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The role of birds in the epidemiology of tick-borne pathogens

Ph.D. thesis

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Introduction

Epidemiological role of birds has been increasingly recognised. Members of the Passeriformes order, especially birds that belong to the ground foraging species, consequently and frequently infested with ticks and may contribute to the transmission of ticks and tick-borne pathogens to urban habitats.

The most common tick species collected from passerines in Central Europe are *Ixodes ricinus* (Taragel'ová et al. 2008; Dubska et al. 2009; Lommano et al. 2014) and *Haemaphysalis concinna* (Špitalská et al. 2011). Tick species that are usually considered strictly specific to birds include *I. frontalis* (Lommano et al. 2014), *I. arboricola* (Dubska et al. 2011; Špitalská et al. 2011) and *I. lividus* (Jaenson et al. 1994). Occurance of ornithophilic ticks is relatively rare in Central Europe, substantiated by *I. arboricola*, that had been reported from Hungary more than 50 years ago (Babos 1965). Another interesting phenomenon is the natural transportation of exotic ticks (e.g. *Hyalomma* spp.) by migratory birds, that may introduce non-endemic agents into European countries (Hornok et al. 2013a). The degree of tick infestation of birds depends on several factors. Significant tick infestation of ground-foraging bird species like Blackbirds (*Turdus merula*) and European Robins (*Erithacus rubecula*) is a well known phenomenon throughout Europe (Taragel'ová et al. 2008; Norte et al. 2015). Weather conditions and local geographical characteristics may influence the activity and survival of ticks (Hasle et al. 2009), hereby influencing the degree of tick infestation of birds. In a Hungarian study the majority of thermophil *Ha. concinna* larvae and nymphs were collected from birds in the summer (Hornok et al. 2013a).

Vector-transmitted pathogens and food-borne hormones in host blood may affect the level of tick infestation and pathogen transmission to birds at the same time. A Czech study revealed that ecdysteroids (hormones that effect cell proliferation and growth in insects) can be present in high amount in the blood of birds after oral administration of ecdysteroid–containing plants (Koudela et al. 1995). Apolysis, the initiative act of moulting in arthropods, is influenced by ecdysteroids. High ecdysteroid concentration is required in tick's haemolymph in order to start separation of the cuticule from the epidermis (Diehl et al. 1982).Three-host ixodid ticks moult to the next stage after finishing their blood meal then they acquire another host. Ticks will be able to infect the new host with the pathogens they received during their previous meal or from their mother.

In Central Europe *Anaplasma phagocytophilum* (Špitalská et al. 2011), *Rickettsia* species (Hildebrandt et al. 2010; Lommano et al. 2014), *Borrelia* species (Taragel'ová et al. 2008; Dubska et al. 2009; Špitalská et al. 2011), *Francisella tularensis* (Franke et al. 2010a), tick-borne encephalitis virus (Lommano et al. 2014) and *Babesia* species (Franke et al. 2010a; Hildebrandt et al. 2010) are the most common zoonotic pathogens in bird ticks. Members of

the Turdidae family may serve as important reservoirs of *B. garinii* and *B. valaisiana* (Dubska et al., 2009). *Borrelia*-prevalence in tick larvae suggest that they became infected during feeding on these birds (Franke et al 2010a). *I. ricinus* is one of the most common ectoparasite of passerine birds in Europe, and it is the main tick vector of *B. burgdorferi sensu lato* at the same time (Heylen et al. 2014b). Birds as tick-hosts and reservoir competent hosts of *Borrelia* bacteria have a significant role in the maintenance of these pathogens. Additionally, *R. helvetica* and *A. phagocytophilum* were detected in blood samples collected from synanthropic birds in Hungary (Hornok et al. 2014b).

Aims of the study

The aims of the study were:

1. to investigate species and genetic diversity of ixodid ticks transported by migratory and non-migratory bird species in Hungary, in particular to analyse molecular taxonomic characteristics of bird ticks in a geographical context.

2. to determine species specific characteristics of birds that influence their tick-infestation.

