UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

PREVALENCE AND GENETIC DIVERSITY OF AVIAN MALARIA AND RELATED HAEMOPARASITES OF WILD BIRDS FROM REHABILITATION CENTRES IN MAINLAND PORTUGAL

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Doutor Luís Manuel Madeira de Carvalho

2024

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2024

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Título da Tese ou Dissertação: Prevalence and genetic diversity of avian malaria and related haemoparasites of wild birds from rehabilitation centres in mainland Portugal

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Prevalência e diversidade genética da malária aviária e de hemoparasitas relacionados em aves selvagens de centros de reabilitação em Portugal continental.

Resumo

Na última década, mais de 40% das espécies de aves na Europa têm apresentado um mau estado de conservação, com mais de 30% das espécies de aves em Portugal continental ameaçadas de extinção. Para além dos fatores antropogénicos, os parasitas e os agentes patogénicos, como os hemoparasitas, têm sido implicados nestes declínios populacionais. Os centros de reabilitação da fauna selvagem desempenham um papel essencial na conservação das espécies selvagens, enquanto também podem fornecer informações valiosas sobre a transmissão e patogenicidade de muitos agentes e respetivas doenças que afetam aves selvagens, raramente amostradas na natureza. Contudo, os relatos sobre hemoparasitas em aves em cativeiro permanecem limitados.

Neste estudo, explorámos a prevalência e diversidade genética de hemosporídeos em 163 aves, incluindo 89 de centros em Portugal continental, 41 pombos selvagens de Lisboa e 33 aves exóticas de clínicas veterinárias em Lisboa. Implementou-se uma abordagem de dupla metodologia, utilizando-se em simultâneo métodos moleculares e análise morfológica de esfregaços de sangue. Nos centros de reabilitação, a prevalência total foi de 30,3%, com 19,1% infetados por *Haemoproteus*, 13,5% por *Leucocytozoon* e 1,1% por *Plasmodium*. Notavelmente, 30% das aves infetadas eram de espécies ainda não registadas como hospedeiras. A prevalência de infeção entre os pombos foi de 100% para *Haemoproteus columbae*, sem outros hemosporídeos detetados, e 0% entre as aves exóticas. Foram detetadas 19 linhagens, incluindo 2 novas linhagens. Além disso, foram identificadas 10 novas interações hospedeiro-parasita e 14 novos registos geográficos de linhagens.

Este é o primeiro estudo em Portugal a usar uma metodologia dupla, resultando na descoberta da morfoespécie para 5 linhagens, e na descoberta do marcador molecular para a espécie *Haemoproteus contortus*. A ordem Strigiformes e as aves admitidas nos centros devido a doenças debilitantes, mostraram as maiores probabilidades de infeção, destacando a relação recíproca entre um estado de debilidade e a infeção por parasitas sanguíneos. Este estudo revelou que as aves infetadas com hemosporídeos necessitaram de períodos de reabilitação mais longos, aumentando os custos para os centros e potencialmente comprometendo a sua sobrevivência após a libertação.

Estes achados sublinham a importância de integrar considerações sobre infeções por hemosporídeos nos protocolos de reabilitação, destacando os desafios colocados por estas infeções na conservação e reabilitação de aves, incluindo exigências económicas e logísticas.

Palavras-chave: Hemosporídeos aviários, duração de tratamento; *Haemoproteus*; *Leucocytozoon*; *Plasmodium*, Portugal

Prevalence and genetic diversity of avian malaria and related haemoparasites of wild birds from rehabilitation centres in mainland Portugal

Abstract

In the last decade, over 40% of bird species in Europe have experienced poor conservation status, with more than 30% of bird species in mainland Portugal threatened with extinction. Alongside anthropogenic factors, parasites and pathogens such as avian haemosporidians have been implicated in these population declines. Wildlife rehabilitation centres play an essential role in species conservation, providing valuable information on the transmission and pathogenicity of many agents and respective diseases affecting wild birds rarely sampled in nature. However, reports on haemosporidians in captive birds remain limited.

In this study, the prevalence and genetic diversity of avian haemosporidians in 163 birds, including 89 birds from rehabilitation centres in mainland Portugal, 41 feral pigeons from Lisbon, and 33 exotic birds from veterinary clinics in Lisbon. A dual-methodology approach was implemented using molecular methods and morphological analysis of blood smears.

An overall infection prevalence of 30.3% in the rehabilitation centres was found, with 19.1% infected with *Haemoproteus*, 13.5% infected with *Leucocytozoon*, and 1.1% infected with *Plasmodium*. Remarkably, 30% of the infected bird species at the centres had not been previously documented to harbour malaria parasites. The infection prevalence among feral pigeons was 100% for *Haemoproteus columbae*, with no other haemosporidians detected, and 0% among exotic birds. Were detected 19 different haemosporidian lineages, including 2 new lineages. Furthermore, we identified 10 new host–parasite interactions and 14 new geographical lineage records.

This is the first study in Portugal to use a dual-methodology approach, resulting in the identification of the morphospecies for 5 lineages whose morphospecies were previously unknown, and the discovery of the molecular marker for the species *Haemoproteus contortus*. Strigiformes and birds admitted to rehabilitation due to debilitating diseases showed the highest probabilities of being infected with haemosporidians among birds brought to wildlife centres in Portugal, highlighting the reciprocal relationship between a debilitating state and blood parasite infection. This study revealed that birds infected with haemosporidians required longer rehabilitation periods, increasing economic costs for the centres and potentially impairing their survival prospects upon release.

These findings underscore the importance of integrating haemosporidian infection considerations into rehabilitation protocols, highlighting the challenges posed by these infections in avian conservation and rehabilitation, including economic and logistical demands.

Keywords: Avian haemosporidians; treatment duration; *Haemoproteus*; *Leucocytozoon*; *Plasmodium*; Portugal

Prevalência e diversidade genética da malária aviária e de hemoparasitas relacionados em aves selvagens de centros de reabilitação em Portugal continental.

Resumo Alargado

Nas últimas décadas, as populações de aves têm sofrido uma redução considerável devido a diversos fatores ambientais e biológicos. Na Europa, mais de 40% das espécies de aves apresentam mau estado de conservação, com 30% das espécies em Portugal continental a serem classificadas como ameaçadas de extinção. Além das ameaças antropogénicas, como a destruição de habitats, poluição e mudanças climáticas, há um reconhecimento crescente da importância de parasitas e agentes patogénicos na saúde das populações de aves. Entre os agentes patogénicos mais relevantes, os hemoparasitas aviários, como os hemosporídeos, destacam-se pelo seu impacto potencial nas aves, tanto em termos de morbilidade como de mortalidade. Estes parasitas, antes considerados relativamente benignos, têm ganho cada vez mais destaque devido ao seu impacto nas populações de aves. Além de estarem associados a uma elevada mortalidade, também têm sido relacionados com a redução da condição corporal das aves, comprometendo a sua capacidade reprodutiva, diminuindo o tamanho das ninhadas, encurtando a esperança de vida e aumentando o risco de predação.

Os centros de reabilitação de fauna selvagem, ao desempenharem um papel crucial na recuperação de aves feridas ou doentes, têm também uma função importante na monitorização de parasitas. Aves que raramente são amostradas na natureza, podem ser estudadas em profundidade quando admitidas nos centros, oferecendo uma oportunidade única para investigar a prevalência e a diversidade genética de parasitas como os hemosporídeos. No entanto, os estudos sobre hemosporídeos aviários em Portugal ainda são escassos, sobretudo no que diz respeito à sua prevalência e diversidade genética em aves de centros de reabilitação. O diagnóstico preciso destes parasitas exige uma combinação de métodos morfológicos e moleculares, no entanto, os estudos em Portugal não têm utilizado esta abordagem dupla, o que deixa lacunas na nossa compreensão da diversidade e prevalência de hemosporídeos. No contexto da reabilitação de vida selvagem, especialmente em centros onde o tratamento eficaz e a rápida libertação são prioritários, a duração da permanência das aves é um indicador crucial para avaliar o sucesso da reabilitação e os custos associados. Embora a parasitose seja frequente nas aves admitidas, o impacto das infeções por hemosporídeos na duração do processo de reabilitação permanece amplamente inexplorado.

Este estudo teve como objetivo colmatar estas lacunas de conhecimento ao detetar e identificar hemosporídeos aviários, analisando a sua prevalência e diversidade genética, determinando a influência de diversos fatores na probabilidade de infeção, bem como

avaliando o impacto das infeções no processo de reabilitação. Utilizando uma metodologia dupla, pioneira em Portugal, que combina métodos moleculares para a identificação genética dos parasitas e análise morfológica de esfregaços sanguíneos para a sua caracterização morfológica, foi possível conectar a informação genética (linhagem) e a morfologia dos parasitas, aprofundando assim a nossa compreensão sobre os hemosporídeos e suas implicações tanto para a conservação da vida selvagem como para os esforços de reabilitação.

A amostragem envolveu um total de 163 aves divididas em três grupos distintos: 89 aves provenientes de centros de reabilitação de fauna selvagem em Portugal continental, 41 pombos selvagens capturados na cidade de Lisboa e 33 aves exóticas provenientes de clínicas veterinárias localizadas também em Lisboa.

Para a colheita de amostras, foram retiradas pequenas quantidades de sangue das aves, seguindo protocolos de segurança e ética animal. Foram produzidos esfregaços sanguíneos e o restante sangue foi colocado em SET-buffer e armazenado a 4ºC até análise posterior.

A análise molecular foi realizada na Faculdade de Biologia da Universidade de Extremadura, Espanha. Das amostras armazenadas em SET-buffer, foi extraído e quantificado o DNA, e foram realizadas reações *nested* em cadeia da polimerase, com foco no gene citocromo b da mitocôndria dos hemosporídeos, conforme descrito por Bensch et al. (2000) e Hellgren et al. (2004). Esta técnica molecular permitiu amplificar o material genético dos parasitas dos géneros *Haemoproteus*, *Leucocytozoon* e *Plasmodium*, possibilitando a sua identificação com base na comparação das sequências de DNA com as bases de dados MalAvi e GenBank.

No Laboratório de Parasitologia e Doenças Parasitárias da Faculdade de Medicina Veterinária da Universidade de Lisboa, os esfregaços foram corados com Giemsa e os hemosporídeos foram identificados morfologicamente até ao género, seguindo Valkiūnas (2005) e Valkiūnas e Lezhova (2018, 2022). A identificação morfológica das espécies do género *Haemoproteus* foi realizada utilizando chaves de identificação de Valkiūnas e Lezhova (2022).

Os dados recolhidos foram inseridos no Microsoft Excel e um Modelo Linear Generalizado foi utilizado para analisar a relação entre variáveis como condição corporal, infeção por hemosporídeos, centro de reabilitação e estação do ano de admissão e o número de dias de tratamento médico necessário para as aves. Uma regressão logística foi aplicada para determinar se a ordem taxonómica, condição corporal, centro de reabilitação, estação do ano e causa de admissão influenciavam a probabilidade de infeção por hemosporídeos.

Os resultados do estudo indicaram uma prevalência geral de infeção por hemosporídeos de 30.3% nas aves provenientes dos centros de reabilitação. Entre os

parasitas identificados, *Haemoproteus* spp. foram os mais prevalentes, responsáveis por 19.1% das infeções, seguido por *Leucocytozoon* spp. (13.5%) e *Plasmodium* spp. (1.1%). Destaca-se que cerca de 30% das espécies de aves infetadas nos centros de reabilitação não tinham sido previamente documentadas como hospedeiras de hemosporídeos.

Nos pombos selvagens de Lisboa, a prevalência de infeção foi de 100%, com todas as aves infetadas por *Haemoproteus columbae*, um parasita altamente específico de Columbiformes e não foram detetados outros tipos de hemosporídeos neste grupo. Por outro lado, as aves exóticas das clínicas veterinárias não apresentaram qualquer infeção por hemosporídeos.

Foram identificadas 19 linhagens diferentes de hemosporídeos, das quais duas eram novas para a ciência. Além disso, foram documentadas 10 novas interações parasita-hospedeiro e 14 novos registos geográficos de linhagens de parasitas. A análise morfológica permitiu identificar as morfoespécies de cinco linhagens cujas morfoespécies eram anteriormente desconhecidas, incluindo a descoberta do marcador molecular para a espécie *Haemoproteus contortus*.

Este estudo também revelou que as aves infetadas com hemosporídeos necessitaram de períodos de reabilitação significativamente mais longos em comparação com as aves não infetadas. Adicionalmente, as aves da ordem Strigiformes e as aves admitidas nos centros devido a doenças debilitantes, mostraram as maiores probabilidades de infeção entre as aves levadas aos centros de reabilitação.

Os resultados obtidos neste estudo reforçam a importância dos centros de reabilitação como locais-chave para a monitorização e estudo de parasitas aviários. A prevalência de infeção de 30.3% nas aves dos centros de reabilitação é um indicativo da elevada exposição destas aves aos vetores, que são os responsáveis pela transmissão de hemosporídeos. A elevada prevalência de *Haemoproteus columbae* nos pombos selvagens de Lisboa está em conformidade com outros estudos que documentam a associação entre este parasita e os Columbiformes, uma vez que é comum encontrar infeções por este parasita em populações de pombos urbanos.

A ausência de infeções por hemosporídeos nas aves exóticas das clínicas veterinárias sublinha a importância do ambiente controlado em que estas aves são mantidas. A falta de exposição aos vetores que transmitem estes parasitas pode explicar a ausência de infeções, realçando a importância de controlar os ambientes em que as aves exóticas são mantidas, particularmente em locais com maior abundância de vetores.

A descoberta de novas linhagens de hemosporídeos, de novas interações hospedeiro-parasita e até de novos hospedeiros para hemosporídeos, são achados de grande relevância científica. Estas novas linhagens, ainda não descritas na literatura, expandem o conhecimento sobre a diversidade genética dos hemosporídeos em Portugal e sugerem que a verdadeira

extensão da diversidade parasitária pode ser ainda maior do que o estimado. A identificação de novas interações entre parasitas e espécies hospedeiras revela também a necessidade de continuar a monitorizar a presença de parasitas em diferentes espécies de aves, especialmente em habitats com elevada diversidade de vetores e hospedeiros.

A observação e conclusão de que as aves infetadas com hemosporídeos necessitam de períodos mais longos de reabilitação, tem implicações práticas significativas para os centros de reabilitação de fauna selvagem. A presença de parasitas pode enfraquecer as aves e prolongar o tempo necessário para a sua recuperação completa. Esse prolongamento acarreta não apenas um aumento nos custos de tratamento, mas também um impacto na capacidade dos centros de reabilitação de gerir eficientemente o seu trabalho. É, portanto, essencial que os protocolos de reabilitação incluam estratégias para a deteção precoce e tratamento de infeções parasitárias, a fim de melhorar a recuperação das aves e otimizar o uso dos recursos disponíveis.

O nosso estudo identificou fatores que influenciam a infeção por hemosporídeos em aves em centros de vida selvagem em Portugal, com destaque para a ordem Strigiformes que apresentou as maiores probabilidades de infeção. A predisposição para a infeção pode ser influenciada por uma combinação de fatores comportamentais, preferência dos vetores e características do hospedeiro, como o tamanho corporal e o tempo de permanência no ninho. Além disso, aves com doenças debilitantes apresentaram maior probabilidade de infeção, destacando a relação recíproca entre um estado de debilidade e a infeção por parasitas sanguíneos.

Este estudo pioneiro em Portugal combina métodos moleculares e morfológicos para identificar hemosporídeos em aves, revelando uma prevalência notável de infeções parasitárias em centros de reabilitação. A descoberta de novas linhagens e interações parasita-hospedeiro sublinha a importância de estudos futuros, sugerindo que a diversidade parasitária em aves selvagens em Portugal pode ser significativamente mais ampla do que se conhece atualmente. As infeções por hemosporídeos prolongam o tempo de recuperação das aves e aumentam os custos de tratamento, sendo crucial que os centros de reabilitação implementem medidas específicas para a deteção e tratamento destas infeções, com o objetivo de otimizar a reabilitação e melhorar as chances de sobrevivência pós-libertação.

Palavras-chave: Hemosporídeos aviários; duração de tratamento; *Haemoproteus*; *Leucocytozoon*; *Plasmodium*; Portugal

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1. Introduction

In 2022, the conservation status of 287 bird species in Portugal was evaluated, revealing a troubling trend: 95 species were classified as threatened with extinction, showing an increase from the 88 species identified as threatened in 2005 (Cabral et al. 2005; Almeida et al. 2022). Simultaneously, avian haemosporidian parasites, once considered relatively benign, have gained attention due to their potential impact on bird populations (Palinauskas et al. 2020). Accurate diagnosis of these parasites requires a combination of morphological and molecular methods (Valkiūnas and Iezhova 2022), yet studies in Portugal have not utilised this dual approach, leaving gaps in our understanding of haemosporidian diversity and prevalence.

In the context of wildlife rehabilitation, particularly within centres where effective treatment and quick release are paramount, the length of stay of birds is a critical metric for evaluating rehabilitation success and cost (Molina-López et al. 2017). Despite frequent parasitisation of admitted birds (Inumaru et al. 2017; Jia et al. 2018; Gomes et al. 2023), the influence of haemosporidian infections on rehabilitation duration remains largely unexplored. This study seeks to bridge these knowledge gaps by detecting and identifying avian haemosporidians, analysing their prevalence and genetic diversity, and assessing the impact of infections on the rehabilitation process. Employing a pioneering dual methodology in Portugal, this research aims to enhance our understanding of haemosporidian parasites and their implications for both wildlife conservation and rehabilitation efforts.

The document is structured as follows: starts with a detailed internship report, outlining the practical experiences and research activities conducted at various institutions. This is followed by an introduction to the topic, which sets the stage for the literature review. The literature review provides a comprehensive overview of the factors contributing to the global decline of bird populations, with a specific focus on avian haemosporidians, their life cycle, pathology, diagnosis, and epidemiology. Subsequent sections detail the materials and methods used in the research, present the findings, and discuss the results in the context of existing scientific knowledge. Finally, the dissertation concludes with a summary of the key findings and their implications for future research and conservation efforts. This structure ensures a logical flow, guiding readers through the various aspects of the study, from the initial practical experiences to the detailed scientific analysis and concluding insights.

2. Externships

2.1. Externship at the Faculty of Veterinary Medicine, University of Lisbon - Parasitology Laboratory

During my externship at the Parasitology Laboratory, Faculty of Veterinary Medicine, University of Lisbon, I trained in several parasitological techniques, including sedimentation, Modified Willis Flotation, the Baermann method, the Mini-Flotac method, the McMaster method, direct fecal smear analysis, and the collection and microscopic analysis of parasites from necropsies, such as fleas, mites, lice, ticks, cestodes, nematodes, and acanthocephalans. Additionally, I analysed blood smears for my thesis.

From 2022 to 2024 I participated in various research projects at the laboratory, leading to the following scientific publications:

- Articles:
    - Marques C, Delgado I, **Cruz JT**, Costa P, Portela G, Munhoz A, Waap H, Pereira A, Ramilo DW. 2022. Ácaros trombiculídeos: Revisão de uma parasitose negligenciada em animais de companhia. Revista Lusófona de Ciência e Medicina Veterinária. 12: 18-33. DOI: https://doi.org/10.60543/rlcmv.v12i1.8344 (Appendix I).
    - Ramilo DW*, **Cruz JT***, Amin O, Fragoso C, Brazio E, Correia J, Cardoso L, Pereira da Fonseca I. 2023. A new definitive host for *Moniliformis cestodiformis*: first report of a naturally infected European hedgehog (*Erinaceus europaeus*). Brazilian Journal of Veterinary Parasitology. 32: 1-5 DOI: 10.1590/S1984-29612023014 (*equal contributers) (Appendix II).
    - Marques G*, **Cruz JT***, Pinto M, Leal M, Flanagan C, Urbani N, Madeira de Carvalho L. 2023. Respiratory Infection by *Cyathostoma* (*Hovorkonema*) *americana* in a Population of Burrowing Owls (*Athene cunicularia*) - A Potential Case of Zoo-Wildlife Cross-Transmission. Journal of Zoological and Botanical Gardens. 4: 788-799. DOI: 10.3390/jzbg4040056 (*equal contributer) (Appendix III).
- Posters:
    - **Cruz JT,** Casero M, Brazio E, Ramilo DW, Marzal A, Madeira de Carvalho L. 2022 First report of *Zachvatkinia larica* in seagulls from the Iberian Peninsula. In: XXII Congress of the Spanish Society of Parasitology; 5–8 July; Madrid; Spain (Appendix IV)
    - Marques C, Costa P, **Cruz JT,** Delgado I, Portela G, Munhoz A, Waap H, Pereira A, Ramilo DW. 2022. Detection of trombiculid mites (Acari: Trombiculidae) in stray cats and the environment in Lisbon and Santarém. In: XVIII International Veterinary

Montenegro Congress; 4–5 November; Santa Maria da Feira; Portugal (Appendix V).

- **Cruz JT,** Madeira de Carvalho L, Correia J, Marzal A, Cardoso L, Pereira da Fonseca I, Ramilo DW. 2022. Helminths found in wild thrushes (*Turdus* spp.) from Portugal. In: CIISA Congress Innovation in Animal, Veterinary and Biomedical Research; 11–12 November; Lisbon; Portugal (Appendix VI).
- **Cruz JT**, Madeira de Carvalho L, Afonso F, Sequeira M, Marzal A, Ramilo DW. 2022. Presence of *Phyllobothrium delphini* in a striped dolphin (*Stenella coeruleoalba*) in the Tagus estuary (Alcochete Portugal). In: CIISA Congress Innovation in Animal, Veterinary and Biomedical Research; 11–12 November; Lisbon; Portugal (Appendix VII).
- **Cruz JT,** Madeira de Carvalho L, Correia J, Marzal A, Serra R, Neves N, Cardoso L, Pereira da Fonseca I, Ramilo DW. 2023. Parasitological survey of the endangered Iberian lynx (*Lynx pardinus*) in Portugal. In: XI FAUNA International Conference; 17–19 March; Lisbon; Portugal (Appendix VIII).
- Marques GN, Pinto MGF, **Cruz JT,** Leal MO, Urbani N, Flanagan CA, Madeira de Carvalho L. 2023. Clinical and environmental management of mixed lung parasite infection in a population of burrowing owls (*Athene cunicularia*) in a zoo context. In: 10th Meeting of Training of the Order of Veterinary Physicians; 14–16 April; Lisbon; Portugal (Appendix IX).

- Article Reviews (Appendix X)
  - Review for the Journal of Zoological and Botanical Gardens (7th May 2024).
  - Two reviews for the Journal Animals (27th June 2024 and 20th August 2024).
  - Review for the Journal of Veterinary Sciences (30th July 2024).

These experiences have significantly enhanced my knowledge in Veterinary Medicine and Parasitology research.

2.2. Externship at the South Florida Wildlife Centre, Fort Lauderdale, Florida – USA

As part of an extracurricular externship, I had the opportunity to join the South Florida Wildlife Centre in Fort Lauderdale, Florida, from 14th November 2022 to 13th January 2023. During this period, I assisted the veterinary staff with the majority of the 749 wildlife clinical cases, involving 114 different species, handled by the centre. This experience provided me with a thorough understanding of wildlife rehabilitation medicine and significantly enhanced my practical skills. I participated in several surgeries, including serving as a hands-on assistant surgeon for various procedures. These included the amputation of an eastern gray squirrel (*Sciurus carolinensis*) tail, the placement of an esophagostomy tube in a Florida soft shell turtle

(*Apalone ferox*), and the orchidectomy of a domestic rabbit (*Oryctolagus cuniculus domesticus*) (Figure 1A). Additionally, I performed numerous x-rays, administered laser therapy, and engaged in physiotherapy sessions.

My responsibilities also included conducting several necropsies, during which I collected samples and investigated various cases to determine the causes of death. This work aimed to enhance the centre's diagnostic capabilities. I also collected blood samples and performed blood smears for 72 different clinical cases for haemosporidian research. Unfortunately, due to customs issues, it was not possible to bring these samples back to Portugal.

In addition, I administered medications, fed animals, prepared bandages, participated in the capture and release of wild animals, and implemented various husbandry practices. These activities further enriched my practical experience and skills in wildlife rehabilitation.

For my externship final project, I researched and presented to all the staff at the centre the first insights into a possible new disease affecting red-shouldered hawks (*Buteo lineatus*). This disease is characterised by similar neurological clinical signs to each (Figure 1B) other and high mortality. All necropsies performed by specialists from the Southeastern Cooperative Wildlife Disease Study (SCWDS) at the College of Veterinary Medicine, University of Georgia, were inconclusive and negative for all tested infectious diseases, pesticides, and heavy metals. This condition has been observed annually and already in more than one hundred cases with no apparent cause until now. This project underscored the need for further investigation into this potential new disease (Appendix XI).

Throughout this externship, I gained experience across a range of activities, significantly enhancing my practical skills and knowledge in wildlife rehabilitation medicine.

Figure 1. Orchidectomy procedure in a domestic rabbit (*Oryctolagus cuniculus domesticus*) (A) and a representative case illustrating the potential new disease studied during my externship final project (B).

2.3. Curricular Externship at the Faculty of Sciences, University of Extremadura, Badajoz – Spain

As part of the final externship for my integrated master's degree in veterinary medicine, I had the privilege of joining the Lab Marzal research team at the Faculty of Sciences, University of Extremadura, from 1st May 2023 to 28th July 2023. Under the supervision of Professor Alfonso Marzal Reynolds, this externship focused on employing molecular methodologies to diagnose haemosporidian infections in birds. I worked with both samples presented in this dissertation and those from projects within the Lab Marzal team, gaining a comprehensive practical and theoretical understanding of this area of study. The externship also included the statistical and bioinformatic analysis of results and fieldwork, specifically the netting, capture, and sampling of a colony of hundreds of swallows as part of an ongoing project that has been sampling the same colony for several decades.

During the externship, I conducted DNA amplification using polymerase chain reaction (PCR) on all samples related to my thesis (Figure 2A). I used electrophoresis in agarose gel to visualise the amplified DNA fragments, interpreted the results, and analysed these sequences with bioinformatics software to accurately identify the lineages present. This experience significantly enhanced my technical and manipulative skills in using laboratory equipment, particularly those related to PCR-based methodologies. It also improved my sampling procedures, scientific investigation, and problem-solving skills. I learned to formulate hypotheses, design and conduct experiments, collect and interpret data, test hypotheses, and draw well-supported conclusions.

A particularly valuable aspect of this externship was acquiring a complete understanding of the diagnostic techniques for haemosporidian infections, from the capture of birds, collection of blood for molecular analysis, and performance of blood smears, to the analysis of both. This included recognising the limitations and strengths of both PCR and microscopy methods.

This externship culminated in several scientific publications:

- Oral presentation (Figure 2B):
    - **Cruz JT**, Madeira de Carvalho L, Ferreira MR, Nunes C, Casero M, Marzal A. 2023. Prevalence, Genetic Diversity, Geographic Distribution and Clinical Findings of Haemoparasites Causing Avian Malaria in Wildlife Centres of Portugal. In: XVIII Congreso Nacional y XV Iberoamericano de Etología y Ecología Evolutiva; 31 October–3 November; Badajoz; Spain (Appendix XII).
- Articles:
    - **Cruz JT**, Madeira de Carvalho L, Ferreira MR, Nunes C, Casero M, Marzal A. 2024. Avian Haemosporidian Infection in Wildlife Rehabilitation Centres of Portugal:

Causes, Consequences, and Genetic Diversity. Animals. 14 (8): 1216. DOI: 10.3390/ani14081216 (Appendix XIII)

- Mora-Rubio C, Garcia-Longoria L, Ferraguti M, Magallanes S, **Cruz JT**, de Lope, F, Marzal A. 2024. The Impact of Avian Haemosporidian Infection on Feather Quality and Feather Growth Rate of Migratory Passerines. Animals. 14(12): 1772. DOI: 10.3390/ani14121772 (Appendix XIV).

Additionally, I joined the Wildlife Malaria Network (WIMANET-CA22108) COST Action as a member and participated in the 1st WIMANET-COST online workshop from 6th-8th December 2023 (Appendix XV). I also attended the 2nd WIMANET-COST workshop in Cluj-Napoca, Romania, from 20th-23rd February 2024, fully funded (Appendix XVI), and I have been accepted to being funded to participate in the WIMANET Summer Training School from 1st-7th September 2024 in Mohelno, Czech Republic.

Figure 2. PCR-based DNA amplification performed during my externship (A) and oral presentation at the XVIII Congreso Nacional y XV Iberoamericano de Etología y Ecología Evolutiva (B).

3. Literature Review

3.1. Global decline of bird populations

The decline of animal populations on a global scale stands as one of the most dramatic repercussions of human impacts on Earth, with 48% of animal species witnessing declines (Finn et al. 2023). Birds, in particular, suffer markedly, with 5412 out of 11162 species (48.5%) experiencing decreasing populations (Finn et al. 2023). While the most significant declines occur in tropical regions (Almond et al. 2022), birds from temperate regions also face negative population trends. In Europe, for instance, the percentage of birds with poor and bad conservation status rose by 7% over the last decade, reaching 39% (Naumann et al. 2020). The conservation status of wild European birds presents an escalating concern, with 13% threatened with extinction (Vulnerable, Endangered, or Critically Endangered), 6% classified as near-threatened, and 30% showing declining trends (BirdLife International 2021). Despite shifts in bird abundance in Europe since the 1980s, declines vary across countries (Rigal et al. 2023). Mainland Portugal is home to a rich diversity of wildlife, hosting 404 confirmed species of wild birds (Matias et al. 2011). An assessment in 2022 revealed that 95 out of 287 evaluated species were classified as threatened with extinction, marking an increase from the 88 species classified as threatened in 2005 (Cabral et al. 2005; Almeida et al. 2022).

3.1.1. Anthropogenic factors

Studies analysing long-term bird population data attribute avian population declines to various anthropogenic factors. Agricultural intensification, particularly the use of pesticides and fertilizers, emerges as a primary pressure for invertebrate feeders (Rigal et al. 2023). Climate change, evidenced by rising temperatures, negatively impacts the reproduction of migratory and larger-bodied avian species (Halupka et al. 2023). Forest degradation contributes to broad-scale declines in most forest bird species in Canada (Betts et al. 2022).

3.1.2. Pathogen factors

Additionally, parasites and pathogens pose significant threats to bird species, exemplified by the emergence of *Trichomonas gallinae* in British finch populations in 2005, resulting in a 35% decline in greenfinch (*Carduelis chloris*) and a 21% decline in chaffinch (*Fringilla coelebs*) populations by 2007 (Robinson et al. 2010). Moreover, the highly pathogenic avian influenza H5 epidemic has recorded the highest number of casualties among wild birds in Europe (Adlhoch et al. 2022).

Given the recognized impact of emerging diseases on native bird populations (Friend et al. 2001), the potential influence of avian malaria on native avifauna, though yet unknown, could explain the reported declines (Tompkins and Jakob-Hoff 2011).

3.2. Avian Haemosporidians

3.2.1. Life cycle and infection phases

Avian haemosporidians, order Haemosporida (Alveolata, Apicomplexa, Sporozoa), are vector-borne intracellular protozoan parasites belonging to the genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, and *Fallisia* (Valkiūnas 2005; Santiago-Alarcon et al. 2012), with *Fallisia* being less studied and relatively rarer (Valkiūnas 2005). The main genera of Haemosporidians (*Plasmodium*, *Haemoproteus* and *Leucocytozoon* spp.) are commonly referred as “avian malaria” due to the malaria-like clinical signs they often cause (Marzal 2012; Ellis et al. 2020). This group of parasites is globally distributed, apart from polar regions (Clark et al. 2014), and a large genetic diversity has been discovered during the past decade with more than 5100 lineages identified (MalAvi database Version 2.5.8, October 2023; Bensch et al. 2009).

In 1884, only 5 years after Charles Louis Alphonse Laveran discovered the agents of human malaria, Vassily Danilewsky reported the first description of the pathological effects of avian malaria on bird hosts belonging to the families Accipitridae, Laniidae and Corvidae, marking the beginning of avian haemosporidian research (Santiago-Alarcon and Marzal 2020).

The life cycle of avian haemosporidians is complex, involving both avian hosts and dipteran vectors (Figure 3). Despite the variations in life cycles among avian haemosporidians, especially during the asexual phase in the infected tissues of the vertebrate host and in the concentration of blood stages, the following developmental stages and their sequence of occurrence are found in all groups of avian haemosporidians. In vectors, both sexual processes (gametogenesis and fertilization) and asexual multiplication occur, signifying their role as definitive hosts where development of gametes, fertilization, and meiosis take place. Vertebrates are intermediate hosts, in which only asexual multiplication occurs. Birds become infected with haemosporidians when vectors release viable sporozoites into susceptible avian hosts while taking a blood meal (1). The sporozoites initiate merogony in cells of fixed tissues and develop into tissue meronts or exoerythrocytic meronts (2). Merozoites are formed in meronts by means of mitotic division (3). The merozoites are asexual stages responsible for the spread of infection within the vertebrate host. After several generations of exoerythrocytic merogony, numerous merozoites develop and invade blood cells (4), developing into gametocytes (or gamonts). Gametocytes are asexual developmental stages possessing sexual potency, and infectivity to vectors and exhibit distinct sexual dimorphic characteristics (5). The sexual dimorphism of gametocytes is the most prominent distinguishing feature of haemosporidians. Shortly after vectors feed on infected birds, mature gametocytes produce gametes in their midgut. The female roundish gametes are fertilized by smaller, motile elongated male gametes and fertilization occurs extracellularly. The resulting zygotes (6) undergo meiosis and then develop into worm-like motile ookinetes (7). Until ookinetes

transform into oocysts, no additional nuclear division occurs. Ookinetes migrate toward the epithelial layer of the midgut, pass through it, and round up extracellularly beneath the basal lamina, forming roundish oocysts. Meiosis occurs in the zygote before the initial stages of ookinete development. Oocysts undergo a final mitotic division, known as sporogony, concluding with the formation of numerous elongated sporozoites, which are released into the haemocoel and are distributed throughout the vector's body. A portion of these sporozoites migrates to the vector's salivary glands (8), where they finalize maturation. The sporozoite represents the infective stage for the vertebrate host when it is injected with saliva through the vector's mouthparts during a blood meal (Valkiūnas 2005; Valkiūnas and Atkinson 2020).

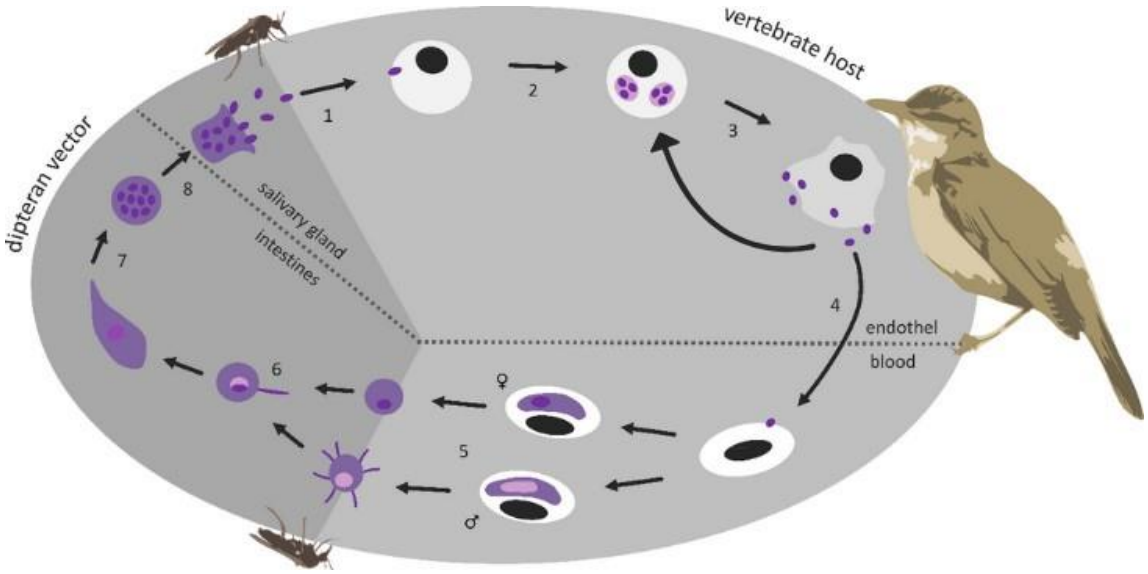


Figure 3. Schematic representation of the general life cycle of haemosporidian parasites.
The vertebrate host becomes infected with haemosporidians when vectors release viable sporozoites into susceptible hosts during a blood meal (1). The sporozoites initiate merogony in cells of fixed tissues, forming tissue meronts or exoerythrocytic meronts (2). Merozoites are produced within meronts through mitotic division (3). After multiple generations, merozoites invade blood cells (4) and develop into gametocytes, which are infective to vectors (5). Following vector feeding, gametocytes produce gametes in the midgut. Female gametes are fertilized by male gametes, forming zygotes (6) that undergo meiosis and develop into ookinetes (7). Ookinetes transform into oocysts, where sporogony produces sporozoites. Sporozoites migrate to the vector's salivary glands (8), finalizing maturation and enabling transmission (Adapted from Valkiūnas 2005 and Emmenegger 2018).

Haemosporidian infections progress through similar phases (Figure 4), differing primarily in duration. The prepatent period, during which parasites exclusively develop within internal organs, involves multiplication before invading blood cells. Exoerythrocytic merogony encompasses at least two generations, culminating in the development of merozoites capable of invading blood cells. The appearance of parasites in blood cells indicates the onset of the patent period, marking the acute phase where parasitaemia intensifies, reaching a peak or crisis. As a result of the host's immune system, survivors of the acute stage transition into the

chronic phase, where only a few parasites persist in the bloodstream. Subsequently, a latency period emerges, during which parasites remain absent from the bloodstream but persist in internal organs throughout the host's life. Infected birds often harbour haemosporidians for many years or even their lifespan, yet the mechanisms underlying this persistence are not fully understood and may vary across different host-parasite associations. This prolonged infection is crucial for parasite survival during unfavourable transmission periods, especially in temperate ecosystems where vectors may be absent for significant portions of the year. Breaks in bird immunity or physiological stressors such as the reproduction period of the vertebrate hosts, can trigger parasite multiplication in fixed tissues, leading to the reappearance of parasites in the blood, designated secondary parasitaemia or relapse (Valkiūnas 2005; Valkiūnas and Atkinson 2020).

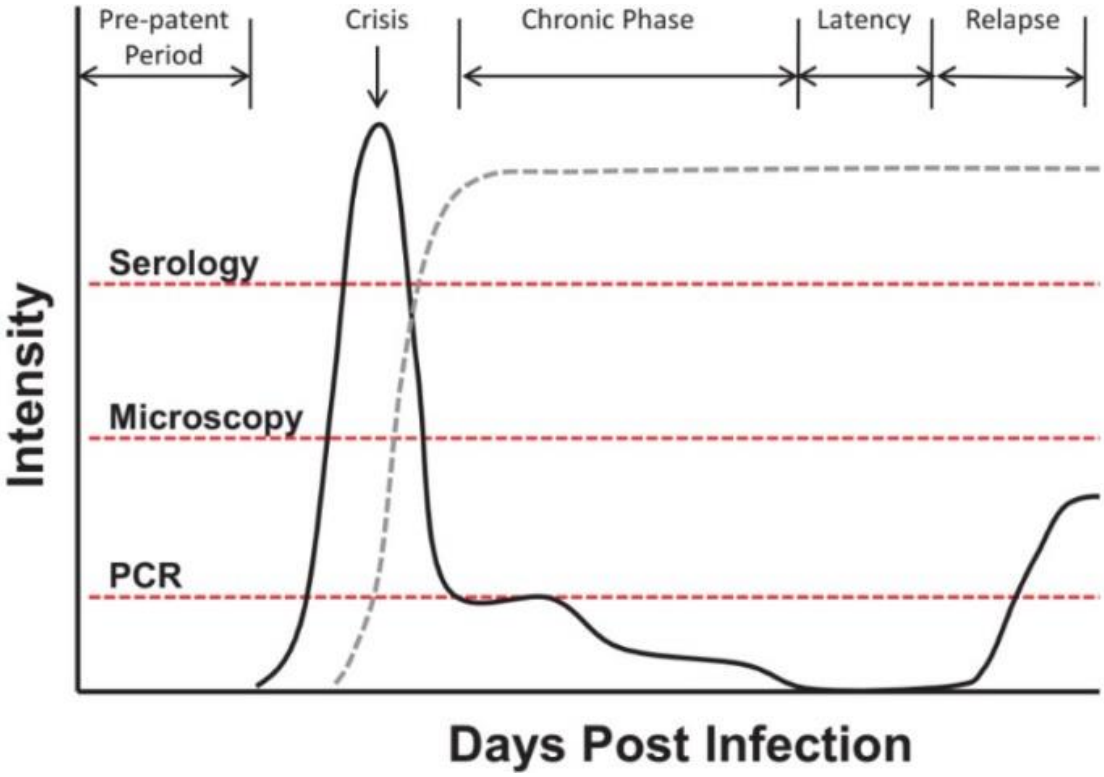


Figure 4. Temporal dynamics of parasitaemia intensity (solid line) and antibody titter (dashed line) in Haemosporidian infections with main phases and diagnostic detection (red dashed lines). The main phases progress from the prepatent period, marked by internal organ parasite development, to the acute phase, distinguished by parasitaemia and a peak or crisis. Following this, the chronic phase exhibits few parasites in the bloodstream, succeeded by a latency period where parasites persist solely in organs. Breaks in immunity can lead to secondary parasitaemia or relapse. Microscopy, PCR, and serology vary in their ability to detect infections depending on the stage of infection. PCR is the first test to be able to detect the infection, but when infections become latent and disappear from circulation, serology is the only method that can detect evidence of infection. None of the three methods detects infections during the prepatent period (initial development in organs), before parasites enter circulating erythrocytes. (adapted from Valkiūnas 2005 and Valkiūnas and Atkinson 2020).

