreta or contact with contaminated animals are the common causes of contagion [18]. *C. burnetii* presents a prevalence of 4.3% in wild boars from Northern Spain [19]. On the other hand, although *Dermacentor, Rhipicephalus, Hyalomma* and *Ixodes* tick genus have been described as vectors [20], some controversy on the vector competence of ticks for *C. burnetii* exists [21].

The aims of this study are (1) **to perform a parasitological study of ticks infesting wild boar in CNP** by (1a) making a description of the tick species parasitizing wild boar and study their seasonality and spatial distribution, (1b) to define the prevalence of infestation, tick abundance and ticks species distribution by hosts and (1c) performing an infestation model using host dependent and environmental variables; and (2) **to screen ticks and wild boar tissue samples for *Rickettsia* spp and *C. burnetii* by RT-PCR.**

# Methods

### Study area

CNP is located inside the Collserola massif (coord: 41° 25′ 28″ N, 2° 6′ 32″ E) and has an extension of 8,000 ha. The highest hill is the Tibidabo Mountain (512m). The park is delimited by rivers Besos and Llobregat in the east and west, respectively, by small watercourses and urban areas in the north and by Barcelona city in the south (Figure 1). The weather is characterized by temperate winters with dry and warm summers with a mean annual temperature of 15 C°. Annual precipitations are relatively high, 620 mm, with two wet periods, spring and autumn, and a very dry summer. There are many microclimates inside the park but we can differentiate two main climates corresponding to the two slopes of the ridge. The southern slope is sunny and has a warm weather influenced by the human activities of the city of Barcelona and the sea. The northern slope is characterized by a cooler and wet climate. Forest and scrubland are the main vegetal communities in the park. Some of the predominant flora species are *Quercus ilex, Pinus halepensis, Quercus cerrioides* or *Arbutus unedo*. Appart from the wild boar, other mammals like red foxes (*Vulpes vulpes*), rabbits (*Oryctolagus cuniculi*) and the common genet (*Genetta genetta*) inhabit the park.

## Sampling

Between 2013 and 2016, 486 either hunter-harvested or captured and euthanized wild boars were examined and sampled throughout the year in several locations within CNP and nearby urban areas. The wild boars were not removed for research purposes but for population control and incidences management within urban areas. All the boars were legally hunted by authorized hunters or captured and euthanized by veterinarians. Just after harvesting, the wild boars were carefully inspected for five minutes to detect and collect ticks. All the ticks found in a single wild boar were removed manually and placed within a 5 ml independent plastic tube and stored at -20ºC until processing. The tubes were identified according to the host (wild boar) reference. Later, tick species and sex (male or female), as well as life cycle stage (adult, nymph or larva) were determined. To study wild boar parasitization, wild boar sex, age class, body condition and weight were recorded. Age class was determined using dentition patterns, classifying the wild boars as piglets (< 7 months), juveniles (7 to 12 months), yearlings (12 to 24 months) and adults (>2 years). Body condition was determined based on palpation and observation of bony prominences by an experienced observer. Animals were classified in four groups: very poor, poor, good or very good body condition. The month of collection, location of each boar inside the park, distribution (northern or southern slope), origin of capture (hunter-harvested or urban wild boars) and louse presence were also registered.

Blood samples were also collected either through intra-cardiac punction or from the cavernous sinus [22] in either anesthetized or dead wild boars using a 20 ml syringe and 18 G needles. The serum was centrifuged at 1.200×g for 15 minutes, removed and stored at −20°C until analysed. Necropsy of all animals were performed and tissue samples were collected in sterile tubes and stored at -20ºC until analysed.

## Ticks identification

We identified tick specimens with binocular lens and according to morphological identification keys [8] [23] [24].*Rhipicephalus* species (*R. sanguineus – R. turanicus*) included within the *R. sanguineus* complex were not differentiated due to their morphological similarities and because molecular techniques would have been required for accurate classification [25]. Hence, all tick

specimens within this complex were grouped. The tubes containing ticks samples were processed one by one to minimize the defrost effect over the potential pathogen DNA integrity. Ticks of each wild boar were classified in 1.5 ml tubes by species, life stage (larva, nymph and adult) and sex (male and female). Pools were identified according the host and tick species. Once performed the classification, we returned the resulting 1.5 ml tubes to freezing conditions at -20ºC until processing for tick-borne pathogen detection.

**Tick-borne pathogen identification**

We selected 180 tick pools of different hosts comprising a variable number of adult parasites (1-6) of the same species, with no sex discrimination. In the case of wild boars co-infested with more than a tick species, we selected only one species per wild boar. The four tick species found in our study were represented in the analyzed pools (*D. marginatus* n=74; *H. lusitanicum* n=62; *R. bursa* n=1; *R. sanguineus* n=43). Tick pools were processed individually and each pool was washed three times with sterile water and once with 70% ethanol. The tick specimens were air dried and collected in sterile tubes. Physical disruption of the ticks was done using sterilized scissors and conical tissue grinders. Spleen samples (10 mg) of 167 out of the 180 wild boars for which tick pools were selected were also individually subjected to mechanical disruption and homogenization of each sample. Next, QIAamp cador Pathogen Mini Kit (Qiagen, Hilden, Germany) was used to extract DNA from ticks and spleen samples in a single step. Commercial kits are a valuable tool and provide an easy method to extract genetic material of disrupted ticks [26] [27]. The extraction process was performed according to the manufacturer instructions and the resulting samples were stored at -20C until further processing.

We screened the 180 tick pools and the 167 wild boar spleen samples by RT-PCR for *Rickettsia* spp. and *C. burnetti*. Specific primers for *gtlA* gene (encoding cytrate synthase protein) of *Rickettsia* spp. and two different gene targets, IS1111 insertion sequences (transposase) and IS30a spacer region of *C. burnetii* have been used (Table 1). Molecular detection of *Rickettsia* spp. was performed using a total PCR volume of 20 µL comprising 5 µL of extracted DNA and 15µL of PCR mix. The mix included 10µL of mix Quantitect QIAGEN, 2 µl (2 pmol/µl) sonde Taqman, 0.5 µl (20 pmol/µl) of primer forward, 0.5 µl (20 pmol/µl) of primer reverse and 2 µL of distilled water. Amplification conditions started with a first step of denaturation at 95ºC for 3 minutes followed by

40 cycles at 92ºC for 1 second, hybridation and elongation at 60ºC for 35 seconds, and one last cycle at 42ºC for 30 seconds. The length of the expected product was less than 200 bp. Distillated water was used as negative control and a laboratory-cultured *Rickettsia conorii* strain was the positive control. For the detection of *C. burnetti* we followed the protocol described in previous studies [28]. A total volume of 20 µL including 5 µL of extracted DNA and 15µL of PCR mix was used. PCR mix included: 10 µl MyTaq™ Mix, 0,5µl (10 pmol/µL) of each primer, 2 µl (2 µmol/ µL) of FAM and TAMRA-labeled probes and 2 µL of distilled water. PCR conditions included a first denaturalization cycle at 95ºC for 15 minutes, 40 cycles at 95ºC for 1 second and a hybridation and elongation cycle at 60ºC for 60 seconds. Positive samples for both genes were considered positive for *C. burnetti*. Distillated water was used as negative control and a known *C. burnetii* strain served as positive control. Detection of both pathogens was performed using DNA Engine Opticon 2 Continous Fluorescence Detector Mod: CFD-3220 (MJ Research, Canada).