3. to investigate the presence of food-borne hormones in host blood and their effect on tick infestations and to compare the findings with population density data of lepidopterans, in order to analyse the influence of exogenous moulting hormones on apolysis.

4. to investigate the occurrence and prevalence of piroplasms in *Ha. concinna* ticks, carried by birds.

Materials and methods

Collection of samples from birds

Ixodid ticks and blood samples were collected from passerine birds from January 2012 until December 2014. Collection took place at ringing stations in Hungary (Ócsa, Fenékpuszta, Bódva-völgy). Birds were captured by standard Ecotone mist-nets (Hornok et al. 2014b). All captured birds were examined for the presence of ticks. Ectoparasites were put into 70% ethanol in separate tubes according to their hosts. Tick species were determined according to standard keys (Babos 1965), and were subsequently stored at room temperature.

Blood samples were taken from the brachial vein of some of the tick-infested birds to detect the presence of food-borne hormones (ecdysteroids). Samples were collected into EDTA-containing microtubes and stored frozen at -20^{o}C until analysis. Eighteen blood samples were randomly selected for the analysis with liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS).

LC-MS/MS studies

After sample preparation the clear supernatant was utilized for LC-MS/MS studies. Calibration for the assay were performed with seven standard ecdysteroids (purity > 95%) including 20-hydroxyecdysone (20E), polypodine B (pB), poststerone (pS), ecdysone (E), 2-deoxy-20-hydroxyecdysone (2d20E), ajugasterone C (ajC) and dacryhainansterone (Ds) (Hunyadi et al. 2007; Tóth et al. 2008). Equipment of the experiments were an Agilent 1200 liquid chromatography system. Chromatographic analysis was carried out using a Kinetex XB-C18 column at 40^{o}C (100 x 2.1 mm, 2.6 µm), with a mobile phase flow rate of 0.5 ml/min. Mass spectrometry detection was carried out on a 6410A triple quadrupole MS equipped with an electrospray ionization source used in positive ionization mode. Analyte detection was performed by multiple reaction monitoring (MRM) using an electron multiplier voltage (EMV) of 700 volts. Fragmentor voltage and collision energy (CE) were optimized individually for each target compound. Data acquisition and qualitative analysis was carried out with MassHunter B.04.01.

Molecular taxonomic and phylogenetic analysis of bird ticks

Tick specific COI gene based conventional PCR (Former et al. 1994) was used as the first target of molecular analysis. Another conventional PCR (Black and Piesman 1994) was chosen to amplify a 460 bp fragment of the 16S rDNA gene from one sample among those that yielded the same COI genotype. DNA was extracted from individual ticks with the QIAamp DNA Mini Kit as reported by Hornok et al. (2014b). PCR products were electrophoresed in a 1.5 % agarose gel, stained with ethidium-bromide and visualised under

ultra-violet light. Purification and sequencing was done by Biomi Inc. (Gödöllő, Hungary) and the sequences were submitted to GenBank. Phylogenetic analyses were conducted with the Tamura-Nei model and Maximum Composite Likelihood method by using MEGA version 5.2 (Tamura et al. 2011).

Piroplasm detection in *Ha. concinna* ticks and phylogenetic analyses of piroplasms

Samples were screened for the presence of piroplasms by a conventional PCR modified from Casati et al. (2006). This method was used to amplify an approximately 500 bp portion of the 18S rRNA gene of *Babesia*/*Theileria* spp. PCR products were subjected to electrophoresis in 1% standard agarose gel and were visualized with ECO Safe nucleic acid staining solution. After purification and sequencing of PCR products, the obtained sequences were manually edited, then aligned with GenBank sequences by nucleotide BLASTN program. Phylogenetic analyses were conducted with the Maximum Likelihood method (Jukes Cantor model) by using MEGA version 6.0 (Tamura et al. 2013).

Ethical approval

The investigations were carried out according to the national animal welfare regulations of Hungary (28/1998). Sampling of birds was approved by the regulation of Conservancy of Environmental and Protection Areas of Central Danube valley (number of the regulation: 27251-1/2014).

