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The Role of Peridomestic Animals in the Eco-Epidemiology of *Anaplasma phagocytophilum*

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Abstract

Anaplasma phagocytophilum is an important tick-borne zoonotic agent of human granulocytic anaplasmosis (HGA). In Europe, the *Ixodes* ticks are the main vector responsible for *A. phagocytophilum* transmission. A wide range of wild animals is involved in the circulation of this pathogen in the environment. Changes in populations of vertebrates living in different ecosystems impact the ecology of ticks and the epidemiology of tick-borne diseases. In this study, we investigated four species, Western European hedgehog (*Erinaceus europaeus*), northern white-breasted hedgehog (*Erinaceus roumanicus*), Eurasian red squirrel (*Sciurus vulgaris*), and the common blackbird (*Turdus merula*), to describe their role in the circulation of *A. phagocytophilum* in urban and periurban ecosystems. Ten different tissues were collected from cadavers of the four species, and blood and ear/skin samples from live blackbirds and hedgehogs. Using qPCR, we detected a high rate of *A. phagocytophilum*: Western European hedgehogs (96.4%), northern white-breasted hedgehogs (92.9%), Eurasian red squirrels (60%), and common blackbirds (33.8%). In the *groEL* gene, we found nine genotypes belonging to three ecotypes; seven of the genotypes are associated with HGA symptoms. Our findings underline the role of peridomestic animals in the ecology of *A. phagocytophilum* and indicate that cadavers are an important source of material for monitoring zoonotic pathogens. Concerning the high prevalence rate, all investigated species play an important role in the circulation of *A. phagocytophilum* in municipal areas; however, hedgehogs present the greatest anaplasmosis risk for humans. Common blackbirds and squirrels carry different *A. phagocytophilum* variants some of which are responsible for HGA.

Keywords Tick-borne diseases · HGA · Urban wildlife · Hedgehog · Squirrel · Blackbird

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Introduction

Anaplasma phagocytophilum is a tick-borne pathogen (TBP) that causes febrile disease in humans as human granulocytic anaplasmosis (HGA) and animals (e.g. pasture fever, equine and canine granulocytic anaplasmosis) [1, 2]. Several *A. phagocytophilum* genetic variants have been reported in Europe in a broad range of free-living and domestic animals [3–5]. Several *Ixodes* spp. are involved in the circulation of this pathogen, but dominant genotypes in Europe are transmitted mainly by *Ixodes ricinus*. The pathogen is transmitted by *Ixodes persulcatus* in eastern Europe and temperate Asia, and by *Ixodes scapularis* and *Ixodes pacificus* in North America [6, 7].

Similar to other Gram-negative TBPs, *A. phagocytophilum* is not transmitted vertically in ticks, and its ecological niche and epidemiology largely result from a multidimensional overlap of the ecology of *Ixodes* spp. vectors and reservoir vertebrate hosts in a given ecosystem [7, 8]. Analysis of several molecular markers has revealed the existence of diverse genetic variants in *A. phagocytophilum*. Moreover, some extent of host specificity at both the tick and vertebrate host levels has been reported for the different genetic variants [9–13]. The genetic structure of *A. phagocytophilum* and its complex epidemiology resemble the diversity of genospecies of the *Borrelia burgdorferi* sensu lato complex [6, 7, 14, 15]. Classification of *A. phagocytophilum* genetic variants based on *groEL* generally provides more robust and consistent results than those based on 16S rDNA, *ankA*, and *msp4* gene sequences [12, 14]. Recently, Jaarsma et al. [7] elaborated an analysis based on the DNA sequence of a fragment from *groEL* and distinguished eight genetic clusters grouped into four ecotypes that differ in geographic range in combination with vertebrate and/or vector species.

Changes in the human lifestyle and in the abundance and distribution of populations of vertebrates living in urban and periurban ecosystems significantly impact the ecology and epidemiology of TBPs [16]. Squirrels and birds are good feeding hosts and, thus, a source of TBP infection [17, 18]. However, hedgehogs also act as propagation hosts for *I. ricinus* and may be responsible for tick maintenance in urban greeneries [19].

The hedgehog is one of the most successful free-living animals colonizing urban environments, as its populations can reach up to 9 times greater densities in urban than rural areas [18]. Similarly, squirrel abundance is significantly higher in urban and rural settlements than in other natural habitats, such as forests [20]. Both, hedgehogs and squirrels, are involved in feeding of subadult tick developmental stages in the urban environment, where they also play an important role as reservoirs of *Borrelia bavariensis, Borrelia spielmanii, Borrelia afzelii, A. phagocytophilum*, and *Rickettsia helvetica* [3, 18, 21–24]. In addition, hedgehogs have a unique ability for hosting immatures as well as adult ticks. This is the reason why, especially in city parks, these animals are able to maintain a large *I. ricinus* tick population in the absence of larger mammals [25]. Urban birds are also important hosts of different tick species, and their infestation by ticks mainly depends on the degree of their movement on the ground [26]. Due to their feeding habits, thrushes and blackbirds are extensively exposed to intensive tick infestation [27, 28].

