Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado em Biologia da Conservação

Dissertação

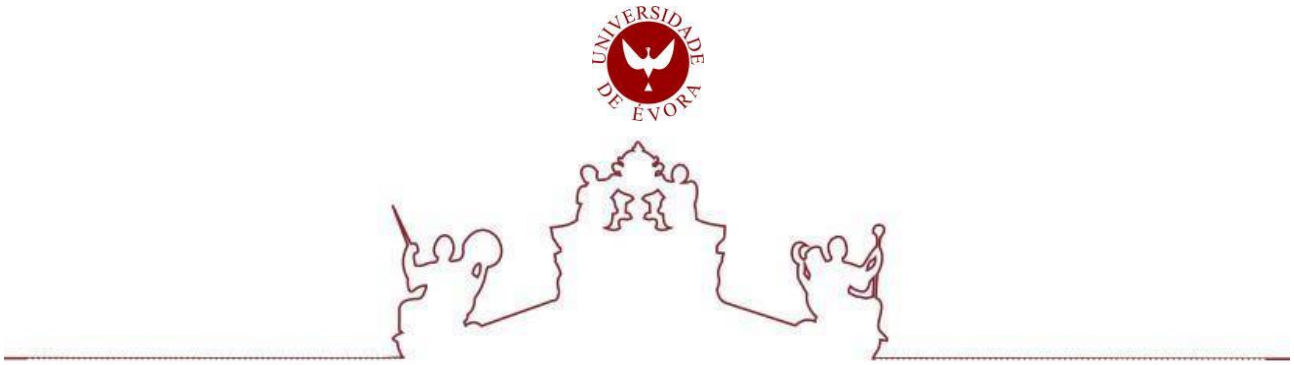
Survey of haemoparasites in owls in mainland Portugal

Ana Rita Bárrio Machado Franco Santos

Orientador(es) | Rui Lourenço

Jacinto José Carneiro Gomes

Évora 2023



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Presidente: Paulo Alexandre da Cunha e Sá de Sousa (UÉvora)

Vogal orientador: Rui do Nascimento Fazenda Lourenço (MED, UÉvora)

Vogal arguente: Inês Margarida Ferreira Roque (MED, UÉvora)

Survey of haemoparasites in owls in mainland Portugal

Abstract

Owls are important avian hosts of haemoparasites, being susceptible of disease and even death when infected. This study aimed to analyse the haemosporidians in owl tissues collected from dead individuals, from different regions of mainland Portugal, obtained mostly in wildlife rehabilitation centres. Nested polymerase chain reactions, targeting the cytochrome b gene of the mitochondria of the haemosporidians, were conducted for each sample. 19 positive samples were selected for purification and further sequencing and species or lineage identification.

Overall, 122 of the 240 collected samples (51%) were positive for at least one haemosporidian species, 31% of the owls (n = 75) were positive for either *Haemoproteus/Plasmodium* spp. and 44% (n = 106) were positive for *Leucocytozoon* spp. Species, age and NUTS II region were significantly associated with haemoparasites' prevalence: *Bubo bubo*, adult owls and Alentejo region presented higher prevalences. Maximum likelihood phylogenetic trees were constructed.

Keywords: Strigiformes, *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, wildlife conservation, Portugal.

Pesquisa de hemoparasitas em aves de rapina noturnas em Portugal Continental

Resumo

As aves de rapina noturnas representam um importante grupo de hospedeiros de hemoparasitas aviários, suscetíveis a doença ou morte quando infetadas. Este estudo analisou hemosporídeos em tecidos de cadáveres de corujas, de diferentes regiões de Portugal Continental, obtidas principalmente em centros de reabilitação de animais silvestres. Reações *nested* em cadeia da polimerase, tendo como alvo o gene citocromo b da mitocôndria dos hemosporídeos, foram conduzidas. 19 amostras positivas foram selecionadas e purificadas para posterior sequenciação e identificação das espécies ou linhagem.

Das 240 amostras, 122 (51%) foram positivas para pelo menos um hemosporídeo, 31% das aves (n = 75) foram positivas para *Haemoproteus/Plasmodium* spp. e 44% (n = 106) foram positivas para *Leucocytozoon* spp. A espécie, idade e região NUTS II foram significativamente associados à prevalência de hemoparasitas: *Bubo bubo*, animais adultos e a região do Alentejo apresentaram prevalências mais elevadas. Árvores filogenéticas por probabilidade máxima foram construídas.

Palavras-chave: Strigiformes, *Plasmodium*, *Haemoproteus*, *Leucocytozoon,* conservação de vida selvagem, Portugal.

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1. Introduction

Nocturnal birds of prey, also known as owls, are distributed worldwide, except in Antarctica and in some remote islands. Owls belong to the order Strigiformes, which includes 220 to 225 extant species divided into two families, namely Tytonidae (barn owls) and Strigidae (true owls) (Ponder & Willette, 2015). Owl species are present in most terrestrial ecosystems, where they play an important role maintaining community structure and balance, not only because of their function as top predators in the food web (Krone et al., 2008), but also because they are pest controllers and offer several ecosystem services (Donázar et al., 2016).

The conservation of owl populations is influenced by many factors, going from better-known ones, such as, habitat loss and degradation, road mortality and prey declines (Martínez & Zuberogoitia, 2004; Salek et al., 2010; van der Horst et al., 2019) to lesser-known ones, such as contamination, parasites, and diseases (Gancz et al., 2004; Ansara-Rosset al., 2013; Rogers et al., 2016). While most attention has been concentrated on how habitat changes, prey declines, and human activities impact owls, less is known about the role of less visible factors, including the role of parasites.

Haemosporidians (Phylum Apicomplexa, order Haemosporida), mainly represented by *Plasmodium*, *Haemoproteus* and *Leucocytozoon,* are a known cause of avian vector-borne diseases and have an impact on biodiversity conservation, playing a role as selective agents in wild bird populations (Marzal et al., 2005; Ishak et al., 2008; Barino et al., 2020). These blood parasites not only lead to morbidity and mortality in bird species (Bennett et al., 1993; Atkinson & Samuel, 2010; Jia et al., 2018), but also may reduce breeding success (Korpimaki et al., 1993; Norte et al., 2009; Asghar et al., 2011). Owls represent an important group of haemosporidian avian hosts, being susceptible of disease and even death when infected (Niedringhaus et al., 2018; Barino et al., 2021; Yoshimoto et al., 2021).

1.1. Owl species occurring in Portugal

Seven species of owls occur regularly in continental Portugal: Barn Owl (*Tyto alba*), Eurasian Scops-owl (*Otus scops*), Eurasian Eagle-owl (*Bubo bubo*), Little Owl (*Athene noctua*), Tawny Owl (*Strix aluco*), Long-eared Owl (*Asio otus*), and Short-eared Owl (*Asio flammeus*). Most of them are species of conservation concern, in accordance with the Portuguese vertebrate red list (Cabral et al., 2005), namely *Asio flammeus* (endangered), *Bubo bubo* (nearly threatened), while *Otus scops* and *Asio otus* are data deficient.

The most concerning threats that Portuguese owl populations face are the agricultural land modifications; woodland deterioration due to forest fires and unsuitable forestry

practices; the reduction of rocky and riparian areas; besides the mortality on roads and due to electrocution or collision with powerlines (Lourenço et al., 2015).

1.1.1. Barn Owl (*Tyto alba*, Scopoli, 1769)

Figure 1 – Photo by Steve Garvie. Barn Owl. Wikimedia Commons License.

In Europe, *Tyto alba* lives in open agricultural habitats and grassland, especially with linear structures (Keller et al., 2020); in Portugal, it can be found in most of the continental territory, as well as in Madeira archipelago (Lourenço et al., 2015).

This species is sedentary, with nocturnal and crepuscular behaviours, feeding on mice, frogs and insects (Svensson et al., 2017). It lives alone or in pairs that remain stable for their whole life (Konig & Weick, 2010), nesting in tree cavities, abandoned buildings, farms and nest boxes (Svensson et al., 2017).

Tyto alba's conservation status is listed as "Least Concern" (LC), in both Portugal (Cabral et al., 2005) and Europe (BirdLife International, 2021g). It presented both population and distribution declines from 2010 to 2021 in Portugal (GTAN-SPEA, 2021; Lourenço et al., 2021).

1.1.2. Eurasian Scops-owl (*Otus scops*, Linnaeus, 1758)

Figure 2 – Photo by Imran Shah. Scops-owl. License by Wikimedia Commons.

This species is migratory occurring during its breeding period in almost all the Portuguese territory, more evenly in Beiras Interiores (Beira Baixa and Beira Alta), Trás-os-Montes and Minho. It uses diverse habitats, such as sparse woodlands, close to open habitats, but also urban gardens and farms. In Northeastern Algarve, it prefers horticultural plantations, holm oak and cork forests (Cabral et al., 2005). *Otus scops* presents nocturnal behaviour, feeding on insects and nesting in cavities and nest boxes (Svensson et al., 2017).

Its conservation status is classified as “Data Deficient” (DD) in Portugal (Cabral et al., 2005) and “Least Concern” (LC) in Europe (Birdlife International, 2021e). In a study conducted between 2010 and 2017, *Otus scops* presented a non-significant decline, however it had the biggest reduction regarding distribution, compared to other owl species (Lourenço et al., 2021).

1.1.3. Eurasian Eagle-owl (*Bubo bubo*, Linnaeus, 1758)

Figure 3 – Photo by Imran Shah. Eurasian Eagle-owl. License by Wikimedia Commons.

Bubo bubo is one of the main top predators in the Iberian Peninsula (Lourenço, 2005); in Portugal, where it is a resident species, it presents a wide distribution throughout most of the territory, especially in the South (Cabral et al., 2005; Lourenço et al., 2015). In Iberian Mediterranean ecosystems, it may have either a diet based on lagomorphs, a diet based on rodents, or diversified diets, where lagomorphs are important prey species (Lourenço, 2005).

Its conservation status is classified as “Near Threatened” (NT), in Portugal (Cabral et al., 2005) and “Least Concern” (LC) in Europe (BirdLife International, 2021d). Particularly, the variation and reduction of the wild rabbit populations due to epizootic diseases, as well as habitat destruction, especially dam construction, where the Portuguese reproductive population is located, are important threats (Cabral et al., 2005).

1.1.4. Little Owl (*Athene noctua*, Scopoli, 1769)

Figure 4 – Photo by Artemy Voikhansky. Little Owl. License by Wikimedia Commons.

Athene noctua inhabits shrubland and grassland habitats, adapting to either natural and artificial areas, breeding in most European countries, the Paleartic east to Korea and North Africa (BirdLife International, 2021a). It is a sedentary species, with partially diurnal habits, feeding on insects, birds, small amphibians and snakes. *A. noctua* nest in cavities in trees and buildings, but also in cliffs, quarries or directly on the ground (Svensson et al., 2017).

This species occurs evenly throughout the Portuguese continental territory and seems to be stable in its distribution, however it is decreasing in density (Lourenço et al., 2021), mainly due to large-scale agricultural changes (Lourenço et al., 2015). In Portugal, its conservation status is classified as “Least Concern” (LC) (Cabral et al., 2005), as well as in Europe (BirdLife International, 2021).

1.1.5. Tawny Owl (*Strix aluco*, Linnaeus 1758)

Figure 5 – Photo by Martin Mecnarowski. Tawny Owl. License by Wikimedia Commons.

Strix aluco is distributed in most of continental Portugal, however it is not found, or is present in low density, inland north and in the agricultural regions of Baixo Alentejo (Lourenço et al., 2015). This species is very versatile and opportunistic regarding habitat selection, occurring over a broad range of elevations and landscapes, microclimate and vegetation types (Marchesi et al., 2006).

It presents nocturnal and sedentary behaviours, feeding on small rodents and insects, captured on the ground, and it nests in cavities and nestboxes (Svensson et al., 2017), but also in cliffs, trees and buildings (Marchesi et al., 2006).

Strix aluco's conservation status is "Least Concern" (LC) in Portugal (Cabral et al., 2005), with stable or slightly decreasing population and distribution trends (Lourenço et al., 2021); in Europe, it is also classified as a "Least Concern" (LC) species (BirdLife International, 2021f).

1.1.6. Long-eared Owl (*Asio otus*, Linnaeus, 1758)

Figure 6 – Photo by Ron Knight. Long-eared Owl. License by Wikimedia Commons.

Asio otus is found throughout the Northern hemisphere, in forest, shrubland, grassland and inland wetland habitats (BirdLife International, 2021b). It is a migratory species within its Northern range, and sedentary in the Southern and Western areas of its distribution (Svensson et al., 2017). In continental Portugal, it presents a very fragmented distribution from North to South, with a broad distribution in Azores. The species occurs mostly in low densities, but it is probable that the nesting distribution is broader, once the current knowledge is very limited, due to its discrete behaviour (Lourenço et al., 2015).

With nocturnal and crepuscular habits, it feeds on small mice and birds, usually captured while in flight. *Asio otus* nests in trees, old nests belonging to other birds, usually on top of coniferous trees (Svensson et al., 2017).

Regarding the conservation status, in Portugal it is classified as “Data Deficient” (DD) (Cabral et al., 2005), and as “Least Concern” (LC) in Europe (BirdLife International, 2021b). The population trend is unknown due to its remarkable fluctuations, however the respective distribution seemed to increase mildly between 2010 and 2017 (Lourenço et al., 2021).

1.1.7. Short-eared Owl (*Asio flammeus*, Pontoppidan, 1763)

Figure 7 – Photo by Dario Sanches. Short-eared Owl. License by Wikimedia Commons.