**Statistical analysis**

Infestation prevalence (number of hosts infested) and tick abundance (number of ticks collected in the same host) were also studied. Percentage of tick species and life cycle stages were studied. Species monthly seasonality and spatial distribution were analyzed using Chi-squared test. Descriptive statistical analyses of infestation were performed.

We used general linear models (GLM) to assess the host and environmental (or no host-dependent) factors that contribute to wild boar infestation. Host variables studied were weight, sex, class of age and body condition. Environmental variables introduced were louse co-infestation, orientation, location, origin and season. Analyses of variance (ANOVA) between each variable and logarithmic-transformed tick abundance were performed to determine their relationships before modeling. Variables with $p<0,01$ were used to build linear models.

Percentage of positive samples for *Rickettsia* spp. and *C. burnetti,* were determined in ticks pools and spleen samples. Chi-squared test was used to investigate differences between tick species. All data were analyzed using R software version 3.3.1 (https://cran.r-project.org/)

# Results

The infestation prevalence was 53.7% (261 wild boars out of the 486 included in the present study carried at least one tick). The average tick load was 8.48 ticks per boar, with a standard deviation was 10.8, a median of 5 and a range between 1 and 70 ticks (Table 3). All louse specimens were identified as *Haematopinus suis*. The prevalence of co-infestation by ticks and louse was 21.5% (56 of 261 wild boars) but no quantification was made. We collected a total of 2,235 ticks and identified four species of three different genera feeding on wild boar, *D. marginatus* (n=533; 23.8%), *H. lusitanicum* (n=1143; 51.1%), *R. sanguineus* (s.l.) (n=558; 25%) and *R. bursa* (n=1; 0.04%). Males were more abundant (n=1335; 59.7%) than females (n=799; 35.7% ) and nymphs (n=101; 4.5%). No larvas were found (Table 2). We collected *H. lusitanicum* specimens in more than a half of the infested wild boars, 55.93% (117/261). This was the most abundant (higher number) and also widespread (higher number of hosts) species in wild boar population. *D. marginatus* appeared in the 44.82% (146/261) of infested animals, *R. sanguineus* (s.l.) in the 33.33% (87/261) and *R. bursa* in the 0.38% (1/261) (Figure 6). Variable number of males and females of each species were identified. More males than females were collected of all species except for *R. sanguineus* (s.l.). We only detected nymphs of *R. sanguineus* (s.l.) and *H. lusitanicum*. Different tick species combinations were observed to feed in the same host (Figure 4) being the *R. sanguineus* (s.l.)-*D. marginatus* the most frequent combination (31%). Pure infestations (samples with only one tick species) were more prevalent (67.05%) than co-infestations (two species 31.42%; three species 1.53%) (Figure 5).

We detected a spatial pattern for tick species distribution in CNP (Chi-squared: 338.77; $p<0.001$). *H. lusitanicum* and *R. sanguineus* (s.l.) were collected more frequently in southern localities whilst *D. marginatus* was collected in the northern slope. The *R. bursa* specimen was collected in the southern area (Figure 2). Monthly seasonality varied significantly amongst species (Chi-square: 1966.59; $p<0.001$). From november to february *D. marginatus* was the predominant species, *R. sanguineus (*s.l.) appeared overrepresented between march and may, and *H. lusitanicum* between july and october (Figure 3).

**Modeling parasitization**

The variables used to build the models were weight, sex, age class, body condition and season. The variables origin, orientation, location, and louse co-infestation were excluded owing to lacking significance (p>0,01). Three different models including these variables were tested. Model 1 included sex, age class, body condition and season covariates. Model 2 included weight, sex, age class, body condition and season and model 3 included weight, sex, age class, body condition, season, sex and age class interaction and sex and body condition interaction. Post-hoc test using Tukey contrasts were performed for all covariates. Model 1 displayed higher adjusted R-squared (0.25) in comparison with models 2 and 3 (0.24). All models were compared using the F-test (Table 5). Model 1 was considered more accurate for its better R-squared and simplicity (included fewer variables), but no significant differences among models were found (p>0.05).

Post-hoc test demonstrates that higher ticks parasitization rates were observed during spring in adult male wild boars with poor or very poor body condition, in comparison with other seasons and either females or younger wild boars with good body condition (Figure 7 and Table 6). Despite host weight variable was excluded of the selected model, it was significant in the previous ANOVA (Table 4). Additional univariate linear regression between logarithmic-transformed variables of tick abundance and host weight was performed. Results showed a $p<0.01$ ($p=1.79x10^{-7}$) and low coefficient of determination (Multiple R-squared: 0.1). Scatterplot of both variables are represented in Figure 8.

**Tick-borne pathogens detection**

A total of 96 out of the 180 tick pools tested resulted positive for *Rickettsia* spp (53.3%). We found differences between species (Chi-square: 73.8; p<0,001). *D. marginatus* (71.62% positives) and *R. sanguineus* (67.44% positives) were the species with more positive samples. *H. lusitanicum* presented a low number of positive samples (3.22% positives) and the *R. bursa* specimen resulted negative for *Rickettsia* spp. (Figure 9). All wild boar spleen samples were negative for *Rickettsia* spp. (0/167). Amplification of C. burnetii was negative for all ticks (0/180) and spleen samples (0/167). Results are summarized in Table 7.

# Discussion

**Infestation characteristics and ticks population**

Three main tick species have been detected parasitizing the wild boar in CNP, *H. lusitanicum*, *D. marginatus and R. sanguineus* (s.l.). We consider anecdotal the detection of a unique specimen of *R. bursa*. Although *R. bursa* has been described to parasite wild boar [7], its main hosts are cattle and small domestic ruminants [8][29]. Our observation could be due to the almost total absence of domestic ruminants in CNP and, hence, low *R. bursa* presence. All four species are common in northeastern Spain [8], displaying distribution differences across the country [30]. The higher abundance of *H. lusitanicum* in our study coincides with other tick surveys in central Spain [7]. It is a Mediterranean three host-species tick [31]. The low number of nymphs detected and the absence of larval stages of all species can be partly explained because immature stages feed on smaller mammals, wild boar being an adult stage host [7][8].

