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DEPARTMENT OF PARASITOLOGY AND ZOOLOGY

Seasonality, morphologic and molecular analyses of ixodid ticks collected in two urban biotopes of Budapest

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1. Introduction and aims

In the era of globalization it is known that by 2030 approximately 70% of human population will live in cities. The surface covered by urban areas is steadily increasing worldwide, and these expand into natural habitats of wildlife. In addition, urbanized regions include preserved, artificially maintained green surfaces, habitats in a mosaic-like arrangement, which may be significant to consider from an epidemiological view, i.e., when their role is considered in the context of pathogen reservoirs.

Hard ticks (Acari: Ixodida: Ixodidae) are haematophagous ectoparasites of terrestrial vertebrates, with outstanding veterinary-medical importance as vectors in the temperate climate zone, as exemplified by Central Europe. Ticks may cause harm to heir host with their blood-feeding activity, secretion of bioactive (toxic) substances, as well as by the inoculation of tick-borne pathogens. In a worldwide context, there are more than 700 ixodid tick species, of which nearly 60 occur in Europe, and at least 26 were reported in Hungary. However, the latter numbers will most likely increase, due to establishment of new species as a result of climate change.

Previously, in the capital city of Hungary various habitats were surveyed for the presence of ticks and tick-borne pathogens. Neglected parts of cemeteries were found to be especially suitable to maintain large tick populations, most likely because of the high density of birds and small mammals (i.e., due to the restricted entry or near absence of medium sized carnivores). In the frame of this study, we collected ticks with the dragging-flagging method from the vegetation in a cemetery during a 28-month period (February 2019 to May 2021), encompassing three spring and two autumn tick seasons. For comparison, ticks were also collected in an urban forest for 24 months (February 2019 to January 2021). These ticks were analyzed morphologically as well as by molecular biological tools (conventional PCRs), taking into account their seasonality.

2. Literature review

2.1 Ixodid tick species reported in Hungary

Adapted from *Checklist of the hard tick (Acari: Ixodidae) fauna of Hungary with emphasis on host-associations and the emergence of Rhipicephalus sanguineus* (Hornok et al. 2020).

Indigenous Ixodid tick species (completing their full life cycle) in Hungary.

Ixodes ricinus is the most common and has a country-wide distribution. The larvae and nymph species use many birds, reptiles, and mammals (including humans) as hosts; while the adult stage is limited to mammals. This species is active in forested areas year-round, with biphasic activity peaking in May and a smaller peak in September. *Ixodes hexagonus* is spread throughout the north, west, and south of Hungary. Hosts are mammals including dogs, cats, foxes, and hedgehogs. This species prefers burrows and caves and is active during the spring and summer, though exact months can vary by host species. *Ixodes canisuga* is spread throughout the north, west, and south of Hungary. Hosts are mammals such as dogs, badgers, martins, and foxes. This tick prefers dark, shaded areas and can be found from March to August depending on host species. *Ixodes kaiseri* is widespread in western and central Hungary. Adults are found on domestic dogs, and nymphs on European badgers. This species prefers shadows and is found in December on badger hosts, as well as in January and April on dogs. *Ixodes acuminatus* is widespread in central and southeastern Hungary. Adults can be found on dogs and hamsters; nymphs are found on hedgehogs. This species is active in May and October. *Ixodes apronophorus* is sporadically distributed in central Hungary. Hosts are small mammals, such as voles and shrews. *Ixodes trianguliceps* is sporadically distributed in the north and west of Hungary. It is active from May to August. Mice, moles, and voles are typical hosts for this species. *Ixodes languri* is widespread in western and eastern Hungary. Typical hosts are squirrels and hamsters. This species is active in July. *Ixodes vespertilionis* is a dark-prefering tick that hosts on bats, mainly of the Rhinolophidae family, and is found in Hungary's mountainous regions and caves where these host are available. This species is active year-round, with populations peaking in the spring. *Ixodes ariadnae* also prefers dark caves and bat hosts, though mainly from the Vespertilionidae family. This tick is active in all seasons, with larvae and nymph numbers peaking in early fall. *Ixodes simplex* host almost exclusively on bats of the Miniopteridae family living in mountain caves. Samples of this species are collected during early fall. *Ixodes frontalis* is widespread in western and northeastern Hungary. This tick host on ground feeding birds, mainly in the early spring. *Ixodes arboricola* prefers arboreal, cavity-nesting birds and is sporadically distributed in north-central and eastern Hungary. *Ixodes lividus* is a host specific parasite of the European sand martin, and is found according the distribution of the host birds (largely along the Tizsa river.) This tick lives in the burrows and nest of its host, it can be found from April to October. *Haemaphysalis parva* is found in southern Hungary, but seasonality and exact habit are unknown due to the rarity of this species. It has been identified from horses and hares. *Haemaphysalis concinna* is distributed in forested areas country wide. The larvae and nymphs host of birds, mainly Passeriformes, and all life stages are found on a variety of mammals. This species has a monophasic activity form late spring to early fall that peaks in May. *Haemaphysalis punctata* is found in the west and northeast of Hungary, largely in dry habitats. It has a biphasic activity with peaks from March to May and in September. Larvae and nymphs typically host on birds, while adults of this species are found on mammals such as dogs, cattle, and roe deer. *Haemaphysalis inermis* is distributed countrywide in forested areas with limited tree cover and dense vegetation. Typical hosts are mammals, including humans and domestic livestock species. *Dermacentor marginatus* was distributed countrywide in the mid-late 1900s, but has not been found in western Hungary during more recent surveys. It has a biphasic activity (spring and fall) with the larger peak being in March. This tick is almost exclusively found on mammals, but has rarely been identified from the Yellowhammer bird. *Dermacentor reticulatus* is present countrywide and has been expanding in range since the mid-1900s. These ticks are found in open country and river basins, and have a biphasic activity (spring and fall) with the higher peak in September to October. Typical hosts are mammals, including domestic livestock and companion species. *Rhipicephalus sanguineus* is sporadically distributed, but has shown increasing numbers of cases in recent years. This tick mainly hosts on domestic dogs and cats, and is particularly associated with kennels.

Ixodid tick species imported by migratory birds

Some of the species of Ixodid ticks found in Hungary do not complete their full life cycle locally, but are instead brought in temporarily when attached to migrating birds. *Ixodes festai* and *Ixodes persulcatus* are found sporadically in central and northern Hungary, respectively. *Hyalomma marginatum* and *Hyalomma rufipes* are carried in by northward bird migration in the spring and after molting can be found on horses and cows, respectively, as adult stages in the fall.

Ixodid tick species brought to Hungary by transportation of their hosts

Amblyomma dissimile was identified in 1959 on reptiles brought to the Budapest Zoo from South America. *Haemaphysalis erinacei erinacei* was found in 2005 from an animal imported for sale as a pet. This is a rare occurrence, with one case reported for each species.

2.2. Ixodid tick species occurring in urban biotopes of Budapest

The urban environment of Budapest has three main biotopes: forests, parks, and cemeteries; each of which varies in species and prevalent tick-borne pathogens. Within Budapest *I. ricinus* and *H. concinna* have been collected in all three biotopes, *D. reticulatus* in park and forest biotypes, and *D. marginatus* (one specimen) in only the forest biotope (Hornok et al. 2014).

A Budapest survey collecting ticks from vegetation (Hornok et al. 2014) found that *I. ricinus* was the most abundant species in urban habitats, being present in 27 out of 29 surveyed habitats, and in habitats closest to the city center compared to other urban species. While population densities of *I. ricinus* were equal between parks and forests from April to June, it was higher in cemeteries than parks in April, and higher than parks and forests in June. *I. ricinus* females also showed varying levels of pathogen prevalence with varying biotopes. *Anaplasma phagocytophilum* was higher in parks, or parks and forests together, than in cemeteries. *Borrelia burgdorferi* was significantly less prevalent in females collected in cemeteries than in forests.

The second most abundant species in urban Budapest was *H. concinna*, which was also collected from all three biotypes. However, this species was much less widespread, being found 6 of the 29 habitats surveyed.

The third most abundant was *D. reticulatus*. While this species was only present in the park and forest biotypes it was more widespread, being present in 9 of 29 habitats. *D. reticulatus* is a major vector of *Babesia canis*, a protozoan parasite of dogs that has transovarial transmission in ticks. While peak activity of *D. reticlatus* occurs in the fall (Hornok 2009) babesiosis infections are most common in spring (Pavlovic et al. 2003). Babesia is more commonly found in smaller ticks that have molted recently, and the questing activity of these freshly molted ticks leads to increased presence of *B. canis* infected ticks, and subsequent cases of babesiosis(Hornok et al. 2016). It is thought that this seasonality and sooner emergence of infected ticks is more relevant to urban environments due to the heat island effect of urban areas (Wickham et al.).

Cohabitation of urban tick species is common. Two park habitats (Hornok et al. 2014) contained all three species, six sites had *I. ricinus* and *H. concinna*, and nine sites had *I. Ricinus* and *D. reticulatus*.

I. ricinus and *H. concinna* both use small mammals and birds as hosts(Hornok et al. 2020), likely leading to their widespread occurrence in the region. *D. reticulatus*, however, is most frequently found on pet dogs (Földvári and Farkas 2005) which are generally banned from cemeteries, which may explain this species absence from the cemetery biotype.

While less common than *I. ricinus, Ixodes frontalis* has also been located in urban areas, specifically under bamboo bushes in urban parks (Plantard et al. 2021). As this is a largely avian tick it is thought that bamboo offers the birds a more favorable overnight roost than other biotypes, leading to an increased density of *I. frontalis* on the ground below.

While ticks are often thought of as a rural issue, this is not accurate. Due to vegetation, host availability, and pathogen reservoirs this risk will vary between urban biotopes; but exposure risk to ticks and the pathogens they carry remains significant.

2.3. *Ixodes ricinus*

Hosts and life cycle

Ixodes Ricinus is considered the most common, well known, and most studied European tick species. It is widely distributed throughout Europe and is a vector of many important pathogens. This species has a wide host range, and has been recorded on over 160 species (Estrada-Pena et al. 2004). Larvae and nymphs generally prefer small mammal hosts, as well as lizards and birds, but the adult ticks are more commonly found on large mammals. *I. ricinus* generally has biphasic activity where adults are active for most of the year, with peak activity of adults and nymphs in spring and fall, and larvae activity peaking in early summer (Hornok et al. 2020). Seasonality may vary depending on climate with adults being present year-round if the weather remains warm enough, or even showing uniphasic activity (Otranto et al. 2017).

Ecology and distribution

I. ricinus is an exophilic tick, with ecological activity independent of domestic human environments. This species is sensitive to desiccation and requires a moist climate, therefore it is commonly found in undergrowth of woodlands and forests where there is moderate to high rainfall (Otranto et al. 2017). This is a wide-ranging species, extending from the United Kingdom to Turkey west-east, and from Finland to North Africa north-south. However, likely due to climate change, this range appears to be expanding northwards and in altitude (Lindgren et al. 2000).

Ixodes ricinus as a vector

I. ricinus is an important pathogen vector for pathogens throughout Europe. This tick is known to carry *Borrelia burgdorferi, Anaplasma phagocytophilum, Francisella tularensis, Rickettsia* species, *Babesia* species, and the tick-borne encephalitis virus (Reye et al. 2010). These diseases have great veterinary significance for both companion and livestock practice, as well as zoonotic potential.

2.4. *Ixodes frontalis*

Hosts and life cycle

Ixodes frontalis is a so-called “three host tick,” meaning that the ticks leave their host to molt after both the larval and nymph stages. The hosts for each stage may be different species depending on what animals are present in the area (Doby and Bigaignon 1997). This is an avian tick, infesting may genus of birds, though passerine birds are the most common host. *I. frontalis* has rarely been reported on other host species, such and humans or other mammals (Pfäffle et al. 2017a).

