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SEASONALLY BIASED OR SINGLE-HABITAT SAMPLING IS NOT INFORMATIVE ON THE REAL PREVALENCE OF *DERMACENTOR RETICULATUS*-BORNE RICKETTSIAE – A PILOT STUDY

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Dermacentor reticulatus is a tick species of high medical and veterinary importance, emerging in several parts of Europe. Up to now most studies focusing on zoonotic rickettsiae in *D. reticulatus* were based on ticks collected in a limited part of the questing period, and did not take into account the potential seasonal variations in the rate of infection with tick-borne rickettsiae. The aim of the present study was to investigate the latter phenomenon, i.e. to screen *D. reticulatus* adults, collected monthly in two urban habitats of Budapest, for the presence of three zoonotic *Rickettsia* spp. Altogether 852 *D. reticulatus* adults were collected, which showed significantly similar seasonal activity in the two evaluated habitats. Among the 413 molecularly analysed ticks, *R. helvetica*-infected *D. reticulatus* were only collected during autumn in habitat-1, in contrast to habitat-2. The overall prevalence of *R. raoultii* in *D. reticulatus* adults was significantly higher in habitat-1 than in habitat-2. In addition, the seasonal distribution of *R. raoultii*-infected ticks was different between the two habitats (in habitat-2 significantly more *R. raoultii*-infected ticks were collected in the autumn, in comparison with winter and spring). *Rickettsia slovacica* was not detected in any of the molecularly analysed ticks. The results clearly indicate that a single-time or seasonally biased collection of *D. reticulatus* adults and their subsequent molecular analysis may not be informative on the real prevalence of rickettsiae. This is because the availability/activity of infected ticks shows significant seasonal fluctuations, both within and between habitats. Instead, for screening *D. reticulatus*-borne rickettsiae, it is important to collect monthly samples and then to assess seasonal prevalence and actual habitat-associated eco-epidemiological risks.

Key words: *Rickettsia*, *Dermacentor reticulatus*, ticks, transstadial, transovarial

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Dermacentor reticulatus (Ixodida: Ixodidae) can be regarded as one of the most important tick species in Europe, taking into account its widespread occurrence and emerging significance in western and eastern countries of the continent (Jongejan et al., 2015; Paulauskas et al., 2015), its high abundance in Central Europe (Hornok and Farkas, 2009) and its potential to bite humans (Estrada-Peña and Jongejan, 1999). Among other zoonotic pathogens, *D. reticulatus* is known to be a carrier and/or vector of several *Rickettsia* spp., including *R. raoultii*, *R. slovacica* (Spitalská et al., 2012) and *R. helvetica* (Dobec et al., 2009).

The genus *Rickettsia* (Rickettsiales: Rickettsiaceae) includes Gram-negative, obligate intracellular bacteria (Dumler et al., 2001). Arthropod-borne rickettsial diseases were reported to probably represent the most complete paradigm for understanding emerging diseases (Raoult and Roux, 1997). Rickettsiae are usually associated with arthropods which may act as vectors, reservoirs and/or amplifiers in the life cycles of these bacteria. In particular, ticks are the main vectors and reservoirs of spotted-fever group rickettsiae (Raoult and Roux, 1997). *Rickettsia* spp. are transmitted by ticks both transovarially and transstadially (Raoult and Roux, 1997), i.e. adult ticks may carry rickettsiae after becoming infected from the previous generation (transovarially, without involving a vertebrate reservoir) or from a previous stage (transstadially, acquiring rickettsiae from a rickettsaemic vertebrate host) (Raoult and Roux, 1997). Consequently, due to transovarial transmission, rickettsiae can be maintained in ticks even in the (short-term) absence of vertebrate reservoirs.

In Europe there are numerous reports on the prevalence of zoonotic rickettsiae in *D. reticulatus*, mostly focusing on the causative agents of tick-borne lymphadenopathy (TIBOLA), i.e. *R. raoultii* and *R. slovacica*. However, to the best of the authors' knowledge, most (if not all) of these studies are based on the molecular analysis of ticks collected in a limited part of the questing period (e.g. Dobec et al., 2009; Reye et al., 2013), not taking into account potential seasonal variations in the rate of infection with tick-borne rickettsiae, or not mentioning seasonal data at all (Wójcik-Fatla et al., 2013). The objective of this study was to provide an example, in which it is shown that for a more accurate determination of the prevalence rates of *D. reticulatus*-borne rickettsiae it is inevitable to include samples from the whole tick season. Therefore, the present study was aimed at assessing monthly fluctuations in the prevalence of rickettsiae in *D. reticulatus* adults during their whole activity period.

Materials and methods

Sample collection

Dermacentor reticulatus was collected in two urban biotopes (habitat-1 and habitat-2) of southern Budapest as reported (Hornok et al., 2016). These two

habitats were selected as the only places in the city where continuous and high activity of *D. reticulatus* was observed from March to June (Hornok et al., 2014a). In brief, these habitats can be characterised by uncut meadow-grass, scattered bushes and few (mainly oak) trees. Among the most important potential hosts of *D. reticulatus* the following are known to be present: rodents (lower number in habitat-2 due to pest control), hedgehogs (in both habitats), hares and pheasants (in habitat-1) and dogs (in habitat-2). Ticks were collected monthly (between August 2014 and June 2015) in both habitats from the grass with the dragging-flagging method along ten 100-m-long transects (i.e. in approx. 1000 m²). All specimens were stored in 70% ethanol until morphological identification by the use of standard keys.