Asio flammeus is a migratory species, breeding across the Holarctic in a diverse variety of climatic conditions in open habitats, semi-natural habitats and sometimes in relatively intensive agricultural areas (Keller et al., 2020). It presents a partially diurnal behaviour and feeds mainly on mice (Svensson et al., 2017).

In continental Portugal, *Asio flammeus* is only found over the winter, in coastal wetlands but also in dams and rice fields in Alentejo; there are a few records in Madeira and Azores (Lourenço et al., 2015). Its conservation status was classified as “Endangered” (EN) in Portugal, while in Europe is “Least Concern” (BirdLife International, 2021c).

1.2. Owls' haemosporidians

Regarding avian vector-borne haemoparasites, *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are the three main genera within haemosporidians (Sporozoa: Haemosporida), belonging to the Apicomplexa phylum. Another haemosporidian, *Fallisia,* is less studied and relatively rarer (Valkiŭnas, 2004; Marzal et al., 2022). Other avian haemoparasites include sporozoan of the genera *Hepatozoon*, *Babesia*, *Atoxoplasma* and *Trypanosoma*, considered less frequent and thus less studied (Valkiŭnas, 2004), but also avian filaroids, which induce either blood-borne or skin-inhabiting microfilariae, that are mostly non-pathogenic or cause mild clinical signs (Bartlett, 2008).

Haemosporidians are obligate heteroxenous protozoans, which means that these parasites require two host groups in order to complete their life cycle: intermediate hosts, namely vertebrates (birds), where the asexual phase occur; and definitive hosts, represented by vectors (blood-sucking dipterans, Insecta: Diptera), where the sexual process takes place (Valkiŭnas, 2004).

While feeding on birds, vectors inoculate sporozoites that undergo asexual division in the cells of fixed tissues of the intermediate hosts – these agamic stages are recognised as exoerythrocytic meronts or schizonts. As a result, multiple division of meronts (merogony or schizogony), unicelular merozoites are formed – these can induce a new cycle of merogony and/or the development of sexual stages in avian blood cells, resulting in gametocytes or gamonts, which are infective for the vectors. After feeding on infected birds, gametocytes initiate gametogenesis in the vector's midgut. The result of the sexual phase, after fertilization, is the development of oocysts (sporogony) that form numerous uninuclear sporozoites, which are infective to birds (Valkiŭnas, 2004).

Haemosporidian infections in birds are described by five main stages:

1. Prepatent, when parasites develop in tissues, but not in the blood;
2. Acute, when parasites appear in the blood and there is an abrupt increase of parasitaemia;
3. Crisis, the moment when parasitaemia reaches the respective peak;
4. Chronic, when there is a rapid decline in intensity of infection to chronic levels and the immune system controls the parasitaemia;
5. Latent, when the parasitaemia decreases and is eliminated by the host's immune response.

Generally, parasites persist in avian hosts for many years or even for life, relapsing during the bird's reproduction period, which enhances not only the transmission to offspring but also vectors' infection (Valkiŭnas, 2008).

Haemosporidians were described for the first time in 1884, by Danilewsky, a Russian physiologist, in Accipitridae, Laniidae and Corvidae's blood samples (Atkinson & Van Riper III,1991; Valkiŭnas, 2004). Presently, it is known that a wide range of avian hosts in all zoogeographical regions are infected by these pathogens (Valkiūnas, 2005; MalAvi database, 2022).

An important group of haemosporidian hosts is represented by raptors, being susceptible to disease and even death (Krone et al., 2008). In numerous studies, owls presented higher prevalences of haemosporidians compared with diurnal birds of prey (Krone et al., 2001; Santos et al., 2008; Baptista et al., 2010). Santos et al. (2008) hypothesised that the elevated prevalence in owls could be a result of the nocturnal activity, as well as the nesting sites, that overlap with the greater activity of the parasites' vectors. According to Forrester et al. (1994), the high diversity of haemoparasites registered in Strigiformes, related to the high diversity of vectors to which they are exposed, may be justified not only by the nocturnal behaviour but also by the use of hidden and shady perches throughout the day. Several different species of haemosporidians have been described in owls worldwide (MalAvi, 2022), and this information in presented on Table 1, focusing on the studied owl species.

Table 1 – Number of lineages of haemosporidians described in the studied owl species (MalAvi database, 2022).

	Haemoproteus	*Plasmodium*	*Leucocytozoon*
Athene noctua	5	3	2
Asio flammeus	2	0	1
Asio otus	5	1	9
Bubo bubo	7	3	2
Otus scops	5	4	12
Strix aluco	5	1	2
Tyto alba	8	2	2

1.2.1. *Haemoproteus*

Species from the genus *Haemoproteus* (phylum Apicomplexa, class Aconoidasida, order Haemosporida, family Plasmodiidae) are some of the most widespread haemoparasites of wild birds, with a worldwide distribution in temperate and tropical climates, however the impact as a disease agent in wild populations is mostly unknown. Some species may be significantly pathogenic, causing severe myositis in birds (Atkinson, 2008b). Over 160 species of *Haemoproteus* have been reported (Fecchio et al., 2020).

Ceratopogonid flies from genus *Culicoides* and ectoparasitic hippoboscid flies (Diptera: Hippoboscidae) are the vectors of avian *Haemoproteus* species (Atkinson, 2008b). The merogony occurs in endothelial cells and possibly in fixed macrophages; in certain species it occurs in myofibroblasts as well. Within tissues, most meronts are found in lungs, less often in the liver, spleen, kidneys, heart and skeletal muscle. Oppositely, gametocytes develop in blood cells (Valkiŭnas, 2004).

Within the Strigiformes group, *Haemoproteus syrnii*, *H. noctuae* and *H. tytoni* have been described (Valkiŭnas, 2004).

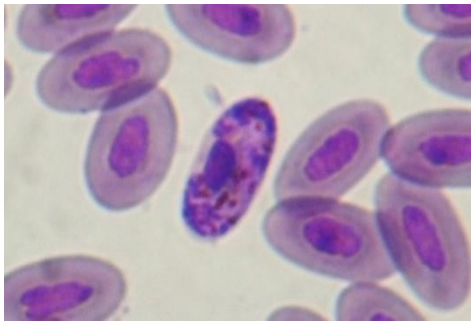

Figure 8 – *Haemoproteus* spp. gametocyte in *Strix aluco* erythrocyte (Diff-Quick, x1000). Gently ceded by Tomás (2014).

1.2.2. *Plasmodium*

The members of this genus (phylum Apicomplexa, class Aconoidasida, order Haemosporida, family Plasmodiidae), also known as malarial parasites, occur in all major groups of terrestrial vertebrates (Valkiūnas & Iezhova, 2018). Fifty-five species of *Plasmodium* were identified in avian hosts (Fecchio et al., 2020), differing extensively in host range, geographic distribution, vectors and pathogenicity (Atkinson, 2008a). These haemosporidians are mainly distinguished by their intraerythrocytic development and asexual reproduction in circulating blood cells (Peirce, 2000).

Plasmodium species that infect birds are found in all zoogeographic regions, except for Antarctica, and present a cosmopolitan distribution. Reports of epizootics are uncommon, being mainly associated with captive birds' infections and with abnormal host-parasite combinations following introduction of infected mosquitoes to remote islands (Atkinson, 2008a).

The vectors of *Plasmodium* are blood-sucking mosquitoes (Diptera: Culicidae), belonging to the genera *Culex*, *Aedes*, *Culiseta* and *Anopheles*, although only the dipteran females feed on blood and, therefore, spread the infection (Valkiŭnas, 2008).

In birds, merogony occurs in endothelial cells lining the capillaries, in the cells of hemopoietic and lymphoid macrophage systems, while there are also erythrocytic meronts. Gametocytes develop mostly in mature erythrocytes (Valkiŭnas, 2008). The progression of the disease and clinical signs increase proportionally to the number of parasites in peripheral circulation (van Riper et al., 1994).

P. subpraecox, *P. fallax* and *P. gundersi* may infect birds from the Strigidae group (Valkiŭnas, 2008).

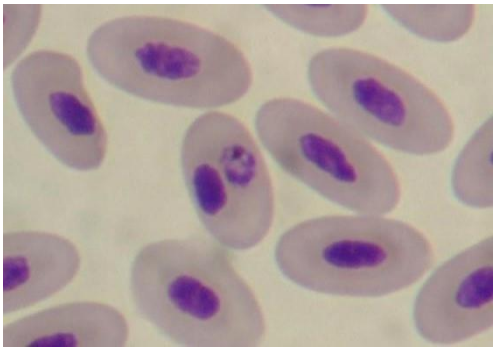

Figure 9 – *Plasmodium* spp. trophozoite in *Strix aluco* erythrocyte (Diff-Quick, x1000). Gently ceded by Tomás (2014).

1.2.3. *Leucocytozoon*

Species of the genus *Leucocytozoon* (phylum Apicomplexa, class Aconoidasida, order Haemosporida, family Leucocytozoidae) are parasitic protozoans distributed worldwide, except in the Antarctic (Valkiŭnas, 2005). In the Holarctic, there is the highest prevalence and species diversity of leucocytozoids (Forrester & Greiner, 2008), with fifty species of Leucocytozoon infecting avian hosts (Fecchio et al., 2020). Waterfowl, pigeons, galliforms, raptors and ostriches are the main avian groups at risk (Valkiŭnas, 2005), however young ducks and geese seem to be the most susceptible to disease and death (Wobeser, 1997).

Blood-sucking simuliid flies (Diptera: Simuliidae) are the vectors of *Leucocytozoon*, with the exception of *L. caulleryi*, in which biting midges (Diptera: Ceratopogonidae) are the vectors. Merogony occurs in avian hepatocytes, macrophages and other reticuloendothelial cells, while gametocytes develop in erythroblasts, erythrocytes and mononuclear leucocytes (Valkiŭnas, 2004).

Leucocytozoon danilewskyi may be found in both Strigidae and Tytonidae groups (Forrester & Greiner, 2008).

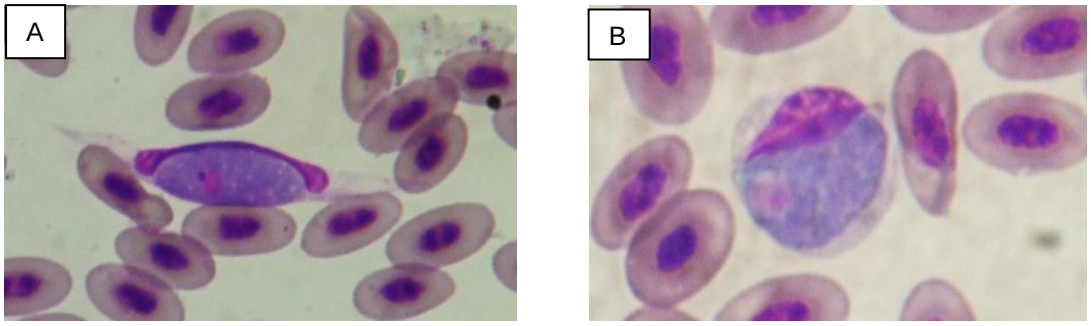

Figure 10 – Fusiform (A) and round (B) macrogametocytes of *Leucocytozoon* spp. in *Strix aluco* blood (Diff-Quick, x1000). Gently ceded by Tomás (2014).

1.3. Pathogenicity and clinical signs

In general, haemoparasites seem to have little pathogenic impact on avian hosts and they are frequently found in healthy birds of prey, however, associated with other factors, such as concomitant infections, chronic stress, environmental and nutritional drivers, they may interfere with the bird's health status. Therefore, parasitaemia may indicate an underlying disease or lead to the prognosis of a severe disease process (Matta & Rodríguez, 2001; Remple, 2004; González et al., 2021).

Anorexia, lethargy, depression, and jade-green urates are described as clinical signs displayed by infected birds; laboured breathing and concomitant anaemia may arise in terminal stages of the disease (Willette et al., 2009). Anaemia results from erythrocyte rupture, due to developing gametocytes, while cardiac and skeletal muscle, liver, lung and kidneys inflammation, necrosis and haemorrhage are a consequence of meronts and megalomeronts rupture (Niedringhaus et al., 2018). Hepatomegaly and splenomegaly are common necropsy findings, as well as general pallor of all tissues (Valkiŭnas, 2004; Niedringhaus et al., 2018; Barino et al., 2021).

Several owl species seem to be particularly susceptible to the effects of disease, for example, Snowy Owls (*Bubo scandiacus*) present signs ranging from anaemia, anorexia, depression, lethargy, to death due to haemosporidians' infection (Baker et al., 2018; Lee et al., 2018; Yoshimoto et al., 2021). Mortality was associated with *Leucocytozoon* spp. infection in fledgling Great Horned Owls (*Bubo virginianus*), from Canada (Hunter et al., 1997), and in nestling and fledgling great horned owls, from multiple regions of the USA (Niedringhaus et al., 2018). In a Brazilian wildlife rehabilitation centre, owl species such as the Striped Owl (*Asio clamator*), Burrowing Owl (*Athene cunicularia*) and Tropical Screech-owl (*Megascops choliba*) were found infected with *Haemoproteus syrnii*, presenting clinical signs prior to death, including lethargy, ataxia, pallor skin and mucous membranes (Barino et al., 2021).

1.4. Haemosporidian diagnosis

According to Krone et al. (2008), traditional microscopic blood smear examination and molecular techniques such as polymerase chain reaction (PCR) are valid methods for the diagnosis of avian haemosporidians, with similar levels of detection. However, Krone et al. (2008) recommend a combination of both methods to minimize the deficiencies in the respective capability to detect haemoparasites.