Wild animals living in cities often become accidental victims of urban infrastructure, and their carcasses can serve as a valuable source of data for epidemiological studies addressing zoonoses in urban environments [24]. Our goal was to investigate *A. phagocytophilum* in sentinel vertebrates that reach high population densities in the peridomestic environment and are commonly affected by ticks and accessible for screening. Within a larger study with a citizen participatory science component, we collected and examined cadavers of two hedgehog species, *Erinaceus roumanicus* and *Erinaceus europaeus*, Eurasian red squirrels (*Sciurus vulgaris*), and common blackbirds (*Turdus merula*) from numerous localities in the Czech Republic. A smaller set of the same host species was trapped and sampled alive. The goal of our study was to analyse the genetic diversity of *A. phagocytophilum* using the partial *groEL* gene sequence and the multiple loci variable-number tandem repeats analysis (MLVA) method, and to assess the role of the selected peridomestic vertebrates in the circulation of *A. phagocytophilum* ecotypes in Central Europe.

Materials and Methods

Hosts, Dissections, Sampling

Cadavers of the Western European hedgehog (*E. europaeus*), the northern white-breasted hedgehog (*E. roumanicus*), the Eurasian red squirrel (*S. vulgaris*), and the common blackbird (*T. merula*) were collected within a larger project addressing the sentinel potential of these species for TBPs. Employees of research centres where the project was carried out, as well as the public in the cities mentioned further, were asked for collaboration to bring carcasses of targeted urban vertebrate species. The information was spread out by using leaflets and though a website hosted on the server of the Biology Center of Czech Academy of Sciences (https://jez-ko-ve.bc.cas.cz). The studied animals were found dead as roadkill, killed by other animals (dogs and cats), after crashing into glass buildings (birds), or received dead from rescue centres in 2016–2018, mainly from Brno, Prague, České Budějovice, and surroundings. Cadavers were kept at −20°C until processing. Thawing the cadaver before necropsy took 4–18 h depending on the cadaver size. After thawing, the grade of autolysis was determined at stages 1–3 (low degradation of

tissues, intermediate degradation, and progressed disintegrating of tissues, respectively). Only cadavers with grades 1 and 2 autolysis were processed further. The identification of each animal was based on the characteristic morphological features [29–31]. The identification of hedgehogs was confirmed by mitochondrial DNA sequencing [19] using primers ProL-He/DLH-He for amplification of the 5' end of the mitochondrial region (410 bp) including the hypervariable domain. Each cadaver was weighed, the foot length measured, and age categories assigned: juvenile (milk sucking babies resp. birds with juvenile plumage), subadult (sexually immature individuals), or adult. The gender of the animal was determined when external or internal sexual organs were present in the cadaver. Infestation with ectoparasites was recorded as presence/absence.

Tissues were removed using sterile surgical instruments (scissors and forceps) and cross-contamination of DNA or RNA prevented by PCR Clean™ (Minerva Biolabs, Germany). An approximate 5×5-mm piece of each tissue was placed into a 2-ml Eppendorf tube, where it was further cut onto approximately 1-mm pieces. We collected samples of ear tissue or skin (in birds or when the ears were absent), muscle, lungs, liver, spleen, urinary bladder, kidney, and brain. A blood coagulum or liquid blood (50–500 μl) was obtained from the heart or thoracic cavity of the cadaver using a sterile Pasteur pipette and placed in a sterile 2-ml Eppendorf tube. RLT buffer (1 ml; Qiagen) was added to each Eppendorf tube with a blood or tissue sample and stored at −70°C until further analysis.