Molecular analyses

Approximately 23 ticks (randomly sampled 11 males and 12 females, if available) per month from both habitats were processed for molecular analyses (exception: 46 ticks in October from habitat-1; 22 ticks in January from habitat-2; Table 1). The DNA from these altogether 413 ticks was extracted by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) as reported (Hornok et al., 2014b). The presence of amplifiable DNA was confirmed for each sample extracted from ticks using a real-time TaqMan PCR assay specific for the 18S rRNA gene (Applied Biosystems, Rotkreuz, Switzerland) as previously described (Boretti et al., 2009).

All tick DNA extracts were screened for rickettsiae with TaqMan real-time PCRs. The presence of *R. helvetica* was evaluated by amplifying a 65-bp fragment of the 23 rRNA gene as reported (Boretti et al., 2009). The detection methods for *R. raoultii* and *R. slovaca* were based on amplifying 107-bp and 129-bp fragments of the outer membrane protein B (ompB) gene, respectively, as reported (Jiang et al., 2012). Both of these tests have 98.8–100% sensitivities and specificities, therefore prevalence data were used uncorrected (Reiczigel et al., 2010).

Statistical analyses

Exact confidence intervals (CI) for the prevalence rates were calculated at the level of 95%. Spearman rank correlation was used to compare the seasonal activity of ticks (i.e. their monthly number collected in standard areas) in the two habitats. Prevalence rates of *Rickettsia* spp. between habitats and between different seasons in the same habitat were compared by using Fisher's exact test. Differences were considered significant if $P < 0.05$.

Results and discussion

In habitat-1 and habitat-2 altogether 621 and 231 *Dermacentor reticulatus* adults were collected, respectively. The seasonal (monthly) number of collected ticks showed a significantly similar pattern in the two habitats (Spearman's rank correlation: $r = 0.74$, $P = 0.009$), reflecting a peak activity in October/November and March (Fig. 1). This is in line with the previously reported seasonality of *D. reticulatus* in Central Europe (Hornok, 2009).

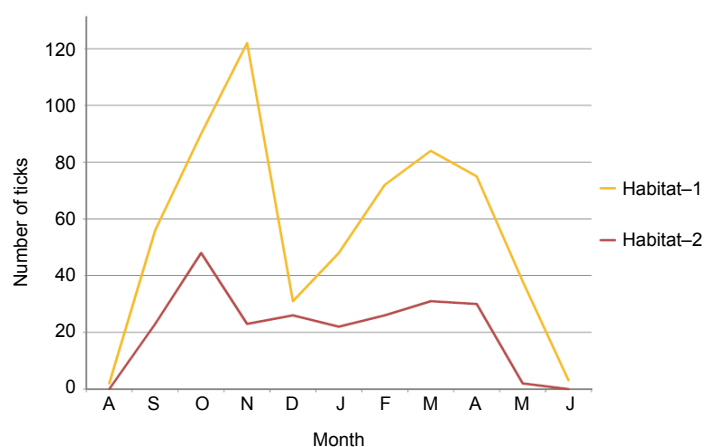


Fig. 1. The number of ticks collected monthly (from August to June) in the two habitats

Concerning the 413 molecularly analysed ticks, there was no significant difference between the proportions of rickettsia-carrier male vs. female ticks in the two habitats (prevalence in males: 34.4%, i.e. 63 of 183 PCR positive; prevalence in females: 34.3%, i.e. 79 of 230 PCR positive).

The prevalence rates of *R. helvetica* in *D. reticulatus* adults were significantly higher in habitat-1 than in habitat-2 in September and October ($P = 0.004$ and 0.045 , respectively) (Table 1). In habitat-1, *R. helvetica*-infected ticks were only collected during autumn (16 of 92 ticks: 17.4%, CI = 10.3–26.7%), which is a significant difference in comparison with the remaining seasons of *D. reticulatus* adult activity ($P < 0.0001$). At the same time, in habitat-2 the presence of *R. helvetica*-carrier ticks (although sporadically) was observed in all three seasons of *D. reticulatus* adult activity (Table 1).

For *R. helvetica*, *Ixodes ricinus* is both a vector and a reservoir, while domestic and wild ruminants (Jilintai et al., 2008), dogs (Wächter et al., 2015), wild boars, rodents (Sprong et al., 2009) and birds (Hornok et al., 2014b) may act as vertebrate reservoirs. Differences in the number of these reservoirs (e.g. the presence of small game animals and the higher number of rodents in habitat-1) may have contributed to the above results.

