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SUMMARY

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

KEY WORDS

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

INTRODUCTION

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

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

Tick density is also heavily influenced by the presence of suitable hosts, most notably wild ungulates that sustain adults, thus enabling tick population renewal (Gilbert *et al.*, 2012; van Wieren and Hofmeester, 2016). However, it's important to emphasise that immediate tick survival and questing 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*.
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MATERIAL AND METHODS

Tick collection

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

Tick washing, crushing and DNA extraction

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

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

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

Tick-borne pathogens detection

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

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

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

Primers and probes

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

DNA pre-amplification

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

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

High throughput real time PCR

For each pre-amplified sample, the BioMark™ real-time PCR system (Fluidigm, USA) was used for high-throughput microfluidic real-time PCR amplification using the 48.48 microfluidic dynamic array (Fluidigm Corporation, USA). Amplifications were performed using FAM- and black hole quencher (BHQ1)-labeled TaqMan probes with TaqMan Gene Expression Master Mix in accordance with manufacturer's instructions (Applied Biosystems, France). Thermal cycling conditions were as follows: 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 BioMark Real-Time PCR system and analysed using the Fluidigm Real-Time PCR Analysis software to obtain crossing point (CP) values. Three tick species control (*I. ricinus*, *Dermacentor reticulatus*, *Dermacentor marginatus*), one negative water control and one positive *E. coli* control were included per chip.

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 that 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).

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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 model 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).

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188 *Statistical modelling of tick-borne pathogens associations*

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

RESULTS

Tick temporal dynamics

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

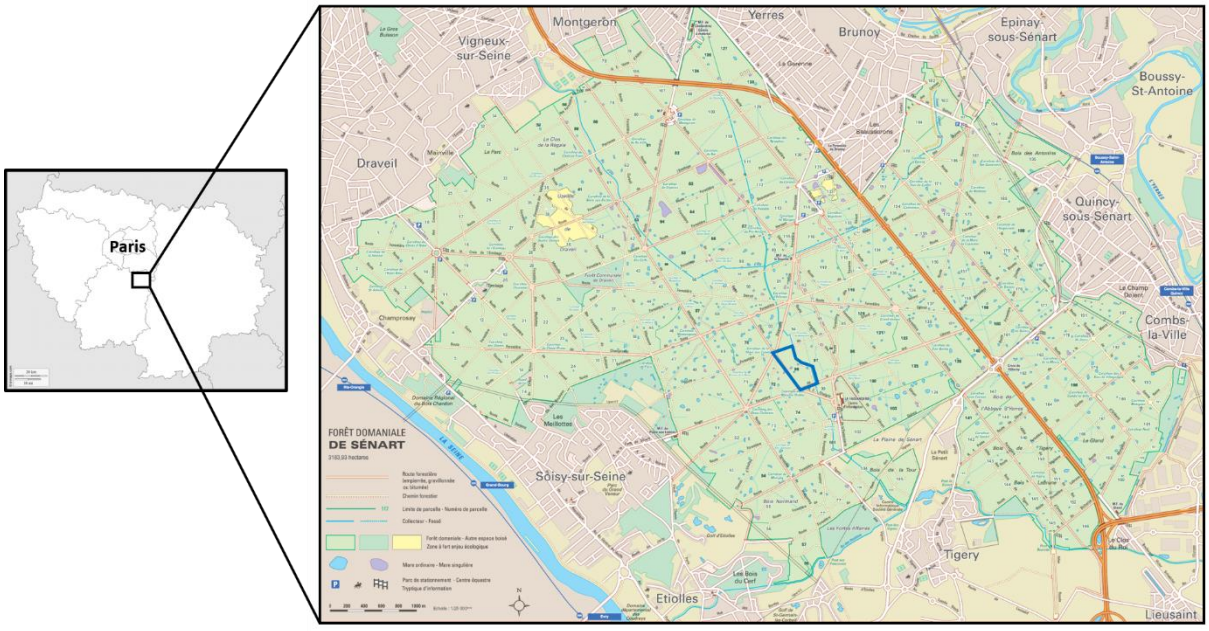

Figure 1. Senart forest, location and parcel map. Sampling was made on the blue framed parcel.

