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Detection of Avian Haemosporidian Parasites in Wild Birds in Slovakia

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Abstract: Haemosporidians are a group of vector-borne parasites belonging to the order Haemosporida. They infect avian hosts and require blood-sucking insects (Diptera) for their transmission. The occurrence and diversity of haemosporidian parasites are shaped primarily by the specificity of the parasite and the susceptibility of the host/vector. In this study, the presence and distribution of haemosporidians in blood samples from birds in urbanized and natural habitats were estimated using microscopic and molecular approaches. Birds in urbanized habitats were infected with four different species of *Plasmodium*: *Plasmodium relictum, P. vaughani, P. matutinum, P. circumflexum*, two different species of *Haemoproteus: Haemoproteus majoris, H. parabelopolskyi*, and *Leucocytozoon* sp. The species, *H. attenuatus, H. concavocentralis, H. minutus, H. pallidus, H. noctue* and *H. tartakovskyi* were additionally identified in birds in natural habitats. Typically, juvenile birds are essential markers of parasite species transmitted in the study area. The juveniles in the urbanized habitats carried the *P. relictum, P. vaughani, P. circumflexum, H. parabelopolskyi, H. majoris*, and *Leucocytozoon* species. The most abundant parasite was *H. parabelopolskyi*, which was found in both types of habitats. The prevalence of the *Haemoproteus*/*Plasmodium* species by nested PCR in birds in natural habitats (in totally 43.80%; 53/121) was significantly higher than that in birds in urbanized habitats (in totally 21.94%; 43/196) ($p < 0.05$), even in spring. There was no statistically significant difference between habitat types in overall infection rate of *Leucocytozoon* sp. ($p > 0.05$; 10/121 vs. 19/196), neither overall nor in the spring.

Keywords: *Plasmodium*; *Haemoproteus*; *Leucocytozoon*; avian diseases; Slovakia; adults; juveniles

1. Introduction

Avian haemosporidians belonging to order Haemosporida (Alveolata, Apicomplexa, Sporozoa) are classified in four families Haemoproteidae (*Haemoproteus*), Plasmodiidae (*Plasmodium*), Leucocytozoidae ((*Leucocytozoon*), Garniidae. They are a group of vector-borne parasites that infect avian hosts and require blood-sucking insects (Diptera) as vectors and to complete their life cycle. Species of the genus *Haemoproteus* are transmitted by biting midges (Ceratopogonidae) and hippoboscid flies (Hippoboscidae), *Plasmodium* species by mosquitoes (Culicidae), *Leucocytozoon* species by simuliid flies (Simuliidae). Their life cycle is complicated as they change hosts, and ways of reproduction, and morphologically form different developmental stages [1,2]. Parasites can persist in birds for many years or in chronic stage even a lifetime, and they serve as a source of infection for vectors, which can also lead to the infection of offspring at the site during the breeding season/reproduction period. [1,3]

Most related studies in the past are based on blood smear microscopy, where a set of morphological characteristics served as the basis for species identification [1,2]. After the use of

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molecular methods in parasitology, the number of studies increased rapidly. The most frequently used protocol for the detection of haemosporidians is based on the *cytochrome b* (*cyt b*) gene, which was established by Hellgren et al. [4]. The routine application of PCR, followed by sequencing for detection and identification of haemosporidian lineages has led to a massive expansion in parasite genetic diversity of the parasites. Bensch et al. [5] created the MalAvi database [6], which summarizes unique 5123 parasite lineages and knowledge about their host ranges and geographical distributions facilitating understanding the evolutionary and ecological factors that drive this complex multi-host-multiparasite system.

Information on the occurance of haemosporidian parasites in Slovakia is currently limited. In the past, most studies on the presence of haemosporidians in Slovakia were based on the microscopy analysis of blood smears [3,7–10]. PCR and sequencing were used for the first time in Slovakia by Berthová et al. [11] and expanded by Šujanová et al. [12].

Here, we investigated the presence of haemosporidians in wild birds, both adults and juveniles, in Slovakia using microscopic and molecular approaches. In order to enrich the knowledge of the occurrence and distribution of haemosporidians in Slovakia, it is necessary to identify the species whose transmission takes place here; we compared the occurrence of haemosporidians in urbanized and natural habitats with local bird populations and migrating bird populations, respectively.

