

# Journal of the Hellenic Veterinary Medical Society

Vol 74, No 2 (2023)



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doi: [10.12681/jhvms.29714](https://doi.org/10.12681/jhvms.29714)

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### To cite this article:

Zerek, A., Erdem, I., Alakus, H., Yaman, M., & Altuğ, M. (2023). Prevalence of Haemoproteus and Leucocytozoon spp. in Wild Birds in Hatay, Turkey. *Journal of the Hellenic Veterinary Medical Society*, 74(2), 5651–5656. <https://doi.org/10.12681/jhvms.29714> (Original work published July 6, 2023)

## Prevalence of *Haemoproteus* and *Leucocytozoon* spp. in Wild Birds in Hatay, Turkey

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**ABSTRACT:** Hemosporidian parasites that cause infections in poultry and death in susceptible animals show a global distribution. Infected wild birds play an important role in this distribution due to seasonal migration among different regions. Hatay, where migratory birds enter Turkey, is an active region regarding vectors, disease agents and hosts. This study aimed to investigate the prevalence of *Haemoproteus* and *Leucocytozoon* parasites in wild birds of Hatay Province by microscopic and molecular methods. Blood samples were taken from a total of 50 wild birds belonging to three orders, eight species. In the microscopic examination, *Haemoproteus* spp. infection 18% (9/50) and coinfections (*Haemoproteus* spp. and *Leucocytozoon* spp.) 4% (2/50) were detected while in the PCR examination, coinfections 4% (2/50), *H. columbae* 20% (10/50) and *Leucocytozoon* spp. 2% (1/50) were detected. As a result, haemosporidian parasites were detected in approximately one of every four wild birds (microscopic 22%, molecular 26%).

**Keywords:** *Haemoproteus*; Hatay; *Leucocytozoon*; Turkey; wild bird

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Date of initial submission: 23-02-2022  
Date of acceptance: 19-03-2023

## INTRODUCTION

Worldwide, haemosporidian blood parasites are vector-borne parasites that infect mammals, amphibians, reptiles, and poultry. More than 200 haemosporidian parasites have been identified in the strains of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* that infect domestic and wild birds (Valkiunas, 2004). The vectors of the *Plasmodium* species are mosquitoes; vectors of the *Haemoproteus* species are *Pseudolynchia*, *Microlynchia* and *Culicoides*; vectors of the *Leucocytozoon* species are *Culicoides* and *Simulium*, which are hematophage flies. Species belonging to the *Haemoproteus* and *Leucocytozoon* strains are prevalent in poultry. *Haemoproteus meleagridis* in domestic and wild turkeys; *H. nettionis* in domestic and wild ducks, geese and a variety of poultry; *H. iophoryx* in quails; *H. sacharovi* in pigeons and doves; *H. columbae* in domestic and wild pigeons, doves and other wild birds are parasitized. *Leucocytozoon simondi* from waterfowl, *L. marchouxi* from pigeons and doves, *L. toddi* from birds of prey have been reported (Derinbay Ekici, 2017). Although it is stated that haemosporidian parasites are not very harmful to poultry, it has been stated that they cause severe infections resulting in death in wild birds when high parasitemia occurs (Palinauskas et al., 2016). These parasites can cause anemia, fever, weakening, loss of appetite, difficulty in breathing, limping and decrease in lifespan, loss of productivity, changes in metabolic activities such as migration and reproduction, impairments in thermoregulation, and even deaths in poultry (Atkinson et al., 2000). In pathological findings, liver, spleen and kidney enlargement has been encountered (Valkiunas, 2004).

Microscopic and molecular studies on the presence of haemosporidian parasites have been carried out in domestic (Öz and Turut, 2007; Sürsal et al., 2017; Tasci et al., 2018) and wild birds (Balkaya et al., 2016; Ciloglu et al., 2016; Ciloglu et al., 2020; Marzal and Albayrak, 2012; Özmen et al., 2006; Özmen et al., 2009; Yildirim et al., 2013) in different provinces of Turkey.