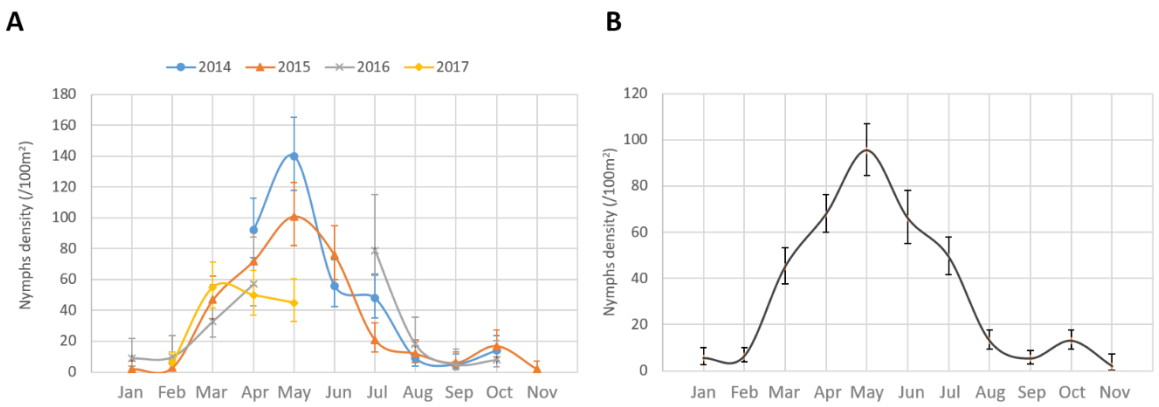

Figure 2. Ixodes ricinus nymph density. A: *Ixodes ricinus* nymphs monthly density (/100m^2) in 2014, 2015, 2016 and 2017. Ticks were sampled from April 2014 to May 2017. Please note that May and June 2016 were unfortunately not sampled. B: Average of nymph density per month (/100m^2). The calculation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