1.4.1. Microscopic blood smear examination

The microscopic blood smear examination is based on morphological features presented by each genus and/or species of haemoparasite (Fromont, 1993), besides the little experimental data on their vertebrate host specificity. This method allows both qualitative and quantitative assessments (Krone et al., 2008). However, haemoparasite identification is not always possible through blood smear, since some species are morphologically identical and younger phases of the parasite are often similar (Fromont, 1993). Additively, parasites periodically sequester in tissues, for example, *Plasmodium* species (Remple, 2004).

In stained blood smears, the halter-shaped pigmented gametocytes with an absence of schizonts in blood smears diagnoses *Haemoproteus* infection; cell-distorting nonpigmented gametocytes are typical of *Leucocytozoon* infection; pigmented gametocytes together with schizonts in peripheral blood confirm the diagnosis of *Plasmodium* (Remple, 2004). It is also possible to distinguish *Leucocytozoon* from *Plasmodium* and *Haemoproteus* morphologically, by the lack of hemozoin (exuberant golden-brown or black pigment granules from host's haemoglobin digestion) in all stages of its life cycle (Valkiūnas, 2005; Atkinson, 2008a).

Initial microscopic examination with low power magnification allows the viewing of distorted leucocytes containing *Leucocytozoon* spp. and distorted erythrocytes containing *Haemoproteus* spp. Afterwards, using a higher magnification (40 or 100x), it may be possible to recognise parasites with lower dimensions, such as *Plasmodium* spp. This observation enables the quantification of haemoparasites, in order to calculate parasitaemia levels, and also the detailed examination of the several structures of the haemoparasite forms (Clark et al., 2009).

1.4.2. Molecular techniques

Molecular methods that allow to study mitochondrial DNA sequence variation became a key part of avian haemosporidian detection, particularly at early stages of infections and

throughout chronic infections, when parasitaemia is low and parasites can be missed in blood smear examinations (Jarvi et al., 2003; Bensch & Hellgren, 2020). Furthermore, molecular diversity is presumably higher than morphological diversity, taking into account genetic variation among individuals within populations and species (Sehgal et al., 2006).

A 479 bp fragment of the cytochrome b (*cytb*) gene, a particular region of the haemosporidians' mitochondrial DNA, has been the target in several studies, therefore becoming the "barcoding" region for these avian parasites. Unique haplotypes of this gene region are known as "lineages" and can be amplified by a few primer pairs. Thus, molecular methods allow parasites' identification and phylogenetic reconstruction, but also to know further about the genetics and molecular biology of haemosporidians (Bensch & Hellgren, 2020).

However, these methods fail to detect some infections, even though parasites were observed in smears and vice versa (Krone et al., 2008) and, used alone, PCR techniques alone may underestimate simultaneous infections by haemosporidians (Valkiŭnas et al., 2006).

1.5. Prevention and management

Despite the difficulties posed to eradicate haemoparasites in the wild (Levin & Parker, 2012) some preventative measures are preconized for captive avian collections, namely related to vector control (mosquito netting and eliminating mosquito breeding grounds). Other options are keeping birds indoors when mosquitoes are active and the application of prophylactic measures, such as mefloquine hydrochloride at 30 mg/kg, PO, q7d (Willette et al., 2009; Ponder & Willette, 2015).

The treatment of this disease is based on the oral administration of antimalarial drugs, associated with supportive therapy, including oxygen, fluids and blood transfusions as required (Willette et al., 2009). Oral treatment options, described for raptor species, are summarised in Table 2.

Table 2 – Antimalarial treatment options, described for raptor species.

Treatment	Reference
Mefloquine hydrochloride – 30 mg/kg, PO, at 0, 12, 24 and 48h	Tavernier et al., 2005; Willette et al., 2009
Mefloquine hydrochloride – 50 mg/kg, PO, q24h x 7 days	Chitty & Lierz, 2008
Chloroquine phosphate – 10 mg/kg at 0h, then 5 mg/kg at 6, 24 and 48h Primaquine – 0.3 mg/kg, PO starting at 24h, q24h x 7 days	Hawkins, et al., 2013
Chloroquine phosphate – 10 mg/kg, PO, q7d Primaquine – 1 mg/kg, PO, q7d	Hawkins, et al., 2013

1.6. Epidemiology

The irregular distribution of hosts and their ecological traits, adding to abiotic factors, such as landscape and climate, result in a patchy distribution of most haemosporidian species, however there is a general tendency to cosmopolitanism (Valkiŭnas, 2008, Fecchio et al., 2021). In mainland Portugal, there are several studies and reports on haemosporidian infections in diverse wild bird species (Tomás et al., 2014; Zacarias, 2017; Leitão, 2020).

In a study conducted in 1999, in southern Portugal, 41% of the sampled *Athene noctua* were infected with *Leucocytozoon ziemanni* and one individual was infected with *Trypanosoma* sp. (Tomé et al., 2005).

A survey in two Portuguese wildlife rehabilitation centres, located in Parque Nacional da Peneda-Gerês and Parque Natural da Ria Formosa (RIAS), reported that 64% of the studied Strigiformes were infected with haemoparasites and this prevalence was significantly higher compared with Falconiformes (20%). Within the nocturnal raptors, 50% were positive for *Leucocytozoon* spp. and 14% for *Haemoproteus* spp. (Santos et al., 2008).

In the Wildlife Rehabilitation Centre of Lisbon (LxCRAS), Martinho & Melo (2006) found that 31% of the sampled Strigiformes (n = 16) were infected with *Leucocytozoon* spp., 38% were infected with *Haemoproteus* spp. and 50% were infected with *Plasmodium* spp. Later, in 2010, in the same wildlife centre, a study based on 692 raptors' blood smears, collected throughout two years, revealed that 22% of the sampled birds were positive for haemoparasites, from which 25% were respecting to Falconiformes samples and 75% to Strigiformes samples. Four different genera were found, namely *Leucocytozoon* (the most commmon), *Haemoproteus*, *Plasmodium* and *Babesia*; mixed infections of two or three distinct genera were identified in both diurnal and nocturnal birds. Taking into consideration

the region of capture, Central Portugal registered the highest prevalence, especially in spring and summer seasons (Baptista et al., 2010).

Other study, developed at the Wildlife Rehabilitation and Investigation Centre of the Ria Formosa (RIAS), owls presented a prevalence of 32% regarding to haemoparasite infection. *Leucocytozoon* spp*., Haemoproteus* spp*.*, and *Plasmodium* spp*.* were identified on blood smears with a decreasing prevalence, respectively; parasitic associations of *Leucocytozoon* sp. and *Haemoproteus* sp. were identified in *Athene noctua* and *Bubo bubo*'s samples (Zacarias, 2017).

In a study developed at the Wildlife Ecology, Rehabilitation and Surveillance Centre of Gouveia (CERVAS), several avian species' liver and spleen samples were collected for DNA extraction and nested-PCR. About 62% of the owls' samples were positive either for *Leucocytozoon* and/or *Haemoproteus/Plasmodium*, in species such as *Athene noctua*, *Bubo bubo*, *Strix aluco* and *Tyto alba* (Leitão, 2020).

1.7. Impacts of haemoparasites on the conservation of owls

Parasites are organisms that tend to reach a state of balance with the respective hosts, resulting in minimal consequences when the environmental conditions are stable. However, sudden changes, such as genetic mutation or recombination of the parasitic agent and variations in the host's immune response or in the environment (loss of habitat or prey) may result in disease. Associated with other ecological factors that may decrease birds' populations, parasitic diseases may represent an important cause of mortality for these groups, especially if there is the cumulative effect of different diseases in the same population (Saggese, 2007a; Wobeser, 2013).

Chronic haemosporidian infections play an important role as selective agents in wild bird populations, with negative fitness effects (Marzal et al., 2005; Asghar et al., 2011). Other consideration is the potential competitive advantage of avian species that have lower prevalence of haemoparasites infection, which also reflects individual and population's fitness (Valkiŭnas, 2004; Ishak et al., 2008). Furthermore, the infections by blood parasites increase the risk of predation in their avian hosts (Møller & Nielsen, 2007).

The populations more prone to the negative effects of emergent diseases are those geographically restricted and isolated from specific pathogens (Atkinson & LaPointe, 2009), that present limited genetic diversity (Saggese et al., 2007b), and the ones that breed in colonies (Tella, 2002). Individual avian host features, such as age, reproductive status and immunity seem to affect the infection probability and haemoparasitaemia (Knowles et al.,

2011). For example, the lack of immunity from no previous exposure to the pathogen may result in greater sequelae in immature birds (Sol et al., 2003).

Migratory birds are more exposed to diverse pathogens (Hubálek, 2004) and may carry haemoparasites between distinct geographical regions, therefore being important carriers and arising cross-species transmission to resident birds (Tanigawa et al., 2012; Inumaru et al., 2017). Vector distribution and habitat requirements are also key factors affecting spatiotemporal patterns of avian haemoparasites (Knowles et al., 2011).

Haemoparasitism, combined with food availability, may impact the life cycle of owls. A study conducted with Boreal Owls (*Aegolius funereus*) breeding pairs, in western Finland, reported that the infection with *Leucocytozoon ziemanni* reduced the clutch size of females when the abundance of the main food was intermediate, with no effect when the food abundance peaked (Korpimaki et al., 1993). A study with *Strix aluco*, developed in Northern England, revealed that parasite burdens in adults were negatively correlated with the food abundance on their territories (Appleby et al.,1999).

1.8. Aims of the study

The main goal of this study was to analyse the haemosporidians in owl tissues from different regions of mainland Portugal. Owl samples were collected from dead individuals, obtained mostly in wildlife rehabilitation centres. Specifically, it was intended to compare the occurrence of these parasites in different owl species, age classes and sexes, but also to evaluate variations in parasite occurrence between the several regions where the birds were collected. Temporal variations, namely year and season, were also studied.

2. Materials and methods

2.1. Sampling

Owl samples corresponded either to liver tissue, previously frozen and kept in a samples archive or collected after bird necropsies (stored at -20ºC). Samples were collected from four distinct institutions in Central and Southern Portugal (Table 3, Figure 11):

a) Wildlife Ecology, Rehabilitation and Surveillance Centre of Gouveia (CERVAS), Central Portugal region, NUTS II (PT16);
b) Centre for Studies and Rehabilitation of Wild Animals of Castelo Branco (CERAS), Central Portugal region, NUTS II (PT16);
c) Laboratory of Ornithology from the University of Évora (LabOr), Alentejo region, NUTS II (PT18);
d) Wildlife Rehabilitation and Investigation Centre of the Ria Formosa (RIAS), Algarve region, NUTS II (PT15).

Most of the samples, with the exception of those from LabOr, corresponded to owls that were admitted into wildlife centres and died after tentative treatment. The samples from LabOr were roadkill casualties collected in the district of Évora. These samples correspond to animals collected from 2010 to 2021, being organised and processed between September 2021 and June 2022.

The time dedicated to necropsy work and sample collection represented around 50 hours, while laboratory work represented approximately 100 hours.

Table 3 – Number of samples collected per institution.

Place of collection	Number of samples (n)
CERAS	17
CERVAS	44
LabOr	15
RIAS	164
Total	240

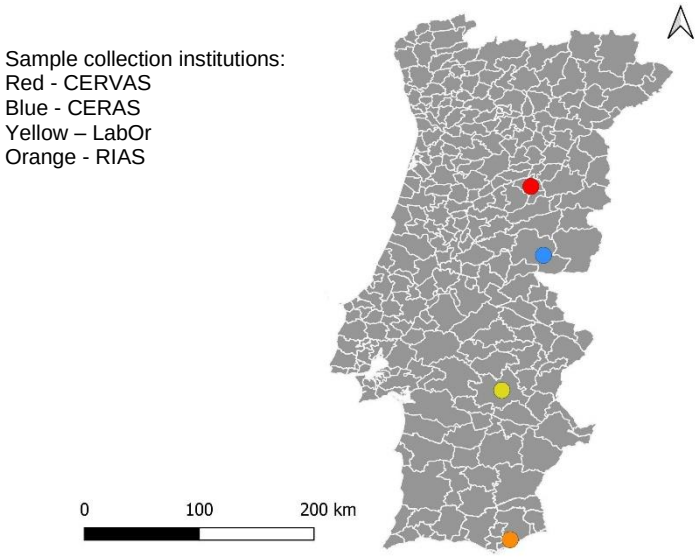

Figure 11 – Map of continental Portugal, with the sample collection institutions and respective locations (coloured circles).

2.2. Necropsies and sample collection

The necropsy technique was based and adapted from Peleteiro (2016), in which the coelomic cavity was opened and the owl's gastrointestinal tract was inspected, and a liver fragment was collected using sterilised scalpel blades (Figure 12), forceps and scissors into a sterilized recipient, and frozen at – 20ºC until further processing.

Each sample was identified and associated with the respective bird species, date of admission in the wildlife centre/laboratory, geographical region where the bird was found, age and sex (the latter when it was possible to identify, based on biometry/morphology or by inspection of gonads during the necropsy; Martínez et al., 2002).

