

FIRST ASSESSMENT OF THE PREVALENCE OF HAEMOSPORIDIAN INFECTIONS IN ACCIPITRIFORMES RAPTORS IN GREECE

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Introduction

The common buzzard (*Buteo buteo*) and the Eurasian sparrowhawk (*Accipiter nisus*), both belonging to the order Accipitriformes (the biggest group of raptors which includes the most endangered avian species in the world) are the most numerous raptor species in Europe and they are endemic in Greece, a country-crossroad to the migration of birds (Harl et al., 2022).

Raptors host many parasites. Important endoparasites are haemosporidians (Apicomplexa: Haemosporida), single-celled eukaryotic protozoa that infect the blood cells and other tissues of birds globally, using blood-sucking dipteran insects as vectors (Hellgren et al., 2004). Species belonging to the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are the ones of most wildlife importance (Valkiūnas, 2005).

Although haemosporidian infections were usually associated with asymptomatic infections, there are more recorded cases of birds suffering from severe clinical symptoms or even dying (Valkiūnas et al., 2023). It is reported that avian blood parasites may potentially cause significant declines in bird populations and even extinctions (Dadam et al., 2019). Haemosporidiosis may also have a negative effect on aviculture with important financial consequences (Valkiūnas, 2005). Studying the blood parasites in wild birds, which are characterized as living sentinels, is important as the avifauna is a key-factor for the maintenance and transmission of many pathogens in other animals and humans (Hamer et al 2012).

Data is currently limited worldwide and lacking in Greece. The objectives of this study were to determine, for the first time, the prevalence and the lineages diversity of haemosporidian infections of common buzzards and Eurasian sparrowhawks in Greece and to subsequently correlate them with sex, age and region of origin.

Materials & Methods

This study involved 62 common buzzards and 26 Eurasian sparrowhawks. These birds were admitted for treatment to “ANIMA, Association for Wildlife Care and Protection” in Athens, Greece.

Blood samples (100 µL) were collected from each bird which and stored in SET buffer (0.015M NaCl, 0.05M Tris, 0.001M ethylenediaminetetraacetic acid, pH 8, 1:10).

Moreover, blood smears were prepared and stained with Giemsa for the morphological identification of the parasites and the assessment of the infections' intensity. DNA was extracted following the Quick-DNA™ Miniprep Kit by Zymo Research. Samples were screened for positive infections following a modification of the nested PCR protocol (Waldenström et al., 2004), developed by Perez-Rodriguez et al. (2013), which amplifies a fragment of the mitochondrial cytochrome b gene of all three haemosporidian genera. The protocol involves a first PCR step using the primers Plas1F (5'-GAGAATTATGGAGTGGATGGTG-3'; Duval et al., 2007) and HaemNR3 (5'- ATAGAAAGATAAGAAATACCATTCC-3'; Hellgren et al., 2004), followed by a nested PCR step with the internal primers 3760F (5'- GAGTGGATGGTGTTTTAGAT-3'; Beadell et al., 2004) and the HaemJR4 (5'- GAAATACCATTCTGGAACAATATG-3'). Those that were positive were subjected to a second nested PCR using initially the primers HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') and HaemNR3 (5'- ATAGAAAGATAAGAAATACCATTCC-3') to amplify parasite mtDNA and then HaemF (5' – ATGGTCTTTTCGATATATGCATG-3') and HaemR2 (5'- GCATTATCTGGATGTGATAATGGT-3') primers (Bensch et al., 2000) for *Plasmodium* spp. and *Haemoproteus* spp. (Hellgren et al., 2004). Second PCR products were sequenced using Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISMTM 3100 capillary sequencing robot (Applied Biosystems, Foster City, CA, USA).

• Binary logistic regression model: $YG = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3$

YG = the probability of a bird being positive for i) at least one haemosporidian species, ii) specific haemosporidian genera, β_1 to β_3 the regression coefficients of bird species (X_1 , 0 = *Buteo buteo*, 1 = *Accipiter nisus*), age (X_2 , 0 = young, 1 = adult), and sex (X_3 , 0 = male, 1 = female)

Results

	MALES				FEMALES											
	ADULTS		SUBADULTS		ADULTS		SUBADULTS									
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative								
	P	L	P+L		P	L	P+L		P	L	P+L					
Common buzzards	0	10	1	5	1	9	1	10	0	8	0	7	0	7	2	1
Eurasian sparrowhawks	0	0	1	0	0	0	1	0	0	11	3	0	0	6	4	0

Table 1. Summary of the molecular (PCR) results (L: Infection by *Leucocytozoon* spp., P: Infection by *Plasmodium* spp., P+L: Mixed infection by *Leucocytozoon* spp. & *Plasmodium* spp.)

Eurasian sparrowhawks were 11.1 times more likely to be infected by *Plasmodium* spp. (95% CI, 2.0-50.0; $p < 0.01$). The likelihood of the Eurasian sparrowhawks being infected by a mixed haemosporidian infection was 9.1 times higher (95% CI, 2.3-33.3; $p < 0.01$). In all cases, age and sex had no significant effect on molecular positivity.