Detected pathogens and their prevalence in tick population

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

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

Two species of *Rickettsia* were detected in questing *I. ricinus* nymphs. *Rickettsia helvetica* was the most prevalent and was detected in 4.5% [3.3%, 6.0%] of nymphs. *Rickettsia felis* was detected in only one nymph (0.1% [.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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	Months	Analysed nymph number	*B. burgdorferi ss*	*B. garinii*	*B. afzelii*	*B. valaisiana*	*B. spielmanii*	*B. burgdorferi sl*	*B. myiamotoi*	*Borrelia spp*	*A. phagocytophilum*	*Anaplasma spp*	*R. helvetica*	*R. felis*	*Rickettsia spp*	*B. venatorum*	*B. diveregens*	*Babesia spp*	Co-infections (2 TBP)	Co-infections (3 TBP)	Total
2014	Apr-14	89	0	0	0	0	0	0	0	**0**	10	**10**	7	0	**7**	0	1	**1**	0	0	**18**
	May-14	127	1	0	0	0	0	1	1	**2**	12	**12**	2	0	**2**	4	0	**4**	2	0	**18**
	Jun-14	54	0	1	1	1	0	1	0	**1**	4	**4**	9	1	**10**	2	0	**2**	1	1	**16**
	Jul-14	38	0	1	0	0	1	1	0	**1**	6	**6**	1	0	**1**	0	0	**0**	1	0	**8**
	Aug-14	9	0	0	0	0	0	0	0	**0**	0	**0**	1	0	**1**	0	0	**0**	0	0	**1**
	Sep-14	5	1	0	0	0	0	1	0	**1**	0	**0**	0	0	**0**	1	0	**1**	0	0	**2**
	Oct-14	13	0	0	0	1	0	1	0	**1**	1	**1**	0	0	**0**	0	0	**0**	0	0	**2**
2015	Jan-15	2	0	0	0	0	0	0	0	**0**	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
	Feb-15	3	0	0	1	0	0	1	0	**1**	0	**0**	0	0	**0**	0	0	**0**	0	0	**1**
	Mar-15	43	0	3	1	1	3	3	0	**6**	1	**1**	5	0	**5**	0	0	**0**	1	1	**12**
	Apr-15	69	2	0	0	0	0	2	0	**2**	3	**4**	4	0	**4**	0	0	**0**	0	0	**10**
	May-15	88	0	0	0	0	0	0	1	**1**	1	**1**	3	0	**3**	2	0	**2**	0	0	**7**
	Jun-15	78	1	0	0	0	1	2	2	**4**	2	**2**	1	0	**1**	2	0	**2**	1	0	**8**
	Jul-15	21	0	2	2	0	0	3	1	**4**	0	**0**	0	0	**0**	0	0	**0**	1	0	**4**
	Aug-15	6	0	0	0	0	0	0	0	**0**	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
	Sep-15	6	0	0	0	0	0	0	0	**0**	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
	Oct-15	17	4	0	0	0	0	4	0	**4**	0	**0**	0	0	**0**	0	0	**0**	0	0	**4**
	Nov-15	2	0	0	0	0	0	0	1	**1**	0	**0**	0	0	**0**	0	0	**0**	0	0	**1**
2016	Jan-16	9	0	0	1	0	0	1	0	**1**	0	**0**	1	0	**1**	0	0	**0**	0	0	**2**
	Feb-16	10	0	0	0	0	0	0	0	**0**	0	**0**	1	0	**1**	0	0	**0**	0	0	**1**
	Mar-16	26	2	1	2	0	0	4	0	**4**	1	**1**	0	0	**0**	0	0	**0**	1	0	**5**
	Apr-16	33	0	0	0	1	0	1	0	**1**	0	**0**	2	0	**2**	0	0	**0**	0	0	**3**
	Jul-16	78	2	2	2	0	1	5	0	**5**	2	**2**	4	0	**4**	1	0	**1**	1	1	**11**
	Aug-16	11	2	0	0	0	0	2	0	**2**	0	**0**	2	0	**2**	0	0	**0**	1	0	**3**
	Sep-16	5	0	0	0	0	0	0	0	**0**	0	**0**	1	0	**1**	0	0	**0**	0	0	**1**
	Oct-16	6	0	0	0	0	0	0	1	**1**	0	**0**	0	0	**0**	0	0	**0**	0	0	**1**
2017	Feb-17	6	0	0	0	0	0	0	1	**1**	0	**0**	0	0	**0**	0	0	**0**	0	0	**1**
	Mar-17	53	0	1	0	1	0	2	0	**2**	3	**3**	0	0	**0**	2	0	**2**	0	0	**7**
	Apr-17	50	0	0	1	1	0	2	0	**2**	3	**3**	0	0	**0**	1	0	**1**	0	0	**6**
	May-17	41	0	0	0	0	0	0	1	**1**	4	**4**	1	0	**1**	0	0	**0**	0	0	**6**
	Apr-14 to May-17	**998**	**15**	**11**	**11**	**6**	**4**	**37**	**12**	**49**	**53**	**54**	**45**	**1**	**46**	**15**	**1**	**16**	**10**	**3**	**159**
	Number of positive months	30	8	7	8	6	4	18	9	**23**	14	**14**	16	1	**16**	8	1	**9**	9	3	**27**

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

Temporal patterns of TBP in nymphal I. ricinus

TBP prevalence at the monthly scale

Global infection rates fluctuated over the three years from a minimum of 8% [3.3%, 15.7%] in May 2015 to a maximum of 29.6% [18.0%, 43.6%] in June 2014, yet monthly variation was not significant probably due the low sample sizes ($p > 0.05$ according to Chi^2 test; Figure 3).