*Dermacentor* specimens were more numerous in the northern slope of CNP, where the climate is cooler and wetter, whereas *Hyalomma* and *Rhipicephalus* samples were bigger in the southern slope. Previous studies based on environmental sampling of ticks have associated climate preferences with spatial differences of tick distribution [32]. However, our study is based on sampling of ticks feeding on the host. Hence, despite the similarities, our results must be interpreted with caution as factors such as prolonged feeding time of ticks over the host [33] and a relatively large wild boar home range [34] could have biased our results. Further studies to conclude on tick species distribution in CNP will be necessary. With regard to seasonality, although ticks parasitizing wild boar in CNP presented activity during all year round, species abundance varied along year. *D. marginatus* was the most abundant species during the cold months whereas its apparition was minimal in the warm months. *R. sanguineus* (s.l.) species were the most active during spring but almost disappeared at the end of summer. Finally, *H. lusitanicum* was overrepresented in summer although maintained its activity during all the year. These observations agree with the described seasonality patterns for these tick species [8][30]. Despite ticks are generalist parasites [35], certain host preferences are described for some species [36]. For that reason, we cannot extrapolate the tick species distribution found in wild boar neither to other host species nor be interpreted as an approach to the CNP tick population.

The tick burdens in wild boar from CNP observed in the present study are low. We found higher prevalence of infestation but lower tick abundance than studies performed in central Spain [7]. Pure infestations by one tick species are prevalent in wild boar of CNP. *H. lusitanicum* was the most abundant species followed by *D. marginatus* and *R. sanguineus* (s.l.). Similar results have been reported in central Spain [37]. Co-infestations with two tick species were not uncommon but so it was the presence of three species. These results could be due to differences in tick species seasonality or even to inter-specific competition among tick species [38]. Although it is out of the scope of this work to assess how these unknown relationships could affect the presence of certain tick species, we must consider them.

**The model: conclusions and limitations**

Host-dependent factors such as sex, age class, body condition and season of sampling were the variables included in the most explicative model built to explain tick infestation of wild boar in CNP. This model explains the 25% of variability in tick abundance on wild boar. These results are comparable with studies performed in other species [39][40]. Tick parasitization is determined by the presence of the host and the parasite in the environment. We collected more parasites in the warm months coinciding with the season of more activity of the species found. The infestation rate on wild boar could reflect the tick activity in the park. Climatic factors seem to be determinant in tick abundance on hosts [41] and in the environment [42]. Males and adult individuals resulted more infested than females or young animals, which agrees with studies on other species [41] [43] including pigs [44]. Conversely, cattle females were more infested than males in another study [45]. The high mobility of solitary male wild boars [34] could explain their higher parasitization because of increased exposition to different habitats in the park. The lack of grooming behavior [45], a mechanism of defense against ectoparasites described in ungulates [46] [47], and lowered immunity response due to testosterone [45] could contribute to higher tick infestations in male wild boars. However, additional studies are needed to confirm or reject this assumption.

In our results, body condition appears as an important factor to explain boar tick burden. Animals with poor body condition appeared more parasitized, coinciding with studies done in cattle [48] [49] and pigs [50] but it contrasts with findings on reptiles [39]. However, it may be underlying causes that determine this association, such as reduced time dedicated to grooming behaviors that contribute to remove parasites [46] or immunosuppression due to the detrimental effects of other

health problems or pathogens that make these individuals more prone to tick infestation. In fact, studies on hedgehogs demonstrate a predilection of ticks for diseased individuals [51] and also have been documented a negative correlation between tick abundance and innate immunity in buffaloes [52].

Hence, the host-dependent factors considered in this work, despite their predictive value, could be correlated with other not considered factors. On the other hand, individual host characteristics such as body condition, sex or age can be considered poor predictive variables to determine the tick burden despite differences among groups (sex or age class) can be significant [53]. The complex relationships between the same individual characteristics can influence over the tick abundance, moreover has been shown that body weight can influence differently depending the sex of the host [41] [54].

Regarding the variables excluded of the model, although higher infestations appeared in weighed boars (as seen in additional univariate model), host weight variable was excluded of the model selected model as its inclusion did not increase the explained variability. Perhaps, could exist collinearity between age and weight variables in the model due to the positive correlation between these two variables (adult animals are weighed and also are more infested). Although this fact was not studied, this phenomenon could explain the lack of improve of the model adding the weight variable. Geographic factors did not influence wild boar tick burden, so we do not expect to find differences in tick abundance among urban and hunted wild boars. On the contrary, geographic factors influenced tick species implicated, probably due to correlation with climate conditions more suitable for every tick species [30]. Finally, the presence of louse did not had a significant influence in tick abundance. All louse specimens collected were identified as *Haematopinus suis,* a common domestic and wild swine ectoparasite capable to act as vector of some pathogens [55] [56].

**Tick-borne pathogens detection**

RT-PCR allows a quick and specific determination of *Rickettsia* [57] and the use of highly conserved gene targets like gltA permits an accurate screening [58]. We demonstrate the presence of *Rickettsia* spp. DNA in ticks parasitizing half of the wild boars tested (53.3%) in CNP. Previous studies on rickettsial prevalence in ticks demonstrate prevalences ranging from 71.1% [59] to

