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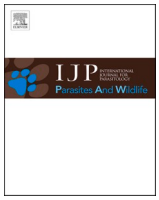

Prevalence and local transmission of haemosporidian (Haemosporida) parasites in nestlings of birds of prey (Aves, Accipitriformes) in the temperate forests in Lithuania

Dovilė Bukauskaitė *, Carolina Romeiro Fernandes Chagas, Mélanie Duc, Margarita Kazak, Rimgaudas Treinys

Nature Research Centre, Akademijos 2, 08412, Vilnius, Lithuania

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ABSTRACT

Wild birds of prey (Accipitriformes) are infected with haemosporidian (Haemosporida) parasites worldwide, and it is known that these parasites can negatively affect their health. These birds are less studied due to their low densities in ecosystems, conservation status, and difficulty of accessing them in the wild. Therefore, in this study, we focused on nestlings of birds of prey that are accessible in their nests during late breeding stages in temperate forests in Lithuania. Investigating haemosporidian parasites in nestlings is crucial for understanding local parasite transmission. To identify the haemosporidian parasite species transmitted in Lithuania, we sampled nestlings of the white-tailed eagles (*Haliaeetus albicilla*), lesser spotted eagles (*Clanga pomarina*), and common buzzards (*Buteo buteo*) in 2019–2022. Blood samples were collected from the nestlings, and molecular methods were employed to sequence a fragment of the parasite's cytochrome *b* (cyt *b*) gene using specific primers (Plas1F/HaemNR3 and 3760F/HaemJR4). In addition to molecular techniques, microscopy was used to examine blood smears for the presence of parasites. Our results revealed that nestlings of birds of prey were infected only with *Leucocytozoon* spp., with an overall prevalence of 30.5%. The prevalence was similar between years, but it was significantly species-dependent. The common buzzard nestlings had the highest prevalence (80%), followed by the lesser spotted eagle (29.2%) and the white-tailed eagle (13.2%). A total of nine genetic lineages were identified, with five of them being novel. Our study demonstrates that *Leucocytozoon* parasites are actively transmitted to nestlings of birds of prey in Lithuania, with a high prevalence.

1. Introduction

Avian haemosporidian parasites (Haemosporida) are a group of pathogens that infect wild birds worldwide, except Antarctica (Valkiūnas, 2005). Within this group of bird hosts, birds of prey (Accipitriformes) are important to study due to their ecological role as predators in ecosystems and vulnerability to environmental and anthropogenic stressors (McClure et al., 2018). It is known that these parasites can cause illness and mortality in avian hosts (Donovan et al., 2008; Garvin et al., 2003), as well as having a negative impact on the health of birds of prey (Chakarov and Blanco, 2021).

Most studies about haemosporidian parasites in birds of prey come from zoos and rehabilitation centres. These studies are important for disease management, understanding host-parasite interactions, and assessing their impact on avian health and welfare (Chagas et al., 2017; Harl et al., 2022; Ombugadu et al., 2019; Woods et al., 2022). Also, birds in zoos are at higher risk of being infected with non-usual parasites, as a long-term study (8 years) conducted at Beijing Zoo has shown that young cranes are susceptible to *Plasmodium* and *Leucocytozoon* infections, leading to high mortality of juveniles. Juvenile cranes in the zoo were exposed to infections that they probably would not encounter in the wild (Jia et al., 2018). Birds in captivity experience different stressors and environmental conditions compared to the wild ones (Woods et al., 2022). Studying the infection of birds with haemosporidian parasites in zoos and rehabilitation centres is significant (Ombugadu et al., 2019), however, in order to understand the natural transmission and distribution of parasites, it is necessary to conduct studies on wild birds.

* Corresponding author.
E-mail addresses: dovile.bukauskaite@gamtc.lt (D. Bukauskaitė), carolina.chagas@gamtc.lt (C. Romeiro Fernandes Chagas), melanie.duc@gamtc.lt (M. Duc), margarita.kazak@gamtc.lt (M. Kazak), rimgaudas.treinys@gamtc.lt (R. Treinys).

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Adult birds of prey are infected during the breeding season when they are incubating or roosting, while adults of migratory species may also be infected during migration or at wintering sites, where they spend a considerable part of their annual cycle. Some studies have determined that migratory bird species, including birds of prey, have a higher prevalence and diversity of haemosporidian parasites compared with resident species (Bukauskaitė et al., 2024; de Angeli Dutra et al., 2021). This could be because infection with haemosporidian parasites is influenced by biological and ecological factors. Migration is an energy demanding process that could weaken the hosts' immune system (Nebel et al., 2012), making them more susceptible to parasites. Infection with haemosporidian parasites in migratory birds or those already in wintering areas is increased by the fact that they usually stay in flocks (Agostini et al., 2004), which can attract more vectors and facilitate parasite transmission. One of the most important factors in the transmission of haemosporidian parasites is suitable vectors (mosquitoes (Culicidae), biting midges (Ceratopogonidae), louse flies (Hippoboscidae), and black flies (Simuliidae)). Migratory birds are exposed to a wider variety of vectors than non-migratory birds, mainly due to their journey, which increases the possibility of finding a suitable vector (Valkiūnas, 2005). All the factors mentioned above lead to a greater prevalence and variety of haemosporidian parasites in adult migratory bird species, such as birds of prey, in comparison to resident species.

