Current Research in Parasitology & Vector-Borne Diseases 6 (2024) 100213

Contents lists available at ScienceDirect

Current Research in Parasitology & Vector-Borne Diseases

journal homepage: www.sciencedirect.com/journal/current-research-in-parasitology-and-vector-borne-diseases

Temporal dynamics of the *Hyalomma marginatum*-borne pathogens in southern France

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ARTICLE INFO

Keywords:
Hyalomma marginatum
Tick-borne pathogens
Temporal patterns
Rickettsia aeschlimannii

ABSTRACT

Spatio-temporal scales have a clear influence on microbial community distribution and diversity and should thus be applied to study the dynamics of microorganisms. The invasive tick species *Hyalomma marginatum* has recently become established in southern France. It may carry pathogens of medical and veterinary interest including the Crimean-Congo haemorrhagic fever virus, *Rickettsia aeschlimannii*, *Theileria equi* and *Anaplasma phagocytophilum*. Pathogenic communities of *H. marginatum* have been identified and their spatial distribution characterized, but their temporal dynamics remain unknown. *Hyalomma marginatum* ticks were collected from hosts at monthly intervals from February to September 2022 in a site in southern France to study their presence and temporal dynamics. Of the 281 ticks analysed, we detected pathogens including *R. aeschlimannii*, *Anaplasma* spp. and *T. equi* with infection rates reaching 47.0%, 4.6% and 11.0%, respectively. A total of 14.6% of ticks were infected with at least *Theileria* or *Anaplasma*, with monthly fluctuations ranging from 2.9% to 28.6%. Strong temporal patterns were observed for each pathogen detected, particularly for *R. aeschlimannii*, whose infection rates increased dramatically at the beginning of summer, correlated with monthly mean temperatures at the site. Based on these results, we hypothesise that *R. aeschlimannii* may be a secondary symbiont of *H. marginatum* and could be involved in the stress response to temperature increase and mediate thermal tolerance of *H. marginatum*. Analysis of monthly and seasonal fluctuations in pathogens transmitted by *H. marginatum* led us to conclude that the risk of infection is low but persists throughout the period of *H. marginatum* activity, with a notable increase in summer.

1. Introduction

The scale at which an epidemiological study is conducted affects how the results will be interpreted. When identifying tick-borne diseases of medical and veterinary interest, detecting spatial and temporal scales requires precise mapping of the occurrence and dynamics of tick-borne pathogens (TBP) (Halos et al., 2010; Crowder et al., 2014; Pollet et al., 2020; Lejal et al., 2019a, 2019b, 2021). This information is crucial to assess the risk ticks represent to both humans and animals. The tick lifespan, density, duration of contact between ticks and their hosts and pathogen acquisition can be affected by variations in environmental factors, including temperature, humidity, levels of canopy cover and forest fragmentation, across the seasons (Randolph and Rogers, 1997; Wimberly et al., 2008; Schulz et al., 2014; Paul et al., 2016; Marchant et al., 2017; Pollet et al., 2020; Diuk-Wasser et al., 2021). Even though longitudinal surveys may be limited by resources and time constraints, such surveys are indispensable not only to identify fluctuations in the occurrence of tick-borne pathogens but also potential emergence events (Sormunen et al., 2018). Several studies have already demonstrated that temporal trends affect the occurrence of pathogens in several tick species in different countries (Reye et al., 2010; Coipan et al., 2013; Takken et al., 2017; Chvostáč et al., 2018; Keesing et al., 2021; Foster et al., 2022; Tran et al., 2022; Kazimírová et al., 2023). In France, a three-year study of *Ixodes ricinus*-borne pathogens revealed fluctuations in the prevalence of several common and less common TBP at monthly, seasonal and yearly scales (Lejal et al., 2019a). That study underlined the

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https://doi.org/10.1016/j.crpvbd.2024.100213
Received 17 April 2024; Received in revised form 26 August 2024; Accepted 6 September 2024
Available online 7 September 2024

limits of sporadic tick sampling and the need for regular monitoring to determine pathogen prevalences.

In southern France, a new invasive tick species, *Hyalomma marginatum*, recently became established in the Mediterranean area (Stachurski and Vial, 2018; Vial et al., 2016). This tick species is known to carry several animal and human pathogens including *Rickettsia aeschlimannii*, *Theileria equi*, *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Ehrlichia minasensis* and West Nile virus (Bernard et al., 2024b; Joly-Kukla et al., 2024). *Hyalomma marginatum* is known to be the reservoir and vector of the Crimean-Congo haemorrhagic fever virus, which severely affects humans (Fillâtre et al., 2019; Bernard et al., 2022; Hawman and Feldmann, 2023). The virus was detected in southern France for the first time in ticks collected from cattle and horses in 2022 and again in 2023 (Bernard et al., 2024a).

Assessing the risks linked to *H. marginatum* not only requires knowledge of the circulating pathogens and their infection rates but also of their dynamics in space and over time. Although the spatial scale was recently shown to influence the distribution of *H. marginatum* pathogens (Joly-Kukla et al., 2024), the influence of the temporal scale remains unknown. Because *H. marginatum* is an invasive tick species, its spread and the pathogens it carries need to be surveyed over a period of several years. Furthermore, since pathogen dynamics is probably influenced by environmental factors that vary with the seasons, it is advisable to assess the tick-borne pathogen dynamics at least at monthly intervals. The temporal scale could also provide further information on the potential role of the maternally transmitted bacterium *Rickettsia aeschlimannii* in *H. marginatum* ticks, which has already been detected at high prevalence in this tick species (Oteo and Portillo, 2012; Wallménius et al., 2014; Portillo et al., 2015; Azagi et al., 2017; Grech-Angelini et al., 2020; Bernard et al., 2024b), for example 87.3% (Joly-Kukla et al., 2024). In this paper, we develop our hypothesis that *R. aeschlimannii* is a secondary symbiont in *H. marginatum*, whose role remains to be determined (Bernard et al., 2024b; Joly-Kukla et al., 2024). The vector of transmission between *H. marginatum* ticks and its hosts is not yet clear, but this bacterium is responsible for spotted fever in humans (Beati et al., 1997; Raoult et al., 2002; Bonnet et al., 2023). Thus, analysis of seasonal trends in the occurrence of *R. aeschlimannii* could provide valuable insights into its function in *H. marginatum* ticks. In this context, the objective of this study was to characterize the temporal patterns of *H. marginatum*-borne pathogens by collecting ticks at monthly intervals in a specific site in the South of France (Occitanie Region).