Leucocytozoon lineages	Plasmodium lineages
BUBT2 (13), BUBT3 (8), MILANSO4 (2), MILVUS01 (8), BUBO01 (1), ACNI1 (1), BUTBUT17 (2) NEW!	TURDUS1 (4), DONANA2 (1)
ACNI1 (11), ACNI04 (3), MILANSO4 (6)	TURDUS1 (7), BT7 (2)

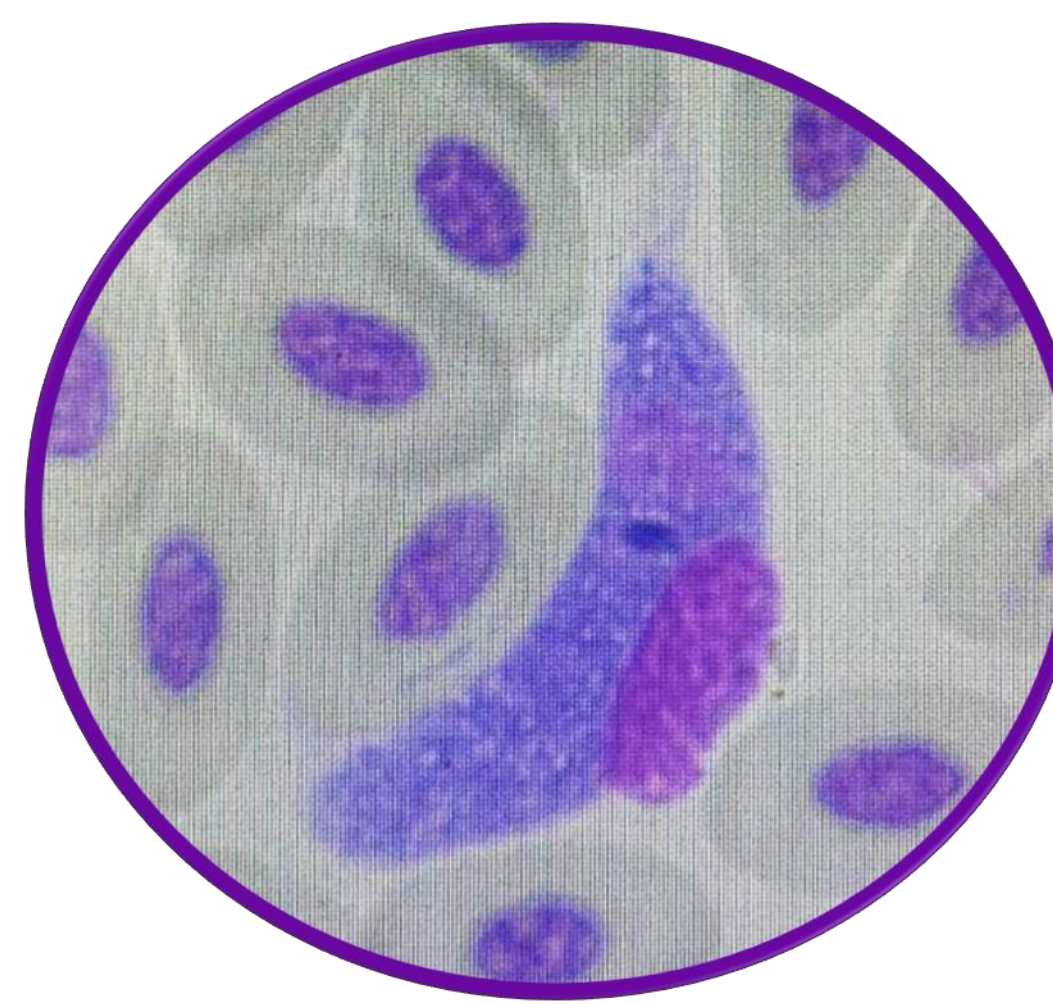


Table 2. Genetic lineages of haemosporidians isolates

Figure. Geographical regions of Greece were infected birds originated from.



In all cases, the intensity of the microscopically detected infections was < 1 parasite/20,000 erythrocytes.

Discussion / Conclusion

The occurrence of *Leucocytozoon* spp. infection detected by PCR was remarkably high (72.7%). *Leucocytozoon* spp. are cosmopolitan parasites, thriving in hot and cold climates (Valkiūnas et al., 2023) and their vectors cover extremely large distances (Chakarov et al., 2021). This probably explains their high prevalence, in addition with a high dispersion of the black flies in the studied regions of Greece (Adler et al., 2016). Regarding sex as a risk factor of infection, the “parent-to-offspring” infection pattern that has been noted (Chakarov et al., 2015) is not verified by our findings. Moreover, according to this study, the bigger size of the females is not significant to affect the infection prevalence between sexes. Furthermore, despite the fact that the excessive production of kairomones by females was speculated to be appealing to the vectors, sex differences are more probable in species with uniparental incubation (Grieves et al., 2022), whilst raptors are generally biparental (Wreford, 2014). *Plasmodium circumflexum* was the most frequent *Plasmodium* species isolated. It is not an Accipitriformes specific parasite and this highlights migration as another factor that seems to affect the haemosporidian infection of wild birds, and the possible exchange of lineages among them, and it has to be investigated. Both common buzzards and Eurasian sparrowhawks are recorded to migrate (Panuccio et al., 2021).

In the current study, no *Haemoproteus* spp. infections were detected (0%). Given that their vectors have been recorded in Greece (Alexiou et al., 2021), the low infection prevalence can be explained by the fact that the primers used might not be able to attach to the mitochondrial cytochrome b gene of species from that genus (Harl et al., 2022), or it may also be linked to the hosts' immune system's sufficiency (Gutiérrez-López et al., 2015). Another potential explanation is that a high parasitaemia causes high mortality rates to the infected birds during the acute phase, thus, reducing the detected occurrence (Ortego et al., 2007). This study underlines the necessity of further investigation of the prevalence, the diversity and the consequences of haemosporidiosis in raptors, a group of birds that is underrepresented in studies of haemosporidians.

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