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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. These parasites infect avian hosts and require blood-sucking insects (Diptera) for 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*, *P. relictum*, *P. vaughani*, *P. matutinum*, and *P. circumflexum*, and one species of *Haemoproteus*, *H. parabelopolskyi*, and *Leucocytozoon* sp. The species *H. attenuatus*, *H. concavocentralis*, *H. minutus*, *H. pallidus*, *H. noctuae,* 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 *P. relictum*, *P. vaughani*, *P. circumflexum*, *H. parabelopolskyi*, and *Leucocytozoon* species. The most abundant parasite was *H. parabelopolskyi*, which was found in both habitat types. The prevalence of *Haemoproteus*/*Plasmodium* species determined by nested PCR in birds in natural habitats (43.80%; 53/121) was significantly greater than that in birds in urbanized habitats (21.94%; 43/196) ($p < 0.05$). There was no significant difference in the infection rate of *Leucocytozoon* sp. between the habitat types ($p > 0.05$; 10/121 vs. 19/196).

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



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## 1. Introduction

Avian haemosporidians belonging to the order Haemosporida (Alveolata, Apicomplexa) are classified into four families: Haemoproteidae (*Haemoproteus*), Plasmodiidae (*Plasmodium*), Leucocytozoidae (*Leucocytozoon*), and Garniidae. They are a group of vector-borne parasites that infect avian hosts and require blood-sucking insects (Diptera) as vectors 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), and *Leucocytozoon* species by simuliid flies (Simuliidae). The life cycle of these parasites is complicated because they change hosts and reproduction modes and develop into different morphological stages [1,2]. Parasites can persist in birds for many years or in the chronic stage throughout their 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 past studies have been based on blood smear microscopy, where a set of morphological characteristics served as the basis for species identification [1,2]. With the use of molecular methods in parasitology, the number of studies has 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 the detection and identification of haemosporidian lineages, has led to a massive expansion in the identification of parasite genetic diversity. Bensch et al. [5] created the MalAvi database [6], which summarizes 5123 unique parasite lineages and knowledge about their host ranges and geographical distributions, facilitating understanding of the evolutionary and ecological factors that drive this complex multi-host, multi-parasite system.

Information on the occurrence of haemosporidian parasites in Slovakia is currently limited. In the past, most studies on the presence of haemosporidians in Slovakia were based on microscopic 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. To enrich the knowledge of the occurrence and distribution of haemosporidians in Slovakia, it is necessary to identify the species whose transmission occurs here; thus, 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, and in natural habitats in April 2012). The birds were captured, ringed, blood-sampled, and released under permit No. 9368/2011-2.2 from the Ministry of Environment of the Slovak Republic. Bratislava and Prievidza represent urbanized habitats in city areas, with anthropogenic activities such as walking, cycling, hiking, and horse riding. Bratislava is located in the Small Carpathian Mountains in southwestern Slovakia at 202–334 m above sea level (asl) (259 m asl, 48°10′ N 17°03′ E). It is characterized by deciduous woodlands, oaks at lower altitudes, and beech at 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 characterized by local bird populations. Drienovec (181 m asl, 48°10′ N, 17°03′ E) represents a natural habitat with an ornithological observatory for research on migratory birds and is 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 sub-Mediterranean xerothermophilous oak woods and colline limestone grasslands [13,14].

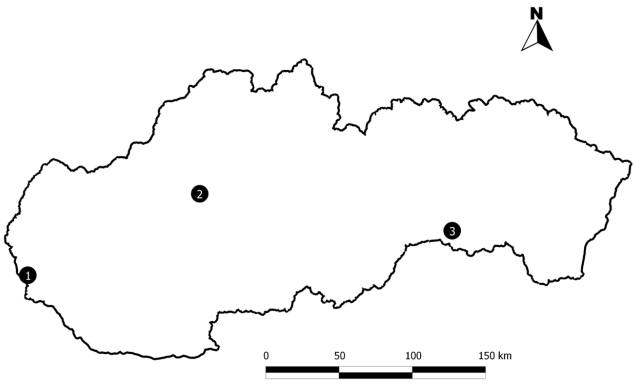



**Figure 1.** Map showing study areas: urbanized habitat (1—Bratislava, 2—Prievidza) and natural habitat (3—Drienovec) [14].