Statistical analysis

Confidence intervals (CI) for the prevalence rates were calculated at the 95% level according to Sterne's method (Reiczigel, J. 2003). Prevalence data were analyzed by Fisher's exact test. Mean values for the intensity of tick infestation were compared between bird categories by Mann-Whitney *U*-Test. Differences were considered significant when $P < 0.05$.

Activity of caterpillars was deduced from the population density data of moths (Insecta: Lepidopterans), based on the Hungarian Plant Protection and Forestry Light Trap Network records, that were collected between 1974 and 2006 (Gimesi et al. 2012). To test the association between the monthly proportion of apolytic ticks and the population density of lepidopterans Spearman rank correlation was used. The association of blood ecdysteroids with season and tick apolysis was compared by using Fisher's exact test.

Fisher's exact test was used also to compare the proportion of piroplasm positive *H. concinna* ticks. Confidence interval (CI) for the overall prevalence was calculated at the 95% level according to Sterne's method (Reiczigel, J. 2003).

Results

Species diversity of ixodid ticks collected from migratory and non-migratory birds

A total of 3339 ixodid ticks were collected from 1167 infested passerines. Bird ticks belonged to six species. *Ixodes* ricinus and *Ha. concinna* were the most common species with 2296 and 989 specimens (larvae and nymphs). There were 48 specimens of *I. frontalis* (including three adults) and three nymphs of *Hy. rufipes*. Three adult female ticks, two *I. festai* and one *I.lividus* were also collected.

Thirty-eight *I. frontalis* specimens were collected from European Robins (*E. rubecula*). *Hyalomma* ticks were collected from a Common Whitethroat (*Sylvia communis*), the two *I. festai* females were found on a Greenfinch (*Carduelis chloris*) and a Dunnock (*Prunella modularis*). The *I. lividus* female was collected from a Sand Martin (*Riparia riparia*).

Genetic diversity of less common ixodid ticks in a geographical context

Results from the investigation of four tick species were notable. Two separated genetic lineages were discovered among the 46 *I. frontalis* specimen for which part of the COI gene was sequenced ("A": KU170492-500, and "B": KU170501-9). The separation of the two lineages had a high bootstrap support on the phylogenetic tree. The following 16S rDNA gene analysis revealed only two distinct genetic variants (KU170518: genotype A-Hu16S, KU170519: genotype B-Hu16S). These two 16S rDNA genotypes had 100 % sequence identity to South-Western European isolates from the Azores (KP769863 and KP769862).

The COI sequence of *I. lividus* (KU170510) had 100% identity with an isolate of the same tick species from the UK (GU124743), and the partial 16S rDNA sequence had 99.7% identity with an isolate from Belgium (KJ414461).

The partial COI sequence of the one investigated *Hyalomma* nymph (KU170491) showed the highest degree of identity to a *Hy. rufipes* x *Hy. dromedarii* hybrid from Ethiopia (AJ437079). Based on the partial sequence of its 16S rDNA gene (KU170517) it showed the highest degree of identity to *Hy. rufipes*.

Among the 12 specimens of the analysed *Ha. concinna* ticks six different COI genotypes were found (KU170511-6), and clustered in two lineages on the phylogenetic tree. These COI genotypes represented three 16S rDNA genotypes (KU170523-5), that clustered together with the Far Eastern isolates.

Investigation of characteristics of birds influencing their tick-infestation

The majority of *I. ricinus* and *I. frontalis* larvae and nymphs (78.4%, CI: 76.7-80.1% and 91.7%, CI: 80-97.7%, respectively) occurred on birds that preferentially feed from the ground. However, the majority of *Ha. concinna* larvae and nymphs (73.1%, CI: 70.2-75.9%) were

found on birds that feed above the ground level. The difference of the host seeking habit between the two tick genus was significant (Fisher's exact test: $P < 0.0001$). The mean intensity of tick infestation had no significant association with the lower (6-38 g) or higher (39-140 g) body weight of host species (arbitrary threshold: 39 g), or with long vs. short distance (or no) migration route of hosts (Mann-Whitney *U*-Test: $P > 0.05$).

