



ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

**MOLECULAR CHARACTERIZATION OF AVIAN MALARIA HEMOPARASITES IN
WILD BIRDS**

Marcelo Francisco Leitão

Coimbra, Outubro de 2020



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Marcelo Francisco Leitão

Aluno do Mestrado Integrado em Medicina Veterinária

Constituição do Júri

*Presidente do Júri: Prof. Doutora Inês
Rebelo Crespo*

Arguente: Prof. Doutor Nuno Santos

*Orientador: Prof. Doutora Teresa Letra
Mateus*

Orientador Interno

Prof. Doutora Teresa Letra Mateus

Orientador Externo

Prof. Doutor Jacinto José Carneiro Gomes
(INIAV)

Dr.º Ricardo Brandão (CERVAS)

Eng.^a Ana Paula Sançana

“It is important to draw wisdom from many different places. If you take it from only one place, it becomes rigid and stale.”

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Lista de abreviaturas

BLAST – *Basic Local Alignment Search Tool*

CE - *Critically Endangered*, criticamente em perigo

CERVAS – Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens

CEVDI - Centro de Estudos de Vectores e Doenças Infecciosas Dr. Francisco Cambournac

DD - *Data Deficient*, informação insuficiente

EN – *Endangered*, em perigo

ICNF – Instituto da Conservação da Natureza e das Florestas

INIAV – Instituto Nacional de Investigação Agrária e Veterinária

NCBI – *National Center for Biotechnology Information*

NT - *Near Threatened*, quase ameaçado

VU – *Vulnerable*, vulnerável

® - marca registrada

Molecular Characterization of Avian Malaria Hemoparasites in Wild Birds

Marcelo Leitão^a, Ricardo Brandão^b, Jacinto Gomes^{c,d}, Teresa Letra Mateus^{a,e,f}

^a Departamento de Medicina Veterinária, Escola Universitária Vasco da Gama (EUVG), Av. José R. Sousa Fernandes 197, Campus Universitário- Bloco B, Lordemão, 3020-210, Coimbra, Portugal (marcelofranciscoleitao@gmail.com)

^b CERVAS / Associação ALDEIA, Apartado 126 6290-909 Gouveia (brandaoric@gmail.com)

^c National Institute for Agrarian and Veterinary Research (INIAV IP), Av. da República, Quinta do Marquês, Edifício Principal, 2780-157, Oeiras, Portugal (jacinto.gomes@iniav.pt)

^d Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Portugal

^e Epidemiology Research Unit (EPIUnit), Instituto de Saúde Pública da Universidade do Porto, Porto, Portugal (tlmateus@gmail.com)

^f Center for Research and Development in Agrifood Systems and Sustainability (CISAS), Escola Superior Agrária, Instituto Politécnico de Viana do Castelo, Refoios do Lima, Portugal

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Resumo

A malária aviária é uma doença por vezes subestimada no que diz respeito ao seu impacto e patogenicidade em aves selvagens. Os estudos epidemiológicos e de caracterização molecular de hemoparasitas em aves selvagens, permitem avaliar a sua presença nestes animais e atuar em termos de medicina da conservação. Em Portugal cerca de 150 espécies de aves são consideradas vulneráveis ou em perigo de extinção, e apesar da maioria das causas de morte não serem de origem infecciosa, é necessário realizar estudos sobre a presença de hemoparasitas, já que as consequências indiretas da sua presença na saúde de aves selvagens não são completamente conhecidas. Além disso, estes estudos são uma fonte de informação que pode ajudar centros de recuperação, onde várias espécies co-existem num espaço, mas com o objetivo de serem libertadas. O objetivo deste estudo é avaliar a presença de *Plasmodium*, *Haemoproteus* e *Leucocytozoon* em aves selvagens, que deram entrada num centro de recuperação da zona centro de Portugal. Para o efeito foram recolhidas amostras de tecido hepático e esplênico, maioritariamente através de necrópsia, para posterior extração de DNA e testagem através de *nested-PCR* a presença dos géneros referidos. Num grupo de 70 amostras de animais diferentes, 17 (24,3%) amostras foram positivas para *Plasmodium*/*Haemoproteus* e 30 (42,9%) para *Leucocytozoon*, sendo que 15 (21,4%) foram infeções mistas. Por conveniência, dando foco a espécies em perigo, algumas amostras foram sequenciadas e organizadas em árvores filogenéticas, observando-se várias linhagens de *Leucocytozoon* sp. com diferentes características em termos dos hospedeiros que afetam. Também foram sequenciadas amostras com *Plasmodium matutinum* e *Plasmodium relictum* que apresentaram diferentes grupos de infeção em diferentes espécies. Algumas das amostras testadas foram os primeiros casos reportados nas espécies *Aquila pennata* (Águia-calçada), *Gyps fulvus* (Grifo), *Motacilla alba* (Álveola-branca), *Neophron percnopterus* (Abutre-do-egipto) e *Picus viridis* (Pica-pau-verde), onde se incluem duas espécies de aves necrófagas, grupo onde não estava referido a presença de malária aviária. As amostras de aves com presença de lesões hepáticas e esplênicas encontradas em necrópsia apresentaram elevada frequência de hemoparasitas. Também foi frequente a presença de hemoparasitas em aves recém-nascidas até juvenis. A análise filogenética evidenciou linhagens que afetam grupos específicos de aves e outros que afetam uma enorme variedade de ordens de aves.