### 2.2. Mist-Netting and Sampling Birds

After ringing the birds, the sex and age of each individual were determined using the field guides of 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, Warwick, UK), and 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 Haemosporidians

A drop of blood was used to prepare blood smears, and 2 thin smears were prepared per individual of a total 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 using a Leica DM4500B microscope (Wetzlar, Germany), and images were taken with a Leica DFC480 camera (Wetzlar, Germany). Approximately 100 fields were scanned under ×400 magnification, and at least 100 fields were subsequently examined under high magnification (×1000) (oil immersion). The intensity of parasitemia was determined according to the count of infected blood cells per 10,000 erythrocytes as follows: low (1–10 parasites), medium (11–100 parasites), or high (more than 100 parasites) according to Valkiūnas [1]. Images for the identification of parasites were edited using the program Leica Image Manager (Wetzlar, 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, Slovakia.

### 2.4. Molecular Analysis of Haemosporidians

DNA from the 317 blood samples was extracted using a NucleoSpin® Tissue commercial kit (Macherey–Nagel, Düren, 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 h. The following procedure was performed according to the manufacturer's instructions. The concentration and quality of the DNA were assessed using a NanoPhotometer Pearl (Implen, Munich, Germany). The DNA samples were stored at −20 °C until analysis. The presence of blood parasites was examined using 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, the HaemNFI and HaemNR3 primers were used to amplify the 617-bp fragment. In the second step, nested PCR with the 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, Heidelberg, Germany) was used. The PCR products of the expected size were considered positive and were sent for the purification and sequencing of both DNA strands at Macrogen (Amsterdam, Netherlands;). All the *cyt b* sequences were edited in MEGA6 version software [20] and identified by performing a nucleotide BLAST search in the GenBank NCBI database [21]. The obtained sequences were compared with those in the MalAvi database [5,6]. Poor-quality sequences 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 haemosporidians were examined for the intensity of haemosporidian infection using quantitative real-time PCR (qPCR), which targeted an 182 bp fragment of the *cyt b* gene [22]. All the reactions were carried out using

GoTaq qPCR Master Mix (Promega, Madison, WI, USA) on a CFX96 real-time thermocycler (Bio-Rad, Hercules, CA, USA). The following cycling conditions were used: 95 °C for 2 min, 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, Ebersberg, Germany) 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 duplicate [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 is 2.8 pg [24]. An average of 1–1000 DNA copies per 100 avian red blood cells was marked as low parasite intensity, 1000–10,000 DNA copies per 100 avian red blood cells was marked as medium parasitemia, and more than 10,000 DNA copies per 100 avian red blood cells was marked as high parasitemia.

### *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 $\chi^2$ test ($p = 0.05$) using Past version 2.17b software [25]. We calculated 95% confidence intervals (CIs) for each proportion individually [26].

## 3. Results

### *3.1. Haemosporidian Infections Based on Microscopic 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 rubecula* (42/121; 34.71%), and of the birds captured in urbanized habitats, the great tit, *Parus major* (37/225; 16.44%), was the most common (Figure 2).

The presence of haemosporidian parasites in the blood smears was detected in 52/302 individuals (17.22%). The most abundant species was *H. parabelopolskyi* in blood smears of 21 birds (Figure 3a), which is a host specific to the black cap *S. atricapilla*, and it was detected in birds from both habitat types. The second most abundant species was *H. attenuatus* (Figure 3b), which was detected in blood smears of 13 bird samples obtained from only 1 host species, the European robin *E. rubecula*, and only in birds from the natural habitats. No haemosporidians were identified in 18 bird samples. *Plasmodium* meronts were detected in the blood smears of five birds. *Leucocytozoon* was only detected in the blood smears of two individuals.