2. Materials and Methods

2.1. Study sites

Birds were mist-netted in urbanized and natural habitat types (Figure 1) in Slovakia from April to August 2012 and from April to October 2013 in the morning (in urbanized habitats in April, June, July, August 2012 and from April to October 2013; in natural habitat in April 2012). The birds were captured, ringed, blood sampled, and released under the permission of the Ministry of Environment of the Slovak Republic No. 9368/2011-2.2. Bratislava and Prievidza represent urbanized habitats in city areas, with anthropogenic activities such as walking, cycling, hiking, and horsing. Bratislava is located in the Small Carpathian Mountains in the southwest of Slovakia at 202 – 334 m above sea level (asl), (259 m above sea level, 48°10' N 17°03' E),. It is characterized by deciduous woodlands, oaks in lower altitudes and beech in higher altitudes. The Prievidza district (289 m asl 48°47' N 18°34' E), located in central Slovakia, is a forest-steppe rural area with Carpathian oak-hornbeam woods. Both study sites are characteristic by local bird populations. Drienovec (181 m asl, 48°10´N, 17°03´E) represents a natural habitat with an ornithological observatory for research of migratory birds, situated far from a city area and free from significant human activity. It is a forest-steppe natural area (181 m asl) in the Slovak Karst National Park in southeast Slovakia with submediterranean xero-thermophilous oak woods and colline limestone grasslands [13,14].

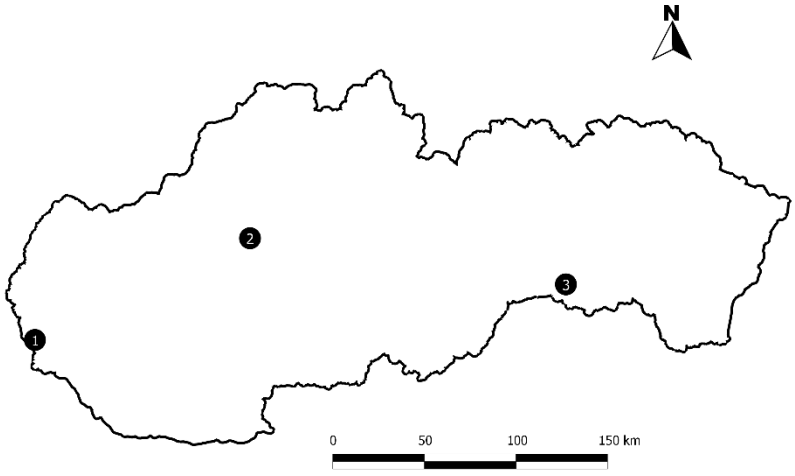

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Figure 1. Map showing study areas: urbanized habitat (1 – Bratislava, 2 – Prievidza), and natural habitat (3 – Drienovec) [14].

2.2. Mist-netting and sampling birds

After ringing the birds, the sex and age of each individual were determined using the field guide by Svensson [15] and Hromádko et al. [16–18]. A small amount of blood (approximately 50 µl) was taken by puncture of the *vena ulnaris cutanea* using a 1 ml X-tra-fine needle (HMD Healthcare, UK), and the blood smears were prepared, while the rest of the blood was stored in 96% ethanol. All birds were released immediately after sampling. All first-year individuals captured in the calendar year of hatching were considered juveniles. A total of 346 birds representing 42 species from 18 families were caught, including 114 juveniles [14]. Blood was collected from 317 individual birds.

2.3. Microscopic analysis of haemosporidian

A drop of blood was used to prepare the blood smears, and two thin smears were prepared for each individual of 302 birds. All smears were air-dried, fixed in methanol (96%), and stained with Giemsa stain as described in Valkiūnas [1]. The smears were examined by a Leica DM4500B microscope (Germany), and pictures were taken with a Leica DFC480 camera (Germany). Approximately 100 fields were scanned under ×400 magnification, and then at least of 100 fields were examined under high magnification (×1000) (oil immersion). The intensity of parasitaemia was determined according to the count of infected blood cells per 10,000 erythrocytes as follows: low (1-10 parasites), medium (11-100 parasites), and high (more than 100 parasites) according to Valkiūnas [1]. Pictures for the identification of parasites were edited using the program Leica Image Manager (Germany). Determination of the haemosporidians was performed based on keys for morphological identification developed by Valkiūnas [1,2]. The smears were deposited at the Institute of Virology, Biomedical Research Center, SAS, Bratislava.

2.4. Molecular analysis of haemosporidian

DNA from the 317 blood samples was extracted using a NucleoSpin® Tissue commercial kit (Macherey–Nagel, Germany). Approximately 50 µl of the blood stored in ethanol was transferred to a sterile 1.5 ml Eppendorf tube, and the ethanol was allowed to evaporate. When the blood was completely dry, 200 µl of lysis buffer B3 and 25 µl of proteinase K were added. The mixture was gently vortexed and incubated at 70 °C for 1 hour. The following procedure was performed according to the manufacturer's instructions. The concentration and the quality of the DNA were assessed using a NanoPhotometer Pearl (Implen, Germany). The DNA samples were stored at -20 °C until the analyses. The presence of blood parasites was examined by a nested PCR assay targeting the *cyt b* gene mtDNA according to Bensch et al. [19] and Hellgren et al. [4]. Briefly, in the first step, HaemNFI and HaemNR3 primers were used to amplify the 617 bp fragment. In the second step, the nested PCR with primers HaemF and HaemR2 amplified 480 bp fragments of *Haemoproteus* and *Plasmodium*, and the set of HaemFL and HaemR2L primers amplified a 478 bp fragment of *Leucocytozoon*. Sterile distilled water was used as a negative control, and the samples previously detected as PCR-positive and confirmed by sequencing [11] were used as positive controls. For the separation and visualization of the PCR products, 1 % agarose gel stained with DNA Stain G (Serva, Germany) was used. The PCR products of the expected size were considered positive and sent for the purification and sequencing of both DNA strands to Macrogen (The Netherlands; http://www.macrogen.com). All the *cyt b* sequences were edited in MEGA6 software [20] and identified by performing a nucleotide blast in GenBank on NCBI [21]. The obtained sequences were compared with those in the MalAvi database [5,6]. Sequences of poor quality were marked as unusable sequences (*us*), and sequences with double peaks, which denote co-infections, were marked as *dp*.