Unlike other *Ixodes* species, who take several years to complete a life cycle, *I. frontalis* takes only one year. It is an exophilic species, naturally independent of humans and the environments they create (Monks et al. 2006). This tick is most abundant in early spring (Hornok et al. 2020).

Ecology and distribution

Because *I. frontalis* is an avian parasite it is most commonly found in and around bird nests, and rarely collected via flagging methods. However, specimens have also been collected from vegetation and ground cover beneath popular roosting sites (Plantard et al. 2021) in a variety of forest biotypes. This species has a wide range of distribution throughout Europe, Asian, and northern Africa, and can be transported between regions and biotypes by the migration of hosts (Drehmann et al. 2019).

Ixodes frontalis as a vector

I. frontalis has the capacity to act as a carrier of bacteria and viruses, but this area is not widely researched. Because this is an avian-investing tick it is unlikely that it poses significant risk of pathogen transmission to humans or other mammals. However, adult *I. frontalis* females have been associated with arboviruses and the avian illness “tick-related syndrome,” or TRS (Drehmann et al. 2019; Monks et al. 2006). TRS is a condition wherein birds rapidly become depressed or die, as well as showing hemorrhage and edema on the head and neck, with a present tick bite nearby.

2.5. *Haemaphysalis concinna*

Hosts and life cycle

Like *I. ricinus* and *I. frontalis*, *Haemaphysalis concinna* is an exophilic three-host tick. In a natural environment this species has a three-year life cycle, though this can be accelerated in a controlled laboratory (Pfäffle et al. 2017b). *H. concinna* ticks feed mainly on mammals, with the larval and nymph stages attaching to small mammals and the adults preferring ungulates (Pfäffle et al. 2017b). Occasional infestation of humans has been reported (Bursali et al. 2012).

Ecology and distribution

Activity of *H. concinna* varies depending on the geographical region, and ticks can be active year-round. In Hungary, *H. concinna* is generally active from April to November with a peak in May (Hornok 2009). Larvae are active from May to November and nymph April to October (Pfäffle et al. 2017b). *H. concinna* ticks are well suited to several types of forest habitats, as well as water-adjacent vegetation such as lake coasts and shorelines. This is a species that seeks humid habitats and is largely thermophilic. The range of *H. concinna* is notably smaller than that of *I. ricinus* and *I. frontalis*, this being a Eurasian species (Pfäffle et al. 2017b).

Haemaphysalis concinna as a vector

A number of pathogenic *Rickettsia* species can be carried and transmitted by *H. concinna*, as well and some *Borrelia* species, though it is a less competent *Borrelia* vector and cannot maintain it as a transstadial infection. This species is also a known vector of tick-borne encephalitis. (Pfäffle et al. 2017b).

2.6. Seasonality of ixodid ticks in Hungary

Hungary has a traditional continental climate involving humid, cold, and cloudy winters; as well as warm to hot summers. This climate pattern leads to high levels of tick activity in the spring and early summer, as well as a possible peak in the fall (Széll et al. 2006). The main species of tick present in Hungary each have a different, species-specific seasonal distribution. A 2006 study collecting ticks from killed foxes (Széll et al. 2006) showed *I. ricinus* ticks present year-round but most active April and June with peak activity occurring in May; as well as a second, smaller peak in September to October. *Dermacentor reticulatus* ticks were also biphasic, being present from September to November with and October peak, but also a marked activity increase in April. *H. concinna*, on the other hand, was active from May to July with a peak in June, but was not found at all between October and March. However, possibly due to climate change and mild winters, a study of questing ticks just two years later (Hornok 2009) showed peak activity of these species 1-2 months earlier than previously seen, with the exception of *I. ricinus* remaining the same.

Seasonality can also be affected by specific biotope conditions. A flagging survey of Hungarian questing ticks (Hornok and Farkas 2009) that seasonality and activity peak of all ixodid ticks to show variation depending on biotopes. Forest ticks, especially *Haemaphysalis* species, developed better in areas with high humidity. Open country ticks, such as *Dermacentor* species, peaked later in cooler mountain meadows than warmer dry pastures areas, implying a temperature-dependent temporal variation.

Different stages of tick development are also present at different times of the year. This is thought to play a significant role in seasonality of tick-borne infections, as different stages may pose a higher or lower risk of pathogen transmission. In 2008 (Hornok 2009) *H. concinna* showed simultaneous presence of adults and nymphs on vegetation in late spring, while *I. ricinus* is present year-round but nymphs were not collected in midsummer and midwinter, and no nymphs were collected for *D. reticulatus*.

Sex ratio of active ticks can also vary by season. Throughout the year females are predominant in the questing tick populations, with the exception of *Haemaphysalis* species, which show roughly equal male-female ratios (Hornok 2009). For tick species that overwinter the population found on vegetation in the spring represents the same cohort that was present the previous fall, and for most species the proportion of questing males is non-significantly higher in fall than spring. The exception is *I. ricinus*, which shows a lower percentage of questing males in the fall. It is thought that these sex ratios are influenced by species-specific mating strategies and whether the species mates on or off of hosts (Hornok 2009).

2.7. Pathogens detected in questing ticks collected in Hungary

The most important, and most well studied, tick-borne pathogens present in Hungary are tick-borne encephalitis (TBE), *Borrelia*, *Babesia*, *Anaplasma*, and *Reckettsia* species.

TBE is an flavivirus infection that attacks the central nervous system of hosts who receive via tick bites. It mainly effects small rodents, wild birds, carnivores, deer, and domestic animals, though rodents and small insectivores are the main reservoirs. The European subtype of TBE is carried by *I. ricinus* as a transstadial and sometimes transovarial infection, making ticks an additional reservoir for the virus. These ticks can transmit the infection to mammalian hosts during feeding at each life stage. Humans can be incidental hosts, but do not participate in natural circulation of the virus (Donoso-Mantke et al. 2011).

Borrelia species, the causative agents of Lyme disease, are also carried as a transstadial infection by *I. ricinus* ticks. In Hungary, the *B. burgdorferi* genospecies is the most common (Egyed et al. 2012), with up to 40% of adult *I. ricinus* females PCR positive (Hornok et al. 2014). Biotopes in urban areas have a notable effect on the presence of *B. burgorferi* as ticks in cemeteries show significantly lower infection rates than those in forests and parks, as well as a negative correlation between tick abundance and tick infection rates (Hornok et al. 2014).

Protozoan *Babesia* species, specifically *Babesia canis*, are present in Hungary, and have veterinary significance in canine medicine. *D. reticulatus* is present in urban biotopes of Budapest and can carry *B. canis* transovarial infections as a competent vector of this disease. Despite the peak activity of *D. reticulatus* occurring in fall cases for babesiosis are most frequent in the spring. This seasonality is thought to be due to the effect of *Babesia* infection on the behavior of ticks, causing earlier questing activity (including sooner after molt) and an increased presence of infected ticks active in winter and spring (Hornok et al. 2016).

Anaplasma phagocytophilum is a pathogenic bacteria transmitted in Hungary mainly by *I. ricinus* ticks. In urban Budapest, biotopes infection rates are higher than those recorded in natural habitats, and significantly lower in ticks collected from cemeteries than in parks. This is thought to be due to a combination of decreased biodiversity and increased density of potential tick hosts (Hornok et al. 2014).

Pathogenic *Rickettsia* bacteria species in Hungary have both human and veterinary significance as emerging diseases and are transmitted by *I. ricinus* and *D. reticulatus* (Szekeres et al. 2016). These pathogens have been detected in *I. ricinus* ticks from multiple biotypes in Hungary, though overall tick infection rates were low to moderate (Egyed et al. 2012). Infection rates of *D. reticulatus* ticks have been shown in 57% precent of specimens collected from urban Budapest biotopes, a significant increase from previous studies collecting throughout the country (Szekeres et al. 2016).

The Study

3. Materials and methods

3.1. Sample collection

Two tick collection sites were chosen, based on the results of a large-scale survey of urban biotopes in Budapest (Hornok et al., 2014). The main collection site was a cemetery, where neglected parts had dense lower vegetation (grass, weed) and sparse covering with bushes and trees. The auxiliary collection site was a forest with a mixture of deciduous trees and focally continuous ivy covering on the ground. These sites were visited at monthly interval, at the end of each month between February, 2019 and May, 2021 (cemetery: 28 months) or between February and January, 2021 (forest: 24 months). Tick collections were performed under similar (dry) weather conditions and at the same time of the day. Ticks were collected from the vegetation by the dragging-flagging method, i.e., a white towel, measuring 1 m × 1 m, was drawn over the vegetation for 1 hour, during which an approx. 300 m long transect was sampled in both tick habitats (i.e., 300 m^2). Ticks attached to and removed from the collecting device were immediately put into and stored in 96% ethanol. Species were identified by using standard morphological keys (Estrada-Peña et al., 2017). Pictures were made and measurements were performed with a VHX-5000 digital microscope (Keyence Co., Osaka, Japan). Tick activity was calculated from the monthly number of ticks, expressed as a percentage of all conspecific ticks of the same developmental stage collected in the relevant biotope during one year. When only few ticks were collected during the tick season (less than ten), the activity period was considered instead of the activity peak (Supplementary Table 1).

3.2. Molecular analyses

DNA was extracted individually, from two legs of ticks selected for morphological analysis, and from the whole body of ticks used for screening piroplasms. Ticks were disinfected on their surface with sequential washing for 15 s in 10% NaClO, 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 used for screening piroplasms, in order to monitor cross-contamination.

The 16S rRNA gene was chosen for the molecular-phylogenetic analyses of *Ixodes ricinus* (n=24) and *Ixodes frontalis* (n=24) selected for morphological analysis, because this target gene has a broad spectrum of corresponding sequences available for comparison from other studies in GenBank (e.g., Noureddine et al., 2011; Hornok et al., 2016). The method used in this study amplifies an approx. 460-bp-long fragment of the 16S rRNA gene of Ixodidae (Black and Piesman, 1994) with the primers 16S+1 (5’- CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3’) and 16S-1 (5’-CCG GTC TGA ACT CAG ATC AAG T-3’) (IDT) as reported (Hornok et al., 2016).

The cytochrome *c* oxidase subunit I (*cox*1) gene was chosen for the confirmation of *I. frontalis* haplotypes ("A" or "B"). A 710-bp-long fragment of this gene was amplified with a conventional PCR using the primers LCO1490 (forward: 5’ -GGT CAA CAA ATC ATA AAG ATA TTG G-3’) and HCO2198 (reverse: 5’ -TAA ACT TCA GGG TGA CCA AAA AAT CA-3’) (Integrated DNA Technologies, IDT, Leuven, Belgium) (Folmer et al., 1994) as reported (Hornok et al. 2016).

In addition, *Haemaphysalis concinna* specimens (n=161) collected in 2019 were screened for the presence of piroplasms by a conventional PCR modified from Casati et al. (2006). The primers BJ1 (forward: 5′-GTC TTG TAA TTG GAA TGA TGG-3′) and BN2 (reverse: 5′-TAG TTT ATG GTT AGG ACT ACG-3′) were used to amplify an approximately 500-bp-portion of the 18S rRNA gene of *Babesia*/*Theileria* spp. The reaction volume was 25 µl, i.e. 5 µl of extracted DNA was added to 20 µl reaction mixture containing 0.5 U HotStarTaq DNA Plus polymerase (5 U/µl), 200 µM of PCR nucleotide mix, 1 µM of each primer and 2.5 µl of 10×

CoralLoad PCR buffer (15 mM $MgCl_2$ included). Cycling conditions included an initial denaturation step at 95 °C for 10 min, 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. The final extension was performed at 72 °C for 5 min.

All PCRs were run with sequence-verified positive control and negative control (non-template reaction mixture). Extraction controls and negative controls remained PCR negative.