Turkey is a crucial Europe region due to its diverse ecosystems and its location on the migration routes of migratory birds. They constitute approximately 20% of the recorded bird species in Turkey. The province of Hatay is a critical region where migratory birds enter Turkey in the spring period and leave in the autumn period (Yaman et al., 2018). The prevalence of haemosporidian parasites in migratory birds that have

the potential to encounter more vectors is higher than in those that do not migrate (Møller and Erritzøe, 1998). Hatay Province has a crucial position in migratory birds, yet no study on this subject is up to date.

This study aimed to investigate the prevalence of *Haemoproteus* and *Leucocytozoon* parasites in wild birds in Hatay by microscopic and molecular methods and contribute to the literature in this field.

## MATERIALS AND METHODS

### Ethical Statement

This study was performed with the Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee decision. It was decided that ethics committee approval was not required. (Approval no: 2019/06-4, date: 31.05.2019).

### Features of the Work Areas

The province of Hatay, where the temperate Mediterranean climate prevails, has an area of approximately 6,000 km<sup>2</sup> at 36 ° 14 North and 36 ° 10 East coordinates (Muz et al., 2015).

### Collection of Blood Samples

In the study, 1 ml of blood samples was taken from vena subcutanea ulnaris to blood tubes with anticoagulant substance from a total of 50 wild birds belonging to three orders, eight species (determined with the help of the relevant literature) (Kiziroğlu, 2013; Porter et al., 2009). They were brought to the HMKU Veterinary Health, Practice and Research Center as wounded between May 2018 and January 2020.

### Microscopic Method

The smears prepared from each of the blood samples brought to the laboratory of Parasitology were stained with the Giemsa solution and examined under the light microscope with the help of the relevant literature (Valkiunas, 2004). The remaining parts of the blood samples were stored at -20 °C for molecular analysis.

### Molecular Method

The genomic DNA was extracted using the protocol of the commercial kit of Roche. The extracted DNA was stored at -20 °C until it was used in PCR. Then, PCR was performed using primers specific to the mitochondrial *cytochrome b* (mt-cytb) gene region of *Haemoproteus* and *Leucocytozoon* parasites.

The primers of PCR reaction for detection of *Haemoproteus columbae* were as follows: *H. clom-F*: 5'-TTA GAT ACA TGC ATG CAA CTG GTG-3' and *H. clom-R*: 5'-TAG TAA TAA CAG TTG CAC CCC AG-3'. PCR amplification was carried out in 20 µl reaction mixtures containing 4 µl 5x Master Mix (Solis BioDyne), 1 µl of each primer (10 pmol), 1 µl DNA (100 ng/µl) and 13 µl water (PCR grade). PCR was performed as follows: initial denaturation at 94 °C for 5 min followed by 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. PCR products were separated by electrophoresis on 1% agarose gels and were visualized under UV light in terms of the presence of specific bant. The size of *H. columbae* applicant was 207 base pairs (bp) (Doosti et al., 2014).

The extracted DNA was used in nested PCR reactions to amplify *Leucocytozoon* spp. For the first amplification, primers were used DW2: 5'-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3', and DW4: 5'-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3'. The primers were described by Perkins and Schall (Perkins and Schall, 2002). PCR amplification was carried out in 20 µl reaction mixtures containing 4 µl Master Mix (5x, Solis BioDyne), 1 µl of each primer (10 pmol), 1 µl DNA (100 ng/µl) and 13 µl water (PCR grade). The first PCR reaction was performed as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles consisting of denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, extension at 72 °C for 1 min and final extension at 72

°C for 10 min. For the second PCR reaction, primers were used belonging to the mitochondrial *cytochrome b* (mt-cytb) gene region specific to *Leucocytozoon* spp. These primers were Leuco-F: 5'-TCT TAC TGG TGT ATT ATT AGC AAC-3' and Leuco R: 5'-AGC ATA GAA TGT GCAAAT AAA CC-3'. PCR amplification steps were carried out to be the same as the first PCR reaction. PCR products were separated by electrophoresis on 1% agarose gels and were visualized under UV light in terms of the presence of specific bant. The size of *Leucocytozoon* spp. applicant was 865 bp (Sehgal et al., 2006).