3.2.1.1. *Haemoproteus*

Haemosporidians belonging to the genus *Haemoproteus* exclusively infect avian and reptilian hosts (Garnham 1966; Valkiūnas 2005). Despite their prevalence, these protists have been overlooked as significant blood pathogens largely due to the traditional assumption of their benign nature towards their hosts (Bennett et al. 1993). Nonetheless, recent histopathological studies have revealed the development of large megalomeronts, some reaching sizes of more than 300 μm, during the exoerythrocytic phase of *Haemoproteus* infections, leading to organ damage and, in severe instances, mortality (Atkinson et al. 1988; Atkinson et al. 2008; Valkiūnas and Iezhova 2017; Kelly et al. 2018; Himmel et al. 2019; Ilgūnas et al. 2019; Duc et al. 2021; Yoshimoto et al. 2021). These discoveries underscore the necessity for further research aimed at enhancing our understanding of haemoproteids and their implications for animal health (Valkiūnas and Iezhova 2022).

Haemoproteus species exhibit a cosmopolitan distribution and are frequently encountered in avian populations (Valkiūnas 2005). Molecular analyses have identified over 2000 distinct cytochrome b gene lineages of *Haemoproteus*, with the likelihood of many more yet to be discovered (MalAvi database Version 2.5.8, October 2023; Bensch et al. 2009). During the past two decades alone, the description of *Haemoproteus* species has expanded to include 49 newly identified species among the documented total of 177 (Valkiūnas and Iezhova 2022).

Transmission of *Haemoproteus* parasites primarily occurs through *Culicoides* biting midges (Ceratopogonidae), while a select few species are vectored by louse flies (Hippoboscidae) (Valkiūnas 2005, Valkiūnas and Atkinson 2020). The prepatent period of *Haemoproteus* parasites ranges from 10 days to 3 weeks, marking an inability to diagnose infections before this period (Valkiūnas 2005; Valkiūnas and Iezhova 2022).

Molecular phylogenetic analyses indicate that *Haemoproteus* parasites are very closely related to the genus *Plasmodium* (Bensch et al. 2016). Both genera of haemosporidians incompletely digest haemoglobin, leading to the accumulation of residual pigment (hemozoin) in their blood stages (Garnham 1966; Valkiūnas 2005; Atkinson et al. 2008). This shared characteristic unites species of *Haemoproteus* and *Plasmodium*, setting them apart from the Leucocytozoidae and Garniidae families, which lack residual pigment production during red blood cell development (Valkiūnas and Iezhova 2022).

3.2.1.2. *Plasmodium*

Based on available morphological data and DNA sequence information, 55 species of avian *Plasmodium* parasites can be readily distinguished (Valkiūnas and Iezhova 2018) with more than 1550 lineages already identified (MalAvi database Version 2.5.8, October 2023; Bensch et al. 2009).

The life cycle of the *Plasmodium* genus stands out with unique characteristics compared to other Haemosporidians. Firstly, alongside merozoites originating from exoerythrocytic meronts that invade blood cells and mature into sexual stages (gametocytes or gamonts), there is also the ability for some merozoites to perpetuate merogony within circulating blood cells. Erythrocytic merogony is observed not only in *Plasmodium* but also in select species of Garniidae. Secondly, merozoites derived from erythrocytic meronts may initiate secondary exoerythrocytic development, giving rise to secondary exoerythrocytic meronts or phanerozoites. However, our understanding of this aspect of the life cycle remains limited, and its prevalence remains uncertain. Thirdly, while relapses can occur in parasites belonging to the families Haemoproteidae and Leucocytozoidae, as well as Plasmodiidae, triggered by the activation of exoerythrocytic merogony resulting in heightened erythrocytic parasitaemia, recrudescences are exclusive to haemosporidians that replicate within blood cells. Recrudescence is characterized by an increase in parasitaemia due to the multiplication of persistent circulating blood stages (Valkiūnas 2005; Valkiūnas and Atkinson 2020).

Culicidae mosquitoes are responsible for the transmission of avian *Plasmodium* parasites, although belonging to different genera (*Culex*, *Coquillettidia*, *Aedes*, *Mansonia*, *Culisetta*, *Anopheles*, *Psorophora*) (Valkiūnas and Iezhova 2018).

While closely genetically related, *Haemoproteus* and *Plasmodium* diverge not only in the characteristics outlined above but also in terms of host specificity. *Haemoproteus* species typically fail to complete their life cycle and generate invasive stages (gametocytes) in birds across different orders (Valkiūnas 2005; Valkiūnas and Iezhova 2022). In contrast, *Plasmodium* species exhibit a broad vertebrate host range, with the same species capable of infecting birds that are distantly related. For instance, *Plasmodium* (*Haemamoeba*) *relictum* infects and completes its life cycle in birds spanning over 300 species and 11 orders, while *Plasmodium* (*Huffia*) *elongatum*, *Plasmodium* (*Novyella*) *vaughani*, and numerous other species also demonstrate a wide array of avian hosts (Valkiūnas 2005; Bensch et al. 2009; Zehtindjiev et al. 2012; Palinauskas et al. 2016; Valkiūnas and Iezhova 2018). Therefore, vertebrate host identity cannot serve as a taxonomic feature during the identification of *Plasmodium* parasites (Garnham 1966, Valkiūnas and Ashford 2002; Valkiūnas and Iezhova 2018).

3.2.1.3. *Leucocytozoon*

Leucocytozoon parasites have seen notably less research progress over the past 30 years when compared to the remarkable advancements made in apicomplexan parasites belonging to the Haemoproteidae and Plasmodiidae families (Valkiūnas and Iezhova 2023).

The genetic diversity within *Leucocytozoon* species is substantial, with over 1500 distinct lineages documented in the MalAvi database (Version 2.5.8, October 2023; Bensch et

al. 2009), alongside significant morphological diversity, as evidenced by descriptions of 45 morphospecies (Valkiūnas and Iezhova 2023).

In addition to previously discussed traits such as their unique capacity for complete haemoglobin digestion, leading to the absence of hemozoin across all blood stages, and their distinct lack of multiplication within blood cells, *Leucocytozoon* parasites exhibit several other remarkable characteristics setting them apart from other haemosporidians. These include their profound impact on host cell nuclei, their exclusive parasitic relationship with avian hosts, and the occurrence of sporogony in black flies (Simuliidae). (Valkiūnas 2005; Valkiūnas and Iezhova 2023).

While lacking convincing experimental evidence to confirm that an isolate of the same species of *Leucocytozoon* can infect and produce gametocytes in birds belonging to different orders, the validity of natural host range as a taxonomic character at the species, genus, or family level of host is not supported (Valkiūnas and Ashford 2002; Valkiūnas and Iezhova 2023).

3.2.2. Impact, Pathology and Clinical Signs

Although avian haemosporidian parasites were considered as relatively benign for a long time (see review in Palinauskas et al. 2020), they have been reported to negatively affect host condition (Merino et al. 2000; Marzal et al. 2008), clutch size (Marzal et al. 2005), reproductive success (Merino et al. 2000, Marzal et al. 2005, Knowles et al. 2010; Bosholn et al. 2016), increase the risk of predation (Møller and Nielsen 2007), and decrease lifespan (Marzal et al. 2016), subsequently diminishing host fitness (Lachish et al. 2011; Asghar et al. 2015; Eastwood et al. 2019).

The clinical signs and pathology of bird haemosporidian infection can include vomiting, anorexia, depression, motor incoordination, convulsions, paralysis, anoxia as a result brain capillaries blockage, tissue necrosis (e.g., liver and spleen), regenerative anaemia due to haemolytic processes, splenomegaly and hepatomegaly that can lead to organ rupture, pulmonary oedema (Valkiūnas 2005; Pugliese et al. 2023). Moreover, haemosporidian infections can also lead to a reduction in haematocrit levels that may result in death (Palinauskas et al. 2008, 2011), to an increase in stress proteins (heat shock proteins) (Tomás et al. 2005), acidosis, higher plasma protein concentrations, reduced haemoglobin oxygen-binding capacity and low complete blood counts (Ritchie et al. 2000).

The variability observed in infected birds, ranging from asymptomatic cases to mortality, is believed to be influenced by parasite lineages, reflecting the adaptation of the host species to specific haemosporidian lineages (Dimitrov et al. 2015; Valkiūnas and Iezhova 2018; Pugliese et al. 2023).

3.2.3. Diagnosis

3.2.3.1. Morphological Diagnosis Methods

Traditionally, the standard method for distinguishing infected from non-infected individuals has been the ocular investigation of Giemsa-stained blood smears using light microscopy (Waldenström et al. 2004). The detection and identification of parasites through microscopic blood smear examination rely on their specific morphological characteristics present in the blood circulation of birds (Clark et al. 2009). This method enables the calculation of parasitaemia levels and the detailed examination of the various structures of haemoparasite forms (Clark et al. 2009), enabling for both qualitative and quantitative assessments (Krone et al. 2008) and at low costs (Waldenström et al. 2004).

However, it suffers from several drawbacks, including being time-consuming and requiring well-trained personnel to obtain reliable data, as it is often considered challenging to use for accurate parasite identification (Waldenström et al. 2004). Furthermore, haemoparasite identification is also not always possible, since some species are morphologically identical and younger phases of the parasite are often similar (Santos 2023). Additionally, the detection of blood parasites using optical microscopy is also not always possible due to its low sensitivity. Typically, parasitaemia levels are low, often less than 1 parasite per 1000 erythrocytes (<0.001%) (Valkiūnas 2005), while some researchers suggest that more reliable identification requires higher parasitaemia levels (> 0.1%) (Clark et al. 2009; Tomás 2014). Consequently, blood smear investigations may significantly underestimate the prevalence of blood parasites, especially in mild infections (Waldenström et al. 2004).

3.2.3.2. Molecular Diagnosis Methods

Molecular methods, particularly those based on mitochondrial DNA sequence variation, have become crucial for detecting avian haemosporidians, especially in cases of low parasitaemia levels where detection through blood smear examinations is challenging. A 479 base pairs (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" (Bensch and Hellgren 2020; Santos 2023).

Molecular analysis methods enhance the detection of early haemosporidian infections, thereby improving diagnostic sensitivity. Additionally, they enable the identification of cryptic species and lineages, thereby contributing to a better understanding of the genetic diversity of these parasites (Valkiūnas and Atkinson 2020).

However, due to their high sensitivity, PCR methods can detect parasitaemia as low as one infected erythrocyte in 100,000 or less, making them susceptible to laboratory contamination and resulting in false positives (Waldenström et al. 2004). Despite their sensitivity, these methods can also fail to detect some infections, even though parasites were

observed in blood smears (Krone et al. 2008) and, used alone, PCR techniques may underestimate co-infections of parasites belonging to same genus (Valkiūnas et al. 2006). Furthermore, molecular markers are absent for 58% of *Plasmodium* and *Haemoproteus* species (Valkiūnas and Iezhova 2018, 2022), and *Leucocytozoon* species have minimal molecular characterization (Valkiūnas and Iezhova 2023). Therefore, the development of molecular markers is an imperative task for current haemosporidian parasite researchers (Valkiūnas and Iezhova 2022). The temporal dynamics of parasitaemia intensity and the ability of each diagnostic method to detect infection is shown in Figure 4.

3.2.3.3. Morphological versus Molecular Diagnosis Methods

The diagnosis of avian haemosporidians necessitates the integration of both morphological and molecular methods (Valkiūnas and Iezhova 2022). While morphological examination via blood smear provides valuable qualitative and quantitative data (Krone et al. 2008), its limitations in sensitivity (Waldenström et al. 2004; Clark et al. 2009) and species differentiation (Santos 2023) underscore the importance of molecular techniques. Conversely, molecular methods, while enhancing sensitivity and enabling the identification of cryptic species and lineages, may underestimate co-infections, pose false positive risks, and are currently limited by the absence of species-specific molecular markers (Waldenström et al. 2004; Valkiūnas et al. 2006; Krone et al. 2008; Valkiūnas and Iezhova 2018, 2022, 2023). Therefore, by leveraging the strengths of both approaches, a comprehensive approach that combines both morphological and molecular data is essential for obtaining a true understanding of haemosporidian diversity in avian populations (Valkiūnas and Iezhova 2018, 2022).

Over the last two decades, scientific interest in avian malaria haemoparasites in Portugal has grown, reflected in a substantial increase in related articles. Among the 15 studies conducted in Portugal, 11 utilized molecular analysis methods (Jenkins and Owens 2011; Ventim et al. 2012a, 2012b, 2012c; Drovetki et al. 2014; Pardal et al. 2014; Neto et al. 2015; Rojo et al. 2015; Emmenegger et al. 2020; Santos. 2023; Gomes et al. 2023), while 4 employed morphological analysis methods (Tomé et al. 2015; Santos et al. 2008; Tomás 2014; Zacarias 2017). Remarkably, none of the studies conducted in Portugal to date have employed a dual methodology approach.

3.2.4. Prevention and treatment

Mosquitoes play a crucial role in the life cycle of avian malaria, acting as the bridge between infected and uninfected birds (Miranda Paez et al. 2022). In captive settings, the prevention of haemosporidioses primarily concerns shielding birds from vectors during their active seasons, particularly in warmer weather. Achieving this involves housing birds indoors or in enclosures fitted with fine-mesh netting. Essential measures also include regular testing

and the isolation of infected birds (Valkiūnas 2005). In wild populations, mitigating Haemosporidian infections primarily demands managing mosquito populations. Initially, insecticides were extensively used, proving effective in certain cases. However, this approach has also selected resistant populations of mosquitos to commonly used insecticides, therefore hindering the ability to control mosquito populations in those areas (Miranda Paez et al. 2022). The observed efficacy concerns regarding insecticide-based vector control programmes have urged interest in transitioning towards the use of sterile insect techniques (SITs) (Beebe et al. 2021; Miranda Paez et al. 2022). These methods were successful in combating various insect pest species, including the New World screwworm fly (*Cochliomyia hominivorax*) and the Tsetse fly (*Glossina* spp.) (Beebe et al. 2021). Sterile insect techniques involve releasing sterilised males of the target species into the wild. These males are sterilised through methods such as radiation, chemical sterilisation, genetic modification with lethal genes, or hosting incompatible endosymbionts (e.g., *Wolbachia*). They then mate with wild females, leading to the production of infertile eggs (Miranda Paez et al. 2022).

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; Santos 2023). In the chemical treatment of *Plasmodium*, medications used for human malaria have been employed: chloroquine phosphate, primaquine phosphate, mefloquine, pyrimethamine–sulfadoxine combinations and atovaquone-proguanil have considered to be effective (Atkinson 2008; Zacarias 2017). In a more recent study, the combination of atovaquone and proguanil hydrochloride, commercially known as Malarone®, was considered among the most effective and safe pharmacological choices for the treatment of avian malaria (Pugliese et al. 2023). For *Haemoproteus*, effective treatments include atebrine, chloroquine sulphate, primaquine, mefloquine, and buparvaquone (Atkinson 2008). In *Leucocytozoon* infections, pyrimethamine has been effective, as well as its combination with sufamonomethoxine when administered orally (Atkinson 2008; Zacarias 2017).

3.2.5. Epidemiology

3.2.5.1. Predictors of infection

Several studies have attempted to identify predictors of haemosporidian infection prevalence (Ellis et al. 2020). The variation in the prevalence of haemosporidian infections within host species is determined by ecological factors (e.g., annual differences), individual factors (e.g., age) or individual state, such as poor body condition, nutritional status, or elevated stress levels (Crommenacker et al. 2011; Cornet et al. 2014). These conditions can diminish host defences and increase the likelihood of infection (Valkiūnas 2005). Contrarily, between-species prevalence is determined by vector preferences (Pulgarín-R et al. 2017) or

by unknown host factors such as behaviour (Beadell et al. 2004; Pulgarín-R et al. 2017; Eastwood et al. 2019).

One of the intriguing aspects of haemosporidian parasites lies in their significant variability concerning host specificity and parasite exploitation tactics. Research has shown that haemosporidians can infect either a limited number of related host species or a wide range of further-related bird species, being categorized as specialist parasites or generalist parasites, respectively (Clark et al. 2015; Moens and Pérez-Tris 2016). Additionally, it has been noted that a single parasite lineage may alter its exploitation strategy based on the biodiversity within the community (Garcia-Longoria et al. 2019, 2021).

3.2.5.2. Geographical distribution

The study of bird haemosporidians primarily focuses on a zoogeographical scale due to inconsistent exploration within smaller regions, with these parasites found across all zoogeographical regions except Antarctica (Valkiūnas 2005). Among these regions, the Holarctic, Ethiopian, and Oriental regions exhibit the highest overall prevalence of avian haemosporidians, respectively, while the Neotropical region shows a lower prevalence (Table 1). In the Neotropics, *Leucocytozoon* infections are rare, possibly due to limited suitable vectors. In contrast, the Holarctic stands out with the highest prevalences of *Leucocytozoon* and *Haemoproteus* parasites, while the Ethiopian region shows particularly high infection rates of *Plasmodium* (Table 1). There is a notable trend of increasing *Leucocytozoon* infection prevalence from tropical to high latitudes in the Holarctic, likely influenced by factors such as host population density and the ability of parasites to develop in vectors at lower temperatures. Certain *Leucocytozoon* species have even spread beyond the North Polar Circle, whereas species from other haemosporidian genera are constrained by environmental conditions like temperature and vector availability. For example, *Haemoproteus* is absent from tundra regions due to limited vectors and insufficient warmth for development. Most *Plasmodium* parasites prefer even warmer climates, explaining the Ethiopian region's high overall prevalence of *Plasmodium*, while the Holarctic's relatively high prevalence of infection with *Plasmodium* can be attributed partly to the infection of Holarctic birds in their wintering areas (Table 1) (Valkiūnas 2005).

Table 1. Prevalence of haemosporidian parasites in birds in different zoogeographical regions (Adapted from Valkiūnas 2005).

Zoogeographical region	Number of investigated birds	Infected		
		***Haemoproteus* %**	***Plasmodium* %**	***Leucocytozoon* %**
Holarctic	102590	17.9	2.9	16.2
Ethiopian	11507	16.4	3.2	4.6
Oriental	45091	13.1	0.8	2.9
Neotropical	54101	7.1	1.6	0.1

3.2.5.3. Host distribution

Among the avian species investigated for haemosporidians, roughly 50% were found infected with *Haemoproteus*, while around 30% were found infected with *Plasmodium* and *Leucocytozoon* (Valkiūnas 2005). The highest number of *Plasmodium* species infecting the same host order is found in Galliformes (17), Passeriformes (16), and Columbiformes (11), respectively. Similarly, the maximum number of *Leucocytozoon* species is observed in Passeriformes, Galliformes, and Coraciiformes, each with seven species (Valkiūnas 2005), while the orders Podicipediformes, Procellariiformes, Phoenicopteriformes, Pterocliformes, and Tinamiformes have not been found to be infected with *Leucocytozoon* (Valkiūnas and Iezhova 2023).

Recent reviews focusing on haemosporidian fauna of host orders are only available for the *Haemoproteus* genus, due to its higher host specificity. The most diverse *Haemoproteus* fauna is found among Passeriformes, with 86 described species, followed by Galliformes and Piciformes with 10 species each, and Charadriiformes with nine species. Notably, about 50% of all known *Haemoproteus* species parasitize Passeriformes (Valkiūnas and Iezhova 2022), thereby highlighting Passeriformes and Galliformes as the two bird orders with the richest haemosporidian fauna (Valkiūnas 2005).

Recent studies indicate an expansion of host ranges due to climate change, facilitating increased interactions among vectors, parasites, and hosts, thereby disrupting pre-existing natural and environmental barriers (Fecchio et al. 2019).

3.2.5.4. Epidemiology in Portugal

In mainland Portugal, similar studies have been conducted on haemosporidian infections in diverse wild bird species at wildlife rehabilitation centres (Tomás 2014; Zacarias 2017; Gomes et al. 2023).

Research conducted by Tomás (2014) at both RIAS and CERVAS, revealed an overall prevalence of haemosporidian infection at 33.59%. *Leucocytozoon* was the most prevalent (18%), followed by *Haemoproteus* (15.6%) and *Plasmodium* (2.3%). Significant differences in infection prevalence were observed among taxonomic orders, with Strigiformes exhibiting the highest prevalence (77.8%). Rehabilitation centres also showed significant differences, with CERVAS recording the highest prevalence (88.9%) compared to RIAS (13%). Phenological status also influenced infection rates, with resident birds showing a higher prevalence compared to exclusive migratory birds.

In another study conducted at RIAS, Zacarias (2017) showed of total haemosporidian parasite prevalence of 22.6%. Similarly, *Leucocytozoon* was the most prevalent (15.1%), followed by *Haemoproteus* (13.2%) and *Plasmodium* (3.8%). Significant differences in infection rates were observed among different habitats, seasons, and phenological status.

Among these variables, notably higher prevalence rates were observed among terrestrial birds (30.6%), samples collected during summer (40%) and resident birds (34.5%).

In a recent study conducted at CERVAS by Gomes et al. in 2023, it was found that 48.6% of all sampled birds were positive for haemosporidians, with 44.3% positive for *Leucocytozoon* and 24.3% positive for *Plasmodium*/*Haemoproteus*. Infection significantly varied between the sex of birds, with males (52.4%) being more infected than females (21.7%).

3.2.6. Wild birds vs. exotic birds

When compared to their impact on native avian populations, haemosporidians can pose significant threats for non-indigenous bird species, particularly exotic birds kept in human care (Huijben et al. 2007; Meister et al. 2021). While avian haemosporidians and their hosts have co-evolved since ancient times in Europe, various native bird species can show no clinical signs during infection (Huijben et al. 2007). However, exotic birds brought in from regions where haemosporidian parasites are typically absent or where different groups of haemosporidians are present, are particularly vulnerable to haemosporidian infection (Valkiūnas 2005; Meister et al. 2021). The presence of such birds, particularly in large collections such as zoos, creates favourable conditions for outbreaks (Valkiūnas 2005).

It has been suggested that exotic species may acquire haemosporidians not native to them, which may only develop up to the stage of exoerythrocytic meronts without forming gametocytes. The previously described cases of mortality among exotic Psittaciformes and other birds, in which meronts are found in their skeletal muscles and/or in the heart, despite the absence of parasitaemia, further support this hypothesis (Valkiūnas 2005). This suggests that haemosporidians may play a role in enhancing ecosystem stability by impeding the infiltration of exotic organisms into certain ecosystems (Valkiūnas 2005).

3.2.7. Feral Pigeons

Feral pigeon populations (*Columba livia domestica*) are typical inhabitants of urban landscapes, where they coexist with humans, drawn by the availability of nesting sites and human-provided food sources (Nebel et al. 2020). Their undomesticated relative, the rock pigeon (*Columba livia*), is native to Western and Southern Europe. Over several millennia since their domestication, feral pigeons have successfully expanded their habitat globally, particularly thriving in urban settings (Nebel et al. 2020).

While studies in suburban and urban areas often show a high prevalence of *Haemoproteus* in feral pigeons, primarily attributed to their dense populations, natural populations of *Columba livia* remain largely understudied, leaving a lack of comparative data (Nebel et al. 2020). Their widespread distribution across the globe has made them propagators of *Haemoproteus columbae* and other haemosporidian parasites (Chagas et al. 2016; Nebel et al. 2020). Although *Haemoproteus columbae* typically infects only birds of the order

Columbiformes and thus may not directly threaten other avian groups, the high number of pigeons worldwide can facilitate its spread into naïve new hosts and potentially endanger other Columbiformes species (Chagas et al. 2016; Nebel et al. 2020).

Typically, *Haemoproteus columbae* infections are not regarded as highly harmful to their hosts (Sol et al. 2003). Nevertheless, elevated infection levels can lead to adverse physiological and behavioural effects. In certain cases, particularly among young individuals lacking a fully developed immune response, infections can even be lethal (Sol et al. 2003; Nebel et al. 2020).

3.3. Wildlife centres

Wildlife rehabilitation centres involve the treatment and temporary care of injured, diseased and displaced indigenous animals, and the subsequent release of healthy animals to appropriate habitats in the wild (Miller 2012). The primary objective of wildlife rehabilitation is to mitigate the negative impact of human activity on species demographics and individual animal welfare (Mullineaux 2014; Garcês 2022).

3.3.1. Wildlife centres impact in avian conservation

Human-wildlife interactions emerge as the primary cause driving admissions to wildlife rehabilitation centres (Molina-López et al. 2017). Veterinarians are increasingly involved with Wildlife rehabilitation (Paterson et al. 2021). Wildlife rehabilitation and wildlife veterinarians are essential components of Conservation Medicine, an interdisciplinary field that recognizes and integrates the links between human health, wildlife health, and ecosystem health (Jakob-Hoff and Warren 2012; Paterson et al. 2021). Inserted within the One Health framework (Cleaveland et al. 2014), wildlife rehabilitation not only addresses individual animal welfare but also serves as a platform for monitoring population health indicators (Garcês 2022). Moreover, wildlife rehabilitation remains an undervalued yet potentially useful tool for stabilizing some declining populations and could be targeted to support *in situ* interventions (Paterson et al. 2021). These centres not only fulfil a crucial role in the conservation and preservation of wild animals but also serve as vital sources of information on emerging diseases and epidemiological surveillance. Additionally, they significantly contribute to public education efforts aimed at species conservation, particularly for endangered species (Garcês 2022).

3.3.2. Wildlife centres and parasitological research

The pathogenic impact of haemosporidians on free-living birds is most pronounced during the peak stages of initial development or acute relapses, leading to the debilitation and inactivity of the birds (Valkiūnas 2005). This period is brief, but it is responsible to the elimination of many haemosporidian infected individuals in the wild. Notably, during this acute stage, birds, especially young ones, are debilitated and less active and thus are not captured

using traditional methods like mist nets or traps (Valkiūnas 2005). Consequently, much of the information gathered over the past two decades primarily reflects chronic infections, offering only a partial understanding of host-parasite dynamics in nature. Notably, the influence of haemosporidians on birds during the acute stage of infection remains inadequately studied in wildlife (Valkiūnas 2005). Valkiūnas (2005) highlights a critical limitation in studies relying solely on traditional capture methods, emphasizing the need to also consider birds brought to wildlife rehabilitation centres, which are typically injured or sick. These studies present a complementary perspective, given that healthy animals are less likely to be admitted to the centres.

Additionally, captive wildlife birds serve as an excellent model for parasite research and provide valuable results for wildlife management and conservation of endangered or less-studied species (Inumaru et al. 2017; Jia et al. 2018). Important advances in avian haemosporidian research, including the discovery of the life cycle of some avian haemosporidians (Valkiūnas 2005), the discovery of the negative impact of the parasite infection on their hosts health (Valkiūnas 2005; Santiago-Alarcon and Marzal 2020), or the development of many anti-parasite drugs and therapies (Palinauskas et al. 2020) have been possible thanks to the use of individuals from zoos and rehabilitation centres. Despite this importance, reports of haemosporidians in captive birds are still scarce, with only 277 avian haemosporidian linages recorded in captive birds, representing a mere 6% of the identified haemosporidian lineages available in the MalAvi database (MalAvi database Version 2.5.8, October 2023, Bensch et al. 2009).

3.3.3. Length of stay in rehabilitation centres

In wildlife rehabilitation centres, ensuring adequate treatment and prognosis assessment is crucial for facilitating a swift and effective release into the wild, as prolonged captivity can result in the loss of survival skills among wildlife (Victoria 2001; Molina-López et al. 2017). Consequently, the duration of stay in rehabilitation centres, defined as the period between admission and termination of an individual's stay, has been proposed as a valuable metric for evaluating centre effectiveness and estimating the rehabilitation process's cost, encompassing expenses related to staff, food, and medicines (Molina-López et al. 2017). Given the significant financial constraints faced by wildlife rehabilitation centres, as they often rely on self-funding or substantial subsidies for their operations (Englefield et al. 2019; Paterson et al. 2021), leading to constraints due to insufficient funding, limited staff availability, and restricted access to veterinary care (Cerda et al. 2023), analysing the duration of stay becomes essential.

However, this parameter is barely reported in studies of wildlife rehabilitation (see Tribe et al. 2014; Molina-López et al. 2017; Lukesova et al. 2021 for some exceptions). Furthermore,

even though individuals admitted to rehabilitation centres are frequently parasitized by blood parasites (Inumaru et al. 2017; Jia et al. 2018; Gomes et al. 2023), the impact of avian haemosporidian infections of the length of stay in the rehabilitation centres is largely unknown.

4. Prevalence and Genetic Diversity of Avian Malaria of Wild Birds from Rehabilitation Centres in Mainland Portugal

4.1. Dissertation objectives

This study aims to achieve the following objectives:

- Analyse the prevalence, genetic diversity, and geographic distribution of the haemosporidian parasites causing avian malaria in both wild and pet exotic birds.
- Determine the influence of diverse factors in the haemosporidian infection probability (infected/uninfected), including avian taxonomic order, body condition, rehabilitation centre (locality), reason for admission to the rehabilitation centre, season of admission to rehabilitation centres.
- Conduct a comprehensive study of Haemosporidian parasites in Portugal, employing a pioneering approach in Portugal that combines both morphological and molecular methods, to correlate both methodologies and to identify new molecular markers for species already described morphologically.
- To evaluate the impact of haemosporidian infection in the rehabilitation of wild birds by analysing the association of the haemosporidian infection and the number of days wild birds admitted to the rehabilitation centres required medical treatment.

4.2. Material and Methods:

4.2.1. Sample provenience and study period

The study was conducted in mainland Portugal from November 2022 to May 2023 and incorporates three distinct bird groups that were sampled simultaneously: (1) Wild birds sampled from three Wildlife Rehabilitation centres: Centre for Studies and Rehabilitation of Wild Animals of Castelo Branco (CERAS) in Castelo Branco, Wildlife Rehabilitation Centre of Santo André (CRASSA) in Setúbal and Wildlife Rehabilitation and Research Centre of Ria Formosa (RIAS) in Faro; (2) Pet exotic birds sampled from three veterinary clinics of Lisbon: Exoclinic in Algés, the exotic animal service of the Veterinary School Hospital of FMV-ULisboa and VetEX veterinary service in Lisbon; (3) Peri-urban pigeons sampled in collaboration with the Lisbon City Council and the FMV-ULisboa research project to assess heavy metal contamination in pigeons at two locations in Lisbon: Alameda Afonso Henriques and Jardim da Estrela. A total of 163 birds were sampled, comprising 89 individuals from Wildlife Rehabilitation Centres, 33 from Veterinary Clinics, and 41 peri-urban Pigeons.

4.2.2. Sample collection technique, storage and shipment

In different birds, considering species-specific anatomical features, size, body condition, and clinical situation, samples were collected from different veins, namely the brachial vein, superficial ulnar vein, and medial metatarsal vein.

Pigeon samples were obtained through puncture of the medial metatarsal vein. The blood collection was done before euthanasia, which was carried out as part of a separate project aimed at assessing and comparing heavy metal contamination in the city of Lisbon. The heavy metal contamination screening project was conducted in partnership with the Lisbon City Council and the FMV-ULisboa. It is important to note that the sampling for the present work did not affect the welfare or outcomes of the pigeons.

For each bird, a small blood volume (50 µL) was collected and added to 0.5 mL of SET-buffer and a small blood drop was also used to do a peripheral blood smear. However, due to time constraints at the veterinary clinics, blood smears were not performed for the 33 birds sampled there. The minimal blood volume was collected to minimize any physiological consequences (Clark et al. 2009). Syringes of 1 mL and hypodermic needles of 25-gauge (0.5x16 mm) or 23-gauge (0.6x25 mm) were used.

The SET-buffer was prepared in the laboratory of parasitology and parasitic diseases of the FMV-ULisboa, following the formula: 0.015 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0. Half a millilitre of buffer was added to each 1.5 mL Eppendorf tube and sent to the respective rehabilitation centres or veterinary clinics.

The Eppendorf tubes with blood samples and SET-buffer were stored in the refrigerator at 4°C until they were sent to the Faculty of Biology of the University of Extremadura for further processing and analysis. Meanwhile, blood smears were rolled into paper blocks after proper air-drying and subsequently sent for processing and analysis at the FMV-ULisboa. Detailed instructions for sample collection, storage and packaging for shipment were provided to all centres, clinics, and projects involved in the sample collection (Appendix XVII).

4.2.3. Host data collection and analysed variables

For each sample, a Google Forms sheet was filled, and a unique ID was attributed. That ID was written in both the Eppendorf tube and the blood smears of the same sample. In the Google Forms, data regarding the sample and the bird sampled were added, such as: bird species, date of blood collection, date of admission to the rehabilitation centre, sex, age, body condition, reason for admission to the rehabilitation centre, the district and municipality that the bird was caught and the rehabilitation centre or veterinary clinic that was treating that bird.

Each individual's body condition was evaluated upon admission through palpation of pectoral muscle mass and assessment of the prominence of the keel bone, graded on a scale

of 1 to 5. A score of 1 indicated extreme emaciation, while a score of 5 indicated overweight condition (Tully 2009).

4.2.4. Sample processing

4.2.4.1. Preparation for morphological analysis: Preparation, fixation, and staining of blood smears

Following blood collection, a blood smear using the wedge smear technique was done for each animal sampled. Utilizing a ground-edge frosted slide (76x26 mm) (VWR International®, Radnor, Pennsylvania, USA), a drop of blood was placed at one end, and a coverslip (22x22 mm) (VWR International®, Radnor, Pennsylvania, USA) was used to extend the blood. The coverslip, positioned at a 45º angle, was gently moved backwards until it touched the blood drop, spreading it along its edge. With a continuous and smooth forward motion, the drop was stretched, forming a monolayer zone where blood constituents are defined and closely positioned but not overlapped, maximizing observation under an optical field (Clark et al. 2009).

Before performing the blood smear, all slides were properly labelled with the sample ID by pencil in the frosted part of the slide because ink gets diluted with the staining process. Following the initial fixation, corresponding to air-drying of the smear, blood smears were stained using Giemsa staining, following the protocol utilized in the Laboratory of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine of the University of Lisbon.

The Giemsa staining protocol involved placing the dried smears on a support on a tray, covering the smears with methanol (Merck® 1.06009.2511) for 1 minute, ensuring the second fixation. After that step, without removing the methanol from the slides, Giemsa stain (Merck® 1.09204.2500) was applied onto the slide, covering the smear, and left to act for 1 minute. Being a neutral stain, Giemsa is responsible for staining the acidic and basic components of blood, namely nuclear and cytoplasmic components of cells and potential parasites (Clark et al. 2009). Excess stain was then removed by passing the slide under running water, and the slide was left vertically in a support until it was completely dry.

4.2.4.2. Preparation for molecular analysis: DNA extraction, quantification and storage

All the samples preserved in SET-buffer were sent to STAB "Servicio de Técnicas Aplicadas a la Biociencia", a laboratory of the University of Extremadura, for DNA extraction and quantification. The DNA was extracted using MAGMAX PATHOGEN RNA/DNA KIT (Applied Biosystems™, Waltham, USA, reference: 4462359). The DNA was also quantified, using NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific™, Waltham, USA), according to the manufacturer's recommendations. The DNA was then stored at -20°C until further examination.

4.2.5. Sample analysis

4.2.5.1. Morphological analysis: Haemosporidian identification and molecular analysis confirmation

The morphological identification of haemosporidian parasites was conducted through the observation of blood smears using the binocular Olympus BX50 optical microscope. The search for haemosporidians was performed at magnifications of ×400 and ×1000, observing the monolayer area of the smear and the immediately adjacent areas. This ensured that all slides were examined with the same rigor and within the same area. Blood smear examination was performed for every sample apart from the 33 exotic birds sampled at the veterinary clinics that the blood smears were not performed. The blood smears of all samples were analysed, and the parasites were identified up to the parasitic genus following Valkiūnas (2005), Valkiūnas and Lezhova (2022), and Valkiūnas and Lezhova (2018). Species morphological identification of *Haemoproteus* was performed when the molecular matches were only identified at the genus level using identification keys from Valkiūnas and Lezhova (2022).

Photographic records of parasitic forms were captured using the iPhone 13 camera.

4.2.5.2. Molecular analysis

4.2.5.2.1. Polymerase chain reaction (PCR) amplification

For the genetic analysis, a nested-PCR protocol was conducted to amplify a segment of the mitochondrial cyt b gene of haemosporidians, as described by Bensch et al. (2000) and Hellgren et al. (2004). This technique enables the screening of haemosporidians within the *Plasmodium* and *Haemoproteus* genera, as well as the *Leucocytozoon* genus (Hellgren et al. 2004). In the first PCR, general haemosporidian primers, HaemNFI and HaemNR3, were utilized to amplify DNA from all three haemosporidian genera (Hellgren et al. 2004). In the second PCR, specific primers for *Haemoproteus* and *Plasmodium* spp., namely HaemF and HaemR2 (Bensch et al. 2000), as well as primers specific to *Leucocytozoon* spp., HaemFL and HaemR2L (Hellgren et al. 2004), were used.

To address some methodologic problems found, namely the false positives for *Haemoproteus*/*Plasmodium* in some samples intensely infected with *Leucocytozoon*, the specific primers to *Haemoproteus* and *Plasmodium* were used in the first PCR: HaemNF and HaemNR2 (Waldenström et al. 2004). In the second PCR of these samples, the same primers were used as described above (HaemF and HaemR2, Bensch et al. 2000). All primers were diluted to 10 µM. Primer sequences are described in Table 2.

For the 72 samples with DNA concentration of above 20 ng/µL and less than 100 ng/µL, the first PCR was carried out in a final volume of 25 µL, including 15.9 µL of H2O, 2.5 µL of Buffer (10x Ex Taq Buffer TaKaRa™, Shiga, Japan), 2.5 µL of dNTPs (dNTP Mix TaKaRa™,

Shiga, Japan) 1 µL of Primer HaemNFI, 1 µL of Primer HaemNR3, 0.1 µL of Taq (Ex Taq TaKaRa™, Shiga, Japan) and 2 µL of DNA.

The two second PCRs were also done in a final volume of 25 µL, with 15.9 µL of H2O, 2.5 µL of Buffer, 2.5 µL of dNTPs and 2 µL of the product of the first PCR was added. The primers added to the reactions depended on the target genus of the PCR reaction, with 1 µL of Primer HaemF and 1 µL of Primer HaemR2 added to the reactions targeting *Haemoproteus* and *Plasmodium* spp., and 1 µL of Primer HaemFL and 1 µL of Primer HaemR2L were added to the reactions targeting the *Leucocytozoon* genus.

The 3 samples with DNA concentration above 100 ng/µL were diluted to 25 ng/µL and in the 3 samples with DNA concentrations below 8 ng/µL the DNA extraction protocols were repeated. The samples were excluded from the study when the concentrations did not improve.

The 89 samples with DNA concentrations below 20 ng/µL but above 8 ng/µL underwent a different first PCR protocol. In those cases, instead of the 15.9 µL of H2O, were used 13.9 µL and instead of the 2 µL of DNA, were used 4µL in all amplification reactions. The second PCR protocol was the same as described above for the samples with DNA concentrations above 20 ng/µL and under 100 ng/µL.

In each PCR run, negative and positive control samples were included, namely one negative control with sterilised water for every eight samples, and two positive controls containing DNA from previously identified haemosporidian strains for every 24 samples. Additionally, all blood smears were analysed, and molecular analysis was repeated for any molecular false negatives identified through blood smear examination.