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

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

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

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

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

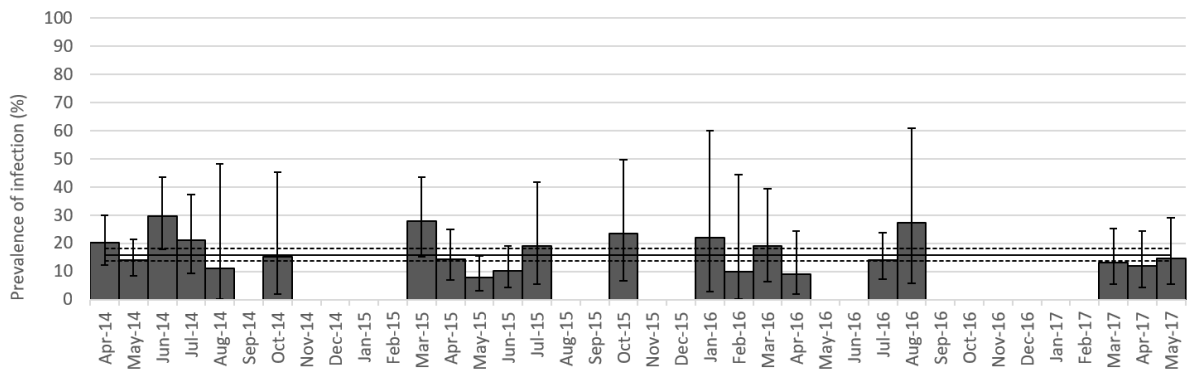

Figure 3. Nymph infection rate per month for at least one tested pathogen. Months with less than 9 nymphs sampled have not been considered for percentage calculation. Error bars represent confidence intervals of the percentage.

