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Short communication

Uneven temporal distribution of piroplasms (Piroplasmida: Babesiidae, Theileriidae) in *Haemaphysalis concinna* in an urban biotope of the Western Palearctic focus region of this tick species

Gergő Keve [a,b,*], Ciara Reynolds [a], Nóra Takács [a,b], Sándor Hornok [a,b]

[a] Department of Parasitology and Zoology, University of Veterinary Medicine, Budapest, Hungary
[b] HUN-REN-UVMB Climate Change: New Blood-sucking Parasites and Vector-borne Pathogens Research Group, Hungary

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ABSTRACT

Haemaphysalis concinna is a Palearctic tick species known as a potential or proven vector of several pathogens, including a broad spectrum of *Babesia* and *Theileria* species. The aim of this study was to examine the monthly presence of these piroplasms in *H. concinna* specimens collected from the vegetation of an urban habitat in Budapest, Hungary, in 2019 and 2020. The questing abundance of *H. concinna* was highest in June. By contrast, the occurrence of *T. capreoli* in unfed *H. concinna* peaked in April, and was significantly more common in the spring, than in the rest of the year. Among the detected eleven *Babesia* genotypes, two were present only in nymphs and adults of *H. concinna*. These were identical in the amplified part of their 18S rRNA gene to piroplasms reported from the Far East. Three further *Babesia* genotypes, however, showed genetic heterogeneity and were also carried by larvae. *Babesia*-infected nymphs and adults were most common in May and July.

In conclusion, the results of this study show that in a questing population of *H. concinna* the highest monthly prevalence of *Babesia* and *Theileria* spp. may be different from each other and from the peak abundance of carrier ticks. Based on previous reports on the effect of tick-borne pathogens on other species of ticks, the factors that may influence this phenomenon in *H. concinna* may include changes in the metabolism and behavior (host finding and feeding success) as well as survival rate of infected ticks. Further studies will be necessary to clarify this.

1. Introduction

Haemaphysalis concinna is a three-host tick species prevalent throughout the northern Palearctic region. Notably, this tick demonstrates a much higher abundance in the Carpathian Basin compared to other regions of Europe (Rubel et al., 2018). While the adults are rather common parasites of medium-sized mammals, larvae and nymphs are frequently found on smaller vertebrates, such as rodents, birds, and rarely on reptiles (Estrada-Peña et al., 2017). *Haemaphysalis concinna* is thermophilic, thriving in habitats with high relative humidity (Hubálek et al., 2003). Its activity spans from April (sometimes March) to November, peaking during the summer months (Estrada-Peña et al., 2017; Keve et al., 2024; Nosek, 1971).

There are several species of small to medium-size wild mammals that could potentially serve as hosts for *H. concinna* in rural and urban areas, for example roe deer (*Capreolus capreolus*), red foxes (*Vulpes vulpes*), beech martens (*Martes foina*), European badgers (*Meles meles*), and northern white-breasted hedgehogs (*Erinaceus roumanicus*) (Estrada-Peña et al., 2017; Nosek, 1971). In addition, domestic cats (*Felis catus*) and dogs (*Canis lupus familiaris*) that are either stray or owned and taken to walks or kept extensively can also become infested with this tick species. Among birds, reed-associated species, e.g. the Savi's warbler (*Locustella luscinioides*), the Sedge warbler (*Acrocephalus schoenobaenus*) and the Eurasian reed warbler (*Acrocephalus scirpaceus*) and several members of the family Turdidae, such as the blackbird (*Turdus merula*) and the song thrush (*Turdus philomelos*) are important hosts of *H. concinna* (Keve et al., 2024).

Haemaphysalis concinna is a proven vector of pathogens of high medical importance, e.g. tick-borne encephalitis virus, *Coxiella burnetii, Francisella tularensis*, and several species of rickettsiae (Estrada-Peña et al., 2017). A large variety of protozoa from the order Piroplasmida were detected in questing *H. concinna* as well, including, *Babesia canis*

* Corresponding author.
E-mail address: keve.gergo@univet.hu (G. Keve).