Samples that were microscopically positive for haemosporians were examined for the intensity of haemosporidian infection using quantitative real-time PCR (qPCR) that targeted 182 bp fragment of the *cyt b* gene [22]. All reactions were carried out using GoTaq qPCR Master Mix (Promega, USA) on a CFX96 real-time thermocycler (Bio-Rad, Hercules, CA). The following cycling conditions were

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used: 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s and 64 °C for 35 s with a plate read, followed by a final melt curve analysis using the instrument's default settings [12]. A synthetic double-stranded DNA product (Eurofins Genomics, France) designed from a 220 bp fragment of the conserved rDNA region of *Plasmodium relictum* (accession number NC012426) was used as the positive control [22]. The synthetic DNA was diluted to a starting concentration of 10^6 copies/µl. The starting solution was serially diluted 10-fold to prepare a series of solutions from the 10^6 copies of genomic DNA (gDNA) per µl down to 1 copy/µl. All samples were run in duplicates [13]. Parasite intensity, which refers to the number of parasite DNA copies per 100 avian red blood cells, was determined for each sample based on the qPCR data and sample DNA concentration following the method of Friedl & Groscurth [23], with the assumption that the average genome size of a passerine bird was 2.8 pg [24].

2.5. Statistical analysis

Statistical analysis was conducted to test the differences between the prevalence of birds trapped in urbanized and natural habitats with the χ^2 test ($p = 0.05$) using Past version 2.17b software [25]. We calculated 95 % confidence intervals (CI) for each proportion individually [26].

3. Results

3.1. Haemosporidian infections based on the microscopical and molecular analyses

Overall, the most frequently captured bird species was the Eurasian blackcap, *Sylvia atricapilla* (60/346; 17.3 %). However, of the birds captured in natural habitats, the most frequently captured species was the European robin *Erithacus rubeculla* (42/121; 34.71%), and of the birds captured in urbanized habitats the great tit *Parus major* (37/225; 16.44%), (Figure 2).

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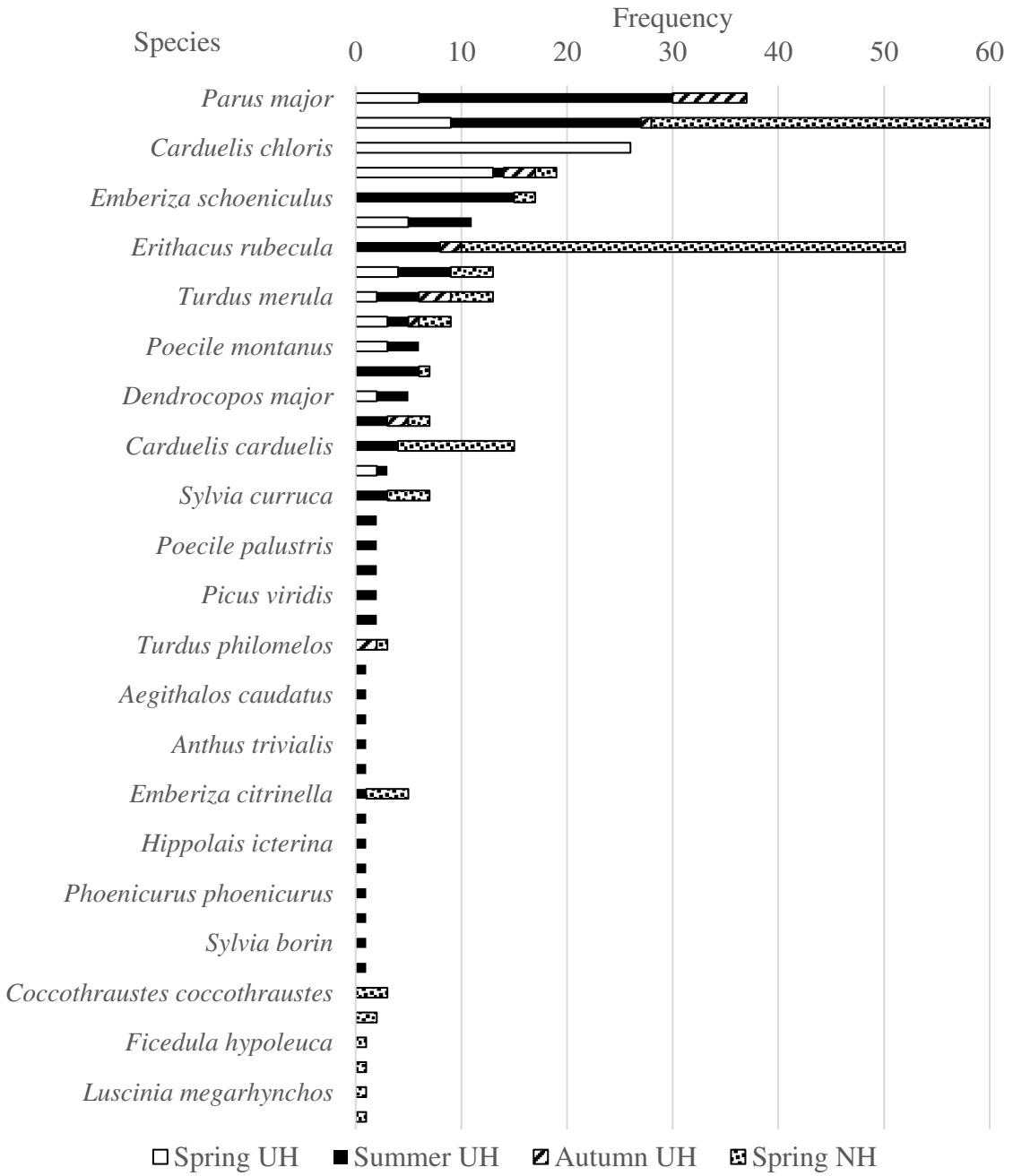