2. Materials and methods

2.1. Tick collection

The ticks were collected at a site called Mas de la Lauze, in Pompignan, located in the department of Gard in southern France (Fig. 1). The study site is a 200-ha area of scrubland where 13 mares of a local breed of horses (Camargue), aged between 2 and 30 years, on which ticks are collected, live in semi-liberty. On each collection date, we sampled approximately 30 ticks from several horses, not always from the same horses on each occasion. The horses were vaccinated and remained untreated for several weeks before tick collection began. Tick collection took place at monthly intervals between February and September 2022, during the active period of *H. marginatum* adults, giving a total of 10 collection dates of which three were in March. We did not collect any ticks in January 2022 or from October to December 2022, as *H. marginatum* is not active during these periods (behavioural diapause in the floor litter). Back in the laboratory, the collected ticks were examined under a stereomicroscope, morphologically identified according to Estrada-Peña et al. (2018), and separated by sex. *Hyalomma marginatum* ticks were kept frozen at −80 °C until further use. The dates of collection and the number of *H. marginatum* that were analysed were as follows: February 24th (n = 21 ticks); March 11th (n = 31); March 17th (n = 24); March 31st (n = 34); April 28th (n = 34); May 25th (n = 34); June 23rd (n = 31); July 21st (n = 33); August 19th (n = 28); and September 16th (n = 11), giving a total of 281 *H. marginatum*. We chose to analyse a maximum of 34 ticks per collection date, and if fewer than 34 ticks were collected on the date concerned, they were all analysed. A 50:50 sex ratio was respected whenever possible (in fact, a total of 153 males and 128 females were analysed). Based on the tick collection dates, we defined three seasons as follows: winter (February 24th, March 11th, March 17th, and March 31st); spring (April 28th and May 25th); and summer (June 23rd, July 21st, August 19th, and September 16th).

Fig. 1. Map of the Occitanie region where the monthly tick sampling was conducted in the site "Pompignan" indicated by a red cross. The four administrative departments where *Hyalomma marginatum* populations are established are in white.

2.2. DNA and RNA extraction

Ticks were crushed and nucleic acid was extracted in a BSL3 facility. The ticks were washed for 30 s in 1% hypochlorite solution that was diluted from a bottle containing 2.6% of active chlorine (Orapi Group, Saint-Vulbas, France), then rinsed three times for 1 min in Milli-Q water to remove any environmental microbes present on the tick cuticle (Binetruy et al., 2019). The ticks were then cut into pieces using a scalpel blade and crushed individually in a Precellys®24 Dual homogenizer (Bertin, Montigny le Bretonneux, France) at 5500 *rpm* for 40 s, using three steel beads (2.8 mm, OZYME, Saint-Cyr-L'École, France) in 400 µl of DMEM (Dulbecco's Modified Eagle Medium, Eurobio Scientific, Les Ulis, France) with 10% foetal calf serum. Total DNA and RNA was extracted using the NucleoMag VET extraction kit (Macherey-Nagel, Hoerdt, France) following the manufacturer's instructions using the IDEAL™ 96 extraction robot (Innovative Diagnostics, Grabels, France). Nucleic acids were eluted in 90 µl of elution buffer and stored at −20 °C for DNA and −80 °C for RNA until further analyses.

2.3. Detection of tick-borne pathogens from tick DNA and RNA extractions

2.3.1. Microfluidic PCR detection

2.3.1.1. Reverse transcription. Samples were retrotranscribed in cDNA using 1 µl of extracted RNA with 1 µl of Reverse Transcription Master Mix and 3 µl of RNase-free ultrapure water provided with the kit

(Standard BioTools, South San Francisco, USA) using a thermal cycler (Eppendorf, Montesson, France) with the following cycles: 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C with a final cooling cycle at 10 °C.

2.3.1.2. Targeted tick-borne pathogens. A set of 48 primers/probes (designs) was used to target tick-borne pathogens (bacteria, parasites and viruses) commonly reported in *H. marginatum* (Supplementary file S1: Table S1). *Francisella*-LE positive samples were confirmed using a specific *Francisella*-LE qPCR (Duron et al., 2018). The Crimean-Congo haemorrhagic fever virus was not tested for in the present study.

2.3.1.3. Pre-amplification. Each sample was pre-amplified using 1.25 µl of DNA mix (1:1 vol ratio of DNA and cDNA) with 1 µl of the PreAmp Master mix (Standard BioTools, South San Francisco, USA), 1.5 µl of ultra-pure water and 1.25 µl of the pooled primers. PCRs were performed using a thermal cycler with the following cycles: 2 min at 95 °C, 14 cycles at 95 °C for 15 s and 60 °C for 4 min and finally a 4-min extension at 4 °C. A negative control was used for each plate with ultra-pure water. Amplicons were diluted 1:10 with ultra-pure water and stored at −20 °C until further use.

2.3.1.4. BioMark assay. The BioMark real-time PCR system (Standard BioTools) was used for high-throughput microfluidic real-time PCR amplification using the 48.48 dynamic array (Standard BioTools). The chips dispense 48 PCR mixes and 48 samples into individual wells, after which on-chip microfluidics assemble PCR reactions in individual chambers prior to thermal cycling resulting in 2304 individual reactions. In one experiment, 47 ticks and one negative control were tested (see Michelet et al., 2014; Gondard et al., 2018, 2020 for details).