3.3. Sequencing and phylogenetic analyses

Purification and sequencing of the PCR products were done by Biomi Ltd. (Gödöllő, Hungary). Quality control and trimming of sequences were performed with BioEdit program, then alignment with GenBank sequences by the nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). Sequences from other studies (retrieved from GenBank) included in the phylogenetic analyses had nearly 100% coverage with sequences from this study. This dataset was resampled 1,000 times to generate bootstrap values. Phylogenetic analyses were conducted with the Neighbor-Joining or the Maximum Likelihood method and model selection by the program using MEGA version 7.0.

4. Results

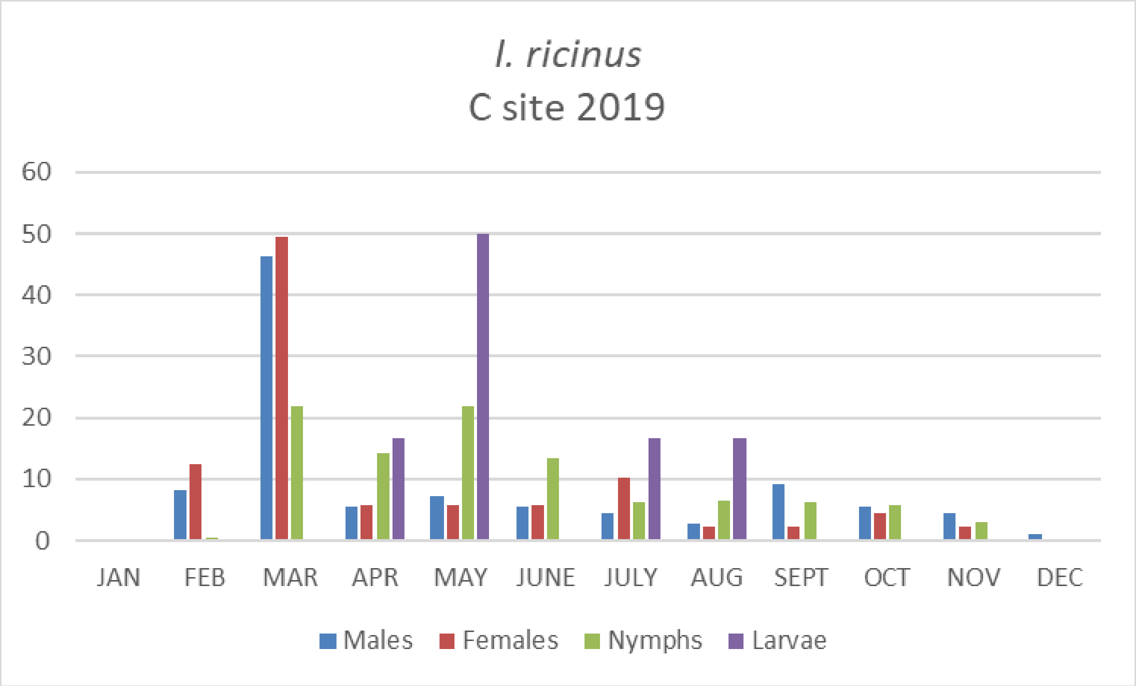
4.1. Species and seasonality of ticks in two urban habitats

In the cemetery, 3818 ticks were collected, including *Ixodes ricinus* (n=2772), *Ixodes frontalis* (n=350) and *Haemaphysalis concinna* (n=696). *Ixodes ricinus* showed activity throughout the year. However, *H. concinna* was not active during winter months and *I. frontalis* was not found questing on the vegetation during late spring and summer months. In the forested habitat only *I. ricinus* was collected (n=292).

At the cemetery, *I. ricinus* males and females showed their peak activity in March in 2019, with a smaller peak of males in September and of females in July, whereas the spring peak of adults was later (in May) in 2020, and again earlier (in March-April) in 2021 (Graph 1).

Nymphs of this species also showed year-round questing activity, reaching the highest level in the spring (March and May) in 2019, and, similarly to the adults, later (in June) in 2020, and earlier (in April) in 2021. Larvae if *I. ricinus* were collected in peak numbers in May, in July and in May in 2019, 2020 and 2021, respectively. In this tick habitat, adults of *H. concinna* showed activity between April and July, while nymphs and larvae of this species initiated their questing, respectively, in March and April of 2019, in May and June in 2020, and in April of 2021. Both developmental stages were active until November in 2019 and in 2020 (Graph 2). The single *I. frontalis* female was found in March, 2019. Nymphs of this species were collected in March (n=3), but not in the autumn during 2019, then (after an absence in the spring) in September of 2020 (n=2), followed by activity in February, March and April of 2021 (n=10, 4 and 1, respectively). Larvae of *I. frontalis* were collected in October (n=111) and November (n=69) in 2019, then in October 2020 (n=136), finally in March and April, 2021 (10 and 3, respectively). This means that while the larvae were significantly associated with the autumn, nymphs predominated in the spring (Fisher exact test: $P < 0.0001$).