The PCRs were conducted using a SimpliAmp™ Thermal Cycler (Applied Biosystems™, Foster City, California). The first PCRs were performed using the following conditions: 30 seconds of denaturation at 94°C, 30 seconds of annealing at 50°C, and 45 seconds of extension at 72°C, all repeated for 25 cycles. The samples were incubated before the cyclic reaction for an initial denaturing step at 94°C for 2 minutes and after the cyclic reaction for a final extension step at 72°C for 10 minutes.

The thermal profile of the second PCRs were identical to the initial PCRs but performed for 35 cycles instead of 25 cycles.

For amplification evaluation, agarose gel electrophoresis was performed. After preparing a 2% agarose gel in tris-borate-EDTA (TBE) buffer, 2.5 µL of the final PCR product was loaded into the wells. A 1000 bp DNA Ladder (TaKaRa™, Shiga, Japan) was used as a size marker in each gel to evaluate the size of the amplicons, which were expected to be 479 base pairs. 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 using Molecular Imager® Gel Doc™ XR System (Bio-Rad™, Hercules, California).

Table 2. Primers used in the study, their sequences, and the corresponding Hemosporidian targets.

Primer name	Sequence	Amplification	Reference
HaemNFI	5'-CATATATTAAGAGAAITATGGAG-3'	*Haemoproteus*, *Plasmodium* and *Leucocytozoon*	Hellgren et al. 2004
HaemNR3	5'-ATAGAAAGATAAGAAATACCATTC-3'	*Haemoproteus*, *Plasmodium* and *Leucocytozoon*	Hellgren et al. 2004
HaemF	5'-ATGGTGCTTTCGATATATGCATG-3'	*Haemoproteus* and *Plasmodium*	Bensch et al. 2000
HaemR2	5'-GCATTATCTGGATGTGATAATGGT-3'	*Haemoproteus* and *Plasmodium*	Bensch et al. 2000
HaemFL	5'-ATGGTGTTTTAGATACTTACATT-3'	*Leucocytozoon*	Hellgren et al. 2004
HaemR2L	5'-ATTATCTGGATGAGATAATGGIGC-3'	*Leucocytozoon*	Hellgren et al. 2004
HaemNF	5'-CATATATTAAGAGAATTATGGAG-3'	*Haemoproteus* and *Plasmodium*	Waldenström et al. 2004
HaemNR2	5'-AGAGGTGTAGCATATCTATCTAC-3'	*Haemoproteus* and *Plasmodium*	Waldenström et al. 2004

4.2.5.2.2. Sequencing

All positive samples for the second PCRs were sent to STAB, a laboratory of the University of Extremadura, to be purified and sequenced on both ends, following the procedures described by Bensch et al. (2000).

All fragments for *Haemoproteus* spp. or *Plasmodium* spp. and for *Leucocytozoon* spp. identification were sequenced from the 5' end with the primers HaemF and HaemFL, and from the 3' end with the primers HaemR2 and HaemR2L, respectively.

4.2.5.2.3. Lineage identification

Sequences were edited and aligned using the program BioEdit (Hall 1999). The 'Basic Local Alignment Search Tool' (Blast) of GenBank (Accessed 28 July 2023) and the MalAvi database (Version 2.5.8, October 2023, Bensch et al. 2009) were used to determine the lineage of detected parasite sequences. Parasites with sequences differing by at least 1 nucleotide from the already described lineages of both databases were considered new evolutionary independent lineages (Bensch et al. 2000).

New lineages (sequences not previously published in GenBank) were coded following the nomenclature of the MalAvi database (Bensch et al. 2009) and deposited in GenBank under the accession numbers PP457803-PP457804 (Table 4). New host–parasite relationships were established by comparison of the results with the public database (MalAvi database Version 2.5.8, October 2023, Bensch et al. 2009) showing avian haemosporidian

lineages (based on mitochondrial cytochrome b lineages) and host range. A new host record was established when the avian haemosporidian lineage had not been previously reported infecting this bird species (according to MalAvi database Version 2.5.8, October 2023, Bensch et al. 2009), as it has been done in previous studies (Muriel et al. 2021; Garcia-Longoria et al. 2022).

4.2.6. Data collection, computerization and statistical analysis

Microsoft Excel® (Microsoft Corporation©) was used for data descriptive analysis and graph production. A General Linear Model (GLM) was used to analyse the relationship between the selected variables: body condition, haemosporidian infection (infected vs. uninfected), rehabilitation centre (CERAS, CRASSA and RIAS) and the season of admission (winter, spring and autumn), on the number of days requiring medical treatment for birds admitted to rehabilitation centre. The dependent variable was log transformed to fit a linear model. A logistic regression analysis was used to explore whether avian taxonomic order, body condition, rehabilitation centre (locality), the season of admission to rehabilitation centres (winter, spring or summer) and the reasons of admission to rehabilitation centres (debilitating disease, trauma or other causes of admission) influenced haemosporidian infection probability. A backward stepwise procedure was used to eliminate all non-significant terms ($P > 0.05$) from the starting maximal model.

All analyses were performed using PASW Statistics 22 statistical package for Windows.

4.2.6.1. Samples used for statistical analysis

The prepatent period is the elapsed time from the inoculation of sporozoites by the vector into the bird until the appearance of blood stages (see Chapter 3.2.1 for more details). This period varies from 11–21 days for *Haemoproteus*, approximately five days for *Leucocytozoon*, and possibly as short as five days in the case of *Plasmodium relictum* (Valkiūnas 2005). Additionally, from those birds sampled at wildlife rehabilitation centres, fifty-six birds were sampled at admission on the centre or before the prepatent period of haemosporidian parasites was over, whereas the remaining 33 individuals were sampled when they were already housed in the centres for longer periods. Hence, for statistical analyses exploring the effect of haemosporidian infection on the required medical treatment for birds admitted to a rehabilitation centre and the factors influencing haemosporidian infection probability, were only considered birds sampled in the centre for less than the corresponding prepatent period of the observed parasite infecting the bird (n = 56), therefore ensuring that all analysed infections were acquired in the wild and not in the wildlife rehabilitation centre.

For statistical analysis purposes, due to their prevalence, only birds from the wildlife rehabilitation centres were considered (see Chapter 4.3.2). All samples (n = 163) were considered for the identification of parasite lineages and lineage–host interactions.

4.3. Results

4.3.1. Sample description

4.3.1.1. Wild birds from Wildlife Rehabilitation centres

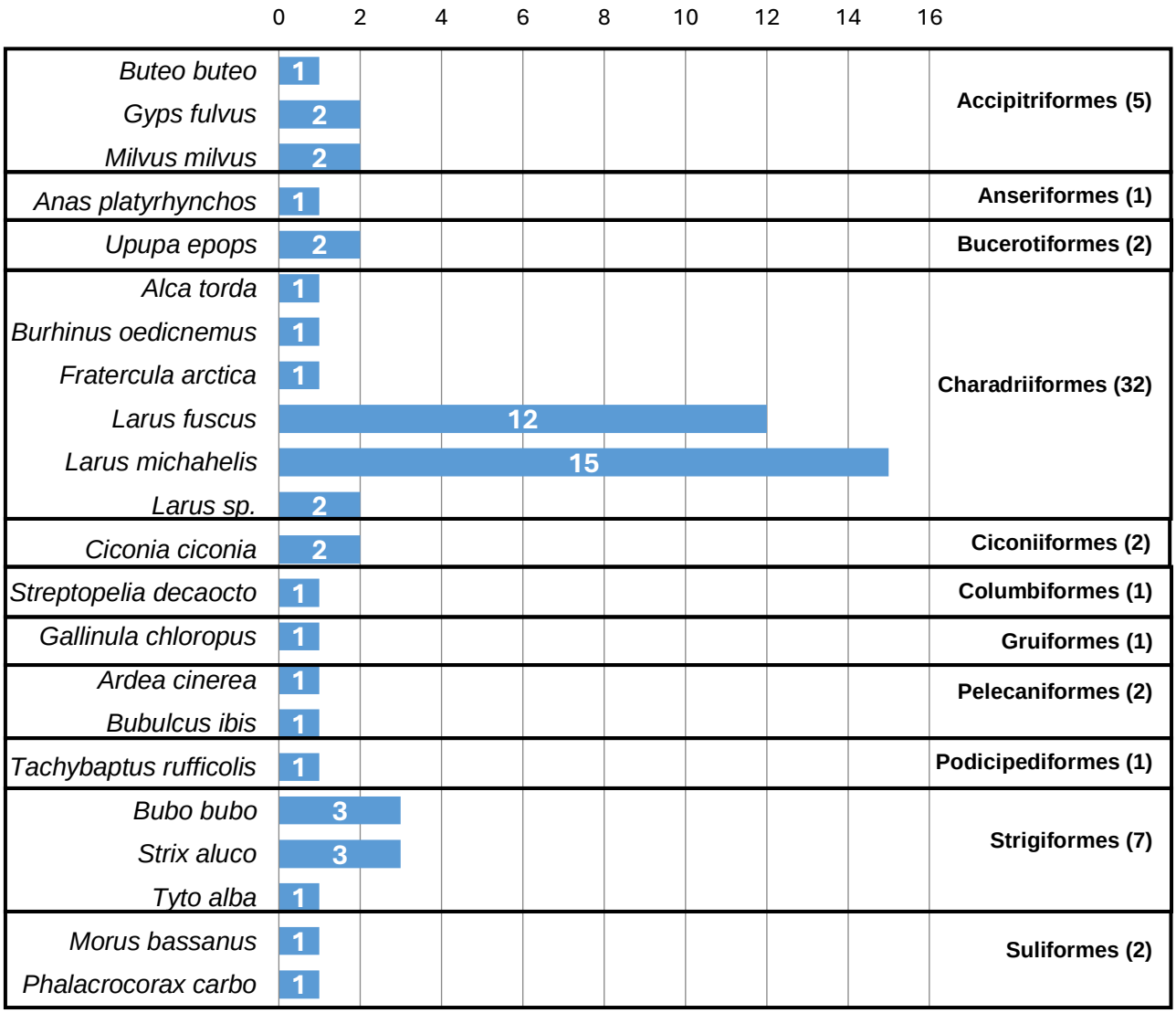
A total 89 wild birds were sampled at the wildlife rehabilitation centres, belonging to 28 distinct species spanning across 13 taxonomic orders (see Table 3 for the complete list of species sampled). From the species sampled, 8 are considered threatened with extinction in mainland Portugal (6 Vulnerable: *Bubulcus ibis*, *Burhinus oedicnemus*, *Larus fuscus*, *Asio flammeus*, *Accipiter gentilis*, *Falco tinnunculus*; 1 Endangered: *Aegypius monachus* and 1 Critically Endangered: *Milvus milvus*), representing 8.4% of all avian species considered threatened with extinction in mainland Portugal (Almeida et al. 2022).

For statistical analysis purposes a subsample was used, only considering birds sampled in the centre for less than the corresponding prepatent period of the observed parasite infecting the bird as explained at Chapter 4.2.6.1.

The subsample comprises 56 birds, belonging to 21 species, with *Larus michahelis* being the most predominant with 15 individuals (26.8%). A smaller representation was observed in 13 species, each with a single individual (1.8%).

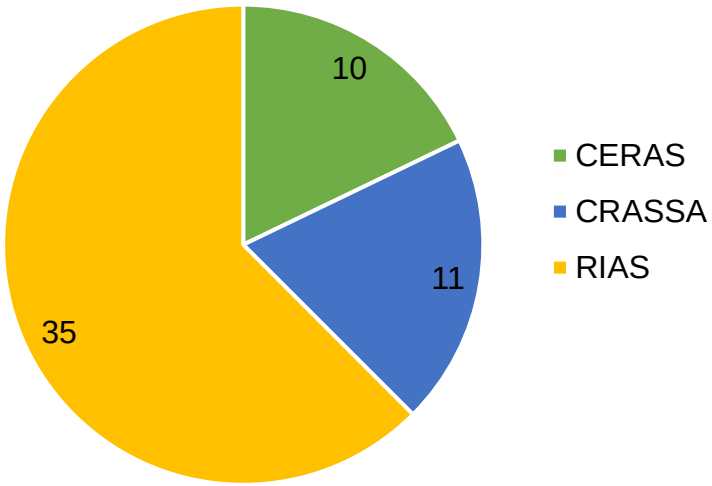
Considering the orders of birds, the subsample is representative of 11 orders, with Charadriiformes (57.1%) being the most predominant, and with lesser representation in 4 orders, namely Anseriformes, Columbiformes, Gruiformes, Podicipediformes, each with a single individual (1.8%). The data related to the number of sampled birds per species, grouped in their respective orders, are summarised in Graphic 1.

Graphic 1. Number of samples collected at the wildlife rehabilitation centre by species and order (n=56).



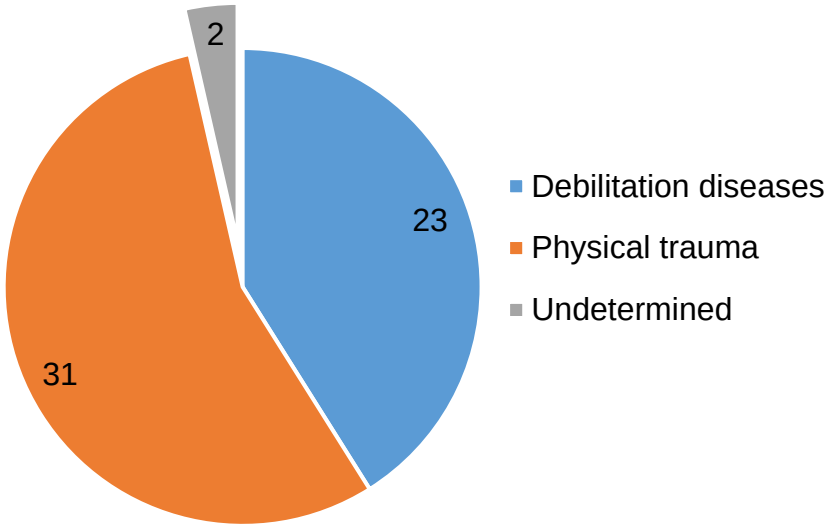
From the 56 birds of the subsample, 35 (62.5%) were sampled at Wildlife Rehabilitation Centre RIAS, 11 (19.6%) were sampled at CRASSA, and 10 (17.9%) were sampled at CERAS (Graphic 2).

Graphic 2. Number of samples collected by Wildlife Rehabilitation Centre (n=56).



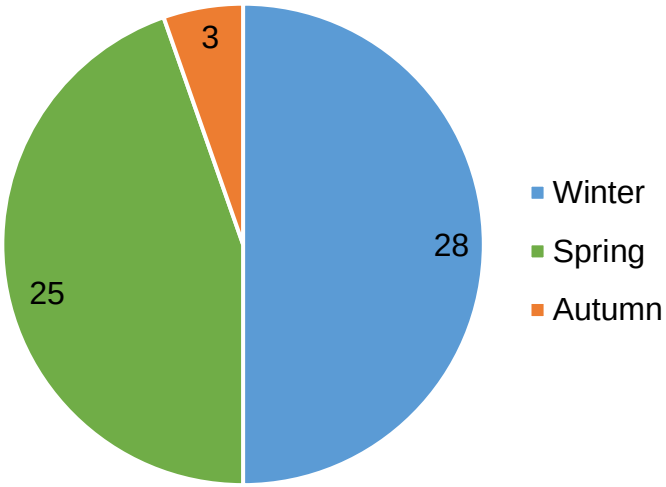
Regarding the reason for admission to the wildlife rehabilitation centres for the sampled birds, 23 (41.1%) were admitted due to debilitating diseases, 31 (55.4%) were admitted due to physical trauma related causes, and 2 (3.6%) for undetermined causes (Graphic 3).

Graphic 3. Number of samples collected by reasons for admission (n=56).



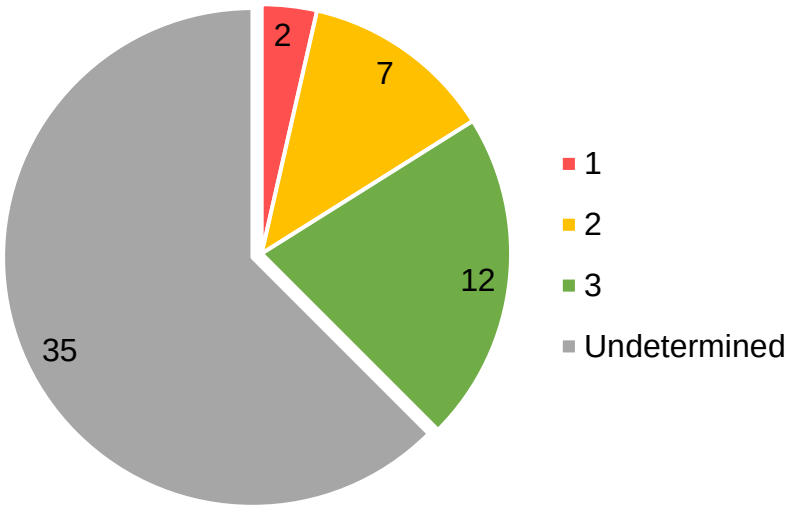
The samples were collected across different seasons. Birds were sampled in the winter (n = 28, 50.0%), spring (n = 25, 44.6%), and autumn (n = 3, 5.4%) (Graphic 4).

Graphic 4. Number of samples collected by season (n=56).



The body condition of the birds was rated on a scale of 1-5 as follows: 3.6% of the birds were in condition 1 (n = 2), 12.5% were in condition 2 (n = 7), and 21.4% were in condition 3 (n = 12). For 62.5% of the birds sampled, the body condition was not determined (n = 35) (Graphic 5).

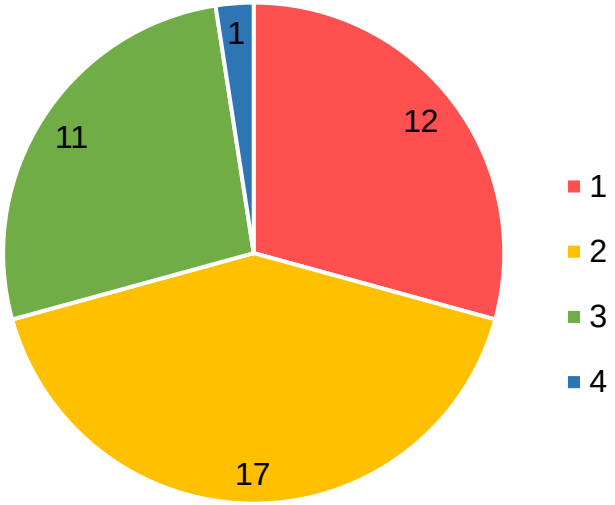
Graphic 5. Number of samples collected by body condition (n=56).



4.3.1.2. Feral Pigeons from Lisbon

The feral pigeon sample comprised 41 birds, with 21 from Alameda Afonso Henriques and 20 from Jardim da Estrela. Their body conditions, rated on a scale of 1 to 5, were distributed as follows: 1 (n = 12, 29.3%), 2 (n = 17, 41.5%), 3 (n = 11, 26.8%), and 4 (n = 1, 2.4%), with no birds having a body condition of 5 (Graphic 6).

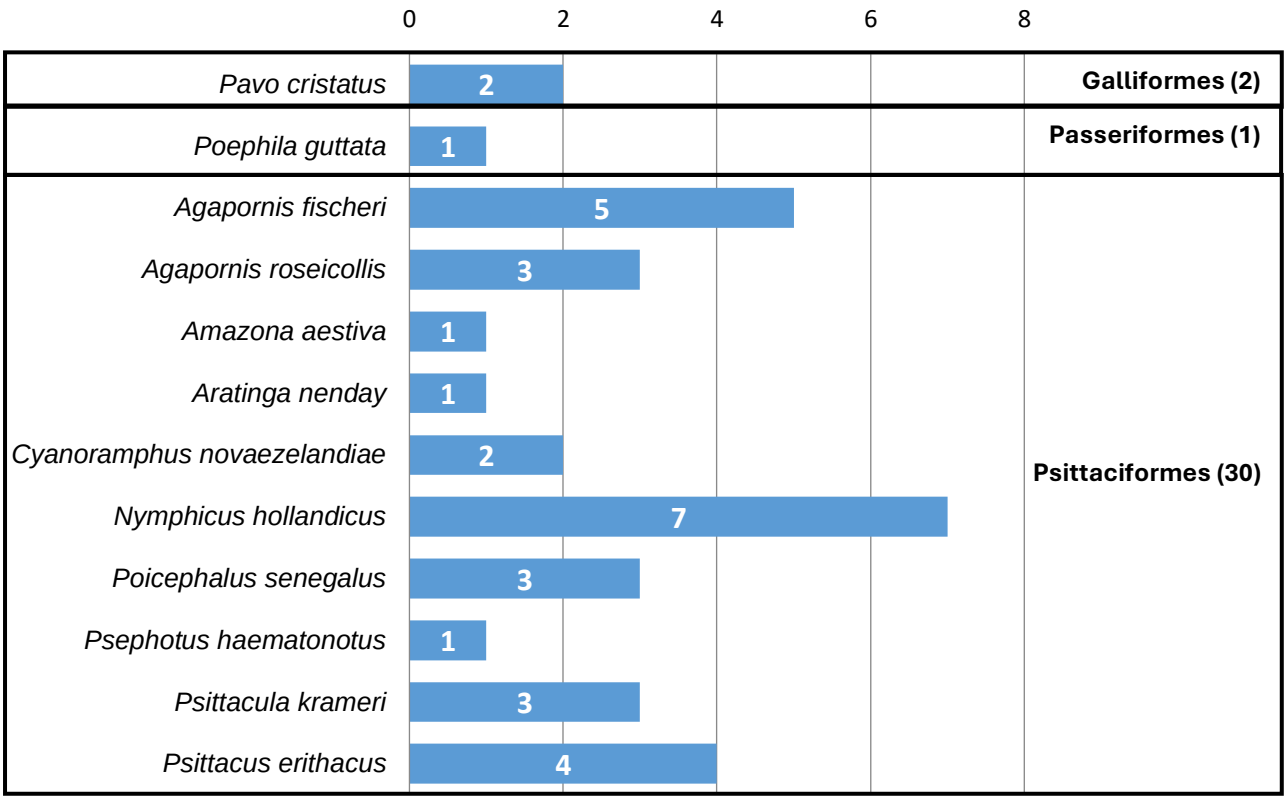
Graphic 6. Number of samples collected by body condition (n=41).



4.3.1.3. Pet exotic birds from veterinary clinics

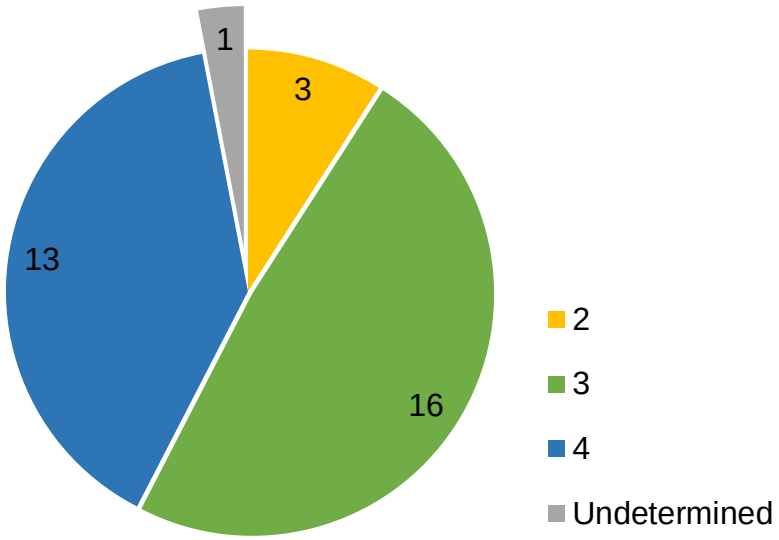
A total of 33 exotic birds from 12 different species and 3 distinct orders were sampled from three veterinary clinics in Lisbon. The most prevalent species were *Nymphicus hollandicus* (n = 7, 21.2%), *Agapornis fischeri* (n = 5, 15.2%), and *Psittacus erithacus* (n = 4, 12.1%), respectively. Four species were represented by a single individual each (3.0%). Most of the birds belonged to the order Psittaciformes (n = 30, 90.1%), followed by Galliformes (n = 2, 6.1%) and Passeriformes (n = 1, 3.0%). The data related to the number of birds sampled per species, grouped in their respective orders, are summarised in Graphic 7.

Graphic 7. Number of samples collected at veterinary clinics by species and order (n=33).



Their body conditions (1-5) ranged between 2 and 4. Three birds (9.1%) were categorized as a 2, 16 birds (48.5%) as a 3, and 13 birds (39.4%) as a 4 (Graphic 8).

Graphic 8. Number of samples collected by body condition (n=33).



4.3.2. Prevalence and Genetic Diversity of Haemosporidian Parasites

4.3.2.1. Wild birds from Wildlife rehabilitation centres

A total of 89 bird individuals belonging to 28 bird species were screened for haemosporidian parasites. Twenty-seven out of the 89 individuals were infected with haemosporidians (overall prevalence = 30.3%) (Figure 5). Of these sampled birds, 19.1% were infected with *Haemoproteus*, 13.5% with *Leucocytozoon*, and 1.1% with *Plasmodium*. Additionally, three individuals (3.6% of prevalence) harboured mixed infections with *Leucocytozoon* and *Haemoproteus* (Table 3).

The prevalence of haemosporidian infection of the 56 birds sampled at the time of admission to the rehabilitation centres or before the prepatent period of haemosporidian parasites was over 41.1%: 25% prevalence for *Haemoproteus*, with a 16.1% prevalence for *Leucocytozoon* and 1.8% prevalence for *Plasmodium*. Moreover, three out of the nine bird species that tested positive for infection in this study had not been previously documented as infected by haemosporidian parasites in molecular studies (Table 3).

Table 3. Number of individuals uninfected, and infected with *Haemoproteus* (H), *Plasmodium* (P), and *Leucocytozoon* (L), per bird species.

Bird Species	Bird Order	Uninfected	H	P	L	TOTAL
Accipiter gentilis	Accipitriformes	0	0	0	1 (0)	1 (0)
Aegypius monachus	Accipitriformes	3 (0)	0	0	0	3 (0)
Alca torda	Charadriiformes	1 (1)	0	0	0	1 (1)
Anas platyrhynchos	Anseriformes	1 (1)	0	0	0	1 (1)
Ardea cinerea	Pelecaniformes	0	0	0	1 (1)	1 (1)
Asio flammeus	Strigiformes	1 (0)	0	0	0	1 (0)
Bubo bubo	Strigiformes	0	1[$] (1[$])	0	4 (3)	4 (3)
Bubulcus ibis	Pelecaniformes	1 (1)	0	0	0	1 (1)
Burhinus oedicnemus	Charadriiformes	1 (1)	0	0	0	1 (1)
Buteo buteo	Accipitriformes	1 (0)	0	0	1 (1)	2 (1)
Ciconia ciconia	Ciconiiformes	10 (2)	0	0	0	10 (2)
Falco tinnunculus	Falconiformes	2 (0)	0	0	0	2 (0)
Fratercula arctica	Charadriiformes	1 (1)	0	0	0	1 (1)
Gallinula chloropus	Gruiformes	1 (1)	0	0	0	1 (1)
Garrulus glandarius	Passeriformes	0	1[$] (0)	0	1 (0)	1 (0)
Gyps fulvus	Accipitriformes	2 (2)	0	0	0	2 (2)
*Larus fuscus**	Charadriiformes	6 (4)	8 (8)	0	0	14 (12)
*Larus michahelis**	Charadriiformes	17 (10)	5 (4)	1 (1)	0	23 (15)

Table 3. Number of individuals uninfected, and infected with Haemoproteus (H), Plasmodium (P), and Leucocytozoon (L), per bird species (cont.).

Bird Species	Bird Order	Uninfected	H	P	L	TOTAL
Larus sp.	Charadriiformes	2 (2)	0	0	0	2 (2)
Milvus migrans	Accipitriformes	1 (0)	0	0	0	1 (0)
Milvus milvus	Accipitriformes	3 (2)	0	0	0	3 (2)
Morus bassanus	Suliformes	1 (1)	0	0	0	1 (1)
Phalacrocorax carbo	Suliformes	1 (1)	0	0	0	1 (1)
*Streptopelia decaocto**	Columbiformes	0	0	0	1 (1)	1 (1)
Strix aluco	Strigiformes	1 (0)	1$ (1$)	0	3 (3)	4 (3)
Sturnus unicolor	Passeriformes	1 (0)	0	0	0	1 (0)
Tachybaptus rufficolis	Podicipediformes	1 (0)	0	0	0	1 (0)
Tyto alba	Strigiformes	2 (1)	0	0	0	2 (1)
Upupa epops	Bucerotiformes	2 (2)	0	0	0	2 (2)
Total		**63 (33)**	**16 (14)**	**1 (1)**	**12 (9)**	**89 (56)**

Numbers in brackets represent the number of individuals for each bird species that were sampled at the time of admission on the rehabilitation centres or before the prepatent period of haemosporidian parasites was over. An asterisk (*) after bird species denotes that these bird species were not previously documented to be infected by haemosporidians (according to MalAvi database Version 2.5.8, October 2023, Bensch et al. 2009), where the symbol $ in *Haemoproteus* infection (H) indicates mixed infection with *Leucocytozoon*.

Fifteen different parasite lineages were found, infecting nine out of the 28 avian species sampled (n = 89). Among these, nine *Leucocytozoon* lineages were found infecting seven bird species, five *Haemoproteus* lineages were found infecting five bird species, and one *Plasmodium* lineage was found infecting one bird species. By comparison of genetic diversity of haemosporidian parasites, it was shown that two out of the 15 lineages identified had not been previously recorded in former studies. Only three of the lineages were found multiple times, with the newly described lineage LARFUS01 being the most frequently detected, occurring 12 times: seven times in *Larus fuscus* and five times in *Larus michahellis*. The remaining two lineages found multiple times were BUBO01, detected in three *Bubo bubo*, and an unnamed lineage with GenBank accession number ON950078, found twice. Furthermore, it was also identified nine new host–parasite interactions, which represent new bird host records for these haemosporidian parasites and nine new lineage geographical reports, representing the first times these lineages were found in Portugal (Table 4).

Table 4. MalAvi parasite lineages, Parasite, genus (H *Haemoproteus*, P *Plasmodium*, L *Leucocytozoon*), GenBank accession numbers, recorded host in this study (Host), and alternative hosts and alternative location in which parasite lineages were previously recorded.

MalAvi lineage	Parasite	Genus	GenBank	Host	Alternative host	Alternative location
Not defined [#]	*Leucocytozoon* sp.	L	OL897562	*Bubo bubo*[$]	-	-
Not defined [#]	*Haemoproteus* sp.	H	ON950078	*Larus fuscus*[$]*; Larus michahellis*[$]	-	-
ARCIN01* [#]	*Leucocytozoon* sp.	L	PP457803	*Ardea cinerea*[$]	-	-
ATNO1	*Leucocytozoon* sp.	L	KJ488699	*Streptopelia decaocto*[$]	*Athene noctua*	Europe (Portugal), Africa (Morocco)
BUBO01 [#]	*Leucocytozoon danilewskyi*	L	MK330142	*Bubo bubo*	*Bubo bubo*	Europe (Spain)
CIAE02	*Leucocytozoon* sp.	L	MK330160	*Buteo buteo*	Falconiformes, Gruiformes, Charadriiformes, Piciformes, Strigiformes, Ciconiiformes, Cuculiformes, Coraciiformes, Phoenicopteriformes	Europe (Spain, Germany, Poland, Portugal, Sweden, Austria), Asia (Philippines, Turkey, Russia, Mongolia, Japan, China, Thailand), Africa (South Africa)
COCOR02 [#]	*Leucocytozoon* sp.	L	JX867111	*Garrulus glandarius*	*Garrulus glandarius, Corvus corax*	Europe (Bulgaria, Slovakia, Germany)
GAGLA05	*Haemoproteus* sp.	H	KJ488735	*Garrulus glandarius*	*Garrulus glandarius, Corvus corax*	Europe (Bulgaria, Portugal), Africa (Morocco)
LARFUS01* [#]	*Haemoproteus* sp.	H	PP547804	*Larus fuscus*[$]*; Larus michahellis*[$]	-	-
LINN1	*Plasmodium matutinum*	P	MK330156	*Larus michahellis*[$]	Passeriformes, Apterygiformes, Sphenisciformes, Gruiformes, Strigiformes, Charadriiformes, Falconiformes	Europe, Oceania, Asia, North America

Table 4. MalAvi parasite lineages, Parasite, genus (H Haemoproteus, P Plasmodium, L Leucocytozoon), GenBank accession numbers, recorded host in this study (Host), and alternative hosts and alternative location in which parasite lineages were previously recorded (cont.).

MalAvi lineage	Parasite	Genus	GenBank	Host	Alternative host	Alternative location
MILVUS02 #	*Leucocytozoon* sp.	L	JN164717	*Accipiter gentilis*$	*Buteo buteo, Buteo lagopus, Milvus milvus, Haliaeetus albicilla*	Europe (Austria, Spain)
STAL2	*Haemoproteus syrnii*	H	KJ488773	*Strix aluco*	*Strix aluco, Strix nebulosa, Strix uralensis, Bubo bubo*	Europe (Portugal, France, Germany), Africa (Morocco)
STAL3 #	*Leucocytozoon* sp.	L	MK652258	*Strix aluco*	*Strix aluco*	Europe (Austria)
STAL5 #	*Leucocytozoon* sp.	L	KC876042	*Strix aluco*	*Strix aluco*	Asia (Turkey)
STRURA03 #	*Haemoproteus* sp.	H	KJ488826	*Bubo bubo*	*Bubo scandiacus, Bubo bubo, Strix nebulosa, Strix uralensis*	Europe (Austria, France, Switzerland)

Asterisk (*) in parasite lineage indicates new haemosporidian lineages not recorded in previous studies, where symbol $ represents new host record for this haemosporidian lineage and where symbol # represents new geographical record for this haemosporidian lineage (according to MalAvi database, Version 2.5.8, October 2023, Bensch et al., 2009).

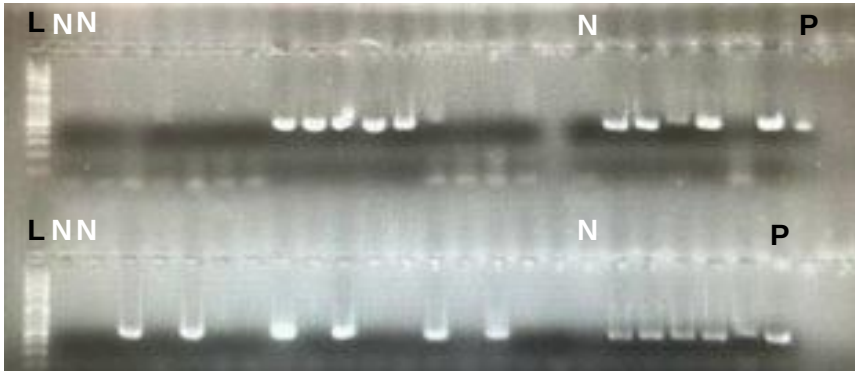


Figure 5. Agarose gel electrophoresis results showing a high prevalence of positive amplification (Original).

L: Ladder; N: Negative controls; P: positive controls; and the samples between them. The amplified DNA fragments measure 479 base pairs, corresponding to an approximate molecular weight of 311 kDa.

4.3.2.2. Feral Pigeons from Lisbon

The prevalence of haemosporidian infection among the 41 sampled pigeons was 100% for *Haemoproteus* sp. and 0% for *Plasmodium* sp. or *Leucocytozoon* sp. One bird, despite testing negative in the PCR, was positive in the blood smear. This case was considered a molecular analysis false negative and was excluded from the genetic diversity analysis. Four different lineages were identified: COLIV03, COQUI05, CXNEA02, and HAECOL1. The HAECOL1 lineage was the most common, with an 85% prevalence. All four lineages represent new geographical records, with COLIV03, COQUI05, and CXNEA02 also being reported in Europe for the first time. Notably, CXNEA02 had previously only been found in the invertebrate host *Culex neavei*, marking a new host record for *Columba livia* and identifying this lineage's vertebrate host for the first time (Table 5).

Table 5. MalAvi parasite lineages, Parasite, GenBank accession numbers, prevalence in percentage, alternative hosts and alternative location in which parasite lineages were previously recorded.

MalAvi lineage	Parasite	GenBank	Prevalence	Alternative host	Alternative location
COLIV03#	*Haemoproteus columbae*	MN065191	7.5 (3/40)	*Columba livia*	Africa (Nigeria, South Africa), Asia (Turkey), North America (USA), South America (Brazil)
COQUI05#	*Haemoproteus columbae*	GQ150192	5 (2/40)	*Columba livia*	Africa (South Africa), Asia (Turkey), South America (Brazil)
CXNEA02#	*Haemoproteus* sp.	HM179163	2.5 (1/40)	$	Africa (Cameroon)
HAECOL1#	*Haemoproteus columbae*	AF495554	85 (34/40)	*Columba livia, Alcippe poioicephala, Garrulax delesserti,*	Africa (Botswana, Nigeria, South Africa), Asia (Singapore, India, Turkey), North America (USA), South America (Brazil, Colombia), Europe (Italy)

Symbol $ represents new host record for this haemosporidian lineage and symbol # represents new geographical record for this haemosporidian lineage (according to MalAvi database, Version 2.5.8, October 2023, Bensch et al. 2009).

4.3.2.3. Exotic birds from veterinary clinics

All 33 exotic birds sampled were negative to PCR.

4.3.3. Morphological analysis results

Among the 19 lineages discovered, only 14 have been assigned to a morphological species. The morphological species of the remaining five lineages were analysed, accompanied by photographs and with the distinctive characteristics of each species highlighted (Figures 6-10). The GAGLA05 lineage was identified as belonging to the species *Haemoproteus homopicae* (Figure 6), the LARFUS01 lineage was identified as *Haemoproteus jenniae* (Figure 7), the STRURA03 lineage as *Haemoproteus syrnii* (Figure 8), the CXNEA02 lineage as *Haemoproteus columbae* (Figure 9), and finally, the GenBank number ON950078 lineage, which does not yet have a name, was identified as belonging to the species *Haemoproteus contortus* (Figure 10).

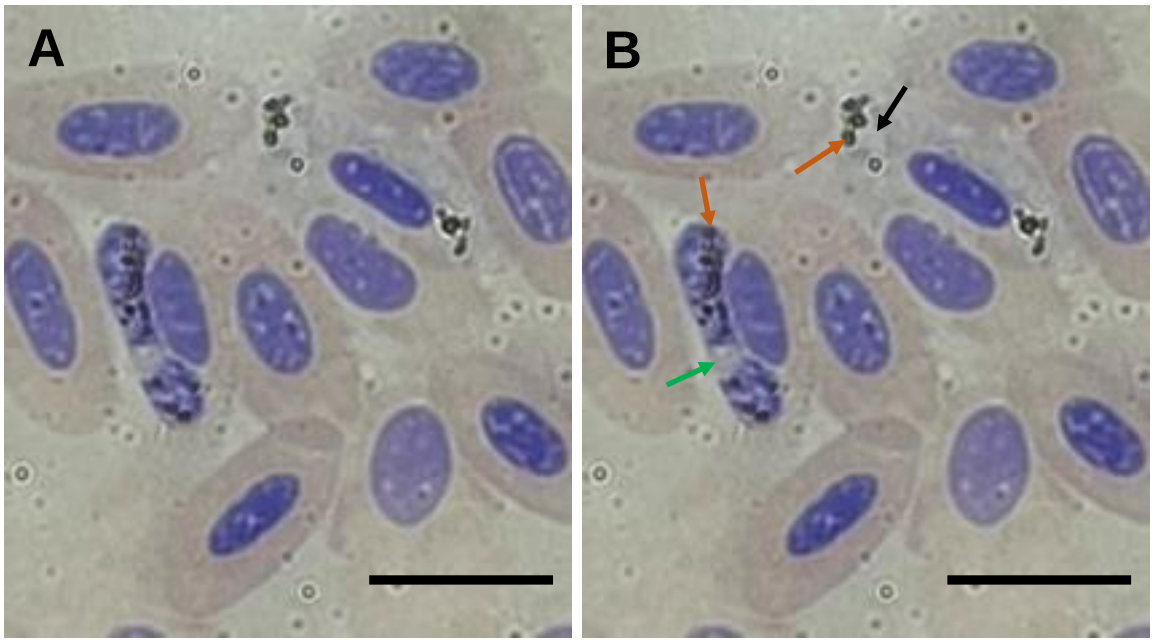


Figure 6. Macrogametocyte (left) and microgametocyte (right) of *Haemoproteus homopicae*.

(A) Original image. Scale bar: 10 µm. (B) Annotated version of (A), with arrows pointing to highlighted features. Black arrow: species-specific large vacuole (measuring more than 1µm in diameter), measuring 1.9 by 1.7 µm. Orange arrows: pigment granules. Green arrow: parasite nuclei. Scale bar: 10 µm. (Original).

