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1 A three-years assessment of *Ixodes ricinus*-borne pathogens in a French peri-urban forest

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17 **SUMMARY**

18 *Ixodes ricinus* is the predominant tick species in Europe and the primary pathogen vector for
19 both humans and animals. These ticks are frequently involved in the transmission of
20 *Borrelia burgdorferi* sensu lato, the causative agents of Lyme borreliosis. While much more is known
21 about *Ixodes ricinus* tick-borne pathogen (TBP) composition, information about temporal TBP patterns
22 remain scarce. These data are crucial for predicting seasonal/annual patterns which could improve
23 understanding and prevent tick-borne diseases (TBD). We examined TBP dynamics in *Ixodes ricinus*
24 collected monthly in a peri-urban forest over three consecutive years. In total, 998 nymphs were
25 screened for 31 pathogenic species using high-throughput microfluidic real-time PCR. We detected
26 *Anaplasma phagocytophilum* (5.3%), *Rickettsia helvetica* (4.5%), *Borrelia burgdorferi* s.l. (3.7%),
27 *Borrelia miyamotoi* (1.2%), *Babesia venatorum* (1.5%), *Babesia divergens* (0.1%), and the agent of the
28 cat-flea typhus, *Rickettsia felis* (0.1%). Among all analysed ticks, 15.9% were infected by at least one of
29 these microorganisms, and 1.3% were co-infected. Co-infections with *B. afzeli*/*B. garinii* and
30 *B. afzeli*/*B. spielmanii* were significantly over-represented. Moreover, significant variations in seasonal
31 and/or inter-annual prevalence were observed for several pathogens (*R. helvetica*, *B. burgdorferi* s.l.,
32 *B. miyamotoi*, and *A. phagocytophilum*), emphasising that regularly monitoring ticks and TBP is
33 essential to appropriately assess acarological risk.

34 **KEY WORDS**

35 Tick-borne pathogens, dynamics, temporal patterns, pathogen co-occurrence

36 INTRODUCTION

37 Ticks are obligatory hematophagous arthropods and consequently, are one of the most
38 important pathogen vectors (Jongejan and Uilenberg, 2004; de la Fuente *et al.*, 2008; Dantas-Torres *et*
39 *al.*, 2012). Lyme borreliosis (LB) is the most commonly reported tick-borne disease (TBD) in the
40 northern hemisphere and is caused by bacteria belonging to the *Borrelia burgdorferi* s.l. complex. In
41 Western Europe, *Ixodes ricinus* is known to be involved in the transmission of these bacteria to both
42 humans and animals. This tick species has also been reported to be a vector for many other tick-borne
43 pathogens (TBP) with potentially significant consequences for human and animal health (*Anaplasma*,
44 *Rickettsia*, *Bartonella*, *Babesia*...) (Strle, 2004; Bonnet *et al.*, 2007a; Bonnet *et al.*, 2007b; Cotté *et al.*,
45 2008; Bonnet *et al.*, 2009; Sprong *et al.*, 2009).

46 While multiple different pathogens have been identified and confirmed in *I. ricinus* ticks, very
47 little is known about their seasonal and inter-annual variations. Time-series studies are thus crucial to
48 understanding natural variability in microbial communities over time. Over the last decade, only a
49 handful of surveys have assessed seasonal and monthly TBP variation patterns (Gassner *et al.*, 2010;
50 Reye *et al.*, 2010; Coipan *et al.*, 2013; Takken *et al.*, 2016; Chvostáč *et al.*, 2018). Although these results
51 have heightened our general understanding of TBP dynamics, several of these studies were performed
52 over short periods of less than two years, rendering it impossible to infer inter-annual discrepancies or
53 to detect bias due to a particularly exceptional year. Only Coipan *et al.* (2013) analysed several
54 pathogenic genera in ticks sampled over more than two years. This study did demonstrate relationships
55 between seasons and TBP prevalence (*Borrelia*, *Rickettsia*, *Anaplasma*, *Neoehrlichia*, and *Babesia*) in
56 questing tick populations. These variations were mainly attributed to the varying availability of
57 reservoir hosts.

58 Tick density is also heavily influenced by the presence of suitable hosts, most notably wild
59 ungulates that sustain adults, thus enabling tick population renewal (Gilbert *et al.*, 2012; van Wieren
60 and Hofmeester, 2016). However, it's important to emphasise that immediate tick survival and questing
61 activities are highly dependent on suitable and specific environmental conditions (temperatures

62 comprised between 8 to 24°C; and up to 80% humidity). Simultaneously, several studies have
63 investigated whether pathogen presence influences tick behaviour. Herrman and Gern (2010, 2012)
64 suggested that *I. ricinus* infected with *B. burgdorferi* s.l. can tolerate increased levels of desiccation,
65 and Neelakanta *et al.* (2010) demonstrated that *I. scapularis* infected with
66 *Anaplasma phagocytophilum* are more resistant to cold. The presence of these TBP could therefore
67 enhance survival or questing activities of the infected ticks under challenging abiotic conditions,
68 suggesting the existence of a potential link between pathogen prevalence in questing ticks and seasons.

69 Tick density and TBP prevalence can thus be influenced by several variables, and can therefore
70 potentially fluctuate both seasonally and annually. Studying these dynamics is essential to better
71 understanding and anticipating TBP risk.

72 Peri-urban forests containing both TBP-reservoir hosts and ticks, and which are highly
73 frequented by people and their pets, represent a particularly interesting area to study tick and TBP
74 dynamics. The Sénart forest, located to the south of Paris, harbours many large ungulates and abundant
75 and diverse populations of other TBP reservoir hosts (bank voles, wood mice, Siberian chipmunks, roe
76 deer, hedgehogs,...), and accommodates more than three million visitors every year. This forest is
77 therefore particularly adapted to studying ticks and tick-borne pathogen dynamics.

78 In this study, we assessed the seasonal and inter-annual variability of *I. ricinus*-borne pathogens
79 in the Sénart forest over three consecutive years (from April 2014 to May 2017), and determined
80 whether any significant associations existed between these pathogens. We investigated a total of 31
81 pathogenic species (bacteria and parasites), belonging to 11 genera: *Borrelia*, *Anaplasma*, *Ehrlichia*,
82 *Neoehrlichia* (only Candidatus *Neoehrlichia mikurensis*), *Rickettsia*, *Bartonella*, *Francisella*, *Coxiella*,
83 *Theileria*, *Babesia*, and *Hepatozoon*.

84

85

86 MATERIAL AND METHODS

87 *Tick collection*

88 *I. ricinus*, nymphs and adults, were monthly collected during three years, from April 2014 to May
89 2017, in the Sénart forest in the south of Paris. Samplings were performed by dragging (Vassallo *et al.*,
90 2000) on 10 transects of 10 square meters, localized on the parcel 96 (48°39'34.6"N 2°29'13.0"E, Figure
91 1). Flagging was always performed 3 consecutive times on each transect. After morphological
92 identification, ticks were stored at -80°C. In total 1167 *I. ricinus* (nymphs and adults) were collected.

93

94 *Tick washing, crushing and DNA extraction*

95 Ticks were first washed once in ethanol 70% for 5 minutes and rinsed twice in sterile MilliQ water
96 for 5 minutes each time, before performing mechanical crushing. Ticks were individually crushed in
97 375µL of DMEM with decomplexed Foetal Calf Serum (10%) and six steel beads using the
98 homogenizer Precellys®24 Dual (Bertin, France) at 5500 rpm for 20 seconds.

99 DNA extraction was then performed on 100µL of tick crushing, using the DNA extraction kit
100 NucleoSpin® Tissue (Macherey-Nagel, Germany), and following the standard protocol for human or
101 animal tissue and cultured cells, from the step 2. DNA extracts were eluted in 50µL of elution buffer
102 and then stored at -20°C until further use.

103 Two controls were performed: (1) the crushing control, corresponding to a DMEM tube in which
104 crushing and DNA extraction were performed in the same conditions than on samples; and (2) the
105 extraction control, corresponding the DNA extraction step performed on water.

106

107 *Tick-borne pathogens detection*

108 A high-throughput screening of the most common bacterial and parasitic species of tick-borne
109 pathogens, known to circulate in Europe was performed, allowing us to detect simultaneously the
110 presence of 31 pathogenic species, 7 genera and 1 phylum: the Borrelia genus and eight Borrelia
111 species (*B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. spielmanii*, *B. lusitaniae*, *B. bissettii* and

112 *B. miyamotoi*); the Anaplasma genus and five Anaplasma species (*A. marginale*, *A. phagocytophilum*,
113 *A. platys*, *A. centrale*, *A. bovis*); the Ehrlichia genus and *E. canis*; *Candidatus* Neoehrlichia mikurensis;
114 the Rickettsia genus and six Rickettsia species (*R. conorii*, *R. slovaca*, *R. massiliae*, *R. helvetica*,
115 *R. aeschlimanii* and *R. felis*); the Bartonella genus and *B. henselae*; *Francisella tularensis*;
116 *Coxiella burnettii*; the apicomplexa phylum and seven Babesia species (*B. divergens*, *B. microti*,
117 *B. caballi*, *B. canis*, *B. venatorum*, *B. bovis*, *B. ovis*), but also the two parasitic genus Theileria and
118 Hepatozoon.

119 TBP DNA was detected using the BioMark™ real-time PCR system (Fluidigm, USA), that is a
120 microfluidic system allowing to perform 48 or 96 real-time PCR reactions on 48 or 96 different samples
121 as described in (Michelet *et al.*, 2014; Moutailler *et al.*, 2016). Briefly, each samples and primers/probe
122 set are deposited in individual wells. A pressure system allowed to load them on the chip, via
123 microchannels, in individual reaction chambers of 10nL, where each sample will meet individually each
124 primers/probe set.

125

126 *Primers and probes*

127 Primers and probes used for this analysis are coming from Michelet *et al.* (2014) and Gondard *et*
128 *al.* (2019). They have been designed to specifically amplified pathogens (bacteria and parasites) which
129 are usually found in ticks in Europe. Their sequences, amplicon size, as well as targeted genes and
130 pathogens are registered in Table S1. It is important to note that, because of cross-reaction of
131 primers/probe combination (i.e. design) targeting *B. burgdorferi* s.s. and *B. spielmanii* with respectively
132 *B. garinii*/*B. valaisiana* and *B. afzelii* DNA, positive samples for the two formers were considered as
133 negative when associated to the latter. Therefore, potential associations between
134 *B. burgdorferi* s.s./*B. garinii*, *B. burgdorferi* s.s./*B. valaisiana* and *B. spielmanii*/*B. afzelii* cannot be
135 detected and the co-infection percentage may be under-estimated.

136

137

138 *DNA pre-amplification*

139 Before high throughput real time PCR step, each sample was first pre-amplified using the TaqMan
140 PreAmp Master Mix (Applied Biosystems, France). Basically, the different primer pairs, used for the real
141 time PCR, were pooled combining equal volume of primers with a final concentration of 0.2 μ M. Due
142 to the high concentration of *Escherichia coli* (positive control) or tick DNA, the pre-amplification step
143 was not needed and the corresponding targeting primers were thus not added into the pool. The only
144 one exception was the primer pair named "Tick_spp_16S", targeting mitochondrial DNA, which was
145 added to the 0.2x pool.

146 For each sample, 1.25 μ L of DNA extract was pre-amplified using the Perfecta PreAmp SuperMix
147 reagent (1x) and the 0.2x pool (0.05 μ M), in a final reactive volume of 5 μ L. PCR cycle comprised a first
148 cycle at 98°C for 2 minutes, followed by 14 cycles with 2 steps, the first one at 95°C for 10 seconds and
149 the second one at 60°C for 3 minutes. Pre-amplified DNA were then diluted (1:10) by addition of 45 μ L
150 of sterile deionised water before use.

151

152 *High throughput real time PCR*

153 For each pre-amplified sample, the BioMark™ real-time PCR system (Fluidigm, USA) was used for
154 high-throughput microfluidic real-time PCR amplification using the 48.48 microfluidic dynamic array
155 (Fluidigm Corporation, USA). Amplifications were performed using FAM- and black hole quencher
156 (BHQ1)-labeled TaqMan probes with TaqMan Gene Expression Master Mix in accordance with
157 manufacturer's instructions (Applied Biosystems, France). Thermal cycling conditions were as follows:
158 95°C for 5 min, 45 cycles at 95°C for 10 s, 60°C for 15 s, and 40°C for 10s. Data were acquired on the
159 BioMark Real-Time PCR system and analysed using the Fluidigm Real-Time PCR Analysis software to
160 obtain crossing point (CP) values. Three tick species control (*I. ricinus*, *Dermacentor reticulatus*,
161 *Dermacentor marginatus*), one negative water control and one positive *E. coli* control were included
162 per chip.