At the forested habitat, compared to 2019, males of *I. ricinus* showed peak activity one month earlier in 2020 (in April instead of May), while females in the same month (March) both in 2019 and 2020 (Graph 3). During the autumn, their number was also the highest earlier in 2020 (males: September, females: October) than in 2019 (August). Nymphs of *I. ricinus* initiated their activity earlier in 2020 (February) than in 2019 (May). Larvae were not found during the 24 months in this habitat.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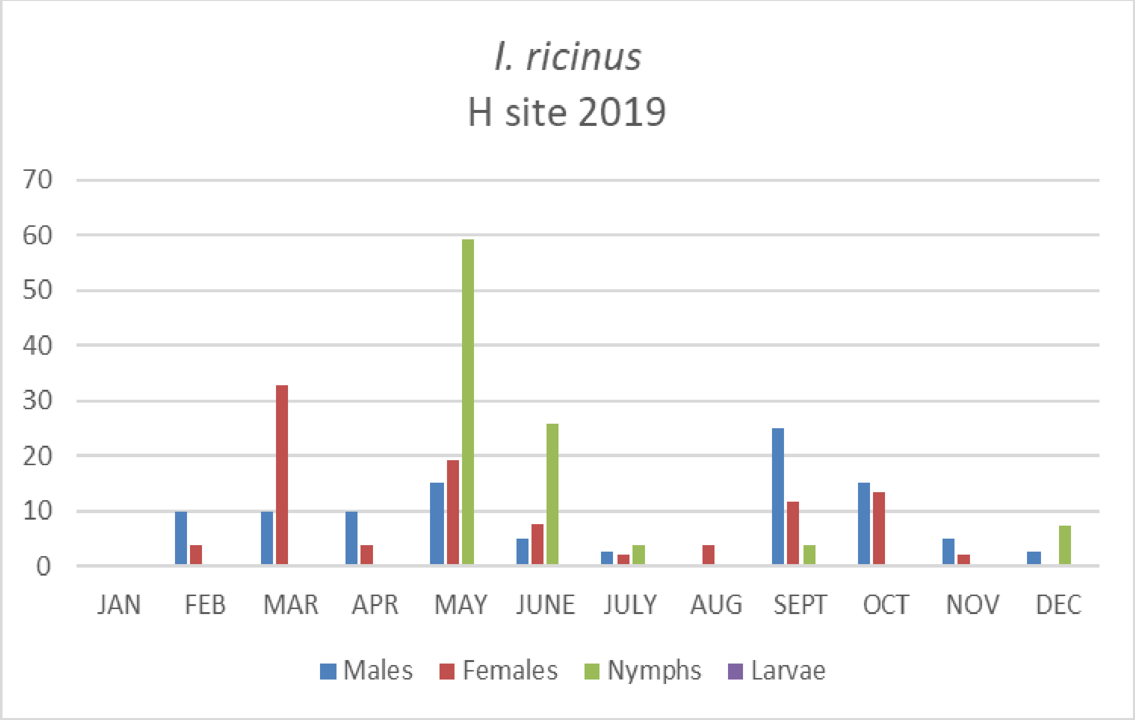
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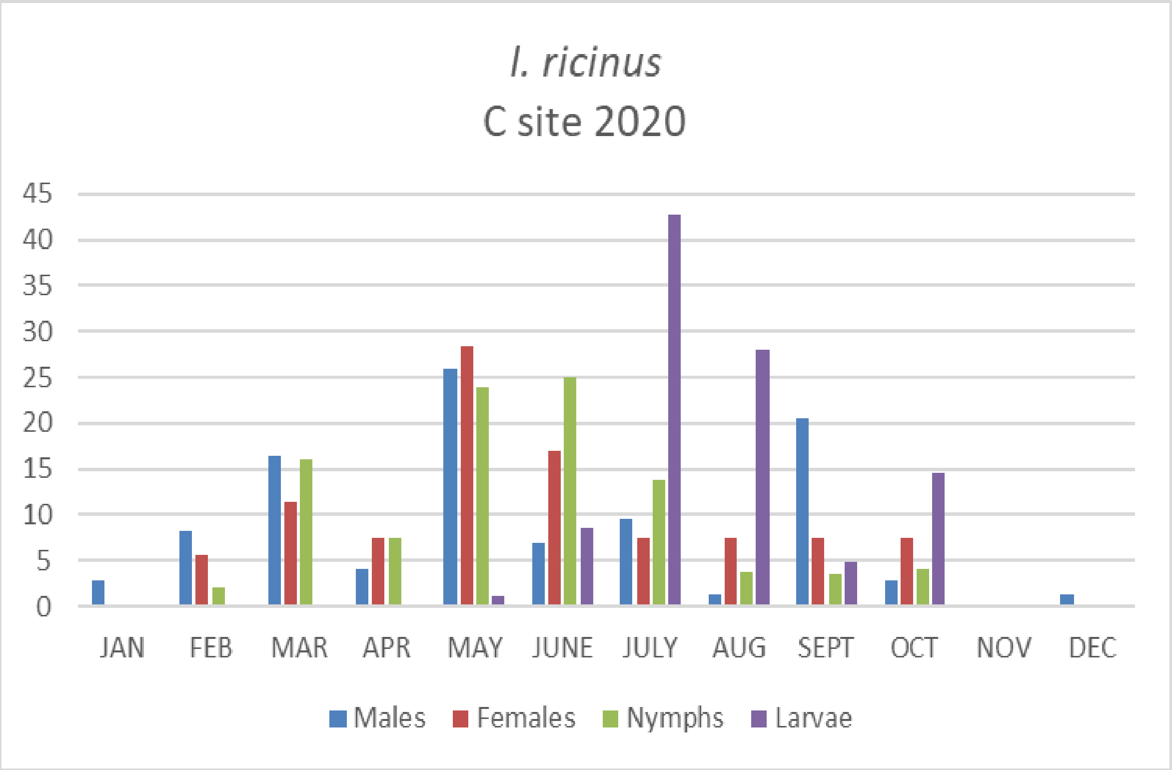
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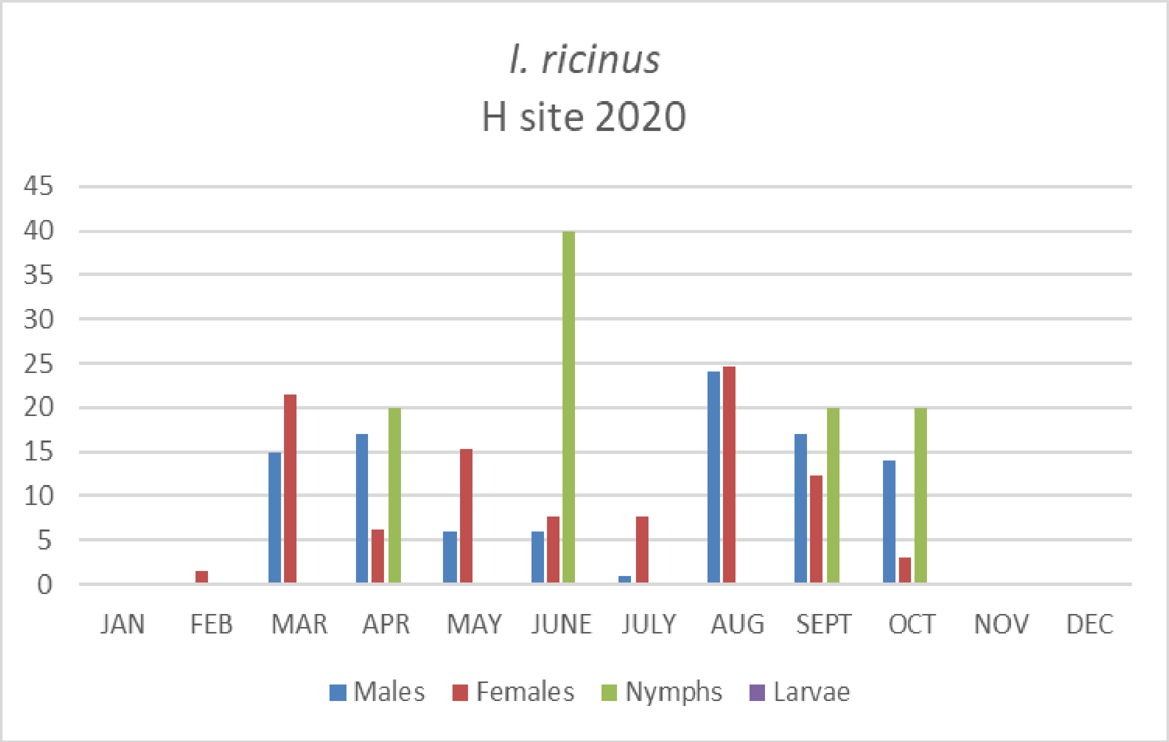
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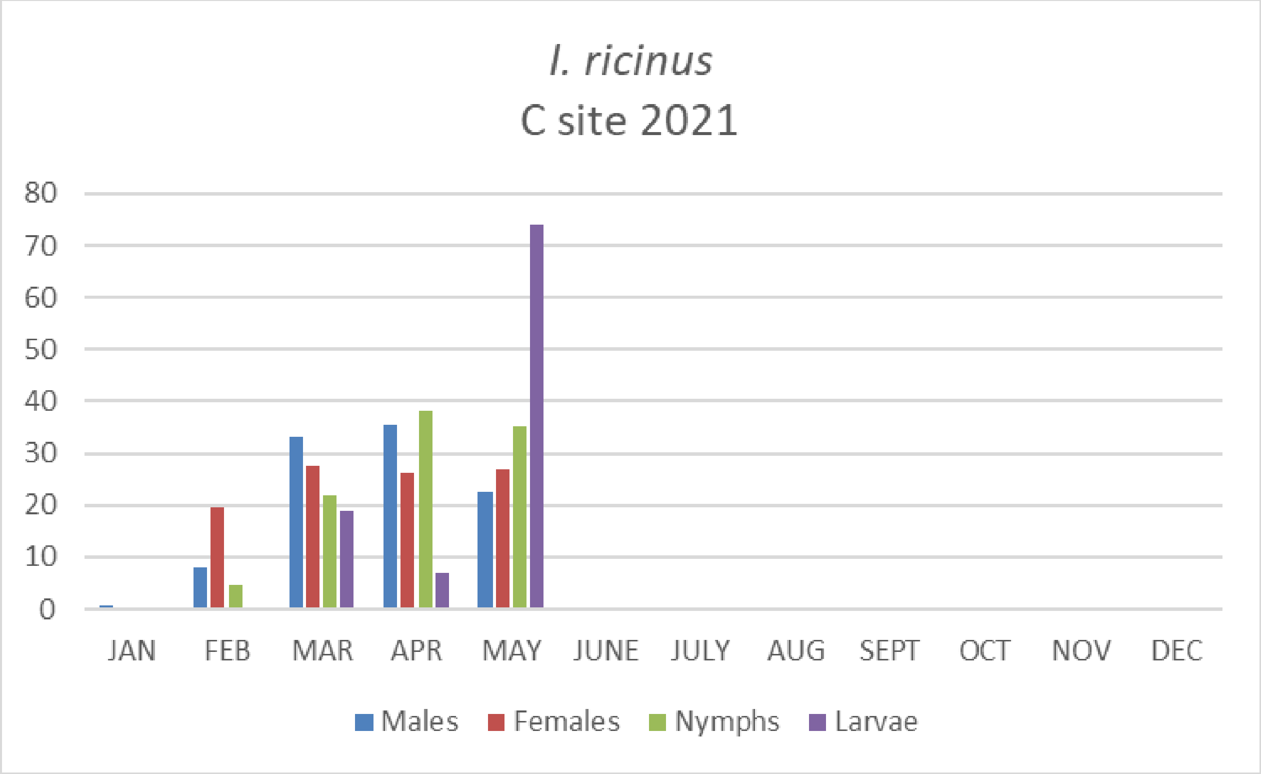
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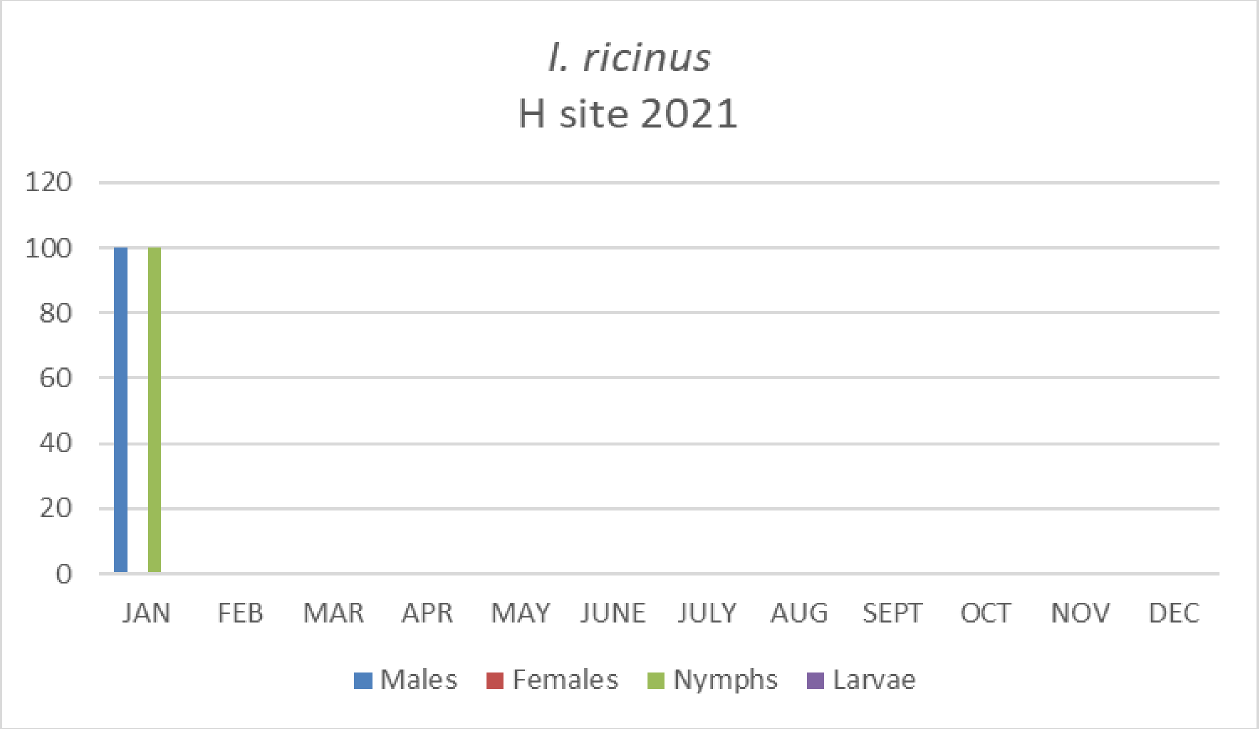
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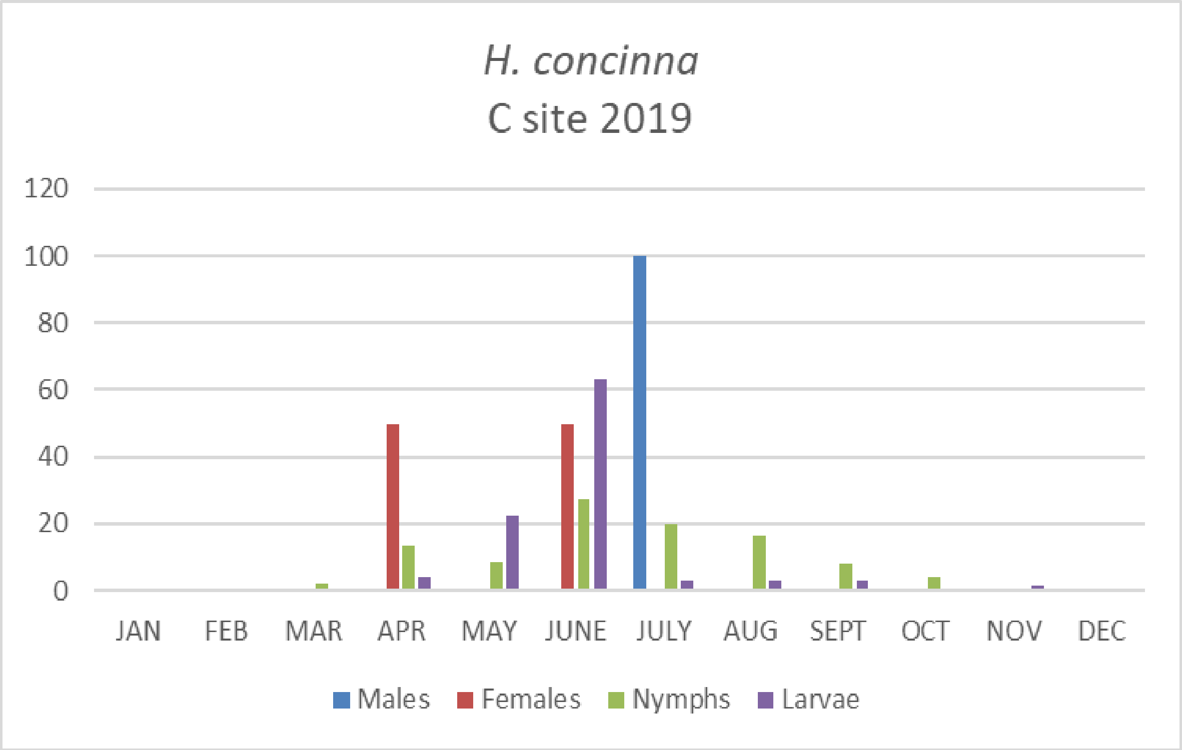
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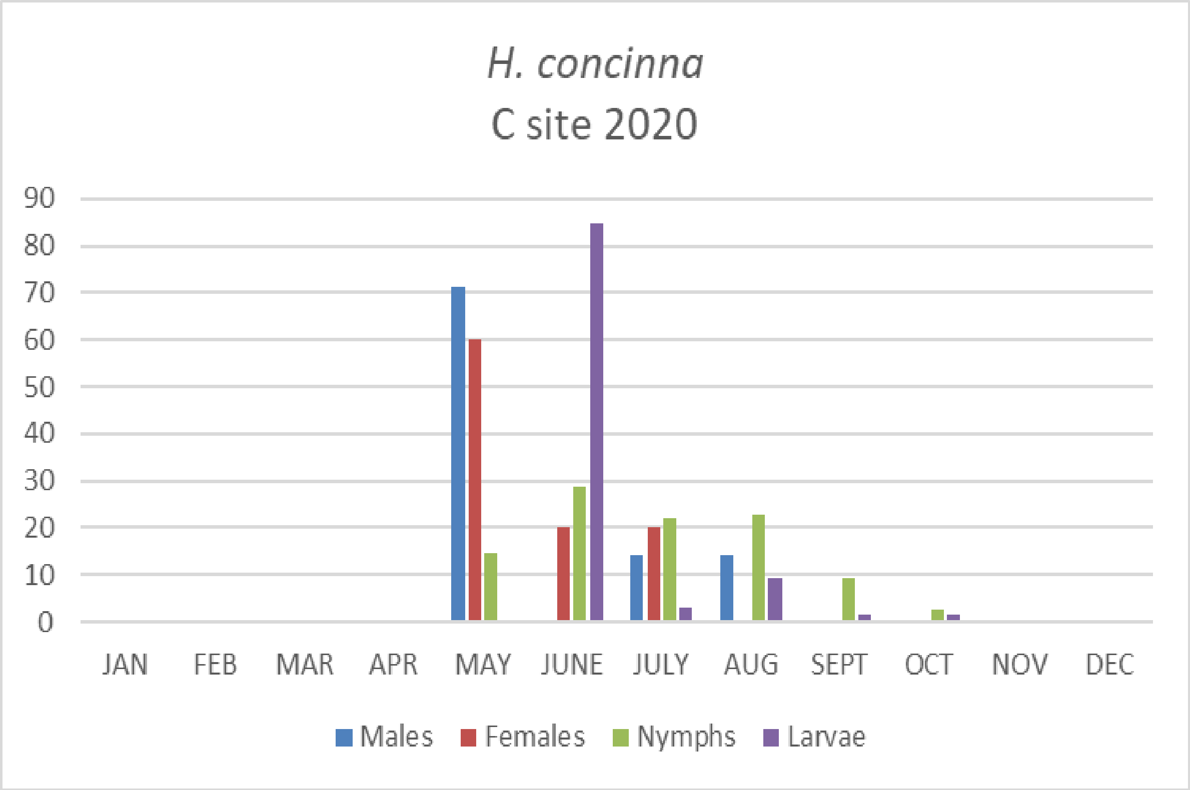
H. concinna
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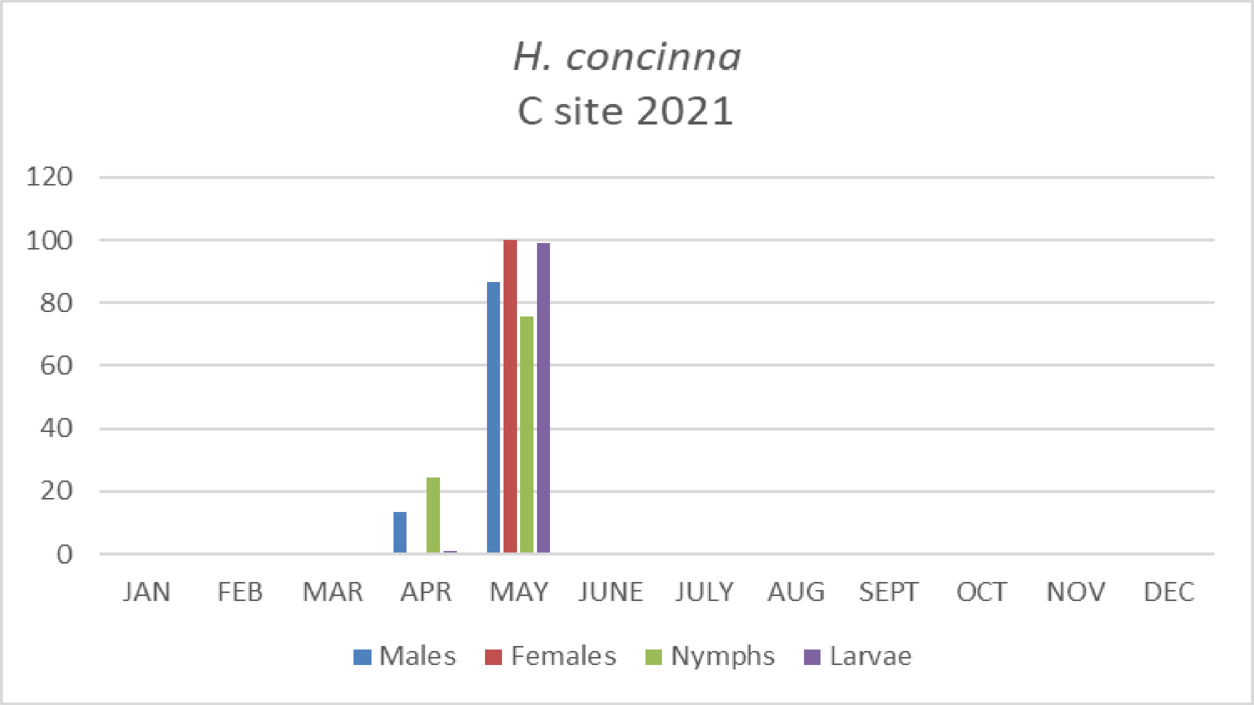