2.3.1.5. Validation of the results by PCR and sequencing. Conventional PCRs or qPCR were then performed on TBP positive-samples using different sets of primers than those used in the BioMark assay to confirm the presence of pathogenic DNA (for details see Joly-Kukla et al., 2024). Amplicons were sequenced by Azenta Life Sciences (Germany) using Sanger-EZ sequencing and assembled using Geneious software (Biomatters, Auckland, New Zealand). An online BLAST (National Center for Biotechnology Information) was conducted to compare results with sequences published in GenBank sequence databases (Michelet et al., 2014; Gondard et al., 2018, 2020; Grech-Angelini et al., 2020).

2.3.2. Detection and quantification of T. equi and R. aeschlimannii by duplex real-time fluorescence quantitative PCR

Tick samples were also screened to detect and quantify *T. equi* and *R. aeschlimannii* using a qPCR approach with primers and probes targeting different genes than those used in the BioMark assay, i.e. the 18S rRNA gene and the *Ompb* gene respectively (Supplementary file S1: Table S2). Five different genotypes of *T. equi* (designated A-E) are known to circulate in Europe, so we wanted to be sure that, when they are present, we are able to detect all five genotypes (Nagore et al., 2004; Bhoora et al., 2009; Salim et al., 2010; Qablan et al., 2013). The Takyon™ No ROX Probe 2× MasterMix Blue dTTP (Eurogentec, Seraing, Belgium) was used with a final reaction volume of 20 µl containing 10 µl of Master Mix 2× (final concentration 1×), 5 µl of RNase-free water, 1 µl of each primer (0.5 µM), probes (0.25 µM), and 2 µl of DNA template. The reaction was carried out using a thermal cycler with the following cycles: 3 min at 95 °C, 45 cycles at 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. Positive controls for both *R. aeschlimannii* and *T. equi* were prepared using a recombinant plasmid from the TA cloning® kit (Invitrogen, Waltham, USA). A 10-fold serial dilution of the plasmid (from an initial concentration of 0.5×10^{8} copy number/µl) was used to generate standard positive plasmids from 2.5×10^{5} copy number/µl to 2.5×10^{-1} copy number/µl. Samples were detected and quantified in duplicate using the standard plasmids. For *R. aeschlimannii*, we considered negative samples with Cq > 37. This limit of detection was established using the last dilution of the standard curve that could be detected by qPCR. For *T. equi*, most samples were close to or below the limit of detection established with the *T. equi* standard curve. Because the protozoan *T. equi* could be circulating at low levels, all positive samples were included.

2.4. Statistical analyses

Multivariate analysis was performed with R software 4.3.0. Generalized linear models (R package *Lme4*; Bates et al., 2015) were used to test the effect of both the season (winter/spring/summer) and the sex (male/female) of the tick on the infection rates of *R. aeschlimannii*, *T. equi*, *Anaplasma* spp. and *Francisella*-LE (presence/absence, binomial distribution). We decided to include *Francisella*-LE in the analysis as a control since this bacterium has been identified as a *H. marginatum* primary endosymbiont (Duron et al., 2017, 2018; Azagi et al., 2017). A gamma distribution was used for *R. aeschlimannii* loads. Akaike's information criterion (AIC) was used to assess the models. Significance of variables was assessed using the 'ANOVA' procedure in the *car* package, which performs a type III hypothesis (Fox and Weisberg, 2018). *Post-hoc* tests (Tukey's HSD test) were conducted using the function 'emmeans'. The data used in the analyses were generated using the BioMark data for *Anaplasma* spp. and using the qPCR data for *R. aeschlimannii* and *T. equi.* The correlation between *R. aeschlimannii* and temperature was performed using a linear regression. A Spearman correlation analysis was then performed in R to statistically assess the significance of the correlation.

3. Results

3.1. Detected microorganisms and their infection rates in H. marginatum

The pathogens detected and their infection rates are listed in Table 1. Among all the ticks collected from February to September, 132 tick samples tested positive for *R. aeschlimannii* by qPCR (*OmpB* gene) and 125 samples using the BioMark assay (*glta* gene). The presence of *R. aeschlimannii* was confirmed by sequencing the *OmpB* gene with 100% homology. One sequence has been deposited in GenBank (accession number: PP663276). The infection rates estimated by qPCR (*OmpB* gene) and BioMark (*glta* gene) were 47.0% (95% CI: 41.1–52.8%) and 44.5% (95% CI: 38.6–50.3%), respectively. The qPCR data were selected for the statistical analysis of *R. aeschlimannii*.

Concerning the other pathogens, 14.6% (95% CI: 10.4–18.7%) of the ticks were infected with at least *Theileria equi* or *Anaplasma* spp. Specifically, 31 ticks were found to be positive for *T. equi* by qPCR, giving an infection rate of 11.0% (95% CI: 7.3–14.7%). Concerning *Anaplasma*, eight ticks were positive for *Anaplasma* spp. alone and five for both *A. phagocytophilum* and *Anaplama* spp. (BioMark assay); these were all categorized as *Anaplasma* spp.-infected samples, with an infection rate of 4.6% (95% CI: 2.2–7.1%) as we were not able to obtain a sequence to confirm the species. Finally, using the BioMark assay, the endosymbiont *Francisella*-LE was detected in 279 samples, with an average infection rate of 99.3% (95% CI: 98.3–100%).

Table 1
Detected pathogens, number of ticks infected, and corresponding infection rates.

Pathogen/Endosymbiont	No. of positive samples	Infection rate (%)
Francisella-LE	279	99.3
R. aeschlimannii (qPCR)	132	47.0
R. aeschlimannii (BioMark)	125	44.5
Anaplasma spp.	13	4.6
T. equi (qPCR)	31	11.0

3.2. Temporal patterns in H. marginatum-borne pathogens

From the ticks collected at monthly intervals (n = 281), we determined the temporal patterns of each *H. marginatum*-borne pathogen (Fig. 2, Table 2). Each month, ticks were recurrently infected with at least one pathogen (*T. equi* or *Anaplasma* spp.), at rates ranging from a minimum of 2.9% of ticks infected in April to a maximum of 28.6% in August. At a seasonal scale, 2.6 times more ticks were infected with at least one pathogen in summer [19.4% (95% CI: 11.6–27.2%)] than in spring [7.4% (95% CI: 1.0–13.7%)]. Interestingly, percentage infection in winter was quite high [14.5% (95% CI: 7.9–21.2%)].