163

164 *Nested PCR and sequencing*

165 Samples that were positive only for species-specific design but not for the genus design or only
166 genus design and not for species designs were all re-analysed by nested PCR using primer pairs allowing
167 to target another gene than the one tested into the fluidigm experiment and that is specific to the TBP
168 genus. Their sequences, amplicon size, as well as targeted genes and pathogen genus are registered in
169 Table S2. Amplicons were then sequenced by Eurofins company. Sequences obtained were assembled
170 using the Bioedit software and compared to the database NCBI (National Center for Biotechnology
171 Information) by sequence alignment using nucleotide BLAST (Basic Local Alignment Search Tool).

172

173 *Statistical analysis*

174 *TBP prevalences at the seasonal and multi-annual scale*

175 We tested if the prevalence of TBP was different within and between years of sampling by using a
176 multivariable logistic regression model. We considered the calendar season level for the within-year
177 variability. Seasons were considered as following: Winter = January to February; Spring = March to May;
178 Summer = June to August and Autumn = September to November. A logistic regression model was
179 developed using the TBP status of each nymph as the outcome measure and season, year and the
180 interaction between season and year as explanatory variables. We performed four specific models for
181 the following group/species of TBP: (1) *B. burgdorferi* s.l. (considering *B. burgdorferi* s.s., *B. garinii*,
182 *B. afzelii*, *B. valaisiana* and *B. spielmanii*), (2) *B. miyamotoi*, (3) *A. phagocytophilum*, and (4)
183 *R. helvetica*. The models were constructed with a generalized linear model (GLM, McCullagh and
184 Nelder, 1989) using a binomial distribution (logit link). Model assessment was based on Akaike
185 information criterion (AIC). Results were expressed as odds ratios (OR) and 95% confidence intervals.
186 Statistical computations were performed in R 3.5.1. (R Core Team, 2018).

187

188 *Statistical modelling of tick-borne pathogens associations*

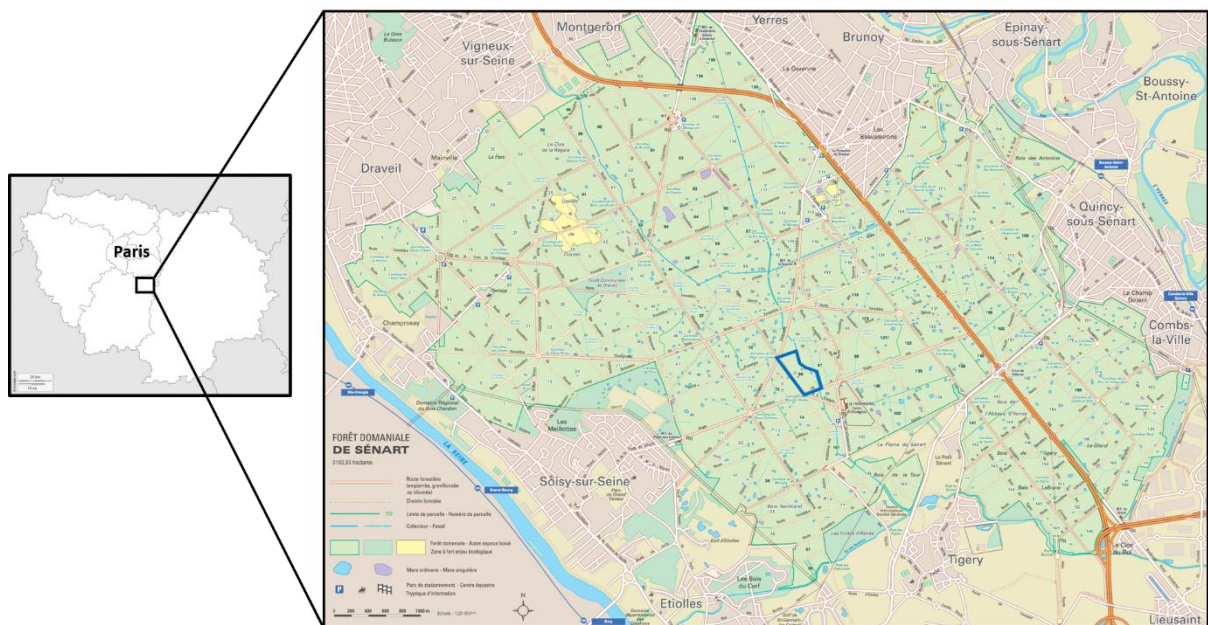
189 We tested the associations between the TBP species that belonged to the co-infection profiles of
190 nymphs found in this study. To do this, we used the association screening approach (Vaumourin *et al.*,
191 2014), which is based on the statistic distribution of the occurrence count of each possible combination
192 of pathogens under the null hypothesis H0 that pathogens associations are random. For a given number
193 of pathogen species tested (NP), the number of possible combination (NC) was calculated as $NC = 2^{NP}$.
194 Assuming similar pathogen prevalence as those observed, a simulated dataset was built as an
195 absence/presence matrix with hosts in lines and pathogen combinations in columns. With 5 000
196 simulations, we obtained the NC statistic distributions. We estimated a 95% confidence envelope to
197 obtain a profile that includes simultaneously all the combinations. From this profile, we inferred for
198 each combination two quantiles, Q_{inf} and Q_{sup} . A global test was based on the 95% confidence
199 envelope. When H0 was rejected, the local tests were based on the NC confidence intervals: [Q_{inf} ;
200 Q_{sup}] (Vaumourin *et al.*, 2014).

201

202 RESULTS

203 *Tick temporal dynamics*

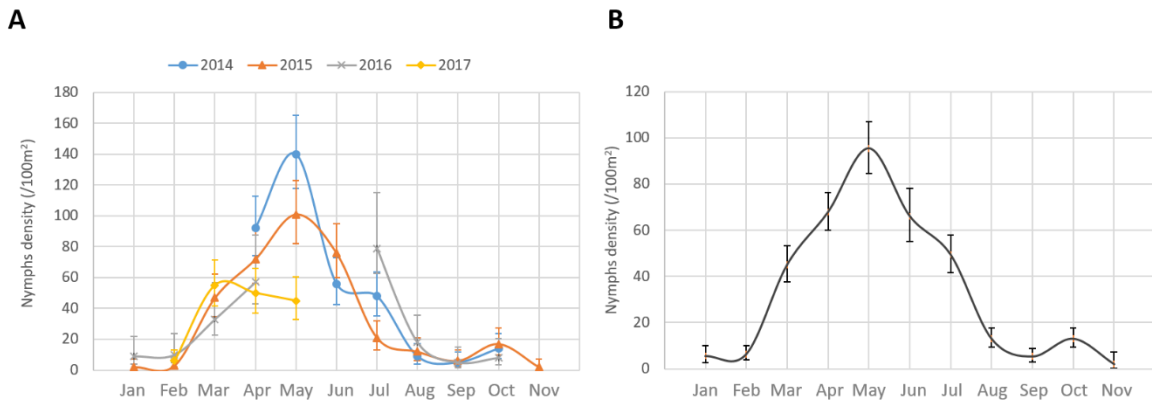
204 From April 2014 to May 2017, a total of 1167 *Ixodes ricinus* ticks were collected in the Sénart forest
205 in the south of Paris (Figure 1). Collected ticks were composed of 1098 nymphs, 35 females and 34
206 males. We focused our temporal analysis on nymphs due to the low number of adults collected over
207 the three years. The temporal dynamics of nymph densities over the three years is shown in Figure 2A.
208 Nymph densities follow similar patterns from one year to another, with a main peak of activity observed
209 every year during spring months, a strong decrease in tick densities during summer and a second peak,
210 smaller, observed in October (Figure 2A). These patterns are more intensively highlighted in the figure
211 2B which presents the average density of nymphs per month. In January and February, the average
212 density is less than 10 questing nymphs, a clear rise is observed from March to May reaching a peak of
213 95.3 nymphs/100m² in May [84.6, 107.0]. Densities then decrease to a minimum of 5.3 nymphs/100m²
214 in September [3.0, 8.7], rise slightly in October (13 nymphs/100m² [9.2, 17.8]) before decreasing again
215 in November (2 nymphs/100m² [0.2, 7.2]).



216
217 **Figure 1. Senart forest, location and parcel map.** Sampling was made on the blue framed parcel.

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225 **Figure 2. *Ixodes ricinus* nymph density.** A: *Ixodes ricinus* nymphs monthly density (/100m²) in 2014, 2015, 2016
226 and 2017. Ticks were sampled from April 2014 to May 2017. Please note that May and June 2016 were
227 unfortunately not sampled. B: Average of nymph density per month (/100m²). The calculation of the average
228 nymph monthly density was made between samples corresponding to the same months through the three years
229 of sampling. Error bars represent the Confidential Interval of the mean, calculated using the Poisson distribution.

230

231 ***Detected pathogens and their prevalence in tick population***

232 Due to technical problems, DNA was extracted and analysed only from 1044 nymphs among the
233 1098 previously mentioned. 46 of them were negative for at least one positive control and thus have
234 been removed from the analysis. From the 998 remaining nymphal DNA samples, 15.9% [13.7%, 18.3%]
235 were positive for at least one tested pathogen, which belong to three bacterial and one parasite genera:
236 *Anaplasma*, *Borrelia*, *Rickettsia* and *Babesia* (Table 1).

237 Pathogens DNA belonging to the *Anaplasma* genus were detected in 5.4% [4.1%, 7.0%] of collected
238 ticks. Most of them were positive for *Anaplasma phagocytophilum* (5.3% of all the samples) and one
239 DNA sample was only positive for the primers/probe combination specific to *Anaplasma* spp.. This
240 sample was retested by nested PCR and the amplicon was then sequenced. The BLAST analysis on NCBI
241 showed that this sequence matched at 99% of identity with four different *Anaplasma* species
242 (*A. phagocytophilum*, *A. marginale*, *A. ovis* and *A. centrale*). Therefore, this sample was only
243 considered as positive for *Anaplasma* spp..

244 Two species of *Rickettsia* were detected in questing *I. ricinus* nymphs. *Rickettsia helvetica* was the
245 most prevalent and was detected in 4.5% [3.3%, 6.0%] of nymphs. *Rickettsia felis* was detected in only
246 one nymph (0.1% [0.003%, 0.6%]). The presence of *R. felis* DNA was confirmed by nested PCR and

247 sequencing as the obtained sequence of 248bp matched with the *R. felis* genome with 100% of identity
248 and 98% of query cover.

249 The genus *Borrelia* was represented by six different species detected in 4.9% [3.7%, 6.4%] of the
250 surveyed nymphs. Five of them belonged to the LB group (3.7% [3.7%, 6.4%]), including
251 *B. burgdorferi* s.s. (1.5% [0.8%, 2.5%]), *B. garinii* (1.1% [0.6%, 2.0%]), *B. afzelii* (1.1% [0.6%, 2.0%]),
252 *B. valaisiana* (0.6% [0.2%, 1.3%]) and *B. spielmanii* (0.4% [0.1%, 1.0%]). DNA of *Borrelia miyamotoi*,
253 belonging to the relapsing fever group, was detected in 1.2% [0.6%, 2.1%] of the surveyed nymphs.

254 DNA from two species of protozoans belonging to the *Babesia* genus were detected in questing
255 nymph: *Babesia venatorum* (1.5% [0.8%, 2.5%] of ticks) and *Babesia divergens* (0.1% [.003%, 0.6%]),
256 detected in one tick).