32.7% [60] and 19.0 % in ticks from Spain [37]. Concretely in ticks removed from wild boars, there has been previous evidence of rickettsial infection [60] [61] [62]. *Rickettsia* DNA amplification varied depending on the tick species in our study; *D. marginatus* and *R. sanguineus* (s.l.) were the species with more positives whereas *H. lusitanicum* presented very low number of amplifications. These results agree with other studies on rickettsial prevalence in Spain, which demonstrate higher prevalence of *Rickettsia* infection in *D. marginatus* and *R. sanguineus* and absence or low prevalence in *H. lusitanicum* [39] [63]. Far from being resistant to rickettsial infection, *Hyalomma* genus (*H. marginatum* species) can be associated with specific spotted fever group *Rickettsia* [64] [65] [66] also described in Spain [67]. Although the *R. bursa* specimen was negative, rickettsial infection is this species has been described in Spain [63]. However, prevalence of rickettsial infection in ticks seems to depend on several factors besides tick species. Presence of reservoirs or the poorly known relationships between *Rickettsia* and ticks could influence their prevalence. For example, some rickettsial species have been described to provoke mortality in its tick vector [68] [69]. In addition, screening for rickettsial infection in ticks must be interpreted with caution as non-pathogenic rickettsial organisms can be present in ticks, overestimating the molecular detection for *Rickettsia* spp. and even interacting with pathogenic *Rickettsia* [70]. Consequently, posterior characterization through sequencing of positive samples is needed to determine the species implicated. On the other hand, all the wild boar spleen samples tested were negative for *Rickettsia* spp., also in agreement with other studies performed in wild boars [71] and other wild mammals [72]. Presence of rickettsial specific antibodies in wild boar has been documented and implication of wild boar in the lifecycle of *Rickettsia* pathogens has been demonstrated [61][11]. However, tissue samples of seropositive animals (spleen and blood) have resulted negative for *Rickettsia* spp DNA [61]. Hence, our results cannot confirm nor discard circulation of *Rickettsia* between ticks and wild boar in CNP and further studies will be needed to clarify the role of wild boar in rickettsial lifecycle.

All ticks and spleen samples tested were negative for *C. burnetti*. The role of ticks in *C. burnetti* epidemiology is controversial. In Spain, differences in *C. burnetii* infection in ticks have been described, prevalence ranging from zero to 3.4% in ticks removed from wildlife [19], and up to 7.7% in questing ticks [72]. *H. lusitanicum* and *D. marginatus* are the main vectors in central Spain, although the infection has been observed also in *R. sanguineus* [72]. Methodology of molecular surveys must be critically assessed as the presence of *Coxiella*-like endosymbionts in ticks can led

to cross reactions in the amplification of *C. burnetti* DNA [73].

Domestic ruminants are the main reservoir of *C. burnetii* [74], but horses [75] or rabbits [79] have also been observed to participate in its epidemiology. Wild ruminants have been postulated as additional reservoirs of the *C. burnetii* [77] despite differences among territories have been observed [78]. The role of wild boar is not clear but low prevalence (4.3%) has been described [19]. We could not demonstrate the presence or circulation of *C. burnetti* in ticks and wild boar in CNP. Since either domestic or wild ruminants are almost absent in CNP, and both ticks and wild boar can harbor the bacteria, our results may derive of true absence or very low presence of *C. burnetii* in the study area

# Conclusions

To the best of our knowledge, this is the first description of wild boar tick infestation in CNP. Wild boars inhabiting CNP are parasitized by four tick species that differ in spatial patterns and present marked seasonality. Ticks abundance on wild boars in CNP depends partially on host-dependent factors such as sex, age class or body condition and season of the year, but another unknown factors are involved. This work also constitutes the first molecular-based screening of *Rickettsia* spp. and *C. burnetii* infection in ticks and wild boars from CNP, resulting in the first rickettsial DNA isolation in ticks feeding on wild boar in CNP.

# Abbreviations

bp= Base pair
CNP= Collserola natural park
GLM= General linear model
RT-PCR= Real time polimerase chain reaction
s.e.= Sensu estricto
s.l.= Sensu lato

# Declarations

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# ANNEX

The elements of this annex are classified in order of apparition in the paper

## Figures

**Figure 1: Study area**

Collserola natural park surrounded by the metropolitan area of Barcelona. Note its relatively small area and lack of connectivity to other "green areas". The names of the surrounding municipalities are marked in the map.

**Figure 2: Relative abundance of tick species according orientation**

Abundance of every tick species (*Dermacentor marginatus:* D. marg.- *Hyaloma lusitanicum:* H.lusit., *Rhipicephalus sanguineus:* R.sang. and *Rhipicephalus bursa:* R.bursa) on wild boars from the northern (N) and southern (S) slopes of Collserola Natural Park.

**Figure 3: Relative seasonality of tick species**

Monthly evolution of abundance of every tick species (*Dermacentor marginatus:* D. marg.- *Hyaloma lusitanicum:* H.lusit., *Rhipicephalus sanguineus:* R.sang. and *Rhipicephalus bursa:* R.bursa) on wild boars from Collserola Natural Park sampled all year round.

**Figure 4: Tick species combinations found in wild boar samples** (*Dermacentor marginatus:* D. marg.- *Hyaloma lusitanicum:* H.lusit., *Rhipicephalus sanguineus:* R.sang. and *Rhipicephalus bursa:* R.bursa).

**Figure 5: Number of species of each sample**

**Figure 6: Tick species prevalence on infested wild boars** (*Dermacentor marginatus:* D. marg. *Hyaloma lusitanicum:* H.lusit., *Rhipicephalus sanguineus:* R.sang., and *Rhipicephalus bursa :* R.bursa). Note that blue bars represents the percentage of infested wild boars positives for the corresponding tick species (x axis) and orange bars the percentage of infested animals negative for the same species (but parasitized by other species).

**Figure 7: Plots tick abundance (log) – co-variables**

Variables included in the definitive model. Higher number of ticks were removed of males, animals with very poor body condition, and during spring. Piglets were less parasitized than other ages.

**Figure 8: Scatterplot between tick abundance (log) – host weight (log)**

Despite higher infestations appear in weighed boars, host weight was excluded of the definitive model.

**Figure 9: *Rickettsia* spp. in tick species**

*D. marginatus* and *R. sanguineus* were the species with more positive results for *Rickettsia* spp. The 71.62% of *D. marginatus* pools and 67.44% of *R. sanguineus* pools amplified rickettsial DNA instead only the 3.23% of *H. lusitanicum* pools resulted positives. The *R. bursa* specimen resulted negative.

## Tables

**Table 1: Primers and probes used for tick-borne pathogens molecular detection**

**Table 2: Number of tick specimens collected on wild boar by tick species, sex and biological stage**

**Table 3: Descriptive statistics of infestation**

**Table 4: ANOVA variables**

Analysis of variance between each co-variable and tick abundance was performed independently.

**Table 5: Comparison between models:** Model 1: log (tick abundance) = sex + class of age + body condition + season; Model 2: log (tick abundance) =weight + sex + class of age + body condition + season; Model 3: log (tick abundance) = weight + sex + class of age + body condition + season + sex:class of age + sex:body condition.

**Table 6: Post-hoc Tukey test**

Significative differences were found between Sex $p<0,05$; Class of age $p<0,01$; Season $p<0,01$ and Body condition $p<0,01$ (except for very poor -poor animals $p>0,05$).

**Table 7: RT-PCR results for *Rickettsia* spp. and *C. burnetii***

Rickettsial DNA was detected in ticks but it was not amplified in wild boar spleen samples. All samples were negative for *C. burnetti*.