Studies on haemosporidian parasite infection in the white-tailed eagles (*Haliaeetus albicilla*), lesser spotted eagles (*Clanga pomarina*), and common buzzards (*Buteo buteo*) have been conducted in several countries, both in free-living and captive animals. According to the MalAvi database (Bensch et al., 2009), only *Leucocytozoon* parasites were found in the white-tailed eagles as well as in the lesser spotted eagles. In contrast, all three parasite genera (*Leucocytozoon*, *Plasmodium*, and *Haemoproteus*) were found in the common buzzard.

Some nestlings of birds of prey species might be heavily infected with haemosporidian parasites. Wiegmann et al. (2021) reported a prevalence of 51.9% for *Leucocytozoon* parasites among nestlings of common buzzards, red kites (*Milvus milvus*), and northern goshawks (*Accipiter gentilis*). Similarly, Chakarov et al. (2008) reported a 40% infection rate in common buzzard nestlings of *Leucocytozoon*. Hanel et al. (2016) observed a lower prevalence (13.6%) of *Leucocytozoon* in northern goshawk nestlings. In contrast, Gutiérrez-López et al. (2015) found that Eleonora's falcon (*Falco eleonorae*) was not infected with any haemosporidian parasites.

Investigation of nestlings is important to determine the local distribution of haemosporidian parasites by identifying the parasite species whose transmission takes place at the breeding sites (Dimitrov et al., 2018), indicating that there are suitable vectors in the studied area (Valkiūnas, 2005). Also, monitoring haemosporidian parasites in nestling birds in populations can help track changes in parasite prevalence and diversity over time in breeding areas. Overall, studying nestling birds provides a valuable opportunity to explore the ecology, epidemiology, and evolutionary biology of haemosporidian parasites in avian populations.

Recently, we studied haemosporidian parasites of adult birds of prey breeding in temperate forests of Lithuania using blood drop from moulted feathers (Bukauskaitė et al., 2024). However, in Lithuania, the prevalence of parasite infection and transmission in nestlings of birds of prey remains unknown. Thus, in this study, using PCR-based and microscopic methods, we have examined them and identified parasites that are transmitted in Lithuania in the nestlings of three species of birds of prey. We 1) investigated the prevalence of haemosporidian parasites and their differences among three bird species and across years; 2) identified parasites genetic lineages; 3) employed molecular phylogenetic analyses to identify evolutionary relationships among the parasite lineages found; and 4) compared the analysis between molecular data and traditional microscopic observations.

2. Materials and methods

2.1. Study site, collection of blood samples, and microscopic examination

In May–July of 2019–2022, we checked the nests of the white-tailed eagle (strictly resident), the common buzzard (short-distance migrant wintering in Europe) and the lesser spotted eagle (long-distance migrant) breeding in the temperate forests of the north-eastern part of Lithuania (55° 31′, 25° 36′). Trees with nests, consisting of 1–3 nestlings, were climbed, the nestlings were put into a bag and landed to the ground by the rope to be ringed and for blood sampling. We sampled well grown nestlings, usually between four to six weeks of age.

Approximately 40 μL of blood was collected from the brachial vein and stored on filter paper and in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0). Three blood smears were done without any anticoagulant, air dried immediately, fixed with absolute methanol, and stained with Giemsa as described by Valkiūnas (2005).

An Olympus BX-41 light microscope with an Olympus DP12 digital camera, and DP-SOFT image software were used for the microscopic investigation (Tokyo, Japan). For 15–20 min, blood smears were examined at low magnification (400×), then 100 fields were studied at high magnification (1000×). The percentage of parasitaemia was calculated by counting the number of infected cells per 10000 red blood cells. Parasites were described based on Valkiūnas (2005) and Valkiūnas and Iezhova (2023). When parasites were not found using the previously described method, the entire blood smear was checked by microscope to confirm the infection.

2.2. DNA extraction, PCR amplification, and sequencing

DNA was extracted using an ammonium-acetate protocol (Sambrook and Russell, 2001). A fragment of the mitochondrial cytochrome *b* gene (cyt *b*) of haemosporidian parasites was amplified using a nested polymerase chain reaction (PCR) procedure. For the initial PCR, the primer pair Plas1F [5′-GAGAATTATGGAGTGGATGGTG-3'] and HaemNR3 [5′-ATAGAAAGATAAGAAATACCATTC-3'] was applied. For the second PCR, we used the primer pair 3760F [5′-GAGTGGATGGTGTTTTA-GAT-3'] and HaemJR4 [5′-GAAATACCATTCTGGAACAATATG-3']. These primers amplify a 542 bp fragment of the cyt *b* gene present in all three genera: *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (Pérez-Rodríguez et al., 2013). The same temperature conditions were used as in Waldenström et al. (2004). In total, 25 μL of PCR mix was used: 12.5 μL of Dreamtaq Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania), 8.5 μL of nuclease free water, 1 μL of each primer, and 2 μL of template DNA. To identify potential contaminations and false amplifications, negative (nuclease-free water) and positive (sample infected with *Plasmodium* sp. parasite) controls were used every 10 samples. All samples were tested on a 2% agarose gel with 2 μl of the final PCR product. Gel was stained using Midori green Advance DNA stain (Nippon Genetics Europe, Germany), the PCR products were stained with TrickTrack DNA loading dye (Thermo Fisher Scientific, Lithuania). And to estimate the bands length the GeneRuler was used as a ladder (Thermo Fisher Scientific, Lithuania). The Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISMTM 3100 capillary sequencing robot were used to sequence amplified samples from the 3′ and 5′ ends (Applied Biosystems, Foster City, California). Geneious Prime® v.2023.2.1 (https://www.geneious.com) was used to align and examine the sequences. Co-infection was defined as the presence of double-base calling in chromatogram.