Parasite DNA was detected by nested PCR 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).

The infection rate of the genera *Haemoproteus* and *Plasmodium* in the birds caught in natural habitats was 43.80% (53/121; CI 34.8–53.1%). The overall infection rate of the birds from urbanized habitats was 21.94% (43/196; CI 16.4–28.4%). The prevalence of *Haemoproteus/Plasmodium* species in birds in natural habitats was significantly greater than that in birds in urbanized habitats ($p < 0.05$). Among the birds in natural habitats, 8.26% (10/121; CI: 4.0–14.7%) were infected with *Leucocytozoon* overall, while 8.00% (6/75; CI 3.0–16.6%) of the birds in urbanized habitats were infected with *Leucocytozoon*. There was no significant difference in infection rates for *Leucocytozoon* ($p > 0.05$). Among the birds in urbanized habitats, the co-infection rate was 3.57% (7/196; CI 1.5–7.2%). In natural

habitats, 4.96% (6/121; CI 1.8–10.5%) of the birds had co-infections. There was no significant difference between the two habitat types ($p > 0.05$).

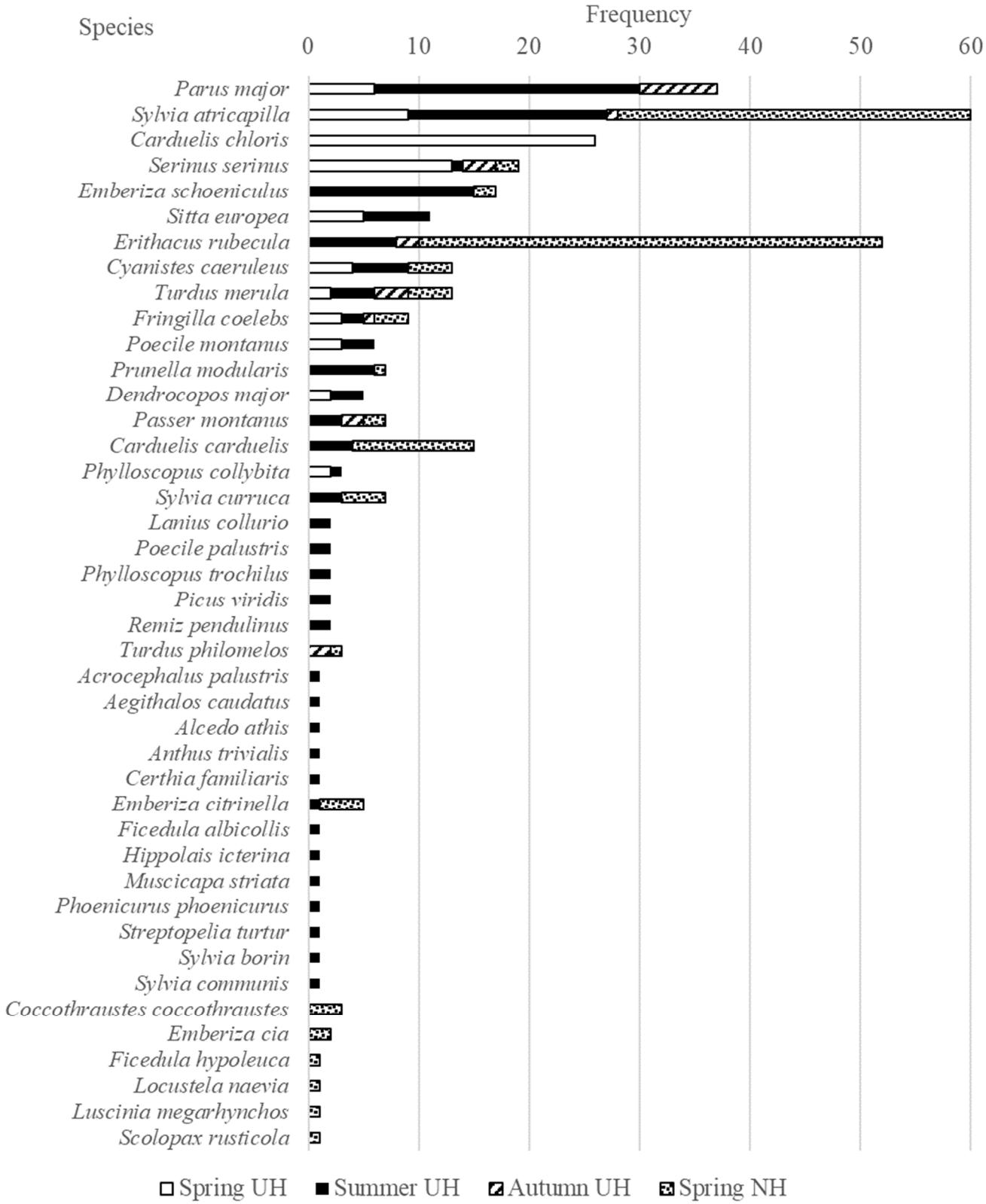
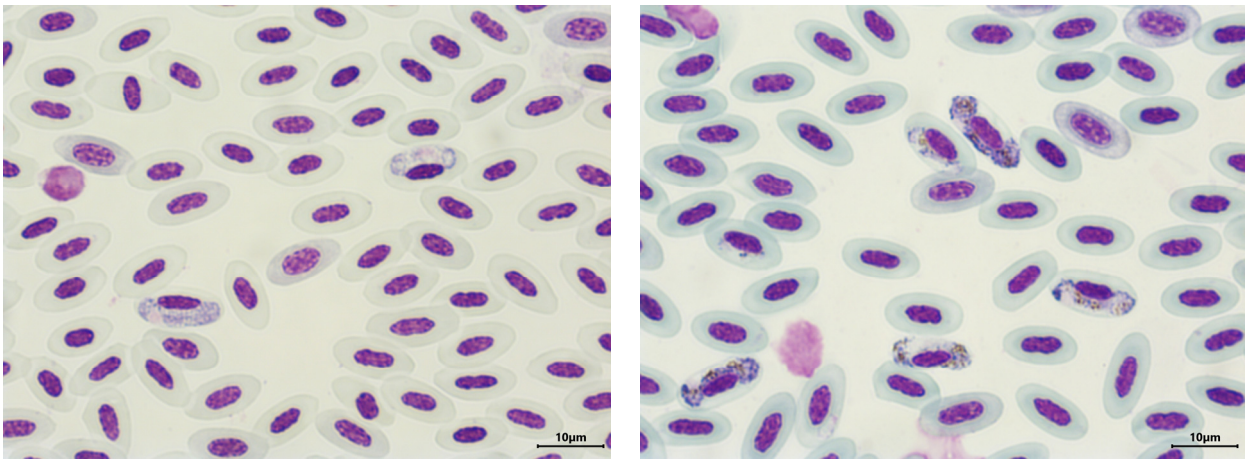



**Figure 2.** Frequencies of the bird species captured in urbanized (UH) and natural (NH) habitats in Slovakia.




(**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*.

**Table 1.** *Haemoproteus/Plasmodium* and *Leucocytozoon* infections in birds based on nested PCR. Birds with long-distance migration are marked with the asterisk symbol (*).