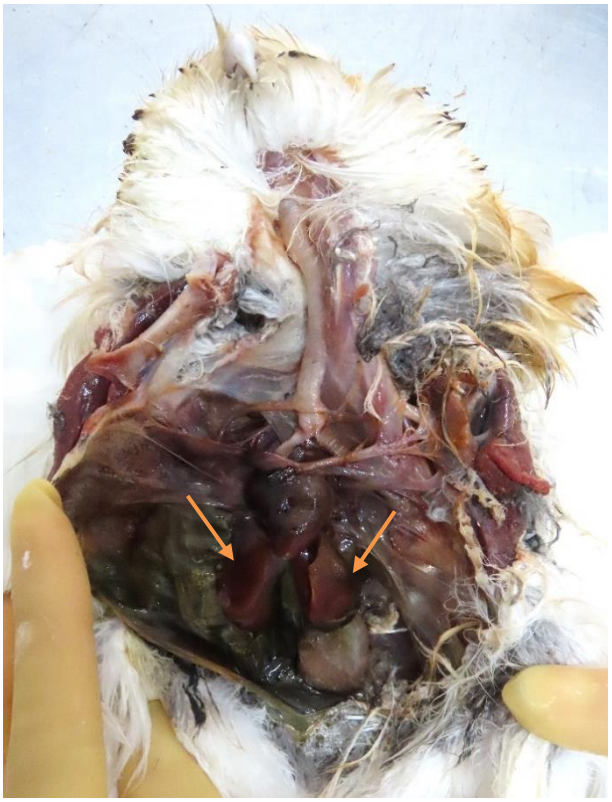

Figure 12 – Barn Owl (*Tyto alba*) necropsy: liver lobes indicated with orange arrows (original).

2.3. DNA extraction

For DNA extraction, approximately 100 milligrams of hepatic tissue was cut in small fragments with a scalpel blade and mixed with 200 microlitres of phosphate-buffered saline (PBS). The tissues were homogenized individually after a brief centrifugation, the supernatant of each homogenized tissue was subjected to nucleic acid extraction using the IndiMag Pathogen Kit (Indical, Leipzig, Germany) in a King Fisher Flex extractor (ThermoScientific, Waltham, EUA), following manufacturer's instructions.

2.4. Polymerase chain reaction (PCR) amplification

A nested-PCR was conducted for each sample, targeting the *cytb* gene of the mitochondria of the haemosporidians, enabling the screening of *Plasmodium* and *Haemoproteus* alongside with *Leucocytozoon* genus. The protocol includes a first PCR, which amplifies the DNA of haemoparasites from all three genera, followed by a second PCR with two primer pairs to either amplify *Haemoproteus* and *Plasmodium* spp. DNA and a third to amplify *Leucocytozoon* spp. DNA (Bensch et al. 2009). Primer sequences are described in Table 4.

The first PCR was carried out in a final volume of 25 µL, including 12.5 µL of MasterMix Taq II 2x Green (NZYTech, Portugal), 1 µL of Primer HaemNFI, 1 µL of Primer HaemNR3 and 5.5 µL of H_2O; 5 µL of DNA was added. The second amplification reaction was

accomplished in a final volume of 25 µL, with 12.5 µL of MasterMix Taq, 1 µL of Primer HaemF, 1 µL of Primer HaemR2 and 8.5 µL of H_2O; 2 µL of DNA was added. For the third PCR, a final volume of 25 µL was achieved, including 12.5 µL of MasterMix Taq, 1 µL of Primer HaemFL, 1 µL of Primer HaemR2L and 8.5 µL of H_2O; 2 µL of DNA was added.

In each PCR, negative and positive control samples were added, namely negative controls with sterilised water, and positive controls, containing DNA from previously identified haemosporidian DNA.

Thermal cycling (BIO-RAD T100 Thermal Cycler) had an initial denaturing step (one cycle) for 3 minutes at 95ºC, followed by 35 cycles of denaturation for 20 seconds at 94ºC, annealing for 20 seconds at 50ºC, and extension for 30 seconds at 72ºC; with a final extension step, for 7 minutes, at 72ºC.

For amplification detection, agarose gel electrophoresis was used. After preparing a 1.5% agarose gel in tris-borate-EDTA (TBE) buffer, 8 µL of PCR product was loaded into the wells. A 100 bp NZY DNA Ladder V was chosen as DNA size marker in each gel, in order to evaluate the size of the amplicons. Agarose gel electrophoresis allowed the separation of DNA fragments, resultant from the PCRs, in accordance with their size. The gels were observed and photographed under UV light.

Table 4 – Primers used on the current study.

Primer name	Sequence	Reaction	Amplification	Reference
HaemNFI	5'-CATATATTAAGAGAAITATGGAG-3'	1st	*Haemoproteus*, *Plasmodium* and *Leucocytozoon*	Hellgren et al., 2004
HaemNR3	5'-ATAGAAAGATAAGAAATACCATTC-3'	1st	*Haemoproteus*, *Plasmodium* and *Leucocytozoon*	Hellgren et al., 2004
HaemF	5'-ATGGTGCTTTCGATATATGCATG-3'	2nd	*Haemoproteus* and *Plasmodium*	Bensch et al., 2000
HaemR2	5'-GCATTATCTGGATGTGATAATGGT-3'	2nd	*Haemoproteus* and *Plasmodium*	Bensch et al., 2000
HaemFL	5'-ATGGTGTTTTAGATACTTACATT-3'	3rd	*Leucocytozoon*	Hellgren et al., 2004
HaemR2L	5'-CATTATCTGGATGAGATAATGGIGC-3'	3rd	*Leucocytozoon*	Hellgren et al., 2004

(*) The letter "I" in the primer sequences of HAEMNFI and HAEMR2L is associated to the modified base Inosine.

2.5. DNA purification for sequencing

Ten samples which were found positive for the second PCR (*Haemoproteus*/*Plasmodium* spp.) and nine samples positive for the third PCR (*Leucocytozoon* spp.) were selected, with the goal to represent the widest variety of species and sample collection locations, for purification and further sequencing.

DNA purification was achieved with NZYGelpure kit, followed by DNA quantification, performed from the PCR products, using a NanoDrop microvolume spectrophotometer, according to the manufacturer's recommendations. Afterwards, the purified PCR products were sent to Eurofins Genomics Europe Sequencing GmbH, for Sanger sequencing on both ends.

2.6. Phylogenetic analyses

MEGA 11 software (Tamura et al., 2021) was used to edit manually the obtained sequences, by removing the primers. For each sample, the forward and reverse sequence were aligned using the MUSCLE method, from the MEGA 11 software and the consensus sequence was obtained using the EMBOSS web server (European Molecular Biology Laboratory, 2022). The Maximum Likelihood algorithm was designated to conduct phylogenetic analysis on the IQ-TREE web server, version 1.6.12 (Trifinopoulos et al., 2016; Minh et al., 2019): the substitution models were determined based on the ModelFinder, which automatically uses Bayesian Information Criterion to select the best-fit model for each analysis (Kalyaanamoorthy et al., 2017). The trees were visualized and edited using FigTree 1.4.4 software (Rambaut, 2018).

2.7. Statistical analysis

Microsoft Excel ® was used for data descriptive analysis and graph production, while the difference between the frequencies of the presence or absence of the studied haemosporidians was analysed, using chi-square tests (significance level considered at $p < 0.05$). The software R studio version 1.2.5042 was used for statistical analysis. Four binomial response variables were considered in the analyses: a) presence/absence of *Plasmodium/Haemoproteus*; b) presence/absence of *Leucocytozoon*; c) presence/absence of any of the three haemoparasites; d) presence/absence of both haemoparasites. The first analysis focused on differences according to owl species, and the explanatory variable was categorical: *Tyto alba*, *Bubo bubo, Athene noctua* and *Strix aluco*. The other owl species were not considered in this analysis due to very small sample size. The effect of the age of the owl sample was analysed for the same four response variables mentioned above, using three categories, namely fledgling, juvenile and adult. Fledglings included all the nestling and fledgling individuals, still dependent of parental care; juveniles were all the individuals, independent of parental care, until the first moults of flight feathers (occurring at the end of the first year of life); adults were classified based on flight feather appearance, namely if they had moulted feathers, with a few or all adult feathers (i.e., individuals with more than one year). The effect of the region from where the sample was collected was also studied, based on the Nomenclature of Territorial Units for Statistics (NUTS), specifically in NUTS II (regions) (Eurostat, 2019), and used as an explanatory variable: a) Centre, Setúbal and North regions; b) Alentejo; and c) Algarve. To check for temporal effects, the explanatory variable “year interval” (interval 1, from 2011 to 2016; interval 2, from 2017 to 2021) was used with the same four response variables. Finally, the effect of the season (categorical

variable: winter; spring; summer; autumn) was studied for the four response variables. Subsequently, the effects mentioned above (age, NUTS II, year interval and season) were studied for each owl species.

The Geographical Information System QGIS 3.26 Buenos Aires was used to prepare maps, showing the distinct proportions between positive/negative samples, for each one of the studied regions, namely NUTS II.

3. Results

3.1. Sample description

Overall, samples collected from 240 owls were included in the present study, classified by species, age and sex. Most of the samples (n = 34, 39%) corresponded to *Athene noctua*, followed by *Tyto alba* (n = 77, 32%); the most represented age group was juvenile (n = 110, 46%) and it was not possible to determine the sex of most birds (n = 136, 57%) (Table 5).

Regarding spatial and temporal features, Algarve was the most represented region (Table 8), with 67% of the overall samples (n = 133), and 45% of the samples corresponded to summer season (n = 108, Table 7). The majority (n = 203, 85%) of the samples were associated with interval 2 (2017-2021), especially with the year of 2021 (n = 53, 22%) (Table 6).

In the NUTS II analysis, the samples from Setúbal (n = 1) and North regions (n = 1), were considered together with the Centre region (Table 8). In all the following statistical analysis, samples with undetermined information were eliminated.

Table 5 – Owl samples classified by species, age and sex.

Species	**Age**				**Sex**			**Overall**
	Fledgling	Juvenile	Adult	Undetermined	Female	Male	Undetermined	
Asio flammeus	0	0	0	1	0	0	1	1
Asio otus	0	0	2	2	0	1	3	4
Athene noctua	14	51	25	4	10	14	70	94
Bubo bubo	0	2	12	4	2	3	13	18
Strix aluco	6	8	23	9	7	5	34	46
Tyto alba	7	49	18	3	17	45	15	77
Total	27	110	80	23	36	68	136	**240**

Table 6 – Number of samples collected per year (2010-2021).

	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	Undetermined
No. of samples	2	7	1	1	3	9	8	27	40	40	43	53	6

Table 7 – Number of samples collected by season.

	Spring	Summer	Autumn	Winter	Undetermined
No. of samples	65	108	30	31	6

Table 8 – Number of samples collected by region NUTS II.

	Alentejo	Algarve	Centre+Set+N*	Undetermined
No. of samples	52	133	54	1

(*) – Centre + Setúbal + North regions.

3.2. Variation of the prevalence of haemosporidian parasites among owl species

Significant differences were found between the proportions of owl species infected with either haemosporidian ($\chi^2 = 15.472$, $p = 0.0035$, figure 13), with *Plasmodium/Haemoproteus* sp. ($\chi^2 = 31.569$, $p < 0.001$, figure 14), with *Leucocytozoon* spp. ($\chi^2 = 23.919$, $p < 0.001$, figure 15) and with both haemosporidians ($\chi^2 = 37.196$, $p < 0.001$, figure 16). *Bubo bubo* was the species with a clear higher proportion of positive samples for all haemosporidians.

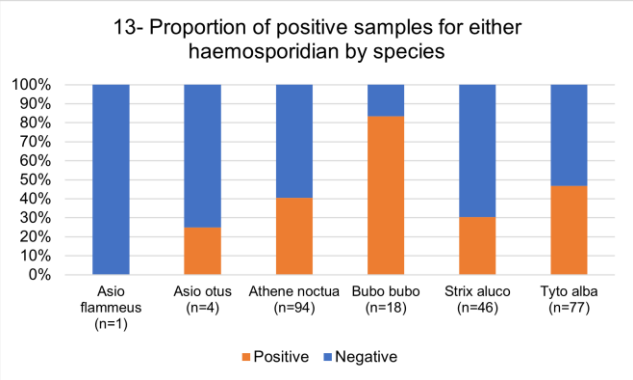

Figure 13 – Proportion of positive samples for either haemosporidian by owl species.

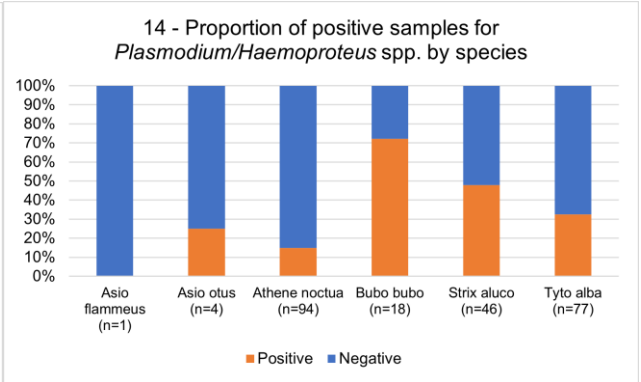

Figure 14 – Proportion of positive samples for *Plasmodium/Haemoproteus* spp. by owl species.

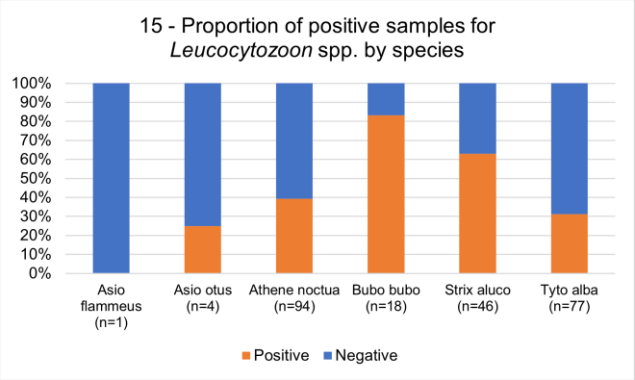

Figure 15 – Proportion of positive samples for *Leucocytozoon* spp. by owl species.