2.3. Phylogenetic analysis

A Bayesian phylogenetic tree was built using the nine obtained *Leucocytozoon* spp. cyt *b* gene sequences with 46 *Leucocytozoon* spp. and one *Plasmodium relictum* (pSGS1) (outgroup) sequence retrieved from GenBank. The best fit model (GTR + I + G) was suggested by the

software jModeltest-2.1.10 (Darriba et al., 2012; Guindon and Gascuel, 2003). The phylogenetic tree was generated using Geneious Prime® v.2023.2.1 (https://www.geneious.com) with MrBayes plugin v2.2.4 (Ronquist and Huelsenbeck, 2003). The analysis was run for 5 million generations with a sampling frequency of every 100th generation, with 25% of the initial trees discarded as a 'burn-in' period before the consensus tree was created. The tree was finalized in CorelDraw (2019) (RRID: SCR_014235, https://www.coreldraw.com/en/).

2.4. Statistical analysis

The relationship between the infection detection probability of the nestlings (binary dependent variable: 0 – not infected, 1 – infected) and bird species (explanatory categorial variable with the three species included) were analysed using a generalised linear model fitted by the binomial family and logit link function. We also analysed the year effect for infection probability of the lesser spotted eagle and white-tailed eagle nestlings because annual samples were available for these two species, representing four and three years, respectively. The model (fitted by binomial family and logit link function) built relating eagles nestlings infection probability (binary dependent variable: 0 – not infected, 1 – infected) and year factor (explanatory categorical variable). To determine whether the models with both categorical explanatory variables provided a better fit to the data compared to corresponding models with only an intercept, we used the Akaike information criterion corrected for small sample sizes (AICc) with the threshold delta ≥ 2. All regression analyses were performed in the R statistical environment (version 4.3.2; R Core Team, 2023), with the following packages used: *lme4* (Bates et al., 2015), *MuMIn* (Barton, 2019).

3. Results

3.1. Prevalence of haemosporidian parasites in nestlings

We analysed blood samples from nestlings of birds of prey ($n = 118$), including the lesser spotted eagle ($n = 65$), white-tailed eagle ($n = 38$), and common buzzard ($n = 15$), and found haemosporidian parasites in all three species. The overall prevalence of haemosporidian parasites by PCR in nestlings was 30.5%. The common buzzard had the highest prevalence (80%, 12 out of 15 individuals) of haemosporidian parasites, while the white-tailed eagle had the lowest (13.2 %, 5 out of 38 individuals). Meanwhile, the prevalence of parasites in lesser spotted eagles was 29.2% (19 out of 65 individuals).

The relationship between infection detection probability of the nestlings (dependent variable) and bird species (explanatory variable) was analysed using a generalised linear model, which was highly supported by the data (i.e., AICc = 129 vs. corresponding null model AICc = 147) indicating that infection detection probability in the nestlings is significantly species dependent. The result of the model indicates that infection detection was statistically more frequently observed in common buzzard nestlings compared to the lesser spotted eagle (2.27 ± 0.7 SE, $P < 0.002$) and white-tailed eagle nestlings (3.27 ± 0.8 SE, $P < 0.0001$). Also, the detection probability of infection was nearly significantly smaller in the white-tailed eagle nestlings compared to the nestlings of the lesser spotted eagle (-1 ± 0.55 SE, $P = 0.07$) (Fig. 1).

We also analysed the year effect for the infection probability of the lesser spotted eagle and the white-tailed eagle nestlings. The model relating eagle nestling infection probability (dependent variable) and year factor (explanatory variable) was not supported by the data (i.e., the null model AICc = 114 vs. the model including year factor AICc = 120). These results indicate the lack of significant variation in infection detection probability in nestlings of eagles across years, and best evident in the similarly sampled lesser spotted eagle nestling infection frequency in three subsequent years, 2019, 2020 and 2021 (Fig. 2).