4.2. Molecular and phylogenetic analysis of *Ixodes ricinus* morphotypes

All specimens of *I. ricinus* collected during the study period that showed significant, unusual morphologic character(s) were analyzed by sequence alignment and phylogenetic comparison of their 16S rRNA gene with ticks showing "usual morphology", characteristic of this species. The ticks examined in this context included three unusual morphotypes of females: morphotype I (with flattened front of basis capituli around the hypostome making the head more rectangular than pentagonal, with medially curved palpal article III: Figures 1-2), morphotype II (smoother scutum, with more scattered small pores: Figure 3) and morphotype III (with wavy edge of the genital pore: Figure 3). In addition, the following morphological anomalies were noted: deformity of the scutum in a female (Figure 4a); absence of groove separating adanal shields in a male (Figure 4b) and circumanal groove in a male (Figure 4c) and a nymph (Figure 4d).

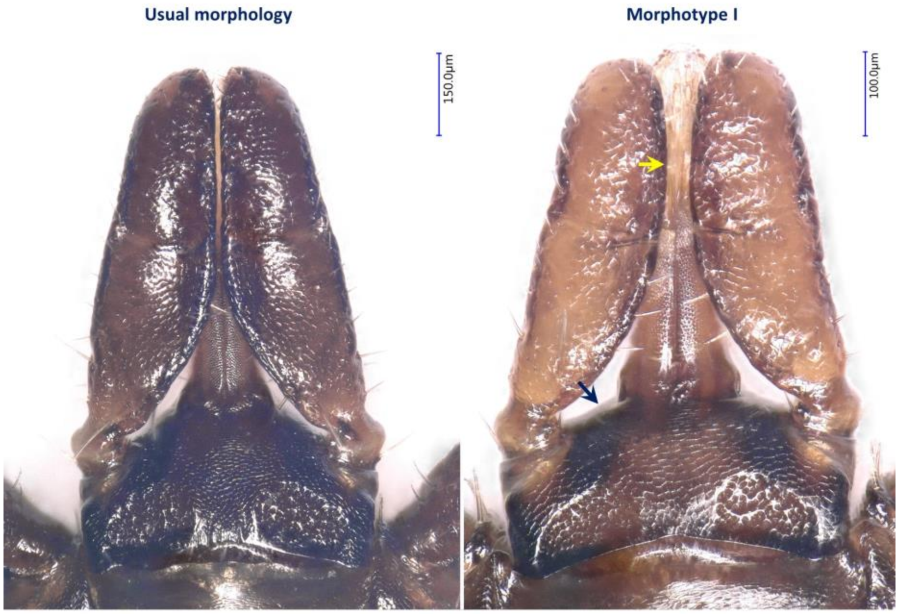

Figure 1 Morphotype I comparison

Figure 2 Morphotype II dorsal view

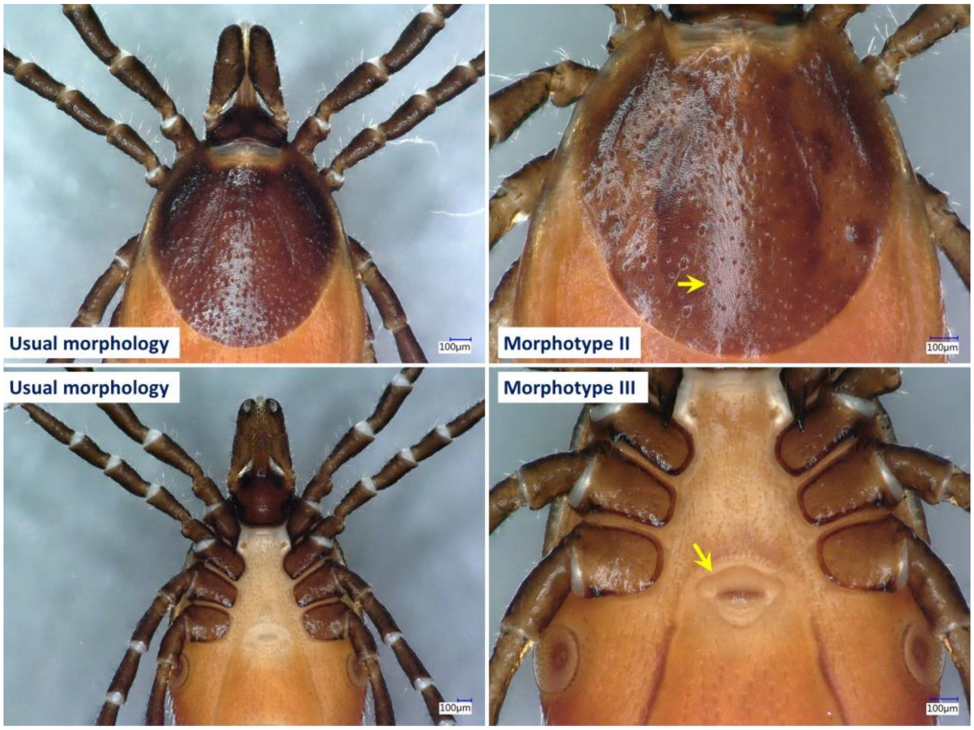

Figure 3 Scutum and genital pore differences

Figure 4 Morphological abnomalities

A standard tick (isolate RIC16) was chosen on account of its 16S rRNA sequence identity with a tick collected in neighboring Slovakia (GU074590). The ticks used for the molecular-phylogenetic comparison included altogether nine ticks (six females, three males) showing "usual morphology" of *I. ricinus* (RIC3, RIC7, RIC9, RIC11, RIC12, RIC13, RIC16, RIC17, RIC21). These had up to 6 bp sequence divergence from each other in the amplified part of their 16S rRNA gene. Specimens belonging to morphotype I (nine females: RIC4, RIC5, RIC6, RIC8, RIC10, RIC18, RIC19, RIC20, RIC24) showed up to 4 bp sequence divergence from each other and from the group showing "usual morphology". Morphotype II (a single female: RIC15) had 4 bp difference, whereas morphotype III (another female: RIC23) had only 1 bp difference from the standard tick (RIC16). Ticks showing morphological anomalies (I-IV.: RIC1, RIC22, RIC14, RIC2) had 1 bp, 1 bp, 5 bp and 2 bp sequence divergence form the standard tick, respectively. The phylogenetic tree on Figure 5 shows the relationships of these specimens.

Taken together, the three morphotypes and four malformed specimens of *I. ricinus* were not more different in their 16S rRNA haplotypes from the typical representatives of this species, than from each other, or the ticks with "usual morphology" from each other. In addition, these unusual specimens did not show consistent phylogenetic clustering.