Abstract

Avian malaria is a disease that is sometimes underrated on its impact and pathogenicity in wild birds. Epidemiologic studies and molecular characterization of hemoparasites in wild birds, allows the evaluation of its presence in these animals and act for conservation medicine. In Portugal around 150 species of birds are considered vulnerable or in danger of extinction, and although the majority of their causes of death don't have an infectious origin, it is necessary to control the presence of hemoparasite whose indirect consequences in the health of wild birds are not completely known. These surveys are a source of information that can help rescue centers, where multiples species co-exist in one space but

with the goal of being released. The objective of this study is to evaluate the presence of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in wild birds, admitted in a rescue center in the center region of Portugal. For this purpose, liver and spleen samples were collected, mostly during necropsy, for DNA extraction and nested-PCR. From the 70 samples collected from different birds, 17 (24,3%) were positive for *Plasmodium*/*Haemoproteus*, 30 (42,9%) for *Leucocytozoon*, and 15 (21,4%) were mixed infections. Focusing on endangered species, some samples were sent for sequencing and phylogenetic studies, showing different lineages of *Leucocytozoon* sp. with different characteristics of different hosts that can affect. Other samples were positive for *Plasmodium matutinum* and *Plasmodium relictum* that showed different infection behaviour in different species. This study provided first reports, specifically on *Aquila pennata*, *Gyps fulvus*, *Motacilla alba*, *Neophron percnopterus* e *Picus viridis*. That includes two species of necrophage birds, a group where avian malaria were not referred previously. Samples from birds with hepatic and splenic lesions found in necropsy presented a high frequency of hemoparasites. It was also frequent the presence of hemoparasites in nestlings. The phylogenetic analysis showed the capacity of different lineages to affect very specific groups and others that affect a high variety of bird's orders.

Key-words: Avian malaria; *Plasmodium*; *Haemoproteus*; *Leucocytozoon*; *Gyps fulvus*; *Neophron percnopterus*;

Highlights:

In a total of 70 samples from different birds, 17 (24.3%) presented *Plasmodium*/*Haemoproteus*, 30 (42.9%) presented *Leucocytozoon*, and 15 (21.4%) were mixed infections.

The confirmed first reports were in Booted eagle (*Aquila pennata*), Griffon vulture (*Gyps fulvus*), White wagtail (*Motacilla alba*), Egyptian vulture (*Neophron percnopterus*) and European green woodpecker (*Picus viridis*).

The Egyptian vulture is the specie with higher risk considering the conservation classification, and their population has a continuous decline with a lot of causes reported. One of the measures made by Portuguese government to control the absence of feeding areas, were the creation of protected places where corpses are available just for necrophage birds.

Different connections in species of hemoparasites, such as *Plasmodium relictum* and *Plasmodium matutinum*, show the diversity in the relationship between parasite and host, since those species can be focused in one specific genus, or can infect very different orders of wild birds.

1. Introduction

Even now we still do not know the full extent of biodiversity present on earth, nevertheless, a lot of species are being made extinct or at least injured by anthropogenic actions (Olah et al., 2016). Given that, concepts like conservation medicine and “One Health” should be present in our mind set, allowing us to better observe ecological interactions and danger for public health, as well as to have the tools to overcome the obstacles and challenges that planet earth is facing (Fernandes and Pinto, 2019).

This role in veterinary medicine was most associated with zoo veterinarians, and although their work is in line the conservation medicine guidelines, normally we are talking about an ex situ condition (Deem, 2015), creating a different situation than a veterinarian in a rescue and rehabilitation center. In a rescue and rehabilitation center, from a conservation and ecology theory, the main and preferred objective is to reintroduce a rescue animal back to their natural habitat and for that reason, it is necessary to ensure a safe release in the long term, but also a minimal time staying in the center with limited human contact (Sós-Koroknai et al., 2020).

In Portugal nearly 150 species of birds are considered in danger or vulnerable of becoming extinct by the National Institute of Nature and Forest Conservation, ICNF (ICNF, 2005). One wild animal rescue center in Portugal report that 12% of their entries are from endangered species, and from all entries the most common birds are of the orders Passeriformes, Falconiformes and Apodiformes (CERVAS, 2018). At the same time, multiple studies have been done in Portugal, with focus on birds, showing that collisions are the most common reported cause of death, and therefore the direct consequences of anthropogenic activity on biodiversity are real and present (Garcês et al., 2020).

The importance biodiversity's knowledge in each region, is not only to help our own environment and present issues, but to preserve knowledge, create the possibility of comparison of different regions or countries, and to create previsions so humanity can prevent future extinctions, using, for example, cryopreservation practices (Breithoff and Harrison, 2020).

Avian malaria, a global vector-borne disease, causes a low mortality in birds, however, this disease is not fully understood yet.

This disease is caused by protozoan parasites from the genera *Plasmodium* and *Haemoproteus*; these two are the more researched genus, but also *Fallisia* and *Leucocytozoon* (Santiago-Alarcon et al., 2016).

The indirect consequences of this parasitosis may help the decline of the specimens of vulnerable species of birds, becoming a challenge in conservation medicine (Eastwood et al., 2019).

The most common vectors present in Portugal, that are associated with avian malaria, associated with *Plasmodium* spp., are mosquitoes species like *Culex pipiens*, *Culex theileri* and *Ochlerotatus caspius*, since there are already studies showing the presence of hemoparasites in those species using molecular testing (Ventim et al., 2012). More recent reports, showed *Aedes aegypt* and *Anopheles maculipennis*

as vectors associated with the presence of hemoparasites too (CEVDI, 2020). Meanwhile, *Leucozytozoon* spp. vectors are reported from the family Simuliidae (Jumpato et al., 2012).