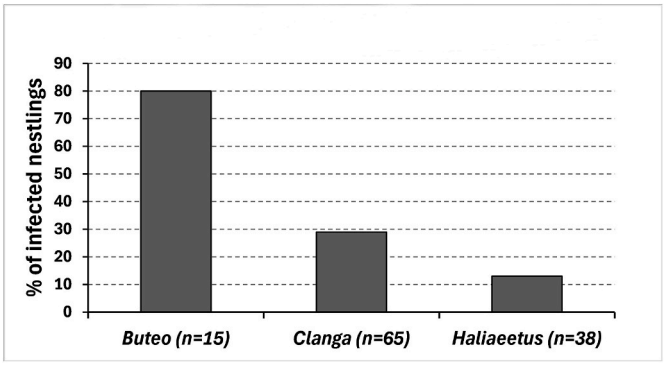

Fig. 1. Infection frequency of common buzzard ("*Buteo*"), lesser spotted eagle ("*Clanga*"), and white-tailed eagle ("*Haliaeetus*") nestlings.

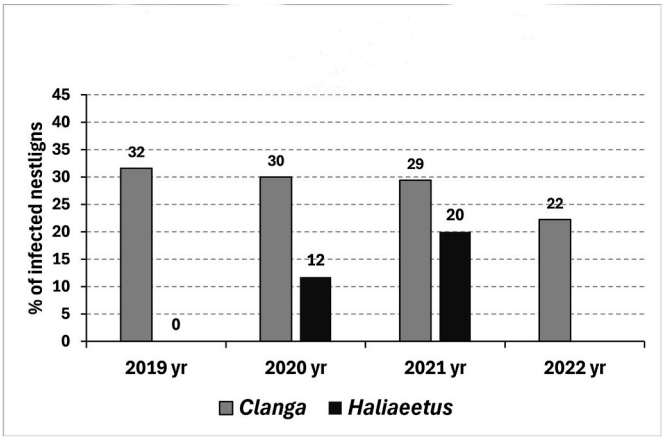

Fig. 2. Annual infection frequency of the lesser spotted eagle ("*Clanga*") and white-tailed eagle ("*Haliaeetus*") nestlings in 2019–2022. The annual sample for the lesser spotted eagle was 19 nestlings in 2019, 20 in 2020, 17 in 2021, and nine in 2022; for the white-tailed eagle, six nestlings were sampled in 2019, 17 in 2020, and 15 in 2021. White-tailed eagles in 2022 were not sampled. The common buzzard was not included in this analysis due to too small sample size.

3.2. Haemosporidian parasites in nestlings

Nestlings of all three sampled birds of prey species were infected only with the genus *Leucocytozoon* (Table 1). The white-tailed eagle was infected only with the lineage lMILVUS01. Meanwhile, lesser spotted eagle nestlings were infected with the lineages lMILVUS01, lMILANS04, four new lineages (lCLAPOM09, lCLAPOM10, lCLAPOM11, lCLAPOM12), and one redescribed lineage (lCLAPOM13, previously described as Raptor16). The most prevalent lineage in the lesser spotted eagle nestlings was lMILVUS01 (42.1 %, 8 out of 19 infected individuals), other lineages were detected only once. The common buzzard was infected with four *Leucocytozoon* spp. lineages (lMILVUS01, lMILANS04, lBUTBUT03, and lBUTBUT18), one of them being new (lBUTBUT18).

We detected co-infections in five lesser-spotted eagle nestlings and in two common buzzards. One of them was infected with a combination of lBUTBUT03 and the new lineage, lBUTBUT18. The co-infection could be distinguished because these two lineages have only one base pair of difference and they were clearly distinguishable in the chromatogram. No co-infection was detected in the white-tailed eagles.

3.3. Microscopic analysis

Twenty-eight individuals were positive both by microscopy and molecular analyses, while eight were positive only by molecular assays

Table 1
Leucocytozoon spp. lineages found in nestlings of birds of prey in Lithuania.

Bird species	Tested/positive	Prevalence %	Lineage name	2019	2020	2021	2022	GenBank accession no.
Clanga pomarina	65/19	29.2 %	lMILVUS01	4	1	2	1	PP975489
			lMILANS04	1	–	–	–	PP975490
			lCLAPOM09	–	1	–	–	PP975491
			lCLAPOM10	–	1	–	–	PP975492
			lCLAPOM11	–	1	–	–	PP975493
			lCLAPOM12	–	1	–	–	PP975494
			lCLAPOM13	–	–	1	–	PP975495
			Co-infection	1	1	2	1	
Buteo buteo	15/12	80 %	lMILVUS01	X[a]	–	4	X[a]	PP975496
			lMILANS04	X[a]	1	5	X[a]	PP975497
			Co-infection	X[a]	1[b]	1	X[a]	PP975498 (lBUTBUT03) PP975499 (lBUTBUT18)
Haliaeetus albicilla	38/5	13.2 %	lMILVUS01	–	2	3	X[a]	PP975488
Total	118/36			6	10	18	2	

[a] No samples were collected that year.
[b] Co-infected lineages were separated (lBUTBUT03 and lBUTBUT18). The difference was 1 bp.

Table 2
Microscopic results and intensity of *Leucocytozoon* spp. parasitaemia in PCR-positive nestlings of birds of prey.

