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The role of urban and wild-living small mammals in the epidemiology of ticks and tick-borne pathogens

Thesis of the dissertation

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2018

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Introduction and aims

Ticks are ectoparasitic mites feeding on blood of several vertebrate hosts. These arthropods have important vector role in the epidemiology of several causative agents with major economic losses (in case of livestock) and causing severe symptoms, even death of the host (including humans and pets). The epidemiology of tick-borne diseases is more complex and divergent than the direct dispersal of some other pathogens. The different stages and species of tick vectors and also the host species have marked effect on this process.

In the natural habitats, the pathogens have a so called “sylvatic cycle” involving many different host species. Additionally, some of these vertebrates have reservoir potential which means they do not just spread the agents, but can also maintain pathogens (which means the pathogens can multiply within the host) (Földvári, 2016; Szekeres et al., 2016). In rural habitats, the several tick and host species could indicate higher diversity in tick borne pathogens. In urban habitats ticks and also vertebrate hosts occur, but with only few dominant species. For example, in Budapest, the capital and the biggest city of Hungary, forty-eight different mammal species from bats to wild boars have been recorded, since 1990 (Tóth-Ronkay et al., 2015). Hedgehogs and squirrels found a niche with many resources, thus they can live in higher densities in cities compared to the forests. This multi levelled host-vector-pathogen-environment system is the most fascinating part to investigate and also gave several paths in this complex labyrinth.

Ticks have altogether four developmental stages. The first egg stage and further three parasitic stages such as the larva, nymph and sexual dimorph adult stage. The life cycle of hard ticks is similar in the whole family. Larvae emerging from eggs have only three pairs of legs, while the further stages have four pairs. After the first blood meal, these larvae search for a shelter. Unlike other mites Ixodid ticks have only a single nymphal stage. Nymphs and adults pose the highest risk for humans to become infected, but it is known that also the larvae have epidemiological role via transovarial transmission of various pathogens (Földvári et al., 2016)

The emergence of Lyme-borreliosis (LB) and other tick-borne diseases with veterinary and medical importance and their association with leisure activities has brought the subject of ticks as vectors of pathogens and methods how to avoid tick bite, to general attention. The number of tick-borne pathogens are the greatest among any other arthropods. Several viruses, bacteria, fungi and protozoa are transmitted via tick bite or contamination with secretion, faeces or crushed bodies of ticks. Ticks can acquire pathogens directly from the host (during the blood

meal) or vertically from the female tick (from the ovary to the eggs) and also pathogen transmission between feeding individuals via feeding pool without infesting the host (called co-feeding).

In the wild ticks, tick-transmitted organisms and their host live in natural balance called enzootic/synanthropic cycle. These hosts usually do not show any sign of infection unless they are in stressed conditions or with low immunity.

People living in urban areas love to be in “green” for leisure activities or just to enjoy the calmness of nature, therefore, cities and houses are designed with some kind of green areas; like alleys, smaller or bigger city parks and nicely cared front or back gardens. These green areas could serve as suitable habitat for some urban animal species. Some of these urbanised mammal species, such as hedgehogs (*Erinaceus* spp.) and squirrels (*Sciurus* spp.), can even reach higher densities in urban/suburban habitats than usually in rural environments (Reeve, 1994; Tóth-Ronkay et al., 2015)

There are several pathogens transmitted by tick bite e.g. tick-borne encephalitis virus, *Coxiella burnetti, Francisella tularensis*, *Borrelia burgdorferi* s.l., *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, *Can*. Neoehrlichia mikurensis, *Hepatozoon* spp. and several *Rickettsia* species. These pathogens can affect humans and our companion animals as well.

The aim of this study was to investigate the occurrence of tick-borne human pathogens in small mammals and ticks from a natural habitat in Southern Hungary, where forestry works, hunting and recreational activities are intensive; and from accidentally killed urbanised, city dwelling mammals and ticks removed from them. With the gained data we wanted to shed light on some interesting parts of some well-known and some new pathogens in our natural study site and also the less investigated researched side of the tick-borne pathogens within cities.