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273	Months	Analysed nymph number	TBP species											Total							
274			Co-infections (2 TBP)			Co-infections (3 TBP)			B. burgdorferi ss			B. burgdorferi sl									
275			<i>B. burgdorferi</i> ss	<i>B. garhii</i>	<i>B. offeii</i>	<i>B. valaisiana</i>	<i>B. speikmani</i>	<i>B. burgdorferi</i> sl	<i>B. myyamotoi</i>	<i>Borrelia</i> spp	<i>A. phagocytophylum</i>	<i>Anaplasma</i> spp	<i>R. helvetica</i>	<i>R. felis</i>	<i>Rickettsia</i> spp	<i>B. venatorum</i>	<i>B. divergens</i>	<i>Babesia</i> spp	Co-infections (2 TBP)	Co-infections (3 TBP)	
276	2014	89	0	0	0	0	0	0	0	0	10	10	7	0	0	0	1	1	0	0	18
277	Apr-14	127	1	0	0	0	0	1	2	12	12	2	0	2	0	0	4	4	2	0	18
278	May-14	54	0	1	1	0	1	0	1	4	4	9	1	10	2	0	2	0	2	1	16
279	Jun-14	38	0	1	0	1	1	0	1	6	6	1	0	1	0	0	0	0	0	1	8
280	Jul-14	9	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
281	Aug-14	5	1	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	0	2
282	Sep-14	13	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	2
283	Oct-14	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
284	2015	3	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
285	Jan-15	43	0	3	1	1	3	3	6	1	1	5	0	5	0	0	0	0	0	1	12
286	Feb-15	69	2	0	0	0	2	0	2	3	4	4	0	4	0	0	0	0	0	1	10
287	Mar-15	88	0	0	0	0	0	1	1	1	1	3	0	3	0	2	0	0	0	0	7
288	Apr-15	78	1	0	0	1	2	2	4	2	2	1	0	1	0	2	0	2	0	0	8
289	May-15	21	0	2	2	0	3	1	4	0	0	0	0	0	0	0	0	0	0	1	4
290	Jun-15	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
291	Jul-15	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
292	Aug-15	17	4	0	0	0	4	0	4	0	0	0	0	0	0	0	0	0	0	0	4
293	Sep-15	2	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
294	Oct-15	9	0	0	1	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	2
295	Nov-15	10	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	2
296	2016	26	2	1	2	0	0	4	4	1	1	0	0	1	0	0	0	0	0	0	1
297	Jan-16	33	0	0	0	1	0	1	1	0	0	2	0	2	0	0	0	0	0	0	5
298	Feb-16	78	2	2	2	0	1	5	5	2	2	4	0	4	0	1	0	1	1	1	11
	Mar-16	11	2	0	0	0	2	0	2	0	0	2	0	2	0	0	0	0	0	1	3
	Apr-16	5	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1
	May-16	6	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
	Jun-16	6	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
	Jul-16	53	0	1	0	1	0	2	2	3	3	0	0	0	0	2	0	2	0	0	7
	Aug-16	50	0	0	1	1	0	2	2	3	3	0	0	0	0	1	0	1	0	0	6
	Sep-16	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	Oct-16	6	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
	Nov-16	6	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
	Dec-16	6	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
	2017	53	0	1	0	1	0	2	2	3	3	0	0	0	0	2	0	2	0	0	7
	Jan-17	50	0	0	1	1	0	2	2	3	3	0	0	0	0	1	0	1	0	0	6
	Feb-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	Mar-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	Apr-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	May-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	Jun-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	Jul-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	Aug-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	Sep-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	Oct-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	Nov-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	Dec-17	41	0	0	0	0	0	1	1	4	4	1	0	1	0	0	0	0	0	0	6
	2018	998	15	11	11	6	4	37	12	49	53	54	45	1	46	15	1	16	10	3	159
	Apr-14 to May-17	30	8	7	8	6	4	18	9	23	14	14	16	1	16	8	1	9	9	3	27
	Number of positive months																				

Table 1. Summary table of the TBP detection study results.

299 **Temporal patterns of TBP in nymphal *I. ricinus***

300 *TBP prevalence at the monthly scale*

301 Global infection rates fluctuated over the three years from a minimum of 8% [3.3%, 15.7%] in May
302 2015 to a maximum of 29.6% [18.0%, 43.6%] in June 2014, yet monthly variation was not significant
303 probably due the low sample sizes ($p > 0.05$ according to Chi² test; Figure 3).

304 At the genus level, variations in TBP prevalences and the number of months for which at least one
305 tick was positive for each tested TBP are presented in Figure 4 and Table 1.

306 DNA from pathogens belonging to both genera *Rickettsia* and *Anaplasma* were detected
307 respectively in 16 and 14 of the 30 sampled months respectively. When detected, prevalence fluctuated
308 from 1.3% [0.03%, 6.9%] (June 2015) to 18.5% [9.3%, 31.4%] (June 2014) for *Rickettsia* and from
309 1.1% [0.03%, 6.2%] (May 2015) to 15.8% [6.0%, 31.3%] (July 2014) for *Anaplasma*. Both genera are
310 mainly represented by one species: *R. helvetica* and *A. phagocytophilum* that are the most frequently
311 detected species (16 and 14 /30 months respectively). These two species were found each sampled
312 year.

313 DNA from members of the *Borrelia* genus was detected in 23 of the 30 sampled months. This
314 bacterial genus displayed the highest variability with monthly prevalences fluctuating from
315 1.1% [0.03%, 6.2%] (May 2015) to 23.5% [6.8%, 49.9%] (October 2015). DNA from members of the LB
316 group was detected in 18 of the 30 sampled months with prevalences ranging from 0.8% [0.03%, 6.2%]
317 in May 2014 to 23.5% [6.8%, 49.9%] in October 2015. The most frequently identified species were
318 *B. burgdorferi* s.s. (8 / 30 sampled months), *B. afzelii* (8 / 30) and *B. garinii* (7 / 30). DNA from these
319 species was regularly detected over the three studied years. Conversely, *B. valaisiana* (6 / 30) and
320 *B. spielmanii* (4 / 30) DNA were not detected during 11 (from April 2015 to March 2016) and 9 (from
321 July 2015 to April 2016) consecutive sampled months respectively. *Borrelia miyamotoi* (relapsing fever
322 group) DNA was detected 9 times over the 30 sampled months with prevalences ranging between
323 0.8% [0.02%, 4.3%] in May 2014 and 7% [1.5%, 19.1%] in March 2015. Despite a prevalence and a
324 frequency of detection that is comparable to *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*, this species

325 DNA was not detected during two main periods: from June 2014 to March 2015 (7 samplings) and from
326 January to September 2016 (7 samplings, but two sampling months were missing in this period). It was
327 detected 5 times over 9 samplings from March to November 2015 and detected 3 times over 5
328 samplings from October 2016 to May 2017.

329 For parasites, DNA from the genus *Babesia* was detected in 9 months out of 30 sampled months.
330 Prevalences presented the lowest variability ranging from 1.1% [0.03%, 6.1%] in April 2014 to
331 3.8% [0.5%, 13.0%] in March 2017 (Figure 4). The main detected species DNA was those of
332 *B. venatorum* that was detected 9 times over 30 samplings and not detected during 9 consecutive
333 sampled months, from June 2015 to April 2016.

334

335

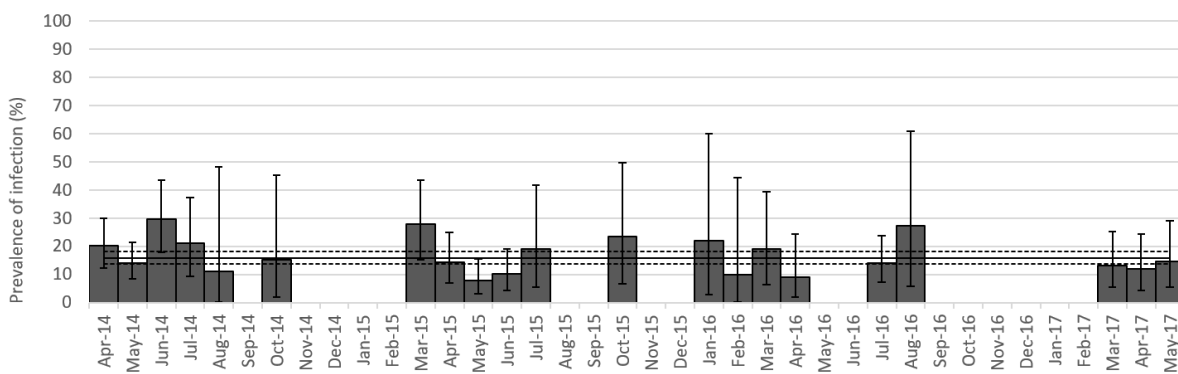
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341 **Figure 3. Nymph infection rate per month for at least one tested pathogen.** Months with less than 9 nymphs
342 sampled have not been considered for percentage calculation. Error bars represent confidence intervals of the
343 percentage.

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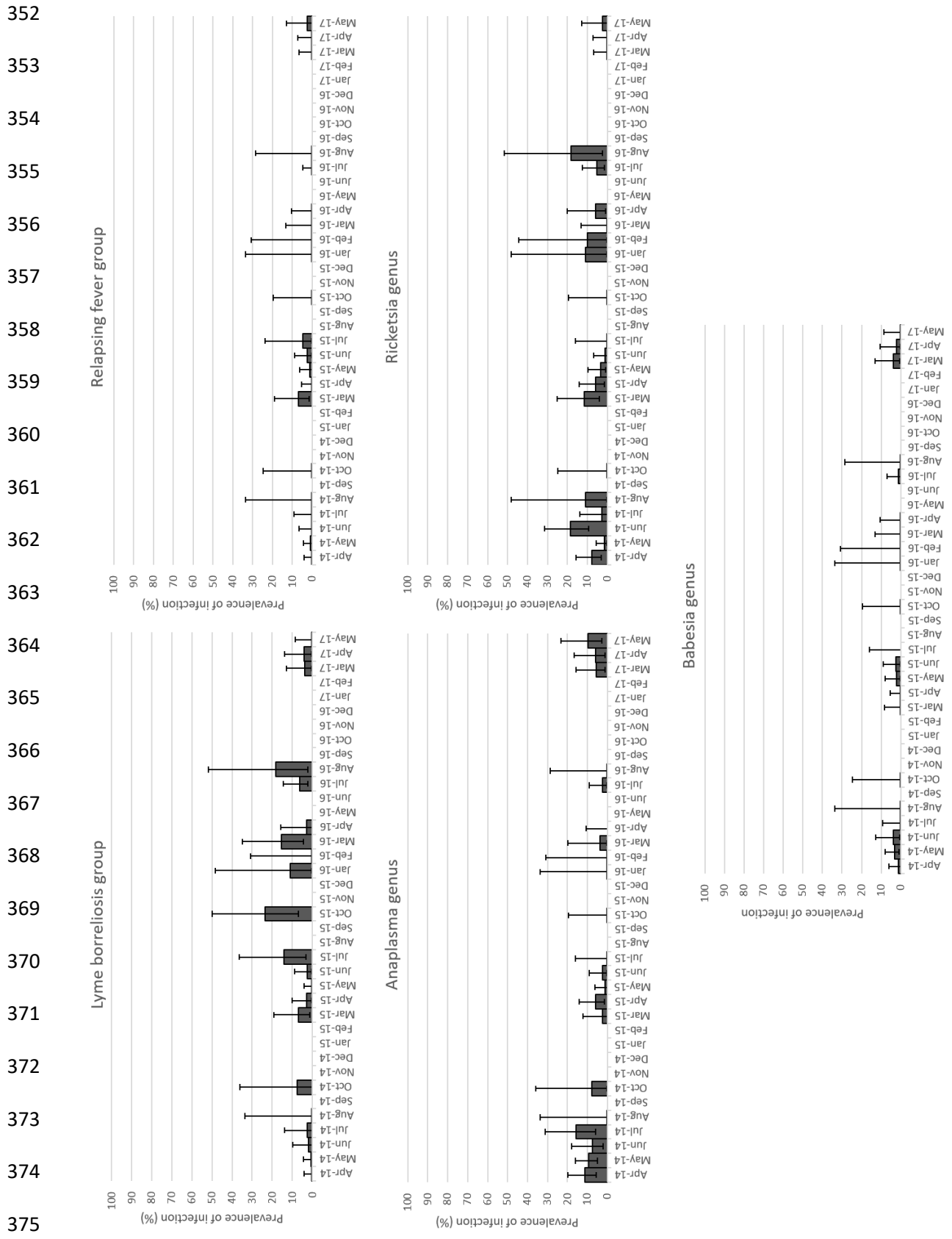
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376 **Figure 4. Nymph infection rate and confidence intervals per month for the different TBP. Months with less than**
 377 **9 nymphs sampled have not been considered. Error bars represent confidence intervals of the percentage.**

378

379 *TBP prevalence at the seasonal and multi-annual scale*

380 In order to determine if the prevalence of TBP was different within and between years of sampling,
381 a multivariable logistic regression model was performed. Because some samplings were missing and
382 because of the low number of nymphs collected in some months, the statistical analysis was performed
383 according to calendar seasons. Spring season and year 2014 have been considered as references for
384 the seasonal and yearly effect respectively. Because some TBP had too low prevalence in the nymph
385 population producing unreliable statistics, analyses were only performed on the most prevalent TBP:
386 *A. phagocytophilum*, *R. helvetica*, *B. burgdorferi* s.l., *B. miyamotoi* and *B. venatorum*.

387 Significant differences were observed at the seasonal scale (Table 2, Figure 5) for *R. helvetica*
388 (higher in summer compared to spring), *B. burgdorferi* s.l. (higher in autumn compared to spring) and
389 *B. miyamotoi* (higher in winter than in spring). It must be noticed that winter is the season with the
390 smallest number of sampled ticks (30 in total), and that the difference observed for *B. miyamotoi* in
391 winter correspond to only one tick infected sampled in February 2017.