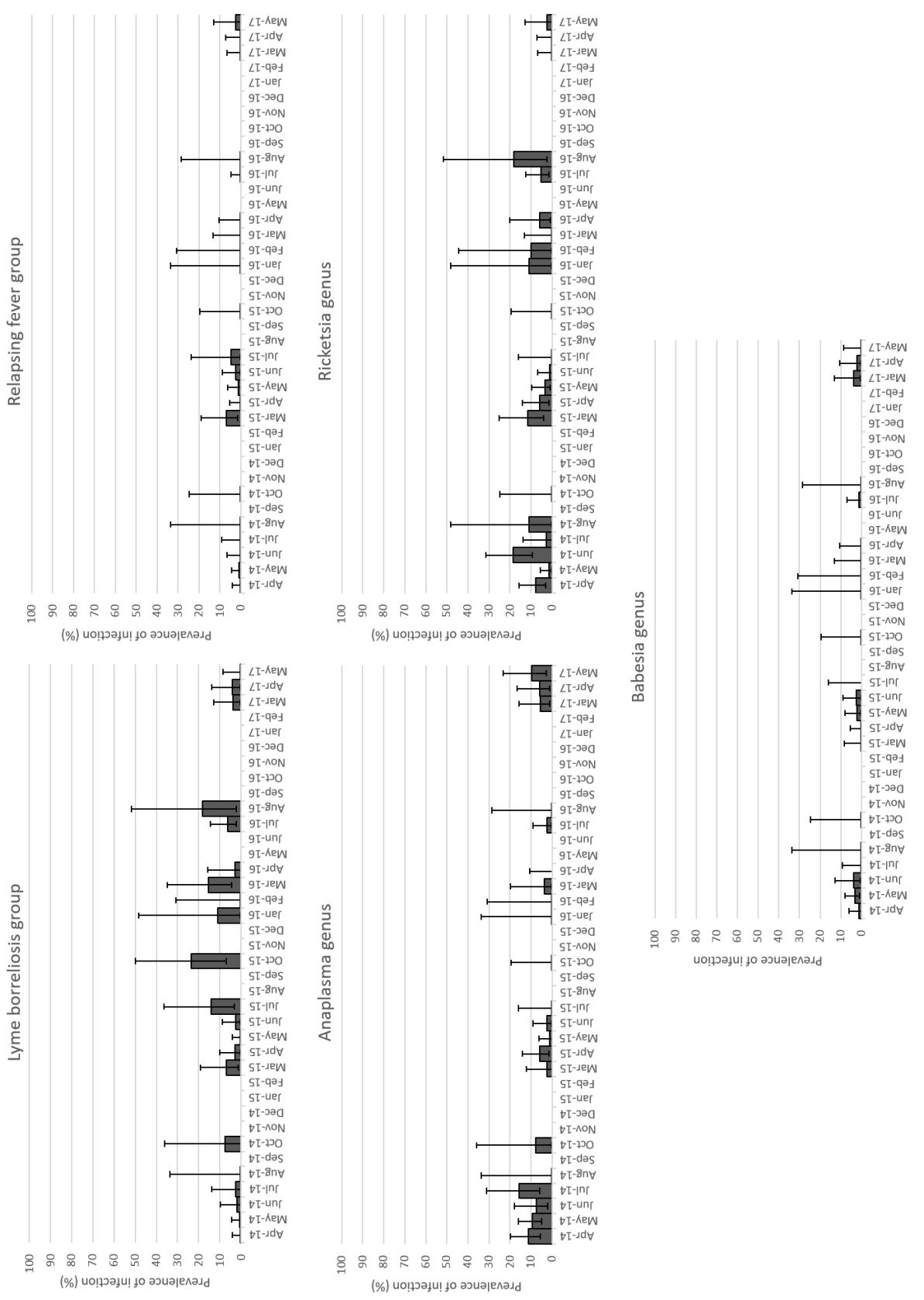

Figure 4. Nymph infection rate and confidence intervals per month for the different TBP. Months with less than 9 nymphs sampled have not been considered. Error bars represent confidence intervals of the percentage.

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

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

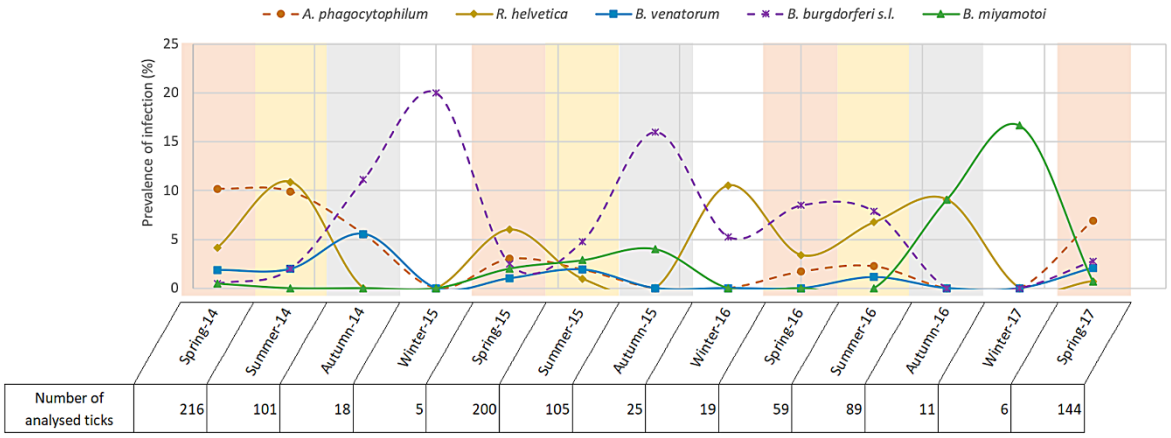

Figure 5. Percentage of positive nymphs per season for the most prevalent TBPs. Winter (white background)= January to February – Spring (orange background)= March to May – Summer (yellow background)= June to August – Autumn (grey background)= September to November.

Pathogen associations

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

B. burgdorferi ss	*B. garinii*	*B. afzelii*	*B. valaisiana*	*B. spielmanii*	*A. phagocytophilum*	*R. helvetica*	*B. venatorum*	**Co-occurrences number**
	X	X						3
	X			X				2
	X	X	X					1
	X		X	X				1
					X		X	3
					X	X		1
X						X		1
	X	X				X		1

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

DISCUSSION

Ixodes ricinus density and seasonal dynamics

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

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

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

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

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

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

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

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

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

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

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

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 (Tijsse-
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.

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

CONCLUSIONS

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

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: TP, MVT, JFC, KCM, EL. Performed the experiments: EL. Analysed the data: EL, TP, SM, MM, KCM. Wrote the paper: EL, MM, KCM, JFC, SM, MVT, TP

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859 **SUPPORTING INFORMATION**