Bird species	PCR result	Microscopy result %
Clanga pomarina	lMILVUS01	+[b]
	lMILVUS01	+[b]
	lMILVUS01	+[b]
	lMILVUS01	+[b]
	lMILVUS01	+[b]
	lMILVUS01	+[b]
	lMILVUS01	+[b]
	lMILVUS01	—[a]
	lMILANS04	—[a]
	lCLAPOM09	+[b]
	lCLAPOM10	0.85
	lCLAPOM11	0.05
	lCLAPOM12	+[b]
	lCLAPOM13	—[a]
	co-infection	+[b]
	co-infection	—[a]
	co-infection	—[a]
	co-infection	—[a]
	co-infection	+[b]
Buteo buteo	lMILVUS01	0.07
	lMILVUS01	0.06
	lMILVUS01	0.025
	lMILVUS01	0.13
	lMILANS04	0.11
	lMILANS04	1.47
	lMILANS04	1.53
	lMILANS04	0.18
	lMILANS04	0.12
	lMILANS04	0.99
	co-infection	+[b]
	co-infection	+[b]
Haliaeetus albicilla	lMILVUS01	—[a]
	lMILVUS01	—[a]
	lMILVUS01	+[b]
	lMILVUS01	+[b]
	lMILVUS01	+[b]

[a] No parasites were detected in blood smears.
[b] Parasitaemia was too low to count, only a few parasites were detected in blood smear.

(Table 2). All PCR-negative slides were also negative by microscopy. The intensity of parasitaemia ranged from 0.025% to 1.53%. In the case of co-infections, either we did not see any parasites in the smear, or we saw only *Leucocytozoon* gametocytes, but the intensity was too low to count. Microscopic and morphological analysis of the samples that were positive for the lCLAPOM10 and lCLAPOM11 lineages revealed that these parasites belong to the *L. toddi* group (Fig. 3). The other novel parasite lineages could not be assigned to any morphological group due to the too low intensity of parasitaemia.

3.4. Phylogenetic analysis

A phylogenetic tree was created to represent both newly described and previously known *Leucocytozoon* spp. parasites (Fig. 4). In total, five new lineages were detected in nestlings (lCLAPOM09, lCLAPOM10, lCLAPOM11, lCLAPOM12 and lBUTBUT18) and one redescribed (lCLAPOM13). The lineage lCLAPOM10 formed a separated clade with the lineages lCLAPOM04 and lCLAPOM05. There is a difference of 21 bp (4.4%) between lCLAPOM10 and lCLAPOM04 and 22 bp (4.6%) between lCLAPOM10 and lCLAPOM05. The lineages lCLAPOM11 and lCLAPOM12 clustered (the difference is 1 bp or 0.21%) and formed a separated clade. One lineage (lCLAPOM13) was separated from the other lineages. The lineage lCLAPOM09 clustered with lMILVUS01 with a 1 bp difference (0.21%), the same as for the lineages lBUTBUT18 and lBUTBUT03, which have also grouped together. The closest lineage to lMILANS04 was lCLAPOM06, with a genetic difference of 41 bp (8.58 %).

4. Discussion

In this study, we have shown that transmission of *Leucocytozoon* parasites of birds of prey occurs in temperate forests in Lithuania, as revealed by sampling the nestlings. Infection of nestlings shows that suitable vectors (Simuliidae) are available in this region, and they are able to reach nestlings in the nests, located in the tree canopies, usually within 12–30 m above the ground. Therefore, our findings indicate that 1) nestlings of birds of prey are infected with *Leucocytozoon* parasites in temperate forests in Lithuania with a prevalence of 30.5%, but with significant differences among bird species and similar prevalence across years; 2) investigated nestlings (white-tailed eagle, lesser spotted eagle, and common buzzard) are infected only with the genus *Leucocytozoon*. Nine genetic lineages were detected in sampled individuals, five of which are new; 3) phylogenetic relationships were determined between the newly identified lineages and other genetic lineages found in birds of prey; and 4) combining both molecular data and traditional microscopic observations provided a more comprehensive evaluation of parasite infections in birds of prey.

In our study, we determined that nestlings of birds of prey are infected with *Leucocytozoon* parasites in Lithuania. Frequent detection of *Leucocytozoon* parasites in nestlings of different birds of prey species and similar prevalence across years indicate the high availability of appropriate vectors in Lithuania (Valkiūnas, 2005). The white-tailed eagle nestlings had the lowest prevalence (13.16%), lesser spotted eagles had intermediate (29%), and common buzzards had the highest prevalence (80%). Despite that, all three species breed sympatrically in the region