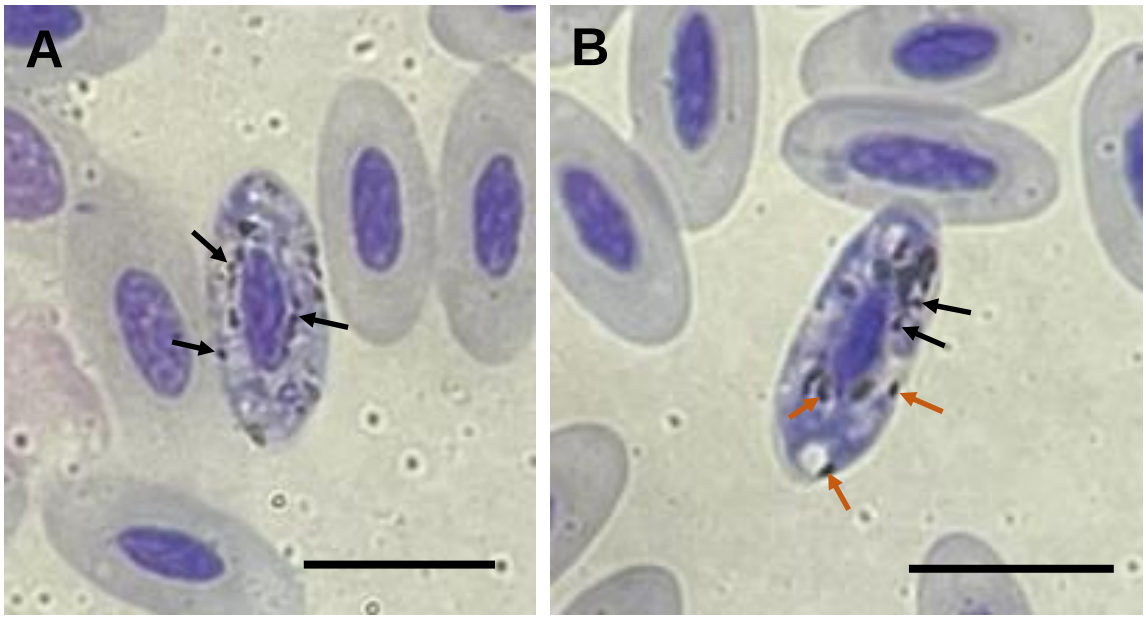


Figure 7. Macrogametocytes of *Haemoproteus jenniae* (A and B).

Circumnuclear macrogametocytes closely appressed to the nuclei of the infected erythrocytes, occupying all available cytoplasmic space. Pigment granules are predominantly round (black arrows) or oval (orange arrows), rather than the thin, rod-like granules, which is a characteristic feature of this species. (Original).

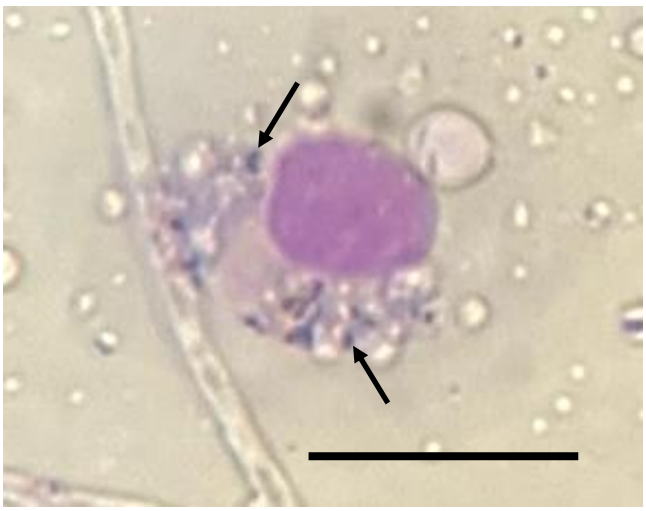


Figure 8. Microgametocyte of *Haemoproteus syrnii*.

Volutin arranged in granules (black arrows) distinguish this species. Bar: 10 µm. (Original).

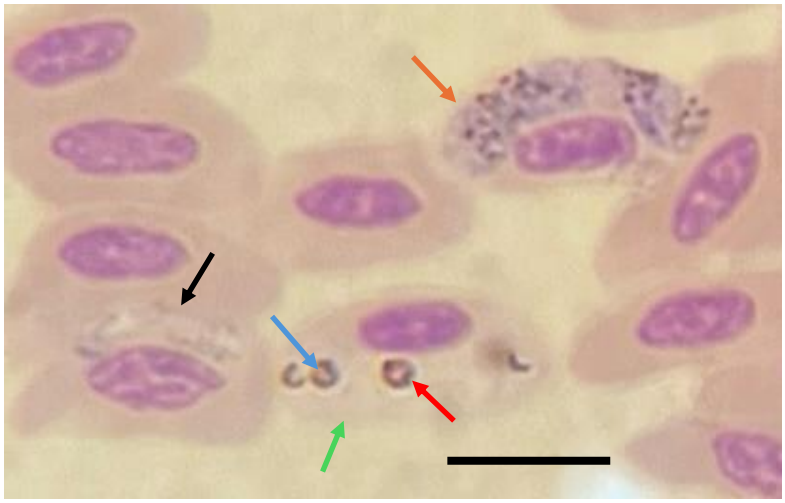


Figure 9. Gametocytes of *Haemoproteus columbae*.

Black arrow: Growing gametocyte. Green arrow: Fully grown microgametocyte. Orange Arrow: Fully grown macrogametocyte. The species characteristic large volutin granules are present in fully grown microgametocytes (red arrow) with the hemozoin granules present inside them (blue arrow). Bar: 10 µm. (Original).

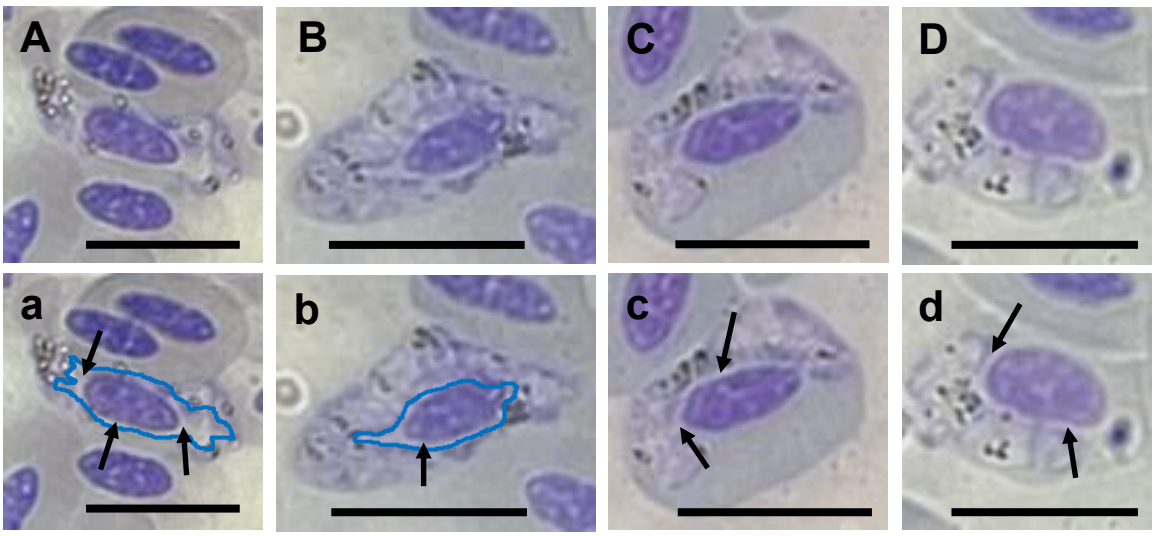


Figure 10. Macrogametocytes of *Haemoproteus contortus*.

(A, B, C, and D) Original images. (a, b, c, and d) Annotated versions of the original images, with highlighted features. Fully grown circumnuclear macrogametocytes do not occupy all available cytoplasmic space in the erythrocytes and exhibit a markedly irregular (wriggled) shape, as indicated by the blue highlights in (a) and (b). All types of fully-grown gametocytes do not completely adhere to the nuclei of infected erythrocytes (black arrows). These characteristics are specific to this parasite species. All scale bars measure 10 µm. (Original).

In some of the samples from the HAECOL1 lineage, several double gametocyte infections (DGI) were discovered, including male-female DGI (Figure 11).

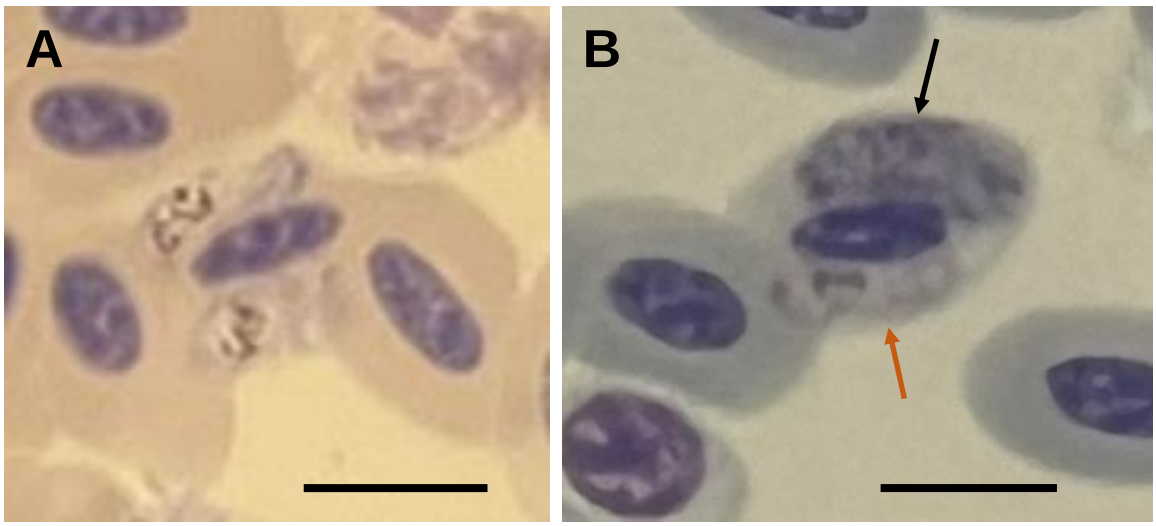


Figure 11. Double gametocyte infections (A and B) of *Haemoproteus columbae*.

(A) Double gametocyte infection with two macrogametocytes. (B) Double gametocyte infection with one macrogametocyte (black arrow) and one microgametocyte (orange arrow). (Original).

4.3.4. Factors Determining the Length of Medical Treatment

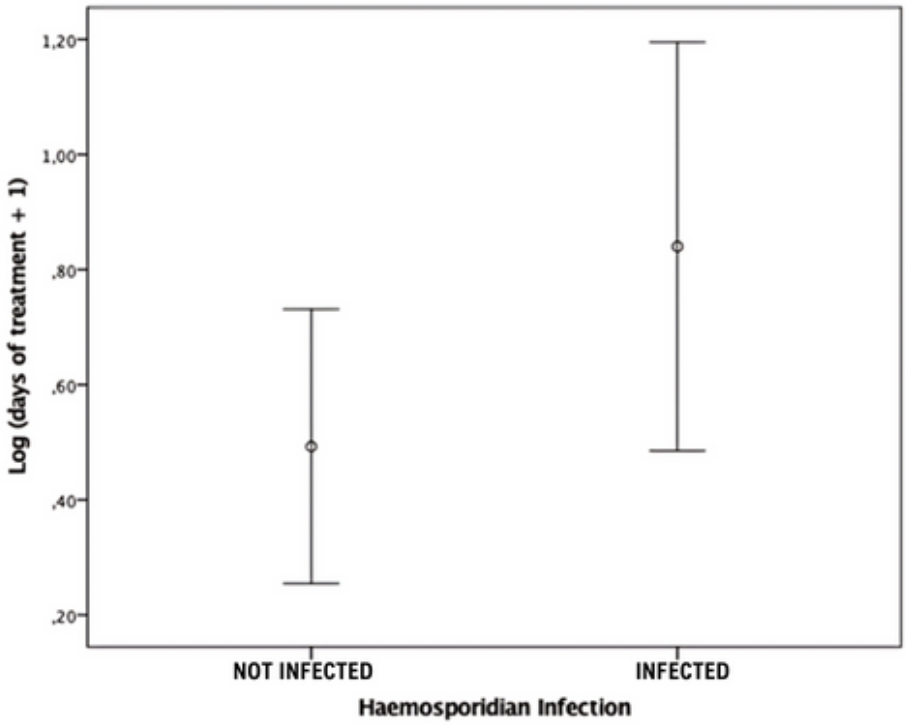
The length of medical treatment required by the birds admitted to the rehabilitation centres differed according to the haemosporidian infection and the season when they were admitted (Table 6). Specifically, haemosporidian-infected birds significantly required longer treatments than uninfected individuals (mean days of treatment (SD): infected = 28.22 (56.89); uninfected = 9.85 (16.81)) (Graphic 9). Also, birds admitted to rehabilitation centres during winter (n = 28) received medical care for longer periods than birds admitted during spring (n = 25) or autumn (n = 3) (mean days of treatment (SD): winter = 30.43 (51.55); spring = 4.24 (11.98); autumn = 6.50 (11.67)) (Graphic 10).

Table 6. Factors explaining variation in the number of days those wild birds admitted to the rehabilitation centre required treatment.

Variable	type III SS	d.f.	F	*p*
Body condition	0.017	1	0.038	0.846
Haemosporidian infection	2.172	1	4.747	**0.034**
Rehabilitation centre	0.010	1	0.021	0.884
Season	6.112	1	13.358	**0.001**

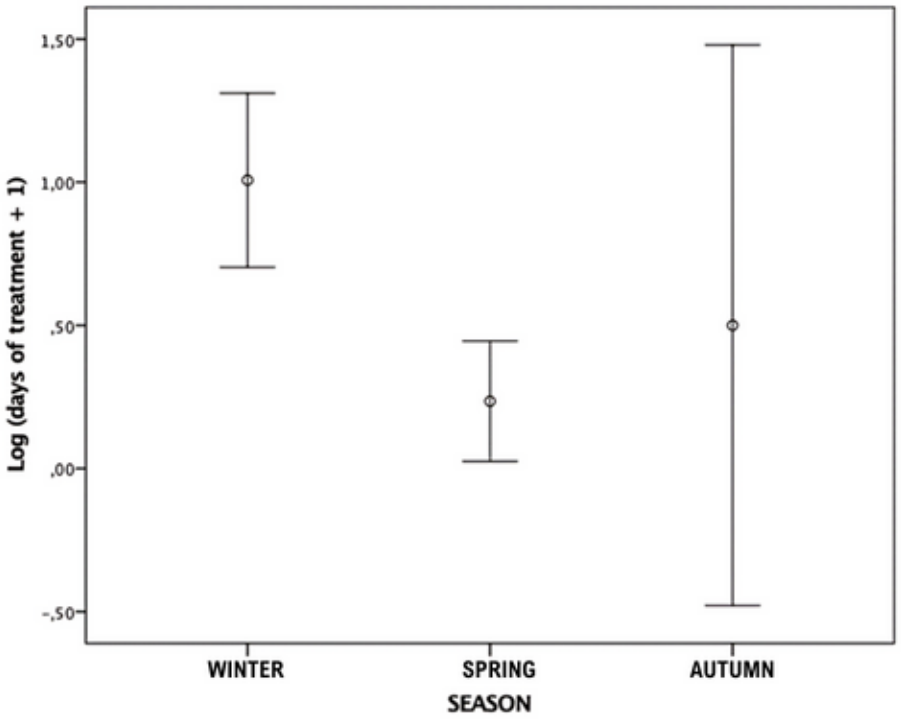
A General lineal model was used with body condition, haemosporidian infection, rehabilitation centre (locality), and the season of admission to rehabilitation centres as predictor variables. Sample size was 56 individuals. Type III SS (Sum of Squares), d.f. (degrees of freedom), F (F-statistic), and p (p-value). Significant factors are highlighted in bold.

Graphic 9. Error bar plots (mean ± 95% CI) showing the number of days (log-transformed) that haemosporidian-infected and uninfected birds admitted to rehabilitation centre required medical treatment.



The infected birds represented a total of 23 individuals and the uninfected were 33.

Graphic 10. Error bar plots (mean ± 95% CI) showing the number of days (log-transformed) requiring medical treatment for birds admitted to rehabilitation centre with respect to the season when they were admitted.



The sampled birds were admitted in the winter (n = 28), spring (n = 25), and autumn (n = 3).

4.3.5. Factors Determining Haemosporidian Infection

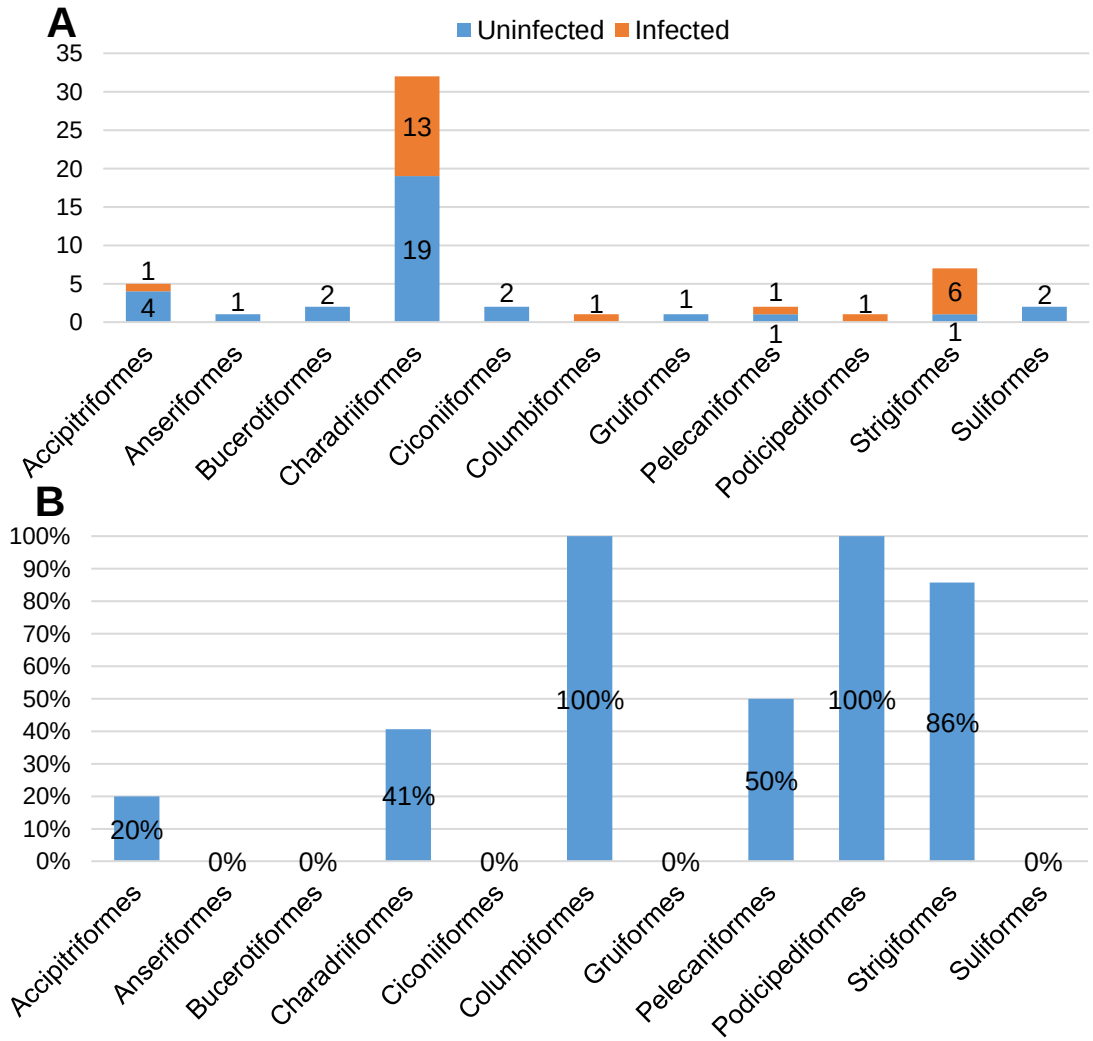
The haemosporidian infection was also analysed in relation to body condition, avian taxonomic order, the rehabilitation centre where birds were admitted, the season of admission to rehabilitation centres, and the reasons for admission to rehabilitation centres. Avian taxonomic order significantly explained variation in haemosporidian infection (Table 7). Notably, six out of seven (85.7%) of Strigiformes showed haemosporidian infection. Also, 40.6% of Charadriiformes were infected with haemosporidian parasites, whereas only 20% of birds belonging to the order Accipitriformes showed haemosporidian infection (Graphic 11). Remarkably, the order Strigiformes was also the only order having double infections (*Leucocytozoon* and *Haemoproteus*) (Table 3). In addition, the prevalence of haemosporidian infection also varied with the reasons for admission to rehabilitation centres (Table 7). Birds admitted due to debilitating diseases had a significantly higher prevalence of haemosporidian infection (52.2%, n = 23) compared to cases of admission due to physical trauma (29.0%, n = 31), with the remaining two individuals admitted for undetermined reasons (Graphic 12).

Table 7. Factors explaining variation in the probability of haemosporidian infection.

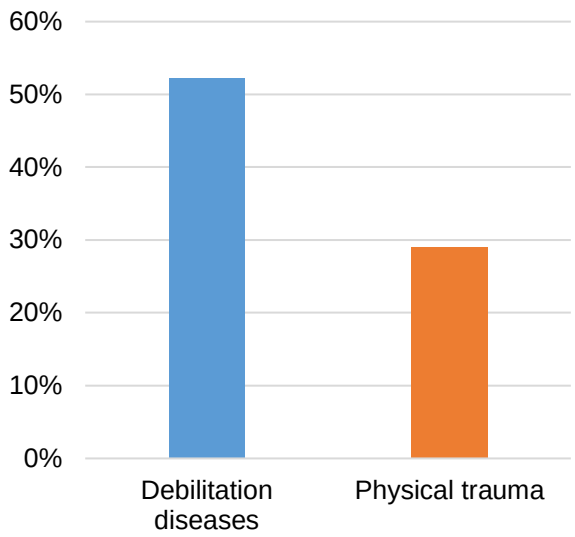
Variable	*B*	S. E.	Wald	d.f.	Exp (B)	*p*
Avian taxonomic order	0.180	0.091	0.943	1	1.197	**0.047**
Reason for admission	-1.256	0.524	5.741	1	0.285	**0.017**
Constant	0.442	0.868	0.259	1	1.555	0.611

A backward stepwise procedure was used in a logistic regression analysis with avian taxonomic order, body condition, rehabilitation centre (locality), the season of admission to rehabilitation centres, and the reasons for admission to rehabilitation centres as predictor variables. Only independent variables selected by the stepwise procedure are listed. Type III SS (Sum of Squares), d.f. (degrees of freedom), F (F-statistic), and p (p-value). Significant factors are highlighted in bold.

Graphic 11. Number of infected and uninfected birds by order (A) and prevalence of infected birds by order (B) (n=56).



Graphic 12. Prevalence of Infection by Reason for Admission excluding undetermined reasons (n=54).



4.4. Discussion

Over a third of the approximately 500 bird species living in Europe are threatened or have a poor conservation status (BirdLife International 2021). In mainland Portugal, more than 30% of bird populations have been recently categorised as threatened with extinctions (Almeida et al. 2022). Hence, preserving and restoring avian populations is one of the cornerstones of EU biodiversity policy. Wildlife rehabilitation is an undervalued and potentially useful tool for stabilising some declining populations and could be targeted to support in situ interventions (Paterson et al. 2021). In this line, rehabilitation centres and other captive breeding facilities play an essential role in species conservation and preservation. Here, the prevalence and genetic diversity of avian haemosporidians in birds admitted to rehabilitation centres in Portugal were analysed to explore the factors explaining variation in haemosporidian infection and to evaluate features influencing the length of required treatment. The haemosporidian prevalence and genetic diversity were also studied in exotic birds and pigeons of Lisbon. The main findings of this study were: (i) approximately 40% of birds sampled upon admission to the wildlife rehabilitation centres were infected with haemosporidians, exhibiting a great genetic diversity among haemosporidian lineages, including two novel lineages, numerous new host–parasite associations, and new lineage geographic reports; (ii) birds infected with haemosporidians required longer medical treatments during their stay in the rehabilitation centres; (iii) the prevalence of avian haemosporidian infection was higher in Strigiformes and in those admitted to rehabilitation centres due to debilitating diseases; (iv) all sampled pigeons from the city of Lisbon were infected with *Haemoproteus columbae*, with new host–parasite interactions and lineage geographic

reports identified, and no other haemosporidian parasites detected; (v) none of the exotic birds sampled at the veterinary clinics in Lisbon showed haemosporidian infection despite the diverse sample used. Next, these results will be discussed in detail.

4.4.1. Prevalence and Genetic Diversity of Haemosporidian Parasites

A haemosporidian prevalence of 41% was observed in wild birds sampled at wildlife rehabilitation centers upon admission or before the end of the parasites' prepatent period. This probability of infection is similar to those found in recent studies using molecular methodologies to analyse haemosporidian infections in birds admitted to rehabilitation centres. For example, Nourani et al. (2022) examined the infection with haemosporidian parasites in captive raptors in two rehabilitation facilities in North and Northeast Iran, determining an overall prevalence of 36%. Likewise, Pornpanom et al. (2019) reported a haemosporidian prevalence of 34% in 12 owl species in a raptor rehabilitation unit in Thailand. Additionally, Gomes et al. (2023) explored the occurrence of blood parasites in wild birds from a wildlife rehabilitation centre in Central Portugal, revealing that 48% of sampled birds were positive for haemosporidians.

Other studies conducted at Wildlife Rehabilitation Centres in Portugal, such as those by Tomás (2014) and Zacarias (2017), have also investigated haemosporidian prevalence. These studies, particularly at the Wildlife Rehabilitation Centre RIAS, indicate an increasing trend in prevalence over the years: 13% in 2014 (Tomás 2014), 22.6% in 2017 (Zacarias 2017), and 45.7% in the present study, further supporting this upward trend. This rise in prevalence, observed in the south of Portugal, coincides with the increase in mean temperatures and the intensification of extremely high temperatures that Portugal is experiencing (Cardoso et al. 2019). The positive correlation between avian malaria and rising temperatures associated with global climate change (Garamszegi 2011) suggests that climate change could be a significant influencing factor. As temperatures continue to rise, this trend may become increasingly evident and problematic in the future.

Wildlife rehabilitation likely generates millions of animal records annually worldwide (Pyke and Szabo 2018). Yet, despite an increasing acknowledgement of the usefulness of wildlife rehabilitation centre data, it remains a prevailing tendency to underutilise this valuable source of information (Kwok et al. 2021). Moreover, rehabilitation centres provide an excellent opportunity to explore the host range, genetic diversity, and geographic distribution of haemosporidian infections in wild birds which are rarely sampled in nature (Stauber 2002; Ilgūnas et al. 2022). For example, Gomes et al. (2023) have recently described the first occurrence of *Leucocytozoon* sp. in the booted eagle *Hieraaetus pennatus*, the short-toed snake eagle *Circaetus gallicus*, and

the European honey buzzard *Pernis apivorus*, and *P. relictum* in the European honey buzzard. Also, Pornpanom et al (2019) reported 17 new lineages of haemosporidian parasites in owls from Southern Asia. Here, were detected 15 haemosporidian lineages infecting a third of bird species sampled. Importantly, the sequences found in this study were compared with those in the MalAvi database (Version 2.5.8, October 2023, Bensch et al. 2009) and it was discovered that two of these 15 haemosporidian lineages detected had not been previously obtained in other studies. Moreover, of these 15 identified haemosporidian lineages, only five (ATNO1, CIAE02, STAL2, GAGLA05, and LINN1) had previously been reported in Portugal, with STRURA03, COCOR02, and STAL3 being also first documented in the Iberian Peninsula, and STAL5 representing the first report outside of Turkey. Furthermore, 30% of the infected bird species had not been found to harbour malaria parasites in preceding studies. Such numbers of newly discovered lineages and the new records of bird hosts infected with blood parasites suggest that the diversity of avian haemosporidians infecting some species of Strigiformes, Charadriiformes, Columbiformes, Pelecaniformes, and Accipitriformes has been insufficiently investigated.

In addition, our analyses also revealed nine new bird–parasite interactions, thus identifying new host records for these haemosporidian parasites. These new bird–haemosporidian associations are made up of the two newly described haemosporidian lineages (ARCIN01 and LARFUS01), plus seven parasite lineages previously identified as infecting alternative hosts.

Parasite mitochondrial DNA evolves 6-10 times slower than the parasite's nuclear genes (Nilsson et al. 2016), with one mutation within the MalAvi fragment being estimated to occur every 154,000 to 2 million years (Bensch and Hellgren 2020). This highlights the significance of identifying new lineages, even those differing by only one base pair, such as the newly described lineage LARFUS01 in the present work. The discovery of the new lineage ARCIN01 is particularly significant due to its substantial genetic divergence from other known lineages. ARCIN01 differs by 21 base pairs from its closest match, an unnamed lineage found in the invertebrate host *Simulium meridionale* (GenBank: OL897554.1), indicating a divergence of 3.2 to 42 million years. It also differs by 41 base pairs from its closest named lineage, AFR251 (a lineage of *Merops apiaster*), suggesting a genetic divergence of up to 82 million years from it. This discovery considerably contributes to our understanding of the genetic diversity of haemosporidian parasites.

Additionally, parasites sharing the same cytb lineage could consist of populations that have been isolated for up to 2 million years. Despite sharing the same mitochondrial

lineage, that populations may also exhibit a noteworthy divergence in their nuclear genomes due to a higher rate of nuclear gene mutations (Bensch and Hellgren 2020).

Some other new host–parasite associations are worthy of being underlined. First, a *Leucocytozoon* parasite (GenBank acc. Number OL897562) that was found infecting *Bubo bubo,* was previously identified in pooled samples of the blackfly *Simulium meridionale* captured throughout Mississippi, USA (Ber et al. 2022). Second, *Streptopelia decaocto*, an avian host that had not been reported as infected by haemosporidians in previous studies, was found infected by *Leucocytozoon* ATNO1, a parasite lineage that had been previously recorded exclusively infecting the little owl (*Athene noctua*) (MalAvi database Version 2.5.8, October 2023, Bensch et al. 2009). Finally, the other detected haemosporidian lineages had been identified in related hosts in previous studies. For example, the *Haemoproteus* lineage (GenBank acc. Number ON950078) infecting *Larus fuscus* and *Larus michahellis* was recently described in other larid hosts (Włodarczyk et al. 2022), the *Leucocytozoon* lineage MILVUS02 is commonly found in other Accipitriformes (Pérez-Rodríguez et al. 2013; Harl et al. 2022), and *Plasmodium matutinum* LINN1 is a generalist haemosporidian lineage found infecting species of a wide range of avian orders, including Charadriiformes (MalAvi database Version 2.5.8, October 2023, Bensch et al. 2009). The new diversity records on host–parasite interactions provided in this study will be valuable for detecting host range and transmission areas of haemosporidian parasites and will improve our knowledge of the mechanisms of adaptation of avian haemosporidians to new hosts.

The high prevalence of *Haemoproteus* and the absence of *Leucocytozoon* and *Plasmodium* in the study population of feral pigeons in Lisbon aligns with the findings of Chagas et al. (2016) in São Paulo, Brazil, and Nebel et al. (2020) in Cape Town, South Africa. *Leucocytozoon* and *Plasmodium* use different dipteran vectors than *Haemoproteus*, with *Leucocytozoon* mainly transmitted by blackflies and *Plasmodium* by mosquitoes (Valkiūnas 2005) (See Chapter 3.2 for more details). Blackflies require fast-flowing streams (Rivers-Moore et al. 2007), which might be absent in highly urbanized areas, potentially explaining the absence of *Leucocytozoon* in this case (Nebel et al. 2020). In contrast, the absence of vectors is unlikely to explain the absence of *Plasmodium* in feral pigeons. *Plasmodium* infections have been rarely reported in wild feral pigeons, and none of these records were confirmed by molecular screening methods (Nebel et al. 2020). The similarity between *Plasmodium* and *Haemoproteus* during certain developmental stages and the variability in blood slide quality (Valkiūnas 2005) support the hypothesis that feral pigeons may not be native hosts for *Plasmodium* parasites (Nebel et al. 2020). This hypothesis, noted as the reason for the absence of

Plasmodium in the study by Nebel et al. (2020), could also explain its absence in the present study.

High prevalence values of *Haemoproteus* in pigeons have also been found in various cities, such as 96.9% in Cape Town, South Africa (Nebel et al. 2020); 97–100% in Madrid, Barcelona, and Granollers, Spain (Sol et al. 2000; Vázquez et al. 2010); 100% in São Paulo, Brazil (Chagas et al. 2016); and 100% in Hyattsville, Maryland, USA (Knisley and Herman 1967).

The high prevalence and intensity of *Haemoproteus* infections in pigeons are not surprising, as pigeons live and roost in flocks, which facilitates easy parasite transmission (Johnston and Janiga 1995). This high infection rate is likely influenced by population density, which is typically high in suburban and urban areas (Chagas et al. 2016; Nebel et al. 2020). Additionally, the common hippoboscid fly *Pseudolynchia canariensis*, which transmits *Haemoproteus columbae* to feral pigeons (Valkiūnas 2005), contributes to the high prevalence of this parasite in densely populated pigeon communities.

Female gametes are very small relative to the bloodmeal volume ingested by vectors, and male gametes lack a mechanism to locate female gametes (Gaillarg et al. 2003). To address this, hypotheses suggest mechanisms that may have evolved to increase gametocyte proximity in the vertebrate host and consequently the proximity of gametes within the bloodmeal. Jovani (2002) proposed that male–female double gametocyte infections (DGIs), where a single blood cell is infected by both a microgametocyte and a macrogametocyte, might facilitate quicker encounters between gametes, enhancing parasite transmission. The "DGI hypothesis" assumes that DGIs, especially male-female DGIs, are common in nature, although this remains unconfirmed (Jovani 2002). Jovani et al. (2004) supported this hypothesis, finding widespread DGIs across various host-parasite relationships, including *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* in birds, and *Haemoproteus* and *Hepatozoon* in reptiles.

In the present study, the presence of DGIs in feral pigeons, including male-female DGIs, might explain the 100% prevalence of *Haemoproteus columbae*. This finding supports the role of DGIs in contributing to high infection rates, as proposed by Jovani (2002).

Despite the high prevalence of infections and the commonality of pigeons, the discovery that all identified lineages were new geographic records in Portugal, with three out of four (COLIV03, COQUI05, and CXNEA02) also being new in Europe, underscores the lack of research on this species, particularly in Europe. This knowledge gap is further highlighted by the first-time identification of the lineage CXNEA02 outside its invertebrate

host (*Culex neavei*) and Cameroon. These findings demonstrate that this species is largely overlooked by researchers.

The absence of haemosporidians in the blood of the 33 exotic birds sampled might be explained by the hypothesis that exotic species acquiring non-native haemosporidians only develop up to the exoerythrocytic meront stage without forming gametocytes (Valkiūnas 2005). Consequently, these infections without parasitaemia are not detected by diagnostic techniques that rely on identifying gametocytes in the blood.

4.4.2. Morphological analysis highlights

Microscopy is a fast and cheap methodology that allows the quantification of parasitemias and to detect mixed infections, which are sometimes difficult to find with molecular screening. Furthermore, although low-intensity avian malaria infections could be difficult to detect solely by microscopic examination of blood smears, the combined use of PCR and traditional microscopy could be especially relevant to connect genetic lineages with morphospecies and describe new species of haemosporidians (Valkiūnas and Atkinson 2020). To the best of the author's knowledge, this study is the first in Portugal to simultaneously employ both PCR and microscopy methods, representing a unique contribution to haemosporidian research in the country. This approach proved effective as it allowed for the identification of molecular false negatives, prompting the repetition of PCR tests, and the identification of several microscopy false negatives. It was also possible to identify DGIs in the samples of feral pigeons, helping to explain their prevalence and further supporting the "DGI hypothesis." Moreover, it was possible to identify the morphological species of five *Haemoproteus* lineages for the first time. One of these, the lineage with the GenBank number ON950078, which does not yet have a name, was identified as belonging to the species *Haemoproteus contortus*, representing a new morphology-molecular correlation for the species *Haemoproteus contortus*, according to the review by Valkiūnas and Ietzhova (2022).

4.4.3. Factors Determining the Length of Medical Treatment

The process of wildlife rescue and rehabilitation encompasses the rescue, treatment, and care of injured, sick, or orphaned native animals, with the goal of their release into their natural habitat or a more suitable environment (Miller 2012). However, a prolonged period in captivity can result in loss of survival skills in wildlife (Victoria 2001). In this line, Cope et al. (2022) have recently conducted a global systematic review and meta-analysis evaluating the factors influencing the success of wildlife rehabilitation, concluding that shorter periods of rehabilitation enhance the probabilities of survival of released animals after the treatments. Wild birds admitted to rehabilitation centres worldwide are frequently parasitized by haemosporidians (Ciloglu et al. 2016; Gomes et

al. 2023; Marzal et al. 2024), although whether haemosporidian infection may extend the rehabilitation period in wild birds remains largely unknown. The findings revealed that birds infected with haemosporidian required longer periods of medical treatment than non-infected birds, which may affect survival up to release or survival post-release (Cope et al. 2022). Therefore, the initial diagnosis of haemosporidian infections in wild birds admitted to rehabilitation centres becomes crucial for an early assignation of a correct anti-malaria treatment that could minimise their length of stay in the centre and thus enhance their survival prospects.

Rehabilitation centres are often self-funded or heavily subsidising their own rehabilitation work (Englefield et al. 2019; Paterson et al. 2021), hence facing constraints due to insufficient funding, staff availability, and access to appropriate veterinary care (Cerda and Webb 2023). Beyond the mentioned increased fitness benefits to the wildlife of shorter rehabilitation periods, a timely assessment and treatment of haemosporidian infection can also have an economic impact on the rehabilitation centre, as it may promote the effective use of limited resources. In this line, the length of medical treatment has been proposed as an indicator of resource usage (Kroch et al. 2007). The daily cost per animal in a wildlife rehabilitation centre has been estimated at EUR 0.19 (Molina-López et al. 2017). According to the mean values of the length of stay of haemosporidian infected and non-infected birds from the present study (28.22 days and 9.85 days, respectively), the average expenses of rehabilitation of a non-infected bird can be estimated at approximately EUR 1.9, whereas these costs rise to EUR 5.4 for an infected bird, representing an additional cost of EUR 3.5 per animal.

The results also show a significant increase in the duration of medical treatment of the birds admitted to rehabilitation centres during the winter months. This finding could be attributed to the reluctance to release rescued birds into the wild during winter, when seasonal environmental conditions are less favourable (Willette et al. 2023), consequently prolonging their stay in the centres. Alternatively, because metabolic disorders are associated with high recovery times in wild birds (Molina-López et al. 2017), the extended period of rehabilitation during winter can also be explained by the higher metabolic costs associated with thermogenesis in winter (Swanson 1991; Arens and Cooper 2005). The large variation in days of treatment shown in Graphic 10 for autumn can be explained because birds admitted to rehabilitation during that season are released either quickly, before winter, or held over winter (Willette et al. 2023). However, these data should be interpreted with caution because of the low number of sampled birds admitted to rehabilitation centres during that season (n = 3).

4.4.4. Factors Determining Haemosporidian Infection

The present study showed differences in the prevalence of infections among avian orders in birds brought to wildlife centres in Portugal, with birds from the order Strigiformes exhibiting the highest probabilities of being infected, followed by the order Charadriiformes. Similarly, Santos et al (2008) and Tomás (2014) reported significant variation in infection rates between taxonomic orders, with Strigiformes showing the highest prevalence, further supporting the results of the present study.

Several factors have been proposed to explain why some bird species are prone to becoming infected with haemosporidians. For example, colonial bird species (such as most species of Charadriiformes), or those with larger body sizes or prolonged stays of their nestlings on the nests, usually show a high prevalence of infections (Valkiūnas 2005). Also, these differences in haemosporidian prevalence have been suggested to be determined by vector preferences (Pulgarín-R et al. 2017) or host behaviour characteristics (Beadell et al. 2004; Pulgarín-R et al. 2017; Eastwood et al. 2019). For example, the higher prevalence of haemosporidians in owls compared to diurnal birds of prey has been well documented (Krone et al. 2001; Santos et al. 2008; Baptista et al. 2010; Gao, et al. 2021), and it has been attributed to two primary factors. First, Strigiformes have nocturnal behaviour, which coincides with the crepuscular or nocturnal hours in which mosquito vector species perform host-seeking behaviour (Santos et al. 2008; Marzal et a. 2022). Second, their preference for concealed and shaded perches during the day, as well as their nesting sites, may expose Strigiformes more frequently to a variety of haemosporidian vector species (Forrester et al. 1994).