Although most of the parasites that cause malaria are different between humans and birds, avian malaria studies are also important tools to help the research on human malaria, as Sir Ronald Ross, used information on birds to fundement and further his research from which he was awarded the Nobel Prize in Medicine in 1902 (Marzal, 2012).

They have heteroxenous life cycles with sexual stages between birds and mosquitoes with asexual stages being in the host's blood and organs, with a high concentration in the spleen and liver (Bowman, 2013). The exo-erythrocytic stages, where the parasite can invade other organs like eyes, brain or skeletal muscles continues to be the least studied part of their life cycle (Valkiūnas and Iezhova, 2017).

The interaction between the animal and the parasite is complex with the immune system having an important role as well as the parasitaemia. Generic symptoms from an acute infection are anorexia, anaemia and lethargy (Lapointe et al., 2012), and also common in infected animals is hepatomegaly and splenomegaly (Sjibrandta et al., 2017).

Most lethal infections come from indirect mortality since destruction of habitat, collision with cars or other structures like windmills, but even if the animal survives these types of trauma and environment alterations, their capacity of recuperation is limited. With a chronic infection, the bird becomes a source of parasites for the vector, with few direct effects on the host (Lapointe et al., 2012).

Although most wild species don't have specific trials for treatments, studies refer that vector prefer to bit non-treated infect birds and this proves another factor that contributes to reduce the spread of the disease focusing in vector control, since it continues to be one of the most efficient ways to control it (Yan et al., 2018).

For decades, microscopy detection of hemoparasites were worldwide used in different studies, but molecular testing like PCR has shown to be a more specific and sensitive way to confirm their presence. Although regular molecular testing can be difficult in cases of mixed infections, new techniques provide the tools to differentiate hemoparasite species (Ciloglu et al., 2019).

The study, characterization and detection of hemoparasites in wild birds are a way to help the scientific community to understand, describe and control the presence of Avian Malaria in different regions and species (Pornpanom et al., 2019). The aim of this study is to evaluate the presence of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in wild birds admitted in a rescue center in Portugal.

2. Materials and method

2.1. Sampling

The study started in CERVAS (*Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens*), a wildlife rescue center based in Gouveia, Portugal, where wild animals are rehabilitated with the objective to release them again back into nature. Besides that, the center has an important role for environmental awareness and as an intermediary for universities and research centers, since they perform necropsies and collect samples for future studies.

When entering the center already dead, or if the animal succumbs to the injuries during their stay, their supposed cause of death will be added to their information, as it can be a support during the necropsy. In this rescue center, dead animals are preserved frozen to be used for research purposes.

The selection of frozen (and fresh) bird carcasses were chosen to be subject to necropsy and sample collection by convenience, but we collected bird samples mainly from species with vulnerable conservation status or higher.

2.2. Necropsy

The necropsy technique used was based and adapted from Peleteiro (2016). Briefly, during the inspection of the gastrointestinal tract, the entire liver of each animal was collected, and when possible, the entire spleen is also collected. Samples were frozen in sample sterilized recipients, identified with: the specie of the bird, organs collected, and the number in order of the necropsies. During the necropsy data was collected like the sex (when possible) and age group (between nestling, juvenile and adults), the presence of lesions in the spleen and liver.

2.3. Molecular testing

DNA extraction was performed using NZY© Tissue gDna isolation Kit (NZYTech, Portugal), followed by quantification with Nanodrop™ 2000. The samples, after extraction and quantification, were tested using a nested-PCR that targets the cytochrome *b* gene of parasites from *Plasmodium*, *Haemoproteus* and *Leucocytozoon* genus as described by Bensch et al. 2009.

A first PCR using the primer HaemNFI [5'-CATATATTAAGAGAA[*I*]TATGGAG-3'] and HaemNR3 [5'-ATAGAAAGATAAGAAATACCATTC-3'] (Bensch et al., 2009), was followed by two PCR, one specific for *Plasmodium* and/or *Haemoproteus*, with primer HaemF [5'-ATGGTGCTTTTCGATATATGCATG-3'] and primer HaemR2 [5'-GCATTATCTGGATGTGATAATGGT-3'] (Bensch et al., 2000) and other for *Leucocytozoon* with primer HaemFL [5'-ATGGTGTTTTAGATACTTACATT-3'] and HaemR2L [5'-CATTATCTGGATGAGATAATGG[*I*]GC-3'] (Hellgren et al., 2004). The PCR reactions were performed in an automated DNA thermal cycler (MJ Mini, Biorad) with the following program: (i) one initial denaturing step (one cycle) for 3 min at 95°C; 35 cycles of denaturation for 20s at 94°C, annealing for 20s at 50°C, and extension for 30s at 72°C; ending with a final extension step for 70 min at 72°C. Amplified products were visualized under UV light after electrophoresis in a 1.5%(w/v) agarose gel (in 1×TBE buffer) stained with GreenSafe.

A subset of positive samples was purified using NZY© Gelpure Kit (NZYTech, Portugal) from the gel and sent for DNA sequencing (Eurofins Genomics).

For phylogenetic analysis, *cytochrome b* genes sequences available from NCBI were used. Both trees were constructed by using Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993), and the evolutionary analyses were conducted in MEGA X (Kumar et al., 2018) For the *Plasmodium* tree (Figure 1), three sequences of *Haemoproteus columbae* were included as outgroup. For the *Leucocytozoon* tree (Figure 2), a sequence from *Plasmodium* was used as outgroup. Samples from this study are highlighted in grey.