**Figure 1: Study area**

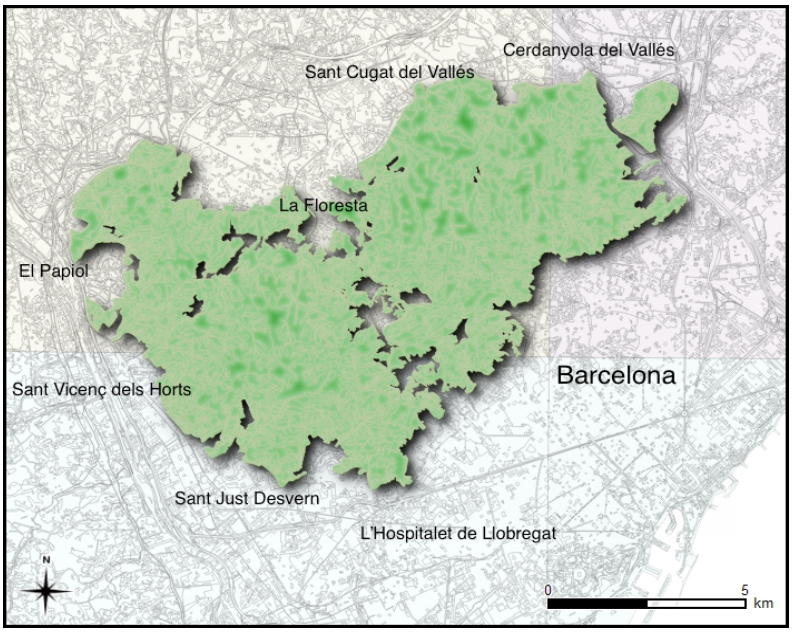


**Table 1: Primers and probes used for tick-borne pathogens molecular detection**

| | | |
|---|---|---|
| *Rickettsia* spp. | Primer RKND03F | 5'-GTG-AAT-GAA-AGA-TTA-CAC-TAT-TTA-T-3' |
| | Primer RKND03R | 5'-GTA-TCT-TAG-CAA-TCA-TTC-TAA-TAG-C-3' |
| | Probe RKND03 | 6-FAM-CTA-TTA-TGC-TTG-CGG-CTG-TCG-GTT-C-TAMRA |
| *C. burnetti* | Primer IS1111F | 5'-GCGTCATAATGCGCCAACATA-3' |
| | Primer IS1111R | 5'-CGCAGCCCACCTTAAGACTG-3' |
| | Probe IS111 | 6FAM-TGCTCAGTATGTATCCACCG-TAMRA |
| | Primer Cbis30aF | 5'-AATGTCTGCGGGAAATAGGC-3' |
| | Primer Cbis30aR | 5'-GAGGCCTTTTACCGGAATTC-3' |
| | Probe IS30a | 6FAM-TCGAGATCATAGCGTCATT-TAMRA |

**Table 2: Descriptive statistics of infestation**

| N hosts | Mean Ticks | Std. Dev. | SEM | Median | Range |
|---|---|---|---|---|---|
| 261 | 8,48 | 10,8 | 0,67 | 5 | 1-70 |

**Table 3: Tick species collected on wild boar**

| | Female | Male | Nymph | Larva | Total Number |
|---|---|---|---|---|---|
| *D. marginatus* | 244 | 289 | 0 | 0 | 533 |
| *H. lusitanicum* | 256 | 793 | 94 | 0 | 1,143 |
| *R. sanguineus* | 299 | 252 | 7 | 0 | 558 |
| *R. bursa* | 0 | 1 | 0 | 0 | 1 |
| Total Number | 799 | 1,335 | 101 | 0 | 2,235 |