Finally, it was shown that the prevalence of infection was higher in birds admitted to the rehabilitation centres due to debilitating diseases than in birds admitted for other causes. Several experimental studies have demonstrated that haemosporidian parasites may impair the physiology of their avian hosts, provoking anaemia (Palinauskas et al. 2015), the blockage of brain capillaries (Ilgūnas et al. 2016), a diminished body condition, a decrease in fat reserves and atrophy of pectoral muscles (Carlson et al. 2016), and reduced haematocrit (Coon et al. 2016). All these negative effects may explain the observed association between infection and debilitating disease in birds. Alternatively, a poor body condition, inadequate nutritional status, or heightened stress levels in birds may compromise their immune system (Crommenacker et al. 2011; Cornet et al. 2014), and thus increase the likelihood of haemosporidian infection of these debilitated birds (Valkiūnas 2005).

4.5. Conclusion

This study assessed the prevalence and genetic diversity of haemosporidian parasites in birds of mainland Portugal, also analysing their effect on the required rehabilitation period and the factors explaining their infection. This study revealed newly discovered parasite lineages and new records of bird hosts infected with blood parasites, thus confirming that the diversity of avian haemosporidians is still insufficiently investigated in some avian species. This includes both species threatened with extinction in Portugal, such as the *Accipiter gentilis*, and very common species like feral pigeons (*Columba livia domestica*). The new geographical records also highlight that wild birds in Portugal and Europe are still insufficiently studied.

In addition, these findings are also relevant because host-switching of blood parasites is relatively frequent among birds housed in zoos and rehabilitation centres, provoking fatal infections (Bueno et al. 2010; García-Del-Río et al. 2021). It was also identified that Strigiformes and birds admitted to rehabilitation due to debilitating disease showed the highest probabilities of being infected with haemosporidians, highlighting the reciprocal relationship between debilitating state and blood parasite infection. Moreover, this study sheds light on the largely unknown impact of avian haemosporidian infections on the length of stay in rehabilitation centres. Haemosporidian-infected individuals required nearly three times more days in veterinary care compared to non-infected counterparts, impairing their survival prospects and exacerbating resource constraints in wildlife rehabilitation centres. These findings emphasise the need for integrating analyses of haemosporidian infection into diagnostic and treatment protocols, also highlighting the importance of blood sampling on the same day of admittance to the rehabilitation centre. Moreover, the seasonal variations observed in the length of veterinary treatment needed, particularly during winter months, stress the importance of adaptive management strategies that account for seasonal fluctuations in rehabilitation demands.

The pioneering approach in Portugal that simultaneously uses both PCR and microscopy methods has proven highly advantageous. It allowed for the identification of molecular and microscopy false negatives, enabled the correlation of known and new parasite lineages to their morphological species, and enabled the discovery of the molecular marker for *Haemoproteus contortus*, filling an important knowledge gap. These findings represent significant contributions to haemosporidian research beyond the national level.

Furthermore, the absence of haemosporidians in the blood of the 33 exotic birds sampled may be attributed to the hypothesis that exotic species acquiring non-native haemosporidians may only develop up to the exoerythrocytic meront stage without

forming detectable gametocytes (Valkiūnas 2005). This suggests that such infections, lacking detectable parasitemia, might remain undetected even when employing both PCR on blood samples and microscopy methods, as both rely on identifying gametocytes in the blood.

In light of the results of this study, some additional recommendations are indicated for further studies exploring factors influencing haemosporidian infection, such as larger sample sizes and incorporating data from summer infections. Also, repeating blood sampling of individuals beyond the day of admittance to the rehabilitation centre would allow for assessment of the effectiveness of treatment and monitoring of whether birds are infected during their stay in the centre. Because the study of blood parasites is also relevant to controlling parasite infections in birds before translocation or liberation (Merino et al. 2002), the insights gained from this study have significant implications for avian conservation and wildlife rehabilitation efforts. By highlighting the challenges posed by haemosporidian infections in avian conservation and rehabilitation, this study emphasises the importance of future research endeavours aimed at enhancing our understanding of avian health and guiding conservation strategies and the need to integrate analyses of haemosporidian infection into diagnostic and treatment protocols.

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Appendix I. Article co-authored published in the Revista Lusófona de Ciência e Medicina Veterinária

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ÁCAROS TROMBICULÍDEOS: REVISÃO DE UMA PARASITOSE NEGLIGENCIADA EM ANIMAIS DE COMPANHIA

TROMBICULID MITES: REVIEW OF A NEGLETED PARASITOSIS IN COMPANION ANIMALS

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Resumo: *A trombiculose é uma parasitose causada por ácaros trombiculídeos (Acari: Trombiculidae) que afeta vertebrados, incluindo cães, gatos e o ser humano. É a fase larvar dos ácaros trombiculídeos que se caracteriza por ser parasita obrigatório e responsável pelo aparecimento de sinais clínicos. Estes incluem prurido, várias lesões cutâneas, podendo, em alguns casos, surgir distúrbios gastrointestinais, neurológicos ou mesmo a morte do animal quando as infestações são massivas. Apesar da sua distribuição mundial, incluindo em Portugal, esta é uma parasitose negligenciada, sobretudo no contexto da medicina veterinária de animais de companhia. Os estudos existentes são escassos, contribuindo para o seu desconhecimento, e a sua verdadeira prevalência nestes animais é pouco conhecida a nível mundial. Apesar do seu papel enquanto vetores de agentes patogénicos não ser claro, alguns estudos já demonstraram a presença de ADN de Anaplasma spp., Bartonella spp., Borrelia spp. e Rickettsia spp., em trombiculídeos. O facto de os ácaros trombiculídeos afetarem várias espécies animais, incluindo os animais de companhia e o ser humano, bem como o seu potencial papel no ciclo de manutenção de vários agentes infeciosos, revela a importância de um maior conhecimento acerca desta parasitose, tanto ao nível da comunidade científica como da comunidade médico veterinária. Assim, esta revisão pretende reunir os dados disponíveis à data quanto à epidemiologia, características clínicas e tratamento desta parasitose, com especial enfoque em Portugal.*

Palavras-chave*: Trombiculidae, ácaro, gato, cão, zoonose*

Abstract: *Trombiculosis is a parasitic disease caused by chigger mites (Acari: Trombiculidae) that affect vertebrate hosts, including dogs, cats and even humans. It is the larval stage of the chigger mites that are obligate parasites and are responsible for the development of clinical signs. These include pruritus, several cutaneous lesions and, in some cases, gastrointestinal and neurological disturbances. When a massive infestation is present, it may even lead to the death of the animal. Despite its worldwide distribution, including in Portugal, this is a neglected parasitosis, especially in the context of veterinary medicine of dogs and cats. The existing studies are scarce, contributing to the lack of awareness about this subject, and its true prevalence in companion animals is still poorly known. Although the role of chigger mites as vectors of infectious disease is unclear, some studies have reported the presence of DNA from several pathogens in mite samples, such as Anaplasma spp., Bartonella spp., Borrelia spp. and Rickettsia spp. The fact that chigger mites affect several vertebrate animal species, including companion animals and humans, as well as their potential role in the maintenance cycle of various infectious agents, reveals the importance of a greater understanding of this parasitosis both by the scientific community and by the veterinary medical community. Thus, this review intends to gather the available data regarding the epidemiology, clinical presentation, and treatment of this parasitosis, with a special focus in Portugal.*

Keywords*: Trombiculidae, mite, cat, dog, zoonosis*

1. TROMBICULOSE: ETIOLOGIA, CICLO DE VIDA E HABITAT

A trombiculose é uma doença cutânea de origem parasitária causada pela fase larvar de ácaros trombiculídeos (Acari: Trombiculidae). Na literatura internacional, esta doença assume diversos nomes, incluindo *grass itch mites*, *chigger mites*, *scrub itch* e *harvest mites* (Takahashi *et al.*, 2004)

Os ácaros trombiculídeos estão presentes em todo o mundo, tendo um vasto leque de hospedeiros vertebrados (Santibáñez *et al*., 2015). Estão descritas cerca de 3000 espécies de ácaros trombiculídeos que podem parasitar animais vertebrados e invertebrados (Kaya & Yilmaz, 2019). É a fase larvar do parasita que afeta todos os grupos de vertebrados, com exceção de peixes, sendo que os pequenos mamíferos (*i.e.,* roedores) e as aves são os seus principais hospedeiros (Santibáñez *et al*, 2015; Weitzel *et al*., 2020).

As ninfas e os adultos têm vida livre (Takahashi *et al.*, 2004). Os adultos reproduzem-se no solo e as larvas eclodem 10 dias após a oviposição (Takahashi *et al.*, 2004). Durante a fase larvar, os ácaros trombiculídeos encontram-se em terrenos desmatados, arbustos, solos com temperaturas quentes e humidade alta (Santibáñez *et al*., 2015; Kaya & Yilmaz, 2019). Estes ácaros também podem ser encontrados em jardins, parques, relva e áreas húmidas próximas a águas superficiais e lagos (Santibáñez *et al*, 2015). As larvas possuem cerca de 250 µm de

comprimento, uma cor alaranjada ou vermelho brilhante característica e três pares de patas (Kaya & Yilmaz, 2019).

As larvas dirigem-se para locais elevados da vegetação rasteira, incluindo a terminação dos talos da relva ou ramos secos de árvores, formando aglomerados, com o intuito de se agarrarem a um hospedeiro que passe perto destes locais, à semelhança do que acontece com as carraças (Heyne *et al*., 2001; Stekolnikov *et al*., 2014).

Uma vez no hospedeiro, as larvas alimentam-se durante 2 a 10 dias (exceção para *Straelensia cynotis,* que se alimenta durante 3 meses) (European Scientific Counsel Companion Animal Parasites [ESCCAP], 2018). Durante a refeição, os ácaros inoculam enzimas líticas nas camadas superiores da pele para conseguirem ingerir as células do tecido liquefeito, as secreções epiteliais e/ou sangue (ESCCAP, 2018; Kaya & Yilmaz, 2019). Ao fim daquele tempo, as larvas caem ao solo e passam pelas restantes fases do ciclo de vida até chegar à fase adulta, vivendo de forma livre (ESCCAP, 2018). O ciclo de vida fica completo em cerca de 50 a 70 dias e as fêmeas conseguem sobreviver por mais de um ano mesmo em condições ambientais adversas (ESCCAP, 2018).

2. TROMBICULOSE ENQUANTO ZOONOSE

A trombiculose não se configura como uma zoonose clássica, uma vez que o ser humano é habitualmente infestado diretamente do ambiente. Contudo, os animais domésticos contribuem para a manutenção do ciclo de vida destes ácaros, e a transmissão direta de *Neotrombicula autumnalis* entre animais e o ser humano, embora rara, já foi reportada (Guarneri *et al*., 2005; Parcell *et al*., 2013). Adicionalmente, os animais domésticos, como cães e gatos, devem ser considerados como potenciais transportadores destes ácaros para locais de maior proximidade com o ser humano.

Mais de 50 espécies de ácaros trombiculídeos que infestam o ser humano foram identificadas, incluindo *Blanciella toldti*, *Blankaartia acuscutellaris*, *Euschoengastia xerothermobia, Kepkatrombicula desaleri* (Ripka & Stekolnikov, 2006; Shatrov & Stekolnikov, 2011; Stekolnikov & Mumcuoglu, 2021; López-Pérez *et al*., 2022), *Hypotrombidium* spp. (Stekolnikov *et al*., 2016), *Eutrombicula* spp. (Faccini *et al*., 2017), *N. autumnalis* e *Neotrombicula inopinata* (di Meo *et al*., 2017; Guarneri *et al*., 2017); provavelmente, as espécies do género *Ericotrombidium* também afetam o ser humano (Stekolnikov *et al*., 2016). Cerca de

20 espécies são importantes em medicina humana, visto que causam dermatite ou são vetores de agentes patogénicos, incluindo *Orientia tsutsugamushi* e *Orientia chuto*, que causam tifo (*scrub typhus* na língua anglosaxónica) na região da Ásia-Pacífico, na Península Arábica e no Chile (Santibáñez *et al*, 2015; Weitzel *et al*., 2020), sendo a doença mais comum do mundo causada por riquétsias (Lin *et al*., 2021).

O. tsutsugamushi possui transmissão transovárica e transestadial no ácaro trombiculídeo e aproximadamente um milhão de casos de tifo ocorrem todos os anos devido a esta bactéria, estando mais de um milhar de milhão de pessoas em risco mundialmente (Candasamy *et al.,* 2016). Esta doença pode provocar falência multiorgânica, levando a uma taxa de mortalidade de até 70% quando não é tratada apropriadamente com doxiciclina (Candasamy *et al.,* 2016).

Os ácaros trombiculídeos podem também transportar uma variedade de outros potenciais agentes patogénicos bacterianos, tais como *Bartonella* spp., *Borrelia* spp., *Rickettsia* spp. e *Anaplasma phagocytophilum* (Literak *et al*., 2008; Kabeya *et al*., 2010; Areso-Apesteguía *et al*., 2019; Jacinavicius *et al*., 2019; Kuo *et al*., 2022), e vírus, tal como o Hantavírus, altamente contagioso e virulento, sendo potencialmente mortal para o ser humano (Yu & Tesh, 2014). Apesar de não existir evidência relativamente à possibilidade destes ácaros transmitirem estes agentes patogénicos, provavelmente terão um papel importante como reservatórios na manutenção dos mesmos no meio ambiente (Yu & Tesh, 2014; Areso-Apesteguía *et al*., 2019).

Os ácaros trombiculídeos também foram implicados como o agente provável da doença canina sazonal, uma doença potencialmente mortal da zona Este da Inglaterra e com casos clínicos semelhantes no Norte de Espanha, cujos cães demonstraram sinais clínicos de vómitos, diarreia, letargia, dor abdominal, anorexia, tremores e pirexia (McGarry *et al*., 2012; Areso-Apesteguía *et al*., 2019; Santibáñez *et al*., 2020).

3. TROMBICULOSE NA EUROPA

No continente europeu, os ácaros trombiculídeos provocam trombiculose sazonal, uma dermatite que afeta vários animais, principalmente cães, gatos e o ser humano. Considerando o ciclo de vida deste ácaro, será expectável que os hospedeiros afetados sejam aqueles que circulam no meio ambiente onde as fases larvares dos ácaros trombiculídeos se encontram. O género *Neotrombicula* inclui as espécies

mais relevantes para medicina veterinária, incluindo as do subgénero *Neotrombicula* e *Eutrombicula*. Na Europa a espécie *N. autumnalis* é comummente identificada embora muitas outras já tenham sido descritas tanto em humanos como em cães e gatos (Tabela 1) (Leone & Han, 2020).

Tabela 1 - Espécies de trombiculídeos que provocam trombiculose reportadas no continente europeu (Segal *et al*., 1972; Ripka & Stekolnikov, 2006; Shatrov & Stekolnikov, 2011; Santibáñez *et al.*, 2015; Stekolnikov *et al.*, 2016; Ramilo *et al.*, 2019; Ramilo *et al.*, 2021; Stekolnikov & Mumcuoglu, 2021; López-Pérez *et al*., 2022)

Espécies	Hospedeiros (Nome científico)	Região
Blanciella toldti	Ser humano (*Homo sapiens sapiens*) Cabra (*Capra aegagrus hircus)*	Suíça, Áustria
Blankaartia acuscutellaris	Aves Mamíferos (incl. *H. s. sapiens*)	Hungria, Espanha, Moldávia, Ucrânia, Rússia
Ericotrombidium geloti	Cão (*Canis lupus familiaris*)	Crimeia
Ericotrombidium hasei	Cão (*C. l. familiaris*) Gato (*Felis catus*)	Roménia, Sul da Europa
Ericotrombidium ibericense	Cão (*C. l. familiaris*) Gato (*F. catus*) Ratos Lagartos	Portugal, Espanha, Itália, Grécia
Euschoengastia xerothermobia	Ser humano (*H. s. sapiens*)	Europa
Kepkatrombicula desaleri	Ser humano (*H. s. sapiens*) Camurça (*Rupicapra rupicapra*) Cabra (*C. a. hircus*) Gaios	Itália, Áustria, Bulgária, Suíça
Neotrombicula autumnalis	Ser humano (*H. s. sapiens*) Cão (*C. l. familiaris*) Gato (*F. catus*) Ouriço (*Erinaceus europaeus*) Coelho (*Oryctolagus cuniculus*) Cavalo (*Equus ferus caballus*) Lebre (*Lepus* spp.) Morcego Aves	Europa (Incluindo as ilhas britânicas e excluindo a Noruega, Suécia, Finlândia e Norte da Rússia)
Neotrombicula inopinata	Ser humano (*H. s. sapiens*) Cão (*C. l. familiaris*) Gato (*F. catus*)	Portugal, Espanha, República Checa, Inglaterra, Áustria, Alemanha, Bulgária, França, Jugoslávia, Ucrânia, Rússia, Roménia, Hungria, Eslováquia e Polónia
Neotrombicula japonica	Roedores Esquilos	Europa (Azerbaijão, Moldávia)
Neotrombicula zachvatkini	Roedores Musaranhos	Europa (República Checa, Bielorrússia, Moldávia, Letónia, Ucrânia)
Straelensia cynotis	Cão (*C. l. familiaris*)	Portugal, Espanha e França

As larvas não têm um hospedeiro específico, podendo afetar vários animais, incluindo o ser humano, o que revela a importância de encarar esta parasitose sob a perspetiva de Uma Só Saúde.

Embora raros, existem alguns trabalhos que referem infestações por ácaros trombiculídeos em cães (Seixas *et al*., 2006; Areso-Apesteguía *et al*., 2019; Santibáñez *et al*., 2020), gatos (Leone *et al*., 2013; Stekolnikov *et al*., 2016) e no ser humano

(di Meo *et al*., 2017; Guarneri *et al*., 2017; Kaya & Yilmaz, 2019) na Europa.

4. TROMBICULOSE EM PORTUGAL

Em Portugal, as seguintes espécies de ácaros trombiculídeos já foram identificados em cães e gatos: *Ericotrombidium ibericense, N. autumnalis* e *N. inopinata* (Ramilo *et al*., 2019; Costa, 2020). Adicionalmente, *S. cynotis* foi também detetada num cão (Seixas *et al.*, 2006). Contudo, a informação relativamente aos animais domésticos em Portugal está limitada a poucos estudos, sendo difícil aferir a prevalência desta parasitose em animais de companhia. Adicionalmente, a prevalência desta infestação em gatos errantes em Portugal é desconhecida.

Trabalhos recentes mostraram que os médicos veterinários não estão a par desta parasitose, nem da melhor forma de a diagnosticar durante a prática clínica (Stekolnikov *et al*., 2014; Costa, 2020). Para além disso, quando estes ácaros são detetados pelos médicos veterinários em animais de companhia, eles são frequentemente identificados como *N. autumnalis* por defeito, quando, na realidade, outras espécies podem estar presentes nestes animais (Stekolnikov *et al*., 2014; Stekolnikov *et al*., 2016; Ramilo *et al*., 2019; Cousandier *et al*., 2021; Ramilo *et al*., 2021). Adicionalmente, devido à escassa disponibilidade de chaves de identificação dos ácaros trombiculídeos e à sua semelhança morfológica, é, por vezes, necessário recorrer à sequenciação do seu genoma para uma correta identificação taxonómica, sendo uma condicionante na prática clínica diária (Elliott *et al*., 2019).

A escassez de estudos realizados em Portugal e a identificação imprecisa dos ácaros trombiculídeos limitam a informação disponível quanto à sua epidemiologia no nosso país. A Tabela 2 mostra a localização geográfica das espécies de trombiculídeos descritos em Portugal até à data, assim como a sua sazonalidade.

Tabela 2 - Distribuição geográfica e sazonalidade das espécies de trombiculídeos, descritos em cães e gatos em Portugal (Ramírez *et al*., 2009; Araújo *et al*., 2013; Stekolnikov *et al*., 2016; Ramilo *et al*., 2019; Costa, 2020; Ramilo *et al*., 2021)

Espécies	Hospedeiros (Nome científico)	Localização geográfica	Sazonalidade
Ericotrombidium ibericense	Cão (*C. l. familiaris*) Gato (*F. catus*)	Paço de Arcos, Santarém, Almancil, São Brás de Alportel	julho a setembro
Neotrombicula autumnalis	Cão (*C. l. familiaris*) Gato (*F. catus*)	Santarém, Almancil	agosto e outubro
Neotrombicula inopinata	Cão (*C. l. familiaris*) Gato (*F. catus*)	Vila Franca do Rosário, Casal de Cambra, Santarém	outubro a fevereiro
Straelensia cynotis	Cão (*C. l. familiaris*)	Norte de Portugal (Ex.: Braga)	setembro a novembro

5. SINAIS CLINICOS E LOCALIZAÇÃO NO HOSPEDEIRO

Os hospedeiros vertebrados podem ser assintomáticos ou apresentar reações de hipersensibilidade devido à ação mecânica dos ácaros e/ou infeções bacterianas secundárias, que resultam em vários níveis de inflamação local (Kabeya *et al*., 2010; Milley *et al*., 2017; Curtis, 2021).

Os principais sinais clínicos em pequenos animais incluem prurido e várias lesões cutâneas, incluindo nódulos eritematosos, pápulas com crostas e escoriações autoinfligidas (Tabela 3) (Leone & Han, 2020). Em alguns casos observam-se distúrbios digestivos, astenia, pirexia ou disfunções neurológicas, por vezes graves (provavelmente devido a um processo neurotóxico), podendo inclusive levar à morte do animal quando a infestação é massiva (Oteo *et al*., 2006; Santibáñez *et al*., 2015; Guarneri *et al*., 2017; Areso-Apesteguía *et al*., 2019; Santibáñez *et al*., 2020). Os locais mais comuns para a presença dos ácaros trombiculídeos incluem o pavilhão auricular externo, em especial na bolsa de Henry, região cervical, pálpebras, lábios, regiões abdominal, inguinal, interocular e interdigital e os membros (Leone *et al*., 2013; Ramilo *et al*., 2019). Segundo Costa (2020), a região interdigital é a zona onde os trombiculídeos ocorrem com maior frequência (65,2%), tendo sido detetados, no mesmo estudo, em 80% dos cães e em 61,1% dos gatos naquela região corporal (Tabela 3).

Tabela 3 - Localização das lesões e sinais clínicos provocados por ácaros trombiculídeos em cães e gatos de Portugal (Costa, 2020; Costa *et al*., 2021)

Hospedeiro	Localização das lesões (% de animais)	Sinais clínicos (% de animais)
Cão	Espaços interdigitais (80-100%) Membros anteriores (60-80%) Membros posteriores, órgãos genitais, abdómen e peito (20-40%) Dorso, cauda e cabeça (0-20%)	Crostas (80%) Eritema (40%) Pústulas (40%) Alopecia (20%) Escoriação (20%)
Gato	Espaços interdigitais e pavilhão auricular (60-80%) Membros anteriores (40-60%) Membros posteriores, abdómen, órgãos genitais e cauda (20-40%) Dorso e cabeça (0-20%)	Eritema (72%) Crostas (44%) Alopecia (22%) Escoriação (11%)

A localização das lesões, assim como os sinais clínicos decorrentes da infestação pelas espécies de ácaros trombiculídeos descritas em cães e gatos em Portugal, podem variar e encontram-se descritas na Tabela 4.

Tabela 4 - Localização das lesões e sinais clínicos em cães e gatos provocados por espécies de ácaros trombiculídeos reportados em Portugal (Le Net *et al*., 2002; Seixas *et al*., 2006; Costa, 2020)

Espécies	Localização das lesões (% de animais)*	Sinais clínicos (% de animais)*
Ericotrombidium ibericense	Região interdigital (75%) Abdómen (58,3%): Cicatriz umbilical e regiões mamilares Pavilhões auriculares (33,3%) Vulva** e face*** (8,3%)	Eritema (75%) Crostas (50%) Pústulas e escoriações (16,6%) Alopecia** (8,3%) Prurido médio: 4,4****
Neotrombicula autumnalis	Região interdigital (85,7%) Pavilhões auriculares e cauda (57,1%)	Eritema (57,1%) Crostas (28,6%) Alopecia (14,3%) Sem sinais clínicos (42,9%) Prurido médio: 3,4****
Neotrombicula inopinata	Face e pavilhões auriculares (75%) Conduto auditivo** (50%) Abdómen e cauda (25%)	Crostas (100%) Alopecia (75%) Eritema (50%) Escoriações (25%) Prurido médio: 6,8****
*Straelensia cynotis****	Todo o corpo (dorso e cabeça mais afetados; abdómen e peito menos afetados)	Dermatite nodular (dor ao toque), alopecia, eritema, pápulas com pús e crostas. Sem prurido

*Valores obtidos por Costa (2020)
**Em gatos
***Em cães
****Escala de 1 a 10. Valores obtidos por Costa (2020)

6. DIAGNÓSTICO

A deteção ou suspeita de uma infeção por ácaros trombiculídeos pode ser acidental, no caso de animais com infestação subclínica, ou resultante da apresentação de sinais clínicos (Leone *et al*., 2013). Os principais diagnósticos diferenciais incluem dermatite atópica, dermatose responsiva a alimentos, dermatite por *Malassezia* e pododemodicoses (Curtis, 2021).

Por vezes, na inspeção da pelagem do animal, é possível a visualização macroscópica de agregados cor de laranja que à microscopia ótica são posteriormente identificados como ácaros trombiculídeos na sua fase larvar (Figura 1). Quando tal identificação não é possível, poderá ser necessária a realização de raspagens de pele nos locais afetados (Curtis, 2021). Mais raramente, a identificação destes ácaros poderá requerer a realização de biópsia de pele e histopatologia, como no caso de infeção por *S. cynotis* (Curtis, 2021).

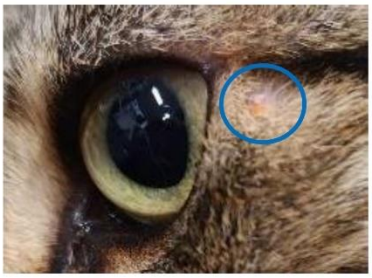

Figura 1 - Ácaros trombiculídeos na comissura ocular de um felino (circunferência).

Para a observação ao microscópio, poder-se-á recorrer a fita-cola por aposição, recolha de pelos contendo ácaros com auxílio de pinças ou raspagens de pele superficiais. Estas amostras deverão ser colocadas entre lâmina e lamela utilizando um meio de montagem (*i.e.,* lactofenol, meio de Hoyer). Na visualização ao microscópio, os ácaros trombiculídeos caracterizam-se genericamente por terem três pares de patas (Figura 2A), muitas sedas na face dorsal e ventral e por

possuírem um pequeno escudo na parte anterior da face dorsal (Figura 2B). Dada a sua dimensão, podem ser observados em objetivas de 10x ou 40x (ampliação total de 100x ou 400x, respetivamente), não sendo necessário o recurso a óleo de imersão para a sua deteção. Por necessitarem de hospedeiro vertebrado apenas na sua fase larvar, caracterizam-se também pela presença de 3 pares de patas, ao contrário de outros ácaros parasitas que podem ser detetados na fase de adulto ou ninfa, com 4 pares de patas.

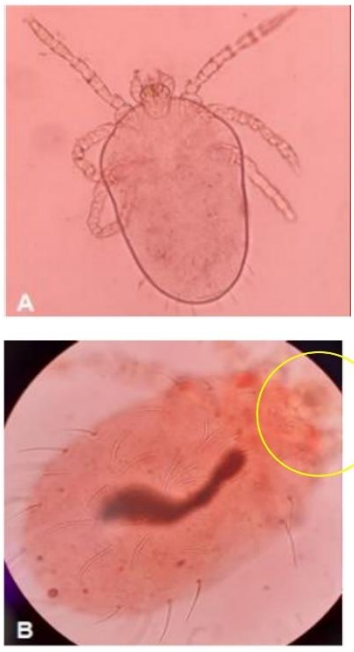

Figura 2 - Larvas de ácaros trombiculídeos observadas ao microscópio. A - Vista geral da larva de um ácaro trombiculídeo; B - Sedas dorsais e escudo (circunferência). Escalas: 60µm.

A identificação da espécie do ácaro trombiculídeo em causa exige a utilização de chaves taxonómicas de difícil acesso e que requerem especialização na identificação de estruturas específicas. Por essa razão, não é de fácil determinação por médicos veterinários no seu dia a dia. Mesmo quando realizada por um especialista em taxonomia, a identificação da espécie de ácaro trombiculídeo pode ser difícil ou impossível utilizando apenas características morfológicas. Nestes casos, a identificação da espécie requer a sequenciação de genes como, por exemplo, aquele que codifica a enzima mitocondrial citocromo oxidase c subunidade I (COI). Contudo, a especiação por sequenciação é realizada essencialmente para fins científicos (Kumlert *et al*., 2018).

7. TRATAMENTO E PROFILAXIA

7.1. TRATAMENTO EM CÃES

A associação de permetrina com piriproxifeno (solução para pulverização cutânea, 50ml/kg ou unção punctiforme, 0,5ml/kg) apresenta resultados positivos no controlo e eliminação de trombiculídeos da espécie *N. autumnalis* em cães afetados, em que 73% dos animais ficam sem parasitas desta espécie com uma só aplicação; os restantes 27% dos animais necessitam de uma segunda aplicação do antiparasitário

14 dias após a primeira aplicação para eliminação total do parasita (Smal *et al*., 2004). A associação de fipronil (6,7mg/kg) com permetrina (60mg/kg) (unção punctiforme) permite igualmente o controlo da parasitose em cães (ESCCAP, 2018; Lecru *et al*., 2019).

Segundo o *European Scientific Counsel Companion Animal Parasites* (ESCCAP) (2018), a ivermectina pode ser utilizada no controlo das infestações causadas por *S. cynotis* quando administrada por longos períodos de tempo, ocorrendo cura clínica ao fim de 6 a 12 meses após o início da terapêutica. A associação de princípios ativos inclui: 1) fipronil (*spray*, a cada semana [q1w]) e ivermectina (300µg/kg, subcutâneo [SC], q1w ou a cada duas semanas [q2w]); 2) ivermectina (SC, q2w), banhos de amitraz (1:200, a cada 4 dias [q4d]) e clindamicina (*per os* [PO], para as infeções secundárias); 3) cefalexina (PO, para as infeções secundárias) e amitraz (colar); 4) aplicação tópica de permetrina (q4d); 5) moxidectina (a cada 3 semanas [q3w]) e doramectina (SC, q2w) e 6) banhos de amitraz (q1w) (Kaufmann *et al*., 2015).

Num outro estudo, a administração única de sarolaner (2-4 mg/kg, PO) permitiu a eliminação total das larvas em 100% dos cães parasitados por *E. ibericense* e *N. autumnalis*, com uma eficácia de prevenção de reinfestações durante, pelo menos, 30 dias (Costa, 2020).

Outros autores referem que o tratamento com fenilpirazol em cães permite a queda de quase todos os ácaros do hospedeiro em 12h (Santibáñez *et al*., 2020). Para além deste princípio ativo, os mesmos autores referem a eficácia das isoxazolinas ao eliminar os ácaros 6 a 8h após a aplicação (Santibáñez *et al*., 2020). Por vezes, é necessário o recurso a prednisolona (10mg/kg/dia, reduzindo até 0,5mg/kg/dia, durante 15 dias) para reduzir o prurido e a inflamação (Santibáñez *et al*., 2015).

As reinfestações podem surgir caso os animais mantenham o acesso a áreas onde os ácaros trombiculídeos possam estar em grande quantidade (Smal *et al*., 2004; Santibáñez *et al*., 2020).

7.2. TRATAMENTO EM GATOS

A utilização de fipronil (unção punctiforme, 50mg/kg) revelou-se eficaz no tratamento de casos de infestação por *N. autumnalis* (Cadiergues *et al*., 2018). Segundo o ESCCAP (2018), o fipronil deve ser usado em forma de *spray* a cada 3-5 dias para evitar reinfestações, sendo aplicado nas patas e abdómen, zonas mais frequentemente afetadas pelos ácaros trombiculídeos.

A selamectina (*spot on*, 6mg/kg) apresenta eficácia de 100% na eliminação de *N. autumnalis* em gatos, reduzindo os sinais clínicos da doença (Leone & Albanese, 2004). Contudo, a associação desta molécula na mesma dose referida com sarolaner (1mg/kg) só conduziu à eliminação completa do parasita em 55,6% dos animais parasitados com *E. ibericense, N. autumnalis* e *N. inopinata* (Costa, 2020).

O tratamento de *S. cynotis* em gatos pode ser conseguido através da associação de moxidectina (25mg/ml, unção punctiforme) com ivermectina (350µg/kg, SC, q1w) e acetato de metilprednisolona (SC, toma única) (Kaufmann *et al*., 2015).

Existem vários princípios ativos/esquemas terapêuticos reportados na literatura como não sendo eficazes no tratamento desta parasitose em gatos: nitenpiram (11,4 mg, PO, toma única) ou ivermectina (0,4 mg/kg, PO, toma única). Outros foram eficazes por 30 dias (fluralaner, 250 mg, transdérmico), mas os animais acabam por apresentar novamente sinais clínicos devido ao seu acesso à rua não ser limitado e estes contactarem com áreas onde a concentração de ácaros trombiculídeos é elevada (Santibáñez *et al*., 2020; Cousandier *et al*., 2021).

7.3. PROFILAXIA

Para além da aplicação dos antiparasitários, é recomendado que os animais não tenham acesso aos locais onde os ácaros trombiculídeos se encontram, pois as reinfestações são frequentes, mesmo aquando da aplicação adequada de acaricidas (Santibáñez *et al*., 2020; Cousandier *et al*., 2021). Os médicos veterinários devem estar informados sobre esta parasitose, assim como dos sinais clínicos associados, por forma a realizar um correto diagnóstico e solucionar esta condição clínica (Santibáñez *et al*., 2020).

8. CONCLUSÃO, LIMITAÇÕES E PERPECTIVAS FUTURAS

Os ácaros trombiculídeos podem afetar um vasto grupo de animais vertebrados, incluindo os animais domésticos e o ser humano, sendo vetores ou potenciais vetores de agentes patogénicos. Existem relatos na literatura de animais domésticos com vários sinais clínicos, inclusive alguns relacionados com disfunções neurológicas, por vezes severas e fatais.

Os ácaros trombiculídeos não foram alvo, nos tempos recentes, de um estudo aprofundado pela comunidade científica, quando comparado com outros parasitas, como carraças e culicídeos (Weitzel *et al*., 2020). Porém, apesar da sua importância enquanto vetores e potenciais vetores de

vários agentes patogénicos bacterianos e virais, tratam-se de parasitas negligenciados (Weitzel *et al*., 2020). Muitos dos estudos publicados até hoje sobre os ácaros trombiculídeos encontram-se escritos em mandarim, japonês, coreano ou russo ou são de difícil acesso, limitando a divulgação científica (Weitzel *et al*., 2020). Alguns estudos recentes ainda referem a espécie *N. autumnalis* como *Trombicula autumnalis* (Kaya & Yilmaz, 2019), mostrando, desta forma, que a informação relativamente a estes ácaros ainda não é de fácil acesso e que não se encontra bem difundida entre a comunidade científica. Para além disso, as *guidelines* da ESCCAP também só mencionam duas das quatro espécies que podem estar presentes nos animais domésticos, sendo crucial a comunicação entre a comunidade científica para abordar esta parasitose de uma forma mais abrangente (ESCCAP, 2018).

Quando os animais de companhia se tornam o foco de estudo, a falta de informação atualizada é bastante evidente, encontrando-se um número de cerca de 15 artigos publicados mundialmente na última década quando se realiza uma pesquisa no Pubmed incluindo o termo 'chiggers' e 'cat' ou 'dog'.

Pelo que foi descrito nesta revisão, é extremamente importante que os clínicos consigam identificar esta parasitose nos animais de companhia, tendo especial atenção àqueles com acesso ao exterior, visto que a presença dos ácaros trombiculídeos pode desencadear apresentações clínicas que variam desde casos assintomáticos até apresentações mais graves. Adicionalmente, mas não menos relevante, o papel dos animais de companhia na manutenção do ciclo de vida destes ácaros em maior proximidade com o ser humano não deve ser negligenciado em virtude da sua baixa especificidade de hospedeiro, sobretudo em animais com acesso à rua e a zonas de grande concentração de vegetação.

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Appendix II. Article published in the Brazilian Journal of Veterinary Parasitology as the primary author ex-aequo

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Short Communication

A new definitive host for *Moniliformis cestodiformis* (Acanthocephala: Moniliformidae): first report of a naturally infected European hedgehog (*Erinaceus europaeus*)

Um novo hospedeiro definitivo para *Moniliformis cestodiformis* (Acanthocephala: Moniliformidae): primeiro relato de um ouriço (*Erinaceus europaeus*) naturalmente infectado

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Abstract

European hedgehogs, *Erinaceus europaeus* (Linnaeus, 1758), are small mammals found in western Europe and also in parts of northern Europe. They can be seen in rural, suburban and urban areas, but are usually found in grassland with edge habitats. These animals are omnivorous and serve as definitive or paratenic hosts for several parasites, including acanthocephalans (phylum Acanthocephala). During necropsy of a European hedgehog, a single adult parasite was collected from the intestinal lumen and preserved in 70% ethanol. After morphological evaluation of the specimen, it was identified as *Moniliformis cestodiformis* (von Linstow, 1904) (Acanthocephala: Moniliformidae). This is the first report of *M. cestodiformis* in a European hedgehog, as well as in Europe. More epidemiological studies need to be carried out to map the location and prevalence of this parasite in Portugal and the European continent.

Keywords: Acanthocephala, *Erinaceus europaeus*, *Moniliformis cestodiformis*.

Resumo

Os ouriços, *Erinaceus europaeus* (Linnaeus, 1758), são pequenos mamíferos que se localizam na Europa ocidental e também em regiões do Norte da Europa. Eles podem ser avistados em áreas rurais, suburbanas e urbanas, mas são geralmente encontrados no campo no limite daqueles hábitats. São animais omnívoros e servem de hospedeiros definitivos ou paratênicos de muitos parasitas, incluindo acantocéfalos (filo Acanthocephala). Durante a necropsia de um ouriço, um exemplar adulto acantocéfalo foi recolhido do lúmen intestinal e preservado em etanol a 70%. Depois de uma avaliação morfológica desse exemplar, este foi identificado como *Moniliformis cestodiformis* (von Linstow, 1904) (Acanthocephala: Moniliformidae). Esta é a primeira referência de *M. cestodiformis* num ouriço, assim como na Europa. Mais estudos epidemiológicos necessitam ser realizados, para localizar este parasita e calcular a sua prevalência em Portugal e no continente europeu.

Palavras-chave: Acanthocephala, *Erinaceus europaeus*, *Moniliformis cestodiformis*.

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Introduction

European hedgehogs, *Erinaceus europaeus* (Linnaeus, 1758) are small nocturnal mammals native to western and northern Europe (Reeve, 1994; Pfäffle, 2011; Sangster et al., 2016; CABI, 2022). They can be seen in rural, suburban and urban areas, but are usually found in grassland with edge habitats (Jahfari et al., 2017). This kind of habitat preferences often results in direct or indirect contact with wildlife, domestic animals and also humans (Amori, 2016). European hedgehogs are omnivorous, feeding mainly on invertebrates including beetles, caterpillars, woodlice and other insects, as well as snails, slugs and earthworms. Hedgehogs can also eat vertebrates, such as snakes, vipers, frogs, toads, fish, birds and their eggs, and small mammals (Pfäffle, 2011; Naem et al., 2015). Because of these food preferences, *E. europaeus* may act as a definitive or paratenic host of several parasites, some of them being zoonotic, like *Trichinella* spp. and *Leptospira* spp. (Jones et al., 2005; Riley & Chomel, 2005; Pozio, 2007; CABI, 2022). Furthermore, several parasites can affect *E. europaeus*, like protozoa, fleas, mites, ticks and helminths, including acanthocephalans (Pfäffle, 2011).

The genus *Moniliformis* (Moniliformida: Moniliformidae) Travassos, 1915 includes 20 recognized species (Amin, 2013; Amin et al., 2016; Gomes et al., 2020; Lynggaard et al., 2021; Dai et al., 2022). The adult forms are medium-sized thorny-headed worms with a very small proboscis when compared to their trunk. The worms are pseudosegmented rounded anteriorly and posteriorly (Golvan, 1962; Amin et al., 2016).