## RESULTS

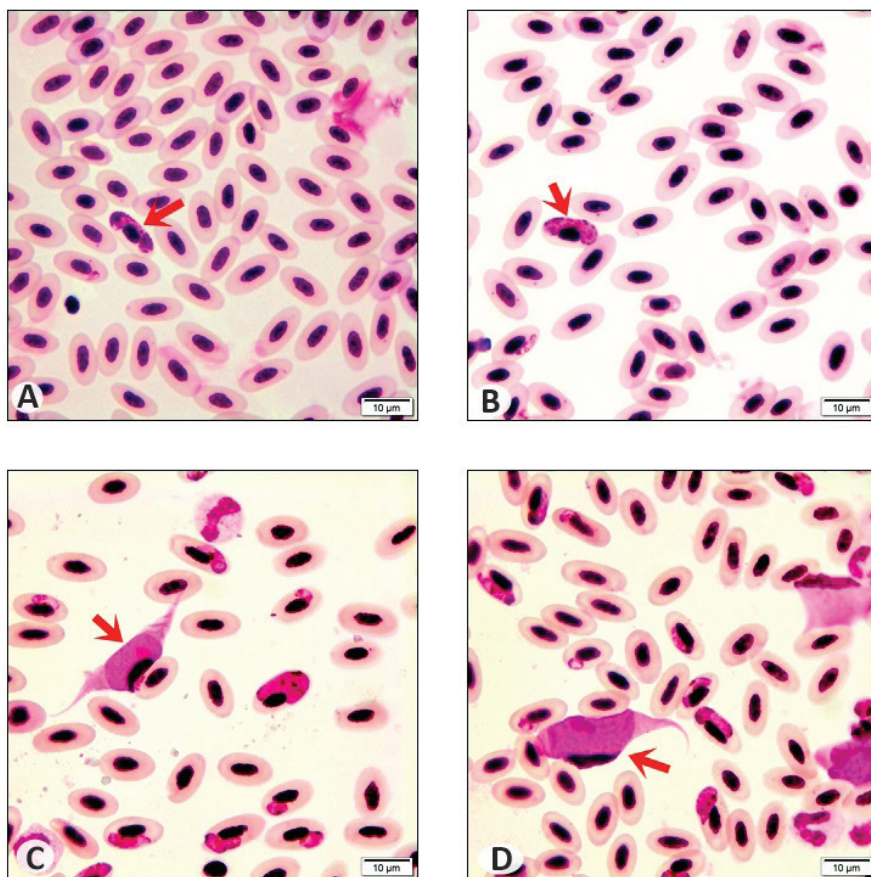
According to the microscopic results, only *Haemoproteus* spp. infection 18% (9/50) and coinfections (*Haemoproteus* spp. and *Leucocytozoon* spp.) 4% (2/50) were detected (Table 1). *Haemoproteus* spp. and *Leucocytozoon* spp. gametocytes were observed in the blood smears (Figure 1). In the PCR examination, *H. columbae* 20% (10/50), *Leucocytozoon* spp. 2% (1/50), coinfections (*H. columbae* and *Leucocytozoon* spp.) 4% (2/50) were determined (Table 1).

As a result, these parasites could not be detected in the Ciconiiformes (*Ciconia ciconia*) and Strigiformes (*Tyto alba*) orders and the *Pernis apivorus* species of the Accipitriformes order by molecular and microscopic methods. Haemosporidian parasites were detected in approximately one of every four wild birds of the Accipitriformes order (microscopic 22%, molecular 26%) (Table 1).