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
Borrelia burgdorferi	rpoB	Forward	GCTTACTCACAAAAGGCGTCTT	83	Michelet *et al*., 2014
		Reverse	GCACATCTCTTACTTCAAATCCT		
		Probe	AATGCTCTTGGACCAGGAGGACTTTCA		
Borrelia garinii	rpoB	Forward	TGGCCGAACTTACCCACAAAA	88	Michelet *et al*., 2014
		Reverse	ACATCTCTTACTTCAAATCCTGC		
		Probe	TCTATCTCTTGAAAGTCCCCCTGGTCC		
Borrelia afzelii	fla	Forward	GGAGCAAATCAAGATGAAGCAAT	116	Michelet *et al*., 2014
		Reverse	TGAGCACCCTCTTGAACAGG		
		Probe	TGCAGCCTGAGCAGCTTGAGCTCC		
Borrelia valaisiana	ospA	Forward	ACTCACAAATGACAGATGCTGAA	135	Michelet *et al*., 2014
		Reverse	GCTTGCTTAAAGTAACAGTACCT		
		Probe	TCCGCCTACAAGATTTCCTGGAAGCTT		
Borrelia lusitaniae	rpoB	Forward	CGAACTTACTCATAAAAGGCGTC	87	Michelet *et al*., 2014
		Reverse	TGGACGTCTCTTACTTCAAATCC		
		Probe	TTAATGCTCTCGGGCCTGGGGGACT		
Borrelia spielmanii	fla	Forward	ATCTATTTTCTGGTGAGGGAGC	71	Michelet *et al*., 2014
		Reverse	TCCTTCTTGTTGAGCACCTTC		
		Probe	TTGAACAGGCGCAGTCTGAGCAGCTT		
Borrelia bissettii	rpoB	Forward	GCAACCAGTCAGCTTTCACAG	87	Michelet *et al*., 2014
		Reverse	CAAATCCTGCCCTATCCCTTG		
		Probe	AAAGTCCTCCCGGCCCAAGAGCATTAA		
Borrelia miyamotoi	glpQ	Forward	CACGACCCAGAAATTGACACA	94	Michelet *et al*., 2014
		Reverse	GTGTGAAGTCAGTGGCGTAAT		
		Probe	TCGTCCGTTTTCTCTAGCTCGATTGGG		
Borrelia spp.	23S	Forward	GAGTCTTAAAAGGGCGATTTAGT	73	Michelet *et al*., 2014
		Reverse	CTTCAGCCTGGCCATAAATAG		
		Probe	AGATGTGGTAGACCCGAAGCCGAGT		
Anaplasma marginale	msp1	Forward	CAGGCTTCAAGCGTACAGTG	85	Michelet *et al*., 2014
		Reverse	GATATCTGTGCCTGGCCTTC		
		Probe	ATGAAAGCCTGGAGATGTTAGACCGAG		
Anaplasma platys	groEL	Forward	TTCTGCCGATCCTTGAAAACG	75	Michelet *et al*., 2014
		Reverse	CTTCTCCTTCTACATCCTCAG		
		Probe	TTGCTAGATCCGGCAGGCCTCTGC		
Anaphasma phagocytophilum	msp2	Forward	GCTATGGAAGGCAGTGTTGG	77	Michelet *et al*., 2014
		Reverse	GTCTTGAAGCGCTCGTAACC		
		Probe	AATCTCAAGCTCAACCCTGGCACCAC		
Anaplasma centrale	groEL	Forward	AGCTGCCCTGCTATACACG	79	Michelet *et al*., 2014
		Reverse	GATGTTGATGCCCAATTGCTC		
		Probe	CTTGCATCTCTAGACGAGGTAAAGGGG		
Anaplasma bovis	groEL	Forward	GGGAGATAGTACACATCCTTG	73	Gondard *et al*., 2019
		Reverse	CTGATAGCTACAGTTAAGCCC		
		Probe	AGGTGCTGTTGGATGTACTGCTGGACC		
Anaplasma spp.	16S	Forward	CTTAGGGTTGTAAAACTCTTTCAG	160	Gondard *et al*., 2019
		Reverse	CTTTAACTTACCAAACCGCCTAC		
		Probe	ATGCCCTTTACGCCCAATAATTCCGAACA		
Ehrlichia spp.	16S	Forward	GCAACGCGAAAAACCTTACCA	98	Gondard *et al*., 2019
		Reverse	AGCCATGCAGCACCTGTGT		
		Probe	AAGGTCCAGCCAAACTGACTCTTCCG		
Ehrlichia canis	gltA	Forward	GACCAAGCAGTTGATAAAGATGG	136	Gondard *et al*., 2019
		Reverse	CACTATAAGACAATCCATGATTAGG		
		Probe	ATTAAAACATCCTAAGATAGCAGTGGCTAAGG		