**Figure 2: Relative abundance of tick species according orientation**

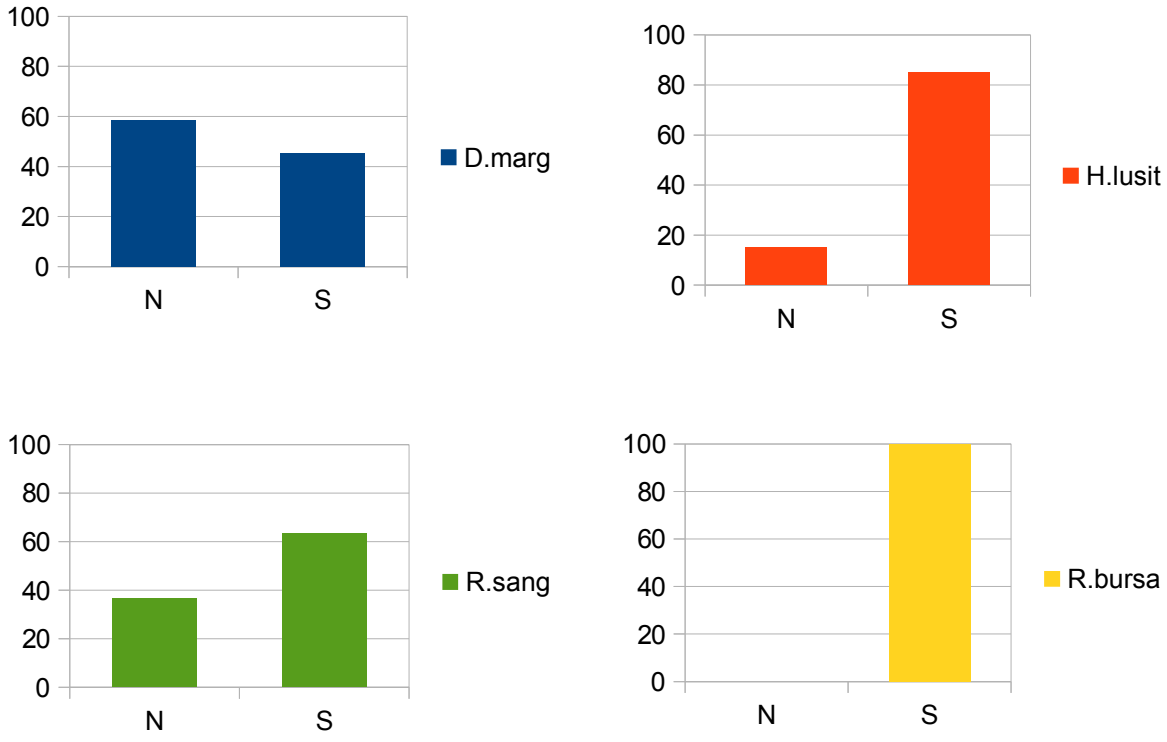

**Figure 3: Relative seasonality of tick species**

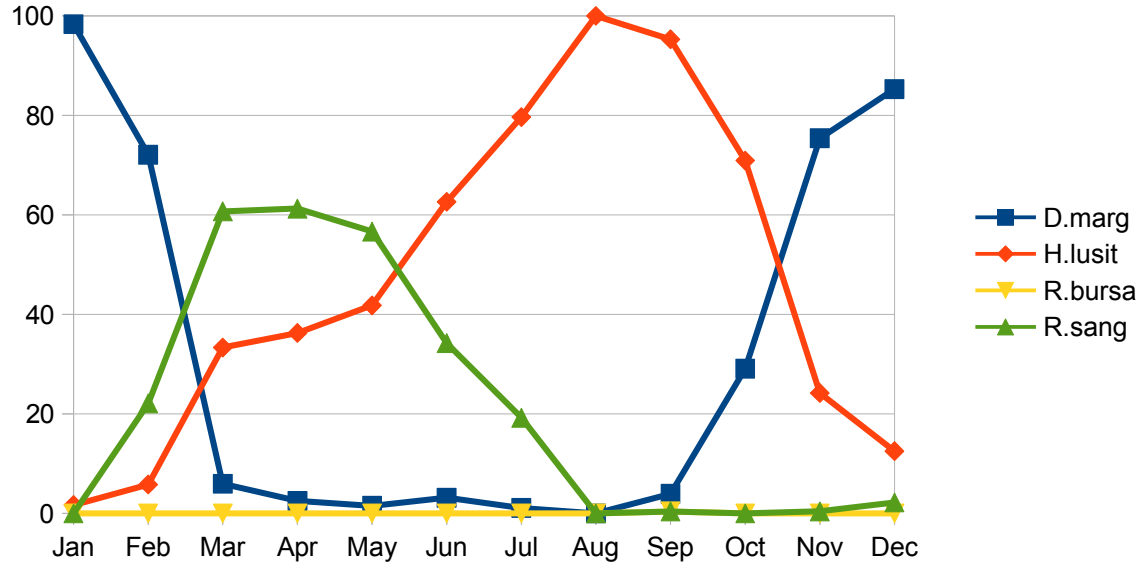


**Figure 4: Tick species combinations found in wild boar samples**

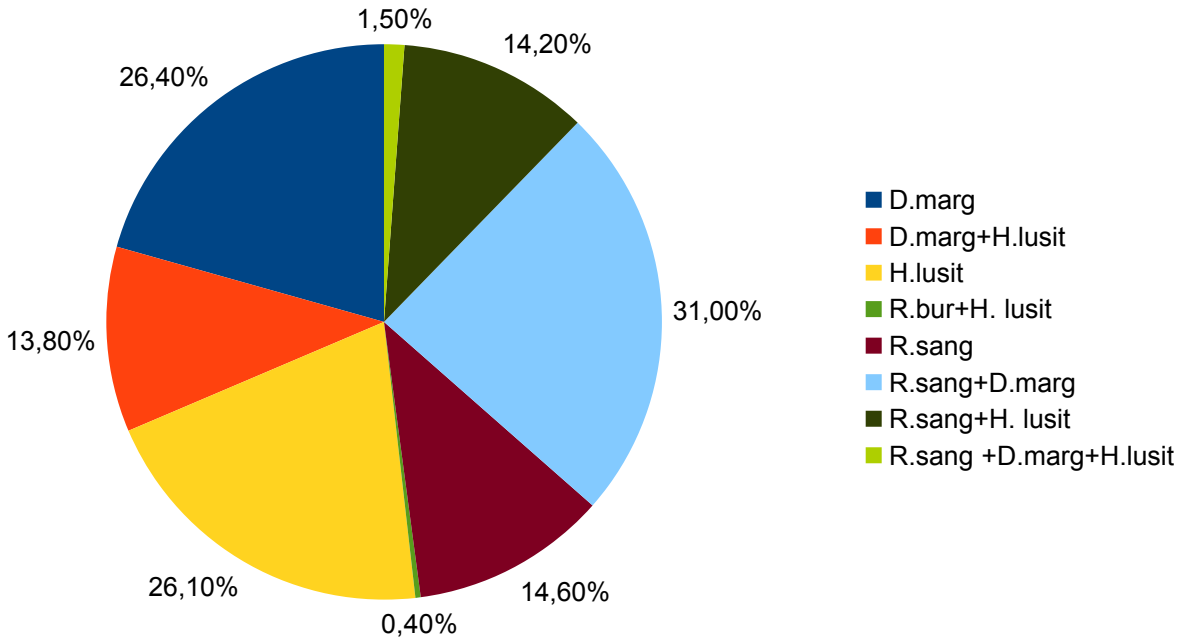

**Figure 5: Number of species of each sample**

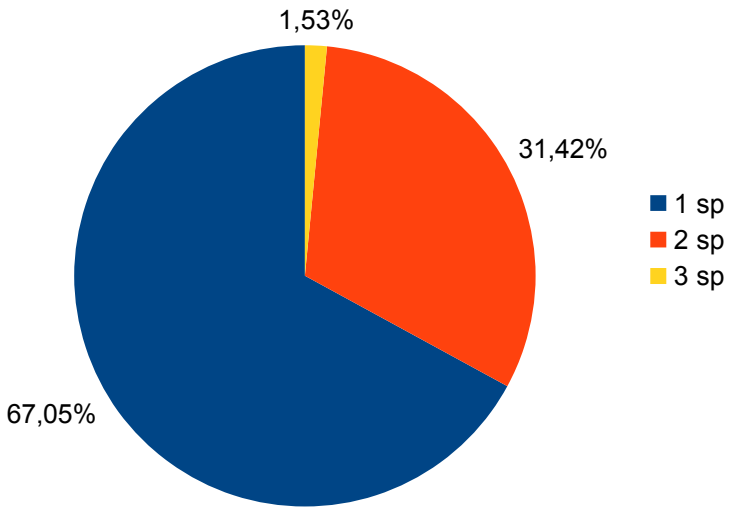


**Figure 6: Tick species prevalence on infested wild boars**

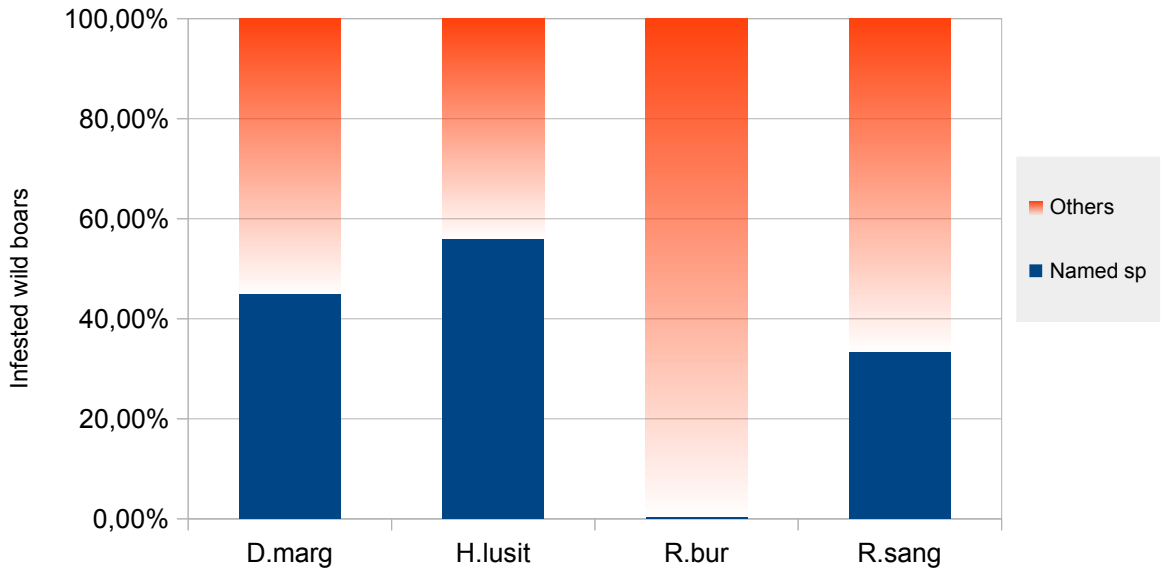

**Table 4: ANOVA variables**

| Variable | Sum sq | Df | F value | Pr(>F) |
|---|---|---|---|---|
| Weight | 23,89 | 1 | 24,36 | $1,43x10^{-6}$ |
| Sex | 7,39 | 1 | 7,07 | $8x10^{-3}$ |
| Class-age | 37,56 | 3 | 13,38 | $3,8x10^{-8}$ |
| BC | 12,01 | 3 | 3,87 | $9,9x10^{-3}$ |
| Location | 12,02 | 5 | 2,3 | 0,04 |
| Origin | 0,81 | 1 | 0,75 | 0,39 |
| Lice | 0,64 | 1 | 0,59 | 0,44 |