I had the following aims:

- assess the tick fauna parasitizing rodents in a natural floodplain forest and hedgehogs in an urban habitat.
- find rodent and ectoparasite species that carry *B. burgdorferi* s.l., *B. miyamotoi*, *A. phagocytophilum*, *Can*. N. mikurenis, *Rickettsia* spp., *Hepatozoon* spp. and *Bartonella* spp. and might be involved in the epidemiology of these pathogens
- find *B. burgdorferi* s.l., *B. miyamotoi*, *A. phagocytophilum*, *Can*. N. mikurenis, *Rickettsia* spp. and *Bartonella* spp. in road hit or accidentally died small and medium sized mammals and their ectoparasites in urban areas
- asses the contribution of Northern white-breasted hedgehogs in the cycle of tick-borne pathogens on Margaret Island.

Materials and methods

Between July 2010 and May 2013, small mammals were live-trapped within the Gemenc area which is a forest covered floodplain near the Danube River, in Southern Hungary. On this study site the sample was started by my colleges from the Department of Parasitology and Zoology, UVM, Budapest; I joined to this process in 2012. The species and sex of trapped rodents was identified and animals belonging to protected species were then released. All the other rodents were euthanized. The carcasses were checked for ticks and other ectoparasites and samples from spleen and skin were collected. The spleen and skin samples in this study did not originate from the same individuals. During the trapping in May 2012, ticks were collected with flagging from the vegetation in several different locations within the Gemenc area. Ectoparasites were stored in 70% ethanol, and were later identified using standard identification keys.

Questing ticks were collected with flagging in 2011–2012 on Margaret-Island (Budapest). The collection was done by my supervisor and his former PhD student and I joined the systematic flagging in 2012. Ear tissue samples were obtained from hedgehogs anesthetized with intramuscular ketamine (5 mg/kg) and dexmedetomidine (50 µg/kg) in 2011.

Between April and August of 2015 we collected road-hit hedgehogs with the help of volunteers mainly from Budapest and some other locations around Hungary. In addition, we also collected some animals died for other reasons (e.g. caught by cats). We collected samples from all the possible identifiable tissues (minimum: skin, maximum: five different tissues). The species, date of collection, location and the degree of degradation were recorded. Before dissection, we collected all the ectoparasites and stored in 70% ethanol at 4°C until the molecular analysis. The ticks were identified using standard identification keys. The carcasses and the collected tissue samples were stored at -20°C.

DNA was extracted from ticks by alkaline hydrolysis from both habitats. Pool samples were prepared from each 10 larvae removed from the same host. Adult ticks were processed individually from both habitats. All nymphs collected from the natural habitat were examined individually, but the nymphs removed from urban road-hit or accidentally died animals were pooled by 5 and nymphs from the same host in this study.

DNA was isolated from tissue samples with commercial kits. We stored extracted DNA in 1.5 ml, 2 ml microcentrifuge tube or 2ml screw capped and rubber band sealed microtube at −20°C in the freezer for further analyses.

The presence of the different pathogens was examined with real-time and conventional PCR and sequencing as well. Statistical and phylogenetic analysis were also performed on the obtained data.

Results

Natural habitat

We trapped altogether 525 rodents in the study sites. Tissue samples of six rodent species (*Apodemus flavicollis*, *A. agrarius*, *Myodes glareolus*, *Microtus arvalis*, *Micromys minutus*, *Mus musculus)* were analysed. Altogether 343 ticks belonging to five species (*Haemaphysalis concinna*, *Ixodes ricinus, I. acuminatus, Dermacentor reticulatus*, *D. marginatus*) were found with flagging (n=162) and on rodents (n = 181). One hundred and thirty-one fleas belonging to three different species (*Ctenophthalmus agyrtes, C. assimilis* and *Megabothris turbidus*) were collected from 81 small mammals.