3. Results

Seventy samples of spleen and/or liver from 34 different bird species were collected: 52 were obtain by necropsy of birds from CERVAS; 18 were from a sample archive created by CERVAS for studies. In total 14 species from the global pool have conservation classification equal or superior to Near Threatened (NT) or DD (Data Deficient) considering the ICNF criteria (Table 1).

Table 1. Order, species, number, sex, age group and conservation classification of the birds sampled.

Order	Species	Common name	Number of Birds sampled	Sex (F / M / I)*	Age Group (N / J / A / I)**	Conservation Classification
Accipitriformes	<i>Accipiter gentilis</i>	Northern goshawk	1	n.i.	n.i.	VU
	<i>Accipiter nisus</i>	Eurasian sparrowhawk	2	0 / 2 / 0	0 / 1 / 1 / 0	-
	<i>Aquila pennata</i>	Booted eagle	2	1 / 1 / 0	0 / 0 / 1 / 1	NT
	<i>Buteo buteo</i>	Common buzzard	5	1 / 4 / 0	0 / 1 / 4 / 0	-
	<i>Circaetus gallicus</i>	Short-toed snake eagle	1	0 / 1 / 0	0 / 0 / 1 / 0	NT
	<i>Gyps fulvus</i>	Griffon vulture	2	0 / 1 / 1	0 / 1 / 1 / 0	NT
	<i>Milvus migrans</i>	Black kite	2	n.i.	n.i.	-
	<i>Milvus milvus</i>	Red kite	1	n.i.	n.i.	CE
	<i>Neophron percnopterus</i>	Egyptian vulture	1	n.i.	n.i.	EN
	<i>Pernis apivorus</i>	European honey buzzard	2	n.i.	n.i.	VU
Apodiformes	<i>Apus apus</i>	Common swift	1	n.i.	n.i.	-
Bucerotiformes	<i>Upupa epops</i>	Eurasian hoopoe	1	0 / 1 / 0	0 / 0 / 1 / 0	VU in Madeira
Caprimulgiformes	<i>Caprimulgus europaeus</i>	European nightjar	1	1 / 0 / 0	0 / 1 / 0 / 0	VU
Ciconiiformes	<i>Ciconia ciconia</i>	White stork	2	n.i.	n.i.	-

Table 1. (cont.) Order, species, number, sex, age group and conservation classification of the birds sampled.

Order	Species	Common name	Number of Birds samples	Sex (F / M / I)*	Age Group (N / J / A / I)**	Conservation Classification
Columbiformes	<i>Columba livia domestica</i>	Rock dove	1	1 / 0 / 0	0 / 0 / 1 / 0	-
	<i>Streptopelia decaocto</i>	Eurasian collared dove	2	1 / 1 / 0	0 / 0 / 2 / 0	-
Coraciiformes	<i>Merops apiaster</i>	European bee-eater	1	1 / 0 / 0	0 / 1 / 0 / 0	-
Falconiformes	<i>Falco naumanni</i>	Lesser kestrel	1	n.i.	n.i.	VU
	<i>Falco tinnunculus</i>	Common kestrel	1	n.i.	n.i.	VU
Passeriformes	<i>Garrulus glandarius</i>	Eurasian jay	4	2 / 1 / 1	1 / 3 / 0 / 0	-
	<i>Carduelis chloris</i>	European greenfinch	1	0 / 1 / 0	0 / 1 / 0 / 0	-
	<i>Corvus corone</i>	Carrion crow	2	0 / 1 / 1	1 / 0 / 1 / 0	-
	<i>Motacilla alba</i>	White wagtail	1	n.i.	n.i.	-
	<i>Passer domesticus</i>	House sparrow	1	0 / 0 / 1	1 / 0 / 0 / 0	-
	<i>Pica pica</i>	Eurasian magpie	1	0 / 1 / 0	0 / 0 / 1 / 0	-
	<i>Sturnus unicolor</i>	Spotless starling	2	1 / 1 / 0	0 / 1 / 1 / 0	-
	<i>Turdus merula</i>	Common blackbird	11	7 / 2 / 2	4 / 7 / 0 / 0	-
Pelecaniformes	<i>Ardea cinerea</i>	Grey heron	1	n.i.	n.i.	DD in Açores
Piciformes	<i>Picus viridis</i>	European green woodpecker	2	1 / 1 / 0	0 / 0 / 2 / 0	-
Strigiformes	<i>Athene noctua</i>	Little owl	5	3 / 1 / 1	1 / 3 / 1 / 0	
	<i>Bubo bubo</i>	Eurasian eagle-owl	2	n.i.	n.i.	NT
	<i>Otus scops</i>	Eurasian scops owl	1	1 / 0 / 0	0 / 0 / 1 / 0	DD
	<i>Strix aluco</i>	Tawny owl	2	n.i.	n.i.	-
	<i>Tyto alba</i>	Barn owl	3	2 / 0 / 1	1 / 0 / 2 / 0	-

* (female / male / indeterminate) ** (nestlings / juveniles / adults / indeterminate)

***(VU- Vulnerable; NT- Near Threatened; CE- Critically Endangered; E- Endangered; DD- Data Deficient)

There were 22 females, 20 males and 28 sex indeterminate birds or no information available. From those ones that we collected data on age group (n=52): 40.4% (n=21) were adults, 38.5% (n=20) were juveniles, 17.3% (n=9) were nestlings and 3.8%(n=2) were age indeterminate.