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(Mihaljica et al., 2012), *Babesia motasi, Babesia* sp. Irk-Hc133 (Hornok et al., 2015), *Babesia* sp. Kh-Hc222, *Babesia* sp. Kh-Hc232 (Hamšíková et al., 2016), *Theileria capreoli* (Hornok et al., 2015), and *Theileria* sp. ZS TO4 (Fuehrer et al., 2013).

Numerous tick-borne pathogens show unequal seasonal distribution in *Ixodes ricinus* ticks, as exemplified by *Anaplasma, Rickettsia,* and *Borrelia* species (Kantsø et al., 2010; Mysterud et al., 2013; Polin et al., 2004). The seasonally biased occurrence of *B. canis* in its tick vector, *Dermacentor reticulatus* has also been reported (Hornok et al., 2016).

Due to the scarcity of comprehensive data on the temporal distribution of piroplasms *H. concinna* may carry, this study aimed to address this gap by investigating their seasonal patterns in an urban biotope and to serve as a basis for future research efforts.

2. Materials and methods

2.1. Sample collection

Haemaphysalis concinna specimens examined in this study were collected between February 2019 and November 2020, as previously reported (Reynolds et al., 2022). In brief, the tick collection site was chosen based on the results of a large-scale survey of urban biotopes in Budapest (Hornok et al., 2014). This biotope is part of a large cemetery, where neglected parts had dense lower vegetation covering [grass, weeds and nearly continuous ivy (*Hedera* sp.)] and sparse distribution of bushes and trees. This site was visited at monthly intervals, at the end of each month. Tick collections were performed under dry weather conditions. Ticks were collected from the vegetation by the dragging-flagging method, i.e., a white towel, measuring 1 × 1 m, was drawn over the vegetation and checked every 10 s. During this the same five, approx. 60 m long parallel transects were sampled regularly (i.e., 300 m^2). Ticks attached to and removed from the collecting device were immediately put into and stored in 96 % ethanol. *Haemaphysalis concinna* specimens were identified by using standard morphological keys (Filippova, 1997).

2.2. DNA extraction

DNA was extracted from unfed *H. concinna* individually, from whole ticks. These were disinfected on their surface with sequential washing for 15 s in water-detergent mix, in tap water and in distilled water. DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instruction, including an overnight digestion in tissue lysis buffer and proteinase-K at 56 °C. A negative control (tissue lysis buffer) was also processed in each set of tick samples, to monitor cross-contamination.

2.3. Molecular analyses

The PCR method described by Casati et al. (2006) was used for detection of piroplasm DNA. A conventional PCR was used with the primers BJ1 5′- GTC TTG TAA TTG GAA TGA TGG– 3′ forward and BN2 5′ –TAG TTT ATG GTT AGG ACT ACG - 3′reverse to amplify a ~500 bp long fragment of the 18S rRNA gene. Five µl of extracted DNA were added to 20 µl of reaction mixture containing 1.0 U HotStar Taq Plus DNA Polymerase (5 U/µl) (QIAGEN, Hilden, Germany), 0.5 µl dNTP Mix (10 mM), 0.5 µl of each primer (50 µM), 2.5 µl of 10x Coral Load PCR buffer (15 mM $MgCl_2$ included), and 15.8 µl DW. An initial denaturation step at 95 °C for 10 min was followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 40 s. Final extension was performed at 72 °C for 5 min. then kept at 40 °C. PCR products were electrophoresed in 1.5 % agarose gel (100 V, 50 min), stained with ethidium-bromide and visualized under ultra-violet light. Negative (non-template) controls and positive (sequence verified, species specific) controls were also processed in the cases of all PCR probes. All extraction and negative controls remained negative in the PCRs.

Purification and sequencing of the PCR products was done by Eurofins Biomi Ltd (Gödöllő, Hungary). Quality control and trimming of sequences were performed using the BioEdit program, followed by alignment with GenBank sequences online by BLASTN (https://blast.ncbi.nlm.nih.gov). Sequences obtained in this study have been submitted to GenBank (PQ040346-PQ040357).