From the Margaret Island 88 Northern white-breasted hedgehogs were caught and ear biopsy was taken under veterinary supervision and anaesthesia. Twenty-three road-killed hedgehogs and twelve other collected mammals from seven different species (e.g. European red squirrel and European mole) were included into the study. We collected carcasses of accidentally killed animals (struck and killed by motor vehicles on highways or e.g. killed by cat) from urbanised habitats, mainly from Budapest, Hungary. From the carcasses, we collected 90 tissue samples for molecular analysis (52 from hedgehogs and 38 from the other species). The degree of degradation of the carcasses was different; some specimens were in “perfect” condition with no sign of degradation (degree of degradation 1) and some were dry and heavily damaged by vehicles (degree of degradation 5).

From nine hedgehogs we removed 417 ticks, 124 samples were created (111 *I. ricinus* and 13 *I. hexagonus*) using adults individually, nymphs pooled by five and larvae pooled by 10 per host.

The prevalence of *B. burdorferi* s.l. in rodent tissue samples was 6.6% in skins and 2.3% in spleens. *Borrelia miyamotoi* was found in 0.3% of skin and 0.5% of spleen samples removed from the captured small mammals. *Borrelia burgdorferi* s.l. was found in *A. flavicollis*, *A. agrarius* and *My. glareolus* samples. *Borrelia miyamotoi* was detected in two *A. flavicollis* males. *Borrelia burgdorferi* s.l. and *B. miyamotoi* was detected in ticks. In the ticks removed from rodents, DNA amplification of both pathogens was successful from *I. ricinus* larvae while from 2 *Ixodes acuminatus* larvae, and the single tested nymph only *B. burgdorferi* s.l. DNA was amplified. There was no significant difference in *B. burgdorferi* s.l. minimum infection prevalence between *I. ricinus* and *I. acuminatus* larvae ($p>0.05$). Three *D. marginatus* larva samples removed from two uninfected *A. flavicollis* and an uninfected *A. agrarius* were also *B. burgdorferi* s.l. positive.

We found 23 and 9 *A. phagocytophilum* PCR positives in the skin and spleen samples of rodents. The prevalence of *A. phagocytophilum* in skin samples of *A. flavicollis* was significantly higher compared to the *Candidatus* N. mikurensis (Fisher test, $p=0.0036$). Five questing ticks were PCR-positive. One *I. ricinus* nymph removed from a PCR-positive male *A. flavicollis* was infected with *A. phagocytophilum*. Six out of 348 rodent skin samples and six out of 176 spleen samples were positive for *Candidatus* N. mikurensis. Only two (*A. flavicollis* and *A. agrarius*) out examined rodent species were infected with *Candidatus* N. mikurensis. Three out of 34 questing *I. ricinus* ticks were infected. The other tick species and the engorged ticks were negative for this pathogen.

Rickettsiae were detected in 57.8 %of *D. reticulatus.* We identified *R. raoultii* infection with sequencing in 31 qPCR-positive *D. reticulatus* samples from the rural habitat.

From 528 trapped small mammals in the early stage of the smear samples were made. During the examination eight of the 36 trapped bank voles (*M. glareolus*) were *Hepatozoon* positive. These were also found positive with apicomplexan-specific primers all tissues from other species were negative.

Thirteen fleas (from three species: *M. turbidus, C, agyrtes, C. assimilis*) were found to be infected with *Hepatozoon* spp. but none of the tick samples. Unfortunately, 18S rDNA sequencing was not successful for any of the PCR-positive flea samples. Therefore partial 18S sequences sequenced. These partial sequences were almost identical with the corresponding regions of the whole 18S sequences from tissue samples. Based on gamont morphology and 18S rDNA sequences, the bank vole as the exclusive host and fleas (and not ticks) as probable vectors, we identified the parasite as *Hepatozoon erhardovae*.

Urban habitat

We detected *A. phagocytophilum* in 67 (76.1%) and *Candidatus* N. mikurensis in 2 (2.3%) of 88 ear tissue samples from urban hedgehogs collected on the Margaret Island.