392 Significant differences were also observed between years for bacteria belonging to the complex
393 *B. burgdorferi* s.l. with higher infection rates in 2015 and 2016 compared to 2014; for
394 *A. phagocytophilum*, which was lower in 2015 and in 2016 compared to 2014 and for *R. helvetica*,
395 which was lower in 2017 than in 2014. However, please note that samplings were only performed from
396 January to May in 2017. No significant differences were observed according to season or year for
397 *B. venatorum*.

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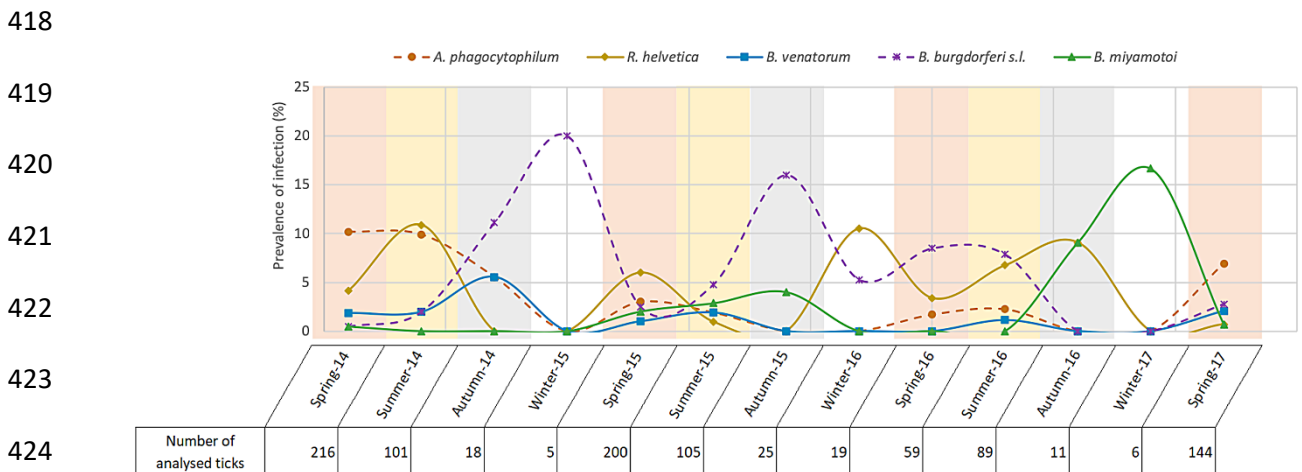
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403

	Model	TBP	Variable	Odds Ratio	95% Confidence Interval		
					Low	High	
404							
405	(1)	<i>B. burgdorferi</i> s.l.	Spring		REF		
			Autumn	4.53	1.50	12.49	**
406			Summer	1.69	0.75	3.89	
			Winter	1.73	0.25	7.01	
407			2014		REF		
			2015	2.93	1.12	9.14	**
			2016	4.48	1.60	14.53	**
408			2017	2.45	0.57	9.95	
409	(2)	<i>B. miyamotoi</i>	Spring		REF		
			Autumn	0.00	NA	8.3275E+218	
			Summer	0.00	NA	2.26397E+88	
			Winter	28.60	1.03	800.00	**
410	(3)	<i>A. phagocytophilum</i>	2014		REF		
			2015	0.20	0.08	0.42	**
411			2016	0.16	0.04	0.45	**
			2017	0.65	0.30	1.32	
412	(4)	<i>R. helvetica</i>	Spring		REF		
			Autumn	0.00	0.00	6.7759E+11	
			Summer	3.10	1.27	7.85	**
413			Winter	0.00	NA	1.1447E+145	
			2014		REF		
			2015	1.34	0.54	3.39	
414			2016	0.81	0.12	3.24	
			2017	0.16	0.01	0.87	**

415 **Table 2. Multivariable logistic regression models assessing the seasonal and yearly TBP prevalence variations**
 416 **in nymphs.** Odds ratios and their associated 95% confidence intervals obtained from the best model of TBP
 417 seasonal and yearly prevalence in questing nymphs.



425 **Figure 5. Percentage of positive nymphs per season for the most prevalent TBPs.** Winter (white background)=
 426 January to February – Spring (orange background)= March to May – Summer (yellow background)= June to August
 427 – Autumn (grey background)= September to November.

428

429 **Pathogen associations**

430 Among all the sampled ticks, 1% [0.5%, 1.8%] were co-infected with two pathogens and
 431 0.3% [0.006%, 0.8%] were co-infected with three pathogens. Eight different co-infection profiles were
 432 found (Table 3). In most of cases (7/13), these co-infections concerned species belonging to the *Borrelia*
 433 genus: *B. garinii/B. afzelii*; *B. garinii/B. spielmanii*; *B. garinii/B. afzelii/B. valaisiana* and
 434 *B. garinii/B. valaisiana/B. spielmanii*. Co-infections profiles with species belonging to different genus
 435 were also observed: *A. phagocytophilum/B. venatorum*; *A. phagocytophilum/R. helvetica*;
 436 *B. burgdorferi s.s./R. helvetica* and *B. garinii/B. afzelii/R. helvetica*. All these associations between
 437 pathogens were tested using the association screening approach (Vaumourin *et al.*, 2014). Compared
 438 to a random analysis, no associations were found to be under represented while two were over
 439 represented: the first one between *B. garinii* and *B. afzelii* (observation = 3; min expected = 0; max
 440 expected = 2), and the second one between *B. garinii* and *B. spielmanii* (observation = 2; min expected
 441 = 0; max expected = 1).

442

443

444

	<i>B. burgdorferi s.s.</i>	<i>B. garinii</i>	<i>B. afzelii</i>	<i>B. valaisiana</i>	<i>B. spielmanii</i>	<i>A. phagocytophilum</i>	<i>R. helvetica</i>	<i>B. venatorum</i>	Co-occurrences number
445	X	X							3
	X			X					2
446	X	X	X						1
	X		X	X					1
447					X		X		3
					X	X			1
448	X					X			1
		X	X			X			1

449 **Table 3. Summary table of the reported co-infection profiles.**

450 **DISCUSSION**

451 ***Ixodes ricinus* density and seasonal dynamics**

452 This three-year survey demonstrated a clear seasonal pattern in *I. ricinus* density, with a marked
453 peak of questing nymphs in spring and a smaller peak in autumn. Low, but present activity was detected
454 in winter, as has been observed in Germany (Dautel *et al.*, 2008). In addition to these general patterns,
455 some unexpected data were observed, the most striking being no peak activity in spring 2017 (April
456 and May) with tick densities very similar to those recorded in March. Abiotic factors such as
457 temperature, relative humidity, and rainfall, or fluctuating host numbers in the sampling area are
458 known to influence questing tick abundance and activity patterns (Perret *et al.*, 2000; Gilbert, 2010;
459 Tagliapietra *et al.*, 2011; Schulz *et al.*, 2014; Vourc'h *et al.*, 2016; Marchant *et al.*, 2017) and could
460 explain these unusual observations. It's important to note that 2017 was distinguished by an
461 abnormally wet March, with total rainfall much higher than that recorded in previous years in the same
462 area (71.3, compared to 11.2, 33.6, and 61.7 mm rain/month in 2014, 2015, and 2016, respectively.
463 Interestingly, the increased March rainfall was followed by an April drought (7.9 mm of rain/month in
464 2017, compared to 48.4, 27.2, and 66.2 mm rain/month in 2014, 2015, and 2016, respectively) (rainfall
465 data estimated from the Orly station, Météo-France data;
466 https://donneespubliques.meteofrance.fr/?fond=produit&id_produit=90&id_rubrique=32). These unusual meteorological
467 characteristics could explain the stable tick density from March to May 2017. Thereby, this finding
468 clearly shows that the bimodal tick activity pattern usually observed during this study can punctually
469 change with exceptional environmental conditions, reinforcing the importance of regular monitoring.

470

471 ***Ixodes ricinus*-borne pathogen composition and prevalence over the three years**

472 Most of the detected pathogen species corresponded to micro-organisms known to circulate in the
473 Western Palearctic (Capelli *et al.*, 2012; Overzier *et al.*, 2013; Pangrácová *et al.*, 2013; Reye *et al.*, 2013;
474 Hansford *et al.*, 2015; Paul *et al.*, 2016; Sormunen *et al.*, 2016; Raileanu *et al.*, 2017). However, several
475 species belonging to the *Bartonella* and *Francisella* genera, previously reported in the studied area

476 (Reis *et al.*, 2010; Paul *et al.*, 2016), were not detected. The most prevalent pathogen species were
477 *A. phagocytophilum* (5.4% of the examined nymphs), *R. helvetica* (4.5%), and *B. burgdorferi* s.l. (3.7%).
478 Both high- and low-prevalence TBP were consistently detected in the sampling area for the duration of
479 the study. Although prevalence varied between different TBP, and some were not detected for long
480 periods, they were all detected recurrently. Continued detection is consistent with the year-round
481 presence of reservoir hosts in the sampling area (wood mice, bank voles, Siberian chipmunks, roe deer,
482 common blackbird, European robin, song thrush...) (Marsot *et al.*, 2012, 2013; Marchant *et al.*, 2017).
483 The continued presence of reservoir hosts could facilitate the circulation of dominant species, and
484 maintain, even at low rates, less prevalent pathogen species. These findings lend further support to
485 regularly studying TBP temporal dynamics to assess and monitor changing infection risks.

486 We also highlighted the infrequent presence of some TBP, such as *B. divergens*, which was only
487 detected in a single tick, as also reported by Paul *et al.* (2016). *I. ricinus* can host and transmit both
488 *B. divergens* and *B. venatorum* (Bonnet *et al.*, 2007a; Bonnet *et al.*, 2007b; Bonnet *et al.*, 2009), the
489 etiological agents of human babesiosis (Centeno-Lima *et al.*, 2003; Herwaldt *et al.*, 2003). Human
490 babesiosis is known to be a rare disease, but is classified as a potential emerging disease in Europe
491 (Hildebrandt *et al.*, 2013; Rizzoli *et al.*, 2014; Oechslin *et al.*, 2017). While *Babesia venatorum* is known
492 to circulate in roe deer (Bonnet *et al.*, 2007a) of which there are many in the Sénart forest, *Babesia*
493 *divergens* circulates in bovine species absent from the Sénart forest, which may explain its low
494 prevalence among tick populations. Similarly, we detected *R. felis* DNA in only one nymph. This bacteria
495 is also considered to be an emergent human pathogen, and has been associated with clinical
496 manifestations in patients from America, Asia, Africa, and Europe (see in Angelakis *et al.*, 2016). Its
497 detection is particularly interesting as this bacteria is known to be mainly transmitted from cat to cat
498 *via* fleas, with human contamination arising from cat or flea bites. Nevertheless, several studies have
499 detected the presence of *R. felis* or *R. felis*-like organisms in hematophagous arthropods (see in Reif
500 and Macaluso, 2009; Brown and Macaluso, 2016), including ticks collected from the natural
501 environment (Oliveira *et al.*, 2008), and notably in *Ixodes* species (Ishikura *et al.*, 2013). *R. felis* has been

502 reported in *I. ricinus* in two previous studies on questing ticks (Vayssier-Taussat *et al.*, 2013; Lejal *et al.*,
503 2018). The Vayssier-Taussat's one was based on RNA detection, demonstrating viability and active
504 replication of this bacteria in *I. ricinus*. Rarely investigated in studies dealing with TBP, the repeated
505 detection of *R. felis* should encourage increased surveillance for this spotted fever-causing pathogen in
506 humans. Let's finally note that all these findings suggest that a punctual sampling would certainly not
507 facilitate the detection of these pathogens, again highlighting the importance of collecting and
508 analysing ticks at a large temporal scale.

509

510 ***Seasonal and inter-annual dynamics of I. ricinus-borne pathogens***

511 Improving the prevention of TBD requires a better understanding of their temporal—and in
512 particular—their seasonal dynamics. However, only a few studies have addressed these issues during
513 a minimum three-year period (Coipan *et al.*, 2013; Takken *et al.*, 2016). As ticks were collected monthly
514 for over three years in this study, we detected significant seasonal or annual infection rate fluctuations
515 for four TBP: *R. helvetica*, *B. burgdorferi* s.l., *B. miyamotoi*, and *A. phagocytophilum*. Note that the
516 statistically significant highest prevalence of *B. miyamotoi* in winter is only due to the detection of one
517 positive tick sampled during winter in 2017. In our opinion, this result alone is insufficient to presume
518 that *B. miyamotoi* have an increased winter prevalence. However, we can observe that even if very few
519 ticks are questing during these periods, they may carry TBP.