Although 18 samples were from the center's samples archive, and that way we don't have the sufficient evaluation for their causes of death, from those 52 obtained by necropsy, the more common were: nest fall (26.9%), collision with motorized vehicles (17.3%), other trauma (9.6%), debility/bad nutrition (7.7%), predation (7.7%), electrocution (3.8%), illegal captivity (1.9%), poisoning (1.9%) and unknown causes (23.1%).

From the 52 necropsies made, lesions were found in liver (23.1%, n=12), spleen (9.6%, n=5) and in both (5.8%, n=3), in a total of 20 (38.5%) carcasses/birds with visible lesions, like hepatomegalia.

From all samples (n=70), regardless of the origin (necropsy or center's sample archive), in 34 (48.6%) of them was possible to identify hemoparasites: in 17 (24.3%) *Plasmodium*/*Haemoproteus*, in 30 (42.9%) *Leucocytozoon*, and in 15 (21.4%) of them for both (Table 2).

Table 2. Bird order, species, total and positive number of samples by hemoparasite.

Order	Species	N.º of Samples	N.º of Positive Samples	<i>Plasmodium</i> / <i>Haemoproteus</i>	<i>Leucocytozoon</i>
Accipitriformes	<i>Accipiter gentilis</i> *	1	1(1)**	1	1
	<i>Accipiter nisus</i>	2	0	0	0
	<i>Aquila pennata</i> *	2	1	0	1
	<i>Buteo buteo</i>	5	1	0	1
	<i>Circaetus gallicus</i> *	1	1	0	1
	<i>Gyps fulvus</i> *	2	1	0	1
	<i>Milvus migrans</i>	2	2(2)**	2	2
	<i>Milvus milvus</i> *	1	1(1)**	1	1
	<i>Neophron percnopterus</i> *	1	1	0	1
	<i>Pernis apivorus</i> *	2	1(1)**	1	1
Apodiformes	<i>Apus apus</i>	1	0	0	0
Bucerotiformes	<i>Upupa epops</i> *	1	0	0	0
Caprimulgiformes	<i>Caprimulgus europaeus</i> *	1	0	0	0
Ciconiiformes	<i>Ciconia ciconia</i>	2	1(1)**	1	1
Columbiformes	<i>Columba livia domestica</i>	1	0	0	0
	<i>Streptopelia decaocto</i>	2	1	0	1
Coraciiformes	<i>Merops apiaster</i>	1	0	0	0
Falconiformes	<i>Falco naumanni</i> *	1	0	0	0
	<i>Falco tinnunculus</i> *	1	1	1	0
Passeriformes	<i>Carduelis chloris</i>	1	0	0	0
	<i>Corvus corone</i>	2	2(1)**	1	2
	<i>Garrulus glandarius</i>	4	2(2)**	2	2
	<i>Motacilla alba</i>	1	1(1)**	1	0
	<i>Passer domesticus</i>	1	0	0	0
	<i>Pica pica</i>	1	0	0	0
	<i>Sturnus unicolor</i>	2	0	0	0
	<i>Turdus merula</i>	11	6(2)**	2	6
Pelecaniformes	<i>Ardea cinerea</i> *	1	1	0	1
Piciformes	<i>Picus viridis</i>	2	1(1)**	1	1
Strigiformes	<i>Athene noctua</i>	5	3(1)**	2	2
	<i>Bubo bubo</i> *	2	2	0	2
	<i>Otus scops</i> *	1	0	0	0
	<i>Strix aluco</i>	2	2	0	2
	<i>Tyto alba</i>	3	1(1)**	1	1

*species with conservation classification equal or superior to NT, or DD.

** (number of mixed infections).

Six of the 20 samples that were from birds with lesions of the spleen/liver, that were observed during necropsy, were identified with the presence of hemoparasites, representing 30% of them.

From the positive pool, choosing by convenience, but mainly from species with critical statue, one from each bird species, were sequenced, and the sequencing best result species characterized in the different genus/species: *Leucocytozoon* sp., *P. matutinum* and *P. relictum* (Table 3).

Table 3. Species DNA identified for each bird species that were sequenced.

Bird Specie	Parasite Species
<i>Accipiter gentilis</i>	<i>Leucocytozoon</i> sp. & <i>P. matutinum</i>
<i>Aquila pennata</i>	<i>Leucocytozoon</i> sp.
<i>Athene noctua</i>	<i>Leucocytozoon</i> sp.
<i>Athene noctua</i>	<i>Leucocytozoon</i> sp.
<i>Circaetus gallicus</i>	<i>Leucocytozoon</i> sp.
<i>Garrulus glandarius</i>	<i>P. matutinum</i>
<i>Milvus milvus</i>	<i>Leucocytozoon</i> sp. & <i>P. matutinum</i>
<i>Pernis apivorus</i>	<i>Leucocytozoon</i> sp. & <i>P. relictum</i>
<i>Picus viridis</i>	<i>Leucocytozoon</i> sp.
<i>Turdus merula</i>	<i>Leucocytozoon</i> sp. & <i>P. matutinum</i>
<i>Tyto alba</i>	<i>Leucocytozoon</i> sp. & <i>P. matutinum</i>