Infection rates of *R. aeschlimannii* ranged from 19% in February to 83.9% in June. A dramatic increase was recorded between the sampling day in May [20.6% (95% CI: 6.3–34.9%)] and the sampling day in June [83.9% (95% CI: 70.2–97.6%)]. Monthly infection rates in summer were all higher [average 74.8% (95% CI: 66.2–83.3%)] than those estimated in winter [average 34.5% (95% CI: 25.5–43.6%)] and spring [average 25.0% (95% CI: 14.4–35.6%)]. Infection rates in summer were statistically higher than in winter (χ^2 = 10.29, df = 1, P = 0.0058) (Table 2, Fig. 2). We also compared variation in *R. aeschlimannii* infection rates assessed using two different targeted genes and techniques, the BioMark assay (target gene: citrate synthase gene), and the qPCR approach targeting the *OmpB* gene. The variation was limited (from 0 to 3.2%) in most months (March, April, May, June and September). Bigger variations were observed in February, July and August (from 12.1 to 17.9%) but monthly trends were similar using the two techniques and targeted genes (Supplementary file S2: Fig. S1). As temporal patterns affecting *H. marginatum* pathogens can be directly linked to abiotic conditions, the average monthly temperature (°C) in the site was plotted (Fig. 2). The linear regression between the monthly infection rates of *R. aeschlimannii* and temperature revealed a significant positive correlation (Spearman's ρ = 0.7619, P = 0.0183) (Fig. 3).

Beyond the seasonal influence, we also observed that infection rates differed significantly between males and females (χ^2 = 10.11, df = 1, P = 0.0015) (Table 2). Infection rates were significantly higher in females throughout the period from February to September [average 57.0% (95% CI: 48.3–65.7%)], than in males [average 38.6% (95% CI: 30.8–46.4%)]. Finally, the interaction between the variables season and tick sex was also significant (χ^2 = 9.51, df = 2, P = 0.0086). Based on these results for *R. aeschlimannii*, we assessed the impact of the season on males and females separately (Fig. 4A). The infection rate of *R. aeschlimannii* in females was significantly higher in summer [71.7% (95% CI: 59.9–83.4%)] than in spring [41.2% (95% CI: 23.7–58.6%)] (P = 0.0122), while in winter, the infection rate was 47.1% (95% CI: 29.4–64.7%). In male ticks, the infection rate of *R. aeschlimannii* was also significantly higher in summer [79.1% (95% CI: 66.4–91.7%)] than in winter [28.9% (95% CI: 18.5–39.4%)] (P < 0.0001) and spring [8.8% (95% CI: 0–18.9%)] (P < 0.0001). While the season had no significant influence on *R. aeschlimannii* loads (Table 3), the loads did differ significantly between males and females (χ^2 = 9.71, df = 1, P = 0.0018), with higher loads in females [9.6×10^4 (1.7×10^4–1.8×10^5)] than in males [1.8×10^4 (3.0×10^3–3.3×10^4)] (P = 0.0018) (Fig. 4B–Table 3). Interestingly, there was a bigger difference between the lowest and the highest monthly infection rate for *R. aeschlimannii* in males [94.1%: from 5.9% in May (n = 17) to 100% in June (n = 14)] than in females [52.9%: from 35.3% in May (n = 17) to 88.2% in August (n = 17)] (Supplementary file S2: Fig. S2A). Finally, 0% infection rates were observed for females in February and males in September.

The presence of *T. equi*, *Anaplasma* spp. and *Francisella*-LE was not influenced by the sex of the tick (Table 2, Supplementary file S2: Figs. S2B–D). The infection rates of *T. equi* varied between months (from 2.9% in April to 25.0% in August), while the differences between the seasons were not significant. However, a significant difference was observed depending on the season for *Anaplasma* spp. (χ^2 = 8.61, df = 2, P = 0.0135) (Table 2). Infection rates of *Anaplasma* spp. ranged from 0% in spring to an average of 7.8% (95% CI: 2.5–13.0%) in summer and an average of 4.5% (95% CI: 0.6–8.5%) in winter. Finally, the primary symbiont *Francisella*-LE was detected in almost all ticks collected (279/281) regardless of the season (Table 2).

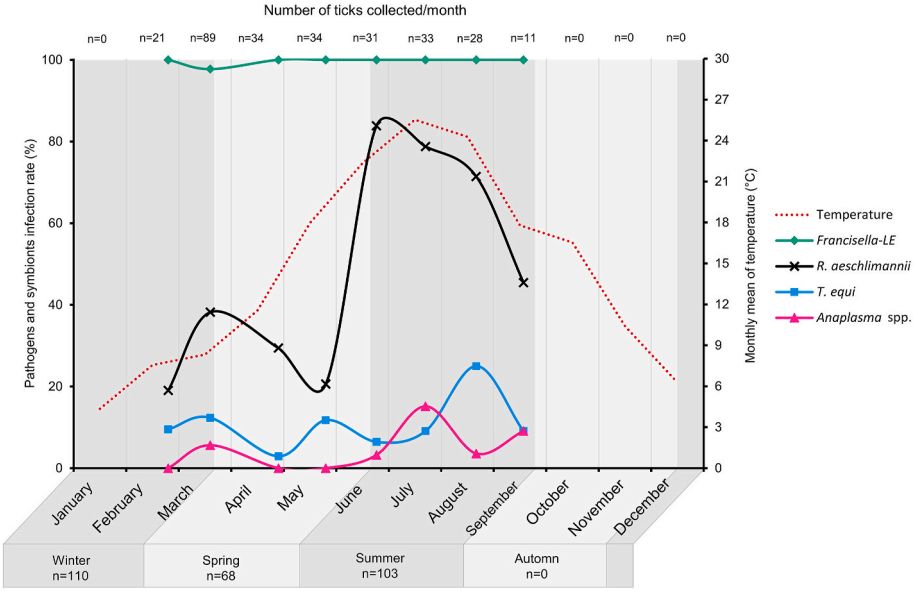

Fig. 2. Infection rates (%) of *Hyalomma marginatum* pathogens and symbionts for each monthly collection (represented by different symbols). The number of collected ticks (n) per month and season is indicated. The monthly mean temperature (°C) at the Pompignan study site was obtained with the R package Open-Meteo (https://open-meteo.com/).