860 *(Continued)*

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
Candidatus Neoehrlichia mikurensis	groEL	Forward	AGAGACATCATTCGCATTTTGGA		
		Reverse	TTCCGGTGTACCATAAGGCTT	96	Michelet *et al*., 2014
		Probe	AGATGCTGTTGGATGTACTGCTGGACC		
Rickettsia conorii	23S-5S ITS	Forward	CTCACAAAGTTATCAGGTTAAATAG		
		Reverse	CGATACTCAGCAAAATAATTCTCG	118	Michelet *et al*., 2014
		Probe	CTGGATATCGTGGCAGGGCTACAGTAT		
Rickettsia slovaca	23S-5S ITS	Forward	GTATCTACTCACAAAGTTATCAGG		
		Reverse	CTTAACTTTTACTACAATACTCAGC	138	Michelet *et al*., 2014
		Probe	TAATTTTCGCTGGATATCGTGGCAGGG		
Rickettsia massiliae	23S-5S ITS	Forward	GTTATTGCATCACTAATGTTATACTG		
		Reverse	GTTAATGTTGTTGCACGACTCAA	128	Michelet *et al*., 2014
		Probe	TAGCCCCGCCACGATATCTAGCAAAAA		
Rickettsia helvetica	23S-5S ITS	Forward	AGAACCGTAGCGTACACTTAG		
		Reverse	GAAAACCCTACTTCTAGGGGT	79	Michelet *et al*., 2014
		Probe	TACGTGAGGATTTGAGTACCGGATCGA		
Rickettsia aeschlimannii	ITS	Forward	CTCACAAAGTTATCAGGTTAAATAG		
		Reverse	CTTAACTTTTACTACGATACTTAGCA	134	Michelet *et al*., 2014
		Probe	TAATTTTTGCTGGATATCGTGGCGGGG		
Rickettsia felis	orfB	Forward	ACCCTTTTCGTAACGCTTTGC		
		Reverse	TATACTTAATGCTGGGCTAAACC	163	Gondard *et al*., 2019
		Probe	AGGGAAACCTGGACTCCATATTCAAAAGAG		
Rickettsia spp.	gltA	Forward	GTCGCAAATGTTCACGGTACTT		
		Reverse	TCTTCGTGCATTTCTTTCCATTG	78	Gondard *et al*., 2019
		Probe	TGCAATAGCAAGAACCGTAGGCTGGATG		
Bartonella henselae	pap31	Forward	CCGCTGATCGCATTATGCCT		
		Reverse	AGCGATTTCTGCATCATCTGCT	107	Michelet *et al*., 2014
		Probe	ATGTTGCTGGTGGTGTTTCCTATGCAC		
Bartonella spp.	ssrA	Forward	CGTTATCGGGCTAAATGAGTAG		
		Reverse	ACCCCGCTTAAACCTGCGA	118	Gondard *et al*., 2019
		Probe	TTGCAAATGACAACTATGCGGAAGCACGTC		
Francisella tularensis	tul4	Forward	ACCCACAAGGAAGTGTAAGATTA		
		Reverse	GTAATTGGGAAGCTTGTATCATG	76	Michelet *et al*., 2014
		Probe	AATGGCAGGCTCCAGAAGGTTCTAAGT		
	fopA	Forward	GGCAAATCTAGCAGGTCAAGC		
		Reverse	CAACACTTGCTTGAACATTTCTAG	91	Michelet *et al*., 2014
		Probe	AACAGGTGCTTGGGATGTGGGTGGTG		
Coxiella burnettii	IS1111	Forward	TGGAGGAGCGAACCATTGGT		
		Reverse	CATACGGTTTGACGTGCTGC	86	Michelet *et al*., 2014
		Probe	ATCGGACGTTTATGGGGATGGGTATCC		
	icd	Forward	AGGCCCGTCCGTTATTTTACG		
		Reverse	CGGAAAATCACCATATTCACCTT	74	Michelet *et al*., 2014
		Probe	TTCAGGCGTTTTGACCGGGCTTGGC		
Apycomplexa	18S	Forward	TGAACGAGGAATGCCTAGTATG		
		Reverse	CACCGGATCACTCGATCGG	104	Gondard *et al*., 2019
		Probe	TAGGAGCGACGGGCGGTGTGTAC		
Babesia microti	CCTeta	Forward	ACAATGGATTTTCCCCAGCAAAA		
		Reverse	GCGACATTTCGGCAACTTATATA	145	Michelet *et al*., 2014
		Probe	TACTCTGGTGCAATGAGCGTATGGGTA		
Babesia canis	hsp70	Forward	TCACTGTGCCTGCGTACTTC		
		Reverse	TGATACGCATGACGTTGAGAC	87	Michelet *et al*., 2014
		Probe	AACGACTCCCAGCGCCAGGCCAC		