520 While significant seasonal and annual differences were observed for *B. miyamotoi* and
521 *A. phagocytophilum*, respectively, the presence of *R. helvetica* and *B. burgdorferi* s.l. varied
522 significantly according to both seasons and years. None of these micro-organisms presented a similar
523 pattern to any others. Comparing our results to the pluri-annual studies previously mentioned, we
524 observe that only *R. helvetica* presented similar seasonal patterns (Coipan *et al.*, 2013). This finding
525 again emphasises how the season, the year or the sampling area can influence TBP presence and
526 prevalence in questing tick populations.

527 The most common explanation for temporal variations in TBP prevalence is the variable availability
528 of reservoir hosts during tick previous stage feeding. Because the tick lifecycle is fundamentally linked
529 to its host, any changes to the available host spectrum will undoubtedly influence TBP prevalence in
530 the tick community (see in Pfäffle *et al.*, 2013). Moreover, contact rates between ticks and their
531 reservoir hosts have been suggested as a major factor driving seasonal TBP prevalence (Estrada-Peña
532 and de la Fuente, 2016). Furthermore, Coipan *et al* (2013) observed that several micro-organisms
533 presumed to share the same reservoir host, also presented similar seasonal detection patterns, thereby
534 supporting the previous hypothesis. However, an alternate hypothesis could also explain these
535 patterns, as carrying certain TBP was shown to improve tick resistance to challenging abiotic conditions.
536 Herrmann and Gern (2010, 2012) demonstrated that ticks carrying *Borrelia* species exhibited higher
537 survival rates in desiccating conditions and a lower affinity for humid conditions than non-infected
538 ticks, suggesting a potentially higher prevalence of *Borrelia*-infected questing ticks during or after
539 summer. Similarly, Neelakanta (2010) demonstrated a higher expression of *iafgp* gene, coding for an
540 antifreeze glycoprotein, in *A. phagocytophilum*-infected ticks. This thus conferred to ticks a stronger
541 resistance to cold that could lead to higher prevalence of *A. phagocytophilum*-infected questing ticks
542 during or just after winter. In this context, TBP prevalence in questing tick populations could be also
543 influenced by abiotic meteorological conditions. Additionally, this hypothesis coincides with
544 observations from Coipan, Takken, and the current study concerning *B. burgdorferi* s.l. infected
545 questing ticks. Indeed, a higher prevalence was always observed during and/or after summer,
546 potentially implying an improved survival rate of infected ticks during hot and dry summer conditions.
547 In contrast, *A. phagocytophilum* was not observed in greater prevalence during the cold season.

548 Our results, in combination with those from the literature, support the hypothesis that TBP
549 prevalence is influenced by both biotic and abiotic factors, and suggest one more time that undertaking
550 regular TBP analysis is an optimal method by which to improve knowledge on TBP dynamics and to
551 better understand TBD epidemiology.

552

553 ***Pathogen co-occurrence***

554 Tick co-infections are being identified more and more frequently (Halos *et al.*, 2005; Schicht *et al.*,
555 2011; Andersson *et al.*, 2013; Cosson *et al.*, 2014; Castro *et al.*, 2015; Moutailler *et al.*, 2016; Raileanu
556 *et al.*, 2017; Lejal *et al.*, 2018). Clinical co-infections with several TBP are commonly reported (Tijssse-
557 Klasen *et al.*, 2013; Moniuszko *et al.*, 2014; Hoversten and Bartlett, 2018) and are known to affect both
558 disease symptoms and severity (Krause *et al.*, 1996; Diuk-Wasser *et al.*, 2016). It is thus essential to
559 investigate TBP associations in ticks, to better identify potential clinical co-infections and to improve
560 epidemiological knowledge of TBD.

561 In this longitudinal three-year study, two TBP associations were significantly over-represented
562 compared to a random distribution: the first was between *B. garinii* and *B. afzelii*, as has been
563 previously observed in studies using similar detection tools (Moutailler *et al.*, 2016; Raileanu *et al.*,
564 2017), or different methods (16s rRNA gene sequencing, Aivelo *et al.*, 2019); the second was between
565 *B. garinii* and *B. spielmanii*. Interestingly, these findings contrast with published results on
566 *Ixodes ricinus* TBP. While performing a meta-analysis on data published from 2010 to 2016, Strnad *et*
567 *al* (2017) observed a negative correlation between *B. garinii* and *B. afzelii*. Similarly, Herrmann *et al.*
568 (2013) also detected a negative co-occurrence between these two species following the analysis of
569 7400 nymphs collected over three years. These results are coherent considering the host specificity of
570 these Borrelia species. Indeed, *B. garinii* doesn't share the same reservoir host (birds) than *B. afzelii* or
571 *B. spielmanii* (wood mice and bank voles, or hazel and garden dormice) (Humair *et al.*, 1998;
572 Kurtenbach *et al.*, 1998; Huegli *et al.*, 2002; Richter *et al.*, 2004, 2011; Taragel'ova *et al.*, 2008), and
573 none of these species are known to be transmitted transovarially.

574 Even though the associations we identified were statistically "over-represented", in actual fact we
575 only observed one more association than the fixed over-representation threshold (i.e. observed
576 associations = 3 and 2; minimum expected = 0 and 0; maximum expected = 2 and 1; for
577 *B. garinii/B. afzelii* and *B. garinii/B. spielmanii* associations, respectively). This indicates that caution
578 should be applied when drawing conclusions about permanent associations between these different

579 bacteria in ticks. Several different hypotheses could potentially explain these associations in the same
580 nymph. Firstly, hosts are likely to carry several adjacent feeding ticks. This phenomenon, known as co-
581 feeding, could promote pathogen exchange between ticks even in the absence of systemic host
582 infection (Randolph *et al.*, 1996). Secondly, as discussed by van Duijvendijk *et al.* (2016), when
583 bloodmeals are disrupted due to host grooming, immune response or death, ticks may feed on more
584 than one host to completely engorge, and consequently be exposed to several pathogens. Thirdly,
585 despite these TBP species segregating between bird and rodent hosts, all of them have been detected
586 in hedgehogs (Skuballa *et al.*, 2007, 2012), and *B. afzelii* and *B. garinii* have been simultaneously
587 detected in one Siberian chipmunk (Marsot *et al.*, 2013). Both of these mammals were found to host a
588 large number of tick larvae (Gern *et al.*, 1997; Marsot *et al.*, 2013), and Siberian chipmunks have been
589 reported to induce higher *B. burgdorferi* s.l. infection rates in nymphs, compared to bank voles and
590 wood mice (Marsot *et al.*, 2013) in the Sénart forest. A last hypothesis might be that our analyses
591 methods are unable to distinguish the rodent-circulating *B. garinii* OspA serotype 4 (corresponding to
592 *B. bavariensis*) (Margos *et al.*, 2009) from other *B. garinii* serotypes.

593 Associations between *B. garinii* and *B. valaisiana* are frequently reported, which isn't surprising
594 as these species share the same reservoir host (Hanincova *et al.*, 2003). This association was the most
595 common TBP association in a meta-analysis of literature published between 1984 and 2003 (Rauter
596 and Hartung, 2005), and has been reported several times since in later studies (Reye *et al.*, 2010;
597 Lommano *et al.*, 2012; Herrmann *et al.*, 2013). While we observed this association twice, both times in
598 association with a third *Borrelia* species, either *B. afzelii* or *B. spielmanii*, it was not significantly over-
599 represented compared to a random distribution. Among the three previously mentioned studies, only
600 Herrmann *et al.* (2013) demonstrated that this association was over-represented when compared to a
601 randomly sampled analysis. However our study was performed on a much smaller dataset (998 versus
602 7400 analysed nymphs), with a halved co-infection percentage (1.3% versus 3%), indicating that our
603 statistical analysis may be less powerful, which could explain why this association wasn't detected.

604 These contrasting tick pathogen association results highlight the complexity in clearly identifying
605 pathogen associations in field-collected ticks. Several other parameters can also potentially influence
606 pathogen association (host spectrum within the studied area, sample size influencing analytical
607 statistical power, identification bias...). In this context, performing investigations under controlled
608 conditions (suitable TBP growing and tick breeding systems...) will be a crucial future step to
609 experimentally test these different associations and improve our knowledge on TBP co-occurrence.

610

611 **CONCLUSIONS**

612 This three-year study of *I. ricinus*-borne pathogens; (1) identified several TBP previously reported
613 in the area, consistent with reservoir host availability; (2) highlighted the surprising presence of micro-
614 organisms not normally reported in questing ticks such as *R. felis*; (3) highlighted significant variations
615 in seasonal and inter-annual pathogen prevalence; and finally (4) identified several unexpected co-
616 occurrences between pathogens belonging to the *B. burgdorferi* s.l. complex. All these data underline
617 the importance of regularly performing tick collections and TBP analysis to improve our knowledge on
618 TBP presence and dynamics, and to better understand the ecology and epidemiology of TBD.

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631

632 **AUTHOR CONTRIBUTIONS**

633 Conceived and designed the experiments: TP, MVT, JFC, KCM, EL. Performed the experiments: EL.

634 Analysed the data: EL, TP, SM, MM, KCM. Wrote the paper: EL, MM, KCM, JFC, SM, MVT, TP

635 **REFERENCES**

- 636 Aivelo, T., Norberg, A., and Tschirren, B. (2019) Human pathogen co-occurrence in *Ixodes ricinus* ticks:
637 effects of landscape topography, climatic factors and microbiota interactions. *bioRxiv* 559245.
- 638 Andersson, M., Bartkova, S., Lindestad, O., and Råberg, L. (2013) Co-Infection with 'Candidatus
639 Neoehrlichia mikurensis' and *Borrelia afzelii* in *Ixodes ricinus* Ticks in Southern Sweden. *Vector-*
640 *Borne Zoonotic Dis.* **13**: 438–442.
- 641 Angelakis, E., Mediannikov, O., Parola, P., and Raoult, D. (2016) *Rickettsia felis*: The Complex Journey of
642 an Emergent Human Pathogen. *Trends Parasitol.* **32**: 554–564.
- 643 Bonnet, S., Brisseau, N., Hermouet, A., Jouglin, M., and Chauvin, A. (2009) Experimental in vitro
644 transmission of *Babesia* sp. (EU1) by *Ixodes ricinus*. *Vet. Res.* **40**: 1–8.
- 645 Bonnet, S., Jouglin, M., L'Hostis, M., and Chauvin, A. (2007a) *Babesia* sp. EU1 from Roe Deer and
646 Transmission within *Ixodes ricinus*. *Emerg. Infect. Dis.* **13**: 1208–1210.
- 647 Bonnet, S., Jouglin, M., Malandrin, L., Becker, C., Agoulon, A., L'hostis, M., and Chauvin, A. (2007b)
648 Transstadial and transovarial persistence of *Babesia divergens* DNA in *Ixodes ricinus* ticks fed
649 on infected blood in a new skin-feeding technique. *Parasitology* **134**: 197–207.
- 650 Brown, L.D. and Macaluso, K.R. (2016) *Rickettsia felis*, an Emerging Flea-Borne Rickettsiosis. *Curr. Trop.*
651 *Med. Rep.* **3**: 27–39.
- 652 Capelli, G., Ravagnan, S., Montarsi, F., Ciocchetta, S., Cazzin, S., Porcellato, E., et al. (2012) Occurrence
653 and identification of risk areas of *Ixodes ricinus*-borne pathogens: a cost-effectiveness analysis
654 in north-eastern Italy. *Parasit. Vectors* **5**: 61.
- 655 Castro, L.R., Gabrielli, S., Iori, A., and Cancrini, G. (2015) Molecular detection of *Rickettsia*, *Borrelia*, and
656 *Babesia* species in *Ixodes ricinus* sampled in northeastern, central, and insular areas of Italy.
657 *Exp. Appl. Acarol.* **66**: 443–452.
- 658 Centeno-Lima, S., Do Rosário, V., Parreira, R., Maia, A.J., Freudenthal, A.M., Nijhof, A.M., and Jongejan,
659 F. (2003) A fatal case of human babesiosis in Portugal: molecular and phylogenetic analysis.
660 *Trop. Med. Int. Health* **8**: 760–764.
- 661 Choi, Y.-J., Lee, S.-H., Park, K.-H., Koh, Y.-S., Lee, K.-H., Baik, H.-S., et al. (2005) Evaluation of PCR-Based
662 Assay for Diagnosis of Spotted Fever Group Rickettsiosis in Human Serum Samples. *Clin Diagn*
663 *Lab Immunol* **12**: 759–763.
- 664 Chvostáč, M., Špitalská, E., Václav, R., Vaculová, T., Minichová, L., and Derdáková, M. (2018) Seasonal
665 Patterns in the Prevalence and Diversity of Tick-Borne *Borrelia burgdorferi* Sensu Lato,
666 *Anaplasma phagocytophilum* and *Rickettsia* spp. in an Urban Temperate Forest in South
667 Western Slovakia. *Int. J. Environ. Res. Public Health* **15**: 994.
- 668 Coipan, E.C., Jahfari, S., Fonville, M., Maassen, C., van der Giessen, J., Takken, W., et al. (2013)
669 Spatiotemporal dynamics of emerging pathogens in questing *Ixodes ricinus*. *Front. Cell. Infect.*
670 *Microbiol.* **3**.
- 671 Cosson, J.-F., Michelet, L., Chotte, J., Le Naour, E., Cote, M., Devillers, E., et al. (2014) Genetic
672 characterization of the human relapsing fever spirochete *Borrelia miyamotoi* in vectors and
673 animal reservoirs of Lyme disease spirochetes in France. *Parasit. Vectors* **7**: 233.
- 674 Cotté, V., Bonnet, S., Le Rhun, D., Le Naour, E., Chauvin, A., Boulouis, H.-J., et al. (2008) Transmission of
675 *Bartonella henselae* by *Ixodes ricinus*. *Emerg. Infect. Dis.* **14**: 1074–1080.
- 676 Dantas-Torres, F., Chomel, B.B., and Otranto, D. (2012) Ticks and tick-borne diseases: a One Health
677 perspective. *Trends Parasitol.* **28**: 437–446.
- 678 Dautel, H., Dippel, C., Kämmer, D., Werkhausen, A., and Kahl, O. (2008) Winter activity of *Ixodes ricinus*
679 in a Berlin forest. *Int. J. Med. Microbiol.* **298**: 50–54.
- 680 Diuk-Wasser, M.A., Vannier, E., and Krause, P.J. (2016) Coinfection by *Ixodes* Tick-Borne Pathogens:
681 Ecological, Epidemiological, and Clinical Consequences. *Trends Parasitol.* **32**: 30–42.
- 682 van Duijvendijk, G., Coipan, C., Wagemakers, A., Fonville, M., Ersöz, J., Oei, A., et al. (2016) Larvae of
683 *Ixodes ricinus* transmit *Borrelia afzelii* and *B. miyamotoi* to vertebrate hosts. *Parasit. Vectors*
684 **9**.