Food-borne hormones in bird blood influencing their tick infestation

During the three year period, 3330 early developmental stages of ixodid ticks (predominantly *Ixodes* spp. and *Ha. concinna*) were collected from 1164 passerine birds that belong to mainly or partly insectivorous species. A noteworthy proportion, 20.5% (683 out of 3330, CI: 19.2-21.9%) of immature ticks collected from birds showed apolysis. The signs of moulting were also observed in the case of unengorged ticks, i.e. at the beginning of their blood meal. Investigation of engorged apolytic nymphs revealed that the place of the genital pore was darker and more visible. The greatest proportion of apolytic ticks collected from birds in July (35.5%, CI: 31.9-39.1%). There was a significant association between the monthly proportion of apolytic larvae and nymphs and the reported monthly regional population density of lepidopterans (Spearman's rank correlation: r=0.93, P=0.00001).

Seven ecdysteroids or their derivates were found (in detectable quantities) in eight out of 18 blood samples of tick-infested birds. There were significantly more positive samples in the summer (61.5%=8/13) than in spring (0.0%=0/5) (Fisher's exact test: P=0.036). Among the birds that carried apolytic ticks, the proportion of ecdysteroid-positive samples (87.5%=7/8) was significantly higher than in birds with no apolytic ticks (10.0%=1/10) (Fisher's exact test: P=0.003).

Piroplasm detection in *Ha. concinna* ticks carried by birds and molecular investigation of piroplasms

Among *321 Ha. concinna* ticks 51 specimens were positive for piroplasms (15.9%, CI: 12.1-20.4%). These parasites were molecularly identified and proven to be 100% identical to three *Babesia* genotypes that have been reported previously from southern Siberia and Far East of Russia (Rar et al. 2014). In the majority of ticks two genotypes were present: "Irk-Hc133" (previously found in Irkutsk, Siberia) and "Kh-Hc222" (reported from Khabarovsk, Far East). An additional genotype: "Irk-Hc130" (Rar et al., 2014) was isolated from three *Ha. concinna* specimens. Phylogenetic analysis showed that all three genotypes belonged to the phylogenetic group formed by *Babesia* spp. of ruminants. The proportions of piroplasm PCR-positive *Ha. concinna* larvae and nymphs were similar, but during summer and autumn significantly more PCR-positive ticks were collected than in the spring. *Haemaphysalis concinna* immature ticks PCR positive for piroplasms were collected significantly more frequently from five bird species. These five bird species with known eastern migratory

connections were Yellowhammer (*Emberiza citrinella*), River Warbler (*Locustella fluviatilis*), Song Thrush (*Turdus philomelos*), Savi's Warbler (*Locustella luscinioides*) and Nightingale (*Luscinia megarhynchos*).

Discussion

Species diversity of ixodid ticks collected from migratory and non-migratory birds

Ixodes ricinus and *Ha. concinna* occur more often on birds in Central Europe, than ornithophilic ticks (Dubska et al. 2009; Špitalská et al. 2011). Based on our data bird ticks revealed the same pattern, as the most abundant tick species found were *I. ricinus* and *Ha. concinna* immature stages. Bird specific tick species occurred less frequently on the birds studied by us, with adult forms also collected besides larvae and nymphs. *Ixodes frontalis* has been previously reported from Hungary more than half a century ago (Janisch 1959). Our present results attest that this may be a quite common tick species in our country. In the present study the great majority were collected from European Robins (*E. rubecula*), a bird species that known to have predominantly south-west to north-east spring migration from the Mediterranean region to Hungary (Hornok et al. 2012b). *Ixodes arboricola*, which is another common ornithophilic tick species in Central Europe (Mihalca et al. 2012; Novakova et al. 2015), was not found in this survey. Concerning exotic ticks, in the present work the transportation of *Hy. rufipes* immature stages by birds in Central Europe has been proved for the first time with molecular methods. Moreover, the presence of *I. festai* in Hungary is reported as a new observation.