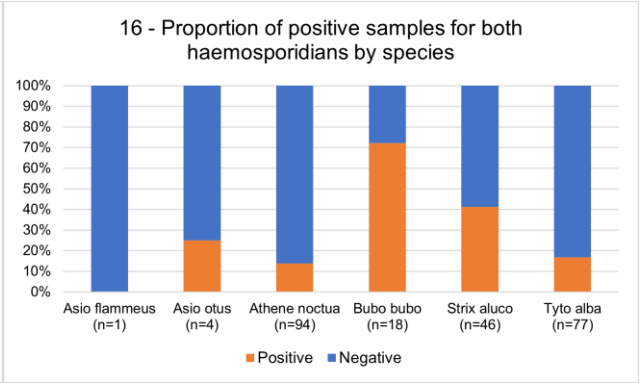

Figure 16 - Proportion of positive samples for both haemosporidians by owl species.

3.3. Variation of the prevalence of haemosporidan parasites between owl age groups

Significant differences were found between the proportions of the distinct age groups, infected with either haemosporidian (χ^2 = 12.162, p = 0.004, figure 17), with *Leucocytozoon* spp. (χ^2 = 24.476, p < 0.001, figure 18) and with both haemosporidian genera (χ^2 = 10.508, p = 0.007, figure 19). The adult owls had a higher proportion of positive samples for all haemosporidians.

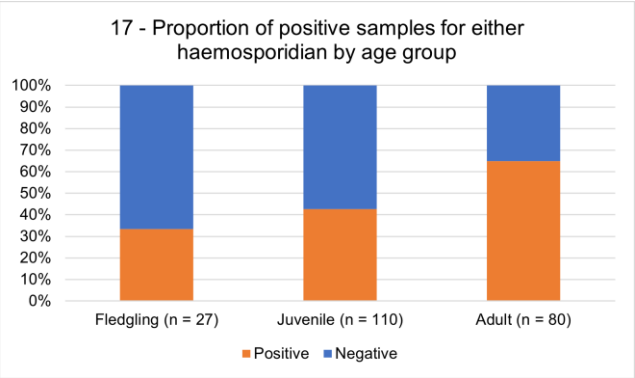

Figure 17 – Proportion of positive samples of either haemosporidian by age group.

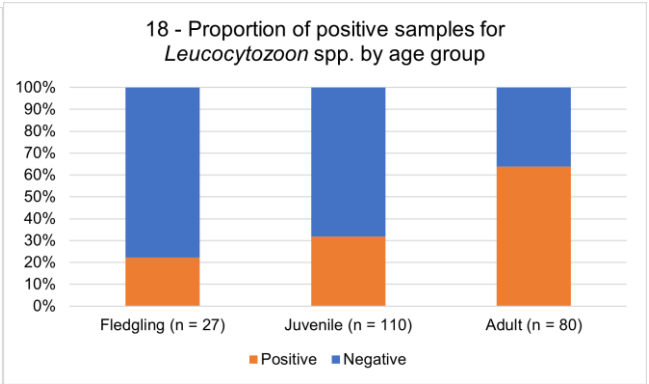

Figure 18 - Proportion of positive samples for *Leucocytozoon* spp. by age group.

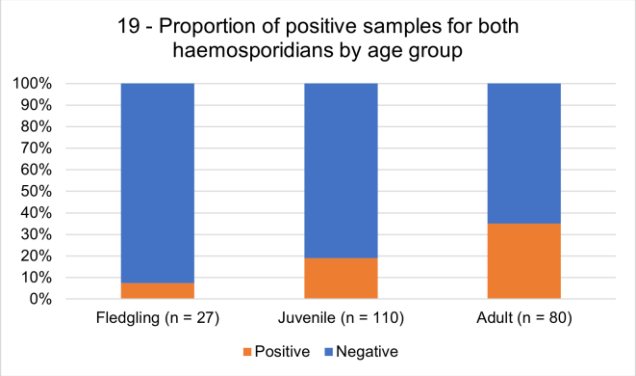

Figure 19 - Proportion of positive samples for both haemosporidians by age group.

3.4. Variation in prevalence of haemosporidian parasites according to NUTS II region

Significant differences were found between the proportions of the samples coming from different NUTS II regions, with Alentejo being the region with higher proportion of samples infected with either haemosporidian (χ^2 = 6.4854, p = 0.048, figure 20), with *Haemoproteus/Plasmodium* spp. (χ^2 = 23.307, p < 0.001, figure 21), with *Leucocytozoon* spp. (χ^2 = 51.805, p < 0.001, figure 22) and with both haemosporidian genera (χ^2 = 17.353, p < 0.001, figure 23). Maps with results per NUTS II were constructed for better visualisation of the proportions of positive samples by region (Figures 24, 25, 26 and 27).

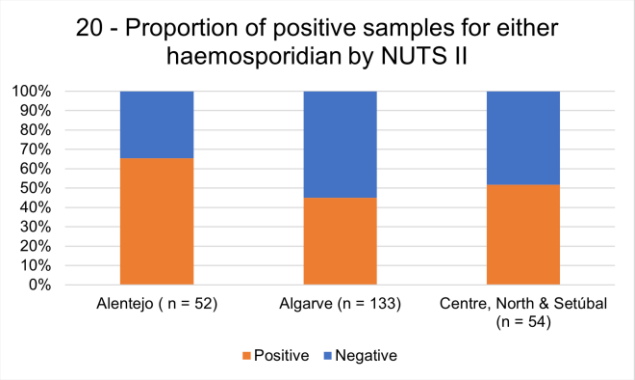

Figure 20 – Proportion of positive samples for either haemosporidian by NUTS II regions.

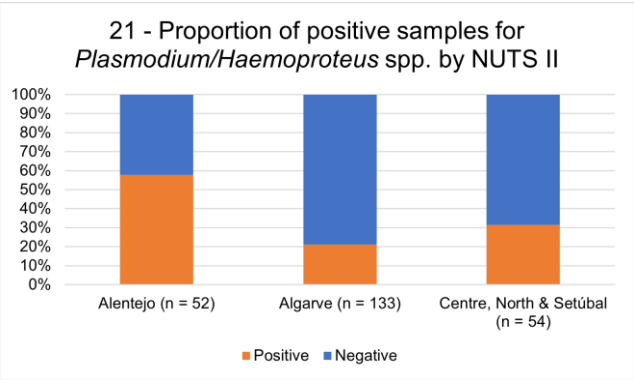

Figure 21 - Proportion of positive samples for *Plasmodium/Haemoproteus* spp., by NUTS II.

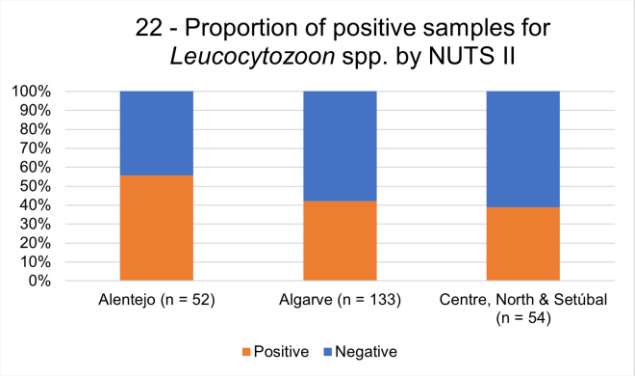

Figure 22 – Proportion of positive samples for *Leucocytozoon* spp. by NUTS II regions.

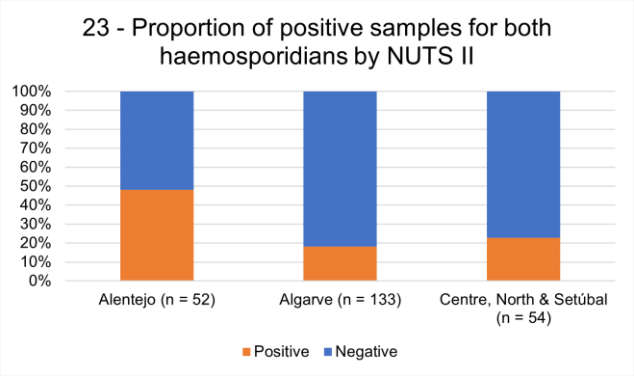

Figure 23 - Proportion of positive samples for both haemosporidians, by NUTS II.

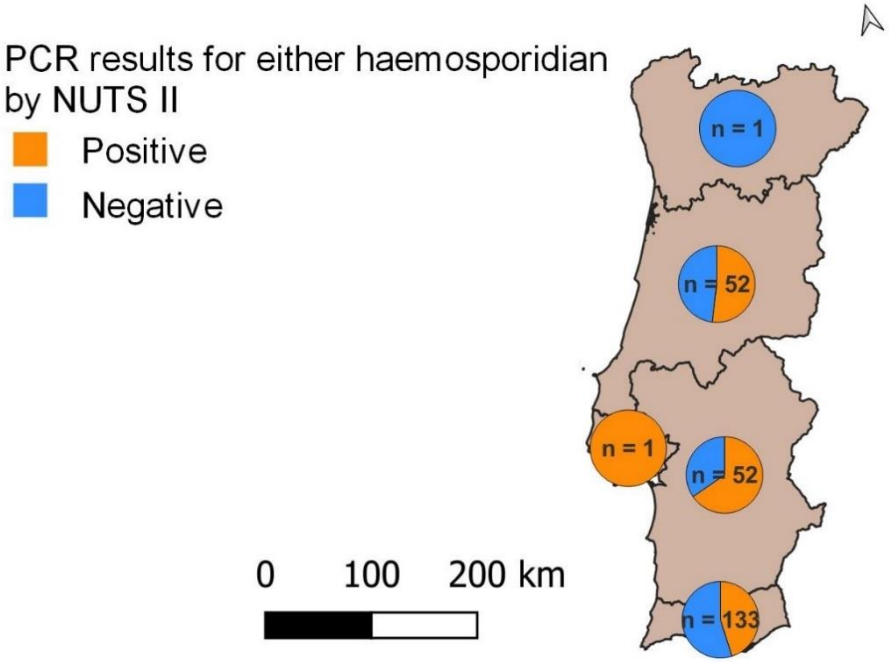

Figure 24 – Proportion of positive samples for either haemosporidian by NUTS II regions of continental Portugal.

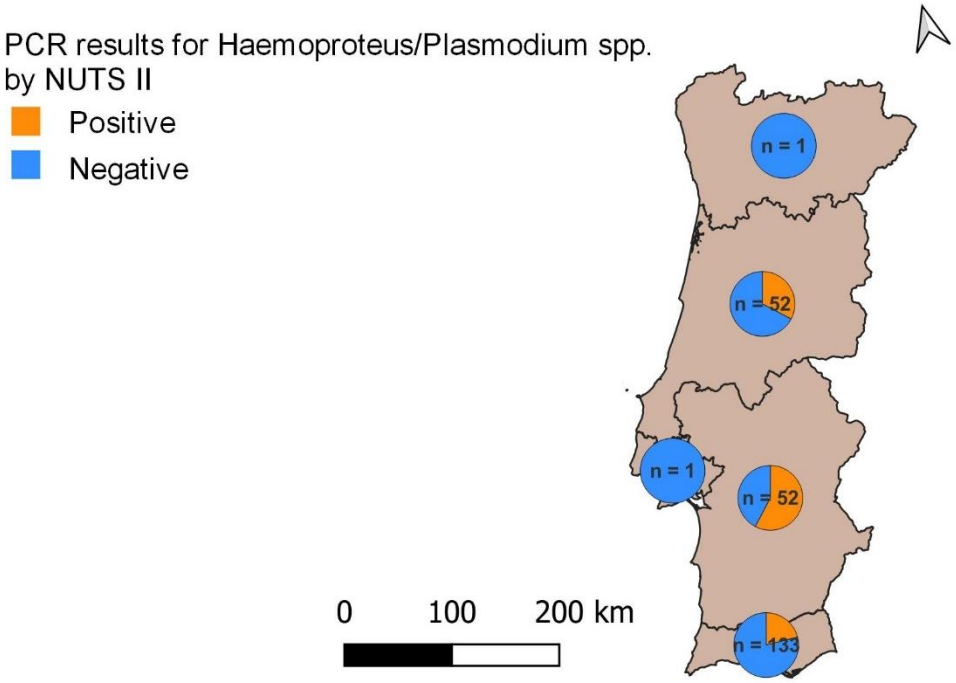

Figure 25 - Proportion of positive samples for *Haemoproteus/Plasmodium* spp. by NUTS II regions of Continental Portugal.