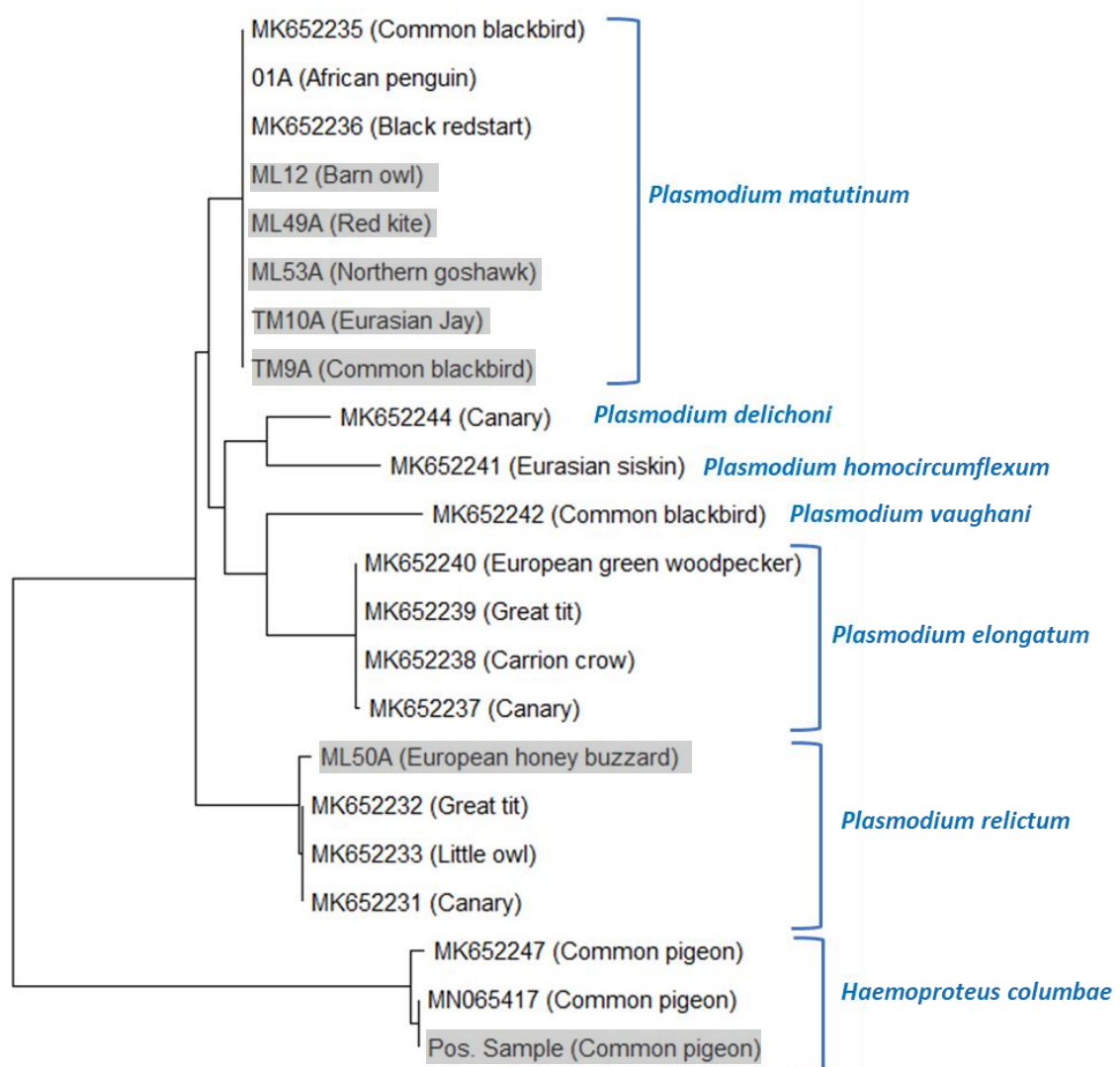


Figure 1. Phylogenetic tree of *Cytochrome b* gene of *Plasmodium* sequences from this study in relation to a selection of sequences from GenBank®, constructed using the Maximum Likelihood method and Tamura-Nei model.