Genetic diversity of less common ixodid ticks in a geographical context

From a molecular taxonomic point of view it may be important to investigate bird ticks, to find out the connections within the species between separated geographical regions. In this work the majority of *I. frontalis* ticks were derived from European Robins (*E. rubecula)*. Molecular studies supported migratory connection of this bird species between South-Western Europe and Central European fields, because the two 16S rDNA genotypes (KU170518: genotype A-Hu16S, KU170519: genotype B-Hu16S) had 100% sequence identity to the corresponding isolates from the Azores. The two 16S rDNA genotypes represented two distinct genetic lineages of *I. frontalis*, similarly to the results of COI gene sequencing, where the two genetic lineages were recognizable. Additionally, the degree of COI sequence divergence between the two lineages (9%) exceeds the proposed approximated sequence difference as species boundary for ticks (6.1 % of COI gene) (Lv et al., 2014). However, morphological differences between the two lineages have not been revealed.

It is remarkable that *I. festai* was reported from Hungary for the first time. It was found on bird species which migrate to the Mediterranean Basin in autumn. In the present study all three *Hyalomma* nymphs morphologically resembled *Hy. rufipes*. One specimen, that was investigated by PCR method showed close identity in its partial COI gene to an Ethiopian *Hy. rufipes* hybrid (Rees et al. 2003). The Common Whitethroat (*S. communis*) that hosted the

Hyalomma ticks is known to overwinter in sub-Saharan Africa (Csörgő et al. 2009) and breeds in Central Europe. Migratory birds are taking part in the transportation of immature stages of exotic ticks. *Haemaphysalis concinna* ticks are broadly distributed in Eurasia, and typically attaching to birds. The COI genotypes of *Ha. concinna* represented three 16S rDNA genotypes. Two of them have a high degree of 16S rDNA gene identity with conspecific ticks from East Siberia (Khasnatinov et al. 2016) and Japan (Takano et al. 2014). The reason for this close identity for some specimens can be the ectoparasite exchange via migratory birds between Europe and East Asia.

Investigation of the factors that influence tick-infestation of birds

Several factors affect the intensity of tick infestation among birds. Similarly to previous observations (Dubska et al. 2009; Hornok et al. 2014b), there were significantly more tick-infested birds among the ground-feeding bird species. *Ixodes ricinus* and *I. frontalis* immature stages were found mostly on these birds. In case of *I. frontalis* association with ground-feeding bird species was demonstrated here for the first time. In a contrast to this, *Ha. concinna* larvae and nymphs were found significantly more frequently on birds that feed higher above the ground level. The difference may be related to the different questing height of the two groups of ticks. In the present study the body weight and length of migration route of birds had no significant association with intensity of tick infestation.

Food-borne hormone in host blood influencing tick infestations

In the present study another interesting factor has been revealed that influences host-parasite relationship. Former laboratory studies reviewed by Rees (2004) proved that exogenous source of moulting hormones accelerate the moulting of ticks, by inducing apolysis. In the current study summer peak of apolytic ticks followed the regional top activity of caterpillars, furthermore significant seasonal correlation was demonstrated between the population density of lepidopterans by month and the ratio of apolytic ticks. Caterpillars may contain high titres of ecdysteroids (Sehnal et al. 1981), and may even appear in the diet, if some passerine species mostly feed on other classes of arthropods (Haraszthy, L. 1998). The high ratio of apolytic ticks feeding on insectivorous birds and the seasonal peak activity of lepidopterans at the same time can be regarded as a consequence of ecdysteroids in caterpillars (and probably other arthropods).

As the duration of feeding gets shorter, the risk of pathogen transmission is decreasing. (Wilhelmsson et al 2013). Our results showed high levels of 20-hydroxyecdysone and ecdysone in the blood samples of insectivorous passerine birds. The ecdysteroid values in our work showed greater accumulation in several individuals in contrast to a previous Czech study (Koudela et al. 1995.), confirming that accumulation of ecdysteroids in birds is possible.