2.4. Phylogenetic analysis

Sequences from other studies, used here for phylogenetic analyses, were retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches (Felsenstein, 1985). All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 445 positions in the final dataset. Phylogenetic analyses were conducted with the Neighbor-Joining method (Saitou and Nei, 1987) and p-distance model (Nei et al., 2000) by using MEGA 11 (Tamura et al., 2021).

2.5. Statistical analysis

Larvae (which typically show aggregated presence during questing activity, originating from the same egg clutch, and were not distributed by hosts before) were excluded from statistical analyses. Statistical analyses were conducted with R (R 4.4.1) (R Core Team, 2023). *Babesia* and *T. capreoli* prevalences were calculated as the ratio of infected ticks proportionate to their total number (Supplementary Table 1). Fisher's exact test was used to compare infection rates of nymphs and adult ticks together. Differences were regarded as significant when $P < 0.05$.

3. Results

In both years of the study period, *H. concinna* questing activity was observed from March to November, but not during winter months. A total of 454 *H. concinna* individuals were collected, including 154 larvae, 287 nymphs, and 13 adults (seven males and six females). Nymphs and larvae reached their peak abundance in June, while the few adults predominated in May.

Eleven distinct *Babesia* genotypes and *T. capreoli* were identified in these ticks (Fig. 1). Regarding developmental stages, the *Babesia* prevalence was 9.7 % (15/154) in larvae, 15.7 %; (45/287) in nymphs, and 46 %; (6/13) in adults. *Babesia* infection in *H. concinna* nymphs and adults was significantly more common in May ($P = 0.0173$) and in July ($P = 0.0067$) when compared to other months combined (Fig. 1). Among *Babesia* genotypes, *Babesia* sp. Bp-Hc2 and *Babesia* sp. Bp-Hc8 were not detected in any *H. concinna* larvae, similarly to *T. capreoli*. On the contrary, *Babesia* sp. Bp-Hc5 and *Babesia* sp. Bp-Hc7 were only found in *H. concinna* larvae (Fig. 1). Another genotype, *Babesia* sp. Bp-Hc3 was detected both in larvae and nymphs of *H. concinna* between April and August (Fig. 1). *Theileria capreoli* was not detected in larvae, only in nymphs ($n = 5$) males ($n = 2$) and a female of *H. concinna*. All PCR-positive ticks occurred in the first five months of the sampling period (April to July). In addition, significantly ($P = 0.0038$) more *T. capreoli*-infected nymphs and adults were identified during the springtime (March-May) (6 infected, 71 uninfected) than in the summer and autumn combined (June-November) (2 infected, 229 uninfected).

Phylogenetically, *Babesia* genotypes identified in this study belong to the phylogenetic group of ruminant-associated species of Babesiidae which also include zoonotic species (Fig. 3). These always clustered together with the corresponding, "Far Eastern" *Babesia* genotypes, with low to moderate support. Importantly, two of these *Babesia* variants were only represented by a single genotype and were never detected in larvae during this study (Fig. 3).

Year	Piroplasm \ Total number in 2019/2020	March	April	May	June	July	August	September	October	November
		L: -/- N: 3/- F: -/- M: -/-	L: 3/- N: 19/3 F: 1/- M: -/-	L: 16/- N: 12/25 F: -/3 M: -/5	L: -/117 N: 23/46 F: 1/1 M: -/-	L: -/3 N: 23/35 F: -/- M: 1/-	L: -/11 N: 23/38 F: -/- M: -/1	L: -/2 N: 11/15 F: -/- M: -/-	L: -/2 N: 6/4 F: -/- M: -/-	L: -/- N: 1/- F: -/- M: -/-
2019 2020	*T. capreoli*		NNN F	 MM	N	N				
2019 2020	*B.* sp. Bp-Hc2		N	 NNNN F	NNNN	NNN	N N	N		
2019 2020	*B.* sp. Bp-Hc3		L	LL N	NN	NN N	N LLL			
2019 2020	*B.* sp. Bp-Hc4			L N						
2019 2020	*B.* sp. Bp-Hc5			LL						
2019 2020	*B.* sp. Bp-Hc12						M			
2019 2020	*B.* sp. Bp-Hc6			L			N			
2019 2020	*B.* sp. Bp-Hc7			LLLL			 L			
2019 2020	*B.* sp. Bp-Hc11				N					
2019 2020	*B.* sp. Bp-Hc8			 NN MM	N NN	N NNNNNNNN	NN N		 NN	
2019 2020	*B.* sp. Bp-Hc10			 F			 N			
2019 2020	*B.* sp. Bp-Hc9					M				