Ticks were only found on hedgehog carcasses. *Borrelia miyamotoi* and *Can*. N. mikurensis DNA were not detected in tick samples. *Borrelia burgdorferi* s.l. was detected in 16% of ticks; all stages of *I. ricinus* and *I. hexagonus* nymphs. *Anaplasma phagocytophilum* was presented in all stages of both tick species. *Rickettsia helvetica* was found in *I. ricinus* females, males, nymphs and larvae and in *I. hexagonus* nymphs with an overall *R. helvetica* prevalence of 20.5% in ticks. *Rickettsia* sp. was found in all stages of *I. ricinus* and *I. hexagonus* nymphs.

Anaplasma phagocytophilum prevalence was significantly lower (Fisher-test, $p<0.00001$) in natural rodents of the present study compared to urban hedgehogs.

From the urban habitat 22 *R. monacensis* and 9 *R. helvetica* out of 534 questing *I. ricinus* were identified with the less sensitive conventional PCR and sequencing.

All examined pathogens occurred in the collected road-killed mammal tissue samples except *Can*. N. mikurensis. *Borrelia burgdorferi* s.l. was detected in hedgehogs and a squirrel. *Borrelia miyamotoi* was only detected in a squirrel spleen sample. *Anaplasma phagocytophilum* was found in several tissue samples from hedgehogs; in a roe deer and a lesser shrew. *Rickettsia helvetica* DNA was amplified in hedgehogs; mouse and stone marten. We found *Rickettsia* sp. positive hedgehogs; house mouse, mole and a lesser weasel. *Bartonella* species were detected in moles, hedgehogs, a house mouse and lesser weasel.

The qPCR-positive samples were also amplified with specific conventional PCR assays and were sequenced if the qPCR was not species specific or it is needed to separate different subgroups within one species (ecotypes in case of *A. phagocytophilum*). *Borrelia afzelii* was found in one *I. ricinus* female, one male and seven nymph pools. *Anaplasma phagocytophilum* ecotype I was found in two females and five *I. ricinus* nymph pools and one *I. hexagonus* female. *Rickettsia monacensis* occurred in four *I. ricinus* nymph and a larva pool. *Borrelia afzelii* and *B. spielmanii* was found only in hedgehog skin and muscle. *Anaplasma phagocytophilum* was detected in liver, skin and spleen samples from hedgehogs. *Bartonella* species DNA was amplified in *E. roumanicus*, house mouse, mole and lesser weasel tissues.

These samples were sequenced; and aligned using GenBank BLAST.

Discussion and conclusion

Rodents have high metabolic and reproduction rate with relatively large body surface compared to their body weight and these small mammals are in high densities in the natural habitats. All these features make rodents suitable hosts for ticks and also suitable reservoirs for many pathogens (Ostfeld et al., 2014). Pathogen cycles differ in urban and natural habitats. If in turn the number of hosts is limited the pathogen has to specialise for these which decreases its genetic diversity. These local limitations in available hosts might have also led to the evolution of different *A. phagocytophilum* ecotypes (Jahfari et al., 2014). The presence of similar processes should also be investigated in *Can*. N. mikurensis, *B. miyamotoi* and other tick-borne pathogen populations.

Borrelia afzelii was the most prevalent among the sequenced LB spirochetes (17/18) in the rural study site, but also *B. lusitaniae* was also presented. *Borrelia afzelii* is the most widespread *Borrelia* species in Europe (Stanek et al., 2012), usually maintained by rodents (Burri et al., 2014; Rigó et al., 2011). Also, this spirochete is probably the most important LB causative agent in Hungary (Földvári et al., 2005). Presence of at least two pathogenic LB spirochetes in the Gemenc area can pose a risk of LB infection to the occupationally exposed persons. *Ixodes acuminatus* ticks have endophilic (or nidicolous) lifecycle. All stages live in rodents' nests, thus, being capable of maintaining a local cycle of pathogens similar to the natural cycle of *A. phagocytophilum* and *Babesia microti* with the endophilic *I. trianguliceps* (Bown et al., 2008, 2006). We found *B. afzelii* in one nymphal and four larval pools of *I. acuminatus*.and Rigó et al. (2011) detected *B. afzelii* in an adult *I. acuminatus* female, also support this hypothesis. This double natural cycle has also been observed in the case of *B. burgdorferi* s.l. and *I. ricinus* vs. *I. hexagonus* (Gern et al., 1997). Both of these cycles have to be considered and monitored in order to predict and prevent human infection risk, because *I. acuminatus* can occasionally bit humans.