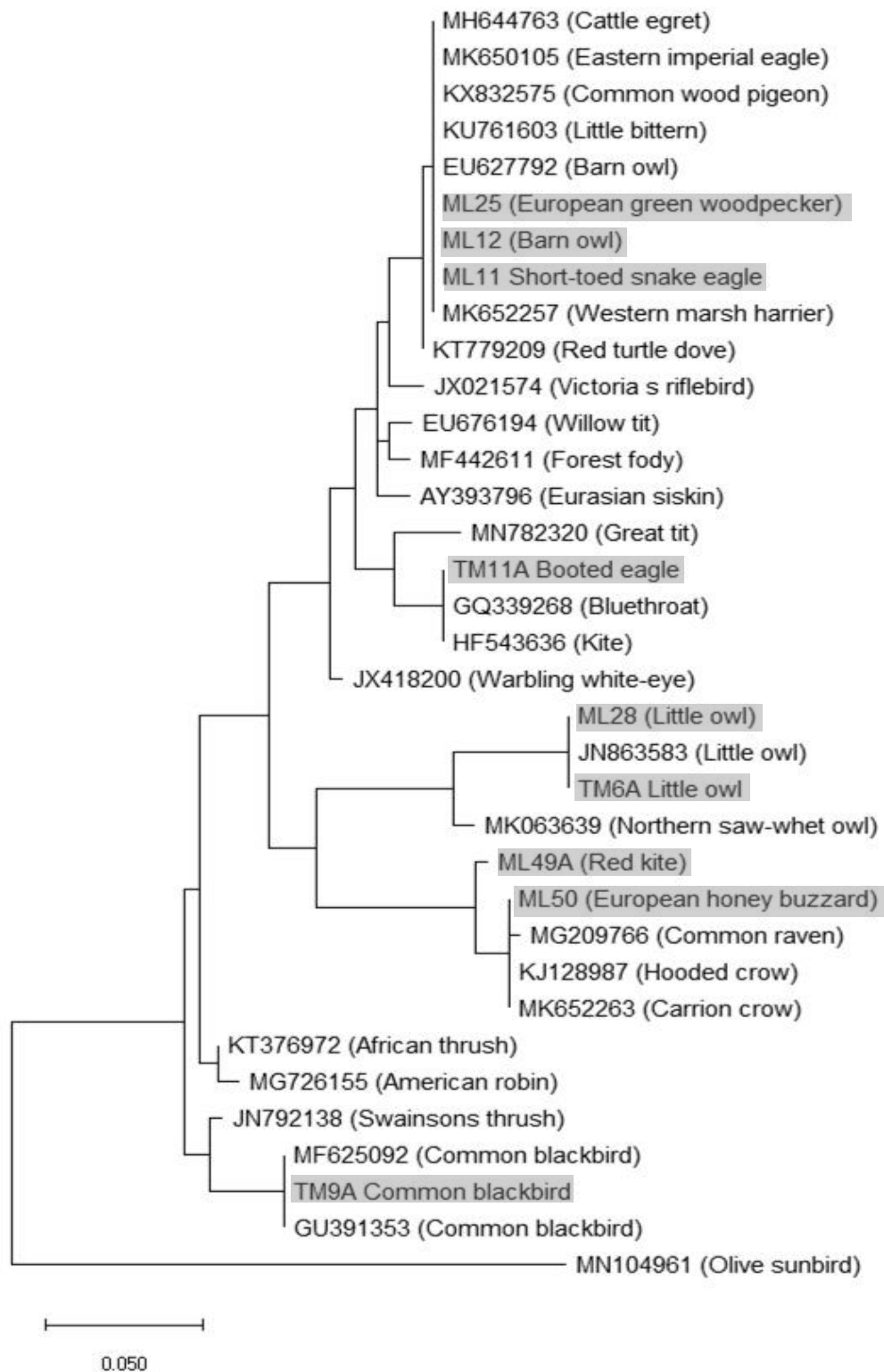


Figure 2. Phylogenetic tree of *Cytochrome b* gene of *Leucocytozoon* sequences from this study in relation to a selection of sequences from GenBank®, constructed using the Maximum Likelihood method and Tamura-Nei model.

4. Discussion

In the total number of 70 samples, 12 different orders were represented being Accipitriformes (10 different species), Passeriformes (8 different species) and Strigiformes (5 different species) the most common. From those, eight orders had positive samples. From the 14 species with conservation classification equal or higher to NT, or DD, in ten (71.4%) of them hemoparasites had been identified.

Eight orders presented hemoparasites, and since each order had a very diverse number of samples is not representative for each order.

Although we don't have much difference between the number of females and males, there is a high number of birds with undetermined sex caused by the number of nestlings and juveniles, since some species are difficult to know the sex in a young age, even with necropsy. While some species have very specific sexual dimorphism in adults, juveniles and new-borns became a challenge, as for birds with few to none differences between sexes, there is a necessity for tools like zoometric measures in big pools of each bird species, to create a way for differentiation of males from females, when necessary (Rathert et al., 2017).

Between those that was possible to determine the sex, 50.0% (n=10) of the 20 males presented hemoparasites, while only 5 of the 22 females presented positive results, but since there are 40.0% (n=28) of all 70 birds that we don't have this information, it isn't possible to make any conclusion, but it is possible to see a tendency.

Nestlings and juveniles are common among the samples, since nest falling is very common, which explains this to be a regular cause of death in the sample pool. This happens from a combination of bad nest location (caused by natural competition and/or alteration of habitat by humans), and sibling competition (Bize and Roulin, 2006). This way, mapping out nest locations in more urban based birds can be a value to rescue new-borns from falls.

Most studies show that hemoparasites are more present in adults (Hanel et al., 2016; Pérez-Rodríguez et al., 2013), but our study present a higher presence of hemoparasites in nestlings, than other age groups.

It is worth to mention that one study suggested that Eurasian blackbirds (*Turdus merula*), the specie most frequent in our study, in urban habitat have fewer cases of hemoparasites presence compared to a forest habitat. In no way this validate any type of intrusion in natural habitats, but presents the idea that species that adapt to urban habitats have less of a chance to be infected (Geue and Partecke, 2008).

Because trauma from collision with motorized vehicles, falls, and other causes that can lead to internal bleeding and therefore hepatic and spleen lesions, it's not possible to perform a relation between them and hemoparasites presence, but the fact that 30% of samples that had lesions compatible with presence of these hemoparasites were positive for their presence makes a bigger interest in future studies based in histopathology.

This relation between lesions and molecular results isn't comparable to other studies, since most studies dedicated to molecular testing and phylogeny don't mention data about organ lesion but some confirmed with microscope detection of hemoparasites (Dimitrov et al., 2010; Pérez-Rodríguez et al., 2013; Svobodová et al., 2015). The biggest difference observed is that most studies used blood as samples, instead of tissue, and for that reason alone can explain the absence of this information, once it is made possible the confirmation by microscope, as adding information about morphology of the hemoparasite (Valkiūnas and Iezhova, 2017), but is important to add that using samples by convenience in frozen cadavers is a major difference too.