| Bird Species | Infected with *Haemoproteus/ Plasmodium*/ Analyzed | Infected with *Leucocytozoon*/ Analyzed | Infected with *Haemoproteus/ Plasmodium*/Analyzed | Infected with *Leucocytozoon*/ Analyzed |
|---|---|---|---|---|
| | Urbanized Habitat | | Natural Habitat | |
| *Parus major* | 5/37 | 12/37 | | |
| *Poecole montanus* | 1/6 | 0/6 | | |
| *Cyanistes caeruleus* | 1/9 | 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 | 0/6 | 1/3 | 0/3 |
| *Emberiza citrinella* | 1/1 | 0/1 | 2/4 | 0/4 |
| *Turdus merula* | 8/9 | 1/9 | 4/4 | 0/4 |
| *Erithacus rubecula* | 1/10 | 0/10 | 19/42 | 5/42 |
| *Emberiza schoeniculus* | 3/15 | 0/15 | 1/2 | 0/2 |
| *Turdus philomelos* | 2/2 | 1/2 | 1/1 | 0/1 |
| *Prunella modularis* | 1/6 | 1/6 | | |
| *Carduelis carduelis* | | | 3/11 | 0/11 |
| *Coccothraustes coccothraustes* | | | 2/3 | 3/3 |
| *Scolopax rusticola* * | | | 1/1 | 1/1 |
| *Emberiza cia* | | | 1/2 | 0/2 |
| *Passer montanus* | | | 1/2 | 0/2 |
| Uninfected | 0/66 | 0/66 | 0/6 | 0/6 |
| Total | 43/196 | 19/196 | 53/121 | 10/121 |

All 125 positive samples (PCR products of the expected size) were sequenced. After comparing the obtained 81 sequences with known sequences in the GenBank and MalAvi databases, we identified 11 haemosporidian species: *H. attenuatus*, *H. concavocentralis*, *H.*

*minutus*, *H. pallidus*, *H. parabelopolskyi*, *H. noctuae*, *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 five *Haemoproteus* spp./*Plasmodium* spp. (evaluated by nested PCR based on *cyt b*), and birds from the natural habitat were infected with eleven *Haemoproteus* spp./*Plasmodium* spp. (Table S1). The most abundant parasite species in the birds (evaluation only included species exceeding 10 individuals) from the urbanized habitats were the *H. parabelopolskyi* SYAT01, SYAT02, SYAT07, and SYAT10 lineages, with a prevalence of 39.29% in *S. atricapilla*; *P. relictum* SGS1 lineage (13.51%) in *P. major*; and *P. circumflexum* SYABOR02 lineage (13.33%) in *E. schoeniculus*. Among the birds from the natural habitat, infections with the *H. parabelopolskyi* SYAT01 and SYAT02 lineages were also the most prevalent (40.63%) in *S. atricapilla*, followed by the *H. attenuatus* ROBIN1/LULU1 lineage (23.8%) in *E. rubecula*, and the *H. pallidus* SYAT03 lineage (3.77%) in *E. rubecula.* The *H. attenuatus* ROBIN1/LULU1 lineage was present in a wide range of hosts (five species), though only in birds captured in the natural habitat (Table S1). Interestingly, we found this parasite in the blood of a seven-year-old female, which was confirmed by ringing data from 2006. No unique lineages were recorded.

Birds with long-distance migration were infected with the *H. parabelopolskyi* SYAT01, SYAT02, SYAT07, and SYAT10 lineages; *H. attenuatus* ROBIN1/LULU1 lineage; *Haemoproteus* sp. LWT1 lineage; *P. relictum* SGS1 lineage; and *Leucocytozoon* sp. PARUS4 and SCORUS01 lineages. In birds with short-distance migration and non-migratory birds, the presence of the *H. attenuatus* ROBIN1/LULU1 lineage; *H. concavocentralis* HAWF2 lineage; *H. minutus* TURDUS2 lineage; *H. pallidus* SYAT03 lineage; *H. noctuae* CIRCUM01 lineage; *H. tartakovskyi* HAWF1 lineage; *Haemoproteus* sp. CCF1, CCF2, CCF6, and EMCIR01 lineages; *P. relictum* SGS1 and COLL1 lineages; *P. vaughani* SYAT05 lineage; *P. matutinum* LINN1 lineage; *P. circumflexum* TURDUS1, BT7, and SYBOR02 lineages; and *Leucocytozoon* sp. PARUS4, PARUS18, PARUS19, PARUS28, PARUS50, STUR1/TURPEL01, PRUMOD01, BT2, BT5, SFC8, and SYCON06 lineages were identified.