Borrelia miyamotoi spirochetes have been previously detected only in wild *A. argenteus* in Asia*, P. leucopus* in North-America and *My. glareolus* in Europe (Cosson et al., 2014; Fukunaga et al., 1995; Scoles et al., 2001). The reservoir role of *A. flavicollis* and *My. glareolus* was proven under xenodiagnostic laboratory conditions. Our study provides the first evidence for the presence of *B. miyamotoi* infection in a wild *A. flavicollis* population.

The present study identified *B. miyamotoi* and *B. burgdorferi* s.l. from samples of different years (2011–2012) indicating that these pathogens seem to have a stable cycle in this area even surviving rodents that usually live for less than a year.

Parks can be considered points of contact for reservoir animals, pathogens, ticks, and humans. Our results indicate that *E. roumanicus* hedgehogs play a role in urban eco-epidemiology of at least two emerging human pathogens. To better understand the urban cycle

of these pathogens, potential reservoir hosts, ticks collected from these hosts, and vegetation in parks should be further investigated.

Our results showed considerable difference between the dominant rickettsial agents in the city park (*R. helvetica* and *R. monacensis*) and natural forest habitat (*R. raoultii*). This is due to the differences of these habitats in their vector and host diversity. Both our study sites have frequent human visitors: Margaret Island is a popular recreational and jogging park in the centre of Budapest and Gemenc is a popular hunting and hiking area with over 50,000 tourists per year.

Our survey showed that accidentally killed animals are valuable resources in the investigation of the eco-epidemiology of tick-borne pathogens. Based on our molecular analyses urbanised mammals are involved in the maintenance of one or several tick-borne pathogens in urban habitats. Using road-killed animals as source of tissue samples and ectoparasites provides both advantages and disadvantages. It is possible to collect samples not only from protected common species but also from inner organs of animals under protection and thus neglected from eco-epidemiological studies. In contrast, the main disadvantage is the varying condition of the carcasses. However, despite the highest degree of degradation it was possible to amplify DNA of *A. phagocytophilum*, *R. helvetica* and *Rickettsia* sp. in dried skin and muscle samples from road- and accidentally killed urban animals with real-time PCR. Therefore, even badly damaged carcasses might provide important eco-epidemiological information.

Some wild animal species (e.g. red foxes, martens) during the recent centuries have become not only urbanised but also became synanthropic species, and the number of these species might grow in the future. The cities take away bigger and bigger areas from the natural habitats while cities with growing food waste also serve as an inexhaustible and easily obtainable food source. With proper management of the urban green areas, the suitable questing substrate and habitats for exophilic ticks can be minimised without harming other species or reducing minerals and organic material of the habitats.

These animals in urban and suburban areas are possible risk factors for humans to get infected from tick-borne pathogens. Nonetheless, with proper usage of repellents and a thorough self inspection after a walk in risky areas the hazard of infection could be minimised.

I hope, I could guide the curious reader out from the complex multi-level maze of the tick-borne pathogens and their relationship with hosts and vectors found in two different habitats. In addition, my dissertation may shed a little bit more light on this interesting topic and will hopefully also generate many good research questions for the future.