Abbreviations: *T – Theileria, B - Babesia*

Fig. 1. Piroplasm species/genotypes according to the developmental stage or sex of *Haemaphysalis concinna* in which they were found in a certain month. Abbreviations: *T – Theileria, B - Babesia.*

4. Discussion

This study aimed to evaluate the seasonal prevalence of *H. concinna*-associated piroplasms in an urban habitat situated in a hotspot of the Palearctic range of this tick species (Rubel et al., 2018). In total, one *Theileria* species, and eleven *Babesia* genotypes were detected, the latter likely representing at least four different species based on their phylogenetic clustering (Fig. 3).

During this study, *T. capreoli* was not detected in questing (pre-feeding) *H. concinna* larvae, but only in nymphs and adult ticks, probably in accordance with its transstadial maintenance. The latter implies that in the absence of transovarial transmission the first opportunity for a tick to obtain theileriae is from the host, during feeding as larvae, ensuring the transstadial survival of these piroplasms when tick larvae molt to the nymph stage (Li et al., 2007). *Theileria capreoli*-infected *H. concinna* nymphs and adults predominated in April and May (Fig. 2), meaning a significant association with the springtime. Interestingly, the peak of horizontal movements of roe deer, the most important mammalian hosts of *H. concinna* (Hornok et al., 2012) also tends to be in May owing to territorial fights (Markolt et al., 2012), thus coinciding with this seasonality. This phenomenon, the seasonally biased occurrence of a tick-borne piroplasm is very similar to what was observed in another urban biotope of the same city, where the presence of *B. canis* in its vector, *D. reticulatus* was almost exclusively noted in the late winter-early spring period (Hornok et al., 2016).

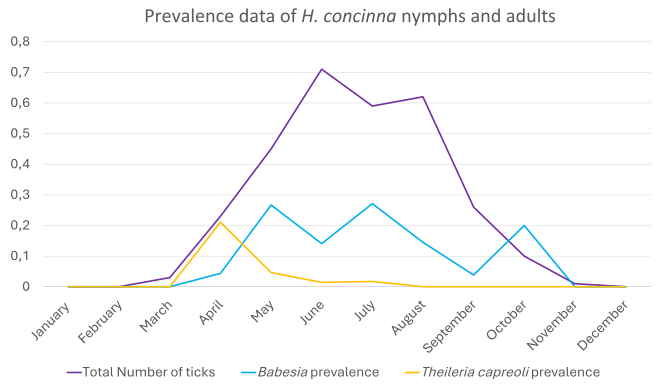

Fig. 2. The *Babesia* prevalence and the *Theileria capreoli* prevalence in *H. concinna* nymphs and adults, and the total numbers of *H. concinna* nymphs and adults. The total numbers of ticks were divided by 100 to adjust them to the graph. The numbers used to generate this figure are included in Supplementary Table 1.

Within the family Babesiidae, all *Babesia* genotypes identified in this study belong to the phylogenetic group associated with ruminant hosts (Fig. 3). These genotypes are commonly referred to as "Far Eastern Babesia genotypes" due to their initial discovery and reporting in the Far East regions of Irkutsk (Siberia) and the Khabarovsk region of Russia (Rar et al., 2014). The majority of these genotypes identified in *H. concinna* were previously reported from this tick species in the Far East (denoted as “Hc” in Fig. 3), with the exception of one genotype from *Ixodes persulcatus* (“Ip” in Fig. 3).