Moniliformis spp. infect warm-blooded vertebrates, including mammals and birds. For example, *M. moniliformis* uses rodents and humans as definitive hosts, being zoonotic in countries where insects, such as cockroaches, are eaten raw (Coomansingh-Springer et al., 2019). Five *Moniliformis* spp. are known to infect hedgehogs, but only *Moniliformis cestodiformis* (von Linstow, 1904) Travassos, 1917 has been reported in *Erinaceus* spp. (Amin et al., 2016).

Knowledge about the parasites of *E. europaeus* from mainland Portugal is scarce. Concerning *M. cestodiformis*, it has only been reported in *Erinaceus* spp. in West Africa in 1925 (Amin et al., 2016). The present study describes for the first time *M. cestodiformis* in an *E. europaeus* specimen and also represents the first report of this parasite in Europe.

Material and Methods

In May 2019, a female European hedgehog in poor physical condition, found in Monsanto forest park, within the municipality of Lisbon, was brought to the Wild Animal Rehabilitation Centre of Lisbon (LxCRAS), together with five offspring. The female stayed with the pups during the first 9 days but, due to her refusal to eat, they were separated. She was treated with natural complementary food (Anima-Strath®, 2.5 ml, *per os* [PO]), a nutritional complement (Duphalyte®, 12 ml/kg, PO) and 3 ml of saline solution subcutaneously (SC), at body temperature. On the next day, the female hedgehog was treated with fenbendazole (Panacur®, 100 mg/kg, PO). On the 11th day the animal was warmed up due to hypothermia, but did not survive.

During necropsy, a single adult parasite was collected from the intestinal lumen and preserved in 70% ethanol. The parasite was measured and some eggs were collected. The obtained material was prepared on a slide with Hoyer's medium and observed under an optical microscope. To identify the parasite specimen, an identification key was used (Amin et al., 2016). Information regarding proboscis hooks and their roots, the female reproductive system and gonopore were collected. To confirm the species, proboscis, proboscis receptacle, lemnisci, hooks and its roots, the terminal part of reproductive system and eggs were observed and measured.

Results

The parasite presented a total length of approximately 13 cm (Figure 1A). Due to its morphological conformation, being round anteriorly and posteriorly and pseudosegmented in-between (Figure 1A), it was identified as belonging to genus *Moniliformis*.

Except where millimeters (mm) are indicated, the following measurements are all in micrometers (µm). Proboscis was 475 long and 195 wide (Figure 1B); it had 16 rows of six to 10 hooks each. Hook blades decreased in size posteriorly. The largest hooks had 23.41 by 9.6 and the smallest 4.78 by 2.06 (Figures 1C and 1D); hook roots were stout and inserted in the ventral middle part (Figure 1E); proboscis receptacle was 1.13 mm long and 396 wide (Figure 1F). Lemnisci was 1.68 mm long and 166 wide posteriorly (Figure 1F).

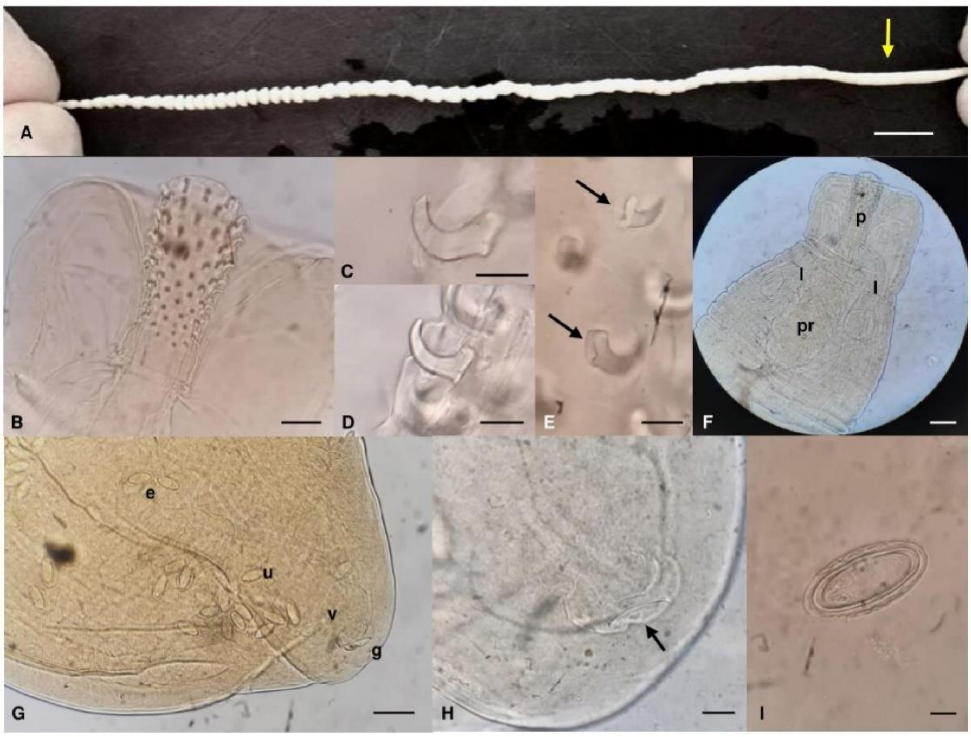

Figure 1. *Moniliformis cestodiformis.* **A:** Adult female specimen. Morphological conformation, with pseudosegmentation of trunk (yellow arrow). **B:** Proboscis. **C-D:** Hooks. **E:** Hook roots (black arrows) with an invagination in the ventral middle part. **F:** Anterior part of the body showing proboscis (p), proboscis receptacle (pr) and lemnisci (l); the diagonal musculature of the proboscis receptacle is also visible. **G:** Posterior part of the body showing the eggs (e), uterus (u), vagina (v) and gonopore (g). **H:** Gonopore and its orifice (black arrow). **I:** Egg. Scale bar on A: 1 cm. Scale bar on B: 100 µm. Scale bar on C-E and I: 20 µm. Scale bar on F and G: 200 µm. Scale bar on H: 40 µm.

Regarding the reproductive system, uterus was 640.5 long, and vagina 135.8 long and 32.5 wide (Figure 1G). Gonopore was nearly terminal (Figure 1G) and its orifice was 67.7 long in its major axis (Figure 1H). Eggs were 87.5-95 long by 45-50 wide (Figure 1I). Likewise, all measurements are in micrometers (µm). Accordingly, the female specimen was identified as *Moniliformis cestodiformis* (von Linstow, 1904).

Discussion

Parasites are known to have a substantial impact on population dynamics of their hosts (Irvine, 2006). They are a threat to debilitated hedgehogs, frequently leading to morbidity and even death. Some parasites can also represent a zoonotic risk and pose a possible cross infection with pets (Wright, 2014). The hedgehog described in this study was in a poor physical condition and the presence of *M. cestodiformis* may have impaired its health improvement.

Although several acanthocephalan species have been reported in hedgehogs (Pfäffle, 2011), only a few earlier publications mention *M. cestodiformis*. This species was originally described in 1904 by von Linstow as *Echinorhynchus cestodiformis* from two different African species of hedgehogs, *Atelerix albventris* and *Atelerix frontalis* (Travassos, 1917). Approximately a century ago, Travassos (1917) transferred it from the genus *Echinorhynchus* to the genus *Moniliformis* (Travassos, 1915). Southwell & Macfie raised *Moniliformis erinacei* as a new species from

measurements obtained from a male and a female specimen in *Erinaceus* spp. from West Africa (Sandground, 1926). According to Amin et al. (2016), these two species, *M. cestodiformis* and *M. erinacei*, are the same and the latter must not be considered as valid species.

None of the previous parasitological surveys in European hedgehogs between 1926 and 2016, e.g., Pfäffle et al. (2014), refers to *M. cestodiformis*. One paper (Amin et al., 2016) includes this species in identification keys and only refers to *M. erinacei* in *Erinaceus* spp. from West Africa. The geographical distribution of this parasite is probably not wide enough to be accounted for during hedgehog necropsies or coprological analysis of their feces, as it happens with some other parasites, such as trematode *Brachylecithum mackoi* in European hedgehogs from Elba island (Casanova & Ribas, 2004).

Conclusion

The present study is a major contribution to the knowledge of the European hedgehog parasitological fauna from Portugal and Europe, providing valuable data concerning *M. cestodiformis* and bringing to light the first reference in *E. europaeus* from Portugal and the European continent. More collections and epidemiological studies must be performed to understand its prevalence and localization in Portugal and also in Europe, since work concerning *M. cestodiformis* is non-existent according to the best of our knowledge.

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Ethics declaration

Authors applied all relevant ethical practices considering the condition of the studied animal.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Appendix III. Article published in the Journal of Zoological and Botanical Gardens as the primary author ex-aequo

Journal of Zoological and Botanical Gardens

MDPI

Article

Respiratory Infection by *Cyathostoma* (*Hovorkonema*) *americana* in a Population of Burrowing Owls (*Athene cunicularia*)—A Potential Case of Zoo–Wildlife Cross-Transmission

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Abstract: A population of burrowing owls (*Athene cunicularia*) under professional care at *Zoomarine* Portugal presented with sudden respiratory clinical signs. Clinical management included a thorough diagnosis plan, including in-house fecal analysis that revealed the presence of ovoid unioperculate eggs. In the *postmortem* examination of one hyperacute dyspneic specimen, adult nematode parasites were collected and identified based on their morphology as *Cyathostoma (Hovorkonema) americana*. Even after a broad-spectrum deworming protocol as part of the treatment and metaphylaxis approach, the incidence of parasitic reinfection was high. The complete clinical resolution was only accomplished after the identification and management of the possible focus of infection, a wild population of cattle egrets (*Bubulcus ibis*) that frequently congregated above the owls' habitat. To the authors' best knowledge, this is the first report of infection by *Cyathostoma* (*Hovorkonema*) *americana* in burrowing owls. Although nematodes of the family Syngamidae are not commonly included in the differential diagnosis of infectious respiratory agents of birds of the order Strigiformes, this report highlights the possibility of opportunistic parasitism in a zoological context, especially where there is a continued proximity to free-ranging avifauna.

Keywords: *Athene cunicularia*; *Cyathostoma* (*Hovorkonema*) *americana*; respiratory disease; opportunistic parasitism; zoo–wildlife cross-transmission

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1. Introduction

Burrowing owls (*Athene cunicularia*) have a wide distribution on the continents of North and South America, and their conservation status is defined by the International Union of Conservation of Nature (IUCN) as of least concern, although there is a decreasing population trend [1,2]. Given the important role of these birds in maintaining a balance in the populations of their prey and also serving as prey themselves for other birds of prey, the decline of some populations may be causing a ripple effect throughout the ecosystem [3,4]. Due to seasonal changes on the population density of their prey, burrowing owls have adaptable food habits. Approximately 90% of burrowing owls' diet is based on arthropods, including grasshoppers, crickets, and beetles, although they also eat small mammals, such as mice, small birds, and ground squirrels [3,5,6]. The main threat to these owls is the loss of habitat, mainly due to human activity [2–4]. Burrowing owls' habitats consist of open-canopied areas, with sparse ground vegetation. These habitats may include agricultural lands, grasslands, prairies, plains, and deserts [3,4].

These small birds of prey have diurnal habits and use burrows in the ground for refuge [3]. They may dig their own burrows or use others abandoned by other animals such as prairie dogs, ground squirrels, badgers, tortoises, coyotes, and foxes. These burrows may reach more than 3 m in length, angled downwards [3,4].

Burrowing owls live 6–8 years in the wild and up to 10 years under professional care in captivity [3,7]. In the wild, two-thirds do not live to adulthood, with mortality frequently happening between fledging and the end of first year of age. The causes of mortality at a young age include low prey density, inexperience in capturing food, predators, and parasitic infections [3].

This species of owl is found in several zoological institutions. A burrowing owl population of 11 resident individuals at *Zoomarine* Portugal inhabited an outdoor walkthrough enclosure adapted for a variety of species of birds and reptiles. As part of the park's zoological collection, these individuals were included in a preventative medicine program and had no relevant clinical history until the sudden and sequential development of respiratory clinical signs.

The subfamily Syngaminae Baylis & Daubney, 1926 includes parasitic nematode organisms found in the respiratory systems of both avian and mammalian hosts, with representatives such as *Boydinema*, *Cyathostoma*, and *Syngamus* for birds and *Mammomonogamus* and *Rodentogamus* for mammals. Notably, the genus *Cyathostoma* was established by Blanchard in 1849 and stands out as the most extensive, boasting a population exceeding 20 distinct species [8]. *Cyathostoma* sp. were reported in several bird species and, even though the occurrence of these nematodes is generally subclinical, heavy infections are often associated with secondary infections and death [8–14]. The ecology of *Cyathostoma* sp. parasites is important for veterinary and conservation reasons and is still a challenging taxonomy and a contentious topic [8].

In the most recent system of Syngaminae proposed by Lichtenfels in 1980 [15], despite being more than 40 years old, the genus *Cyathostoma* is divided into two subgenera based on the structure of the copulatory bursa and spiculae length. On the one hand, *Cyathostoma* (*Cyathostoma*) (Blanchard, 1849) exhibits a dorsal ray that extends beyond the end of the copulatory bursa, forming characteristic thorn-like projections and spicules that measure 0.08–0.4 mm. On the other hand, *Cyathostoma* (*Hovorkonema*) Turemuratov, 1963 features a dorsal ray that does not extend beyond the end of the copulatory bursa, and spicules are within the range of 0.45–0.8 mm.

Cyathostoma (*Hovorkonema*) *americana* was first described by Chapin in 1925, based on material collected from a red-tailed hawk (*Buteo jamaicensis* Gmelin, 1788) in Virginia (USA), and, while there are occasional subsequent reports of this species in birds of prey [8,10,11,13,14,16–18], there are no available reports of *C.* (*Hovorkonema*) *americana* in *A. cunicularia*.

This study aims to describe the diagnostic approach for the respiratory infection in a population of *A. cunicularia* held in a zoo, primarily focusing on parasite identification, exploring the evidence of cross-transmission, and investigating opportunistic parasite infections within the unique environment of a zoological setting.

2. Materials and Methods

The burrowing owls with respiratory clinical signs went through a multimodal diagnostic approach, which included a full physical examination under general anesthesia with isoflurane, when possible, according to the clinical status of each individual. Blood sampling was performed for an in-house complete blood count and general biochemistry panel, the latter through a VETSCAN VS chemistry analyzer (Avian/Reptilian Profile Plus). Both total leukocyte and erythrocyte counts were manually completed, in which a 5 µL blood-filled pipette was inserted into a Natt-Herricks-TIC (Bioanalytic GmbH, Umkirch/Freiburg, Germany) 1:200 stain solution vial, and counting was performed with a Neubauer chamber. Evaluation of blood smears was performed after Diff-Quik staining. Hemoglobin levels were obtained through a hemoglobin analyzer HemoCue (HemoCue AB, Ängelholm, Sweden) and hematocrit after centrifugation of microhematocrit tubes (Centurion Scientific

Ltd.—Pro-Vet, West Sussex, UK) at 12,000 rpm for 5 min. Radiographic studies included ventrodorsal and left lateral views with portable radiographic equipment (GIERTH HF300, GIERTH X-RAY International GmbH, Riesa, Germany). For in-house fecal wet mounts, one drop of new methylene blue was added immediately before microscopic observation.

The adult nematode parasites were sent to the Faculty of Veterinary Medicine at the University of Lisbon for analysis conducted by the Parasitology and Parasitic Diseases Service. The parasites were subjected to morphological identification and measurement and underwent the following procedures: (i) examination under a stereomicroscope for initial observation; (ii) mounting on slides with Hoyer's medium for subsequent examination using a compound microscope.

The identification of parasite subgenera employed the Syngaminae classification system as proposed by Lichtenfels [15]. For the identification of the parasite species, the authors consulted the review of *Cyathostoma* sp. nematodes by Kanarek et al. [8]. Detailed observations and measurements encompassed body length, body width, depth of buccal capsule, spicule length in males, and egg dimensions.

3. Results

A substantial proportion, specifically 45% (5/11), of the population of burrowing owls developed clinical signs of respiratory origin. These clinical signs included dyspnea (5/5), tachypnea (5/5), crackles (3/5), and sneezes (1/5). Other clinical findings included compression of the abdominal air sacs' radiographic silhouette (3/5), mild leukocytosis (2/5), eosinophilia (1/5), and subcutaneous emphysema (1/5) (Figure 1). Additionally, observation of the direct fecal smears revealed the presence of ovoid unioperculate eggs (Figure 2).

The postmortem examination of one individual who presented hyperacute dyspnea showed severe signs of pulmonary congestion. Adult nematodes were found in the parenchyma of the left lung apex. Notwithstanding the signs of air sacculitis, there were no parasites found in the trachea or air sacs. The male parasites exhibited a total length of 1.2 cm, while the females measured 2.3 cm. Notably, the morphological configuration of the dorsal ray of the copulatory bursa, which does not extend beyond the end (Figure 3), was identified as being under the subgenus *Cyathostoma* (*Hovorkonema*) (Turemuratov, 1963).

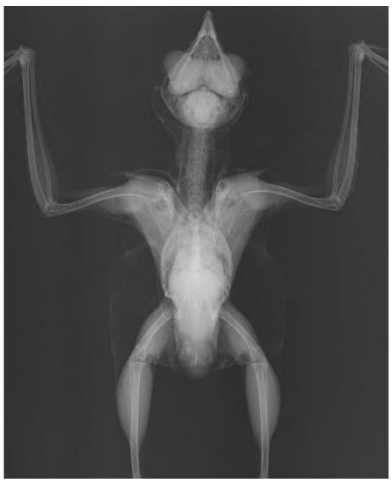

Figure 1. Ventrodorsal radiographic image of an *Athena cunicularia* specimen, depicting a general subcutaneous emphysema.

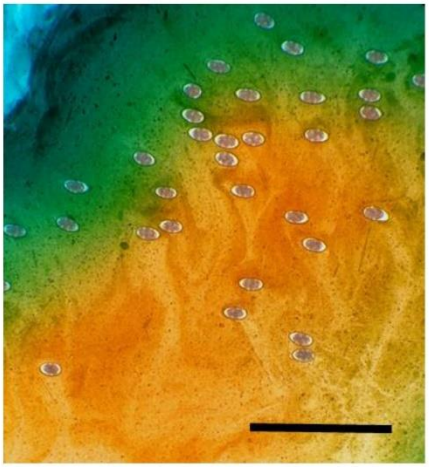

Figure 2. Direct fecal smear showing ovoid unioperculate eggs (new methylene blue, ×100). Bar: 500 µm.

Figure 3. Posterior end of male parasite. Note that no ray of the copulatory bursa extends beyond the end. Bar: 1 mm.

The ensuing measurements are all expressed in micrometers (µm). The dimensions of the male buccal capsule (width × depth) were 255 × 255 (Figure 4), while the female's buccal capsule measured 350 × 210 (Figure 5). The male spicule length measured 435 (Figure 6), and the eggs exhibited dimensions of 85–92.5 in length and 50 in width, with an ovoid form and a single operculum (Figure 7). Notably, given the prevailing attributes, including the smaller spicules and the host being part of the parasite's type group (birds of prey), the specimens were identified as *C.* (*Hovorkonema*) *americana* [16].

Figure 4. Anterior end of male parasite. Bar: 500 µm.

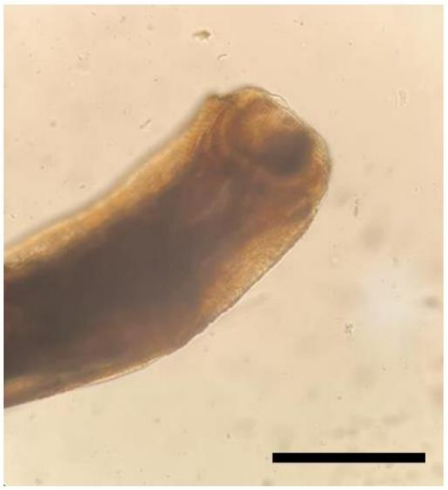

Figure 5. Anterior end of female parasite. Bar: 500 µm.

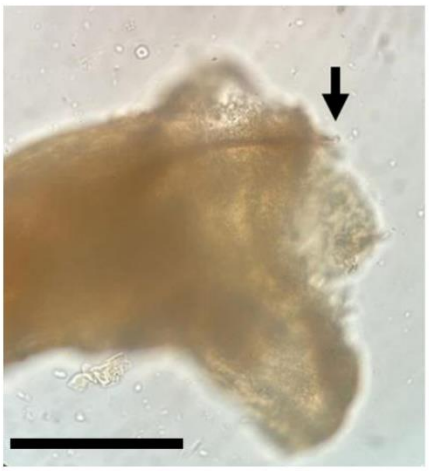

Figure 6. Posterior end of male parasite with copulatory bursa. Note the spicule end (arrow). Bar: 250 µm.

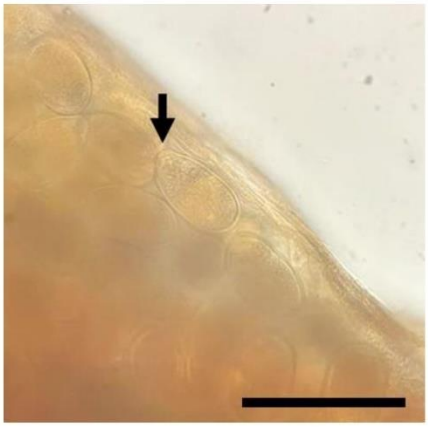

Figure 7. Detail of parasite eggs inside a female specimen. Note the operculum (arrow). Bar: 120 µm.

4. Discussion

Animal welfare assessment is crucial in modern zoological institutions in order to assure the highest possible standards of welfare for animals under professional care. This assessment is based on the Five Domains model, which includes nutrition, environment, health, behavior, and mental state [19–21]. The negative effects of parasitic infections in zoos are numerous and may extensively affect the health dominion (i.e., the absence of diseases, the absence of injuries, and the absence of pain induced by management procedures). The parasitic consequences may vary from the development of secondary infections or nutritional deficiencies to death in the case of severe parasitosis. Moreover, the systematic impact of parasitism may include reproduction impairment, which may be decisive not only for the success of a specific animal collection but also for conservation

purposes. Lastly, in a zoological context, close contact between animals and humans is possible, hence the higher risk of the dispersion and spread of parasitic zoonoses [22,23].

The overall management of parasitic infections in zoos may present itself as a challenge, not only from a diagnostic and treatment perspective but also from an environmental point of view. The original population of burrowing owls under professional care at *Zoomarine* was captive-bred in other European institutions and arrived at *Zoomarine* around six years before the onset of the respiratory parasitic infection. Since their arrival, they inhabited a mixed-species outdoor walkthrough enclosure, which included other bird species (giant wood rail (*Aramides ypecaha*), cattle egret (*Bubulcus ibis*), striated heron (*Butorides striata*), reg-legged seriema (*Cariama cristata*), scarlet ibis (*Eudocimus ruber*), guira cuckoo (*Guira guira*), black-crowned night heron (*Nycticorax nycticorax*), southern wigeon (*Mareca sibilatrix*), ocellated turkey (*Meleagris ocellata*), roseate spoonbill (*Platalea ajaja*), glossy ibis (*Plegadis falcinellus*), and green aracari (*Pteroglossus viridis*)) and reptiles (green iguana (*Iguana iguana*) and red-eared slider (*Trachemys scrypta scrypta*)). This type of natural-looking outdoor habitat is a creative outlet for physical activity and mental stimulation, providing several environmental enrichment opportunities covering both generic (choice, control, variety, and complexity) and specific needs [21,24]. However, one of the logistical challenges of this type of enclosure involves the difficult access to the animals, so the group of owls in *Zoomarine* was trained to enter a crate, allowing easy access for medical reasons whenever needed. This played a key part, as these owls were under a preventative health program, which included but was not limited to fecal sampling for coprological examination (wet mount, Gram stain, Diff-Quik stain, and flotation). However, even under a comprehensive medical protocol, the untimely and low sensitivity of certain diagnostic tools may delay treatment and worsen the overall prognosis of a certain parasitic infection [25,26]. Considering this diagnosis challenge along with the characteristics of the mixed-species outdoor enclosure and the possibility of nearby access for wild fauna and pathogens cross-transmission, all birds were under a deworming protocol every six months, which consisted of fenbendazole (50 mg/kg, *per os* (PO), once), ivermectin (0.2 mg/kg, PO, once, 15 days after fenbendazole) and toltrazuril (10 mg/kg, PO, three administrations, every other day (EOD), 7 days after ivermectin).

Even considering the regular anti-parasitic protocol, respiratory parasitosis was included as a differential diagnosis after the development of respiratory signs in 45% of the owl population. Both the clinical signs and findings from the physical examination and complementary exams fall inside the non-specific signs of respiratory disease in birds [27,28]. Considering the complexity of the avian respiratory tract, disease processes can be located in different anatomic regions, though the distinction between clinical signs is not clear, as many of them (e.g., dyspnea, breathing with an extended neck, tachypnea, respiratory noises, and a change in pitch or voice) may refer to both upper and lower respiratory tract disease. However, upper airway disease does not usually present with severe respiratory distress [27,28]. Due to its specific anatomic characteristics (e.g., air sacs), the avian respiratory system constitutes a substantial target for infection by a myriad of infectious agents [27]. Previous authors reported unspecific clinical signs of infections by *Cyathostoma* sp., such as depression and sudden death, along with syndromes and lesions, such as pneumonia, bronchitis, and air sacculitis, in birds of prey [10,11,13,29].

Hematology abnormalities may include leukocytosis, whereas serum biochemistries are not generally of particular interest in respiratory/parasitic diseases [27]. Indeed, only two individuals, one of them with eosinophilia, showed a slight increase in their total leukocyte counts. Although eosinophilia is commonly associated with mammals with parasitic diseases, this relation in avian species is not straightforward, so more complementary studies are needed on this subject [30]. A physical examination confirmed one case of severe and generalized subcutaneous emphysema, with the typical crackling and air-filled distention, with the common causes being trauma or lung/air sac inflammation associated with parasites or other infectious agents [31–33].

The treatment protocol was adapted according to each individual's clinical condition. The description of the specific clinical approach falls beyond the scope of this work. It is important to note, however, the use of a broad-spectrum protocol that included fenbendazole (50 mg/kg, PO, once a day (SID), 3 to 5 days), in conjunction in some cases with ivermectin (0.2 mg/kg, PO, once, 5 to 10 days after the therapeutic protocol with fenbendazole). Metaphylaxis measures through the same deworming protocol may have prevented the development of a clinical parasitosis in the rest of the owl population. Only one of the five individuals that presented with clinical signs died after a hyperacute clinical respiratory presentation. The literature includes several reports of mortality in birds of prey infected with *Cyathostoma* sp. Fatal parasitic pneumonia was reported in three injured wild owls in Southern Ontario along with four juvenile *A. cunicularia* bred in captivity [10]. A survey in Canada of 394 specimens of Falconiformes and Strigiformes also described fatal infections [13]. Finally, a case of fatal epicarditis associated with *Cyathostoma* species in a hen harrier (*Circus cyaneus*) was also reported [14].

Up until now, the identification of *Cyathostoma* (*Hovorkonema*) nematodes collected from the respiratory tracts of Accipitriformes, Falconiformes, and Strigiformes in Europe and North America has been highly inconsistent among authors [8]. These inconsistencies arose due to several shifts in the taxonomic placement of *Cyathostoma* species, which stem from different interpretations of morphological characteristics, various revisions, and questionable synonymities [34–37]. Furthermore, these discrepancies have been compounded by different authors utilizing distinct classification systems. For instance, Hartwich amalgamated three *C.* (*Hovorkonema*) species—*C.* (*Hovorkonema*) *americana*, *C.* (*Hovorkonema*) *bronchialis*, and *C.* (*Hovorkonema*) *variegatum* sensu stricto—into *C.* (*Hovorkonema*) *variegatum* [37]. This conflation resulted in several identifications of *C.* (*Hovorkonema*) *variegatum* in birds of prey that corresponded to the *C.* (*Hovorkonema*) *americana* species [8].

C. (*Hovorkonema*) *americana*, which is a typical parasite of the trachea and air sacs of raptors, *C.* (*Hovorkonema*) *bronchialis*, typically found in Anseriformes and also detected in Casuariiformes, and *C.* (*Hovorkonema*) *variegatum*, a typical parasite of the trachea of cranes and storks, constitute the three species within this subgenus. According to Kanarek's comprehensive morphological and molecular examination of *Cyathostoma* nematodes parasitizing the respiratory tracts of birds of prey in Europe and North America, only one species of *C.* (*Hovorkonema*) in birds of prey is confirmed to exist: *C.* (*Hovorkonema*) *americana* [8]. However, the possible but rare incidental occurrence of other *C.* (*Hovorkonema*) species, typical for different avian host groups, is acknowledged.

With respect to our samples and focusing on the characteristics highlighted as the most reliable for *Cyathostoma* sp. identification, the subgenus *C.* (*Hovorkonema*) is defined by spicules measuring 0.45–0.8 mm, while *Cyathostoma* (*Cyathostoma*) (Blanchard, 1849) has measurements ranging from 0.08 to 0.4 mm [38]. Our specimens fall between these ranges (0.435 mm). However, the absence of thorn-like projections on the copulatory bursa aligns with classification under the *C.* (*Hovorkonema*) subgenus.

Two of the three species of this subgenera can be clearly distinguished based on spicular length. *C.* (*Hovorkonema*) *variegatum* boasts notably longer spicules (0.58–0.77 mm) [34,39], compared to *C.* (*Hovorkonema*) *americana* (0.47–0.511 mm) [16,40]. The spicules length of *C.* (*Hovorkonema*) *bronchialis* (0.51–0.7 mm) is closely aligned with that of *C.* (*Hovorkonema*) *variegatum* [16,41–43]. Although this matter is still in need of further research, it suggests the potential synonymy of *C.* (*Hovorkonema*) *bronchialis* with *C.* (*Hovorkonema*) *variegatum*, as theorized by Kanarek et al. [8].

Considering these observed spicule lengths and given the close similarity of *C.* (*Hovorkonema*) *americana*'s spicules to our exemplars, along with the fact that it is the parasite of our designated host type (birds of prey), the sole plausible identification is *C.* (*Hovorkonema*) *americana*. Furthermore, the total lengths of the male and female parasites also fall within the species' described measurements.

While *C.* (*Hovorkonema*) *variegatum* in typical hosts exclusively occurs in the trachea, *C.* (*Hovorkonema*) *americana* in birds of prey was mainly recorded in air sacs and less frequently in the trachea [8,10,11,34,39,40]. There are reports on the presence of *Cyathostoma* nematodes in the trachea, bronchi, and air sacs of birds of prey [12,36]. However, there is no specific mention of these parasites being found in the lung parenchyma, least of all exclusively in this site, like in the present case [8].

Hunter et al. reported the presence of a *Cyathostoma* species parasite in an *A. cunicularia* specimen; however, the species of the parasite was not specified [10]. To the best of our knowledge, the current report is the first description of *Cyathostoma* (*Hovorkonema*) *americana* in *A. cunicularia*. The morphological differences and lack of previous reports do not rule out, but rather suggest, that these parasites could be a new species, with *C.* (*Hovorkonema*) *americana* being the closest match among the described species. Further research is needed to confirm this hypothesis, including the molecular characterization of these parasites, comparing the results with the molecular analyses available in the literature [8,11].

Although there are several studies available on parasitism in wild animals, more specifically wild birds, the possibility of free-ranging avifauna acting as a source of infection for captive specimens is a rarely explored subject [25,44]. It has been established, however, that exposure to wild avifauna may result in the parasitism of captive birds, depending if certain conditions for parasitic development are suitable such as parasite–host specificity, parasite life cycle, host resistance, husbandry-related factors, and environmental factors [25,44]. The host specificity of parasites is variable. While some parasite species are only found in a limited number of host species (otherwise known as highly host-specific), other parasites can affect multiple host species (considered to be host generalists, with low host specificity) [44].

There was a potential origin of the parasitic infection in the *A. cunicularia* population, namely, a group of more than 50 wild cattle egrets (*B. ibis*) that used to gather on top of the enclosure net, attracted by individuals of the same species that were part of the zoological collection and in that same enclosure. This gathering followed years of a particularly high birth rate of the captive *B. ibis* population and an increase in the total number of individuals kept in the enclosure. The wild *B. ibis* built nests and stayed above the enclosure throughout the day, and several nestlings and fledglings were seen throughout this period. Moreover, an estimated count of these wild *B. ibis*, not immediately above the enclosure but in the adjacent trees, included more than 600 animals.

Cattle egrets are free-range migratory birds in the family Ardeidae (order Pelecaniformes) with widespread distribution. Inclusively, they are the only member of the genus *Bubulcus* found in the tropics, subtropics, and warm temperate zones [45,46]. They are frequently found in association with cattle, with whom they establish a symbiotic relationship. This species has an extremely large range and does not approach the thresholds for vulnerable conservation status under the range size criterion—cattle egrets are classified as Least Concern under the IUCN conservation status [45,46].

Even though metaphylaxis measures were adopted, and all cleaning and disinfection protocols for the owls' underground burrows were revised, the incidence of reinfection was high. The complete clinical resolution of the parasitic infection of the population of burrowing owls was only accomplished when all the resident cattle egrets were transferred to other zoological institutions, and their wild counterparts immediately stopped congregating above the enclosure. This probable focus of infection cannot, however, be confirmed since a coprological analysis of these wild individuals was not performed. Cattle egrets are not yet known to be definitive hosts of *C.* (*Hovorkonema*) *americana*, but several nematodes were reported in these migratory birds, including from the Syngamidae family [46]. In this study, 40 free-ranging specimens of *B. ibis* were captured and examined for endoparasites and associated ectoparasites. The gastrointestinal content of each specimen was examined for adults and eggs of different helminths and protozoan cysts. The observed endoparasites included *Ascaridia galli* (with the highest prevalence, 35%), *Heterakis* spp. (17.5%), *Capillaria* spp. (12.5%), *Trichostrongylus tenuis* (12.5%), *Fascioloides magna* (10%), and *Syngamus trachea* (7.5%). This study also showed that there was a significantly higher risk of co-infection

by the different types of parasites and that the ubiquitous presence of *B. ibis* may play an important in parasite dispersal in a global perspective. Furthermore, cattle egrets feed daily on a variety of insects, crustaceans, fish, lizards, frogs, spiders, moths, rodents, and earthworms, making it a favorable host through the ingestion of intermediate and paratenic hosts [46,47].

Interestingly, Syngamidae nematodes, including *Cyathostoma* sp., have been reported in free-ranging birds from across Europe, yet, there is only one available occurrence of Syngamidae in wild avifauna in Portugal, namely, the genus *Syngamus*. This study was developed in a wildlife rehabilitation center in Castelo Branco, Portugal, from January 2016 to May 2017, concerning the examination of the fecal samples of a total of 65 birds, in which birds of prey were the most representative group (77% of the samples) [11,34,37,39,40,43,48].

Generalist parasites that tolerate host physiology differences can infect a large variety of avian species [44]. In the present case of monoxenous parasites, owls may have become directly infected by eating soil or other materials contaminated with the feces of wild avifauna. Moreover, since burrowing owls are territorial and tend to stay in the same area, in or close to their underground burrows, there might have been a higher exposure to the feces of the wild *B. ibis* population. The possibility of paratenic hosts (e.g., earthworms) facilitating the chance of infection cannot be excluded, since the owls' habitat included areas of earthy soil. Finally, reinfection is possible through the ingestion of food/substrates contaminated with their own feces.

Parasites seldom lead to subclinical effects in wild avifauna, thus contributing to the dispersion and transmission to birds under human care inhabiting enclosures that allow close proximity to wild birds or paratenic hosts [25,44]. This report highlights the need to consider external sources of excretion and parasitic contamination as part of the management of parasitic infections when the type of zoological enclosures allows the possibility of opportunistic parasitism in susceptible avian collections. More studies are needed demonstrating cases of parasite transmission in a zoological context and describing the species of parasites affecting both captive and free-ranging birds. More studies on zoo–wildlife cross-transmission would benefit the adaptation of specific environment control measures (e.g., architectural designing; the removal of nearby roosts; paratenic host–control programs), as well as enhance effective preventative medical and husbandry protocols.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, due to the absence of interference with the normal animal health program designed by the zoo's veterinarians. This study followed the daily activity of the institution and its normal animal management, in strict collaboration with the zoo owners and assistant veterinarians. No interferences were made during the regular health management of all collections, since all animals were dewormed, and fecal samples were mostly collected from soil after natural excretion by the animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article.

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Appendix IV. Poster and abstract presented at the XXII Congress of the Spanish Society of Parasitology in Madrid, July 2022

XXII congreso de la Sociedad Española de Parasitología

MADRID 5 | 8 julio 2022

First report of *Zachvatkinia larica* in seagulls from the Iberian Peninsula

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INTRODUCTION

Feather mites are bird ectoparasites that show variation in their morphological structures depending on the microhabitat and host specificity [1]. The genus *Zachvatkinia* Dubinin, 1949 comprises 15 species mainly associated with birds from Procellariiformes and Charadriiformes orders [2] and it is usually very abundant on their hosts [3]. The species *Zachvatkinia larica* Mironov, 1989 has been isolated from various gull genera (Subfamiliy Larinae) and was originally described by Mironov [2] based on the specimens collected from *Chroicocephalus ridibundus* (Syn .: *Larus ridibundus*) in the Volga delta, Russia. The present poster reports for the first tme these mites in the Iberian Peninsula and reviews the findings on this ectoparasite on a worldwide basis.

MATERIAL & METHODS

Specimens of feather mites were collected from three different seagull species: *Larus fuscus*, *Larus michahellis* and *Chroicocephalus ridibundus*. This sampling was carried out during parasitological necropsies in RIAS Animal Rehabilitation Center, Olhão, Portugal, in December 2019. Later, in April 2022, specimens of the same feather mite species were collected again from *Larus fuscus* provided by the wildlife rescue center LxCRAS, Lisbon, Portugal. Collected mites were preserved in 70% alcohol, mounted in slides using Hoyer's medium and observed with optical microscopy. Identification of mite genus was performed using the dichotomous key published by Mironov and Dabert [4] and the identification of the species was based on the morphologic data provided by Han et al [5]

Figure 1- Mites collected from the feathers of *Larus fuscus* (A and C) and from the feathers of *Chroicocephalus ridibundus* (B). C- Detail of a male mite

RESULTS & DISCUSSION

This genus has the following diagnostic characteristics: a) the subcapitulum is not gourd-shaped (the anterior part is not narrowed); b) setae mG of genua I are filiform; c) the lateral membrane of the opisthosomal lobe is present; d) epimerites III and IV are closed in males; and e) the anterior part of the hysteronotal shield is extended to a humeral shield in females [2]. *Zachvatkinia larica* is distinguishable from other species in the genus *Zachvatkinia* by several characteristics in male: a) the inner margins of the opisthosomal lobes have ledges near the bases of setae h3; b) three or four dorsobasal spines exist on the tarsi IV; and c) wedded anterior margin of adanal shield without protrusion [2]. All collected mites reported in this work belonged to the species *Zachvatkinia larica*.

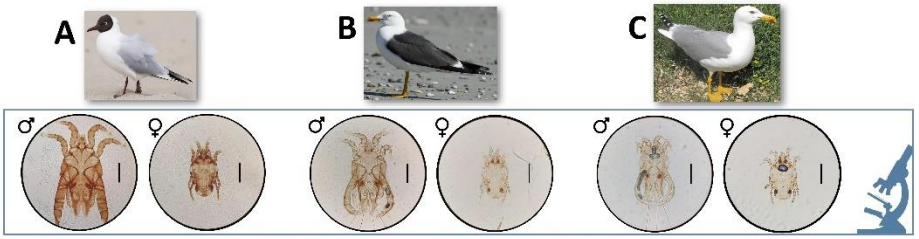

Figure 2- The gull species and their corresponding mites (*Z. larica*), separated by male and female (Scale bar: 0,2 mm).
A- The Black-headed gull (*Chroicocephalus ridibundus*)
B- Lesser black-backed gull (*Larus fuscus*)
C- Yellow-legged gull (*Larus michahellis*)

CONCLUSIONS

To the authors' best knowledge, this is the first report of this mite species in Portugal and Iberian Peninsula, since the only countries where it has been previously detected are: Russia, USA, Colombia, Korea, Chile and The Netherlands, the only European country where this parasite has been isolated [6], although it has recently been reported for the first time in Turkey as well (in this present year) [7]. This parasite has never been reported in Iberian Peninsula, but the vast majority of the necropsied seagulls were parasitized by *Zachvatkinia larica* and with a high rate of infestation, mainly in Olhão.

The fact that there is such a large gap in knowledge of the geographical distribution of this species, points to the need for awareness and the importance of future studies in this field, taken the possible emergence of *Zachvatkinia larica* in some European countries, probably associated with migratory populations of seagulls as definitive hosts.