Table 1
Monthly data of results from molecular analyses of *Dermacentor reticulatus* adult ticks

	PCR positive / all tested (percentage) according to months											
	September	October	November	December	January	February	March	April	May			
<i>R. hevetica</i>												
Habitat-1	8/23 (35%)	8/46 (17%)	0/23	0/23	0/23	0/23	0/23	0/23	0/23	0/23	0/23	0/23
Habitat-2	0/23	0/23	3/23 (13%)	1/23 (4%)	0/22	0/23	0/23	0/23	2/23 (9%)	–	–	–
<i>R. raoultii</i>												
Habitat-1	9/23 (39%)	27/46 (59%)	6/23 (26%)	10/23 (43%)	8/23 (35%)	10/23 (43%)	7/23 (30%)	8/23 (35%)	10/23 (43%)	–	–	–
Habitat-2	6/23 (26%)	5/23 (22%)	3/23 (13%)	8/23 (35%)	0/22	1/23 (4%)	0/23	2/23 (9%)	–	–	–	–

Red, bold numbers indicate sample groups with PCR positivity. Prevalence data of the same *Rickettsia* sp., which are encircled and connected, were significantly different between the two habitats in the same month. Note that tick number declined by May in habitat-2, therefore representative samples could not be included in molecular analyses

The overall prevalence of *R. raoultii* in *D. reticulatus* adults was significantly ($P < 0.0001$) higher in habitat-1 (95 of 230 ticks: 41.3%, CI = 34.9–48%), than in habitat-2 (25 of 183 ticks: 13.7%, CI = 9–19.5%) (Table 1). In particular, the prevalence rates of *R. raoultii* in *D. reticulatus* adults were significantly higher in habitat-1 than in habitat-2 in October, January, February and March ($P = 0.005, 0.004, 0.004$ and 0.009 , respectively) (Table 1). The seasonal distribution of *R. raoultii*-infected ticks was also different between the two habitats. In habitat-1 there was no significant difference in the monthly proportion of *R. raoultii*-infected ticks (Table 1), i.e. its prevalence was equilibrated in all three seasons, whereas in habitat-2 significantly ($P = 0.048$) more *R. raoultii*-infected ticks were collected in autumn (14 of 69 ticks: 20.3%, CI = 11.6–31.7%) than in winter and spring (11 of 114 ticks: 9.7%, CI = 4.9–16.6%).

The vertebrate reservoirs of *R. raoultii* are only partly known. Guinea pigs (Spitalská et al., 2012) and dogs (Wächter et al., 2015) have been reported to be susceptible to this agent. Therefore, differences in the number of rodents and dogs between the two habitats may have contributed to the above results.

In this way, the prevalence rates of *R. helvetica* and *R. raoultii* in *D. reticulatus* were significantly different between the two habitats, as well as between seasons within a habitat. These findings illustrate that the prevalence of *D. reticulatus*-borne rickettsiae may show significant variation over short geographical distances, because the two habitats are only at a 6.5-km distance from each other. Importantly, significantly different prevalence rates of rickettsiae between the two habitats were observed during the two peak activities of *D. reticulatus* (i.e. autumn and spring), in which period this tick species is usually sampled for the detection of rickettsiae (e.g. Dobec et al., 2009). Thus, relevant prevalence rates from single-habitat sampling should be interpreted with caution.

The peak incidence of TIBOLA (in part caused by *R. raoultii*) was reported to be September–November and March to May both in Hungary (Lakos et al., 2012) and in other parts of Europe (Socolovschi et al., 2009). This has been explained by the peak activity of *D. reticulatus*. According to the findings in this survey, risks of acquiring *R. raoultii* were nearly constant in habitat-1, but were associated with autumn in habitat-2. Consequently, the prevalence of rickettsiae may show (significant) seasonal changes in *D. reticulatus*. This confirms that the actual disease risk will depend rather on the availability/activity of infected ticks than on the activity of the vector tick species *per se*. In addition, the present results have important implications for planning tick collections: unless monitored throughout the whole tick season, seasonally biased sampling of *D. reticulatus* (e.g. analysis of ticks collected in one or a few months) may achieve misleading prevalence rates of rickettsiae.

Interestingly, *R. slovaca* was not detected in any of the molecularly analysed ticks. The most likely explanation for the absence of *R. slovaca* in *D. reticulatus* specimens of the present study is that *D. marginatus*, which is both the

vector and reservoir of *R. slovaca* (Rehacek, 1984), does not occur in the two habitats that were evaluated. Therefore, *D. reticulatus* could not have acquired *R. slovaca* from *D. marginatus* through their mutual vertebrate hosts, unlike in biotopes where these two tick species are sympatric (Spitalská et al., 2012).

The above-described spatiotemporal (local and seasonal) differences in the prevalence of *D. reticulatus*-borne rickettsiae seem attributable to two main factors: (1) the seasonally different availability of (amplifying/rickettsaemic) vertebrate hosts, and consequently (2) the significance of transovarial maintenance in competent tick reservoirs/vectors. These factors are interrelated in complex ways and therefore hard to assess in natural ecosystems. Furthermore, it also has to be taken into account that pathogens may alter the activity and behaviour of their tick vector. Relevant to the present results, it was demonstrated that rickettsiae induced higher motility among *Dermacentor* sp. ticks (Kagemann and Clay, 2013), which (theoretically) may cause earlier questing of rickettsia-infected ticks.

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