- 685 Estrada-Peña, A. and de la Fuente, J. (2016) Species interactions in occurrence data for a community of
686 tick-transmitted pathogens. *Sci. Data* **3**: 160056.
- 687 de la Fuente, J., Estrada-Peña, A., Venzal, J.M., Kocan, K.M., and Sonenshine, D.E. (2008) Overview: ticks
688 as vectors of pathogens that cause disease in humans and animals. *Front Biosci* **13**: 6938–6946.
- 689 Gassner, F., van Vliet, A.J.H., Burgers, S.L.G.E., Jacobs, F., Verbaarschot, P., Hovius, E.K.E., et al. (2010)
690 Geographic and Temporal Variations in Population Dynamics of *Ixodes ricinus* and Associated
691 *Borrelia* Infections in The Netherlands. *Vector-Borne Zoonotic Dis.* **11**: 523–532.
- 692 Gern, L., Rouvinez, E., Toutoungi, L.N., and Godfroid, E. (1997) Transmission cycles of *Borrelia*
693 *burgdorferi* sensu lato involving *Ixodes ricinus* and/or *I. hexagonus* ticks and the European
694 hedgehog, *Erinaceus europaeus*, in suburban and urban areas in Switzerland. *Folia Parasitol.*
695 (*Praha*) **44**: 309–314.
- 696 Gilbert, L. (2010) Altitudinal patterns of tick and host abundance: a potential role for climate change in
697 regulating tick-borne diseases? *Oecologia* **162**: 217–225.
- 698 Gilbert, L., Maffey, G.L., Ramsay, S.L., and Hester, A.J. (2012) The effect of deer management on the
699 abundance of *Ixodes ricinus* in Scotland. *Ecol. Appl.* **22**: 658–667.
- 700 Gondard, M., Delannoy, S., Pinarello, V., Aprelon, R., Devillers, E., Galon, C., et al. (2019) Upscaling
701 surveillance of tick-borne pathogens in the French Caribbean islands. *bioRxiv*.
- 702 Halos, L., Jamal, T., Maillard, R., Beugnet, F., Menach, A.L., Boulouis, H.-J., and Vayssier-Taussat, M.
703 (2005) Evidence of *Bartonella* sp. in questing adult and nymphal *Ixodes ricinus* ticks from
704 France and co-infection with *Borrelia burgdorferi* sensu lato and *Babesia* sp. *Vet. Res.* **36**: 79–
705 87.
- 706 Hanincova, K., Taragelova, V., Koci, J., Schafer, S.M., Hails, R., Ullmann, A.J., et al. (2003) Association of
707 *Borrelia garinii* and *B. valaisiana* with Songbirds in Slovakia. *Appl. Environ. Microbiol.* **69**: 2825–
708 2830.
- 709 Hansford, K.M., Fonville, M., Jahfari, S., Sprong, H., and Medlock, J.M. (2015) *Borrelia miyamotoi* in
710 host-seeking *Ixodes ricinus* ticks in England. *Epidemiol. Infect.* **143**: 1079–1087.
- 711 Herrmann, C. and Gern, L. (2012) Do the level of energy reserves, hydration status and *Borrelia*
712 infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks? *Parasitology* **139**: 330–337.
- 713 Herrmann, C. and Gern, L. (2010) Survival of *Ixodes ricinus* (Acari: Ixodidae) Under Challenging
714 Conditions of Temperature and Humidity is Influenced by *Borrelia burgdorferi* sensu lato
715 Infection. *J. Med. Entomol.* **47**: 1196–1204.
- 716 Herrmann, C., Gern, L., and Voordouw, M.J. (2013) Species co-occurrence patterns among Lyme
717 borreliosis pathogens in the tick vector *Ixodes ricinus*. *Appl. Environ. Microbiol.* AEM.02158-13.
- 718 Herwaldt, B.L., Cacciò, S., Gherlinzoni, F., Aspöck, H., Slemenda, S.B., Piccaluga, P., et al. (2003)
719 Molecular Characterization of a Non-*Babesia divergens* Organism Causing Zoonotic Babesiosis
720 in Europe. *Emerg. Infect. Dis.* **9**: 943–948.
- 721 Hildebrandt, A., Gray, J.S., and Hunfeld, K.-P. (2013) Human Babesiosis in Europe: what clinicians need
722 to know. *Infection* **41**: 1057–1072.
- 723 Hoversten, K. and Bartlett, M.A. (2018) Diagnosis of a tick-borne coinfection in a patient with persistent
724 symptoms following treatment for Lyme disease. *Case Rep.* **2018**: bcr-2018-225342.
- 725 Huegli, D., Hu, C.M., Humair, P.-F., Wilske, B., and Gern, L. (2002) Apodemus Species Mice Are Reservoir
726 Hosts of *Borrelia garinii* OspA Serotype 4 in Switzerland. *J. Clin. Microbiol.* **40**: 4735–4737.
- 727 Humair, P.-F., Postic, D., Wallich, R., and Gern, L. (1998) An Avian Reservoir (*Turdus merula*) of the Lyme
728 Borreliosis Spirochetes. *Zentralblatt Für Bakteriologie* **287**: 521–538.
- 729 Ishikura, M., Ando Shuji, Shinagawa Yasuhiro, Matsuura Kumiko, Hasegawa Sumiyo, Nakayama Takashi,
730 et al. (2013) Phylogenetic Analysis of Spotted Fever Group Rickettsiae Based on *gltA*, 17-kDa,
731 and *rOmpA* Genes Amplified by Nested PCR from Ticks in Japan. *Microbiol. Immunol.* **47**: 823–
732 832.
- 733 Jongejan, F. and Uilenberg, G. (2004) The global importance of ticks. *Parasitology* **129**: S3–S14.
- 734 Krause, P.J., Telford, S.R., Spielman, A., Sikand, V., Ryan, R., Christianson, D., et al. (1996) Concurrent
735 Lyme Disease and Babesiosis: Evidence for Increased Severity and Duration of Illness. *JAMA*
736 **275**: 1657–1660.

- 737 Kurtenbach, K., Carey, D., Hoodless, A.N., Nuttall, P.A., and Randolph, S.E. (1998) Competence of
738 Pheasants as Reservoirs for Lyme Disease Spirochetes. *J. Med. Entomol.* **35**: 77–81.
- 739 Lejal, E., Moutailler, S., Simo, L., Vayssier-Taussat, M., and Pollet, T. (2018) Tick-borne pathogen
740 detection in midgut and salivary glands of adult *Ixodes ricinus*. *bioRxiv* 489328.
- 741 Loh, S.-M., Gofton, A.W., Lo, N., Gillett, A., Ryan, U.M., Irwin, P.J., and Oskam, C.L. (2016) Novel *Borrelia*
742 species detected in echidna ticks, *Bothriocroton concolor*, in Australia. *Parasit. Vectors* **9**.
- 743 Lommano, E., Bertaiola, L., Dupasquier, C., and Gern, L. (2012) Infections and co-infections of questing
744 *Ixodes ricinus* ticks by emerging zoonotic pathogens in Western Switzerland. *Appl. Environ.*
745 *Microbiol.* AEM.07961-11.
- 746 Marchant, A., Coupanec, A.L., Joly, C., Perthame, E., Sertour, N., Garnier, M., et al. (2017) Infection of
747 *Ixodes ricinus* by *Borrelia burgdorferi* sensu lato in peri-urban forests of France. *PLOS ONE* **12**:
748 e0183543.
- 749 Margos, G., Vollmer, S.A., Cornet, M., Garnier, M., Fingerle, V., Wilske, B., et al. (2009) A New *Borrelia*
750 Species Defined by Multilocus Sequence Analysis of Housekeeping Genes. *Appl. Environ.*
751 *Microbiol.* **75**: 5410–5416.
- 752 Marsot, M., Chapuis, J.-L., Gasqui, P., Dozières, A., Masségli, S., Pisanu, B., et al. (2013) Introduced
753 Siberian Chipmunks (*Tamias sibiricus barberi*) Contribute More to Lyme Borreliosis Risk than
754 Native Reservoir Rodents. *PLOS ONE* **8**: e55377.
- 755 Marsot, M., Henry, P.-Y., Vourc'h, G., Gasqui, P., Ferquel, E., Laignel, J., et al. (2012) Which forest bird
756 species are the main hosts of the tick, *Ixodes ricinus*, the vector of *Borrelia burgdorferi* sensu
757 lato, during the breeding season? *Int. J. Parasitol.* **42**: 781–788.
- 758 Masatani, T., Hayashi, K., Andoh, M., Tateno, M., Endo, Y., Asada, M., et al. (2017) Detection and
759 molecular characterization of *Babesia*, *Theileria*, and *Hepatozoon* species in hard ticks collected
760 from Kagoshima, the southern region in Japan. *Ticks Tick-Borne Dis.* **8**: 581–587.
- 761 McCullagh, P. and Nelder, J.A. (1989) Generalized Linear Models. 2nd Edition. Chapman et Hall, London.
- 762 Michelet, L., Delannoy, S., Devillers, E., Umhang, G., Aspan, A., Juremalm, M., et al. (2014) High-
763 throughput screening of tick-borne pathogens in Europe. *Front. Cell. Infect. Microbiol.* **4**.
- 764 Moniuszko, A., Dunaj, J., Świącicka, I., Zambrowski, G., Chmielewska-Badora, J., Żukiewicz-Sobczak, W.,
765 et al. (2014) Co-infections with *Borrelia* species, *Anaplasma phagocytophilum* and *Babesia* spp.
766 in patients with tick-borne encephalitis. *Eur. J. Clin. Microbiol. Infect. Dis.* **33**: 1835–1841.
- 767 Moutailler, S., Moro, C.V., Vaumourin, E., Michelet, L., Tran, F.H., Devillers, E., et al. (2016) Co-infection
768 of Ticks: The Rule Rather Than the Exception. *PLoS Negl. Trop. Dis.* **10**: e0004539.
- 769 Neelakanta, G., Sultana, H., Fish, D., Anderson, J.F., and Fikrig, E. (2010) *Anaplasma phagocytophilum*
770 induces *Ixodes scapularis* ticks to express an antifreeze glycoprotein gene that enhances their
771 survival in the cold. *J. Clin. Invest.* **120**: 3179–3190.
- 772 Oechslin, C.P., Heutschi, D., Lenz, N., Tischhauser, W., Péter, O., Rais, O., et al. (2017) Prevalence of tick-
773 borne pathogens in questing *Ixodes ricinus* ticks in urban and suburban areas of Switzerland.
774 *Parasit. Vectors* **10**: 558.
- 775 Oliveira, K., S Oliveira, L., C A Dias, C., Silva, A., R Almeida, M., Almada, G., et al. (2008) Molecular
776 identification of *Rickettsia felis* in ticks and fleas from an endemic area for Brazilian Spotted
777 Fever.
- 778 Overzier, E., Pfister, K., Herb, I., Mahling, M., Böck, G., and Silaghi, C. (2013) Detection of tick-borne
779 pathogens in roe deer (*Capreolus capreolus*), in questing ticks (*Ixodes ricinus*), and in ticks
780 infesting roe deer in southern Germany. *Ticks Tick-Borne Dis.* **4**: 320–328.
- 781 Pangráčová, L., Derdáková, M., Pekárik, L., Hviščová, I., Víchová, B., Stanko, M., et al. (2013) *Ixodes*
782 *ricinus* abundance and its infection with the tick-borne pathogens in urban and suburban areas
783 of Eastern Slovakia. *Parasit. Vectors* **6**: 238.
- 784 Paul, R.E.L., Cote, M., Le Naour, E., and Bonnet, S.I. (2016) Environmental factors influencing tick
785 densities over seven years in a French suburban forest. *Parasit. Vectors* **9**: 309.
- 786 Perret, J.-L., Guigoz, E., Rais, O., and Gern, L. (2000) Influence of saturation deficit and temperature on
787 *Ixodes ricinus* tick questing activity in a Lyme borreliosis-endemic area (Switzerland). *Parasitol.*
788 *Res.* **86**: 554–557.