The parasite intensity was verified via qPCR analysis 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 1–1000 DNA copies per 100 avian red blood cells. Low parasitemia was recorded in the *E. rubecula*, *S. atricapilla*, *T. merula*, *S. curruca*, *F. coelebs*, and *P. major* bird species infected with the *H. parabelopolskyi* SYAT01, SYAT02, SYAT05, and 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 1000–10,000 DNA copies per 100 avian red blood cells. Medium parasitemia was identified in *E. rubecula* birds infected with the *H. attenuatus* ROBIN1/LULU1 lineage and in *S. atricapilla* birds infected with the *H. parabelopolskyi* SYAT01 and SYAT02 lineages. High parasitemia (more than 100 parasites per 10,000 erythrocytes) estimated from blood smears by microscopy corresponded to an average of more than 10,000 DNA copies per 100 avian red blood cells. High parasitemia was found in *E. rubecula* birds infected with the *H. attenuatus* ROBIN1/LULU1 lineage and in *T. merula* infected with the *P. matutinum* LINN1 lineage.

Co-infection with more than one haemosporidian genus 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* parasites were detected in eight individuals by 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 based on nested PCR. Birds with long-distance migration are marked with the asterisk symbol (*).

| Bird Species | Haemosporidian Species |
|---|---|
| Urbanized habitat | |
| *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 habitat | |
| *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 Microscopic and Molecular Analyses*

Important markers of haemosporidians transmitted in Slovakia include juvenile birds captured in urbanized habitats. In our PCR-based study, 26 out of 114 juveniles 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 had co-infections (5.26%; CI 1.2–9.4%). Juvenile great tit *P. major*, Eurasian blue tit *C. caeruleus*, and Eurasian blackcap *S. atricapilla* caught in urbanized habitats carried the *P. relictum* SGS1 lineage, and the common blackbird *T. merula* carried the *P. vaughani* SYAT05 lineage. The song thrush *T. philomelos* was infected with the *P. circumflexum* SYBOR02 lineage, and the Eurasian blackcap *S. atricapilla* was infected with the *H. parabelopolskyi* SYAT02 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*, which were infected with *Haemoproteus* spp. The European robin *E. rubecula* and the common blackbird *T. merula* were infected with *Plasmodium* spp., and *Leucocytozoon* spp. were 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 six Eurasian blackcap *S. atricapilla*, *P. vaughani* in one common blackbird *T. merula*, *Haemoproteus* sp. in one yellowhammer *E. citrinella* and two *S. atricapilla*, and *Plasmodium* sp. in one European robin, *E. rubecula.*

Parasite intensity in juveniles was positive according to nested PCR, and microscopically, it is shown in Table S2.

## 4. Discussion

In the present study, we aimed to extend the body of knowledge about the presence of haemosporidians in the blood samples of birds in urbanized and natural habitats and to identify which species are transmitted in Slovakia using microscopy and molecular detection. Quantitative PCR and microscopical analysis are only supplementary methods applied to a part of the samples. Here, we report eleven species of haemosporidian parasites detected in birds in Slovakia. We detected more different species among the birds in natural habitats, where most of the birds were migratory species, than among those in urbanized habitats with local populations (Table 1). In combination with suitable vectors, infected birds could serve as reservoirs of infection for other birds (because they 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 natural habitats had significantly greater infection rates than birds from local populations in urbanized habitats. The highest prevalence of haemosporidian infections was recorded in the Eurasian blackcap *S. atricapilla*, the great tit *P. major*, the common chaffinch *F. coelebs*, the common blackbird *T. merula*, and the Eurasian blue tit *C. caeruleus*; these results are related to host abundance and are similar to the results of Šujanová et al. [12]. As previously reported, *Haemoproteus* species are the most frequently reported haemosporidian parasites in birds, and these results are not limited to Slovakia [32–35].