Table 2
Multivariate analysis of *Hyalomma marginatum*-borne pathogens (*Rickettsia aeschlimannii*, *Theileria equi*, *Anaplasma* spp., and *Francisella*-LE) according to the season of collection and the sex of the tick.

Pathogen	Season						Sex				
	Winter (n = 110)		Spring (n = 68)		Summer (n = 103)	Significant differences	Male (n = 153)		Female (n = 128)		Significant differences
	n_i	IR% (95% CI)	n_i	IR% (95% CI)	n_i	IR% (95% CI)	Yes/No (statistics, df = 2)	n_i	IR% (95% CI)	n_i	IR% (95% CI)	Yes/No (statistics, df = 1)
R. aeschlimannii	38	34.5 (25.5–43.6)	17	25.0 (14.4–35.6)	77	74.8 (66.2–83.3)	Yes (χ^2 = 10.29, P = 0.0058)	59	38.6 (30.8–46.4)	73	57.0 (48.3–65.7)	Yes (χ^2 = 10.11, P = 0.0015)
T. equi	13	11.8 (5.7–17.9)	5	7.4 (1.0–13.7)	13	12.6 (6.1–19.1)	No (χ^2 = 1.40, P = 0.4967)	19	12.4 (7.1–17.7)	12	9.4 (4.3–14.5)	No (χ^2 = 0.69, P = 0.4052)
Anaplasma spp.	5	4.5 (0.6–8.5)	0	0	8	7.8 (2.5–13.0)	Yes (χ^2 = 8.61, P = 0.0135)	8	5.2 (1.7–8.8)	5	3.9 (0.5–65.7)	No (χ^2 = 0.52, P = 0.4610)
Francisella-LE	108	98.2 (95.6–100)	68	100	103	100	No (χ^2 = 2.83, P = 0.2435)	151	98.7 (96.9–100)	128	100	No (χ^2 = 1.49, P = 0.2214)

Notes: Number of infected ticks (n_i), infection rates (IR%), and the results from statistical analyses of the season of collection and sex of the tick. Infection rates were calculated based on the number of positive ticks in each category and the total number of ticks (n = 281). The data used for the analysis were generated using the BioMark assay for *Anaplasma* spp. and the qPCR assay for *R. aeschlimannii* and *T. equi*.
Abbreviations: df, degrees of freedom; χ^2, Chi-square value; P, P-value; CI, confidence interval.

Fig. 3. Linear correlation analysis of the average monthly *Rickettsia aeschlimannii* infection rates (%) and the average monthly temperature (°C).

4. Discussion

Hyalomma marginatum, which recently became established in southern France, is known to be able to transmit several human and animal pathogens and thus represents a major risk for public and animal health. Assessing the precise risk first requires identifying the composition of *H. marginatum*-borne pathogens and characterising their temporal dynamics. In this context, we examined the dynamics of tick-borne pathogens (TBP) in *H. marginatum* collected at monthly intervals from February to September 2022 in a study site in Pompignan in the South of France.

4.1. Composition and prevalence of Hyalomma marginatum-borne pathogens

Three major pathogens were detected in the 281 *Hyalomma marginatum* analysed: *R. aeschlimannii* (47.0%), *T. equi* (11.0%) and *Anaplasma* spp. (4.6%). In previous studies, these pathogens were already detected in *H. marginatum* in the study region (Occitanie Region), but the vector competence of *H. marginatum* remains unclear (Bernard et al., 2024b; Joly-Kukla et al., 2024). While recent spatial studies in this region detected in *H. marginatum* other pathogens, including *A. marginale*, *E. minasensis* and the West Nile virus, using the same detection technique (Bernard et al., 2024b), none of these pathogens were detected in the present study. The absence of *A. marginale* can be attributed to the fact that infections caused by this bacterium occur in cattle, whereas in the present study, all the ticks sampled were collected from horses. The absence of detection of both *E. minasensis* and West Nile virus, which have previously been detected in cattle (on the island of Corsica) and horses (in mainland France) in a spatial surveillance study of tick-borne pathogens, is likely due to the fact that our study focused on ticks collected from 13 horses in a single area, which may have reduced the probability of detecting a wide diversity of pathogens. Grech-Angelini et al. (2020) used the same detection technique (BioMark assay) to identify the pathogens on *H. marginatum* ticks on the island of Corsica. These authors reported detecting *R. aeschlimannii*, *A. phagocytophilum*, *A. marginale* but did not detect *T. equi* in ticks collected from horses. Although it should be kept in mind that only 4.2% of the ticks collected by Grech-Angelini et al. (2020) were from horses and that more investigations on these particular hosts are needed, this contrasting result could also be due to the primers the authors used for the BioMark assay, which cannot target all the genotypes of *T. equi*.