Figure 3- World map with the countries where *Z. larica* have been detected (according to [6] and [7]), with the addition of this present work.

Figure 4- Map of mainland Portugal with the locations where *Z. larica* was found in this present work.

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XXII CONGRESO DE LA SOCIEDAD
ESPAÑOLA DE PARASITOLOGÍA
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POSTER
#258

Parásitos oportunistas y emergentes

FIRST REPORT OF *Zachvatkinia larica* IN SEAGULLS FROM THE IBERIAN PENINSULA

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ABSTRACT | *RESUMEN*

Feather mites are bird ectoparasites that show variation in their morphological structures depending on the microhabitat and host specificity. The genus *Zachvatkinia Dubinin*, 1949 comprises 15 species mainly associated with birds from Procellariiformes and Charadriiformes orders and it is usually very abundant on their hosts. The species *Zachvatkinia larica Mironov*, 1989 has been isolated from various gull genera (Subfamiliy Larinae).

Specimens of feather mites were collected from three different seagull species: Larus fuscus, Larus michahellis and *Chroicocephalus ridibundus*. This sampling was carried out during parasitological necropsies in RIAS Animal Rehabilitation Center, Olhão, Portugal, in December 2019. Later, in April 2022, specimens of the same feather mite species were collected again from Larus fuscus provided by the wildlife rescue center LxCRAS, Lisbon, Portugal.

Collected mites were preserved in 70% alcohol and were mounted in slides using Hoyer's medium. Identification of mite species was performed using the dichotomous key published by Mironov and Dabert in 1998. All collected mites reported in this work belonged to the species *Zachvatkinia larica*. To the authors' best knowledge, this was the first report of this mite species in Portugal, since the only countries where it has been previously detected are: Russia, Canada, USA, Colombia, Korea, Chile and The Netherlands, the only European country where this parasite has been isolated. This parasite has never been reported in Iberian Peninsula, but the vast majority of the necropsied seagulls were parasitized by *Z. larica* and with a high rate of infestation, mainly in Olhão.

The fact that there is such a large gap in knowledge of the geographical distribution of this species, points to the need for awareness and the importance of future studies in this field, taken the possible emergence of *Z. larica* in some European countries, probably associated with migratory populations of seagulls as its definitive hosts.

KEYWORDS: *Zachvatkinia Larica, Feather Mite, Larus Fuscus, Larus Michahellis, Chroicocephalus Ridibundus.*

FUNDING | *FINANCIACIÓN*

This research was supported by Project CIISA/FMV Project UIDB/00276/2020 and LA/P/0059/2020 - AL4AnimalS (funded by FCT).

Appendix V. Poster and abstract presented at the XVIII International Veterinary Montenegro Congress, November 2022

Deteção de ácaros trombiculídeos (Acari: Trombiculidae) em gatos errantes (*in vivo*) e no meio ambiente (*ex vivo*) em Lisboa e Santarém

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Introdução

Os ácaros trombiculídeos (Acari: Trombiculidae) são responsáveis pelo aparecimento de sinais clínicos dermatológicos, gastrointestinais e/ou neurológicos nos animais afetados. Estes ácaros podem transportar uma grande variedade de agentes patogénicos, como bactérias (*Orientia tsutsugamushi*, transmissível ao ser humano, *Anaplasma phagocytophilum*, *Bartonella* spp. e *Borrelia* spp.) ou vírus (Hantavírus), sendo considerados ácaros zoonóticos.
Estão descritas cerca de 3000 espécies destes ácaros que podem parasitar animais vertebrados e invertebrados [1]. É a fase larvar do parasita que afeta todos os grupos de vertebrados, com exceção de peixes, sendo que os pequenos mamíferos (i.e., roedores) e as aves são os seus principais hospedeiros [2,3].
As larvas possuem cerca de 250 µm de comprimento, uma cor alaranjada ou vermelho brilhante característica e três pares de patas [1].
As larvas após eclosão dirigem-se para locais elevados da vegetação rasteira, incluindo a terminação dos talos da relva ou ramos secos de árvores, formando aglomerados, com o intuito de se agarrarem a um hospedeiro que passe perto destes locais [4,5]. Uma vez no hospedeiro, as larvas alimentam-se durante 2 a 10 dias (exceção para *Straelensia cynotis*, que se alimenta durante 3 meses) [6]. Durante a refeição, os ácaros inoculam enzimas líticas nas camadas superiores da pele para conseguirem ingerir as células do tecido liquefeito, as secreções epiteliais e/ou sangue [1,6]. Ao fim daquele tempo, as larvas caem ao solo e passam pelas restantes fases do ciclo de vida até chegar à fase adulta, vivendo de forma livre [6] (Figura 1).

Figura 1 – O ciclo de vida dos trombiculídeos apresenta 7 estádios de desenvolvimento, sendo que as larvas, após o seu processo de alimentação no hospedeiro, caem para o solo, tornando-se um ácaro de vida livre. O seu ciclo de vida varia entre 2 meses a 1 ano. Adaptado de [2].

Objetivos

O objetivo deste trabalho foi detetar a presença de ácaros trombiculídeos em gatos errantes e no meio ambiente.

Materiais e métodos

Entre o dia 17-01 e o dia 20-05-2022, foram observados 27 gatos errantes capturados para serem castrados na Casa de Animais de Lisboa (CAL). Foram também observados 3 gatos errantes na Clínica Veterinária São Francisco de Assis (Santarém) entre 20-07-2020 e 20-09-2021. Estes animais foram examinados antes do procedimento cirúrgico para a deteção de ácaros trombiculídeos, assim como o ambiente ao redor do gatil da CAL.
Os ácaros detetados foram recolhidos com auxílio de pincéis e pinças cirúrgicas e conservados em etanol a 70% até preparação entre lâmina e lamela com meio de Hoyer e observados ao microscópio.
Os sinais clínicos observados nos animais foram registados, assim como a localização das lesões. Os ácaros foram identificados usando chaves de identificação, tendo em conta as características do escudo dorsal (Figura 2) e a organização das cerdas do idiossoma.

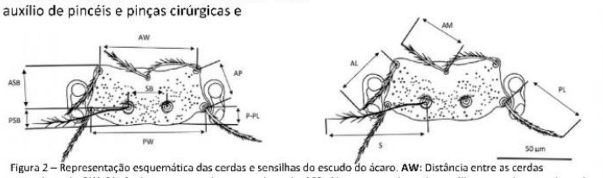


Figura 2 – Representação esquemática das cerdas e sensilhas do escudo do ácaro. **AW**: Distância entre as cerdas anterolaterais; **PW**: Distância entre as cerdas posterolaterais; **ASB**: Altura entre a base da sensilha e a cerda anterolateral; **PSB**: Distância entre a base da sensilha e o extremo posterior do escudo; **SB**: Distância entre as sensilhas; **AP:** Distância entre a cerda anterolateral e posterolateral; **P-PL**: Distância entre a cerda posterolateral e a margem posterior do escudo; **AL:** Cerda anterolateral; **AM:** cerda anteromediana; **PL:** Cerda posterolateral; **S:** Sensilha

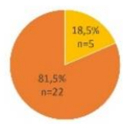
Resultados

Trombiculídeos recolhidos no ambiente

Foram recolhidas larvas e adultos da especie *Neotrombicula inopinata*, na planta *Tamus communis* (syn. *Dioscorea communis*), nas imediações da CAL

Gatos Observados na CAL

Apresentava Trombiculídeos



Espécies encontradas

Neotrombicula inopinata

5 animais

Recolhidos na CAL

Organização das cerdas do idiossoma: 2H-(8-9)-(8-9)-(7-8)-6-4-2

Recolhidos em fevereiro 2022

Ericotrombidium ibericense

3 animais

Recolhidos em Santarém

Organização das cerdas do idiossoma: 2H-8-6-6-4-2-2

Recolhidos entre julho 2020 e setembro 2021

Localização dos ácaros trombiculídeos

Discussão e conclusão

Este é o primeiro estudo em Portugal da identificação de ácaros trombiculídeos em gatos de rua e do meio ambiente. Como esta é uma parasitose negligenciada, de difícil deteção, e geralmente os exemplares são mal identificados taxonomicamente quando detetados (estes são classificados como *Neotrombicula autumnalis* por defeito), é de extrema importância a transmissão de informação relativamente a este parasita à comunidade veterinária, sendo, igualmente, um problema de saúde pública e com potenciais riscos para a saúde humana, uma vez que os ácaros são vetores e potenciais vetores de agentes zoonóticos.
É extremamente importante que os clínicos consigam identificar esta parasitose nos animais de companhia, tendo especial atenção àqueles com acesso ao exterior, visto que a presença dos ácaros trombiculídeos pode desencadear apresentações clínicas que variam desde casos assintomáticos até apresentações mais graves. Adicionalmente, mas não menos relevante, o papel dos animais de companhia na manutenção do ciclo de vida destes ácaros em maior proximidade com o ser humano não deve ser negligenciado em virtude da sua baixa especificidade de hospedeiro, sobretudo em animais com acesso à rua e a zonas de grande concentração de vegetação.
Considera-se fundamental a criação de uma lista de espécies de trombiculídeos existentes em Portugal, assim como a avaliação da existência de casos em humanos, de forma a compreender a potencial importância zoonótica da parasitose em Portugal. Mais informação sobre a parasitose deve ser disponibilizada aos médicos veterinários de modo a ser mais facilmente reconhecida e tratada pelos clínicos.

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Deteção de ácaros trombiculídeos (Acari: Trombiculidae) em gatos errantes (*in vivo*) e no meio ambiente (*ex vivo*) em Lisboa e Santarém

Cátia Marques[a,b,c], Pedro Costa[d], João T. Cruz[b,c], Inês Delgado[a,b,c], , Gonçalo Portela[a], Ana Munhoz[a], Helga Waap[a,b,c,e], André Pereira[a], David W. Ramilo[a,b,c]

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A trombiculose é uma parasitose causada pela forma larvar de ácaros trombiculídeos (Acari: Trombiculidae) dando origem a sinais clínicos dermatológicos, gastrointestinais e/ou neurológicos. Estes ácaros podem afetar vários hospedeiros vertebrados, incluindo gatos e o homem, e servir de vetores de agentes patogénicos, como bactérias (*Orientia tsutsugamushi,* transmissível ao ser humano, *Anaplasma phagocytophilum, Bartonella* spp. e *Borrelia* spp.) ou vírus (Hantavírus). Contudo, a informação sobre a sua ocorrência em gatos errantes em Portugal é escassa.
O objetivo deste trabalho consistiu na deteção e identificação de ácaros trombiculídeos em gatos errantes e no meio ambiente circundante de um gatil municipal.
De julho de 2020 a maio de 2022, foram analisados, para a presença de ácaros trombiculídeos, um total de 27 gatos errantes que deram entrada na Casa de Animais de Lisboa (CAL) e três gatos errantes da região de Santarém. Foi também avaliado o meio ambiente ao redor do gatil da CAL. Os ácaros detetados foram recolhidos e conservados em etanol a 70% até observação no laboratório, onde foram montados entre lâmina e lamela, usando meio de Hoyer, para identificação taxonómica.
Foi detetada a presença de ácaros trombiculídeos em 5 gatos errantes da região de Lisboa (18,5%), sendo que todos provinham da mesma colónia. Foi possível detetar a presença de ácaros no pavilhão auricular de todos estes animais e pontualmente em outras localizações, como testículos e comissura ocular. A identificação taxonómica dos ácaros ao nível da espécie revelou a presença de *Neotrombicula inopinata* nos 5 gatos. O gatil municipal da CAL encontra-se localizado na Serra de Monsanto, Lisboa, que se caracteriza por uma vegetação densa. A avaliação do meio ambiente junto do gatil permitiu a deteção de ácaros trombiculídeos da espécie *Neotrombicula inopinata* em plantas da espécie *Tamus communis* (sin. *Discorea communis*). Os três gatos observados em Santarém encontravam-se parasitados por ácaros trombiculídeos da espécie *Ericotrombidium ibericense*.
Esta é a primeira referência em Portugal da deteção de ácaros trombiculídeos em gatos errantes e diretamente do meio ambiente. Sendo uma parasitose negligenciada, de difícil deteção, e onde os espécimes tendem a ser incorretamente classificados a nível taxonómico (estes são habitualmente classificados por defeito como *Neotrombicula autumnalis*) estes resultados demonstram o papel potencial dos gatos errantes no ciclo de vida natural destes ácaros. Adicionalmente, estes animais poderão contribuir para uma maior proximidade entre estes ácaros e os seres humanos, sendo crucial sensibilizar a comunidade veterinária para a sua existência.

Financiamento: Projeto de investigação 2021-2022 da FMV-ULHT (Acrónimo: Trombiculidae); Projeto UIDB/00276/2020 da FCT - Fundação Portuguesa para a Ciência e Tecnologia; CM, ID, HW e DR financiados pelo projeto FCT-LA/P/0059/2020. JTC é bolseiro Ref. UIDB/00276/2020 e MSc22Jul-04.

Appendix VI. Poster and abstract presented at the CIISA Congress Innovation in Animal, Veterinary and Biomedical Research in Lisbon, November 2022

INNOVATION IN ANIMAL, VETERINARY AND BIOMEDICAL RESEARCH

CIISA CONGRESS 2022

Helminths found in wild thrushes (*Turdus* spp.) from Portugal

João T. Cruz[1,2], Luís Madeira de Carvalho[1,2], Jorge Correia[1,2], Alfonso Marzal[3,4], Luís Cardoso[2,5], Isabel Pereira da Fonseca[1,2], David W. Ramilo[1,2,6]

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INTRODUCTION

Thrushes (Passeriformes: Turdidae) (Fig. 1) are small to medium-sized songbirds and one of the most popular groups of game birds in Europe [1,2]. Several helminths have already been detected in *Turdus* spp [3,4]. Since these birds are predominantly migratory and feed mainly on invertebrates (insects, earthworms, terrestrial crustaceans and snails), they can act as pathogen reservoirs to other hosts [3,4,5].

Figure 1 – Thrush species: Common blackbird (*Turdus merula*) **(left)** and the song thrush (*Turdus philomelos*) **(right)**.

Figure 2 – *Turdus merula* specimen with a very poor body condition shown in two different angles.

MATERIAL & METHODS

Four *Turdus merula* and one *Turdus philamelos* (Figs. 1 and 2) corpses were taken to the laboratory for parasitological screening. Parasites collected from the coelomic cavity and intestine were preserved in 70% ethanol until visualization and were: i) observed using a stereomicroscope or; ii) mounted in slides with Hoyer's medium and observed with a compound microscope. Using dichotomous keys, helminths were identified based on their morphology.

RESULTS

Several specimens of *Morishitum* genus were found in the coelomic cavity of *Turdus philomelos* (Fig. 3). Several helminths were identified in *Turdus merula*, including trematodes (*Brachylecithum filum*) (Fig. 4), cestodes (*Passerilepis* spp.) (Fig. 5A), acanthocephalans (*Plagiorhynchus cylindraceus*) (Fig. 5B) and nematodes (*Porrocaecum* spp.) (Fig. 5C and D).

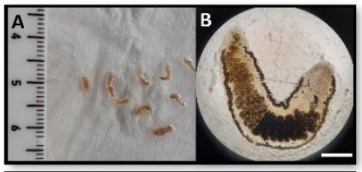

Figure 3 – **A-** *Morishitium* spp. parasites found in the coelomic cavity of *Turdus philamelos*. **B-** *Morishitium* sp. observed with stereomicroscopy. Bar: 134 µm

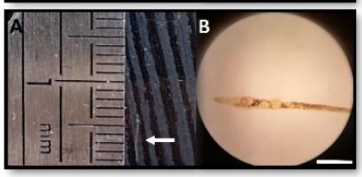

Figure 4 – **A-** *Brachylecithum filum* parasite found in the small intestine of *Turdus merula* (white arrow). **B-** *Brachylecithum filum* observed with compound microscope. Bar: 1.25 mm.

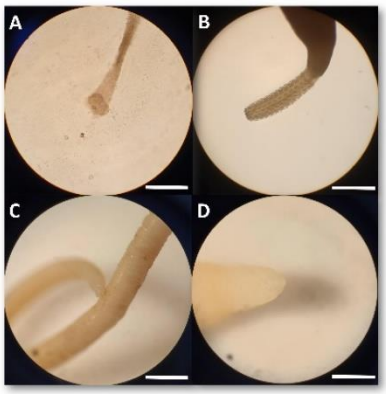

Figure 5 – Parasites found in the intestines of *Turdus merula*. **A-** anterior portion of cestode *Passerilepis* sp. Bar: 500 µm. **B-** Probascis of acanthocephalan *Plagiorhynchus cylindraceus*. Bar: 900 µm. **C-** anterior portion of nematode *Porrocaecum* sp., with a trilobated anterior end, typical of ascarids. Bar: 1.25 mm. **D-** Detail of nematode *Porrocaecum* sp. Bar: 900 µm.

DISCUSSION

Most of the parasites found in this study have intermediary hosts (e.g. molluscs and crustaceans), which are included in thrushes' diet [3,6,7]. The presence of these pathogens can make it difficult for birds to travel long distances during their migration [4]. Even a small number of large-sized parasites may lead to bird's death [4]. To our knowledge, the helminths found in this study are being reported for the first time in our country. Since works regarding parasites in thrushes from Portugal are scarce, this study adds valuable information on the helminthofauna of *Turdus* spp. from our country, being the basis for future epidemiological studies focusing the role of thrushes as dispersals of pathogens to other hosts.

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SUPPORT

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João T. Cruz holds a scientific initiation gran: (Ref. UIDB/00276/2020) and M/SC22/ui-04.

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Helminths found in wild thrushes (Turdus spp.) from Portugal

João T. Cruz[1,2], Luís Madeira de Carvalho[1,2], Jorge Correia[1,2], Alfonso Marzal[3,4], Luís Cardoso[2,5], Isabel Pereira da Fonseca[1,2], David W. Ramilo[1,2,6]

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Introduction: Thrushes (Passeriformes: Turdidae) are small to medium-sized songbirds and one of the most popular groups of game birds in Europe. Several helminths have already been detected in *Turdus* spp. Since these birds are predominantly migratory and feed mainly on invertebrates (insects, earthworms, terrestrial crustaceans and snails), they can act as pathogen reservoirs to other hosts.

Material & Methods: Four *Turdus merula* and one *Turdus philomelos* were taken to the laboratory for parasitological screening. Parasites collected from the coelomic cavity, liver, gallbladder and intestine were preserved in 70% ethanol until visualization and were: i) observed with stereomicroscopy or; ii) prepared in slides with Hoyer's Medium and observed with a compound microscope. Helminths were identified using dichotomous keys based on their morphology.

Results: Several helminths were identified in *Turdus merula*, including nematodes (e.g. *Porrocaecum* spp.), cestodes (e.g. *Passerilepis* spp.), trematodes (e.g. *Brachylecithum filum*) and acanthocephalans (e.g. *Plagiorhynchus cylindraceus*) and a trematode was identified in the coelomic cavity of *Turdus philomelos* (*Morishitium* spp.).

Discussion: Most of the parasites found in this study have intermediary hosts (e.g. molluscs and crustaceans), which are included in thrushes' diet. To our knowledge, the helminths found in this study are being reported for the first time in our country. Since works regarding parasites in thrushes from Portugal are scarce, this study adds valuable information on the helminthofauna of *Turdus* spp. from our country.

Support/interest disclosure: This work was funded by the Portuguese Foundation for Science and Technology (FCT), in the scope of projects UIDB/00276/2020 (CIISA), UIDB/CVT/00772/2020 (CECAV) and LA/P/0059/2020 (AL4AnimalS). J.T.C. holds a scientific initiation scholarship (BIC) and a MSc project grant (MSC22Jul-04) awarded by CIISA.

Keywords: HELMINTHS, PARASITES, PORTUGAL, *TURDUS* SPP.

Appendix VII. Poster and abstract presented at the CIISA Congress Innovation in Animal, Veterinary and Biomedical Research in Lisbon, November 2022

INNOVATION IN ANIMAL, VETERINARY AND BIOMEDICAL RESEARCH

CIISA CONGRESS 2022

Presence of *Phyllobothrium delphini* (Cestoda: Phyllobothriidae) in a striped dolphin (*Stenella coeruleoalba*) stranded in the Tagus estuary (Alcochete, Portugal)

João T. Cruz[1,2], Luís Madeira de Carvalho[1,2], Fernando Afonso[1,2], Marina Sequeira[3], Alfonso Marzal[4,5], David W. Ramilo[1,2,6]

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INTRODUCTION

The world range of striped dolphins (*Stenella coeruleoalba*) extends from warm-temperate to tropical waters and this cetacean is the most frequently dolphin occurring in the Mediterranean Sea [1]. Tetraphyllidean merocercoids have been commonly reported from most cetacean species and some pinnipeds worldwide, including *S. coeruleoalba* [2,3]. Two types of merocercoids have been widely recognized: *Phyllobothrium delphini* (Bosc, 1802), encysted in the subcutaneous blubber, usually in the abdominal area, and *Monorygma grimaldii* (Moniez, 1889), encysted mainly in the peritoneum of the abdominal cavity [3].
Adult worms are found in the spiral valves of elasmobranch and holocephalan fishes, which ingest infected flesh either by predation or by scavenging [4].
The authors report in this work a recent parasitological finding regarding this host and *P. delphini* in Portugal.

Figure 1 - *Stenella coeruleoalba* specimen.

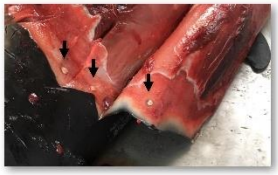

Figure 2 – Parasitic cysts collected from subcutaneous blubber (black arrows).

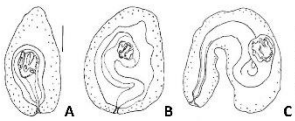

Figure 3 – *Phyllobothrium delphini* morphotypes according to [5].
A - Category "A", all with neck always straight.
B - Category "B", all with curved neck.
C - Category "C", cyst U-shaped or with projections from the cyst wall.
Bar: 1mm.

MATERIAL & METHODS

On 4th April 2019, a *S. coeruleoalba* female specimen was found stranded in Alcochete and was necropsied at the Pathological Anatomy Service of FMV-ULisboa (Fig. 1). Several cystic structures were collected from the subcutaneous blubber of the inguinal area (Fig. 2), preserved in 70% ethanol, and analyzed under a stereoscope. Histological preparations of three of the cysts were made using H&E staining and they were categorized according to Siquier and Le Bas [5] (Fig. 3)

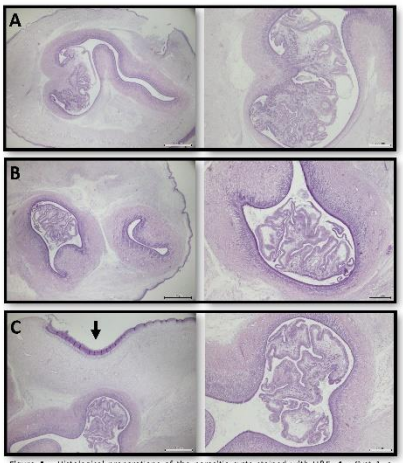

Figure 4 – Histological preparations of the parasitic cysts stained with H&E. **A** - Cyst 1, a morphotype category "B" because of the curved neck **(left)**; Cyst 1 scolex detail **(right)**.
B - Cyst 2, a morphotype category "B" due to the presence of separate scolex and neck in the same transversal cut **(left)**; Cyst 2 scolex detail **(right)**.
C - Cyst 3, a morphotype category "C" due to U-shape of the cyst wall (black arrow) **(left)**; Cyst 3 scolex detail **(right)**.

RESULTS

The observed parasitic cysts had a length between 4-8 mm and were identified as intermediate stages of *P. delphini*. Two morphotype B and one morphotype C were identified (Fig. 4).

DISCUSSION

P. delphini is considered one of the most common parasites of cetaceans. Despite being common, the life cycle of this parasite is not clear and little is known about the biology of its larval forms [5]. Published reports of its presence in *S. coeruleoalba* are available from England, Wales, United States, Spain, Italy and Costa Rica until nowadays [2]. To the best of our knowledge this is the first published reference of *P. delphini* in a striped dolphin from Portugal.
All previous studies with *S. coeruleoalba* in Spain demonstrate a very high prevalence of *P. delphini*. In Galiza it was of 87.5% (n=8) [6] and between Valencia and Múrcia it was 100% (n=11) [3], pointing to a very high expected prevalence in mainland Portugal as well.
This expected high prevalence in our country and the almost non-existent works reporting this parasite species, points to the necessity of additional research in this field, to understand their impact on dolphin health and add more information concerning parasites of these hosts, being of the utmost importance the parasitological information provided by stranded animals.

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SUPPORT

FCT - UIDB/00276/2020 (CIISA) and LA/P/0059/2020 (AL4AnimalS).
João T. Cruz holds a scientific initiation grant (Ref. UIDB/00276/2020) and MSC22Jul 04.

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Presence of Phyllobothrium delphini (Cestoda: Phyllobothriidae) in a striped dolphin (Stenella coeruleoalba) stranded in the Tagus estuary (Alcochete, Portugal)

João T. Cruz[1,2], Luís Madeira de Carvalho[1,2], Fernando Afonso[1,2], Marina Sequeira[3], Alfonso Marzal[4,5], David W. Ramilo[1,2,6]

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Introduction: The range of striped dolphins (*Stenella coeruleoalba*) extends across the warm-temperate to tropical waters of the world and this cetacean is the most frequently occurring dolphin in the Mediterranean Sea. Tetraphyllidean merocercoids have been commonly reported from most cetacean species and some pinnipeds world-wide, including *S. coeruleoalba*. Two types of merocercoids have been widely recognized: *Phyllobothrium delphini* (Bosc, 1802), encysted in the subcutaneous blubber, usually in the abdominal area, and *Monorygma grimaldii* (Moniez, 1889), encysted mainly in the peritoneum of the abdominal cavity. The authors refer to a recent parasitological finding regarding this host and these parasites in Portugal.

Material & Methods: On 4th April 2019, a female *S. coeruleoalba* specimen was found stranded in Alcochete and was necropsied at the Pathological Anatomy Service of FMV-ULisboa. Several cystic structures were collected from the subcutaneous blubber of the inguinal area, preserved in 70% ethanol, and analyzed under a stereoscope.

Results: The observed parasitic cysts had a length between 4-8 mm and were identified as intermediate stages of *P. delphini*.

Discussion: *P. delphini* is considered one of the most common parasites of cetaceans. Despite being common, the life cycle of this parasite is not clear, and little is known about the biology of its larval forms. Published reports of its presence in *S. coeruleoalba* are available from England, Wales, United States, Spain, Italy, and Costa Rica until nowadays. To the best of our knowledge this is the first published reference of *P. delphini* in a striped dolphin from Portugal.

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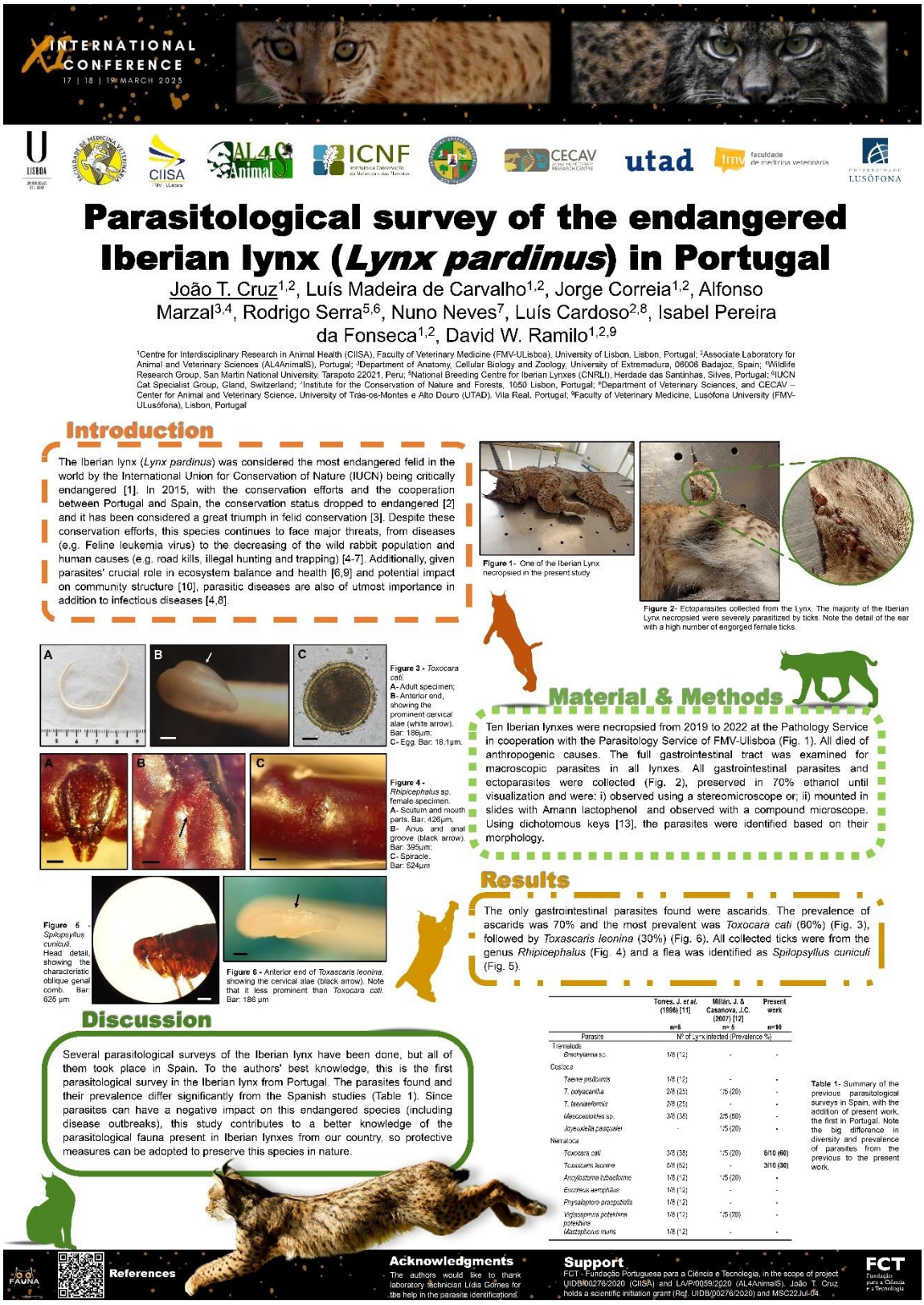
Keywords: HELMINTHS, PARASITES, PORTUGAL, *STENELLA COERULEOALBA, PHYLLOBOTHRIUM DELPHINI.*

Appendix VIII. Poster and abstract presented at the XI FAUNA International Conference in Lisbon, March 2023



Parasite	Torres, J. et al. (1996) [11] n=5	Millán, J. & Casanova, J.C. (2007) [12] n= 5	Present work n=16
	Nº of Lynx infected (Prevalence %)		
Trematoda			
Brachylaima sp.	1/8 (12)	-	-
Cestoca			
Taenia pisiformis	1/8 (12)	-	-
T. polyacantha	2/8 (25)	1/5 (20)	-
T. taeniaeformis	2/8 (25)	-	-
Mesocestoides sp.	3/8 (38)	2/5 (50)	-
Joyeuxiella pasqualei	-	1/5 (20)	-
Nematoda			
Toxocara cati	3/8 (38)	1/5 (20)	6/10 (60)
Toxascaris leonina	5/8 (62)	-	3/10 (30)
Ancylostoma tubaeforme	1/8 (12)	1/5 (20)	-
Excretosoma aemphilus	1/8 (12)	-	-
Physaloptera praeputialis	1/8 (12)	-	-
Vigissynnura poteckhini poteckhini	1/8 (12)	1/5 (20)	-
Mastophorus muris	1/8 (12)	-	-

Parasitological survey of the endangered Iberian lynx (*Lynx pardinus*) in Portugal

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Introduction: The Iberian lynx (*Lynx pardinus*) was once considered the most endangered felid in the world by the International Union for Conservation of Nature (IUCN) with the status of critically endangered. In 2015, with the conservation efforts and the cooperation between Portugal and Spain, the conservation status by the IUCN dropped to endangered and it has been considered a great triumph in felid conservation. Despite these conservation efforts, this species continues to face major threats, from diseases (e.g. Feline leukemia virus) to the decreasing of the wild rabbit population and human causes (e.g. road kills, illegal hunting and trapping).

Material and Methods: Ten Iberian lynxes were necropsied from 2019 to 2022 at the Pathology Service in cooperation with the Parasitology Service of FMV-ULisboa. The full gastrointestinal tract was examined for parasites in all lynxes. All gastrointestinal parasites and ectoparasites were collected, preserved in 70% ethanol until visualization and were: i) observed using a stereomicroscope or; ii) mounted in slides with lactophenol Amann and observed with a compound microscope. Using dichotomous keys, the parasites were identified based on their morphology.

Results: The only gastrointestinal parasites found were ascarids. The prevalence of ascarids was 70% and the most prevalent was *Toxocara cati* (60%), followed by *Toxascaris leonina* (30%). All collected ticks were from the genus *Rhipicephalus* and a flea was identified as *Spilopsyllus cuniculi*.

Discussion/Conclusions: Several parasitological surveys of the Iberian lynx have been done, but all of them took place in Spain. To the authors' best knowledge, this is the first gastrointestinal parasitology survey in the Iberian lynx from Portugal. The parasites found and their prevalence differ significantly from the Spanish studies. Since parasites can have a negative impact on this endangered species (including disease outbreaks), this study contributes to a better knowledge of the parasitological fauna present in Iberian lynxes from our country, so protective measures can be adopted to preserve this species in nature.

Support/interest disclosure: FCT - Fundação Portuguesa para a Ciência e Tecnologia, in the scope of project UIDB/00276/2020 (CIISA) and LA/P/0059/2020 (AL4AnimalS). João T. Cruz holds a scientific initiation grant (Ref. UIDB/00276/2020) and MSC22Jul-04.

Keywords: Iberian lynx, parasites, Portugal

Appendix IX. Poster and abstract presented at the 10th Meeting of Training of the Order of Veterinary Physicians, Lisbon, April 2023

MANEIO CLÍNICO E AMBIENTAL DE INFEÇÃO MISTA POR PARASITAS PULMONARES NUMA POPULAÇÃO DE CORUJAS-BURAQUEIRAS (*Athene cunicularia*) EM CONTEXTO ZOOLÓGICO

Marques, G.N.[1*]; Pinto, M.G.F.[2,3]; Cruz, J.[2,3]; Leal, M.O.[1]; Urbani N.[1]; Flanagan C.A.[1]; Madeira de Carvalho, L.[2,3]

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INTRODUÇÃO

As corujas-buraqueiras (*Athene cunicularia*) são aves com distribuição ampla pelo continente americano, cujo estado de conservação é definido pelo IUCN como pouco preocupante[1]. Estas aves de rapina de pequeno porte têm hábitos diurnos e utilizam frequentemente tocas no solo como refúgio [figura 1]. A população de corujas-buraqueiras residente no Zoomarine encontra-se num habitat de imersão *outdoor* [figura 2], adaptado às diferentes espécies habitantes de aves, répteis e mamíferos. Como parte da coleção zoológica, estes indivíduos estão incluídos num programa de medicina preventiva, sem histórico clínico relevante até ao desenvolvimento súbito e sequencial de sinais clínicos respiratórios (dispneia, taquipneia, ruídos respiratórios, espirros), em alguns indivíduos da população.

MANEIO CLÍNICO

A abordagem clínica multimodal incluiu exame de estado geral, hemograma, análises bioquímicas, exame radiográfico e colheita de amostras biológicas (zaragatoa traqueal, cloacal e fezes) para bacteriologia e coprologia. Os achados clínicos incluíram, não de forma generalizada em todos os indivíduos, enfisema subcutâneo, leucocitose, eosinofilia e compressão da silhueta radiográfica dos sacos aéreos abdominais. A observação do esfregaço fecal [figura 3] permitiu diagnosticar uma provável infeção parasitária mista por *Syngamus trachea* e *Cyathostoma* sp. sendo os primeiros ovos elipsoidais, com um opérculo em ambos os pólos, e os segundos de forma ovóide, com 8-16 blastómeros e sem opérculos. Após declarado o óbito a um dos indivíduos que apresentou um quadro hiperagudo de dispneia, o exame *post mortem* permitiu colher parasitas nematodes adultos a nível pulmonar, que foram submetidos para identificação parasitológica. Os nematodes colhidos eram pertencentes à família Syngamidae e foram identificados como pertencentes à espécie *Cyathostoma* (*Hovorkonema*) *americana*. Este achado é bastante interessante, pois para além da identificação da espécie, os espécimes foram colhidos de uma localização anormal em nódulos pulmonares, quando por norma são sempre descritos apenas na traqueia ou sacos aéreos.

A abordagem médica foi individual de acordo com o quadro clínico apresentado por cada espécime. O tratamento antiparasitário consistiu na administração oral de fenbendazol (50 mg/kg PO SID, 3 a 5 dias), conjugado em alguns casos com ivermectina (0,2 mg/kg PO, administração única, 5 a 10 dias após o protocolo terapêutico de fenbendazol). O tratamento de suporte, quando necessário, baseou-se em oxigenoterapia, antibioterapia (enrofloxacina, 30 mg/kg PO SID), analgesia (meloxicam, 1 mg/kg PO SID) e nebulizações com F10® SC (1:250), aminofilina (3 mg/mL de NaCl 0,9%) e/ou gentamicina (6 mg/mL de NaCl 0,9%).

O potencial foco de infeção parasitária foi identificado como sendo um grupo de indivíduos silvestres da espécie garça-boieira (*Bubulcus ibis*) que comumente repousavam sobre as redes do habitat de imersão, atraídos ao local por elementos da mesma espécie pertencentes à coleção zoológica. Apesar das medidas de metafilaxia adoptadas e a revisão do protocolo de higienização dos esconderijos subterrâneos onde as corujas habitam, a taxa de recidiva de infeção manteve-se elevada. A resolução clínica completa da infeção na população de corujas foi atingida apenas após a transferência da população de *B. ibis* residente e consequente afastamento natural da população de aves silvestres da mesma espécie do habitat de imersão.

CONCLUSÃO

Os parasitas *Syngamus trachea* e *Cyathostoma* spp. têm como hospedeiros definitivos diversas espécies de aves, que se infetam através da ingestão de ovos, larvas ou hospedeiros paraténicos. Estes parasitas não fazem comumente parte dos diagnósticos diferenciais infeciosos em aves da ordem Strigiformes, sendo esta a primeira descrição de infeção em corujas-buraqueiras[2]. Este relato realça a possibilidade de parasitismo oportunista, caso se proporcione o contacto próximo destes animais com aves silvestres, de forma contínua e prolongada. Assim, é importante considerar a existência de fontes externas de excreção e contaminação parasitárias como parte do maneio de infeções parasitárias em aves em contexto zoológico, cujo habitat possibilite o acesso de aves silvestres a zonas com coleções aviárias suscetíveis de se infetarem.

Figura 1
Corujas-buraqueiras (*Athene cunicularia*) e esconderijos onde habitam.

Figura 2
Habitat de imersão "Américas".

Figura 3
Esfregaço fecal: provável infeção por *Syngamus trachea* e *Cyathostoma* sp.

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Maneio Clínico E Ambiental De Infeção Mista Por Parasitas Pulmonares Numa População De Corujas-Buraqueiras (*Athene Cunicularia*) Em Contexto Zoológico

Marques, G.N [1]; Pinto, M.G.F. [2,3]; Cruz, J.T. [2,3]; Leal, M.O. [1]; Urbani, N. [1]; Flanagan, C.A. [1]; Madeira de Carvalho, L. [2,3]

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As corujas-buraqueiras (*Athene cunicularia*) são aves com distribuição ampla pelo continente americano, cujo estado de conservação é definido pelo IUCN como pouco preocupante. A população de corujas-buraqueiras residente no Zoomarine encontra-se num habitat de imersão outdoor, adaptado às diferentes espécies habitantes de aves, répteis e mamíferos. Como parte da coleção zoológica, estes indivíduos estão incluídos num programa de medicina preventiva, não tendo até à data histórico clínico relevante. Alguns indivíduos da população desenvolveram subitamente e sequencialmente sintomatologia respiratória, cuja abordagem clínica multimodal incluiu exame de estado geral, patologia clínica, imagiologia, bacteriologia e coprologia. Os achados clínicos incluíram enfisema subcutâneo, leucocitose, eosinofilia e alterações radiográficas pulmonares e dos sacos aéreos. A observação do esfregaço fecal permitiu diagnosticar uma provável infeção parasitária mista por *Syngamus trachea* e *Cyathostoma* sp. O foco de infeção foi identificado como sendo um grupo de indivíduos silvestres da família Ardeidae que comumente repousavam sobre as redes do habitat de imersão, atraídos ao local por elementos da mesma espécie pertencentes à coleção zoológica. A abordagem médica foi individual de acordo com o quadro clínico apresentado por cada espécime. O tratamento antiparasitário consistiu na administração oral de fenbendazol, conjugado por vezes com ivermectina. O tratamento de suporte, quando necessário, baseou-se em oxigenoterapia, antibioterapia, analgesia e nebulizações com F10®, aminofilina e/ou gentamicina. Apesar das medidas de metafilaxia adoptadas e revisão do protocolo de higienização dos esconderijos subterrâneos onde estes animais habitam, a taxa de recidiva de infeção manteve-se elevada. A resolução clínica completa da infeção na população de corujas foi atingida apenas após a transferência da população Ardeidae residente e consequente afastamento natural da população de aves silvestres da mesma espécie do habitat de imersão. Parasitas como *Syngamus trachea* não fazem comumente parte dos diagnósticos diferenciais infeciosos em aves da ordem Strigiformes. No entanto, este relato realça a possibilidade de parasitismo oportunista, caso se proporcione o contacto próximo destes animais com aves silvestres, de forma contínua e prolongada. É importante considerar a existência de fontes externas de excreção parasitária como parte do maneio de infeções parasitárias em aves em contexto zoológico, cujo habitat possibilite o acesso de aves silvestres.