Since we tested a wide variety of species there were some first reports, although there were reports in others species from the same genera or family, the fact is that this diversity made possible the observation of hemoparasite by molecular techniques in species that sometimes are overlooked.

As far as reviewed from the literature, this is the first report of the presence of hemoparasites in White wagtail (*Motacilla alba*), from the Passeriformes order. But since this order have a massive diversity and the last report of testing in this particular species is from 2012, shows how not every bird species is tested even in bigger studies (Mohammad and Al-Moussawi, 2012).

European green woodpecker (*Picus viridis*) was also a first report in this study, although already tested in past studies but didn't present hemoparasites (Santiago-Alarcon et al., 2016).

Some studies, along the years, report the absence of positives in necrophage birds in Europe (Ciloglu et al., 2016; Martínez-Abraín et al., 2004), but we had presence of hemoparasites in Gryffon vulture (*Gyps fulvus*) and Egyptian vulture (*Neophron percnopterus*).

Those first reports are very important since it goes against prior discussed assumptions of necrophage birds being less vulnerable to hemoparasites due to their immune system or the type of microhabitat they have on their nesting sites (Blanco et al., 1998).

From the first reports in this work, the Egyptian vulture is the specie with higher risk considering the conservation classification, and their population has a continuous decline with a lot of causes reported. One of the measures made by Portuguese government to control the absence of feeding areas, were the creation of protected places where corpses are available just for necrophage birds (ICNF, 2005). This situation created areas where various necrophage birds can be exposed to vectors in a regular situation.

Some species from Accipitriformes order are sometimes absent in recent studies (Valkiūnas et al., 2017), and for that reason some of these positive cases are also first reports in some of the species, especially the ones that were sequenced and identified as *Leucocytozoon* spp., such as Gryffon vulture, Egyptian vulture and Booted eagle.

All but one sequenced samples from *Plasmodium* grouped in a cluster of *Plasmodium matutinum*. This species is clearly euryxenous since it affects birds from different taxonomic orders. One sample clustered with *Plasmodium relictum* that also affects different birds.

Plasmodium matutinum and *Plasmodium relictum* are not considered to be pathogenic in wild birds, but recent discussions are being held about the underestimated pathogenicity of these hemoparasites, giving the possibility of different lineages from different hosts (even if from the same species), to go from no symptoms to high mortality rates (Valkiunas and Iezhova, 2018). Although *Plasmodium* sp. is described as more pathogenic, it's much more studied than other hemoparasites, so this comparison can be questioned.

Leucocytozoon sequences were not identified to species level after BLAST analysis and further studies, including morphological, should clarify the phylogeny of this genus.

A rescue center is a place where different species of wild birds are near each other, can share vectors, they are in a higher stressful environment than nature and normally they are injured or with a low immune system. But is an essential part of the work in conservative medicine, where veterinarians working in research can have access to information and samples of wild life in a controlled environment and can have a direct influence in the life of a singular animal, but especially, contribute to the centers knowledge with genetic information about pathogens that probably doesn't make part of the everyday life of wild bird clinic nowadays.

5. Conclusion

With this study, it was possible to contribute to the knowledge of the presence of hemoparasites in different bird species, some of them being a first report worldwide. Those that weren't first reports, are still a confirmation of hemoparasites presence in wild birds in Portugal.

The confirmed first reports by molecular testing were in Booted eagle (*Aquila pennata*), Griffon vulture (*Gyps fulvus*), White wagtail (*Motacilla alba*), Egyptian vulture (*Neophron percnopterus*) and European green woodpecker (*Picus viridis*).

The presence of hepatic and spleen lesions seen during necropsy can be a suggestion for further studies focused on the correlation between lesions and histopathology and the presence of hemoparasites, keeping in mind of how common is trauma, haemorrhage and other situations as reasons for admission of birds in rescue centers.

This way we conclude that, not only tissue sampling is a valid way to confirm the infection with hemoparasites, and the correlation between visible liver and spleen lesions and hemoparasite infection present in our study shows that this information should be further studied in future avian malaria studies. Also, the underrated pathogeny of avian malaria in wild birds is a reason to call for more studies worldwide since they are present in different species that have a critical conservation classification, especially since it's found even in young birds, suggesting a longer parasite cycle in the host and the possibility of evolve to chronic states of the disease.

The presence of hemoparasites in necrophage birds was not expected as referred in older studies (Blanco et al., 1998; Ciloglu et al., 2016; Martínez-Abraín et al., 2004) and demands further studies.

Samples of Gryffon vulture (*Gyps fulvus*) and Egyptian vulture (*Neophron percnopterus*) presented first reports of hemoparasites, showing the reality of their presence in these birds.

The existence of small areas for feeding necrophage birds can contribute for a higher prevalence of the vector, contributing for the decrease of endangered populations like the Egyptian vulture.

Different connections in species of hemoparasites, such as *Plasmodium relictum* and *Plasmodium matutinum*, show the diversity in the relationship between parasite and host, since those species can be focused in one specific genus, or can infect very different orders of wild birds.

The importance of rescue centers in conservation medicine, is the presence of different species and lineages of hemoparasites that shows a diverse way of interaction with their hosts, making molecular testing, like PCR techniques a fundamental tool to decide future implementations of regular sample collection for control of avian malaria and also to establish prophylactic measures to avoid the infection of vulnerable and endangered bird species.

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