Sampling of Live Animals

A total of 109 live animals were trapped in Brno, Prague, and České Budějovice between 2017 and 2018 and used for this study. Capture and treatment were performed in agreement with the Czech legislation (Act No. 246/1992 Coll.) and protocols (62-2016 UVPS Brno and BC SOS 1520/2017) approved by the responsible authorities of the Czech Republic. Squirrels were not trapped. Birds were captured by mist nets and sampled immediately using manual restraint. Infestation with ectoparasites was recorded, blood obtained from the *vena basilaris* (0.01–0.3 ml), and skin samples obtained from the calamus of feathers loosened during the manipulation. Each individual was identified (species, sex, age category), marked with a leg ring for identification, and released at the same place where it was captured. Hedgehogs were collected manually at night. Sampling was performed after intramuscular administration of anaesthesia with 0.2 mg/kg medetomidine and 10 mg/kg ketamine, which was reversed by intramuscular administration of 1 mg/kg atipamezole. Each hedgehog was identified (species, sex, age category) and marked with a subcutaneous passive integrated transponder (PIT) tag (ID 111 ISO). A bioptic skin tissue sample was obtained from an auricle and 0.1–0.5 ml of blood obtained from a jugular vein. Animals were released where they were captured. Samples were stored at −70°C until analysis.

DNA Isolation

Tissue samples were homogenized in RLT buffer (Qiagen) containing beta-mercaptoethanol (10 μl of 14.3M beta-mercaptoethanol per 1 ml of RLT buffer) using sterile stainless steel beads (Qiagen) and Tissue Lyzer II. Briefly, samples of ear, skin, muscle, lung, liver, spleen, urinary bladder, kidney, and brain were prepared as 30% (w/v) suspensions. After homogenization, 20.0 μl of proteinase K (Qiagen) was added and the samples incubated 30 min at 57°C. The lysate was cleared by centrifugation and the supernatant collected in a clean, sterile microtube. The blood samples (10–100 μl depending on nucleated/non-nucleated blood and sample volume obtained from the particular animal) were resuspended in 220 μl of sterile PBS and the solution used for DNA isolation using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. The elution volume was 200 μl.

Multiplex Real-Time PCR Screening and Nested PCR

Samples were screened for the presence of *A. phagocytophilum* DNA by real-time polymerase chain reaction (qPCR) targeting a 77-bp portion of *msp2* as described previously (Courtney et al., 2004) and analysed using Fluidigm Real-time PCR Analysis software to obtain crossing point (Cp) values. Three negative controls containing water instead of DNA sample were included for each plate.

To determine the *groEL* ecotype, nested PCR was performed on qPCR-positive samples, targeting a 407-bp portion of the variable fragment of *groEL*. Primers EphplgroEL-A.phago-F and EphgroEL-A.phago-R [32] were used in the first round, and primers Ap_GroEl_nested_For and Ap_GroEl_nested_Rev [7] in the second round of PCR. The cycling conditions were as follows: 95°C for 1 min, 35 cycles of 15 s at 95°C, 15 s at 57°C/55°C and 15 s at 72°C, and a final extension at 72°C for 5 min. Whenever possible, a longer fragment of *groEL* (~1297 bp) was amplified following the published nested PCR protocol [33] using slightly modified primers based on currently available sequences: HS1-modified (5'-TGGGCTGGTARTGAAWT) and HS6a (5'-CCICCIGGIACIAIACCTTC) in the first round and HS43-modified (5'-ATAGCTAAGGAAGCATAGTC) and HSVR (5'-CTCAACAGCAGCTCTAGTAGC) in the second round of amplification. The cycling conditions were 95°C for 1 min, followed by 35 cycles of 15 s at 95°C, 15 s at 48°C/52°C, and 45 s at 72°C, with a final extension at 72°C for 7 min. All PCRs were performed using commercial master mix (2× PCRBIO Taq Mix Red, PCR Biosystems, UK) following

the manufacturer's instructions. A total volume of 25 μl was prepared for each reaction, comprising 12.5 μl of the master mix, 10 pmol of each primer, 2 μl of template DNA or 1 μl of PCR product from the first round in the case of nested PCR, and PCR water for the remaining volume. The PCR products were analysed by gel electrophoreses on a 1.5% agarose gel and visualized under UV by Midori Green Advance (Nippon Genetics Europe, Germany). Products from PCRs were purified using the Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taiwan) and directly sequenced using the amplification primers by Macrogen capillary sequencing services (Macrogen Europe, the Netherlands).