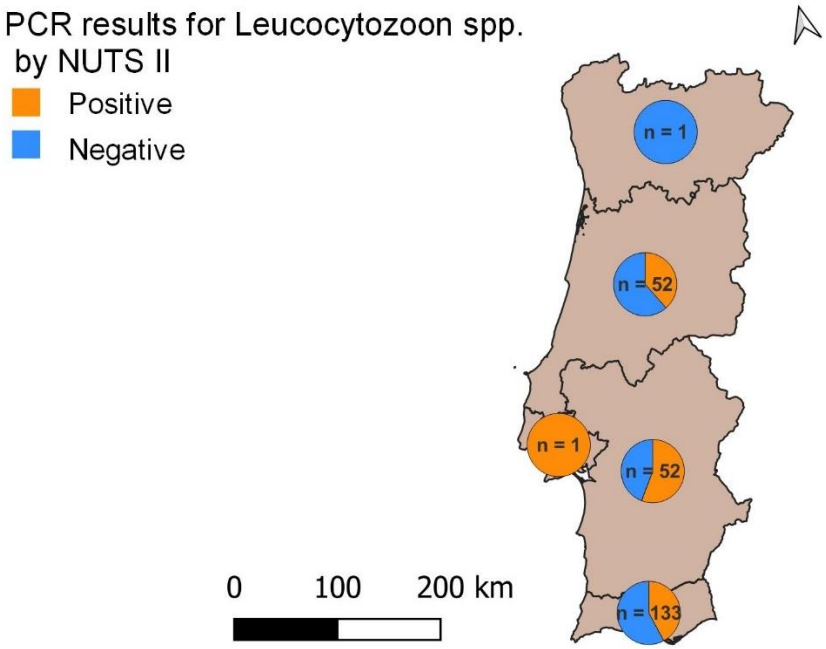

Figure 26 - Proportion of positive samples for *Leucocytozoon* spp. by NUTS II regions of Continental Portugal.

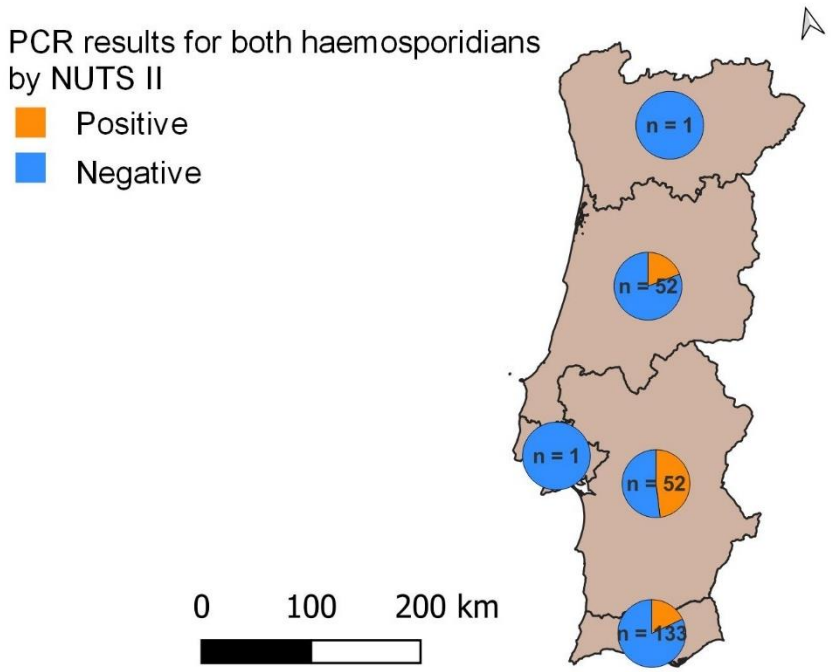

Figure 27 - Proportion of positive samples for both haemosporidians by NUTS II regions of Continental Portugal.

3.5. Variations in the prevalence within the little owl *Athene noctua*

Significant differences in prevalence were found only for the explanatory variable "season", with higher proportions of samples infected with either haemosporidian (χ^2 = 8.5492, p = 0.037, figure 28) and with *Leucocytozoon* spp. found during winter (χ^2 = 9.9518, p = 0.017, figure 29).

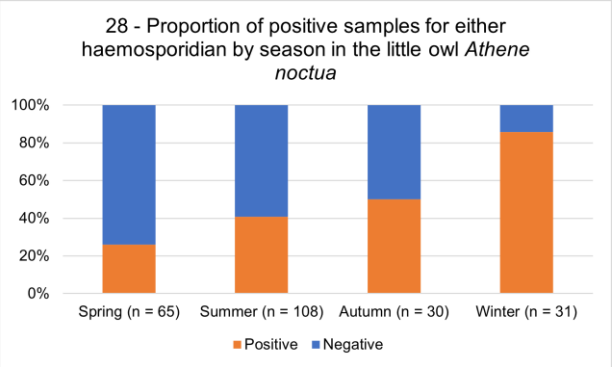

Figure 28 - Proportion of positive samples for either haemosporidian by season in the little owl *Athene noctua*.

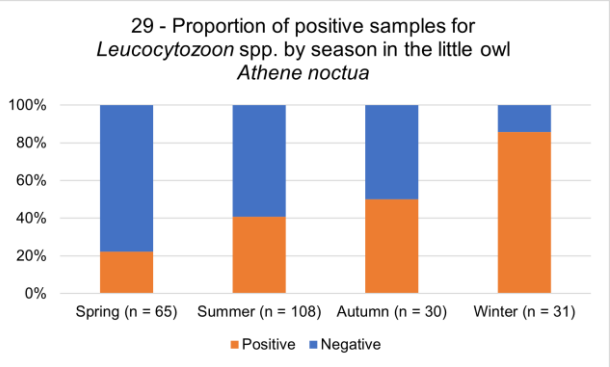

Figure 29 - Proportion of positive samples for *Leucocytozoon* spp. by season in the little owl *Athene noctua*.

3.6. Variations in the prevalence within the tawny owl *Strix aluco*

For *Strix aluco*, significant differences were found only for the explanatory variable "region", with a higher proportion of samples infected with both haemosporidians found in Alentejo ($\chi^2 = 8.7053$, $p = 0.012$, figure 30).

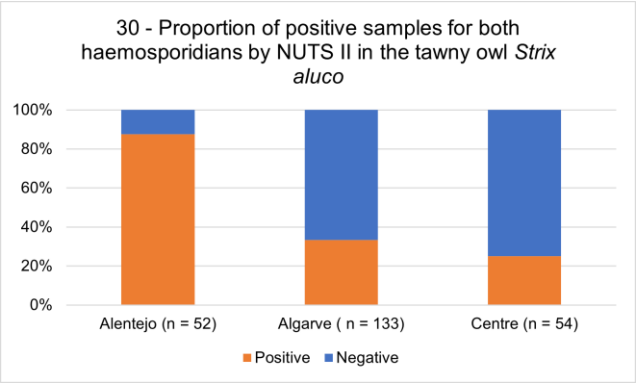

Figure 30 – Proportion of positive samples for both haemosporidians by NUTS II regions of Continental Portugal in the tawny owl *Strix aluco*.

3.7. Variations in the prevalence within the barn owl *Tyto alba*

For *Tyto alba*, significant differences were found for the explanatory variable "age" and "region". There was a higher proportion of samples infected with *Plasmodium/Haemoproteus* spp. in adults ($\chi^2 = 9.2$, $p = 0.011$, figure 31).

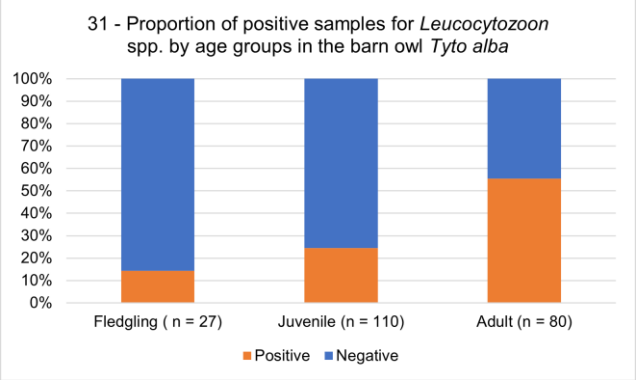

Figure 31 - Proportion of positive samples for *Leucocytozoon* spp. by age groups in the barn owl *Tyto alba*.

Regarding the regions, Algarve had clearly lower proportion of samples infected with *Plasmodium/Haemoproteus* spp. compared to Alentejo and Centre regions ($\chi^2 = 8.1227$, $p = 0.017$, figure 32).

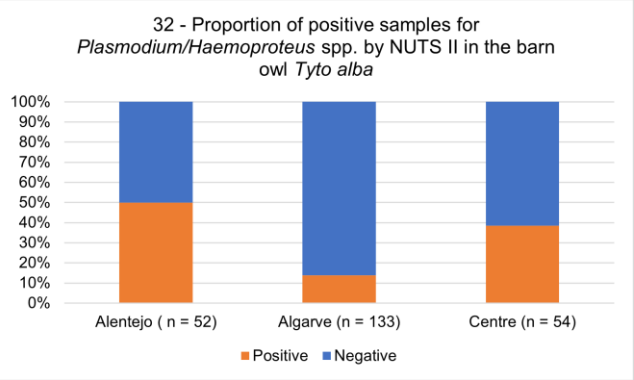

Figure 32 - Proportion of positive samples of *Tyto alba* for either *Plasmodium/Haemoproteus* spp., by NUTS II regions of Continental Portugal.

3.8. Phylogenetic analysis of haemosporidians detected in owls

Maximum likelihood phylogenetic trees calculated using aligned *cytb* sequences were constructed, including the ones from selected positive samples (presented in Table 9 and highlighted in yellow in Figures 33 and 34), and several avian haemosporidians' sequences from GenBank database (National Centre for Biotechnology Information, 2021) for comparison.

In Figure 33 there is a clear separation between *Plasmodium* spp., in the top half of the tree, oppositely to *Haemoproteus* spp., in the bottom half. The samples CVF255C and CVF276C, both from *Athene noctua* hosts, were clustered with *Plasmodium relictum*. KM361491 and CVF255C sequences were identical.

The sequences from samples CVF268C, CVF274C, RF033C, RF042C, RF151C, RF059C, CF299C and LF229C formed a clade, despite being close to *Haemoproteus noctuae* and *Haemoproteus syrnii*, they did not cluster together. RF059C and CF299C sequences were identical.

In Figure 34, three distinct clades were identified, the first one including CVF276C, CVF265C, LF243C, CVF248C and RF222C, the second one containing RF080C and RF209C, and the last one represented by RF036C and CVF274C. CVF265C and CVF276C, both from *Athene noctua* hosts, were identical; CVF274C and RF036C, both from *Tyto alba* hosts, were identical as well.

Tables 10 and 11 present avian haemosporidians' lineages identified and registered in MalAvi BLAST database (Zhang et al., 2000), the percentage of homology with this study's sequences, and the respective hosts and countries where they were identified.

Table 9 – Selected samples for DNA sequencing.

Sample code	*Haemoproteus/Plasmodium* spp.	*Leucocytozoon* spp.	Host species	Origin (NUTS II)
RF033C	X		*Tyto alba*	RIAS (Alentejo)
RF036C		X	*Tyto alba*	RIAS (Algarve)
RF042C	X		*Tyto alba*	RIAS (Alentejo)
RF059C	X		*Strix aluco*	RIAS (Alentejo)
RF080C		X	*Strix aluco*	RIAS (Algarve)
RF151C	X		*Athene noctua*	RIAS (Algarve)
RF209C		X	*Bubo bubo*	RIAS (Alentejo)
RF222C		X	*Bubo bubo*	RIAS (Algarve)
LF229C	X		*Tyto alba*	LabOr (Alentejo)
LF243C		X	*Asio otus*	LabOr (Alentejo)
CVF248C		X	*Strix aluco*	CERVAS (Centre)
CVF255C	X		*Athene noctua*	CERVAS (Centre)
CVF265C		X	*Athene noctua*	CERVAS (Centre)
CVF268C	X		*Tyto alba*	CERVAS (Centre)
CVF274C	X	X	*Tyto alba*	CERVAS (Centre)
CVF276C	X	X	*Athene noctua*	CERVAS (Centre)
CF299C	X		*Strix aluco*	CERAS (Alentejo)

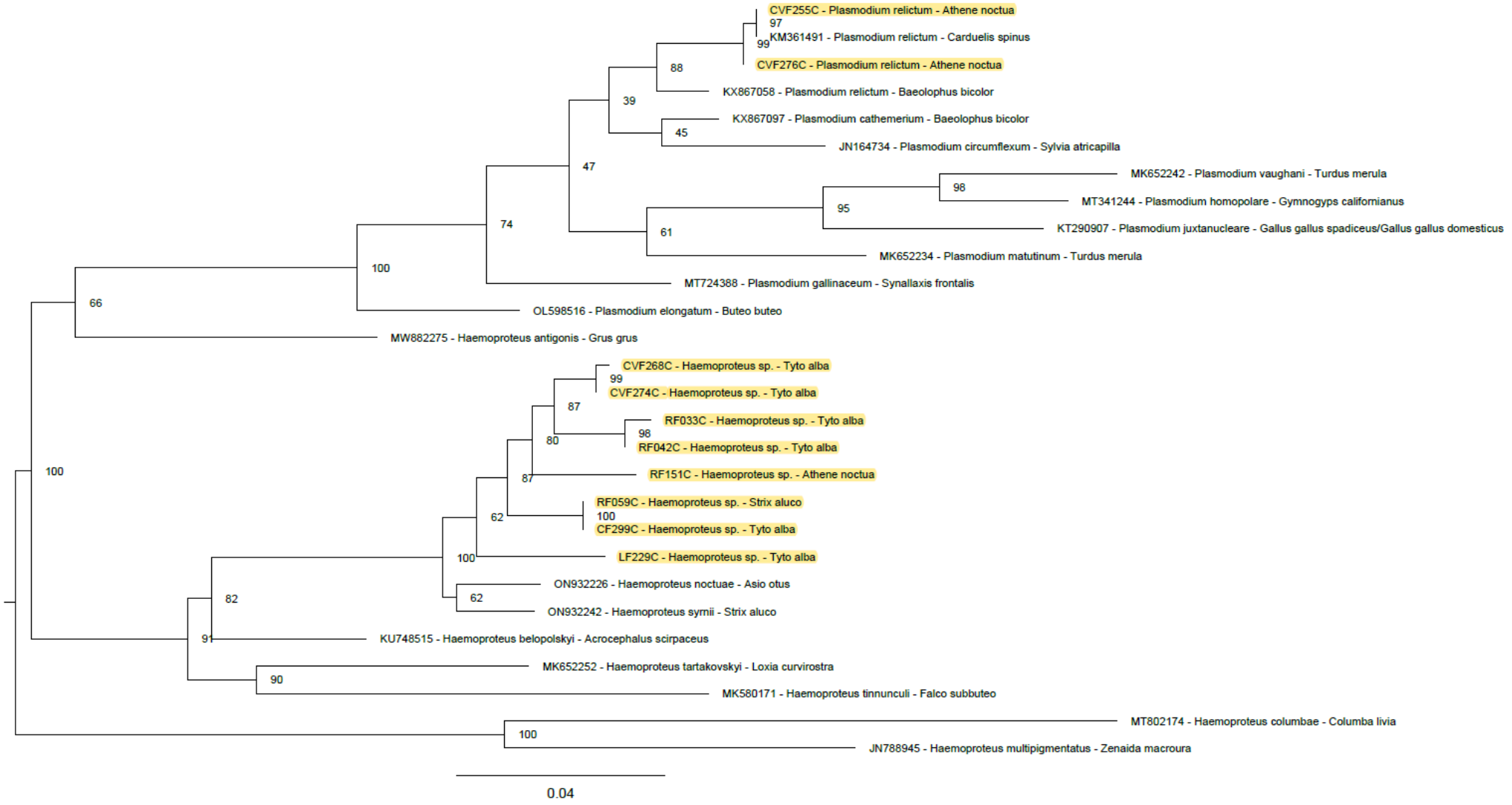

Figure 33 – Maximum likelihood phylogenetic tree calculated using aligned *cytb* sequences. Sequences resulting from samples positive for the 2nd PCR in this study are highlighted in yellow.