Figure 2. Frequencies of the avian species captured in urbanized (UH) and natural (NH) habitats in Slovakia.

The presence of haemosporidian parasites on the blood smears was detected in 52/302 individuals (17.22%). The most abundant species was *H. parabelopolskyi* on blood smears of 21 birds (Figure 3a), which is a host specific primarily to the black cap *Sylvia atricapilla*, and it was detected in birds from both habitat types. The second most abundant species was *H. attenuatus*(Figure 3b), which was detected on blood smears of 13 bird samples obtained from only one host species, the European robin *E. rubeculla,* and only in birds from the natural habitats. No haemosporidians were identified in 18 bird samples. *Plasmodium* meronts were detected on blood smears of five birds. *Leucocytozoon* was detected only on blood smears of 2 individuals.

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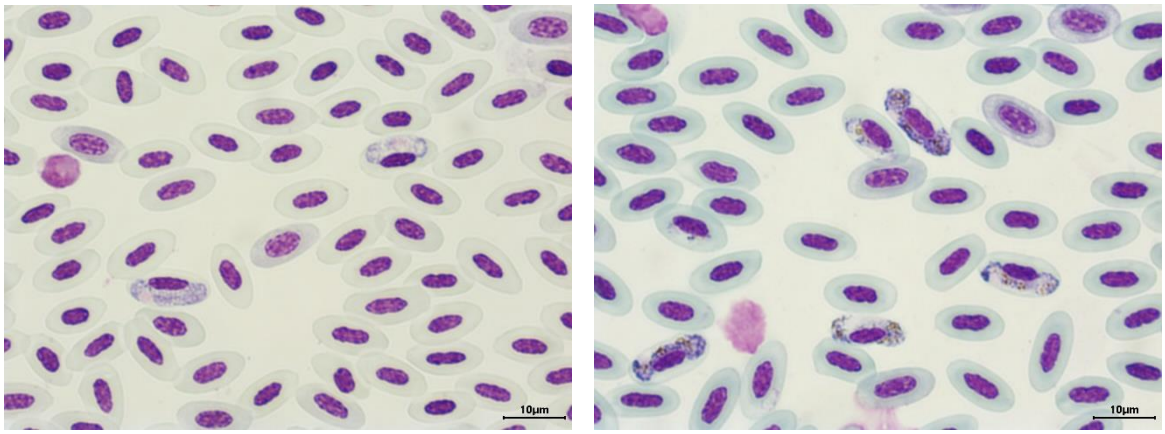

(**a**) (**b**)

Figure 3. Microscopical findings of gametocytes of Haemosporidium (**a**) Gametocytes of *H. parabelopolskyi* (SAYT01 lineage) in the blood of the Eurasian blackcap *Sylvia atricapilla*; (**b**) Gametocytes of *H. attenuatus* (ROBIN1/LULU1 lineage) in the blood of the European robin *Erithacus rubecula*.