Sequence Analysis

All obtained sequences were edited and analysed using Geneious® 9.1.2 software and compared to those available in the GenBank database by BLASTn analysis. For individual animals, sequences from different organs were compared and only unique sequences analysed further. Unique genotypes were identified in the Alignment Transformation Environment (ALTER) [34]. Phylogenetic analysis was performed on a set of unique genotypes from this study, 130 sequences from GenBank representing all four described ecotypes [6], and three sequences of *Anaplasma platys* (AY077621, KY581623, KY987394) used as an outgroup. Due to uneven sequence lengths, the alignment was calculated in two steps using the MAFFT algorithm "Auto" strategy for sequences >1000nt and function–add for implementing sequences <1000nt to the alignment. The phylogenetic tree was generated by the MAFFT online service [35], based on neighbour-joining method and Jukes-Cantor model; branch supports were assessed by 1000 bootstrap replicates; the tree was visualized and edited in FigTree v1.4.1

Multiple Loci Variable-Number Tandem Repeat Analysis

Samples confirmed as positive by qPCR and nested PCR were selected for MLVA [36]. The tandem repeat amplification was performed in a 20-μl volume containing 4.0 μl of purified DNA, 10 pmol of each primer, Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Germany), and PCR water. An initial denaturation step at 98°C for 30 s was followed by 35 cycles of 98°C for 10 s, annealing at 56°C for APVA, APVB, APVC, and APVD primers, or 58°C for APVE for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were separated by electrophoresis on 2% agarose gels in TAE buffer and stained with Midori Green for imaging. Six products from three individuals were chosen and sequenced in both directions using the amplification primers. The identity of amplicons was confirmed by BLASTn analysis.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 software. A chi-squared test was used to assess statistical differences in the prevalence rate among species, age, and sex groups. Fisher's exact test was used to analyse differences in the frequency of positive and negative individuals between cadavers and live animals within each species. $P<0.05$ was considered significant.

Results

A total of 385 individuals (276 cadavers and 109 live animals) were collected and 2055 tissue samples screened by qPCR for the presence of *A. phagocytophilum* DNA. For live animals, ears were more positive than blood (91.2% an 27.8%, respectively), whereas the lungs, ear, and spleen (74.8%, 68.1%, and 64.2%, respectively) had the highest positivity rates in cadavers (Table S1).

An individual animal was considered positive if at least one tissue was positive in qPCR. *A. phagocytophilum* was detected in all studied species, with the highest prevalence in hedgehogs. In *E. europaeus*, the positivity rate was similar in cadavers and live animals (97.6% and 93.1%, respectively). *E. roumanicus* was characterized by a slightly lower prevalence in live animals (85.7% vs. 97.6% for cadavers); however, none of these differences were significant. *A. phagocytophilum* was detected in 60% of *S. vulgaris* cadavers; live squirrels were not collected. Forty-eight (50%) of 96 *T. merula* cadavers were positive, whereas the prevalence among live birds (3.9%) was significantly lower ($p<0.01$). Detailed data on prevalence based on age and gender for each species and group of animals are reported in Table S2.

Sequence Diversity

Nested PCR amplifying 407 bp or 1293 bp of *groEL* resulted in 564 and 99 sequences, respectively, from 209 individuals. Inspection of chromatograms did not reveal any multiple peaks suggestive for mix infections. Ecotype distribution based on *groEL* gene sequences for each species is presented on Fig. 1. For each individual, we aligned the sequences from different tissues and both assays and merged the identical sequences to obtain a consensus sequence. Among all sequences, we identified nine unique genotypes with 90.6–99.7% sequence identity (GenBank accession numbers MW013524-MW013537). The main genotype (H1) was detected in 193 individuals (92.3%), representing all species. The second most common genotype (H9) was detected in nine individuals (4.3%) of *E. roumanicus, S. vulgaris,* or *T. merula*. H2 was found exclusively in *E. europaeus*. The remaining genotypes (H3–H8) were each represented by a

single individual (Table 1). For five genotypes, 100% identical matches from different host species, including human cases, were found in the GenBank database. H2, H5, H6, and H7 were >98.9% identical to the sequences from European hedgehogs (Table 1).

Phylogeny and Ecotype Delineation

A neighbour-joining phylogenetic tree was constructed using the 9 unique genotypes and 121 sequences retrieved from the GenBank, as well as the 9 sequences from Jaarasma et al. (2019). Seven sequences (H1–H7) clustered into a lineage previously marked as ecotype 1 [6], together with sequences originating from human cases from the Netherlands, Poland, Slovenia, and the USA and from wild and domestic animals in both Europe and the USA. Sequences described previously by Jahfari et al. (2014), as ecotype 2, are not monophyletic, forming three separate and insufficiently supported clades. The unique sequence from a squirrel in this study (H8) falls into one of the ecotype 2 subclades together with sequences obtained from ungulates and ticks from Central and Southern Europe. H9 clustered with sequences obtained from *I. frontalis* and *I. ricinus* and was described as ecotype 4 (Jahfari et al., 2014). None of our nine genotypes clustered with clades described as ecotype 3 or within the clade with sequences from *I. ventalloi*.