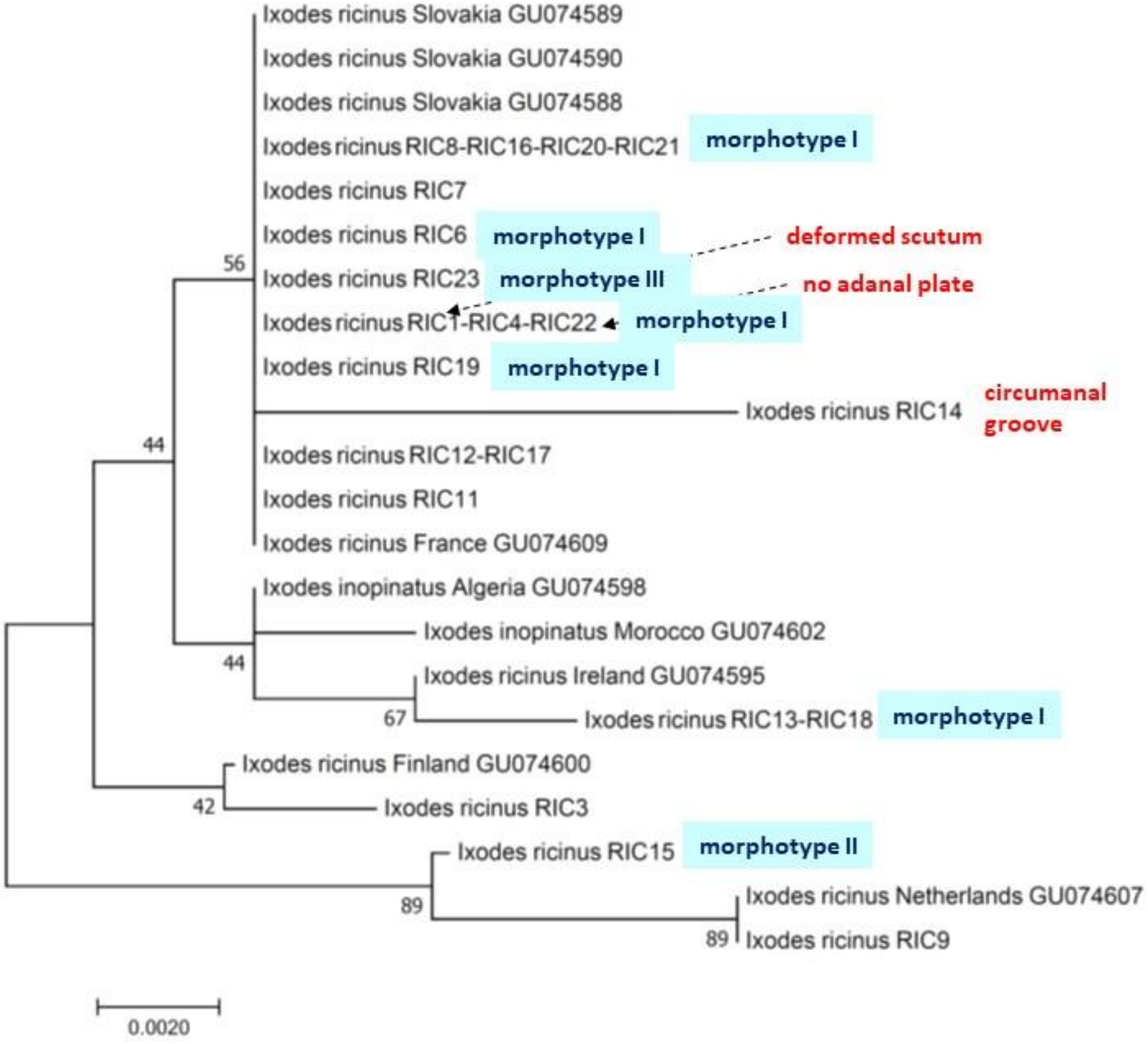

Figure 5 Phylogenetic tree of specimens

4.3. Molecular and morphological comparison of *Ixodes frontalis* haplogroups "A" and "B"

As confirmed by both cox1 (n=24) and 16S rRNA gene (n=25) analyses, *I. frontalis* haplotypes from both groups ("A" and "B") were found in the same habitat (cemetery), but based on the molecularly analyzed 25 ticks haplogroup "A" (n=19) predominated over "B" (n=6). There was no overt difference between their seasonality, although among the molecularly analyzed 7 larvae collected in October and November only haplotype-A was found (Table 1).

Six *I. frontalis* nymphs were selected for detailed morphological analysis: three from haplogroup "A" and three from haplogroup "B". The cox1 sequence of three nymphs representing haplotype-A had 99.8-100% (653-654/654 bp) sequence identity with genotype A-Hu1 (KU170492) reported previously in Hungary. The cox1 sequence of three nymphs representing haplotype-B had 99.7-100% (652-654/645 bp) sequence identity with genotype B-Hu1 (KU170501) also reported in Hungary. Thus, the sequence divergence between three ticks from haplogroup "A" and another three from haplogroup "B" was approx. 8.6% (56/654 bp). This exceeds the "species boundary" (average difference between the cox1 sequences of congeneric ixodid tick species belonging to two closely related species) which is estimated to be around 6.1%.

The following species-specific morphological characters were observed to be uniform between the above six specimens. The idiosoma was lightly colored (e.g., in comparison with nymphs of *I. ricinus*), and the spiracle openings were dark brown. The scutum was slightly broader than long (width to length ratio was in the range of 1.005-1.05), posteriorly with scattered punctuations (Figure 6, blue arrow). Ventrally, the coxae had prominent external spur, and the anal groove was converging behind the anus (Figure 6, yellow arrows). On the gnathosoma, "frontal bumps" were present, and auriculae had narrower anterior and broader posterior divisions (Figure 7, yellow arrows). The hypostome was pointed, the dental formula was 3/3 until mid-length (Figure 7, blue arrow). Anterior median scutal setae (sc1-3) were approx. two times longer (30-35 μm) than posterior scutal setae (sc4-5: 15-20 μm) (Figure 8). Length of lateral alloscutal setae anteriorly 180-200 μm (Figure 12, star). Spiracle opening was round, with 2-3 rows of regularly arranged large goblets (inner circle: approx 10, outer: approx. 20) (Figure 8, yellow arrow).

In summary, the morphological characters listed above did not show definable difference between individuals of the two haplogroups.

	Haplogroup A		Haplogroup B		
	Larvae	**Nymphs**	**Larvae**	**Nymphs**	**Female**
February	NA	4	NA	2	NA
March	2	5	-	1	1
April	ND	-	ND	1	NA
September	NA	1	NA	1	NA
October	5	NA	-	NA	NA
November	2	NA	-	NA	NA

Abbreviations: ND - not done, NA - not available

Table 1. Haplotypes of selected *Ixodes frontalis* specimens according to their month of collection and developmental stage.

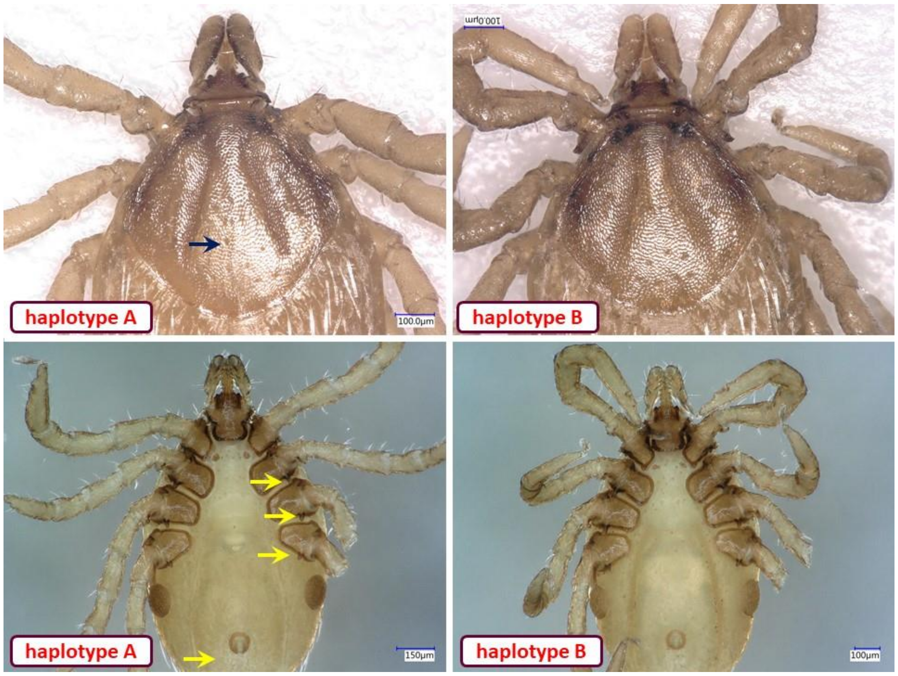

Figure 6 Scutum, external spurs, and anal grove

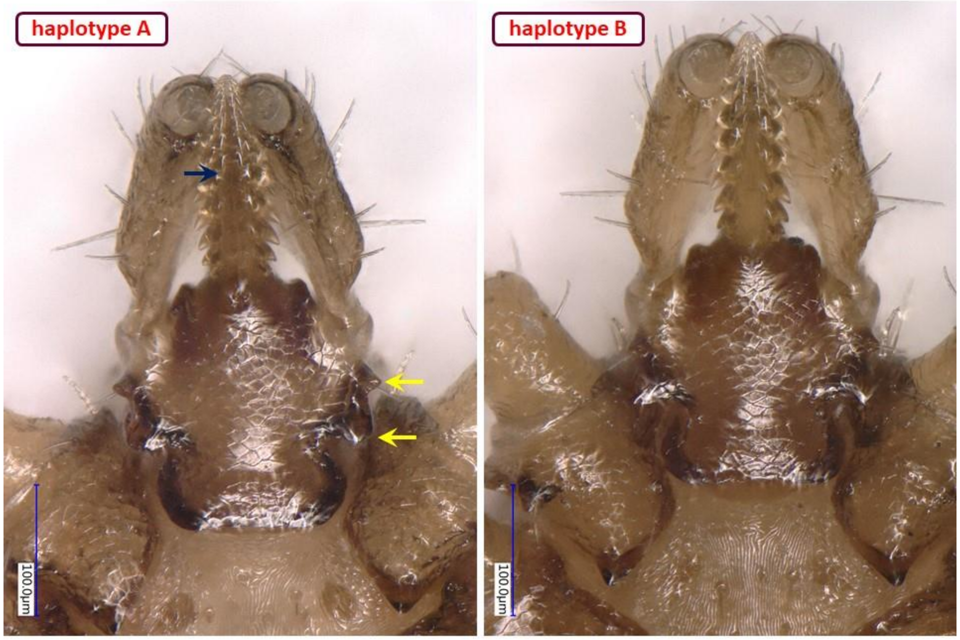

Figure 7 Frontal bumps and hypostome

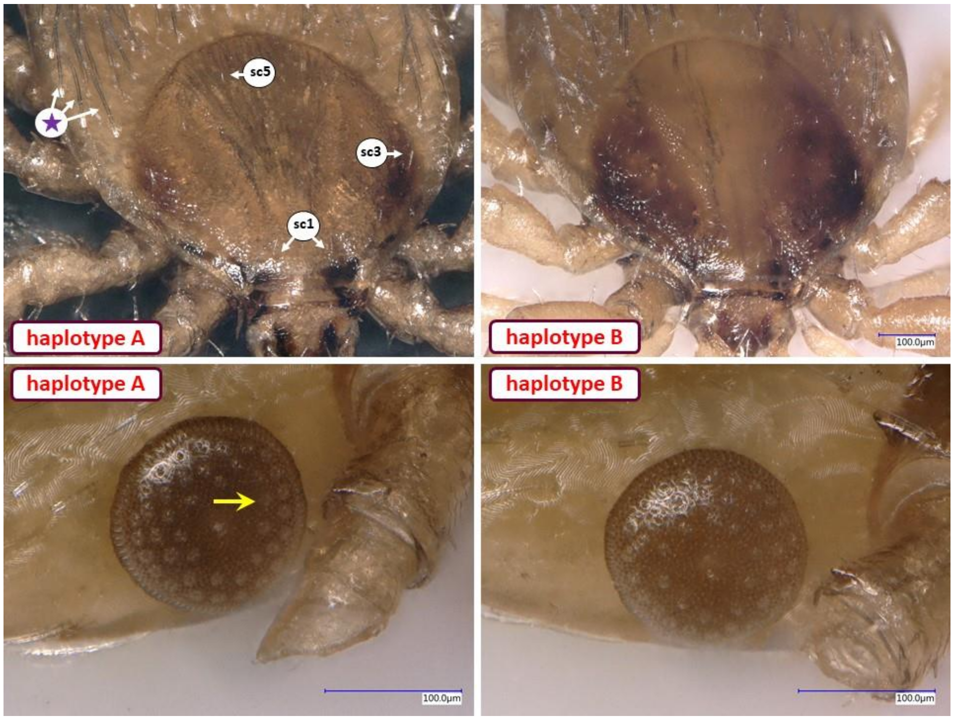

Figure 8 Scutal setae and spiracles

4.4. Piroplasms in *Haemaphysalis concinna* collected over a one-year period

Among the 161 molecularly analyzed *H. concinna* specimens (collected in 2019) 31 were positive in the PCR amplifying part of the 18S rRNA gene of piroplasms. In six samples (one female collected in April and five nymphs collected in April (n=3), June, July *Theileria capreoli* was identified, with 100% (440/440 bp) sequences identity, among the others, to an isolate reported formerly also in northern Hungary (MN611178).