Parasite DNA by nested PCR was detected in 125/317 individuals (39.43%; CI 32.8-47.0%) of 18 bird species. Parasites of the genera *Haemoproteus* and *Plasmodium* were present in 96 individuals (30.28 %; CI 25.2-35.3 %), and parasites of the genus *Leucocytozoon* were present in 29 individuals (9.15 %; CI 6.0-12.3%), Table 1. Thirteen birds (4.10 %; CI 1.9-6.3%) were co-infected, Table 1.

Table 1. *Haemoproteus*/*Plasmodium* and *Leucocytozoon* infections in birds.

Bird species	Infected with *Haemoproteus*/ *Plasmodium*/ Analyzed	Infected with *Leucocytozoon*/ Analyzed	Infected with *Haemoproteus*/ *Plasmodium*/ Analyzed	Infected with *Leucocytozoon*/ Analyzed
	Urbanized habitats		**Natural habitats**	
Parus major	5/37			
Poecole montanus	1/6	12/37		
Cyanistes caeruleus	1/9	0/6 3/9	1/4	1/4
Sylvia atricapilla	15/28	1/28	15/32	0/32
Sylvia communis	1/1	*0/1*		
Sylvia curruca			1/4	0/4
Fringilla coelebs	4/6		1/3	0/3
Emberiza citrinella	1/1	*0/6*	2/4	0/4
Turdus merula	8/9	*0/1*	4/4	0/4
Erithacus rubecula	1/10	1/9 0/10	19/42	5/42
Emberiza schoeniculus	3/15	0/15	1/2	0/2
Turdus philomelos	2/2	1/2 1/6	1/1	0/1
Prunella modularis	1/6			
Carduelis carduelis			3/11	0/11

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Coccothraustes coccothraustes			2/3	3/3
Scolopax rusticola			1/1	1/1
Emberiza cia			1/2	0/2
Passer montanus			1/2	0/2
other	0/66	0/66	0/6	0/6
Total	43/196	19/196	53/121	10/121

The infection rate of the genera *Haemoproteus* and *Plasmodium* in the birds caught in natural habitats was found to be 43.80% (53/121; CI 34.8-53.1%). The overall infection rate of the birds from urbanized habitats was in totally 21.94% (43/196; CI 16.4-28.4%), and in spring 10.67% (8/75; CI 4.7-19.9%). The prevalence of the *Haemoproteus*/ *Plasmodium* species in the birds in natural habitats was significantly higher than that of the birds in urbanized habitats, in total and in spring ($p < 0.05$). Of the birds in natural habitats, 8.26% (10/121; CI: 4.0-14.7%) were infected with *Leucocytozoon;* in total 9.69% (19/196; CI: 5.9-14.7%), in spring 8.00% (6/75; CI 3.0-16.6%) of the birds in urbanized habitats were infected with *Leucocytozoon*. There was no statistically significant difference in overall infection rates for *Leucocytozoon* neither overall nor in the spring ($p > 0.05$). Co-infections were found in total 3.57% (7/196; CI 1.5-7.2%), in spring in 1.33% (1/75; CI 0.0-7.2%) of the birds in urbanized habitats and in 4.96% (6/121; CI 1.8-10.5%) of the birds in natural habitats, and there was no statistically significant difference between the two groups ($p > 0.05$).

All 125 positive samples (PCR products of expected size) were sequenced. After comparing the obtained 81 sequences with known sequences in the GenBank and MalAvi databases, we identified twelve species: *H. attenuatus*, *H. concavocentralis*, *H. majoris*, *H. minutus*, *H. pallidus*, *H. parabelopolskyi*, *H. noctues*, *H. tartakovskyi*, *P. circumflexum*, *P. relictum*, *P. vaughani*, and *P. matutinum*. Sequences from 16 individuals were unusable, and 2 samples showed double peaks, indicating co-infections (Table S1).