The infection rates of *R. aeschlimannii* observed in the present study (47%) are consistent with those estimated in adult *H. marginatum* ticks collected on camels in Israel (54.5%) (Azagi et al., 2017), but contrast

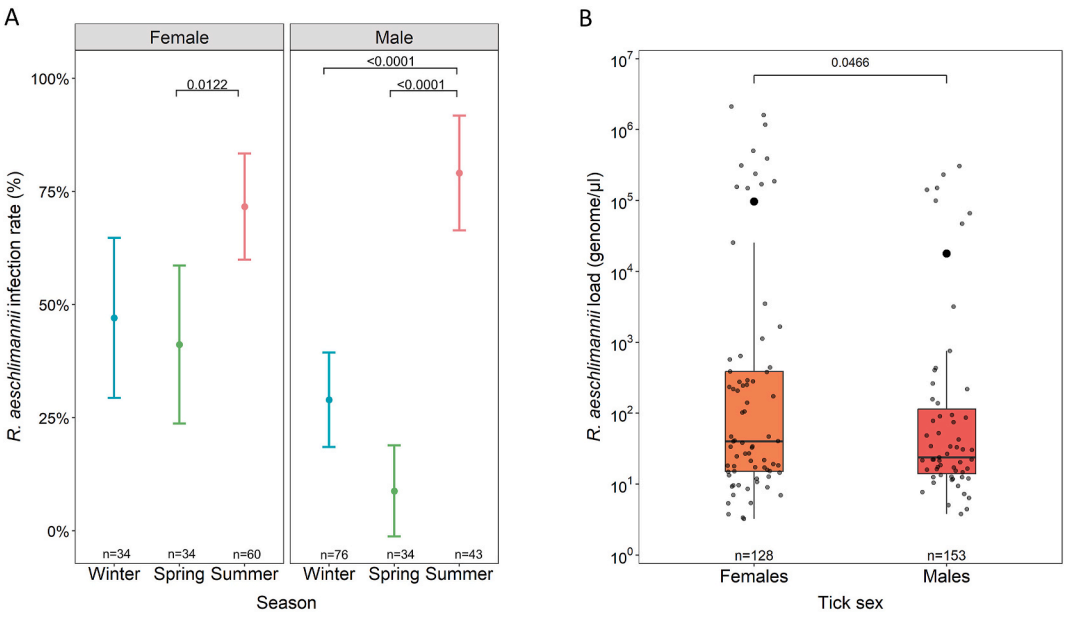

Fig. 4. Seasonal trends of *Rickettsia aeschlimannii*. **A** The average seasonal infection rate (in %) is represented by a dot with the confidence interval. **B** The bacterial loads obtained by qPCR in genome/µl are represented for each tick by grey dots and the means are represented by black dots. The boxplots summarise the median, 1st and 3rd quartiles. The number of collected ticks (*n*) is indicated above each category: winter (February to March); spring (April to May); summer (June to September). Only significant differences and the associated *P*-values (*post-hoc* test) are shown.

Table 3
Multivariate analysis of *Rickettsia aeschlimannii* loads according to the season and the sex of the tick.

	Mean load (genome.µl^{-1}) (95% CI)	Significance of differences
Season		$\chi^2 = 3.74$, $df = 2$, $P = 0.1544$
Winter ($n_i = 38$)	3.5×10^4 (6.5×10^3–6.3×10^4)	
Spring ($n_i = 17$)	6.9×10^4 (0–2.1×10^5)	
Summer ($n_i = 77$)	7.3×10^4 (2.8×10^3–1.4×10^5)	
Sex		$\chi^2 = 9.71$, $df = 1$, $P = 0.0018$
Male ($n_i = 59$)	1.8×10^4 (3.0×10^3–3.3×10^4)	
Female ($n_i = 73$)	9.6×10^4 (1.7×10^4–1.8×10^5)	

Abbreviations: *df*, degrees of freedom; χ^2, Chi-square value; *P*, *P*-value; CI, confidence interval.

with the higher infection rates estimated by Grech-Angelini et al. (2020) in Corsica in ticks collected from a variety of hosts (100%) and with rates observed in our previous study in ticks from horses in the Gard Department at different study sites (78%) (Fig. 1) (Joly-Kukla et al., 2024). On the other hand, these results also contrast with the very low infection rates reported in ticks collected from humans, cows and vegetation in Spain. The infection rates of *R. aeschlimannii* in *H. marginatum* ticks thus appear to be highly variable. It is unlikely that the host is responsible for this variation, as Grech-Angelini et al. (2020) observed a 100% infection rate in ticks collected from multiple hosts including cattle, sheep, goats, horses, wild boars and mouflons. Furthermore, in our previous study, we found no statistical influence of hosts (horses and cattle) on the infection rates of *R. aeschlimannii* (Joly-Kukla et al., 2024). In our opinion, even if we cannot rule out the hypothesis that these differences are explained by the bacterial load being too low to be quantified by qPCR or the BioMark assay, methodological bias is unlikely, as several of these studies used similar detection methods and both the qPCR and the BioMark assay are known to be highly sensitive. The fact we did not detect *R. aeschlimannii* in 100% of ticks despite maternal transmission could have a biological explanation, i.e. the efficacy of the maternal transmission might not reach quite 100% (95.3% in the study of Azagi et al., 2017). In addition, this is in line with our hypothesis that *R. aeschlimannii* is a secondary symbiont of *Hyalomma marginatum* (Joly-Kukla et al., 2024) as facultative symbionts are known to exhibit variable infection frequencies in space and over time (Ferrari et al., 2012; Stefanini and Duron, 2012; Russell et al., 2013; Smith et al., 2015; Duron et al., 2017; Oliver et al., 2010). Ticks may maintain intermediate frequencies because of a cost-benefit balance associated with harbouring these maternally transmitted symbionts (Bonnet et al., 2017). A mixed transmission (vertical and horizontal) is typical in the case of secondary symbionts. We thus cannot rule out the hypothesis of horizontal transmission during the blood meal on the hosts of the immature tick stages (lagomorphs, birds, hedgehogs), or during the blood-feeding of adult ticks on horses, but no information is currently available concerning their susceptibility and their ability to transmit *R. aeschlimannii* to ticks (ANSES, 2023). The extremely efficient maternal transmission (95.3% success) of this bacterium (Azagi et al., 2017) suggests that *R. aeschlimannii* is mainly maintained in *H. marginatum* ticks *via* transovarian transmission rather than horizontal transmission. For this reason, we consider that the hosts are unlikely to influence the presence and dynamics of *R. aeschlimannii*, whose reservoir is the tick *H. marginatum* (Matsumoto et al., 2004).