**Table 1:** Prevalence of haemosporidian parasites in wild birds in Hatay by microscopic and molecular methods

Orders	Species	Number of samples (n)	Number of positive samples (Microscope)				Number of positive samples (PCR)			
			H	L	Coinfections	Total	H*	L	Coinfections	Total
Accipitriformes	<i>Buteo buteo</i> (common buzzard)	17	4	-	-	4	4	-	-	4
Accipitriformes	<i>Clanga pomarina</i> (lesser spotted eagle)	11	2	-	2	4	2	-	2	4
Accipitriformes	<i>Circaetus gallicus</i> (Short-toed snake eagle)	9	1	-	-	1	2	1	-	3
Accipitriformes	<i>Buteo rufinus</i> (Long-legged Buzzards)	5	1	-	-	1	1	-	-	1
Accipitriformes	<i>Milvus migrans</i> (black kite)	3	1	-	-	1	1	-	-	1
Accipitriformes	<i>Pernis apivorus</i> (European honey buzzard)	1	-	-	-	-	-	-	-	-
Ciconiiformes	<i>Ciconia ciconia</i> (white stork)	3	-	-	-	-	-	-	-	-
Strigiformes	<i>Tyto alba</i> (western barn owl)	1	-	-	-	-	-	-	-	-
Total	8	50	9	-	2	11	10	1	2	13

H - *Haemoproteus* spp.; H\* - *H. columbae*; L - *Leucocytozoon* spp.



**Figure 1.** Thin smears from blood samples stained with Giemsa; A-B) *Haemoproteus* spp. gametocyte (arrowed), C-D) *Leucocytozoon* spp. gametocyte (arrowed) (x1000 magnification). Scale bar = 10 µm.

## DISCUSSION

The prevalence of haemosporidian parasites in wild birds in the world has been reported in various studies, and the reasons, such as the difference in bird species, haemosporidian parasites and geographical regions, make it difficult to compare the studies with each other (Inumaru et al., 2017). However, in some molecular studies conducted worldwide, the prevalence rate of *Haemoproteus* and *Leucocytozoon* parasites in wild birds is between 9% and 80.5%. In the Philippine Islands, in 215 wild birds, the infection was detected in a total of 26.4%, and the prevalence rates of *Haemoproteus*, *Leucocytozoon* and coinfections were 14.4%, 8.3% and 3.7%, respectively (Silva-Iturriza et al., 2012). In Slovakia, the rates of *Haemoproteus* and *Leucocytozoon* infections in 251 wild birds were 46.6% and 33.9%, respectively, and a total infection was 80.5% (Berthová et al., 2012). In 475 wild birds in Tokyo, Japan, the total infection rate was determined at 20%, the prevalence of *Haemoproteus* and *Leucocytozoon* was the same ratio as 8.8%, and coinfections were 2.4% (Inumaru et al., 2017). In another study, the total infection rate was found to be 9% in 55 wild birds, the *Haemoproteus*

prevalence rate was 3.6%, and *Leucocytozoon* prevalence rate was 5.4% (Tanigawa et al., 2012). In Indonesia, in 112 wild birds, *Haemoproteus* and *Leucocytozoon* the total infection rate was detected at 8% and 1.8%, respectively, and the total infection rate was 9.8% (Yuda, 2019). The prevalence of the species in the *Haemoproteus* lineage is generally higher than those of the *Leucocytozoon* and *Plasmodium* species in poultry (Atkinson and Van Riper, 1991). In this study, in 50 wild birds, *Haemoproteus columbae* and *Leucocytozoon* spp. were detected by PCR method at 20% and 2%, respectively; coinfections were 4%, and the prevalence rate of total infection was 26%. The *Haemoproteus* lineage was detected as higher than the *Leucocytozoon* lineage and was parallel to some studies conducted in different countries around the world, except for Japan (Inumaru et al., 2017; Tanigawa et al., 2012). In our study, wild birds infected with this blood parasite belong to the Accipitriformes order and generally prefer to live in a habitat of forestry, agriculture and open lands, in which *Leucocytozoon* spp. infection may have low prevalence. According to the literature that draws attention to the vector-host interaction (Krams et al., 2012), the risk of infection

with the *Leucocytozoon* lineage increase significantly with increasing proximity to rivers where blackflies (Diptera: Simuliidae) breed.