- 789 Pfäffle, M., Littwin, N., Muders, S.V., and Petney, T.N. (2013) The ecology of tick-borne diseases. *Int. J.*
790 *Parasitol.* **43**: 1059–1077.
- 791 R Core Team (2018) R: A Language and Environment for Statistical Computing R Foundation for
792 Statistical Computing, Vienna, Austria.
- 793 Raileanu, C., Moutailler, S., Pavel, I., Porea, D., Mihalca, A.D., Savuta, G., and Vayssier-Taussat, M. (2017)
794 *Borrelia* Diversity and Co-infection with Other Tick Borne Pathogens in Ticks. *Front. Cell. Infect.*
795 *Microbiol.* **7**:.
- 796 Randolph, S.E., Gern, L., and Nuttall, P.A. (1996) Co-feeding ticks: Epidemiological significance for tick-
797 borne pathogen transmission. *Parasitol. Today* **12**: 472–479.
- 798 Rar, V.A., Fomenko, N.V., Dobrotvorsky, A.K., Livanova, N.N., Rudakova, S.A., Fedorov, E.G., et al. (2005)
799 Tickborne Pathogen Detection, Western Siberia, Russia. *Emerg. Infect. Dis.* **11**: 1708–1715.
- 800 Rauter, C. and Hartung, T. (2005) Prevalence of *Borrelia burgdorferi* Sensu Lato Genospecies in *Ixodes*
801 *ricinus* Ticks in Europe: a Metaanalysis. *Appl. Environ. Microbiol.* **71**: 7203–7216.
- 802 Reif, K.E. and Macaluso, K.R. (2009) Ecology of *Rickettsia felis*: A Review. *J. Med. Entomol.* **46**: 723–736.
- 803 Reis, C., Cote, M., Paul, R.E.L., and Bonnet, S. (2010) Questing Ticks in Suburban Forest Are Infected by
804 at Least Six Tick-Borne Pathogens. *Vector-Borne Zoonotic Dis.* **11**: 907–916.
- 805 Reye, A.L., Hübschen, J.M., Sausy, A., and Muller, C.P. (2010) Prevalence and Seasonality of Tick-Borne
806 Pathogens in Questing *Ixodes ricinus* Ticks from Luxembourg. *Appl. Environ. Microbiol.* **76**:
807 2923–2931.
- 808 Reye, A.L., Stegny, V., Mishaeva, N.P., Velhin, S., Hübschen, J.M., Ignatyev, G., and Muller, C.P. (2013)
809 Prevalence of Tick-Borne Pathogens in *Ixodes ricinus* and *Dermacentor reticulatus* Ticks from
810 Different Geographical Locations in Belarus. *PLOS ONE* **8**: e54476.
- 811 Richter, D., Schlee, D.B., Allgower, R., and Matuschka, F.-R. (2004) Relationships of a Novel Lyme Disease
812 Spirochete, *Borrelia spielmani* sp. nov., with Its Hosts in Central Europe. *Appl. Environ.*
813 *Microbiol.* **70**: 6414–6419.
- 814 Richter, D., Schlee, D.B., and Matuschka, F.-R. (2011) Reservoir Competence of Various Rodents for the
815 Lyme Disease Spirochete *Borrelia spielmanii*. *Appl. Environ. Microbiol.* **77**: 3565–3570.
- 816 Rizzoli, A., Silaghi, C., Obiegala, A., Rudolf, I., Hubálek, Z., Földvári, G., et al. (2014) *Ixodes ricinus* and
817 Its Transmitted Pathogens in Urban and Peri-Urban Areas in Europe: New Hazards and
818 Relevance for Public Health. *Front. Public Health* **2**:.
- 819 Schicht, S., Junge, S., Schnieder, T., and Strube, C. (2011) Prevalence of *Anaplasma phagocytophilum*
820 and Coinfection with *Borrelia burgdorferi* Sensu Lato in the Hard Tick *Ixodes ricinus* in the City
821 of Hanover (Germany). *Vector-Borne Zoonotic Dis.* **11**: 1595–1597.
- 822 Schulz, M., Mahling Monia, and Pfister Kurt (2014) Abundance and seasonal activity of questing *Ixodes*
823 *ricinus* ticks in their natural habitats in southern Germany in 2011. *J. Vector Ecol.* **39**: 56–65.
- 824 Skuballa, J., Oehme, R., Hartelt, K., Petney, T., Bücher, T., Kimmig, P., and Taraschewski, H. (2007)
825 European Hedgehogs as Hosts for *Borrelia* spp., Germany. *Emerg. Infect. Dis.* **13**: 952–953.
- 826 Skuballa, J., Petney, T., Pfäffle, M., Oehme, R., Hartelt, K., Fingerle, V., et al. (2012) Occurrence of
827 different *Borrelia burgdorferi* sensu lato genospecies including *B. afzelii*, *B. bavariensis*, and *B.*
828 *spielmanii* in hedgehogs (*Erinaceus* spp.) in Europe. *Ticks Tick-Borne Dis.* **3**: 8–13.
- 829 Sormunen, J.J., Penttinen, R., Klemola, T., Hänninen, J., Vuorinen, I., Laaksonen, M., et al. (2016) Tick-
830 borne bacterial pathogens in southwestern Finland. *Parasit. Vectors* **9**: 168.
- 831 Sprong, H., Wielinga, P.R., Fonville, M., Reusken, C., Brandenburg, A.H., Borgsteede, F., et al. (2009)
832 *Ixodes ricinus* ticks are reservoir hosts for *Rickettsia helvetica* and potentially carry flea-borne
833 *Rickettsia* species. *Parasit. Vectors* **2**: 41.
- 834 Strle, F. (2004) Human granulocytic ehrlichiosis in Europe. *Int. J. Med. Microbiol. Suppl.* **293**: 27–35.
- 835 Strnad, M., Hönig, V., Růžek, D., Grubhoffer, L., and Rego, R.O.M. (2017) Europe-Wide Meta-Analysis of
836 *Borrelia burgdorferi* Sensu Lato Prevalence in Questing *Ixodes ricinus* Ticks. *Appl. Environ.*
837 *Microbiol.* **83**:.
- 838 Tagliapietra, V., Rosà, R., Arnoldi, D., Cagnacci, F., Capelli, G., Montarsi, F., et al. (2011) Saturation deficit
839 and deer density affect questing activity and local abundance of *Ixodes ricinus* (Acari, Ixodidae)
840 in Italy. *Vet. Parasitol.* **183**: 114–124.

- 841 Takken, W., van Vliet, A.J.H., Verhulst, N.O., Jacobs, F.H.H., Gassner, F., Hartemink, N., et al. (2016)
842 Acarological Risk of *Borrelia burgdorferi* Sensu Lato Infections Across Space and Time in The
843 Netherlands. *Vector-Borne Zoonotic Dis.* **17**: 99–107.
- 844 Taragel'ova, V., Koci, J., Hanincova, K., Kurtenbach, K., Derdakova, M., Ogden, N.H., et al. (2008)
845 Blackbirds and Song Thrushes Constitute a Key Reservoir of *Borrelia garinii*, the Causative Agent
846 of Borreliosis in Central Europe. *Appl. Environ. Microbiol.* **74**: 1289–1293.
- 847 Tijssse-Klasen, E., Sprong, H., and Pandak, N. (2013) Co-infection of *Borrelia burgdorferi* sensu lato and
848 *Rickettsia* species in ticks and in an erythema migrans patient. *Parasit. Vectors* **6**: 347.
- 849 Vayssier-Taussat, M., Moutailler, S., Michelet, L., Devillers, E., Bonnet, S., Cheval, J., et al. (2013) Next
850 Generation Sequencing Uncovers Unexpected Bacterial Pathogens in Ticks in Western Europe.
851 *PLOS ONE* **8**: e81439.
- 852 Vourc'h, G., Abrial, D., Bord, S., Jacquot, M., Masségli, S., Poux, V., et al. (2016) Mapping human risk
853 of infection with *Borrelia burgdorferi* sensu lato, the agent of Lyme borreliosis, in a periurban
854 forest in France. *Ticks Tick-Borne Dis.* **7**: 644–652.
- 855 van Wieren, S.E. and Hofmeester, T.R. (2016) 6. The role of large herbivores in *Ixodes ricinus* and
856 *Borrelia burgdorferi* s.l. dynamics. In, *Ecology and prevention of Lyme borreliosis*, Ecology and
857 Control of Vector-borne diseases. Wageningen Academic Publishers, pp. 75–89.
858

859 SUPPORTING INFORMATION

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
<i>Borrelia burgdorferi</i>	rpoB	Forward	GCTTACTCACAAAAGCGTCTT	83	Michelet <i>et al.</i> , 2014
		Reverse	GCACATCTCTTACTTCAAATCCT		
		Probe	AATGCTCTTGGACCAGGAGGACTTTCA		
<i>Borrelia garinii</i>	rpoB	Forward	TGGCCGAACCTACCCACAAA	88	Michelet <i>et al.</i> , 2014
		Reverse	ACATCTCTTACTTCAAATCCTGC		
		Probe	TCTATCTCTTGAAGTCCCCCTGGTCC		
<i>Borrelia afzelii</i>	fla	Forward	GGAGCAAATCAAGATGAAGCAAT	116	Michelet <i>et al.</i> , 2014
		Reverse	TGAGCACCTCTTGAACAGG		
		Probe	TGCAGCCTGAGCAGCTTGAAGCTCC		
<i>Borrelia valaisiana</i>	ospA	Forward	ACTCACAAATGACAGATGCTGAA	135	Michelet <i>et al.</i> , 2014
		Reverse	GCTTGCTTAAAGTAACAGTACCT		
		Probe	TCCGCCTACAAGATTTCTGGAAGCTT		
<i>Borrelia lusitaniae</i>	rpoB	Forward	CGAACTTACTCATAAAAAGCGTC	87	Michelet <i>et al.</i> , 2014
		Reverse	TGGAGTCTCTTACTTCAAATCC		
		Probe	TTAATGCTCTCGGGCTGGGGACT		
<i>Borrelia spielmanii</i>	fla	Forward	ATCTATTTCTGGTGAGGGAGC	71	Michelet <i>et al.</i> , 2014
		Reverse	TCCTTCTTGTGAGCACCTTC		
		Probe	TTGAACAGGCGCAGTCTGAGCAGCTT		
<i>Borrelia bissettii</i>	rpoB	Forward	GCAACCAGTCAGCTTTCACAG	87	Michelet <i>et al.</i> , 2014
		Reverse	CAAATCCTGCCCTATCCCTTG		
		Probe	AAAGTCTCCCGCCCAAGAGCATTAA		
<i>Borrelia miyamotoi</i>	glpQ	Forward	CACGACCCAGAAATTGACACA	94	Michelet <i>et al.</i> , 2014
		Reverse	GTGTGAAGTCAGTGGCGTAAT		
		Probe	TCGTCCGTTTTCTCTAGCTCGATTGGG		
<i>Borrelia</i> spp.	23S	Forward	GAGTCTTAAAAGGGCGATTTAGT	73	Michelet <i>et al.</i> , 2014
		Reverse	CTTCAGCCTGGCCATAAATAG		
		Probe	AGATGTGGTAGACCCGAAGCCGAGT		
<i>Anaplasma marginale</i>	msp1	Forward	CAGGCTTCAAGCGTACAGTG	85	Michelet <i>et al.</i> , 2014
		Reverse	GATATCTGTGCTGGCCTTC		
		Probe	ATGAAAGCCTGGAGATGTTAGACCGAG		
<i>Anaplasma platys</i>	groEL	Forward	TTCTGCCGATCCTTGAAAACG	75	Michelet <i>et al.</i> , 2014
		Reverse	CTTCTCCTTCTACATCCTCAG		
		Probe	TTGCTAGATCCGGCAGCCTCTGC		
<i>Anaplasma phagocytophilum</i>	msp2	Forward	GCTATGGAAGGCAGTGTGG	77	Michelet <i>et al.</i> , 2014
		Reverse	GTCTTGAAGCGCTCGTAACC		
		Probe	AATCTCAAGCTCAACCTGGCACCAC		
<i>Anaplasma centrale</i>	groEL	Forward	AGCTGCCCTGCTATACACG	79	Michelet <i>et al.</i> , 2014
		Reverse	GATGTTGATGCCCAATTGCTC		
		Probe	CTTGATCTCTAGACGAGGTAAGGGG		
<i>Anaplasma bovis</i>	groEL	Forward	GGGAGATAGTACACATCCTTG	73	Gondard <i>et al.</i> , 2019
		Reverse	CTGATAGCTACAGTTAAGCCC		
		Probe	AGGTGCTGTTGGATGTAAGTCTGGACC		
<i>Anaplasma</i> spp.	16S	Forward	CTTAGGGTTGTA AAACTCTTTCAG	160	Gondard <i>et al.</i> , 2019
		Reverse	CTTTAACTTACCAAACCGCTAC		
		Probe	ATGCCCTTACGCCAATAATTCCGAACA		
<i>Ehrlichia</i> spp.	16S	Forward	GCAACGCGAAAACTTACCA	98	Gondard <i>et al.</i> , 2019
		Reverse	AGCCATGCAGCACCTGTGT		
		Probe	AAGTCCAGCCAAACTGACTCTTCCG		
<i>Ehrlichia canis</i>	glfA	Forward	GACCAAGCAGTTGATAAAGATGG	136	Gondard <i>et al.</i> , 2019
		Reverse	CACTATAAGACAATCCATGATTAGG		
		Probe	ATTA AAACATCCTAAGATAGCAGTGGCTAAGG		