Overview of the new scientific results

The following scientific results of the presented dissertation are new to science

1. *Borrelia miyamotoi* can infect *Apodemus flavicollis*, the yellow necked field mouse, thus this species is a candidate reservoir.
2. *Ixodes acuminatus* ticks can establish a so called “endofilic pathogen cycle” without the contribution of *Ixodes ricinus* in host nest/borrows within the epidemiology of *Borrelia burgdorferi* s.l.
3. The relatively common but neglected protozoan haemoparasite *Hepatozoon erhardovae* was rediscovered in Gemenc and partial genetic data were obtained.
4. Northern white-breasted hedgehogs (*Erinaceus roumanicus*) are infected with *Anaplasma phagocytophilum* and Can. N. mikurensis, thus this insectivore species is a candidate reservoir for these pathogens, especially in urban habitats where they live in higher density.
5. In urban and natural habitats ticks harbour different *Rickettsia* species composition according to the collection site.
6. Road-killed carcasses are useful source to examine samples from protected species for the presence of tick-borne pathogens for further epidemiological studies.
7. Northern white-breasted hedgehogs (*Erinaceus roumanicus*) are infected with *Borrelia spielmanii* and *Bartonella* spp. which extends its known zoonotic importance.
8. Lesser weasel (*Mustela nivalis*) is infected with *Rickettsia* sp., and stone marten (*Martes foina*) is infected with *Rickettsia helvetica* pathogens. Both pathogens are potential human threat and these species can have role in the cycle of these causative agents.

Scientific publications

Own full text publications in peer-reviewed with impact factor assigned

Papers in the topic of the dissertation:

Szekeres S., Lakos A., Földvári G.: *Borrelia miyamotoi*: egy újabb, humán patogén, kullancs által terjesztett, visszatérő lázat okozó baktérium, (*Borrelia miyamotoi*: a recently identified human pathogenic tick-borne relapsing fever spirochete) ORVOSI HETILAP 158: pp. 1124-1130. (2017) IF (2016): 0,349

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Acknowledgements

Firstly, I would like to thank **Gábor Földvári**, who is my mentor from the beginning of my MSc thesis work. He gave me many opportunities to practice the substantial aspect of the researcher's life. I would also like to thank **Krisztina Rigó**, who was the former PhD student of Gábor, also my supervisor during my MSc work. **Mónika Jablonszky** who showed me some of the DNA extraction methods and also helped us in the sample collection.My former parasitology teacher **Gábor Majoros** was always there when I had some questions about some interesting facts in the life of parasites, animals, geology etc. He and **Alexandra Juhász** helped us in sample collection from the natural and urban habitats.

To my Dutch friends, **Hein Sprong** and his team, **Elena Claudia Coipan, Manoj Fonville, Arieke Docters van Leuween** and **Setareh Jahfari** in the Netherlands was crucial pillar of my work. There I learned how to perform real-time PCR and sequence analysis on my samples. Dank je well!

I would like to thank also for the **Hungarian-Slovakian (HuSk) conference** team in Cape Town, Republic of South Africa and in Cairns, Australia for the best conferences of my life. Hi Bruce! (**Shermanka, Irwinka, Doris, RalPhD** and **Bruce** this greeting is just for You. ☺) **Zsolt Lang**, my biomathematics and biostatistics teacher who was always helpful and supportive with the statistical analysis of the data. **Mária Ronkayné-Tóth** and her many students and volunteers who helped us capturing hedgehogs on Margaret Island. The **many volunteers** who helped the road-killed sample collections all around Hungary from urban habitats. **Boglárka Krizsán, Mónika Gajdos, Evelin Tóth, Jen Lügner** and **Tamás Gabelics** veterinary- and biology MSc students helped me in the procession of the samples (DNA extraction, PCR). Thanks a lot for the nice atmosphere during the laboratory works.

Professor **Róbert Farkas** the former head, nowadays the curator of the Department of Parasitology and Zoology, UVM who provided laboratory to perform the present research and a desk to work in the Department.

Sample collection was approved from both habitats by the Middle-Danube-Valley Inspectorate for Environmental Protection, Nature Conservation and Water Management, Hungary.

I thank **Nóra Takács, Barbara Flaisz, Krisztina Szőke, Anikó Czene, Zsusza Bartos, Veronika Tóth** and **Balázs Tánczos** for the nice working environment in the past and nowadays.

All my BSc, MSc classmates and many friends who were there when we struggled together and we needed each other the most.

Last but not least, I am grateful to **my family** who always supported and encouraged me to achieve my goals.

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