Birds from the urbanized habitats were infected with six *Haemoproteus* spp./ *Plasmodium* spp. (evaluated by nested PCR based on *cyt b*), and birds from the natural habitat were infected with nine *Haemoproteus* spp./ *Plasmodium* spp. The most abundant parasite species in the birds from the urbanized habitats were *H. majoris* CWT4 lineage (100%, 1/1) recorded in *S. communis, P. vaughani* SYAT05 in 6/9 (66.67%) *T. merula* followed by *H. parabelopolskyi* SYAT01, SYAT02, SYAT07, SYAT10 lineages, with a prevalence of 39.29% in *S. atricapilla*, *P. circumflexum* SYABOR02 lineage (21.74%) in *E. schoeniculus*, *P. relictum* SGS1 lineage (10.00%) in *P. major, P. montanus, C. caeruleus, S. atricapilla*, and *P. matutinum* LINN1 lineage (8.82%) in *T. merula, E. rubecula* and *E. schoeniculus* birds. . In the birds from the natural habitats, *H. parabelopolskyi* SYAT01, SYAT02 lineages infections were also the most prevalent (40.63%) in *S. atricapilla*, followed by *H. attenuatus* ROBIN1/LULU1 lineage (15.91%) in *E. rubecula, C. carduelis, E. schoeniculus, S. atricapilla* and *T. philomelos* birds, *P. relictum* SGS1, DP, COLL1 lineages (8.16%) in *E. cia, E. rubecula, P. montanus, F. coelebs*, *P. circumflexum* TURDUS1 lineage (6.52%) in *E. rubecula* and *E. citronella, H. pallidus* SYAT03 lineage (3.77%) in *E. rubecula, C. carduelis* and *H. noctue* CIRCUM01 (2.38%) in *E. rubecula. H. concavocentralis* HAWF2 lineage was recorded in 1/3 (33.33%) *C. coccothraustes, H. tartakovskyi* HAWF1 lineage also in 1/3 *C. coccothraustes* (33.33%), *H. minutus* TURDUS2 lineage in 1/4 (25%) *T. merula, P. vaughaini* SYAT05 lineage in 2/4 (50%) *T. merula,* and *P. matutinum* LINN1 lineage in 1/4 (25%) *T. merula*. *Haemoproteus parabelopolskyi* was detected in birds from both habitat types but only in one host Eurasian blackcap *S. atricapilla*. Interestingly, we found this parasite in the blood of a seven-year-old female, which was confirmed by ringing data from 2006. Otherwise, *H. attenuatus* ROBIN1/LULU1 lineage was present in a wide range of hosts (five species), though only in birds captured in the natural habitats (Table S1). No unique lineages were recorded.

Birds with long-distance migration were infected with *H. parabelopolskyi* SYAT01, SYAT02, SYAT07 and SYAT10 lineages, *H. majoris* CWT4 lineage, *H. attenuatus* ROBIN1/LULU1 lineage,

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Haemoproteus sp. LWT1 lineage, *P. relictum* SGS1 lineage, and *Leucocytozoon* sp. PARUS4 lineage. In birds with short-distance migration and nonmigratory birds was identify the presence of *H. attenuatus* ROBIN1/LULU1 lineage, *H. concavocentralis* HAWF2 lineage, *H. minutus* TURDUS2 lineage, *H. pallidus* SYAT03 lineage, *H. noctue* CIRCUM01, *H. tartakovskyi* HAWF1 lineage, *Haemoproteus* sp. CCF1, CCF2, CCF6, EMCIR01 lineages, *P. relictum* SGS1, COLL1 lineages, *P. vaughani* SYAT05 lineage, *P. matutinum* LINN1 lineage, *Plasmodium circumflexum* TURDUS1, BT7, SYBOR02 lineages, and *Leucocytozoon* sp. PARUS4, PARUS18, PARUS19, PARUS28, PARUS50, STUR1/TURPEL01, PRUMOD01, BT2, BT5, SFC8, SYCON06 and SCORUS01 lineages.

Parasite intensity using qPCR analysis was verified with 52 microscopically positive samples. The intensity varied from 1 to 15,000 DNA copies per 100 avian red blood cells in both types of habitats. Low parasitemia (1-10 parasites per 10,000 erythrocytes) estimated from blood smears by microscopy corresponded on average to 1-1,000 DNA copies per 100 avian red blood cells. Low parasitemia was recorded in *E. rubecula, S. atricapilla, T. merula, S. curruca, F. coelebs, P. major* bird species infected with *H. parabelopolskyi* SYAT01, SYAT02, SYAT05, SYAT10 lineages, *H. attenuatus* ROBIN1/LULU1 lineage, *P. relictum* COLL1 lineage and *Haemoproteus* sp. CCF1 lineage. Medium parasitemia (11-100 parasites per 10,000 erythrocytes) estimated from blood smears by microscopy corresponded on average to 1,000-10,000 DNA copies per 100 avian red blood cells. Medium parasitemia was identified in *E. rubecula* birds infected with *H. attenuatus* ROBIN1/LULU1 lineage and in *S. atricapilla* infected with *H. parabelopolskyi* SYAT01, SYAT02 lineages. And, the high parasitemia (more than 100 parasites per 10,000 erythrocytes) estimated from blood smears by microscopy corresponded on average more than 10,000 DNA copies per 100 avian red blood cells. The high parasitemia was found in *E. rubecula* birds infected with *H. attenuatus* ROBIN1/LULU1 lineage and in *T. merula P. matutinum* LINN1 lineage

Co-infection with more haemosporidian genera was detected in 4.10% of the individuals (13/317; CI 1.9-6.3%). Co-infections with *Haemoproteus* and *Leucocytozoon* parasites were detected in five birds, and co-infections with *Plasmodium* and *Leucocytozoon* were detected in eight individuals, by microscopy and molecular biology (Table 2). Co-infections with *Haemoproteus* and *Plasmodium* were not analyzed in detail in this study.