From the perspective of host species, ecotype 1 was the most prevalent in all four studied species. Ecotype 2 was detected in a single *S. vulgaris* individual and ecotype 4 in *E. roumanicus*, *S. vulgaris*, and *T. merula*. Co-infection with ecotypes 1 and 4 was detected in one *E. europaeus*, four *S. vulgaris,* and two *T. merula* individuals (Fig. 2), though the co-occurring genotypes were detected in different organs.

MLVA

MLVA was performed to search for deeper differences within the detected ecotypes but it did not provide additional information over *groEL* sequencing. A total of 21 cadavers and 10 live animals representing different ecotypes were selected and tested as described by Dugat et al. (2014). MLVA was successful for all loci in only two (9.5%) cadaver samples (both *E. roumanicus*). In contrast, amplification of all five loci from 10 live animals was successful except for loci E from two individuals (Table S3).

Discussion

Many aspects of the ecology of *A. phagocytophilum* in various ecosystems with different compositions of host and tick species are not clear [7]. Geographic variations and pathogen-host-vector associations have been described, distinguishing four ecotypes from the European range [6]. The recent analysis by Jaarasma et al. [8] revealed the presence of eight clusters, partially corresponding to the ecotypes described by Jahfari et al. [6]. *A. phagocytophilum* is known to infect a variety of avian and mammalian hosts, including domestic animals and a broad range of wildlife [18, 37–39]. The main vector of *A. phagocytophilum* in Europe is *I. ricinus*; other ticks involved are *I. hexagonus*, the species that commonly feeds on hedgehogs [6, 40], and bird-specific *I. frontalis* [6, 41].

Green areas in cities are suitable habitats for refuge for many small and medium-sized mammals and resident birds [42–44]. Hedgehogs, squirrels, and common blackbirds are examples of animals achieving higher densities in urbanized areas with favourable conditions than in wild habitats. These

Table 1 Unique *groEL* genotypes of *Anaplasma phagocytophilum detected in the four species and their nearest BLAST hits*

Genotype	Ecotype[b]	Sequence length	*E. europaeus*	*E. roumanicus*	*S. vulgaris*	*T. merula*	GenBank
H1	1	1179	97	48	16	32	100%, AY281823 (*I. ricinus*, Germany)
H2	1	366	2	-	-	-	99.45%, MF372791 (*E. roumanicus*, Hungary)a …
H3	1	930	-	-	-	1	100%, MG570466 (*H. sapiens*, Poland)a …
H4	1	382	-	-	1	-	100%, MF372790 (*E. roumanicus*, Hungary)a …
H5	1	383	-	-	1	-	99.48%, MF372780 (*E. roumanicus,* Hungary)a …
H6	1	277	-	-	1	-	98.92%, KU712133 (*E. europaeus,* Germany)a …
H7	1	367	-	-	1	-	99.73%, KF803998 (*E. roumanicus*, Hungary)
H8	2	934	-	-	1	-	100%, KF031382 (*I. ricinus* tick fed on human, Italy)
H9	4	1009	-	1	4	5	100%, KF031393 (*I. ricinus* tick nymph fed on bird, Italy)

[a] Higher number of hits with the same identity

[b] Ecotypes are described according to Jahfari et al. [6]