To determine 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 our study, juveniles from populations in the natural habitats were not mist-netted, so their infection status remains unknown. In the local populations in the urbanized habitats, blood analysis revealed the presence of *H. parabelopolskyi*, *P. circumflexum*, *P. vaughani*, *P. relictum*, and *Leucocytozoon* sp. in the caught juveniles.

*Haemoproteus* parasites are the most diverse group of avian haemosporidians [2,35]. Due to the limited knowledge of the life cycles and tissue merogony of the majority of *Haemoproteus* species, these parasites have usually been considered benign [36]. However, recent molecular studies indicate that they may cause severe and even lethal disease if infections occur in non-adapted avian hosts [37]. Our findings on *H. parabelopolskyi* and *H. attenuatus* are interesting, as *H. parabelopolskyi* was the most common species found only in the Eurasian blackcap *S. atricapilla*, and *H. attenuatus* was the second most prevalent *Haemoproteus* found in five host species. *H. parabelopolskyi* was the most common species found (24 individuals infected). This species is a strict host specialist for the Eurasian blackcap *S. atricapilla* [38]. However, *H. parabelopolskyi* can be occasionally found in birds of the family Acrocephalidae [2]. We recorded the occurrence of this parasite in both habitat types, likely because the Eurasian blackcap *S. atricapilla* is one of the most common bird species in Slovakia. Evidence of the presence of a suitable vector was confirmed by the infections of eight juveniles. Interestingly, we found a seven-year-old female Eurasian blackcap (*S. atricapilla*) infected with this parasite. The infection was confirmed by microscopy, PCR, and sequencing. Microscopy revealed a very low intensity of infection, and the ability to complete active flight indicated the chronic stage of infection. Although the time of the individual's infection is unknown, this discovery supports the need for monitoring at 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. The *H. attenuatus* ROBIN1 lineage is closely related to several lineages of *H. balmorali* that also parasitize birds of Muscicapidae. In our study, the obtained sequence fragments showed 100% identity with the sequences annotated in the database as both *H. balmorali* and *H. attenuatus*, and microscopic analysis confirmed the presence of *H. attenuatus*. For the first time, Hauptmanová et al. [9] noted the presence of this parasite 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] also identified ROBIN1 lineage in the *C. caeruleus*, *Poecile palustris*, *Turdus iliacus,* and *Prunella modularis* bird species. Thus, the host species reported for *H. attenuatus* has been extended. Hernández-Lara [39] described several species of the orders Passeriformes (Muscicapidae, Certhiidae, Acrocephalidae, Sylviidae, and Turdidae), and Coraciiformes (Alcedinidae) as vertebrate hosts of *H. attenuatus*, and the Diptera (Ceratopogonidae) species *Culicoides festivipennis*, *C. obsoletus*, and *C. nubeculosus* as vectors, 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 further studies are needed to verify this premise.

## 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 *Haemoproteus/Plasmodium* species in birds in natural habitats was significantly greater than that in birds in urbanized habitats ($p < 0.05$), although the prevalence of *Leucocytozoon* was not greater in urban habitats. Among the 42 avian species, 11 haemosporidian species—*H. attenuatus*, *H. concavocentralis*, *H. minutus*, *H. pallidus*, *H. parabelopolskyi*, *H. noctuae*, *H. tartakovskyi*, *P. circumflexum*, *P. relictum*, *P. vaughani*, and *P. matutinum*, were identified. However, the *H. parabelopolskyi*, as well as three *Plasmodium* species, *P. circumflexum*, *P. elictum*, 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: https://www.mdpi.com/article/10.3390/d16020121/s1, 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; Table S2. Parasite intensity in chosen juveniles was determined by qPCR and microscopy.

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**Data Availability Statement:** The datasets used and/or analyzed during the current study are available from the first and corresponding author upon request.

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