In this study, *Babesia* sp. Bp-Hc2 and *Babesia* sp. Bp-Hc8 were detected in nymphs and adults of *H. concinna*. This, together with the absence of these babesiae from larvae likely indicates that these piroplasms arrived in *H. concinna* larvae which fed on migratory birds and are not endemic in the examined urban biotope. This is confirmed by data attesting to the association of ticks harboring these two piroplasms with birds that have historical (evolutionary-phylogenetic) or actual (migratory) connection with the Far East, as exemplified by *Emberiza citrinella* and *Luscinia* spp. (Flaisz et al., 2017; Pitó et al., 2024). Sequence analysis of their 18S rRNA gene segments (PQ040347 and PQ040353) revealed that these sequences are identical to those previously reported from the Far East. (Rar et al., 2014).

On the other hand, *Babesia* sp. Bp-Hc5 and *Babesia* sp. Bp-Hc7 were only found here in questing *H. concinna* larvae, indicating transovarial

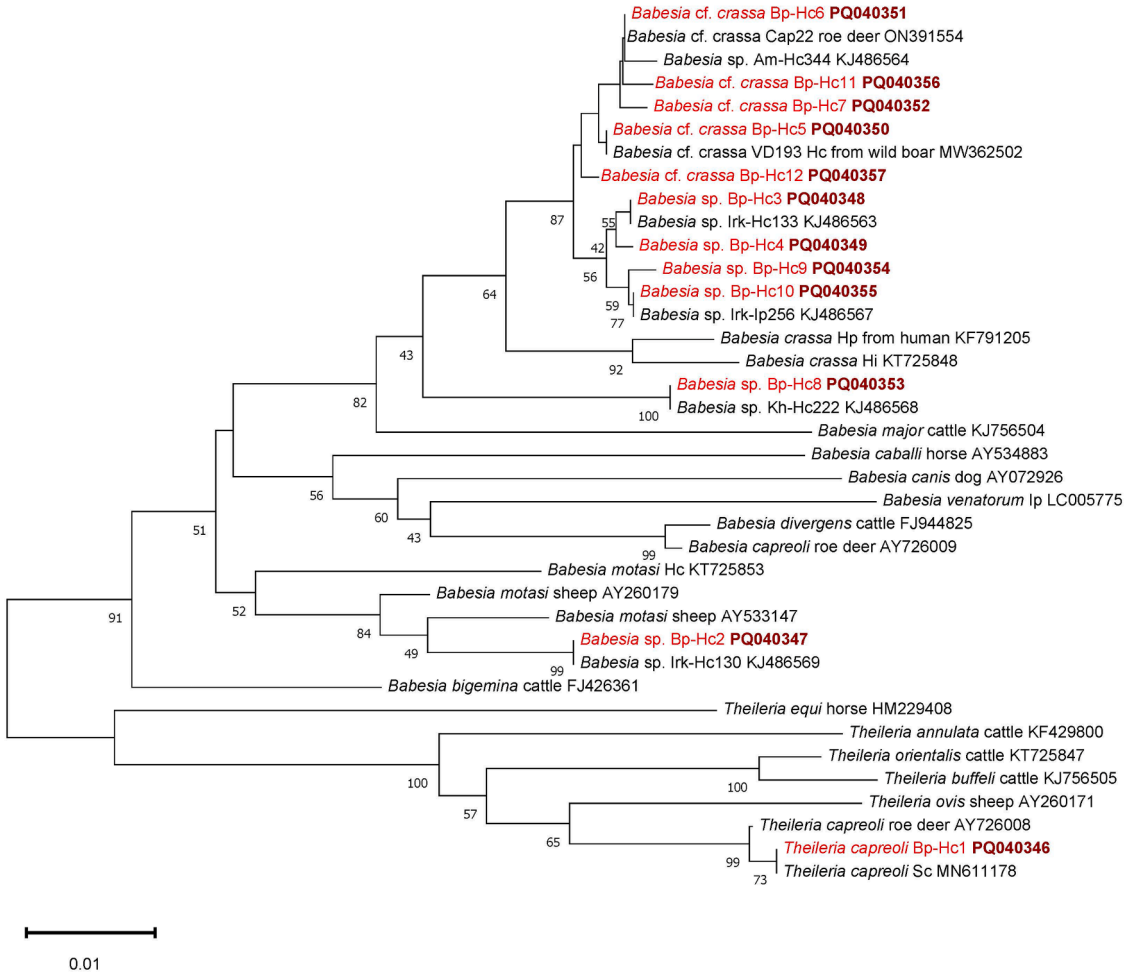

Fig. 3. Phylogenetic tree of Babesiidae and Theileriidae based on the 18S rRNA gene, made with the Neighbor-Joining method and p-distance model. In each row, after the species or genus name, the host (if known) and the GenBank accession number are shown. Sequences obtained in this study are in red and bold accession numbers. The analysis involved 38 nucleotide sequences. There were 445 positions in the final dataset. The scale-bar indicates the number of substitutions per site.

transmission and the likely indigenous status of these piroplasms. This is corroborated by the presence of these species in roe deer (*C. capreolus*) sampled in Italy (Ebani et al., 2022), and in *H. concinna* collected from local wild boars (*Sus scrofa*) in Hungary (Hornok et al., 2022). This finding is especially significant considering the zoonotic nature of the latter *Babesia* genotype (Strasek-Smrdel et al., 2020). The *Babesia* genotypes, of which only larvae or larvae and nymphs of *H. concinna* were found to be PCR-positive (*Babesia* sp. Bp-Hc3), most likely represent well-established, indigenous species, as is also reflected by the different genetic variants with single nucleotid polymorphism (Fig. 1), likely resulting from mutations during reproduction.