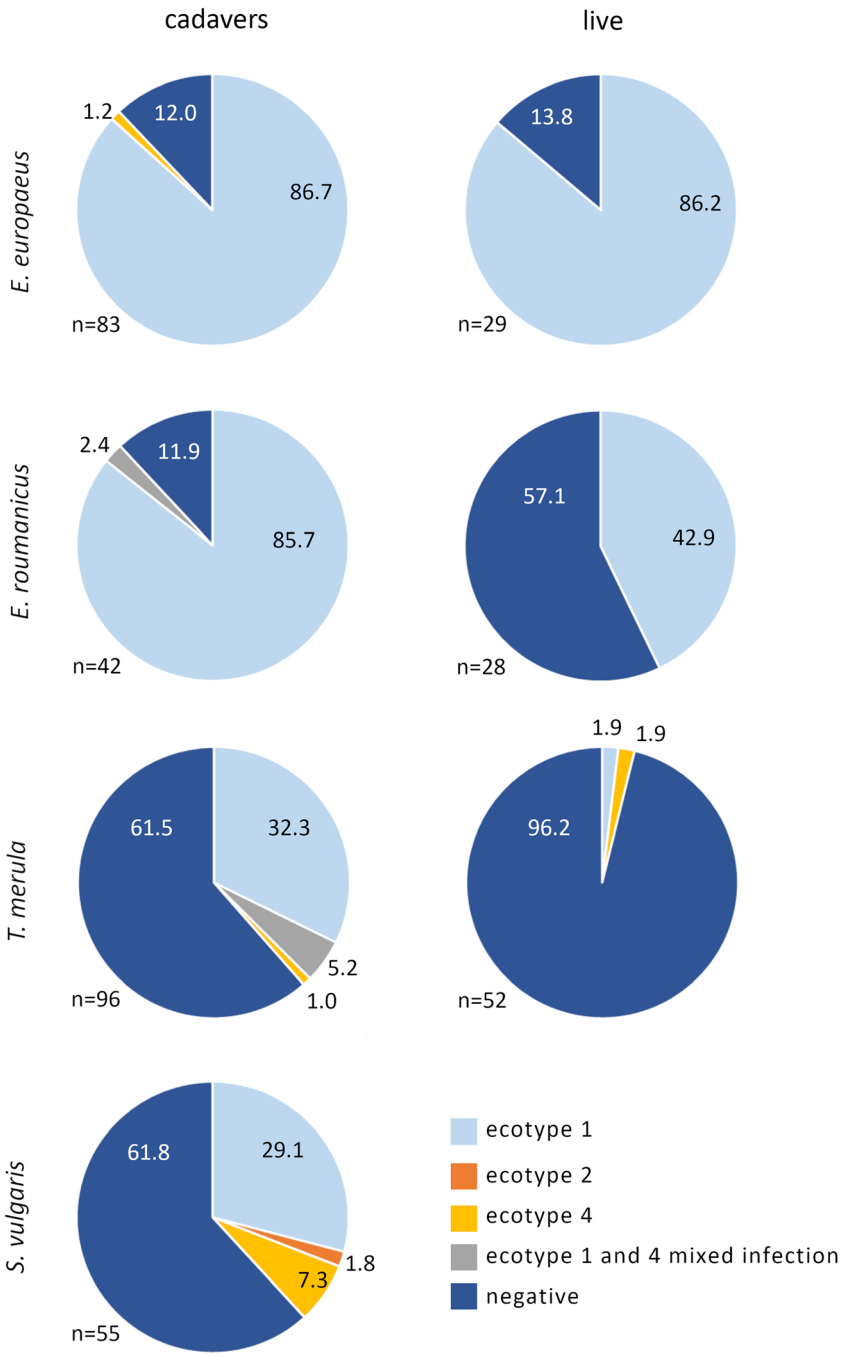

Fig. 1 *Anaplasma phagocytophilum* ecotype distribution based on *groEL* gene sequences from *E. europaeus* (*n*=112), *E. roumanicus* (*n*=70), *T. merula* (*n*=148), *and S. vulgaris* (*n*=55) collected in the Czech Republic

rather isolated populations are important for the maintenance of ticks and TBPs in urban ecosystems [24, 41, 45]. They can also serve as sentinels for monitoring TBPs.

In our study, *A. phagocytophilum* was detected in all four tested species, with the highest prevalence in both hedgehog species, confirming the results of previous studies investigating hedgehogs and other tick-infested animals collected from Central and Southern Europe [3, 24, 46, 47]. The presence of *A. phagocytophilum* was also detected in more than half of red squirrels, suggesting that, along with hedgehogs, *S. vulgaris* plays an important role as a reservoir of *A. phagocytophilum*. The arboricolous life of squirrels regularly exposes them to ticks of terrestrial mammals, as well as to those feeding on birds, and squirrels can harbour many strains of TBPs. In Europe, the red squirrel seems to be regularly infected by several genospecies of *B. burgdorferi* s.l. [48] and has been recognized as a host for *Bartonella washoensis* [49]. Notably, the red squirrel is the most frequently identified rodent species serving as a source of blood-meal for larval ticks in the Czech Republic [50]. Furthermore, common blackbirds are important hosts and sentinels for *A. phagocytophilum* in Europe [41, 51], and *A. phagocytophilum* was frequent in