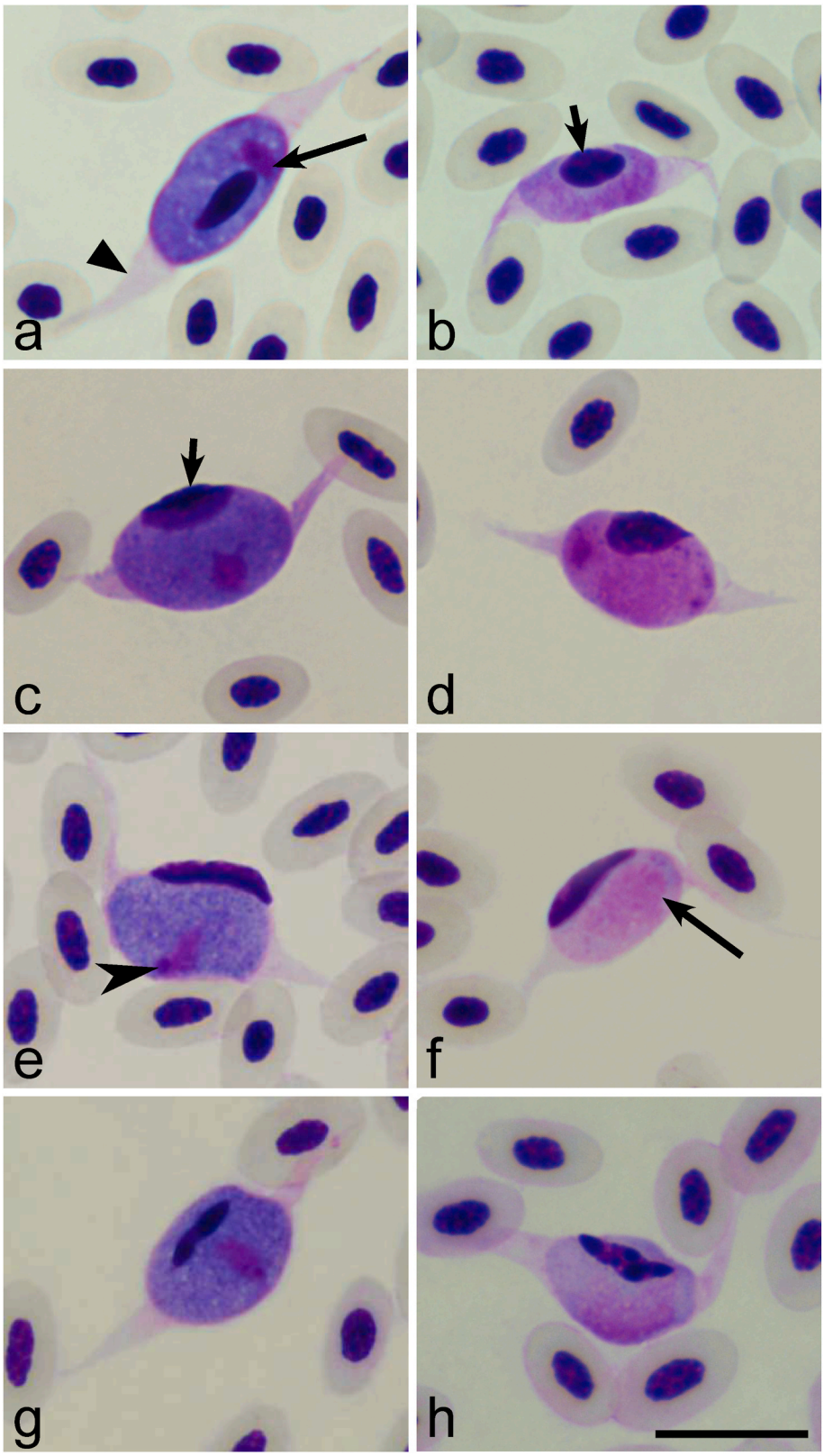

Fig. 3. *Leucocytozoon toddi* group macrogametocytes (a, c, e, g) and microgametocytes (b, d, f, h). A, b – lMILVUS01 genetic lineage, c, d – lMILANS04, e, f – lCLAPOM10 and g, h – lCLAPOM11. Long simple arrow indicates – parasite nucleus, arrowhead – parasite nucleolus, triangle arrowhead – fusiform processors of host cell, short simple arrow – host cell nucleus. Scale – 10 μm. Methanol fixed and Giemsa-stained.