(Continued)

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
Babesia ovis	18S	Forward	TCTGTGATGCCCTTAGATGTC	92	Michelet *et al*., 2014
		Reverse	GCTGGTTACCCGCGCCTT		
		Probe	TCGGAGCGGGGTCAACTCGATGCAT		
Babesia bovis	CCTeta	Forward	GCCAAGTAGTGGTAGACTGTA	100	Michelet *et al*., 2014
		Reverse	GCTCCGTCATTGGTTATGGTA		
		Probe	TAAAGACAACACTGGGTCCGCGTGG		
Babesia caballi	rap1	Forward	GTTGTTCGGCTGGGGCATC	94	Michelet *et al*., 2014
		Reverse	CAGGCGACTGACGCTGTGT		
		Probe	TCTGTCCCGATGTCAAGGGGCAGGT		
Babesia venatorum (sp. EU1)	18S	Forward	GCGCGCTACACTGATGCATT	91	Michelet *et al*., 2014
		Reverse	CAAAAATCAATCCCCGTCACG		
		Probe	CATCGAGTTTAATCCTGTCCCGAAAGG		
Babesia divergens	hsp70	Forward	GCGCGCTACACTGATGCATT	91	Michelet *et al*., 2014
		Reverse	CAAAAATCAATCCCCGTCACG		
		Probe	CATCGAGTTTAATCCTGTCCCGAAAGG		
Theileria spp.	18S	Forward	GTCAGTTTTTACGACTCCTTCAG	213	Michelet *et al*., 2014
		Reverse	CCAAAGAATCAAGAAAGAGCTATC		
		Probe	AATCTGTCAATCCTTCCTTTGTCTGGACC		
Hepatozoon spp.	18S	Forward	ATTGGCTTACCGTGGCAGTG	175	Gondard *et al*., 2019
		Reverse	AAAGCATTTTAACTGCCTTGTATTG		
		Probe	ACGGTTAACGGGGGATTAGGGTTCGAT		
Ixodes ricinus	ITS2	Forward	CGAAACTCGATGGAGACCTG	77	Michelet *et al*., 2014
		Reverse	ATCTCCAACGCACCGACGT		
		Probe	TTGTGGAAATCCCGTCGCACGTTGAAC		
Tick spp	16S	Forward	AAATACTCTAGGGATAACAGCGT	99	Gondard *et al*., 2019
		Reverse	TCTTCATCAAACAAGTATCCTAATC		
		Probe	CAACATCGAGGTCGCAAACCATTTTGTCTA		
Dermacentor reticulatus	ITS2	Forward	AACCCTTTTCCGCTCCGTG	83	Michelet *et al*., 2014
		Reverse	TTTTGCTAGAGCTCGACGTAC		
		Probe	TACGAAGGCAAACAACGCAAACTGCGA		
Dermacentor marginatus	ITS2	Forward	GCACGTTGCGTTGTTTGCC	139	Michelet *et al*., 2014
		Reverse	CCGCTCCGCGCAAGAATCT		
		Probe	TTCGGAGTACGTCGAGCTCTAGCAGA		
Escherichia coli	eae	Forward	CATTGATCAGGATTTTTCTGGTGATA	102	Michelet *et al*., 2014
		Reverse	CTCATGCGGAAATAGCCGTTA		
		Probe	ATAGTCTCGCCAGTATTCGCCACCAATACC		

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

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

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