In addition, eight *Babesia* genotypes that were first reported in the Far-East, were detected. These were as follows. (1) In three larvae (one collected in April and two in May) and five nymphs (two from June, two from July and one from August) *Babesia* sp. Irk-Hc133 (with 100% sequence identity to KY471448). (2) In a larva and a nymph (both from May) a closely related genotype designated as *Babesia* sp. Irk-Hc133-1(with 99.8%, 408/409 bp sequence identity to KY471448) was present. (3) In a single male (collected in July) a genotype closely related to *Babesia* sp. Irk-Ip256 (having 99.8%, 407/408 bp sequence identity to KJ486567) was detected, named here as *Babesia* sp. Irk-Ip256-1. (4) In a larva (from May) and in a nymph (from August) the genotype *Babesia* sp. Kh-Hc232 was demonstrated, with 1 bp difference to the original sequence (KJ486560) i.e. with 99.8% (407/408 bp) identity. (5) In two larvae (collected in May) *Babesia* sp. Am-Hc344-1, and (6) in four further larvae (also collected in May) *Babesia* sp. Am-Hc344-2 were identified, with 99.8% (407/408 bp) and 99.5% (406/408 bp) sequence identity to KJ486564. (7) In four nymphs (one from June, one from July and two from August) *Babesia* sp. Kh-Hc222 was identified (with 100% sequence identity to KJ486568). (8) In two nymphs (from April and August) *Babesia* sp. Irk-Hc130 was detected, with 100% sequence identity to its original genotype (KJ486569).

Regarding the phylogenetic relationships of these isolates, the majority (No. 1-6.) clustered as a sister group to *Babesia* sp. Kh-Hc222, and all these were closely related to ruminant-associated babesiae (*Babesia major* and *Babesia crassa*: Figure 9). On the other hand, *Babesia* sp. Irk-Hc130 occupied a more distant phylogenetic position, together with *Babesia motasi* as a sister group to *Babesia bigemina* (Figure 9).

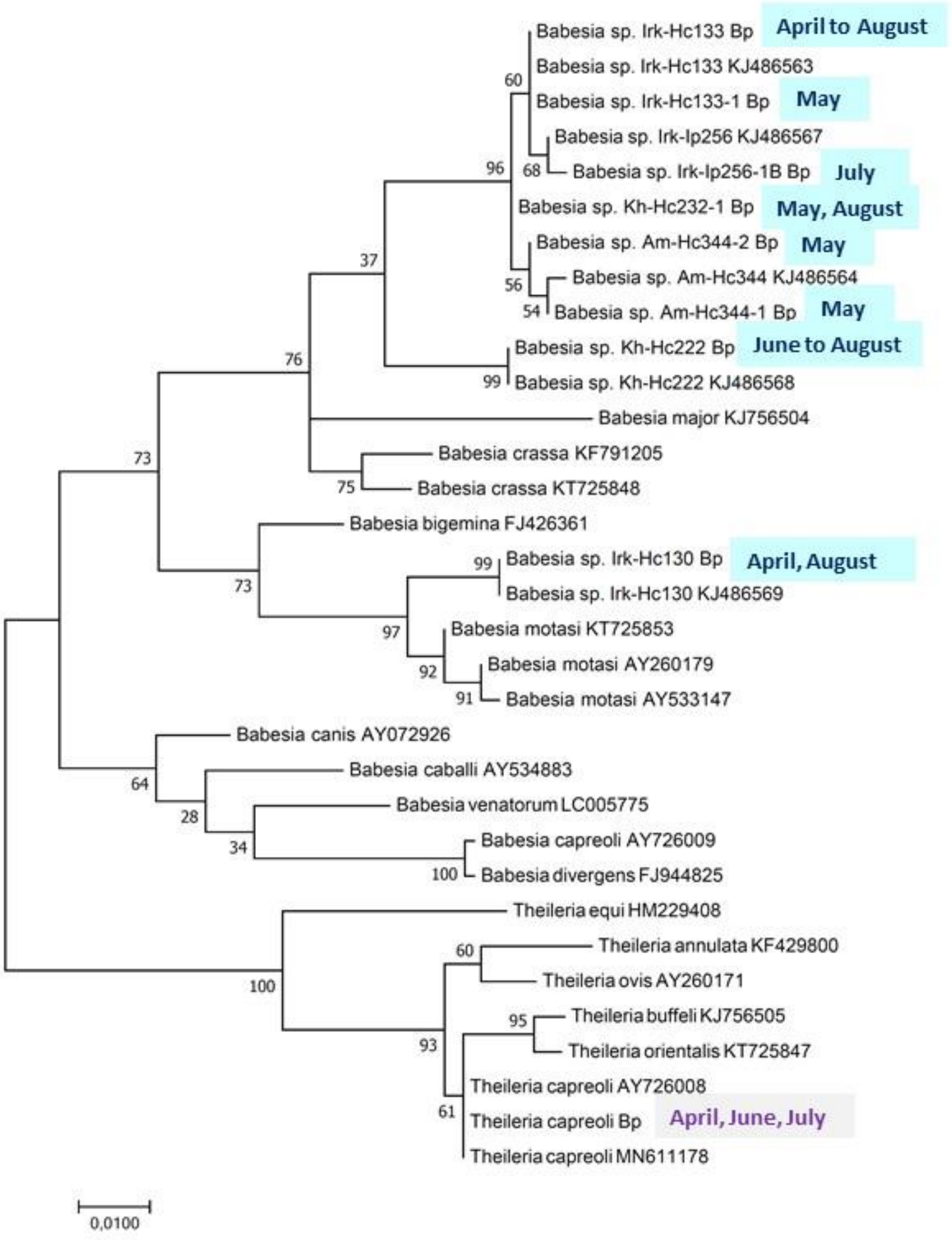

Figure 9 Phylogenetic tree of isolates

5. Discussion

In this study, two *Ixodes* species and *H. concinna* were found. Both sampled tick habitats had sparse or continuous deciduous tree covering, excluding meadows or other open areas, and this explains the complete absence of open country ticks (most notably *Dermacentor* species) among the collected specimens. In addition, the cemetery is surrounded by concrete and wire fences, and dogs are not allowed to enter, therefore important reproduction hosts of *Dermacentor* species were thought to be absent from these areas. However, in the forested habitat, neither *H. concinna*, nor *I. frontalis* were found, only *I. ricinus*. This can be explained by the local absence of the most important host species of *H. concinna* in Hungary, which is the roe deer (Hornok et al., 2012). In case of *I. frontalis*, a highly relevant limiting factor must have been the specific lower vegetation requirement of this species (Plantard, 2021), since its main hosts (i.e., birds) are ubiquitous.

At the same time, this is the first evidence on the presence of questing *I. frontalis* on the vegetation in Hungary. The most likely explanation why this species was formerly not found in the same habitat (cemetery: Hornok et al., 2014) is that the previous study focused on the spring tick season, while here it was demonstrated that *I. frontalis* larvae have their activity peak late autumn. In addition, even during the present study only a single *I. frontalis* female was found and nymphs of this species could not be collected in the spring of 2020.

Regarding seasonality of ixodid ticks in the evaluated urban habitats, the year-round activity of *I. ricinus* males and females in the cemetery is not surprising in light of the fact that even in natural habitats adults of this tick species were found questing in each month of the year (Hornok, 2009). However, in natural habitats the nymphs of this tick species did not show questing activity in most part of the winter (Hornok, 2009), unlike in the cemetery during this study. The nearly continuous presence of *I. ricinus* nymphs questing on the vegetation late autumn and in the winter of 2019/2020 (including November, December and January) may in part be due to the warmest winter of meteorological records in Europe during the relevant months (C3S, 2020).

In the cemetery, all life cycle stages of *I. ricinus* had 1-2 month earlier activity peak in 2019 and in 2021 than in 2020. Based on our data the peak questing activity appeared to be synchronized between developmental stages, thus bi-annual return to the former activity pattern is probably not due to the 2-3 years during which *I. ricinus* usually completes its development under temperate climate (Randolph et al., 2002). Another climate variable may explain this phenomenon. It is known that rapid fall in temperatures causes more *I. ricinus* developmental stages to suspend their activity (Randolph et al., 2000), and the opposite was reported to be relevant in the context of spring tick activity, i.e. extreme temperature increase preceding the spring tick season (as also experienced in 2021) may trigger earlier spring activity peak of tick species (Hornok, 2009). In Central Europe, the increase in extreme temperature values is considerably higher than the corresponding average winter warming, and approximately 2.5 times higher than average global warming (Lorenz et al., 2019). Therefore, we hypothesize that during January/February extreme local warming usually triggers earlier activity peak of *I. ricinus* (all stages), as in 2019 and 2021, but this was neutralized by the permanently warm winter at the beginning of 2020 (C3S, 2020), causing later peaks ("flattened activity curve").

The above yearly shifts in the seasonality of *I. ricinus* was less pronounced (or even showing opposite tendency) in the forest habitat, most likely because temperature fluctuations were more equilibrated there. The cemetery is more open (with sparse higher and dense lower vegetation), while the forested habitat has a closed tree covering, sparse lower vegetation.

In case of *H. concinna*, adult questing activity started in the cemetery as in natural habitats (April-May: Hornok, 2009), but its nymphs initiated their activity 1-2 months earlier in the urban habitat (in both 2019 and 2021) than reported in natural habitats. The most dramatic difference in this comparison was observed at the end of the tick season, because in natural habitats *H. concinna* adults and nymphs were only active until July (Hornok, 2009), whereas in the cemetery until October/November. Since *H. concinna* is a thermophilic tick species (Hubálek et al., 2003), these observations might be explained either by the warm winter at the end of 2019, or by the the so-called heat island effect (warmer average temperatures in cities than in surrounding areas: Wickham et al., 2013) or both.

To the best of our knowledge, this is the first seasonality assessment of *I. frontalis* in Central Europe. This tick species is known for its late autumn and winter activity under suboceanic climate in Western Europe (Agoulon et al., 2019), implying preference for cool weather, as also confirmed here under continental climate. This may be the main cause why in the spring of 2020 (following the mildest winter) *I. frontalis* was not found in the cemetery. In this study, most *I. frontalis* nymphs were collected late winter and early spring (February-April), whereas the peak activity of larvae was during late autumn (October-November), which is a pattern similar to the decline in nymphal activity from October until April next year, and early predominance of nymphs each year as reported in France under suboceanic climate (Agoulon et al., 2019).