Table 2. List of birds co-infected with haemosporidian parasites. Identified lineages are listed.

Avian species	Haemosporidian species
Urbanized habitats	
Turdus merula	*P. vaughani* SYAT05, *L.* sp.
Cyanistes caeruleus	*P. relictum* SGS1, *L.* sp. PARUS19
Parus major	*P. relictum* SGS1, *L.* sp. PARUS4
Parus major	*P. relictum* SGS1, *L.* sp. PARUS18
Parus major	*P. relictum* SGS1, *L.* sp. STUR1/TURPEL01
Sylvia atricapilla	*P.* sp., *L.* sp. PARUS4
Turdus philomelos	*P. circumflexum* BT7, *L.* sp.
Natural habitats	
Erithacus rubecula	*H.* sp., *L.* sp. BT2
Scolopax rusticola	*H.* sp., L. sp.
Erithacus rubecula	*H. attenuatus* ROBIN1/LULU1, *L.* sp. SFC8
Coccothraustes coccothraustes	*H. tartakovskyi* sp. HAWF1, *L.* sp.
Erithacus rubecula	*P. circumflexum* TURDUS1, *L.* sp. SYCON06
Coccothraustes coccothraustes	*H. concavocentralis* HAWF2, *L.* sp.

3.2. Haemosporidian infections in juvenile birds based on the microscopical and molecular analyses

Important markers of haemosporidians transmitted in Slovakia include juvenile birds captured in urbanized habitats. In our study based on PCR, out of 114 juveniles, 26 were infected with *Haemoproteus/ Plasmodium* (22.81%; CI 15.1-30.5%), 8 were infected with *Leucocytozoon* (7.02%; CI 2.3-11.7%) and 6 carried co-infections (5.26%; CI 1.2-9.4%). Juvenile great tit *Parus major*, Eurasian blue

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tit *Cyanistes caeruleus,* and Eurasian blackcap *S. atricapilla* caught in urbanized habitats carried *P. relictum* SGS1 lineage, and the common blackbird *T. merula* carried *P. vaughani* SYAT05 lineage, the song thrush *Turdus philomelos* was infected with *P. circumflexum* SYBOR02 lineage, the Eurasian blackcap *S. atricapilla* was infected with *H. parabelopolskyi* SYAT02 lineage, and the common whitethroat *Sylvia communis* was infected with *H. majoris* CWT4 lineage. The lack of gametocytes made species determination by microscopy impossible for six PCR-positive juveniles the yellowhammer *Emberiza citrinella*, the common blackbird *T. merula*, and the common chaffinch *Fringilla coelebs* that were infected with *Haemoproteus* spp. The European robin *Erithacus rubecula* and the common blackbird *T. merula* were infected with *Plasmodium* spp., and *Leucocytozoon* spp. was found in the great tit *P. major,* the Eurasian blue tit *C. caeruleus,* the common blackbird *T. merula*, the Eurasian blackcap *S. atricapilla*, and the song thrush *T. philomelos*.

Mature gametocytes were confirmed by microscopy in the peripheral blood of 11 juvenile birds: *H. parabelopolskyi* in 6 Eurasian blackcap *S. atricapilla, P. vaughani* in one the common blackbird *T. merula, Haemoproteus* sp. in one yellowhammer *E. citrinella,* two *S. atricapilla* and *Plasmodium* sp. in one the European robin *E. rubecula.*

4. Discussion

In the present study, we aimed to extend knowledge about the presence of haemosporidians in the blood samples of birds in urbanized and natural habitats and identify which species are transmitted in Slovakia using microscopy and molecular detection. Here, we report ten species of haemosporidian parasites detected in the birds in Slovakia. We detected more different species in the birds in the natural habitats, where most of the birds were migratory species compared to those in the urbanized habitats with local populations (Table 1). In combination with suitable vectors, infected birds could serve as reservoirs of infection for other birds (because of share the same habitat for breeding, rest and at night). Differences in prevalence may be due not only to vectors and hosts, but also to abiotic environmental factors such as precipitation and mean annual temperature [27–31]. In our study, birds caught in the natural habitats had significantly higher infection rates than those of the local populations in the urbanized habitats. The highest haemosporidian infections were recorded in the Eurasian blackcap, the great tit, the common chaffinch, the common blackbird, and the Eurasian blue tit, and these results are related to host abundance and are similar to the results of Šujanová et al. [12]. As previously reported, *Haemoproteus* species is the most frequently reported haemosporidian parasite in birds, and this result is not limited to Slovakia [32–35].