Concerning *Anaplasma* spp., the five samples testing positive for both *A. phagocytophilum* and *Anaplama* spp. using the BioMark assay were considered at the genus level (*Anaplasma* spp.), because we could not obtain any readable sequences after the sequencing of confirmation PCR amplicons, although bands were visible in agarose gel and corresponded to the size of the specific band of the *msp2* gene of *A. phagocytophilum*. However, we can hypothesise that samples that tested positive for both *A. phagocytophilum* and *Anaplasma* spp., and the eight others that tested positive for *Anaplasma* spp. (using the BioMark assay) corresponded to *A. phagocytophilum*, since this species was confirmed by sequencing in

H. marginatum ticks from the same geographical area and year in a previous study (Joly-Kukla et al., 2024). Although there are no reports of vector competence by *H. marginatum* for *A. phagocytophilum*, Bernard et al. (2024b) nevertheless suggested transmission is possible. *Anaplasma phagocytophilum* was shown to cause granulocytic anaplasmosis in humans (HGA), as well as in domestic animals (cats, dogs, horses) (Rouxel et al., 2024). Some host species of the immature stages of *H. marginatum*, including birds, are able to transmit *A. phagocytophilum* to ticks (Heylen et al., 2017; Keesing et al., 2021; Rataud et al., 2022). *Anaplasma phagocytophilum* has also been detected in hares in Europe (Rocchigiani et al., 2018; Lesiczka et al., 2021) as well as in hedgehogs (Silaghi et al., 2012; Lesiczka et al., 2021). Based on all these results, we cannot know whether the detection of *A. phagocytophilum* in ticks was due to the blood meal on an infected horse or if *H. marginatum* acquired the bacterium during its immature stages. More investigations of the first development stages of the tick are thus needed.

Theileria equi was found in 11% of ticks. This parasite is the agent of equine piroplasmosis, a widespread disease in France especially in the south (Rocafort-Ferrer et al., 2022). Interestingly, lower percentages were detected in the same study area in another study (Bernard et al., 2024b). As mentioned above, this difference might be explained by the primers used that can target more genotypes of *T. equi*. Even if it seems unlikely, it is not known whether *H. marginatum* is involved in the transmission of this protozoan (Bonnet et al., 2023). Its detection in adult ticks might be mainly due to ingestion of blood from an infected horse or during a blood meal consumed during its immature stages, even if it is still not clear if the hosts of tick immature stages are likely to be infected and transmit the protozoan to ticks (Friedhoff, 1990; Bernard et al., 2024b).

4.2. Seasonal dynamics of Hyalomma marginatum-borne pathogens

Ticks were found recurrently infected with at least one pathogen throughout the collection period. Even though infection rates varied among the different pathogens, they were all detected recurrently from February to September. The presence of *R. aeschlimannii* in ticks varied significantly with the season. These temporal trends followed the same pattern regardless of the detection technique used and the genes targeted were very similar, thereby reinforcing the robustness of this result. While the presence of *R. aeschlimannii* varied with the season, interestingly, this was not the case with its loads. These contrasted results can probably be explained by the high variability in *R. aeschlimannii* loads observed between ticks collected in the same month and emphasise the importance of increasing the sampling effort as much as possible. Both the spatial variation of *R. aeschlimannii* infection rates (Joly-Kukla et al., 2024) and the temporal variations observed in the present study reinforce the hypothesis of a secondary symbiont. Indeed, as observed in our results, a primary symbiont such as *Francisella*-like symbiont in *H. marginatum* was present in almost all the ticks we collected (279 of the 281 ticks analysed) and its infection rates do not vary with time because the characteristics are required for the tick's survival. We observed a marked increase in *R. aeschlimannii* infection rates in all the ticks analysed from June to the end of summer compared to in winter and spring. In parallel, interestingly, we found a significant correlation between *R. aeschlimannii* infection rates and temperature. Under the hypothesis that *R. aeschlimannii* is a secondary symbiont in *H. marginatum*, it could play an important role in the tick's defence against natural enemies (Oliver et al., 2010; Su et al., 2013) or in mediating the thermal tolerance of the host (Dunbar et al., 2007). Based on our results and because an increase in temperature is known to contribute to tick desiccation caused by water loss (Estrada-Peña et al., 2012), we hypothesise that *R. aeschlimannii* in ticks is involved in the stress response to an increase in temperature in the tick's environment and in this way, mediates the thermal tolerance of *H. marginatum*. Experimental investigations are needed to test this hypothesis. It would indeed be interesting to estimate both *R. aeschlimannii* infection rates and loads from ticks experimentally placed in cages located in a shady spot *vs* a sunny spot in the field, with continuous monitoring of temperature. In addition, a genome analysis of this bacterium isolated from our *H. marginatum* ticks or the analysis of the relative expression of some functional genes involved in stress responses to temperature would also help validate or invalidate the hypothesis.

As previously reported (Joly-Kukla et al., 2024) and here (*Section 3.2*), bacterial loads were higher in females than in males. This could be due to the absence of ovaries in males, where *R. aeschlimannii* might predominantly be located since it is maternally transmitted (Matsumoto et al., 2004; Azagi et al., 2017). This hypothesis might also explain the higher overall rates of infection in females than in males. Additional information regarding the location of *R. aeschlimannii* in the different tick organs would enable the formulation of a more precise hypothesis about its potential function in *H. marginatum*.

Concerning *Anaplasma* spp., our findings showed variations in the infection rates over the year with fewer infected ticks in spring than in summer and winter. While *T. equi* and *R. aeschlimannii* were detected in all the months the ticks were collected, *Anaplasma* spp. was only detected in March, and then again between June and September. Because we collected ticks from horses, two scenarios are possible. The first one supposes that ticks were infected by *Anaplasma* spp. during the previous blood meals during the tick immature stages. In this case, let us suppose that *H. marginatum* is able to transmit this bacterium; even if no information is currently available on the vector competence of this tick species for this pathogen, we wish to draw attention to the variations in its infection rates, since there is a higher risk for dogs, horses, and humans, to be bitten by an infected tick in summer (7.8%) than in winter (4.5%) and spring (0%). In this case, the temporal variability would be the result of the dynamics of tick immature stages in the previous year, i.e. before over-wintering, and of their associated hosts which could ensure the continued circulation of this bacterium. The second scenario supposes that adult ticks acquired *Anaplasma* spp. on infected horses. In this case, the observed temporal patterns are more difficult to interpret since each horse has a different infectious status and our ticks were analysed altogether regardless of the individual host. In our opinion, the second hypothesis is less likely because most of the ticks we collected were not or only slightly engorged. However, the second scenario also calls for caution because if horses are positive for *Anaplasma* spp., it means that another tick species, competent for the same pathogen, transmitted it to the horses and would possibly be able to transmit it to other potential hosts including humans.