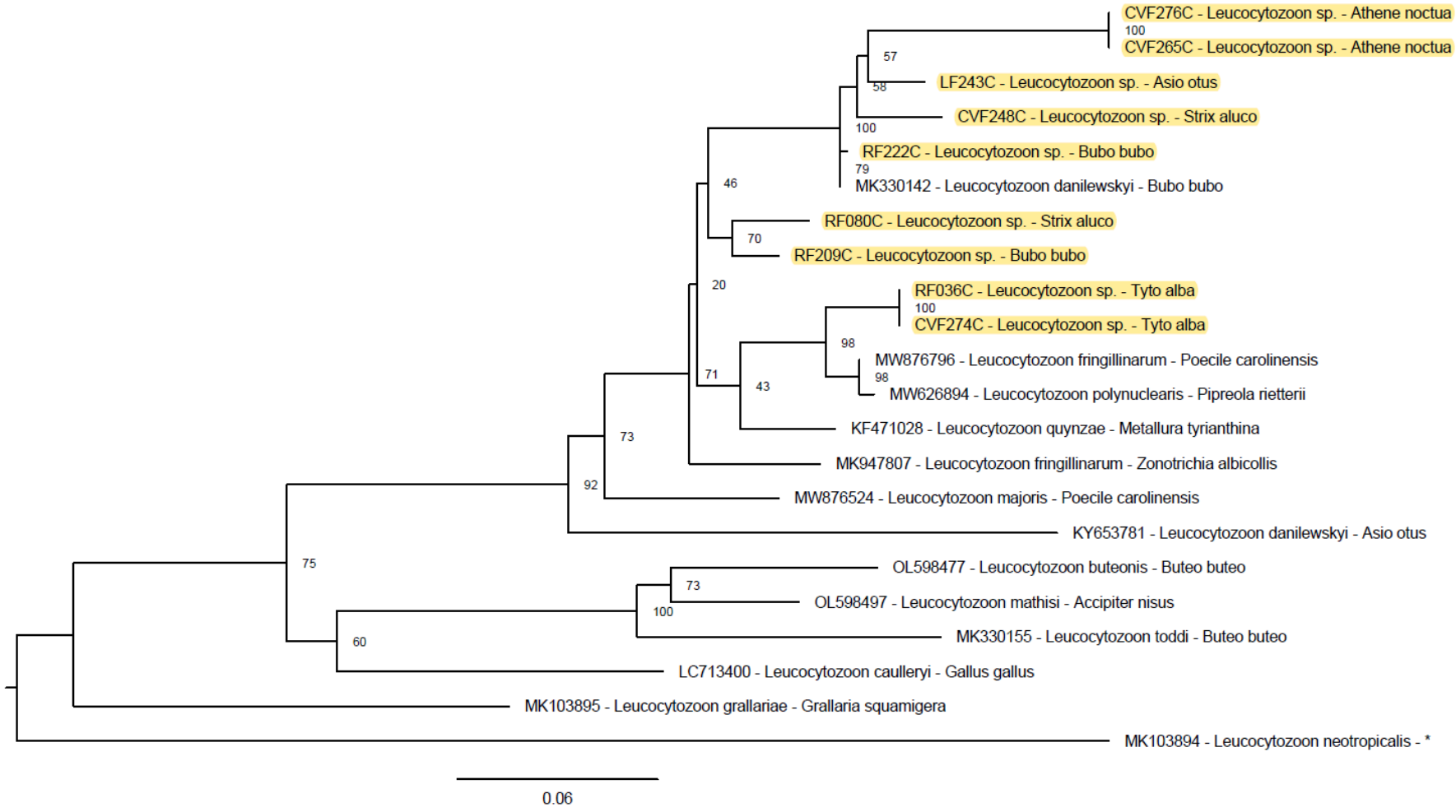

Figure 34 – Maximum likelihood phylogenetic tree calculated using aligned *cytb* sequences. Sequences resulting from samples positive for the 3rd PCR in this study are highlighted in yellow. Note: (*) host not specified, found in North American woodpeckers.

Table 10 – Lineage homologies corresponding to selected samples positive for *Plasmodium* and/or *Haemoproteus*, based on MalAvi BLAST (Zhang et al., 2000).

Sample code	Host	Haemosporidian	MalAvi Match	Hosts	Locations	References
RF033C	*Tyto alba*	*Haemoproteus* sp.	CATGUT01 – H.AK13 (98%)	*Catharus guttatus*	USA	Oakgrove et al., 2014
			ATN02 – H146 (98%)	*Athene noctua*	Morocco	Mata et al., 2015
			OTUING01 (98%)	*Megascops ingens*	Peru	McNew et al., 2021
RF042C	*Tyto alba*	*Haemoproteus* sp.	ATN02 (98%)	*Athene noctua*	Morocco	Mata et al., 2015
			OTUING01 (98%)	*Megascops ingens*	Peru	McNew et al., 2021
RF059C	*Strix aluco*	*Haemoproteus* sp.	STAL2 (100%)	*Strix aluco*	Germany	Krone et al., 2008
			- 154ZI	*S. aluco*	France	Karadgian et al., 2013
			- H219	*S. aluco*	Morocco Portugal	Mata et al., 2015
			- Haplotype A	*Strix nebulosa* *Strix uralensis* *Bubo bubo*	France	Giorgiadis et al., 2020
RF151C	*Athene noctua*	*Haemoproteus* sp.	AEFUN03 (99%)	*Aegolius funereus*	Czech Republic	Synek et al., 2016
			GLACUC03 (99%)	*Glaucidium cuculoides*	Thailand	Pornpanom et al., 2019
			GLACUC04 (99%)	*Glaucidium cuculoides*	Thailand	Pornpanom et al., 2019
LF229C	*Tyto alba*	*Haemoproteus* sp.	TYTAL6 (100%)	*Otus lettia* *Tyto alba* *Diverse Falconiformes*	Thailand	Pornpanom et al., 2019
CVF255C	*Athene noctua*	*Plasmodium* sp.	SGS1 (100%)	*Bubo scandiacus*	France	Giorgiadis et al., 2020
				Athene noctua	China	Huang et al., 2020
				Athene noctua	Portugal	Drovestski et al., 2014 Mata et al., 2015
				Diverse avian Orders	Diverse	Zhang et al., 2000
CVF268C	*Tyto alba*	*Haemoproteus* sp.	STVAR01 (98%)	*Strix varia* *Bubo virginianus*	USA	Ricklefs & Fallon, 2002
				Strix woodfordii	Cameroon	Ishak et al., 2008
				Strix occidentalis *Strix varia* *Bubo virginianus*	USA	Ishak et al., 2008
				Strix varia *Strix occidentalis*	USA	Lewicki et al., 2015
			GLACUC04 (98%) ATN02 (98%)	Check RF151C and RF042C, respectively		
CVF274C	*Tyto alba*	*Haemoproteus sp.*	STVAR01 (98%) GLACUC04 (98%) ATN02 (98%)	Check CVF268C, RF151C and RF042C, respectively		
CVF276C	*Athene noctua*	*Plasmodium*	GRW11 (100%)	*Bubo scandiacus* *Athene noctua*	France	Giorgiadis et al., 2020
				Diverse avian Orders *Note: found in Portugal*	Diverse	Zhang et al., 2000; Ventim et al., 2012a; Mata et al., 2015; Drovestski et al. 2014
CF299C	*Strix aluco*	*Haemoproteus sp.*	STAL2 (100%)	Check RF059C		

Table 11 – Lineage homologies corresponding to selected samples positive for *Leucocytozoon*, based on MalAvi BLAST (Zhang et al., 2000).

Sample code	Host	Haemosporidian	MalAvi Match	Hosts	Locations	References
RF036C	*Tyto alba*	*Leucocytozoon* sp.	CIAE02 (100%)	*Bubo bubo*	China	Huang et al., 2020
				Ninox scutulata	Japan	Inumaru et al., 2017
				Asio otus	Turkey	Ciloglu et al., 2016
				Diverse avian Orders Note: found in Portugal	Diverse	Zhang et al., 2000; Mata et al. 2015
RF080C	*Strix aluco*	*Leucocytozoon* sp.	STAL5 (99%)	*Strix aluco*	Turkey	Ciloglu et al., 2016
			STAL5 AY-2013 (99%)	*S. aluco*	Turkey	Ciloglu et al., 2016
RF209C	*Bubo bubo*	*Leucocytozoon* sp.	BUVIR02 (99%)	*B. virginianus*	United States of America (USA)	Ishak et al., 2008
				B. virginianus	USA	Fecchio et al., 2019
			BUBSUM01 (99%)	*B. sumatranus*	Thailand	Lertwatcharasarakul et al., 2021
RF222C	*B. bubo*	*Leucocytozoon* sp.	BUBO01 (99%)	*B. bubo*	Spain	Ortego & Cordero, 2009
			STOCC16 (99%)	*S. occidentalis B. virginianus*	USA	Ishak et al., 2008
			BUVIR05	*B. virginianus*	USA	Ishak et al., 2008
CVF248	*S. aluco*	*Leucocytozoon* sp.	STAL1 (98%)	*S. aluco*	Germany	Krone et al., 2008
			BUVIR04(98%)	*B. virginianus*	USA	Ishak et al., 2008
				Found in diverse avian Orders	Diverse	Zhang et al., 2000
LF243C	*Asio otus*	*Leucocytozoon sp.*	ASOT06 (99%)	*A. otus & Aegolius funereus*	Lithuania	Ishak et al., 2008
				A. otus & A. noctua	China	Huang et al., 2020
			ASIOTU02 & ASIOTU03 (99%)	*A. otus*	China	Huang et al., 2020
			ASOT2 (99%)	*A. otus*	Germany	Krone et al., 2008
CVF265C	*Athene noctua*	*Leucocytozoon* sp.	ATN01 (100%)	*A. noctua*	Morocco & Portugal	Mata et al., 2015
CVF274C	*T. alba*	*Leucocytozoon sp.*	CIAE02 (100%)		Check RF036	
CVF276	*A. noctua*	*Leucocytozoon sp.*	ATN01 (100%)		Check CVF265C	

4. Discussion

Haemosporidian parasites are very frequent in wildlife, but only a few studies were conducted specifically in Strigiformes: in our study, 122 of the 240 collected samples (51%) were positive for at least one haemosporidian species. Compared with other studies about owls' haemoparasites in Portugal, the prevalence found in this study was higher than the one found by Zacarias (2017), however, lower than the ones registered by Santos et al. (2008), Baptista et al. (2010) and Leitão (2020). The differences between the results from this study and from past studies, already mentioned, may be explained by the higher sample size focused on owls, the diverse samples' origins (other studies targeted specific regions), samples collection in distinct times of the year and several years period and, potentially, the single use of molecular techniques (most of the mentioned studies used only microscopic methods). As recommended by Krone *et al.* (2008), a combination of both microscopic and molecular methods would be ideal to increase haemoparasites detectability.

In general, 31% of the owls (n = 75) were positive for either *Haemoproteus/Plasmodium* spp., 44% (n = 106) were positive for *Leucocytozoon* spp. and about 25% (n = 59) presented mixed infections. Valkiŭnas (2004) considered that avian populations with 50% and greater prevalence of *Haemoproteus* and *Leucocytozoon* are common in the Holarctic region, therefore our results are lower than this threshold.