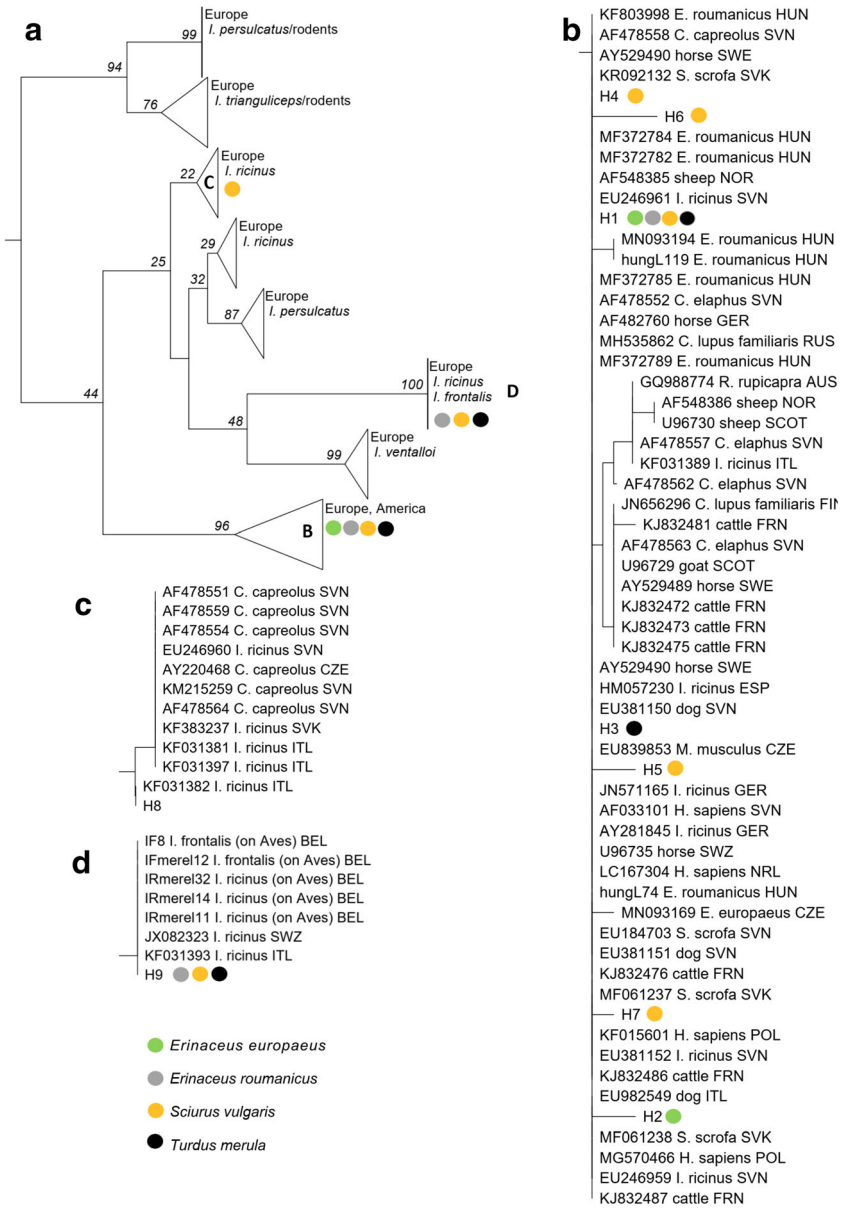

Fig. 2 Phylogenetic trees of *Anaplasma phagocytophilum groEL* gene sequences based on the neighbour-joining method and Jukes-Cantor model. Branch supports were assessed by 1000 bootstrap replicates. The numbers at each node indicate bootstrap values. Particular individuals from each species are marked with colours: *E. europaeus*, green; *E. roumanicus*, grey; *T. merula*, black; and *S. vulgaris*, orange. A, schematic representation of the neighbour-joining phylogenetic tree based on the *groEL* gene sequences of *A. phagocytophilum* longer than 1000 nt representing all ecotypes; tree is rooted with *A. platys* sequences used as an outgroup (not shown); bold letters B, C, and D refer to details of respective branches shown in the following figures. B, uncollapsed branch for ecotype 1; C, uncollapsed branch for part of ecotype 2 that involves our sequences; D, uncollapsed branch for ecotype 4. Genotypes originating from our study are described by numbers H1–H9

our set of common blackbirds (33.8% of individuals). However, the results obtained from this host significantly differed between cadavers (50.0% positive individuals) and live birds (3.9% positive individuals), possibly due to the different sample sources and sample volumes. The low prevalence of Anaplasma sp. in live birds is possibly due to the unavailabilty of invasive tissue samples.

A. phagocytophilum is known for its high intraspecific variability resulting from the range of vertebrate and invertebrate hosts, as well as the extensive geographic range involving higher latitudes of the entire Holarctic realm. Currently, *groEL* is considered to be the most suitable marker for discriminating among *A. phagocytophilum* clades designated as ecotypes or clusters [52]. In our study, we detected nine different *groEL* genotypes. Seven genotypes (H1–7) grouped together in a cluster consistent with an assemblage described as ecotype 1[6] or, more recently, as cluster 1 [7]. Ecotype 1 has the broadest host range, from small mammals, including hedgehogs and squirrels, to wild and domestic ungulates, dogs, and humans. Importantly, strains of *A. phagocytophilum* belonging to this ecotype are responsible for clinical anaplasmosis in human patients. The bacterium from the same ecotype has also been detected in asymptomatic dogs and reported to be the underlying cause of canine granulocytic anaplasmosis cases in Italy and Slovenia [32, 53, 54].