Theileria equi infection rates varied from 2.9% in April to 25% in August, although no significant differences were observed at seasonal scale. Infection rates varied between months of the same season, for example in summer (6.5% in June, 9.1% in July, and 25% in August). The infection rate was particularly high in August (25%). As mentioned above concerning the results for *Anaplasma* spp., at first sight, it is difficult to interpret this increase and more generally the temporal variations in *T. equi*, because we cannot know if the pathogen was acquired during previous blood meals or directly on horses. More investigations are thus required on *T. equi* specifically on questing *H. marginatum*. However, whatever the scenario and because mortality rates due to piroplasmosis can reach 50% for infected horses, our results suggest more care needs to be taken of horses during the summer period. The fact that 2.6× more ticks were infected with at least *Theileria* or *Anaplasma* in summer than in spring, and 1.3× more than in winter calls for more caution given the risk of ticks at specific periods of the year, and particularly that tick densities are not the same across seasons.

As already observed and mentioned for other tick species (Lejal et al., 2019a), our findings confirm that sporadic *H. marginatum* collections are not sufficient to assess the *H. marginatum*-borne pathogen prevalences and thus emphasise the importance of regular monitoring as well as the need to inform the general population about the risk represented by ticks. The fact that our findings were obtained in a one-year study calls for caution before drawing conclusions about the observed pattern, and

a multi-year survey would be necessary to assess potential inter-annual recurrence of this pattern. In the literature, a three-year survey of *Ixodes ricinus*-borne pathogens showed fluctuations of the pathogen dynamics while similar patterns were observed for abiotic conditions from one year to another (Lejal et al., 2019a). In this case, the pathogen dynamics may be linked to host dynamics (Lejal et al., 2019a) rather than to abiotic conditions. In the present study, as previously discussed, the host is unlikely to influence the temporal variation of *R. aeschlimannii*, but it would nevertheless be interesting to know if the temporal pattern we observed in 2022 reoccurs from one year to another. Several studies highlighted similar trends in temporal variations between the prevalence of pathogens and the density of ticks, which is related to the availability and composition of hosts (Coipan et al., 2013; Chvostáč et al., 2018). In order to investigate the influence of tick density and pathogen infection rates in *H. marginatum*, it would be interesting to focus on ticks collected from the same horse each month and to determine tick density by collecting all the ticks present on the animal. In the context of the present study, the scrubland area where horses were sampled for tick collection provides shelter for a limited number of hosts for adult ticks, which are the 13 horses. The remainder of the fauna are immature tick wildlife hosts. As the adult ticks were collected and they only feed on large hosts like horses, which were present in a fixed number, a link between the temporal trends of host density and *H. marginatum*-borne pathogens is unlikely in this case.

In addition to the monthly survey in our specific study site in Pompignan, it would also be interesting to investigate another site in another geographical area. Indeed, it has been reported that *R. aeschlimannii* infection rates differ significantly between sites from one geographical cluster to another even within our study region, with higher infection rates for example in the Aude/Pyrénées-Orientales area (92.3% on average) compared to those estimated in the Hérault/Gard area (78.6% on average) (Fig. 1) (Joly-Kukla et al., 2024). In the present study, while fluctuating infection rates were observed for all the pathogens detected, the most marked temporal variations were detected for *R. aeschlimannii*. A monthly survey in another site located in the Aude/-Pyrénées-Orientales area would definitely be useful to characterize the seasonal dynamics of *R. aeschlimannii* in this second site and to assess the potential recurrence of the temporal pattern observed in Pompignan (Hérault).

5. Conclusions

This study characterized the composition and dynamics of *H. marginatum*-borne pathogens over a period of eight months in 2022. These data are crucial for identifying monthly/seasonal patterns which could improve our understanding of *H. marginatum*-borne pathogen ecology and prevent the occurrence of *H. marginatum*-borne diseases. Our findings allowed us to (i) precisely identify pathogens already suspected to circulate in the study area; (ii) highlight strong temporal patterns at monthly and seasonal scales particularly for *R. aeschlimannii*; our results allow us to propose a hypothesis for the function of *R. aeschlimannii* in *H. marginatum*, which could be involved in the stress response to temperature increase and mediate the thermal tolerance of *H. marginatum*.

Funding

This work was supported by the Holistique project (défi clé RIVOC Occitanie region, University of Montpellier, France): "*Hyalomma marginatum* in Occitanie region: analysis of biological invasion and associated risks". The salary of Charlotte Joly-Kukla, the PhD student working on this project, was funded by INRAE, France.

Ethical approval

Not applicable.

Data availability

The data supporting the conclusions of this article are included within the article and its supplementary files. Nucleotide data were deposited in the GenBank database under the accession number PP663276. Raw data, scripts and code used for bioinformatics, and statistical analysis are available at the project website (https://zenodo.org/records/13628715) (see also Supplementary file S3).

CRediT authorship contribution statement

Charlotte Joly-Kukla: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. **Frédéric Stachurski:** Resources, Validation. **Maxime Duhayon:** Resources, Validation. **Clémence Galon:** Methodology, Resources, Validation. **Sara Moutailler:** Conceptualization, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing. **Thomas Pollet:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Acknowledgments

The authors are grateful to Maud Marsot and Facundo Munoz for their helpful advice on statistics.

Appendix. A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2024.100213.

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