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(Continued)

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
<i>Candidatus Neoehrlichia mikurensis</i>	groEL	Forward	AGAGACATCATTTCGATTTTGGGA	96	Michelet <i>et al.</i> , 2014
		Reverse	TTCCGGTGTAACATAAGGCTT		
		Probe	AGATGCTGTTGGATGTACTGCTGGACC		
<i>Rickettsia conorii</i>	23S-5S ITS	Forward	CTCACAAAGTTATCAGGTTAAATAG	118	Michelet <i>et al.</i> , 2014
		Reverse	CGATACTCAGCAAATAATTCTCG		
		Probe	CTGGATATCGTGGCAGGGCTACAGTAT		
<i>Rickettsia slovaca</i>	23S-5S ITS	Forward	GTATCTACTCACAAAGTTATCAGG	138	Michelet <i>et al.</i> , 2014
		Reverse	CTTAACTTTTACTACAATACTCAGC		
		Probe	TAATTTTCGCTGGATATCGTGGCAGGG		
<i>Rickettsia massiliae</i>	23S-5S ITS	Forward	GTTATTGCATCACTAATGTTATACTG	128	Michelet <i>et al.</i> , 2014
		Reverse	GTTAATGTTGTTGCAGCACTCAA		
		Probe	TAGCCCCGCCACGATATCTAGCAAAAA		
<i>Rickettsia helvetica</i>	23S-5S ITS	Forward	AGAACCGTAGCGTACACTTAG	79	Michelet <i>et al.</i> , 2014
		Reverse	GAAAACCTACTTCTAGGGGT		
		Probe	TACGTGAGGATTTGAGTACCGGATCGA		
<i>Rickettsia aeschlimannii</i>	ITS	Forward	CTCACAAAGTTATCAGGTTAAATAG	134	Michelet <i>et al.</i> , 2014
		Reverse	CTTAACTTTTACTACGATACTTAGCA		
		Probe	TAATTTTCTGGATATCGTGGCGGGG		
<i>Rickettsia felis</i>	orfB	Forward	ACCCTTTTCGTAACGCTTTGC	163	Gondard <i>et al.</i> , 2019
		Reverse	TATACTTAATGCTGGGCTAAACC		
		Probe	AGGGAAACCTGGACTCCATATTCAAAAGAG		
<i>Rickettsia</i> spp.	gltA	Forward	GTCGCAATGTTACGCTACTT	78	Gondard <i>et al.</i> , 2019
		Reverse	TCTTCGTGCACTTTCTTCCATTG		
		Probe	TGCAATAGCAAGAACCGTAGGCTGGATG		
<i>Bartonella henselae</i>	pap31	Forward	CCGCTGATCGCATTATGCCT	107	Michelet <i>et al.</i> , 2014
		Reverse	AGCGATTTCTGCATCATCTGCT		
		Probe	ATGTTGCTGGTGGTTCCTATGCAC		
<i>Bartonella</i> spp.	ssrA	Forward	CGTTATCGGGCTAAATGAGTAG	118	Gondard <i>et al.</i> , 2019
		Reverse	ACCCCGCTTAAACCTGCGA		
		Probe	TTGCAATGACAACTATGCGGAAGCACGTC		
<i>Francisella tularensis</i>	tul4	Forward	ACCCACAAGGAAGTGAAGATTA	76	Michelet <i>et al.</i> , 2014
		Reverse	GTAATTGGGAAGCTTGATCATG		
		Probe	AATGGCAGGCTCCAGAAGTTCTAAGT		
	fopA	Forward	GGCAAATCTAGCAGGTCAAGC	91	Michelet <i>et al.</i> , 2014
		Reverse	CAACACTTGCTTGAACATTTCTAG		
		Probe	AACAGGTGCTTGGATGTGGGTGGTG		
<i>Coxiella burnetii</i>	IS1111	Forward	TGGAGGAGCGAACCATTGGT	86	Michelet <i>et al.</i> , 2014
		Reverse	CATACGGTTTGACGTGCTGC		
		Probe	ATCGGACGTTTATGGGGATGGGTATCC		
icd	Forward	AGGCCGTCCGTTATTTTACG	74	Michelet <i>et al.</i> , 2014	
	Reverse	CGGAAAATCACCATATTCACCTT			
	Probe	TTCAGGCGTTTTGACCGGGCTTGGC			
<i>Apycomplexa</i>	18S	Forward	TGAACGAGGAATGCCTAGTATG	104	Gondard <i>et al.</i> , 2019
		Reverse	CACCGGATCACTCGATCGG		
		Probe	TAGGAGCGACGGCGGTGTGTAC		
<i>Babesia microti</i>	CCTeta	Forward	ACAATGGATTTTCCCAGCAAAA	145	Michelet <i>et al.</i> , 2014
		Reverse	GCGACATTTCCGCAACTTATATA		
		Probe	TACTCTGGTCAATGAGCGTATGGGTA		
<i>Babesia canis</i>	hsp70	Forward	TCACTGTGCCTGCTACTTC	87	Michelet <i>et al.</i> , 2014
		Reverse	TGATACGCATGACGTTGAGAC		
		Probe	AACGACTCCCAGGCCAGGCCAC		

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
<i>Babesia ovis</i>	18S	Forward	TCTGTGATGCCCTTAGATGTC	92	Michelet <i>et al.</i> , 2014
		Reverse	GCTGGTTACCCGCGCCTT		
		Probe	TCGGAGCGGGGTCAACTCGATGCAT		
<i>Babesia bovis</i>	CCTeta	Forward	GCCAAGTAGTGGTAGACTGTA	100	Michelet <i>et al.</i> , 2014
		Reverse	GCTCCGCATTGGTTATGGTA		
		Probe	TAAAGACAACACTGGGTCCGCGTGG		
<i>Babesia caballi</i>	rap1	Forward	GTTGTTCGGCTGGGGCATC	94	Michelet <i>et al.</i> , 2014
		Reverse	CAGGCGACTGACGCTGTGT		
		Probe	TCTGTCCCGATGTCAAGGGGCAGGT		
<i>Babesia venatorum</i> (sp. EU1)	18S	Forward	GCGCGCTACACTGATGCATT	91	Michelet <i>et al.</i> , 2014
		Reverse	CAAAAAATCAATCCCCGTCACG		
		Probe	CATCGAGTTTAATCCTGTCCCGAAAGG		
<i>Babesia divergens</i>	hsp70	Forward	GCGCGCTACACTGATGCATT	91	Michelet <i>et al.</i> , 2014
		Reverse	CAAAAAATCAATCCCCGTCACG		
		Probe	CATCGAGTTTAATCCTGTCCCGAAAGG		
<i>Theileria</i> spp.	18S	Forward	GTCAGTTTTTACGACTCCTTCAG	213	Michelet <i>et al.</i> , 2014
		Reverse	CCAAAGAATCAAGAAAAGACTATC		
		Probe	AATCTGTCAATCCTTCTTTGTCTGGACC		
<i>Hepatozoon</i> spp.	18S	Forward	ATTGGCTTACCGTGGCAGTG	175	Gondard <i>et al.</i> , 2019
		Reverse	AAAGCATTTTAACTGCCTTGATTG		
		Probe	ACGGTTAACGGGGATTAGGGTTCGAT		
<i>Ixodes ricinus</i>	ITS2	Forward	CGAAACTCGATGGAGACCTG	77	Michelet <i>et al.</i> , 2014
		Reverse	ATCTCCAACGCACCGACGT		
		Probe	TTGTGAAATCCCGTCGCACGTTGAAC		
Tick spp	16S	Forward	AAATACTCTAGGGATAACAGCGT	99	Gondard <i>et al.</i> , 2019
		Reverse	TCTTCATCAAACAAGTATCCTAATC		
		Probe	CAACATCGAGGTCGCAAACCATTTGTCTA		
<i>Dermacentor reticulatus</i>	ITS2	Forward	AACCTTTTCCGCTCCGTG	83	Michelet <i>et al.</i> , 2014
		Reverse	TTTTGCTAGAGCTCGACGTAC		
		Probe	TACGAAGGCAAACAACGCAAACCTGCGA		
<i>Dermacentor marginatus</i>	ITS2	Forward	GCACGTTGCGTTGTTGCC	139	Michelet <i>et al.</i> , 2014
		Reverse	CCGCTCCGCGCAAGAATCT		
		Probe	TTCGGAGTACGTCGAGCTTAGCAGA		
<i>Escherichia coli</i>	eae	Forward	CATTGATCAGGATTTTTCTGGTGATA	102	Michelet <i>et al.</i> , 2014
		Reverse	CTCATGCGGAAATAGCCGTTA		
		Probe	ATAGTCTCGCCAGTATTCGCCACCAATACC		

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863 **Table S1. Targeted gene, amplicon size, primers and probe sequences used for TBP and Tick species**
864 **detection.**

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Targeted genus	Targeted gene	Name	Sequence	Reference
Babesia; Theileria; Hepatozoon	18s rRNA gene	BTH 18S 1st F	GTGAAACTGCGAATGGCTCATTAC	Masatani <i>et al.</i> , 2017
		BTH 18S 1st R	AAGTGATAAGGTTCAAAAACCTCCC	
		BTH 18S 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG	
		BTH 18S 2nd R	CGGTCCGAATAATTCACCGGAT	
Anaplasma; Ehrlichia	16s rRNA gene	EHR1	GAACGAACGCTGGCGCAAGC	Rar <i>et al.</i> , 2005
		EHR2	AGTA(T/C)CG(A/G)ACCAGATAGCCGC	
		EHR3	TGCATAGGAATCTACCTAGTAG	
		EHR2	AGTA(T/C)CG(A/G)ACCAGATAGCCGC	
Borrelia	flaB	FlaB280F	GCAATCARTCAGGTAACGG	Loh <i>et al.</i> , 2016
		FlaRL	GCAATCATAGCCATTGCAATTGT	
		flaB_737F	GCATCAACTGTRGTTGTAACATTAACAGG	
		FlaLL	ACATATTCAGATGCAGACAGAGGT	
Rickettsia	rompB	Rc.rompB.4362p	GTCAGCGTTACTTCTTCGATGC	Choi <i>et al.</i> , 2005.
		Rc.rompB.4,836n	CCGTACTCCATCTTAGCATCAG	
		Rc.rompB.4,496p	CCAATGGCAGGACTTAGCTACT	
		Rc.rompB.4,762n	AGGCTGGCTGATACCGGAGTAA	

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867 **Table S2. Targeted gene, amplicon size and primers sequences used for results confirmation.**