Babesia infection in *H. concinna* nymphs and adults was significantly more common in May and in July compared to the other months of the year (Fig. 2). This early predominance of infected ticks and consequent decline of their ratio towards the end of tick season has already been reported in the case of other tick-borne pathogens, as exemplified by *Anaplasma phagocytophilum, Borrelia burgdorferi* sensu lato, *Rickettsia helvetica* and even *B. canis* (Kantsø et al., 2010; Mysterud et al., 2013; Hornok et al., 2016). Considering potential mechanisms underlying these observations, it has been reported that (1) *B. burgdorferi* (s.*l.*) can prolong the survival of infected *Ixodes ricinus* (Herrmann et al., 2013); (2) *Babesia bovis* increases the activity and thus host finding of *Rhipicephalus microplus* by interfering with tick metabolism (Rachinsky et al., 2008); and (3) *Babesia microti* may promote its transmission by enhancing the feeding success of *Ixodes trianguliceps* (Randolph, 1991). Further studies will be necessary to clarify this in the case of babesiae carried by *H. concinna*.

In conclusion, results of this study show for the first time that in a questing population of *H. concinna* the highest monthly prevalence of *Babesia* and *Theileria* spp. may be different from each other and from the peak abundance of carrier ticks, and ticks harboring pathogens predominate early in the annual tick activity period. Based on previous reports on the effect of tick-borne pathogens on other tick species, the factors that may influence this phenomenon in *H. concinna* may include changes in the metabolism, behavior (host finding and feeding success), as well as survival rate of infected ticks.

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CRediT authorship contribution statement

Gergő Keve: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ciara Reynolds:** Writing – review & editing, Validation, Investigation, Data curation. **Nóra Takács:** Writing – review & editing, Validation, Investigation, Data curation. **Sándor Hornok:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project

administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Competing interests

The authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2025.102458.

Data availability

The sequences obtained in the current study were deposited in the GenBank database and are available under accession numbers: (PQ040346-PQ040357) (https://www.ncbi.nlm.nih.gov/genbank/). All other relevant data are included in the manuscript.

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