H9 was detected in nine individuals: five common blackbirds, four squirrels, and a single hedgehog (*E. roumanicus*) . The genotype grouped with sequences originating from ticks (*I. frontalis, I. ricinus*) that fed on birds in an assemblage described previously as ecotype 4 or cluster 7 [7]. Even

though blackbirds were the most numerous host for this genotype in our study, the occurrence in mammals indicates that birds are not the exclusive host for ecotype 4. Throughout Europe, the common blackbird is a common host of *Ixodes* larvae [50, 55] and is commonly suggested to be an important reservoir for *Borrelia* spp. Apparently, ticks feeding on these semiterrestrial birds serve as a bridge for *A. phagocytophilum* ecotype 4 to mammalian hosts.

H8 was found only in a single red squirrel, with 100% sequence homology to *A. phagocytophilum* detected in *I. ricinus* in Italy. This genotype clusters with sequences from *I. ricinus* and the roe deer originating in Central and Southern Europe, previously described as ecotype 2 [6]. However, our analysis revealed that ecotype 2 is paraphyletic, involving three rather isolated subclades that differ in the host and geographic distribution. Low support values for these subclades suggest current undersampling, hiding broader diversity of ecotype 2.

Phylogenetic analysis further demonstrated that the *groEL*-based groups of *A. phagocytophilum* (referred to as clusters/ecotypes) are real entities in the ecological and biogeographical context [2, 6]. However, regardless of this variability, *A. phagocytophilum* is still considered a single species. This contrasts a similar situation in *B. burgdorferi* s.l., which is divided into several operational taxonomic units (referred to as genospecies) with obvious differences in geography, hosts, zoonotic potential, and tissue tropism [56]. Introducing a comprehensive nomenclature for lineages referred to as *A. phagocytophilum* would facilitate a deeper understanding of the pathogen circulation, host affinities, and zoonotic potential of individual haplogroups.

The power of *groEL* in discriminating among *A. phagocytophilum* haplogroups is greater than that of 16S DNA. In addition, MLVA was applied to investigate the within-ecotype variability of selected representatives of *A. phagocytophilum* strains. Our results demonstrate that this approach is not suitable for animal carcasses, most likely due to the poor quality of DNA. In contrast, this genotyping was successful for samples from live animals, though this method did not reveal any additional differences among the ecotypes. In future studies, it will be necessary to examine high-quality DNA samples from other animal species. Recent studies have shown that multilocus sequence typing (MLST) is more appropriate for genotyping *A. phagocytophilum* strains [57, 58], and this should be applied in future studies to better understand the epidemiology of this pathogen.

Using roadkill as a source of tissue samples and ectoparasites has its own strengths and limitations [24]. Numerous cadavers collected from defined areas can be utilized to monitor the abundance of ticks and TBPs. This is especially important in the case of vertebrate species representing major tick hosts in otherwise depauperate urban ecosystems. We demonstrated that cadavers are a suitable source of material, even superior to that collected from live individuals in the case of birds. Unlike *B. burgdorferi* s.l., tissue tropism does not occur in *A. phagocytophilum,* but we recommend using the ear (or skin in the case of birds) for the detection of *A. phagocytophilum*.

We demonstrated that hedgehogs, squirrels, and common blackbirds likely play an important role as reservoir hosts in the circulation of several ecotypes of *A. phagocytophilum*. However, the contribution to anaplasmosis risk is higher for hedgehogs, as they can be the main reservoir of the bacterium in urban environments. The common blackbird and squirrels are also involved in the circulation of *A. phagocytophilum* strains responsible for HGA, though different pathogen variants were found in those animals.

Urbanization is a factor leading to a change in the dynamics of hosts, vectors, and pathogens. Thus, monitoring potential reservoirs, vectors, and pathogens within cities is recommended. Cadavers collected from urban and periurban areas, due to their availability, have a great potential for the elucidation and prediction of transmission dynamics of vector-borne diseases in highly populated areas.

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Declarations

Ethics Approval Capture and treatment were performed in agreement with the Czech legislation (Act No 246/1992 Coll.) and protocols (62-2016 UVPS Brno and BC SOS 1520/2017) approved by the responsible authorities of the Czech Republic.

Consent to Participate Not applicable

Consent for Publication Not applicable

Conflict of Interest The authors declare no conflicts of interest.

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