| Orientation | 1,27 | 1 | 1,19 | 0,27 |
| Season | 16,63 | 3 | 5,45 | $1x10^{-3}$ |

**Table 5: Comparison between models**

| | $R^2$ | Df | Sum of sq | F | Pr(>F) |
|---|---|---|---|---|---|
| Model 1 | 0,25 | | | | |
| Model 2 | 0,24 | 1 | 0,29 | 0,36 | 0,54 |
| Model 3 | 0,24 | 6 | 4,84 | 1 | 0,42 |

**Figure 7: Plots tick abundance (log) - co-variables**

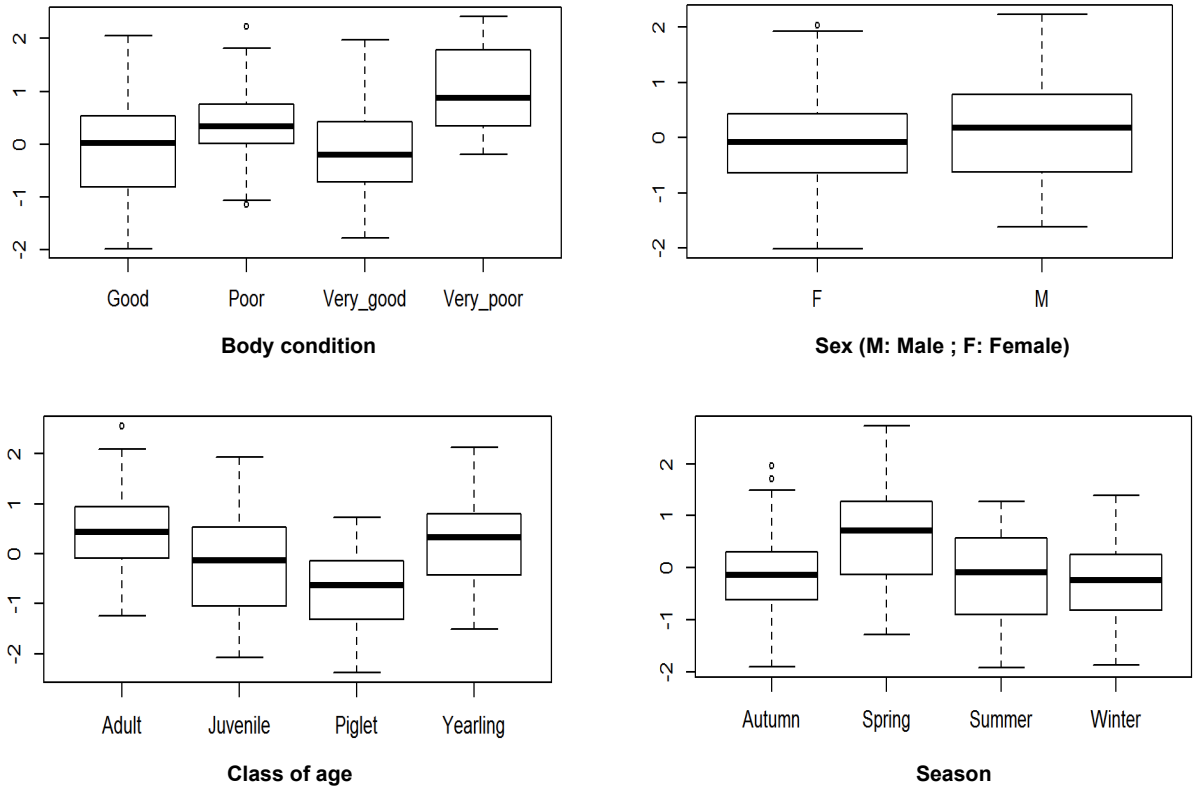


**Table 6: Post-hoc Tukey test**

| Covariables | Linear hypotheses | p-valor |
|---|---|---|
| **Sex** | Male-Female | 0,04 |
| **Class of age** | Piglet-Adult | $5{,}2\times10^{-10}$ |
| | Piglet-Juvenile | 0,001 |
| | Yearling-Piglet | $7{,}8\times10^{-8}$ |
| **Body condition** | Very poor-good | 0,002 |
| | Very poor-poor | 0,06* |
| | Very poor-very good | 0,005 |
| **Season** | Spring-Autumn | $1{,}0\times10^{-6}$ |
| | Summer-Spring | $1{,}2\times10^{-6}$ |
| | Winter-Spring | $4{,}8\times10^{-5}$ |