To find out which parasites are transmitted in Slovakia; it was necessary to carry out an analysis of juveniles. Unlike adult birds, which can become infected in any year or locality, young birds in their first year of life (before autumn migration) can be infected only by parasites actively transmitted by vectors in their breeding area [1,3,7,8]. In the populations in the natural habitats, the juveniles were not mist-netted, so their infection status remains unknown. Unlike in the local populations in the urbanized habitats, in the caught juveniles, the blood analysis showed the presence of *H. parabelopolskyi*, *H. majoris*, *P. circumflexum*, *P. vaughani*, *P. relictum*, and *Leucocytozoon* sp.

Haemoproteus parasites are the most diverse group of avian haemosporidians [2,35]. Due to limited knowledge of life cycles and tissue merogony of the majority of *Haemoproteus* species, these pathogens usually have been considered benign [36]. However, recent molecular studies indicate that they may cause severe and even lethal diseases if infections occur in non-adapted avian hosts [37]. The findings in our study about *H. parabelopolskyi* and *H. balmorali* were interesting whereas *H. parabelopolskyi* was the most numerous species found only in the Eurasian blackcap *S. atricapilla* and *H. attenuatus* was the second most prevalent *Haemoproteus* found in 5 host species. *H. parabelopolskyi* was the most numerous species found (24 individuals infected). This species is a strict host specialist in the Eurasian blackcap *S. atricapilla* [38]. However, *H. parabelopolskyi* can be occasionally found in birds of the Acrocephalidae family [2]. We recorded the occurrence of this parasite in both habitat types, which was likely due to the fact that the Eurasian blackcap *S. atricapilla* is one of the most numerous bird species in Slovakia. Evidence of the presence of a suitable vector was confirmed by the infections in eight juveniles. Interesting for us was the finding of a seven-year-old female Eurasian

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blackcap *S. atricapilla* infected with this parasite. The infection was confirmed by microscopy, PCR, and sequencing. Microscopy showed a very low intensity of infection, and together, the ability of active flight predicted the chronic stage of infection. Although the time of the individual´s infection is unknown, this discovery supports the need for monitoring on the site when birds are migrating. In contrast, *H. attenuatus* (the second most prevalent *Haemoproteus* species, with 14 infected individuals from 5 host species) was detected in the natural habitat only even though these species were common in the other habitats. *H. attenuates* ROBIN1 lineage is closely related to several lineages of *H. balmorali* which also parasitize birds of Muscicapidae. In our study, obtained sequence fragments showed 100% identity with the sequences annotated in the database as both *H. balmorali* and *H. attenuatus*, microscopic analysis confirmed the presence of *H. attenuatus*. Hauptmanová et al. [9] noted the presence of this parasite for the first time in Slovakia in the blood of a European robin (*E. rubecula*) caught in the eastern part of Slovakia. Unfortunately, the authors did not specify the number of infected birds. Šujanová et al. [12] identified ROBIN1 lineage in addition in *Cyanistes caeruleus, Poecile palustris, Turdus iliacus* and *Prunella modularis* bird species. Thus, the host species reported for *H. attenuates* has been extended. As vertebrate hosts of *H. attenuatus*, Hernández-Lara [39] summarized some species of the order Passeriformes (Muscicapidae, Certhiidae, Acrocephalidae, Sylviidae and Turdidae, Coraciformes (Alcenidae), and as vector Diptera (Ceratopogonidae) species *Culicoides festivipennis, C. obsoletus, C. nubeculosus* which also occur in Slovakia and neighboring countries [40]. This could be a marker for the presence of a suitable vector in the east of the country, but to verify this premise, other studies should confirm or disconfirm this proposition.

5. Conclusions

This study contributes to the knowledge about the prevalence, and morphological and molecular richness of haemosporidian parasites circulating in free-living birds in urbanized and natural habitats in Slovakia. The prevalence of the *Haemoproteus/ Plasmodium* species in birds in natural habitats was significantly higher than that in birds in urbanized habitats ($p < 0.05$), although the prevalence of *Leucocytozoon* was not higher for urban habitats, neither overall nor in the spring. In 42 avian species 12 haemosporidian species *H. attenuatus*, *H. concavocentralis*, *H. majoris*, *H. minutus*, *H. pallidus*, *H. parabelopolskyi*, *H. noctue, H. tartakovskyi, P. circumflexum*, *P. relictum*, *P. vaughani*, and *P. matutinum* were identified. However, the species *H. majoris, H. parabelopolskyi*, and three *Plasmodium* species: *P. circumflexum*, *P. relictum*, and *P. vaughani* were also present in the juveniles, indicating the presence of suitable vectors and active local transmission of parasites in Slovakia.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: List of the haemosporidian parasites detected by *cyt b* molecular analysis in the blood samples of the birds from urbanized and natural habitats (us - unusable sequences, dp - double peaks). Numbers in parentheses refer to the number of positive birds of a given lineage.

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Compliance with ethical standards: The experiments presented in this paper comply with the current laws of the Slovak Republic. Birds were captured, ringed, blood sampled and released under the permission of the Ministry of Environment of the Slovak Republic No. 9368/2011-2.2.

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