Concerning morphotypes and morphological anomalies of *I. ricinus* recognized in this work, the majority of these variants were unique in the studied material, except for morphotype I. (with rectangular basis capituli and medially curved palpal article III) which was repeatedly collected and showed the same distinguishing characters consistently. The gnathosoma of nine females that showed this character was significantly different from the usual morphology of *I. ricinus* (e.g., as shown in Bugmyrin et al., 2016; Estrada-Peña et al., 2017). To the best of our knowledge, this is the first evidence for the existence of a different morphotype within *I. ricinus* that, from a medical point of view, is regarded as the most important tick species in the temperate zone of Europe (Gray, 1991).

In addition, morphological anomalies were noted in a minority of ticks, all belonging to *I. ricinus*. These morphological anomalies found among ticks collected in this study were different from those reported in a large scale survey in Germany; and the latter mostly affected the legs of *I. ricinus* (Citimia-Dobler et al., 2017). Although no genetic deviation could be identified in case of the unusual ticks, it should be taken into account that ticks in this study were collected in an urban area where the level of environmental contamination is relatively high. In particular, not only were the chances for morphological abnormalities reported to be higher in urban areas, but the rate of infection with tick-borne pathogens was also shown to be higher in such abnormal ticks, in association with their polluted habitat (Alekseev and Dubinina, 2008).

Previously, when *I. frontalis* specimens collected from birds in Hungary were molecularly analyzed (Hornok et al., 2016a), the results clearly indicated for the first time the existence of two distinct genetic lineages (haplogroups "A" and "B") within *I. frontalis* that are transported by birds in Central Europe. The separation of these mitochondrial lineages was supported by high bootstrap values in both the cox1 and 16S rDNA phylogenetic analyses (Hornok et al., 2016), and by the high rate of cox1 and 16S rDNA sequence divergence in this study. Importantly, the degree of cox1 sequence divergence between the two lineages (8.6-9 %) exceeds the average cox1 sequence difference (6.1 %) separating closely related ixodid tick species (Lv et al., 2014).

To the best of our knowledge, this is the first evidence on the sympatric occurrence of the two haplogroups of *I. frontalis* during questing in the same habitat. Considering the haplotypes of 25 *I. frontalis* specimens analyzed here, the majority belonged to haplogroup-A, limiting the availability of nymphs from haplogroup B for morphological comparison. During the latter all important structures, parameters were examined which serve to recognize this species (Estrada-Peña et al., 2017), and which were regarded as important in separating nymphs of the relatively recently discovered and difficult-to-recognize new species *I. inopinatus* from those of the sibling species *I. ricinus* (Estrada-Peña et al., 2014: including scutal dimensions and the relative size of scutal and alloscutal setae). In the nymph stage of *I. frontalis*, there were no evident morphologic differences between individuals belonging to either haplogroup-A or -B, confirming their status as cryptic species. Nevertheless, morphologic comparison of adult stages should ultimately confirm this.

Among the molecularly analyzed *H. concinna* specimens, six were found to carry *Theileria capreoli*. All *T. capreoli*-infected ticks were nymphs and a female, according to the transstadial mode of transmission of this piroplasm as in case of other theileriae (Schreeg et al., 2016). Taking into account that in the absence of transovarial transmission *T. capreoli* can only exist/survive in nature in the presence of its infected mammalian hosts (cervids, particularly roe deer), this finding is also an indirect evidence that the fencing of the cemetery must be incomplete, allowing roe deer to enter the area.

In addition, from 25 *H. concinna* specimens sequences of eight different *Babesia* genotypes were successfully amplified. PCR-positive ticks included larvae, consistently with the transovarial mode of transmission (Schreeg et al., 2016), confirming that these genotypes belong to the taxonomic-phylogenetic group *Babesia sensu stricto*. Originally, all eight *Babesia* genotypes detected in this study in *H. concinna* were discovered in Asia, in Siberia and the Far

East (Rar et al., 2014). During the last years, some of these newly recognized *Babesia* genotypes, have already been detected in *H. concinna* from Central Europe: in questing specimens of this tick species in Hungary (Hornok et al., 2015), in questing and rodent-attached ticks from Slovakia (Hamšíková et al., 2016) and in *H. concinna* collected from birds in Hungary (Flaisz et al., 2017). Interestingly, while the prevalence of these Central Asian and Far-Eastern *Babesia* genotypes was very similar in Hungary in *H. concinna* collected from birds (Flaisz et al., 1017: 15.9%) and here (25 of 161 ticks: 15.5%), in the former study only three genotypes were detected, whereas their diversity significantly increased (to eight) here. Also in contrast to the bird study (Flaisz et al., 2017) where the presence of these Central Asian and Far-Eastern *Babesia* genotypes in *H. concinna* was significantly more frequent during the summer and autumn compared to spring (suggesting that migratory birds arriving in Hungary from the north or north east are the most important in the dispersal of *H. concinna*-associated piroplasms), in the present study questing *H. concinna* individuals contained these piroplasms only in the spring and the summer. While the Central European arrival (import) of these Central Asian and Far-Eastern Babesia genotypes was attributed to bird species with known eastern migratory habit and/or phylogenetically substantiated connections between their eastern and western Eurasian populations, results of the present study confirm that an increasing diversity (or at least broader spectrum) of these became established in the local tick population.

However, on a monthly level, none of the *H. concinna*-associated piroplasms appeared to show a consistent seasonality in their vector ticks in the evaluated urban habitat. This is in contrast to what was observed for the spring activity of *Babesia canis*-carrier *Dermacentor reticulatus* adults in another urban habitat of Budapest (Hornok et al., 2016b). The most likely explanation for this difference is that while *D. reticulatus* usually has a one-year life cycle in the region which thus can allow the synchronized occurrence of infected ticks, the development of *H. concinna* may last for 3 years in the region of Hungary (Nosek, 1971). Therefore what is detected in terms of PCR-positivity is a mixture of individuals from several generations and life cycle stages.

6. Summary

Hard ticks (Acari: Ixodidae) are regarded as the most important arthropods that are able to transmit pathogens to humans and domestic animals in the temperate zone. Previously, in the capital city of Hungary various habitats were surveyed for the presence of ticks and tick-borne pathogens, and neglected parts of cemeteries were found to be especially suitable to maintain large tick populations, most likely because of the high density of birds and small mammals (i.e., due to the restricted entry or near absence of medium sized carnivores).

In a cemetery, we collected ticks with the dragging-flagging method from the vegetation during a 28-month period (February 2019 to May 2021), encompassing three spring and two autumn tick seasons. For comparison, ticks were also collected in an urban forest for 24 months (February 2019 to January 2021). The ticks were stored in ethanol until identification of their species based on standard morphologic keys. DNA was extracted from selected specimens for either molecular taxonomic analyses (complementing morphologic data) or for screening piroplasms. The former were based on conventional PCRs amplifying the cytochrome *c* oxidase subunit 1 (cox1) gene and the 16S rRNA gene of the ticks, whereas the latter targeted the 18S rRNA gene of piroplasms. The PCR products were sequenced and then compared to GenBank data with the BLASTn program.

In the cemetery, 3818 ticks were collected, including *Ixodes ricinus* (n=2772), *Ixodes frontalis* (n=350) and *Haemaphysalis concinna* (n=696). *Ixodes ricinus* showed activity throughout the year, peaking in March-May and September, while *H. concinna* was not active during winter months and early spring, with activity peak in May-June. Most *I. frontalis* nymphs were collected late winter and early spring (February-April), whereas the peak activity of larvae was during late autumn (October-November). This is the first seasonality assessment of *I. frontalis* in Central Europe. In the forested habitat only *I. ricinus* was found (n=292).

Results of the morphologic and molecular analyses were as follows. Three morphotypes and four malformed specimens of *I. ricinus* were not significantly different in their 16S rRNA haplotypes and phylogenetic clustering from the typical representatives of this species. Considering the haplotypes of 25 *I. frontalis*, the majority (n=19) belonged to haplotype-A, and in the nymph stage there were no evident morphologic differences between haplotypes-A and -B, confirming the status of these as cryptic species.

Among the 161 molecularly analyzed *H. concinna* specimens (collected in 2019) 31 were PCR positive for piroplasms. In these ticks *Theileria capreoli* (n=6, in nymphs and a female) and eight Far-Eastern *Babesia* genotypes were identified (n=25, in larvae, nymphs and a male). None of these appeared to show a consistent seasonality in their vector ticks.

7. Összefoglaló

A kullancsokat (Acari: Ixodidae) állat- és humánegészségügyi szempontból a mérsékelt égöv legfontosabb ízeltlábú kórokozóterjesztőinek tartják. Korábban már felmértük hazánk fővárosának különféle élőhelyeit a kullancsok és kullancs közvetíttette kórokozók előfordulása szempontjából, és leginkább a temetők elhanyagolt részeit találtuk alkalmasnak nagy kullancspopulációk fenntartására, valószínűleg a madarak és kisemlősök nagy egyedsűrűségének köszönhetően (mivel a közepes méretű ragadozók bejutásukban akadályozottak, így csaknem teljesen hiányozhatnak).

Egy temetőben kullancsokat gyűjtöttünk a növényzetről a törülközőhúzási módszerrel 2019 február és 2021 május között (28 hónapig), ami három tavaszi és két őszi kullancsszezont ölelt fel. Összehasonlításképpen egy városi erdőben is gyűjtöttünk kullancsokat 2019 február és 2021 január között (24 hónapig). A kullancsokat etanolban tároltuk, amíg fajukat morfológiai alapon meg nem határoztuk. Válogatott példányokból kivontuk a DNS-t, hogy molekuláris taxonómiai vizsgálatokat végezzünk (a morfológiai vizsgálat kiegészítéseképpen) vagy piroplasmák jelenlétét mutassuk ki belőlük. Az előbbiek olyan konvencionális PCR-en alapultak, amelyek a citokróm c oxidáz I-es alegységét kódoló génszakaszt és a 16S rRNS gént erősítették fel, míg az utóbbi a piroplasmák 18S rRNS génjét célozta. A pCR termékeket szekvenáltuk és a BLASTn programmal génbanki adatokhoz hasonlítottuk.

A temetőben 3818 kullancsot gyűjtöttünk. Ezek a következő fajokba tartoztak: *Ixodes ricinus* (n=2772), *Ixodes frontalis* (n=350) és *Haemaphysalis concinna* (n=696). Az *I. ricinus* egész évben aktív volt, március-május közötti és szeptemberi csúcsidőszakkal, míg a *H. concinna* hiányzott a növényzetről a téli hónapokban és koratavasszal, május-júniusi csúcsidőszakkal. A legtöbb *I. frontalis* nimfát a tél végén és a tavasz kezdetén találtuk (február-április között), ellenben a lárvák aktivitási csúcsa az ősz végén volt (októberben és novemberben). Ez az *I. frontalis* szezonaitásának első felmérése Közép-Európában. A másik, erdős élőhelyen csak *I. ricinus* fordult elő (n=292).

A morfológiai és molekuláris vizsgálatok eredményei a következők voltak. Az *I. ricinus* három morfotípusa és négy rendellenes felépítésű példánya nem különbözött jelentősen 16S rRNS haplotípusában és filogenetikai csoportosulásában e faj tipikus példányaitól. Az *I. frontalis* 25 példányának többsége (n=19) az A-haplotípusba tartozott, és nem volt egyértelmű morfológiai különbség az A- és B-haplotípusú nimfák között, ami megerősíti, hogy ezek kriptikus fajok.

A 161 molekulárisan vizsgált, 2019-ben gyűjtött *H. concinna* példány közül 31 volt PCR pozitív piroplasmákra. E kullancsokban *Theileria capreoli* fajt (n=6, nimfákban és egy nőstényben) és nyolc távol-keleti *Babesia* genotípust azonosítottunk (n=25, lárvákban, nimfákban és egy hímben). Kullancs vektorában ezek egyike sem mutatott következetes szezonalitást.

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