In this study, haemosporidian parasites were not detected in *Ciconia ciconia* (white stork) from the order Ciconiiformes, *Tyto alba* (Barn Owl) in the order Strigiformes, and *Pernis apivorus* (Bee hawk) in the order Accipitriformes. Although *Haemoproteus ciconiae* has been reported from the white stork (Valkiūnas et al., 2016), there are few studies about the diagnosis of blood parasites in these birds due to the wide living area and the difficulty of catching them (Valkiūnas, 2004; Valkiūnas et al., 2002; Jovani et al., 2002). It has been notified that these blood parasites were detected in some of the studies conducted on Barn Owls and Bee hawks (Salakij et al., 2018; Walther et al., 2016), however they were not detected in some of the studies (Krone et al., 2001; Sacchi and Prigioni, 1984). It is believed that these parasites were not found in this study due to examining only a few of the blood samples of wild birds in this order.

Turkey is on the migration routes of wild birds (Yaman et al., 2018), but there are few studies about the prevalence of haemosporidian parasites in wild birds, two of which are case reports (Özmen et al., 2009; Yildirim et al., 2013). While some of the studies used the microscopic method (Balkaya et al., 2016; Özmen et al., 2006; Özmen et al., 2009), some of them were conducted molecularly (Ciloglu et al., 2016; Ciloglu et al., 2020; Marzal and Albayrak, 2012; Yildirim et al., 2013). When looking at the molecular studies conducted in Turkey, *Haemoproteus* infection was detected in one Tawny Owl (*Strix aluco*) (Yildirim et al., 2013). The another study was conducted in 67 Anatolian nuthatches (*Sitta krueperi*), and the prevalence of *Haemoproteus* was 10.44% (7/67) while it was 13.43% (9/67) in *Leucocytozoon*. Coinfections had the same ratio as the last one. The total prevalence was 37,3% (25/67) (Marzal and Albayrak, 2012). *Leucocytozoon* infection was detected in 5 out of 22 (22.7%) predatory bird species in 6 different species (Ciloglu et al., 2016). In a study conducted in Sultan Sazlığı, there were 565 migratory birds from 39 species belonging to 23 families. A total of 193 (34.2%) birds were infected. Birds infected with *Haemoproteus* and *Leucocytozoon* were 168 and 6, respectively. Coinfections were 3.36% (19/565) (Ciloglu et al., 2020). Between our study and conducted the other studies, could not the relationship except for one (Ciloglu et al., 2020). The reason is that bird species, their localities and the

number of blood samples were different. Birds may not have encountered vectors or disease agents.

*H. columbae* parasitizes domestic and wild pigeons, doves and other wild birds (Derinbay Ekici, 2017). Although it is stated that infections with this species are apathogenic for birds, they can cause severe and fatal infections in young individuals whose immune system is not sufficiently developed (Sol et al., 2003). Based on the microscopic methods, *H. columbae* has been reported only in pigeons in Turkey (Karacaoğlu and Karatepe, 2017; Karatepe and Karatepe, 2018). The presence of *H. columbae* has not been reported in wild birds in the world except for pigeons (Al-Rubaie et al., 2020; Doosti et al., 2014; Nematollahi et al., 2012). With this study, the presence and prevalence of *H. columbae* were reported for the first time by PCR method in wild birds worldwide and in Turkey.

Although it is stated that microscopic diagnosis based on its prevalence and morphology in the host can be overlooked at low intensity (Valkiūnas et al., 2008), it has been claimed that it is a reliable tool even at low density and that the PCR test does not make a significant difference compared to microscopy (Krams et al., 2012). However, since the microscopic detection of parasites requires taxonomic expertise to identify the parasite species and stages of development accurately (Krams et al., 2012; Valkiūnas et al., 2008), it is recommended to verify the microscopic methods with molecular studies in today's conditions (Ciloglu et al., 2016; Valkiūnas et al., 2008; Valkiūnas et al., 2014). In this study, there was no significant difference between the microscopic findings and molecular findings. This situation supports the view of Krams et al. (2012).

## CONCLUSION

This study investigates the prevalence of *Haemoproteus* and *Leucocytozoon* parasites in wild birds in Hatay Province, which is located on the crucial migratory bird routes in Turkey. During their migration, wild birds cause to be transport haemosporidian parasites and spread to different parts of the world. For this reason, it is beneficial to carry out more detailed studies on the subject of wild birds in Turkey.

## ACKNOWLEDGEMENTS

This study was supported by the Hatay Mustafa Kemal University Scientific Research Project Coordination with the number 19.M.042.

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