**Figure 8: Scatterplot between tick abundance (log) – host weight (log)**

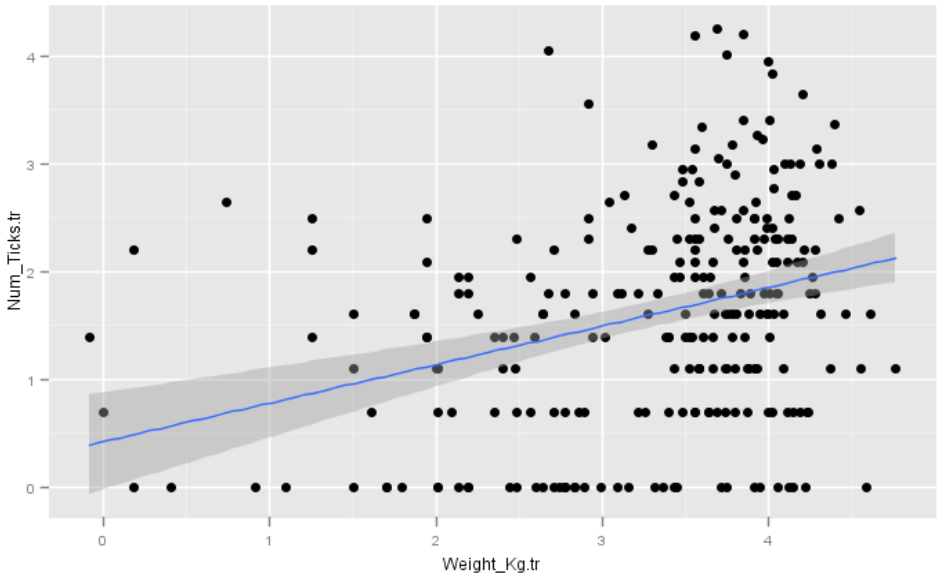


**Figure 9: *Rickettsia* spp. in tick species**

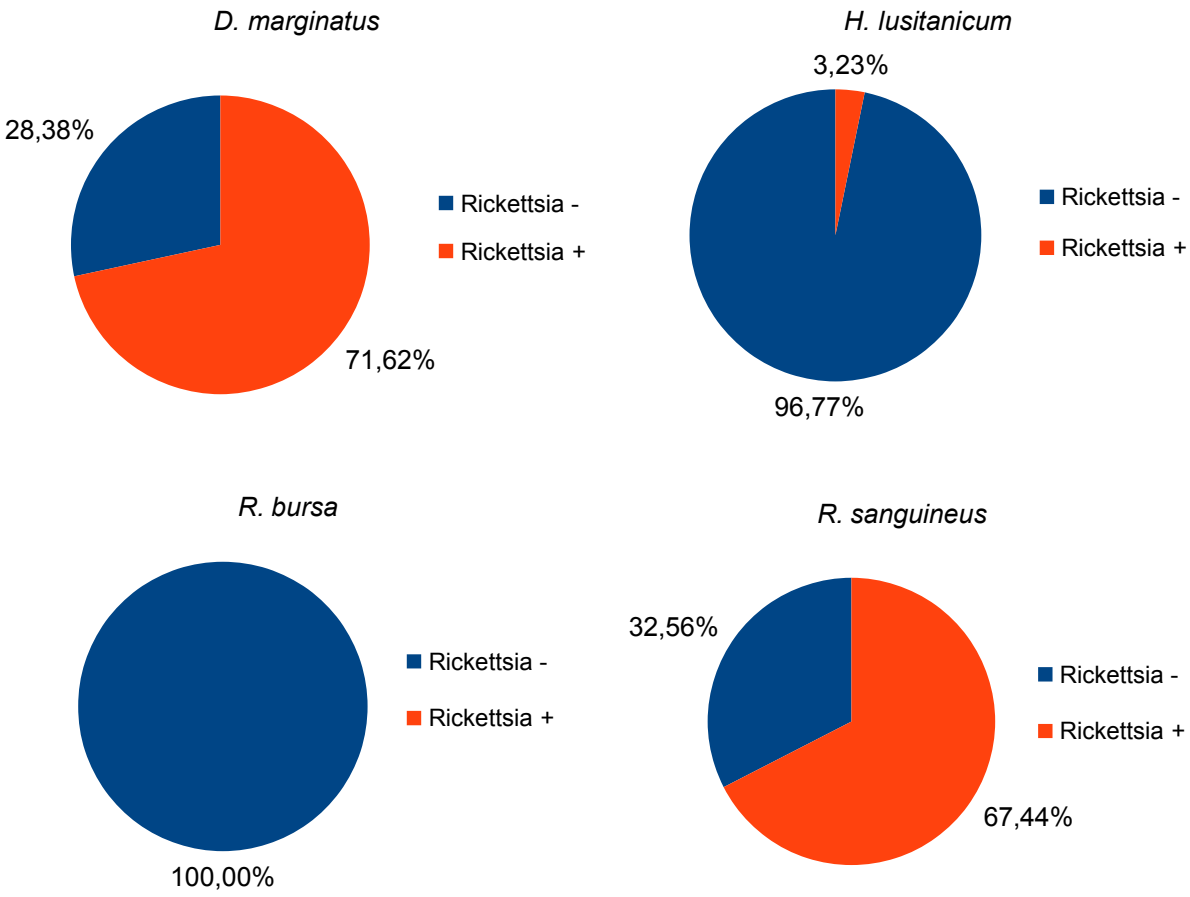

**Table 7: RT-PCR results for *Rickettsia* spp. and *C. burnetii***

| | Spleen samples | Tick pools | Tick species | | | |
|---|---|---|---|---|---|---|
| | | | *D. marginatus* | *H. lusitanicum* | *R. sanguineus* | *R. bursa* |
| **Total analyzed** | 167 | 180 | 74 | 62 | 43 | 1 |
| ***Rickettsia spp.*(+)** | 0/167 (0%) | 96/180 (53.33%) | 53/74 (71.62%) | 2/62 (3.22%) | 29/43 (67.44%) | 0/1 (0%) |
| ***C. burnetii* (+)** | 0/167 (0%) | 0/180 (0%) | 0/74 (0%) | 0/62 (0%) | 0/43 (0%) | 0/1 (0%) |