Piroplasm detection in H. concinna ticks carried by birds and molecular investigation of piroplasms

Haemaphysalis concinna is present throughout Europe and Asia (Lebedeva and Korenberg 1981). Considering that birds are preferred hosts of *Ha. concinna*, transportation of this parasite by migratory birds is also likely to occur frequently. *Haemaphysalis* spp. are important in the transmission of piroplasms in the epidemiology of ruminant babesioses (Alani and Herbert 1988; Yin et al. 1996), and birds are the suspected disseminators of *Babesia*-carrier ticks. In the current study two of the detected *Babesia* genotypes ("Irk-Hc133" and "Kh-Hc222") have been detected before in questing *Ha. concinna* ticks (Hornok et al. 2015b, Hamšíková et al. 2016). The third *Babesia* genotype ("Irk-Hc130"), has not been found in Europe until now. None of these three *Babesia* genotypes have been detected in ticks of birds previously. PCR positivity of larvae suggests that the Siberian, Far Eastern *Babesia* genotypes are transovarially transmitted and thus maintained and dispersed over large geographical distances by *Ha. concinna*.

Based on our data, we presume that autumn migration (from the north or northeast to Central Europe) is more important in the dispersal of *Ha. concinna*-associated piroplasms. *Haemaphysalis concinna* larvae and nymphs PCR positive for piroplasms were collected significantly more frequently from five bird species with known eastern migratory connections (current, or phylogenetic connections), supporting their eco-epidemiological role in the above context. Phylogenetic comparison of current populations of long distance migratory bird species reflected that the direction during post-glacial recolonisation followed eastward or westward directions (Irwin and Irwin 2005).

Overview of the new scientific results

1. In the present work the transportation of *Hy. rufipes* immature stages by birds in Central Europe has been proved by molecular methods, and *I. festai* was collected for the first time in Hungary. Our present results attest that *I. frontalis* ticks are transported by avian hosts frequently in Hungary. Two genetic lineages of *I. frontalis* and *Ha. concinna* are transported by birds in Central Europe, which reflect a high degree of sequence identity to South-Western European and East Asian isolates of the same tick species, respectively. These findings highlight the importance of western and eastern migratory connections by birds, which are also relevant to the epidemiology of tick-borne diseases.

2. In case of *I. frontalis*, association of immature stages with ground-feeding bird species was demonstrated in Hungary for the first time. *Haemaphysalis concinna* larvae and nymphs occured significantly more frequently on vegetation-foraging birds of higher altitude.

3. The presence of naturally acquired ecdysteroids in the blood of passerine birds, which induce on-host apolysis in ticks (not normal to three-host ticks) was reported here for the first time. Investigation of the natural occurrence of this phenomenon, to the best of our knowledge, has never been reported. Exogenous ecdysteroids may reach high levels in the blood of insectivorous passerine birds, and might affect ticks by shortening their parasitism.

4. *Babesia* genotype “Irk-Hc130” has been found in Europe for the first time. This is the first report of “Irk-Hc130”, “Irk-Hc133” and “Kh-Hc222” genotypes in *Ha. concinna* ticks of birds. Findings of the present study indicate that birds may play a significant role in the long distance geographical dispersal of *Babesia* genotypes within *Ha. concinna* ticks. *Babesia*-carrier ticks collected from resident bird species might reflect Central European establishment of Siberian or Far Eastern *Babesia* genotypes.

Scientific publications

In peer-reviewed journals

Flaisz, B., Sulyok, K.M., Kováts, D., Kontschán, J., Csörgő, T., Csipak, Á., Gyuranecz, M., Hornok, S.: ***Babesia* genotypes in *Haemaphysalis concinna* collected from birds in Hungary reflect phylogeographic connections with Siberia and the Far East**, Ticks Tick Borne Dis., 8. 666-670, 2017.

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Hornok, S., Takács, N., Kontschán, J., György, Z., Micsutka, A., Iceton, S., Flaisz, B., Farkas, R., Hofmann-Lehmann, R.: **Diversity of *Haemaphysalis*-associated piroplasms of ruminants in Central-Eastern Europe, Hungary**, Parasit. Vectors., 8. 627., 2015.

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