Bubo bubo presented significantly higher prevalences of either haemosporidian (83%), of *Haemoproteus/Plasmodium* spp. (72%), of *Leucocytozoon* spp. (83%) and mixed infections (72%), as well. This species prefers areas with dense vegetation, close to water streams, overlapping with *Simullidae*'s habitats, which may explain the especially high prevalences of *Leucocytozoon* spp. (Lehane, 2005). This is the largest owl in Portugal, with approximate wingspans of 147 cm in males and 160 cm in females (Martínez et al., 2002), therefore the big body size results in a wider surface of exposure to haemoparasites' vectors attack and for haemoparasites' establishment (Atkinson & Van Riper, 1991).

Strix aluco inhabits a broad range of woodland habitats (Marchesi et al., 2006) and, consequently, may be more exposed to vectors. This is in agreement with the fact that this species presented the second highest prevalence of *Haemoproteus/Plasmodium* spp. (48%), *Leucocytozoon* spp. (63%) and mixed infections (41%), as well.

Approximately half of the sampled *Tyto alba* were infected with either haemosporidian and about 40% of the studied *Athene noctua* were positive for either parasitic agent. Tomé et al. (2014) reported a prevalence of 41% of *Leucocytozoon ziemanni* in *Athene noctua*, which was very similar to the present study (40%); however there were an absence of *Haemoproteus* spp. or *Plasmodium* spp. infections, while we found around 15% of the *Athene noctua* infected.

Asio otus was represented by a relatively small sample size (n = 4), in which only one individual was positive for both haemosporidians. To our current knowledge, it was the first time that the studied haemoparasites were identified in this species, in Portugal.

In this study, there was only one sample of *Asio flammeus* available, most likely because of its relative scarcity in our country and lower probability of admission in a wildlife rehabilitation centre. In other studies conducted in Portugal, *Asio flammeus* were infected with both *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp. in high prevalences, however these studies were based on limited sample sizes (Martinho & Melo, 2006; Santos et al., 2008; Zacarias, 2017). It would be interesting to have more information about this species' haemoparasitism, not only because it has a distinct distribution and behaviour, but also because of its respective Endangered conservation status (BirdLife International, 2021c).

The prevalence of haemoparasites may vary even between taxonomically related avian species that share similar habitats, which may be explained not only by the specific biological traits of each host species, but also by the preference of vector species. For example, *Strix aluco* presents a differential colour-based susceptibility and exposure to flying vectors (Galeotti & Sacchi, 2003), and the distinct owl species micro-habitat preferences result in differential contact with vectors (Valkiŭnas, 2005); other study reported that simuliid flies predominantly prefer larger and widespread avian hosts (Malmqvist et al., 2015).

Vectors' distinct habitat preferences influence avian haemoparasites prevalences, as well: for instance, simuliid flies require clear and well-oxigenated water, namely fast water courses or waterfalls, while Culicoides prefer to lay their eggs in a wide variety of habitats (pools, swamps, streams, marshes, tree holes, rotting vegetation, among others), as long as there is a certain amount of free water or moisture (Mellor et al., 2000; Lehane, 2005). Furthermore, simuliids present mostly diurnal activity, while Culicoides have a distinct nocturnal activity (Mellor et al., 2000; Lehane, 2005). It can

be hypothesised that owls are less active during the day, therefore, more susceptible to diurnal simuliid flies, which might explain the higher *Leucocytozoon* prevalence, compared to *Haemoproteus*, which is transmitted by Culicoides (Taft et al., 1994; Zacarias, 2017).

In general, female owls are bigger and heavier than males, which is known as a reverse sexual dimorphism, however, it is not always possible to distinguish the sexes based on morphological measurements (Martínez et al., 2002). In this study, the birds that were necropsied had sex identification, when possible, since some cadavers presented an advanced state of decomposition, resulting in the impossibility to evaluate the influence of sex on haemoparasites' prevalence. Tomás (2014) did not detect significant differences in haemoparasites' prevalences between *Strix aluco* females and males, while Krone et al. (2001) didn't find significant differences in the infection state of several studied owl species.

Adults were significantly more likely to be infected by haemoparasites than youngsters, which is in accordance with Krone et al. (2001), Leppert et al. (2008) and Tomás et al. (2014), the latter finding that infection prevalence tends to increase with age, however with low parasitaemia levels, possibly explained by acquired immunity. Tomé et al. (2005) found that adult *Athene noctua* were significantly more often infected with *L. ziernni* compared to juveniles. On the opposite, Sol et al. (2003) reported a higher intensity of blood parasites in juveniles rather than in adults, hypothesising that either younger birds with heavy parasitism die before growing into adults, or adult hosts' acquired immunity relatively to the parasite develops and consequently decreases the parasitaemia, or adult hosts' distinct behaviour may decrease vector exposure.

Focusing on the effect of season, spring was related to a significantly lower prevalence of either haemosporidian and *Leucocytozoon* spp., only within *Athene noctua*. This result may be explained by the fact that most of these individuals were represented by nestlings and juveniles (about 70%) that, as explained above, present lower haemoparasites prevalence. Tomé et al. (2005) did not find significant seasonal differences in *Athene noctua*, while Krone et al. (2001) found seasonal differences in distinct birds of prey species, however the prevalence was higher in spring and autumn. The greater prevalence in spring may be explained by the greater infection of breeding birds, when they limit their movement, and the increase of vectors populations; additively, insects such as *Culicoides* and hippoboscid flies prefer warm

and shaded areas, such as nests (Giorgiadis et al., 2020). Valkiŭnas et al. (2005) explain the higher blood parasitaemia of adult birds in spring, due to the increase of hormone levels and stress in the breeding season.

Relatively to the geographical region, Alentejo had significantly higher prevalences of either haemosporidian, of *Haemoproteus/Plasmodium* spp., of *Leucocytozoon* spp. and mixed infections, around 50% or above. Alentejo presents geoclimatic Mediterranean features, with abrupt continental degradation in its interior region: here, the summer is hot and dry, while the winter is humid and cold; precipitation is marked by a high intra and interannual variability (Ferreira, 2001). This region is known by its rolling plains and flat landscapes, oak savannah, olive groves and vineyards, where dryland agriculture stands out: montado, which has anthropogenic origin, is the most representative agroforestry system and it is characterized by parkland forested areas and cork oak that form a wooded matrix with dispersed woodlands, open areas and undisturbed covers of forest and scrublands (Godinho & Rabaça, 2011; Carvalho-Ribeiro et al., 2013; Radke et al., 2015). All owls of the Portuguese fauna, apart from the short-eared owl nest in montado, which presents great landscape heterogeneity, with variable shrub and arboreal coverage, riparian galleries, puddles and dams, as well as rural buildings (Pereira et al., 2015), and consequently present ideal habitats for haemoparasites' vectors to complete their life cycle as well.

For example, several species of Culicoides biting midges were found in Alentejo region, however, the high temperatures combined with dryness, especially in summer, are fatal for some species (Ramilo et al., 2017). A study conducted in Comporta (a coastal wetland area with salt marshes and rice farming fields) and Alqueva dam (multipurpose water resourse project that comprises artificial dams, reservoirs and weirs), both in Alentejo, have a large avian fauna, as well as natural mosquito populations, including several species of *Culex*, *Aedes*, *Anopheles* (Almeida et al., 2010), which represent ideal grounds for avian malaria transmission as well. A study regarding *Anopheles atroparvus*, the only known mosquito that may transmit human malaria in mainland Portugal, suggested that Alentejo is the main hotspot for the disease resurgence in the country (which was eliminated in 1973) (Gomes et al., 2016), which may be explained by the abundance of dry areas in this region, where anopheline mosquitoes tend to be particularly active in the search of a blood meal (de Morais, 2014). As far as the author knows, there are no entomological studies regarding simuliids in Alentejo, nevertheless, a study developed in Eastern Spain, with

intense environmental gradients, representing Mediterranean watercourse landscapes, obtained a wide variety of simuliid species, including ones with biomedical and veterinary importance (López-Peña, et al., 2020).

Some considerations should be made in relation to haemosporidians impact on owls' conservation. *Bubo bubo* had the greatest prevalence of all blood parasite groups and mixed infections, which may be important to further investigate, because this species is classified as "Near Threatened" (NT) in Portugal (Cabral et al., 2005), despite the slightly positive population and distribution trends (Lourenço et al., 2021). More studies should be encouraged in order to understand potential pathological consequences in *Bubo bubo*, since several studies report clinical signs and mortality in other *Bubo* species, namely *Bubo scandiacus* and *Bubo virginianus* (Hunter et al., 1997; Baker et al., 2018; Lee et al., 2018; Niedringhaus et al., 2018; Yoshimoto et al., 2021). It would be also interesting to study the possible conservation consequences, however, according to Valkiŭnas (2005), it is challenging to find association of haemoparasites with fitness effects in avian populations, because there is a selective mortality in the acute phase of the infection, where birds with a considerably acute peak of parasitaemia are expected to die, thus not being caught and sampled. Also, severe infections may restrict bird movements in the wild, becoming problematic to collect and study.

Wildlife rehabilitation centres represent an essential tool to study owls' haemoparasites and the assessment of arthropod vectors preventative measures in these centres should be emphasised, not only because there is a high prevalence in avian hosts in general, but also because animals that stay in captivity for longer periods of time tend to present a greater prevalence and susceptibility to blood parasites' infections (Ziman et al., 2004). Regarding projects with the goal to reintroduce rare or threatened species of birds in their previous habitat, for instance in Portugal, where avian haemoparasites seem to be widespread, there is an increased risk of failure because of the potential transmission of these pathogenic agents (Valkiŭnas, 2005).

Other important note to account for is climate change and respective increasing temperatures, which are expected to influence haemoparasites' prevalence, distribution and diversity, as well as to alter the respective vectors and bird populations distributions. Therefore, it is expected that avian malaria may spread to more northerly and southerly latitudes, but also to higher elevations (Sehgal, 2015), motivating future

studies to predict with more precision these alterations, in order to improve the success of avian conservation efforts in particularly threatened regions.

Focusing on the molecular results, the *Haemoproteus* spp. / *Plasmodium* spp. maximum likelihood tree identified two samples, with distinct sequences, both belonging to *Athene noctua* individuals, as positives for *Plasmodium relictum*. The 100% homologous lineages found for each one of these sequences were reported in several different avian host Orders and were considerably widespread in the globe. Sequences from eight distinct individuals, represented by *Tyto alba*, *Athene noctua* and *Strix aluco hosts*, were identified as *Haemoproteus* spp., however, did not cluster with *H. noctuae* or *H. syrnii* (bootstrap value = 100), probably meaning that these samples were positive for other *Haemoproteus* species, for instance *H. tytoni*, or a distinct species. RF059C (*Strix aluco*) and CF299C (*Tyto alba*) sequences were identical and allowed to infer that this *Haemoproteus* spp. is not host-specific, infecting both Strigidae and Tytonidae; in fact, the respective 100% homologous lineage (STAL2) was reported in several *Strix* species, as well as in *Bubo bubo*, in diverse geographic locations. It is the first time that this lineage was identified in a Tytonidae host.

The *Leucocytozoon* spp. maximum likelihood tree segmented three distinct clades: the first clade had varied Strigidae species as hosts and two sequences (CVF276C and CVF265C) were identical and from the same host (*Athene noctua*). These two sequences had a 100% homologous lineage (ATN01), only reported previously in *A. noctua*, in Portugal and in Morocco, thus probably host-specific. RF222C (*Bubo bubo)* sequence was closely related to MK3330142 – *L. danilewskyi*, also from a *Bubo bubo* host. The first clade can presumably be identified as *L. danilewskyi*, or a closely related species, however, care should be taken to interpret these results, since KY653781 – *L. danilewskyi (Asio otus)* seems to be genetically distant. Some hypotheses may explain these results: *cytb* gene may not be ideal to phylogenetically classify *Leucocytozoon* spp., however it was chosen and used by other authors (Ishak et al., 2008; Ortego & Cordero, 2009; Lertwatcharasarakul et al., 2021); other hypothesis is that *L. danilewskyi* was not identified correctly – this was traditionally thought to be one species, however, genetic sequencing revealed a great diversity of lineages, probably due to a combination of cryptic speciation and intraspecific variation (Ishak et al., 2008). The second clade englobed RF080 sequences belonging to *Strix aluco* and *Bubo bubo* hosts. The third clade was represented by two identical sequences, both

with *Tyto alba* as hosts; the respective 100% homologous lineage (CIAE02) was found in diverse avian species and geolocations, including Portugal.

Relatively to the phylogenetic analysis, it would be interesting to study simultaneously microscopic and molecular aspects of owls' haemosporidians, since it would allow to relate morphological features to species and also to identify cryptic parasite species, similarly to other studies (Bensch et al., 2004; Ishak et al., 2008; Ortego & Cordero, 2009; Barino et al., 2021). Furthermore, it would be valuable to calculate an average uncorrected *p* sequence divergence in the maximum likelihood tree, which enables to distinguish species. Hellgreen et al. (2007) suggested that greater than 5% sequence divergence in the *cytb* gene between two lineages is suggestive of a different species.

Other suggestions for future owls' haemoparasites studies would be to always collect blood smear samples, associated with blood/tissue samples for molecular analysis; to register owls clinical signs/necropsy findings to investigate possible relation with haemoparasitism; to collect samples in order to represent more evenly all the seasons of the year and each year as well – our study had predominantly samples from summer, which is explained by the higher number of admissions in this season; in Portugal, it would be interesting to include Northern and even insular samples, to compare prevalences and to understand the potential effects of parasites in these areas. Furthermore, it would be valuable to know more about haemoparasites' pathogenicity and mortality in threatened owl species, as well as the concurrence of other avian emerging (and re-emerging) infectious diseases, such as West Nile encephalitis and Influenza, but also toxicological contamination in infected birds.

5. References

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