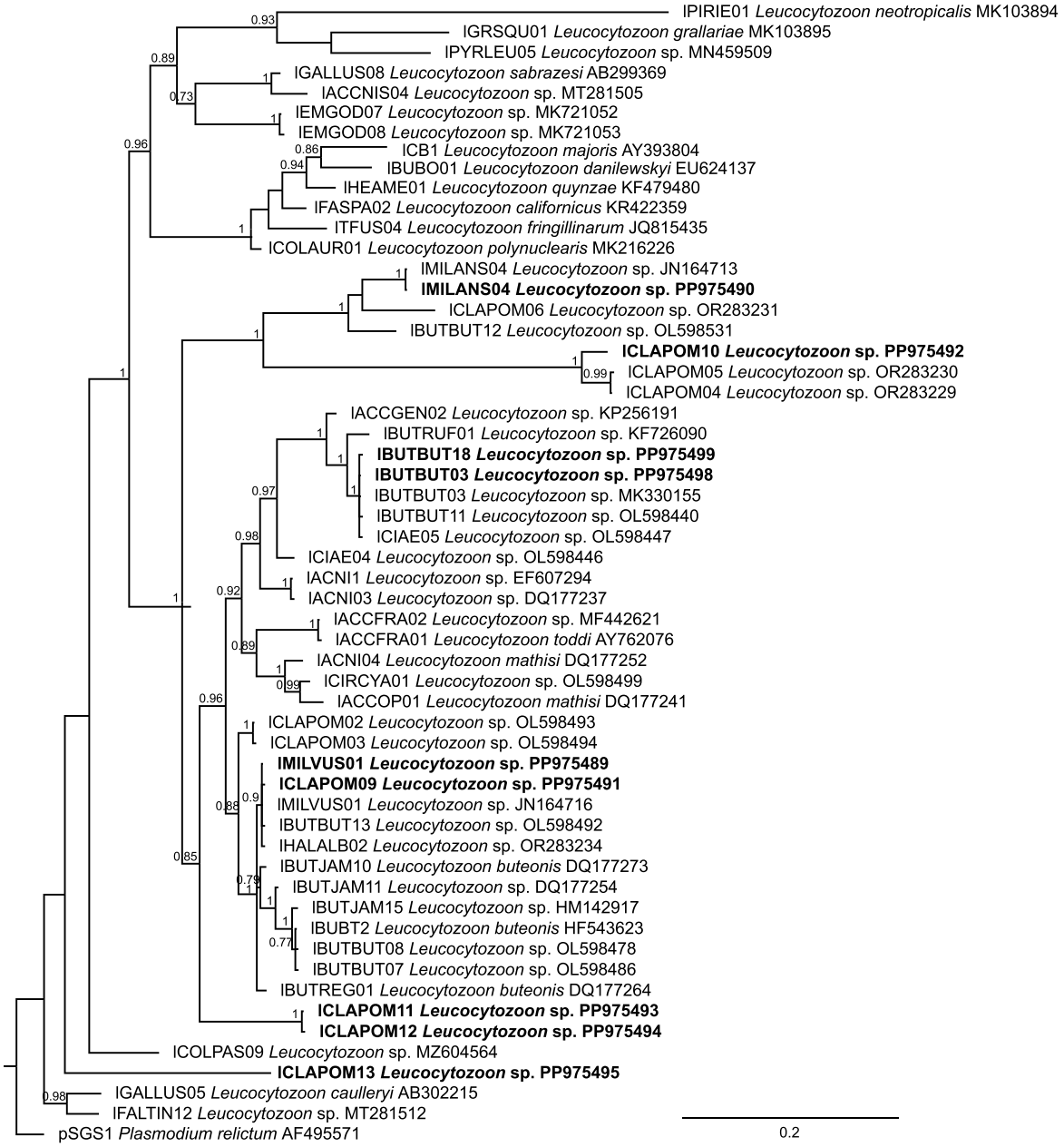

Fig. 4. Bayesian phylogeny of the cyt *b* gene lineages (478 bp) of 46 *Leucocytozoon* spp. retrieved from GenBank among which nine were obtained in this study (indicated in bold). *Plasmodium relictum* was used as an outgroup. Parasite lineages, species when identified and GenBank accession numbers are provided. Posterior probabilities higher than 0.7 are indicated.

and nests are located even in the same forests, it is possible that complementary factors such as fine scale nest site requirements, temporal segregation in breeding phenology, and adult infection rate might explain these differences.

The white-tailed eagle nests are built high in the old trees, usually close to their tops between 20 and 30 m from the ground, and the nests are typically exposed and not covered by the surrounding tree crowns (R.T. personal observation), resulting in windy conditions. This might explain the lower number of infections found in this raptor species. The lesser spotted eagles and the common buzzards usually build their nests in the middle part of trees, 17 m on average from the ground for both species (Kamarauskaitė et al., 2019), and nest trees are mainly located within stands of old trees. Therefore, their nestlings may be more accessible to vectors compared to white-tailed eagle nestlings. Vectors abundance variation in different heights was demonstrated by the results of a recent study in the same area, where marked differences were found in the average number of *Culicoides* females collected at 13 m and 23 m heights from the ground in mature forests (Bernotienė et al., 2024). Also, studies in Sweden (Swanson et al., 2012) have shown that black flies are successfully caught at heights of 1.5, 5, and 10 m and found that their abundance at different heights depends more on the insect species and forest type. Meanwhile, study in Germany showed that Simulidae are quite abundant at medium and high levels of canopy (Chakarov et al., 2020). It is important to note that some studies have determined that mosquitoes are more abundant at ground level, but they are also caught high in the canopy, but in small amount as study in Unites States determined that mosquitoes were more abundant at ground level compared to high canopy (in total 81 mosquitoes were investigated) (Khan et al., 2024). Bernotienė et al. (2024) found that 32.4% of the mosquitoes (in total 34 individuals) were trapped in the

high canopy (about 23 m).

White-tailed eagle nestlings hatch earliest in mid-April, followed by the common buzzards in mid-May (Kamarauskaitė et al., 2020), and the latest hatched nestlings are of lesser spotted eagles in the first half of June (Dementavičius et al., 2019). Following the breeding phenology of these species, we sampled white-tailed eagles between May 25 and June 7, common buzzards between June 15 and June 21, and lesser spotted eagles between July 12 and July 19. Different timing of nestling development in the nests for each bird of prey species may result in different amounts of vectors during their nestling development because of season-dependent peaks in vector abundance. For example, Bernotienė et al. (2024) demonstrated that in old forests of the same area, the numbers of *Culicoides* females collected at 13 m height strongly differed in June and July, supporting the idea of the different number of vectors and thus the probabilities of infection for nestlings of different species.

Vectors can be attracted by infected parent birds, as Díez-Fernández et al. (2020) showed that birds infected with *Plasmodium* parasites are more likely to be bitten by mosquitoes than uninfected birds because *Plasmodium* parasites manipulate the bird's odour to attract more mosquitoes. Indeed, this mechanism could be useful in explaining the results of this study because only 7% of white-tailed eagle adults were infected by blood parasites, while the proportion of infected individuals among adults of lesser spotted eagles was 44% in the same region (Bukauskaitė et al., 2024).

Our research shows that nestlings of birds of prey in Lithuania are infected only with the genus *Leucocytozoon* spp. Wiegmann et al. (2021) also found that nestlings of the common buzzard, red kite, and northern goshawk were infected with *Leucocytozoon* (51.9%), with the highest prevalence found in the common buzzard (54.9%). However, adult birds of prey recently studied in Lithuania were found infected with all three genera of haemosporidian parasites (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) (Bukauskaitė et al., 2024), which is confirmed by the MalAvi database, with *Leucocytozoon* being the most commonly found genus. Krone et al. (2008) also found all three genera (*Leucocytozoon*, *Haemoproteus*, and *Plasmodium*) in adult common buzzards, with a prevalence of 47.6%. Based on above mentioned results, we suggest that parasite diversity in birds of prey could be underestimated if only nestlings are sampled. First, the nestlings sampled in our study were in the nests and usually only four to six weeks old. It is likely that individuals are infected with other genera of parasites later in life, especially during post fledgling dispersal (de Angeli Dutra et al., 2021). Second, it is important to note that the prepatent period of *Leucocytozoon simondi* is five days, whereas the prepatent period of *Haemoproteus* parasites varies from 11 days to three weeks (Valkiūnas, 2005). However, the life cycle of the genus *Leucocytozoon* is relatively poorly investigated compared to *Haemoproteus* and *Plasmodium* and that the prepatent period may vary between different *Leucocytozoon* species. Despite the fact that the prepatent period for parasites belonging to the genus *Plasmodium* is also five days (Valkiūnas, 2005), we were unable to detect their presence in the species studied.

Despite being infected with only one genus of parasite (*Leucocytozoon*), a total of nine different genetic lineages were detected in our study, of which five are new and one is redescribed. Morphology showed that the new lineages (lCLAPOM10 and lCLAPOM11) belong to the *L. toddi* group, just as lMILVUS01 and lMILANS04. This group combines several species that are morphologically very similar but genetically very different (Valkiūnas et al., 2010; Valkiūnas and Iezhova, 2023), and therefore the new lineages cannot be assigned to a particular morphospecies. It is important to mention that the lineage lCLAPOM13 has previously been described as the lineage Raptor16 (GenBank accession number KT944100) and assigned to *Haemoproteus* sp. However, the phylogenetic tree (Fig. 4) showed that this lineage group together with *Leucocytozoon* spp., as an outer and more ancient branch among other *Leucocytozoon* spp. Unfortunately, parasites in blood smears were not seen, and we cannot assign this parasite lineage to any *Leucocytozoon* morphospecies.

While molecular techniques are crucial, microscopy is the only method that allows us to identify parasite species and classify novel lineages within a specific parasite species. However, microscopy has its limitations. At extremely low levels of parasitaemia, it is possible to overlook the presence of parasites in a blood smear. According to the findings of the research conducted in this paper, the PCR approach identified a greater number of infected nestlings compared to microscopy, this shows that PCR is more sensitive than microscopy (Table 2). This demonstrates the significance of combining both molecular and morphological techniques, as they complement each other and provide more comprehensive information.

5. Conclusions

In conclusion, our study provides important information on the infection of nestlings of birds of prey with haemosporidian parasites in temperate forests in Lithuania. Infection of the nestlings indicates that there is a regular local transmission of *Leucocytozoon* spp. in the region and that there are suitable vectors (Simuliidae). We only detected the *Leucocytozoon* genus in the studied individuals; however, we identified nine genetic lineages, five of which were novel, and two of which we assigned to the *L. toddi* group using microscopy. This research highlights the importance of understanding parasite transmission dynamics and the ecological factors of host and vector behaviour in influencing infection rates.

CRediT authorship contribution statement

Dovilė Bukauskaitė: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Carolina Romeiro Fernandes Chagas:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Mélanie Duc:** Writing – review & editing, Visualization, Formal analysis. **Margarita Kazak:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Rimgaudas Treinys:** Writing – review & editing, Visualization, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Conceptualization.

Ethical consideration

Blood samples collection from birds of prey were approved by the Environmental Protection Agency, Vilnius, Lithuania. Permits 2019-04-19 No. SR-156; 2020-06-01 No. (26)-A4E-4679; 2021-06-11 No. (26)-SR-104; 2022-06-03 No. SR-168.

Data availability

The sequences were deposited in the GenBank (PP975488-PP975499) and MalAvi databases. Representative blood films (accession nos. 49808–49811) were deposited at the Nature Research Centre, Vilnius, Lithuania.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

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