Appendix X. Article Reviewer Certificate

REVIEW CONFIRMATION CERTIFICATE

We are pleased to confirm that

João Cruz

has reviewed 4 papers for the following MDPI journals in 2024:

Animals, Veterinary Sciences, Journal of Zoological and Botanical Gardens

Shu-Kun Lin

Dr. Shu-Kun Lin, Publisher and President
Basel, 30 August 2024

MDPI

MDPI is a publisher of open access, international, academic journals. We rely on active researchers, highly qualified in their field to provide review reports and support the editorial process. The criteria for selection of reviewers include: holding a doctoral degree or having an equivalent amount of research experience; a national or international reputation in the relevant field; and having made a significant contribution to the field, evidenced by peer-reviewed publications.

Appendix XI. PowerPoint presentation of the externship final project presented at the South Florida Wildlife Centre, January 2023

Description of the Red-Shouldered Hawk Syndrome

João Tomás Cruz

Why I chose this project

- Very high number of red shouldered hawks with the same neurologic presentation and no apparent cause
- Nothing being published about this disease despite the very high prevalence and mortality
- Contribute to the better understanding of this disease, its etiology, treatment, prevention and impact in other species and human heath
- The first step to that is to describe the disease

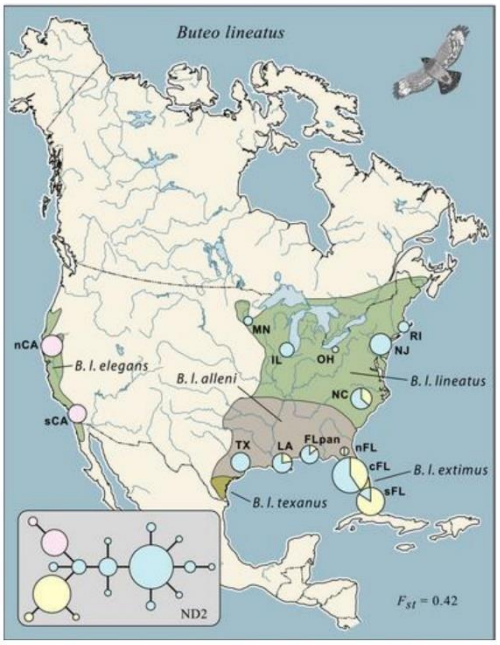
The Red-shouldered Hawk

- Habitat: forests, deciduous swamps. Sometimes suburban areas or mangroves.
- Diet: Mostly small mammals, lizards, snakes, amphibians. Occasionally small birds.
- Nesting: Reuse nests from past years. Often near water and can be in neighborhoods/parks.

The red-shouldered hawk- subspecies

Belongs to the species *Buteo lineatus* and has 5 suspecies:

- *B. l. lineatus*– east North America
- *B. l. alleni* – central south Texas to North Carolina and north Florida (USA)
- *B. l. extimus* – south Florida and the Florida Keys (USA)
- *B. l. texanus*– south Texas (USA) to southeast Mexico
- *B. l. elegans* – south Oregon (USA) to Mexico



George F. Barrowclough *et al* 2019

B. l. lineatus

B. l. extimus

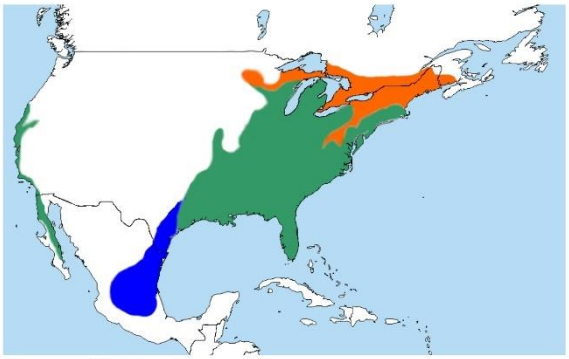
B. l. alleni

B. l. texanus

B. l. elegans

The red-shouldered hawk- range

- It is a permanent resident throughout most of its range
- Only northernmost populations are considered migratory, and the Wintering range is mostly considered to be central Mexico.

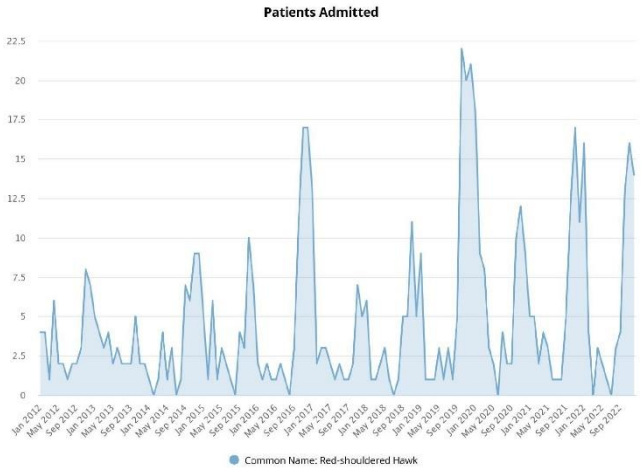


Range of *B. lineatus*

- Breeding range
- Year-round range
- Wintering range

The red-shouldered hawk- range

- Using the data of the intakes from the center in the last 10 years, we can clearly show that we do have a permanent resident population, but the great majority of intakes are in the winter and belong to migratory hawks.



Intakes in SFWC in the last 10 yeras

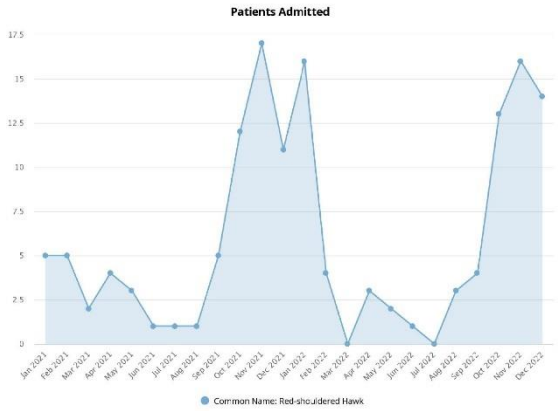
Methods

- Analyzed all cases of 2021 and 2022. N= 143
- Two control groups:

1. Control with similar signs
2. Control without similar signs

- Two Syndrome groups:

1. Mild Syndrome cases
2. Exuberant Syndrome cases



Syndrome presentation

- Generalized paresis (weakness)
- Mental inappropriateness (mostly Obtunded)
- Sternal recumbency to hock siting
- Cervical ventroflexion (cervical retroflexion in rare cases)
- Decreased palpebral response
- Fluctuation between symptomatic to apparently normal
- No signs of trauma

Variables

Positioning and general condition variables:

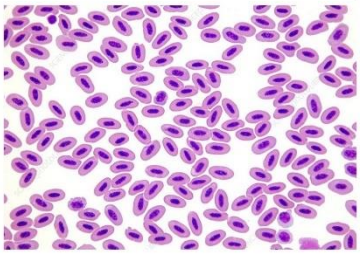
- Sternal
- Hock sitting
- Head Ventroflexed
- Head Retroflexed
- Torticollis
- Normal position
- Atitude
- Blink

- Lower eyelid edema
- Evidence of trauma
- Age
- Body condition
- Dehydration
- Mucous membranes Color
- Mucous membranes texture
- Fecal parasites
- Treatments
- Days in care
- Outcome

Blood Work Variables

Separated the blood results in:

- Blood work in peak clinical signs
- Blood work with less signs than intake
- Blood work without clinical signs



Statistical Analysis

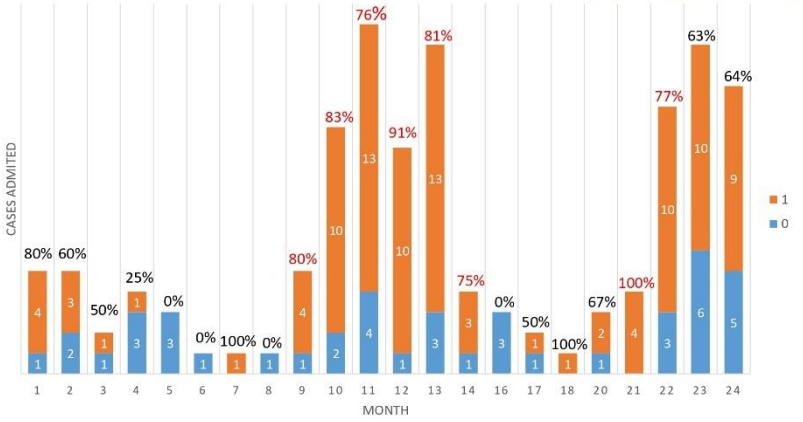
DISCLAIMER

The following slides contains a lot of numbers, graphs and tables and could be considered boring

Do your best not to fall asleep and I'll do my best to summarize the information

Statistical Analysis: cases by month

- The total prevalence was 70%
- The cases peak between September to January in both years
- Higher prevalence in the peaks- pointing to a higher prevalence in the migratory population



Statistical Analysis: body position

	Sternal	Hock sitting	Ventroflexed	Retroflexed	Torticollis
C. Similar	77%	8%	15%	0%	15%
C. Not Similar	8%	0%	0%	0%	0%
Mild Syndrome	25%	17%	8%	0%	0%
Syndrome	74%	19%	67%	1%	2%

- Possible biased to positioning and mental state variables- since that's the criteria to classify as part of the Syndrome
- Sternal- similar between the syndrome and control
- Hock sitting- higher prevalence in the syndrome

- Head Ventroflexed- good identifier of syndrome with high prevalence
- Head Retroflexed- Very low prevalence but seems unique to the syndrome
- Torticollis- seems good indicator of HPAI+ and differs from syndrome

Statistical Analysis: atitude

- C. similar- most prevalent attitude is Depressed but with little amount of Obtunded or Stuporous
- C. not similar- Mostly alert animals
- Mild syndrome- majority depressed
- Syndrome- highest prevalence of Obtunded animals

	C. Similar	C. not similar	Total Controls	Mild Syndrome	Syndrome
Alert	15%	83%	48%	8%	1%
Quiet	15%	8%	12%	25%	14%
Depressed	38%	8%	24%	50%	28%
Obtunded	8%	0%	4%	17%	39%
Stuporous	8%	0%	4%	0%	15%
Non-responsive	15%	0%	8%	0%	3%
Total	100%	100%	100%	100%	100%

Statistical Analysis: blink and lower eyelid edema

	Blink Altered	Lower eyelid edema	Count
Controls	20%	12%	26
Mild Syndrome	30%	33%	12
Syndrome	85%	48%	87
Total	69%	35%	125

- Clear highest prevalence of blink and lower eyelid abnormalities in the Syndrome cases
- Still relevant but not so evident in the mild cases

Statistical Analysis: CBC

- WBC- increased in all syndrome cases besides the asymptomatic and the peak (Leukocytosis)
- Segmented- increased with signs (Heterophilia)
- Presence of band Heterophils in all syndrome variables
- Eosinophils increased in the peak syndrome cases
- Decreased Lymphocytes
- Trombocytes, Toxic heterophils changes and presence of Haemoproteus more common in syndrome cases

		PCV	TP	WBC	Segmented	Band	Eosinophils	Basophils	Lymphocytes	Monocytes	Thrombocytes	Polychromasia	Anisocytosis	Toxic Heterophils	Haemoproteus	Count
NORMAL VALUES		37-48	2,5-4,5	8,2-12,0	4,62-6,60	0	0,33-1,99	0,21-0,72	2,05-4,32	0,21-0,48	0	0	0	0	0	
CONTROL		34,500	3,540	10,480	6,540	-	1,027	0,105	2,243	0,566	-	0,800	-	0,400	-	6,000
MILD SYNDROME		39,571	3,925	12,633	7,812	-	1,305	0,316	2,548	0,674	-	1,167	-	-	100%	8,000
SYNDROME	Average	37,523	4,189	12,320	6,431	0,835	1,816	0,337	2,500	1,164	0,158	1,056	-	0,727	42,1%	45,000
	BW w/o signs	35,941	4,317	11,129	5,520	1,165	1,558	0,360	1,925	1,388	0,235	1,313	-	0,643	41,2%	18,000
	BW less signs	39,158	4,072	14,074	7,798	0,281	1,845	0,367	3,425	0,946	0,111	0,882	-	0,750	38,9%	19,000
	BW at peak	37,000	4,163	9,700	4,559	0,291	2,781	0,065	1,261	0,841	-	0,667	-	1,000	66,7%	8,000

Statistical Analysis: Biochemistry

- No AST increase and CK is only mildly increased in the peak- rule out trauma
- Increased Albumin, Bile acids and potassium
- Decreased uric acid and globulins (artifact?)

		AST	CK	ALB	BA	GLU	CA	P	K	Na	UA	GLOB	Count
NORMAL VALUES		198-640	338-3420	0,6-1,9		297,7-425,5	9,14-13,83	2,91-16,41	1,8-3,2		8,03-49,15	2,5-3,1	
CONTROL		471,800	2 843,60	1,700	-	333,400	9,400	4,800	2,900	152,600	0,720	1,950	6,000
MILD SYNDROME		478,714	2 059,86	2,133	-	316,143	9,750	4,417	4,467	156,286	0,617	1,867	8,000
SYNDROME	Average	547,116	2 802,57	1,983	0,690	332,833	9,693	3,562	3,312	154,571	2,251	1,967	45,000
	BW w/o signs	507,167	2 339,61	2,071	1,647	310,611	9,239	3,733	3,759	156,588	1,728	2,082	18,000
	BW less signs	603,611	2 980,82	1,989	0,056	342,000	10,135	3,247	2,994	152,944	2,117	1,778	19,000
	BW at peak	504,571	3 560,14	1,757	-	372,000	9,786	3,886	3,043	153,857	3,943	2,171	8,000

Statistical Analysis: Outcome- Death

- No deaths in the mild syndrome cases
- 53% of deaths in the exuberant syndrome cases
- Total death in syndrome cases around 50%

- Patients that die, normally die in the first 2 days
- Average of 23 days in care for the released patients

DIED	YES	No	Total
Mild Syndrome	0%	100%	9%
Syndrome	53%	47%	91%
Total Geral	48%	52%	100%

DIED (Syndrome)	YES	NO
Patient days in care	2.37	22.5
Count	46	50

Statistical Analysis: Logistic Regression Models to Predict Death

Model 1

- The significance has to be <0.1 to indicate a variable is a good predictor of death
- In this model only the Glucose value is significant
- For every Glucose unit increase, the chances of dying increase 3.4%
- With a 87% of correct death prediction

Variables in the Equation — <0.1

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	Sternal_I_Position	.371	1.629	.052	1	.820	1.450
	Hock sitting_I_Position	1.388	2.196	.399	1	.528	4.005
	Head_Ventroflexed_I_Position	1.144	1.392	.676	1	.411	3.141
	Nit Blink	-1.508	1.448	1.084	1	.298	.221
	Stuporous	25.225	40192.970	.000	1	.999	9014645005 1.786
	exams_weight	-.002	.008	.037	1	.848	.998
	CK_value	.000	.000	1.120	1	.290	1.000
	GLU_value	.033	.016	4.309	1	.038	1.034
	Constant	-13.937	7.128	3.823	1	.051	.000

a. Variable(s) entered on step 1: Sternal_I_Position, Hock sitting_I_Position, Head_Ventroflexed_I_Position, Nit Blink, Stuporous, exams_weight, CK_value, GLU_value.

Classification Table[a]

			Predicted		
			DIED		Percentage Correct
	Observed		0	1	
Step 1	DIED	0	30	2	93.8
		1	3	4	57.1
	Percentage				87.2

Statistical Analysis: Logistic Regression Models to Predict Death

Model 2

- In this model the sternal position, weight and Obtunded mentation were significant
- The odds of a sternal hawk dying are 11x (1117%)
- The odds of dying are 3x (300%) higher for Obtunded hawks
- Increasing the weight by 1 unit (1 gram) will result in 0.5% increase in the odds of dying
- This model predicts the outcome with 70.2% of correct predictions

Variables in the Equation — <0.1

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	Sternal_I_Position	2.413	.931	6.720	1	.010	11.173
	Hock sitting_I_Position	.662	1.045	.401	1	.526	1.939
	Head_Ventroflexed_I_Position	.705	.546	1.666	1	.197	2.023
	Not Blink	-.196	.594	.108	1	.742	.822
	exams_weight	.005	.003	3.385	1	.066	1.005
	Obtunded	1.100	.542	4.118	1	.042	3.003
	Constant	-5.295	1.883	7.906	1	.005	.005

a. Variable(s) entered on step 1: Sternal_I_Position, Hock sitting_I_Position, Head_Ventroflexed_I_Position, Nit Blink, exams_weight, Obtunded.

Classification Table[a]

			Predicted		
			DIED		Percentage Correct
	Observed		0	1	
Step 1	DIED	0	30	13	69.8
		1	12	29	70.7
	Overall Percentage				70.2

Take home messages

summary for the sleepy ones

- This is a real disease and not just undiagnosed trauma cases
- Prevalence of 70% in all 2021-2022 cases
- Head ventroflexed is the best position to suspect a syndrome case and torticollis is the best to suspect HPAI instead
- Seasonal and more prevalent in the migratory population
- Cities are more prevalent than others (Plantation, Hollywood and Boca)
- Inflammatory response (WBC, Seg, Band, Eo, Toxic Hets., Haemoproteus)
- No Biochemistry signs of trauma but increased Albumin, Bile acids and potassium
- Death rate is around 50%
- Patients that die, normally die in the first 2 days
- Good death predictors- Glucose (increase), sternal position, Obtunded mentation and Weight (increase)

Appendix XII. Abstract of the oral communication presented at the XVIII Congreso Nacional y XV Iberoamericano de Etología y Ecología Evolutiva, November 2023

Prevalence, genetic diversity, geographic distribution and clinical findings of haemoparasites causing avian malaria in wildlife centers of Portugal

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Malaria parasites and other emerging infectious diseases pose significant challenges to global health. Despite significant research on *Plasmodium* spp. parasites, there is still a gap in understanding their diversity, specificity, virulence, and current distribution in Europe. Additionally, more limited knowledge exists about haemoparasites belonging to the *Haemoproteus* and *Leucocytozoon* genera. Avian species, especially migratory birds, serve as crucial reservoir hosts for malaria parasites, making them valuable indicators of ecosystem health giving insights into disease incidence and its distribution in Europe, including Portugal, being especially important in the actual context of climate change. The Wildlife rehabilitation centers play a vital role in studying avian haemoparasites and assessing preventative measures against arthropod vectors. Reintroduction projects for rare or threatened bird species face risks due to potential pathogen transmission. Furthermore, climate change and increasing temperatures are expected to impact haemoparasite prevalence, distribution, vector dynamics, and bird populations health. Blood samples were collected from Wildlife Rehabilitation Centers in Portugal and analyzed using PCR methods and blood smear examinations at the Faculty of Biology of the University of Extremadura in cooperation with the Faculty of Veterinary Medicine of the University of Lisbon. This study aims to investigate the prevalence, genetic diversity, and geographic distribution of haemoparasites causing avian malaria, while comparing haemoparasite prevalence with various host characteristics and the potential relationships between haemoparasitism and clinical findings, contributing to the knowledge and practice of Conservation Medicine in Wildlife Rehabilitation Centers. By combining molecular and microscopic analyses, it's possible to identify cryptic parasite species and to correlate morphological features with species identification. The results contribute to a comprehensive understanding of avian haemoparasites, enhance disease surveillance efforts, and support avian conservation strategies in wildlife species with limited haemoparasites data.

Appendix XIII. Article published in Animals as the primary author

animals

MDPI

Article

Avian Haemosporidian Infection in Wildlife Rehabilitation Centres of Portugal: Causes, Consequences, and Genetic Diversity

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Simple Summary: Over thirty percent of bird species are undergoing population declines and are threatened with extinction in Portugal. Several reasons have been proposed to explain this decrease, such as the impact of human activities on natural environments and pathogens affecting the health of wildlife, domestic animals, and humans. Wildlife rescue and rehabilitation centres play an essential role in the conservation of endangered species. Despite wildlife rehabilitation centres providing valuable information on disease prevalence and transmission, the information on haemosporidian infection is still very scarce for birds admitted in these centres. In this study, we discovered new malaria parasites in birds admitted to wildlife rehabilitation centres in Portugal. We also revealed infection in bird species that were previously unknown to be infected with malaria parasites. Birds admitted to rehabilitation due to debilitating disease were more frequently infected with malaria. Furthermore, we demonstrate that the malaria infection extends the required period for medical treatment in these birds, which imposes additional economic costs for the rehabilitation and reduces the survival probabilities of the bird. These findings stress the importance of the study of malaria parasites in wildlife rehabilitation centres, also helping to design protocols and interventions to preserve endangered species.

Abstract: In the last decade, over 40% of bird species in Europe have experienced poor and bad conservation status, with more than 30% of bird species in mainland Portugal threatened with extinction. Along with anthropogenic factors, parasites and pathogens such as avian haemosporidians have been suggested to be responsible for these avian population declines. Wildlife rehabilitation centres play an essential role in species conservation and preservation. Moreover, animals admitted for rehabilitation can provide valuable information regarding transmission and pathogenicity of many diseases that affect wild birds that are rarely sampled in nature. However, reports of haemosporidians in captive birds are still limited. Here, we explored the prevalence and genetic diversity of avian haemosporidians in 89 birds from 29 species admitted to rehabilitation centres in Portugal, showing an overall infection prevalence of 30.3%. The prevalence of infection was higher in Strigiformes and in birds admitted to rehabilitation centres due to debilitating diseases. Remarkably, 30% of the infected bird species have not been found to harbour malaria parasites in preceding studies. We detected 15 different haemosporidian lineages

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infecting a third of bird species sampled. Notably, 2 out of these 15 detected haemosporidian lineages have not been obtained previously in other studies. Furthermore, we also identified nine new host–parasite interactions representing new host records for these haemosporidian parasites. Finally, our results revealed that birds infected with haemosporidians require longer rehabilitation treatments, which increase the economic costs for rehabilitation and may impair their survival prospects. These findings emphasise the importance of integrating haemosporidian infection considerations into rehabilitation protocols, highlighting the challenges posed by these infections in avian conservation and rehabilitation, including economic and logistical demands.

Keywords: avian malaria; wildlife veterinary medicine; avian conservation; treatment duration; *Haemoproteus*; *Leucocytozoon*; *Plasmodium*

1. Introduction

The global-scale decrease in animal populations represents one of the most dramatic consequences of human impacts on the planet, where 48% of animal species are undergoing population declines [1]. This situation is dramatic for some groups of vertebrates, such as birds, where 5412 out of the 11,162 analysed species (48,5%) face reductions in population size in both temperate and tropical regions [1,2]. For example, the proportion of birds having poor and bad conservation status and the number of avian species threatened with extinction have increased in Europe in the last decade [3,4]. Even though population trends in common bird species in Europe have revealed a change in abundance since the 1980s, the decline is not equally distributed among all countries [5]. In mainland Portugal, there are 404 confirmed wild bird species [6]. In 2022, the conservation status of 287 bird species was evaluated, revealing that 95 of them were classified as threatened with extinction (Vulnerable, Endangered, or Critically Endangered) [7]. This marks an increase from the 88 species of birds classified as threatened in 2005 [8].

Studies analysing long-term data of bird populations have proposed that anthropogenic factors are responsible for these avian population declines. For example, agricultural intensification, in particular, the use of pesticides and fertilisers, is one of the main pressures for invertebrate feeders [5]. Also, the offspring production of migratory and larger-bodied avian species has been negatively affected by the rising temperatures associated with climate change [9]. Moreover, forest degradation has led to broad-scale declines for most forest bird species in Canada [10]. Parasites and pathogens are also major threats facing bird species that can cause rapid declines in wild bird populations. For example, *Trichomonas gallinae* emerged as a novel pathogen of finches in Britain in 2005 and rapidly became epidemic, leading to a decrease in 2007 in breeding populations of greenfinch (*Carduelis chloris*) and chaffinch (*Fringilla coelebs*) by 35% and 21%, respectively [11]. Furthermore, the highly pathogenic avian influenza H5 epidemic has caused the highest number of casualties among wild birds ever recorded in Europe [12].

Avian malaria and related haemosporidian parasites (Apicomplexa; order Haemosporida; *Plasmodium*, *Haemoproteus* and *Leucocytozoon* spp.) represent a highly diverse group of haemoparasites, with over 5100 parasite lineages documented to infect nearly 2300 bird species (MalAvi database Version 2.5.8, 24 October 2023, [13]). They are commonly referred as "avian malaria" due to the malaria-like clinical signs they often cause [14,15]. These parasites are transmitted exclusively by blood-sucking dipteran insects [16]. Culicidae mosquitoes are responsible for the transmission of avian *Plasmodium* parasites, whereas *Haemoproteus* parasites are transmitted by biting midges (Ceratopogonidae) and louse flies (Hippoboscidae), and *Leucocytozoon* are vectored by black flies (Simuliidae) [17]. Although avian haemosporidian parasites were considered as relatively benign for a long time (see review in [18]), they have been reported to negatively affect the host condition [19,20], clutch size [21], reproductive success [19,21–

23], and lifespan [24], subsequently diminishing host fitness [25–27]. The clinical signs and pathology of bird haemosporidian infection include fever, anoxia, tissue necrosis (e.g., liver and spleen), acute anaemia, pneumonia-like symptoms, and excessive enlargement of the spleen and liver that can lead to organ rupture [16]. Moreover, haemosporidian infections can also lead to a reduction in haematocrit levels that may result in death [28,29] and to an increase in stress proteins (heat shock proteins) [30].

Wildlife rehabilitation centres involve the treatment and temporary care of injured, diseased, and displaced indigenous animals, and the subsequent release of healthy animals to appropriate habitats in the wild [31]. In these centres, an adequate treatment and assessment of prognosis is relevant to promote a quick and effective release into the wild. For these reasons, the length of stay in rehabilitation centres (the difference between the date of admission and the date when the stay of the individual in the rehabilitation centre was terminated) has been proposed as an estimator of the cost of the rehabilitation process (cost in staff, food, and medicines) and a useful tool for the evaluation of wildlife rehabilitation centres [32]. However, this parameter is barely reported in studies of wildlife rehabilitation (see [32–34] for some exceptions). Furthermore, even though individuals admitted to rehabilitation centres are frequently parasitised by blood parasites [35–37], the impact of avian haemosporidian infections on the length of stay in the rehabilitation centres is largely unknown. Moreover, wild birds maintained in captivity are an excellent model for parasite research and provide valuable results for wildlife management conservation of endangered of poorly studied species [36,37]. Important advances in the study of avian haemosporidians, such as the discovery of the life cycle of some avian haemosporidians [16], the disclosure of the negative impact of the parasite infection on the health of their hosts [16,38], or the development of many anti-parasite drugs and chemotherapies [18] have been possible thanks to the use of individuals from zoos and rehabilitation centres. Despite this importance, reports of haemosporidians in captive birds are still scarce, with only 277 avian haemosporidian linages recorded in captive birds, which represents 6% of the identified haemosporidian lineages available in the MalAvi database (Version 2.5.8, October 2023, [13]).

Here, we present a molecular-based study to explore the infection by haemosporidian parasites in birds admitted to wildlife rehabilitation centres in mainland Portugal. Our main objectives were (1) to assess the prevalence and genetic diversity of avian haemosporidians in rescued birds; (2) to analyse the factors explaining variation in haemosporidian infection; and (3) to evaluate the association of the haemosporidian infection and the number of days those wild birds admitted to the rehabilitation centre required medical treatment.

2. Materials and Methods

2.1. Sample Collection

The study was conducted in three wildlife rehabilitation centres in mainland Portugal: (1) Centre for Studies and Rehabilitation of Wild Animals of Castelo Branco (CERAS) in Castelo Branco (39°49′27.6″ N 7°27′15.1″ W), (2) Wildlife Rehabilitation Centre of Santo André (CRASSA) in Setúbal (38°04′27.0″ N 8°46′56.8″ W), and (3) Wildlife Rehabilitation and Research Centre of Ria Formosa (RIAS) in Faro (37°02′03.1″ N 7°48′45.7″ W).

From November 2022 to May 2023, we collected blood samples from birds admitted to rehabilitation centres upon intake at the centres and from birds already undergoing medical treatment. Throughout that period, 89 wild birds were sampled, belonging to 29 distinct species spanning across 13 taxonomic orders. For each individual, a small blood volume (50 µL) was collected in heparinised microcapillaries by puncturing the brachial vein and stored at 4 °C in 0.5 mL of SET-buffer (0.015 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) until molecular analysis. The Eppendorf tubes with blood samples in SET-buffer were stored in the refrigerator at 4 °C until they were sent to the Faculty of Biology of the

University of Extremadura, Badajoz, Spain, for further processing and analysis. The body condition of each individual was evaluated upon admission through palpation of pectoral muscle mass and assessment of the prominence of the keel bone, graded on a scale of 1 to 5. A score of 1 indicated extreme emaciation, while a score of 5 indicated overweight condition [39]. The prepatent period is the elapsed time from the inoculation of sporozoites by the vector into the bird until the appearance of blood stages. This period varies from 11–21 days for *Haemoproteus*, approximately five days for *Leucocytozoon*, and possibly as short as five days in the case of *Plasmodium relictum* [16]. Fifty-six birds were sampled at the admission on the rehabilitation centres or before the prepatent period of haemosporidian parasites was over, whereas the remaining 33 individuals were sampled when they were already housed in the centres for longer periods. Hence, for statistical analyses exploring the effect of haemosporidian infection on the required medical treatment for birds admitted to a rehabilitation centre and the factors influencing haemosporidian infection probability, we only considered birds sampled in the centre for less than the corresponding prepatent period of the observed parasite infecting the bird (n = 56), therefore ensuring that all analysed infections were acquired in the wild and not in the wildlife rehabilitation centre. All samples (n = 89) were considered for the identification of parasite lineages and lineage–host interactions.

2.2. Sample Processing and Molecular Determination of Haemosporidian Infection

Blood samples were examined using molecular methods to determine the presence of and genetically characterise haemosporidian parasite lineages at the Faculty of Biology of the University of Extremadura. DNA was extracted from all the samples preserved in SET-buffer using a MAGMAX PATHOGEN RNA/DNA KIT (Applied Biosystems™, Waltham, MA, USA, reference: 4462359). The DNA was also quantified, using a NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific™, Waltham, MA, USA) and diluted to 25 ng/µL. The DNA was then stored at −20 °C until further examination.

For the genetic analysis of haemosporidian infection, a nested-PCR protocol was conducted to amplify a segment of the mitochondrial cytochrome b gene of haemosporidians, as described by Bensch et al. [40] and Hellgren et al. [41]. This technique enables the screening of haemosporidians within the *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* genera [41]. In the first PCR, two general haemosporidian primers, HaemFNI and HaemNR3, were utilised to amplify DNA from all three haemosporidian genera [41]. In the second PCR, a specific set of primers were used for *Haemoproteus* and *Plasmodium* spp. (HaemF and HaemR2) and for *Leucocytozoon* spp. (HaemFL and HaemR3L) [41]. We performed an additional nested PCR, following the procedure of Waldenström et al. [42], to address some methodologic problems found in three samples, namely, the false positives for *Haemoproteus/Plasmodium* in samples intensely infected with *Leucocytozoon*. In these additional nested PCRs, we used two specific primers to *Haemoproteus* and *Plasmodium* in the first PCR: HaemNF and HaemNR2. In the second PCR of these samples, we used the same primers as described above (HaemF and HaemR2).

The PCR mix for all parasites was carried out in a final volume of 25 µL, including 15.9 µL of water, 2.5 µL of Buffer (10× Ex Taq Buffer TaKaRa, Shiga, Japan), 2.5 µL of dNTPs (dNTP Mix TaKaRa, Shiga, Japan), 1 µL of each primer, and 2 µL of DNA. All PCR assays contained 1 negative control (sterilised water) for every 8 samples, and 2 positive controls (samples from infected birds, confirmed by microscopy) for every 24 samples. The PCRs were conducted using a SimpliAmp™ Thermal Cycler (Applied Biosystems™, Foster City, CA, USA), following the thermal profile of Hellgren et al. [41]. The amplification was evaluated by running 2.5 µL of the final PCR on a 2% agarose gel. Haemosporidians detected by a positive amplification were sequenced using the procedures described by Bensch et al. [40]. Sequences were edited and aligned using the program BioEdit [43]. The 'Basic Local Alignment Search Tool' (Blast) of GenBank

(Accessed 28 July 2023) and the MalAvi database (Version 2.5.8, October 2023, [13]) were used to determine the lineage of detected parasite sequences. Parasites with sequences differing by at least 1 nucleotide from the already described lineages of both databases were considered new evolutionary independent lineages [40].

New lineages (sequences not previously published in GenBank) were also sequenced from the reverse end using the primers HaemR2 or HaemR3L to confirm that they were unique. New lineages were coded following the nomenclature of the MalAvi database [13] and deposited in GenBank under the accession numbers PP457803-PP457804 (Supplementary Table S1). New host–parasite relationships were established by comparison of our results with the public database (MalAvi database Version 2.5.8, October 2023, [13]) showing avian haemosporidian lineages (based on mitochondrial cytochrome b lineages) and host range. A new host record was established when the avian haemosporidian lineage had not been previously reported infecting this bird species, as has been done in previous studies [44,45].

2.3. Statistical Analysis

A General Linear Model (GLM) was used to analyse the relationship between body condition, haemosporidian infection (infected vs. uninfected), rehabilitation centre (CERAS, CRASSA and RIAS), and the season of admission (winter, spring, and autumn) on the number of days requiring medical treatment for birds admitted to the rehabilitation centre. The dependent variable was log-transformed to fit a linear model. A logistic regression analysis was used to explore whether avian taxonomic order, body condition, rehabilitation centre (CERAS, CRASSA and RIAS), the season of admission to rehabilitation centres (winter, spring or summer), and the reasons of admission to rehabilitation centres (debilitating disease, trauma, or other causes of admission) influenced haemosporidian infection probability (infected/uninfected). A backward stepwise procedure was used to eliminate all non-significant terms ($p > 0.05$) from our starting maximal model. All analyses were performed using PASW Statistics 22 statistical package for Windows.

3. Results

3.1. Prevalence and Genetic Diversity of Haemosporidian Parasites

A total of 89 bird individuals belonging to 29 bird species were screened for haemosporidian parasites. Twenty-seven out of the 89 individuals were infected with haemosporidians (overall prevalence = 30.3%). Of these sampled birds, 19.1% were infected with *Haemoproteus*, 13.5% were infected with *Leucocytozoon*, and 1.1% were infected with *Plasmodium*. Additionally, three individuals (3.6% of prevalence) harboured mixed infections with *Leucocytozoon* and *Haemoproteus* (Table 1).

The overall prevalence of haemosporidian infection of the 56 birds sampled at the time of admission to the rehabilitation centres or before the prepatent period of haemosporidian parasites was over was 41.1%—25% prevalence for *Haemoproteus*, with a 16.1% prevalence for *Leucocytozoon*—and 1.8% prevalence for *Plasmodium* (Table 1).

We detected 15 different parasite lineages infecting 10 out of the 29 avian species sampled (n = 89). Among these, we found nine *Leucocytozoon* lineages infecting seven bird species, five *Haemoproteus* lineages infecting five bird species, and one *Plasmodium* lineage infecting one bird species (Supplementary Table S1). By comparison of genetic diversity of haemosporidian parasites, we showed that two out of the 15 lineages from our study had not been previously recorded in former studies (Supplementary Table S1). Moreover, we found that three out of the nine bird species tested positive for infection in this study had not been previously documented as infected by haemosporidian parasites in molecular studies (Table 1). Furthermore, we also identified nine new host–parasite interactions, which represent new bird host records for these haemosporidian parasites (Supplementary Table S1).

Table 1. Number of individuals uninfected, and infected with *Haemoproteus* (H), *Plasmodium* (P), and *Leucocytozoon* (L), per bird species. The information on migratory behaviour from each species (Migratory (M), Resident (R), or Undetermined (U)) obtained from [46] is also shown. Numbers in brackets represent the number of individuals for each bird species that were sampled at the time of admission on the rehabilitation centres or before the prepatent period of haemosporidian parasites was over. An asterisk (*) after bird species denotes that these bird species were not previously documented to be infected by haemosporidians (according to (MalAvi database Version 2.5.8, October 2023, [13]), where the symbol $ in *Haemoproteus* infection (H) indicates mixed infection with *Leucocytozoon*.

Bird Species	Bird Order	Migratory	Uninfected	H	P	L	Total
Accipiter gentilis	Accipitriformes	R	0	0	0	1 (0)	1 (0)
Aegypius monachus	Accipitriformes	R	3 (0)	0	0	0	3 (0)
Alca torda	Charadriiformes	M	1 (1)	0	0	0	1 (1)
Anas platyrhynchos	Anseriformes	R	1 (1)	0	0	0	1 (1)
Ardea cinerea	Pelecaniformes	M	0	0	0	1 (1)	1 (1)
Asio flammeus	Strigiformes	M	1 (0)	0	0	0	1 (0)
Bubo bubo	Strigiformes	R	0	1 $ (1 $)	0	4 (3)	4 (3)
Bubulcus ibis	Pelecaniformes	R	1 (1)	0	0	0	1 (1)
Burhinus oedicnemus	Charadriiformes	R	1 (1)	0	0	0	1 (1)
Buteo buteo	Accipitriformes	R	1 (0)	0	0	1 (1)	2 (1)
Ciconia ciconia	Ciconiiformes	R	10 (2)	0	0	0	10 (2)
Falco tinnunculus	Falconiformes	R	2 (0)	0	0	0	2 (0)
Fratercula arctica	Charadriiformes	M	1 (1)	0	0	0	1 (1)
Gallinula chloropus	Gruiformes	R	1 (1)	0	0	0	1 (1)
Garrulus glandarius	Passeriformes	R	0	1 $ (0)	0	1 (0)	1 (0)
Gyps fulvus	Accipitriformes	R	2 (2)	0	0	0	2 (2)
Larus fuscus *	Charadriiformes	M	6 (4)	8 (8)	0	0	14 (12)
Larus michahelis *	Charadriiformes	R	17 (10)	5 (4)	1 (1)	0	23 (15)
Larus sp.	Charadriiformes	U	2 (2)	0	0	0	2 (2)
Milvus migrans	Accipitriformes	M	1 (0)	0	0	0	1 (0)
Milvus milvus	Accipitriformes	R	3 (2)	0	0	0	3 (2)
Morus bassanus	Suliformes	M	1 (1)	0	0	0	1 (1)
Phalacrocorax carbo	Suliformes	M	1 (1)	0	0	0	1 (1)
Streptopelia decaocto *	Columbiformes	R	0	0	0	1 (1)	1 (1)
Strix aluco	Strigiformes	R	1 (0)	1 $ (1 $)	0	3 (3)	4 (3)
Sturnus unicolor	Passeriformes	R	1 (0)	0	0	0	1 (0)
Tachybaptus rufficolis	Podicipediformes	R	1 (0)	0	0	0	1 (0)
Tyto alba	Strigiformes	R	2 (1)	0	0	0	2 (1)
Upupa epops	Bucerotiformes	R	2 (2)	0	0	0	2 (2)
Total			63 (33)	16 (14)	1 (1)	12 (9)	89 (56)

3.2. Factors Determining the Length of Medical Treatment

The length of medical treatment required by the birds admitted to the rehabilitation centres differed according to the haemosporidian infection and the season when they were admitted (Table 2). Specifically, haemosporidian-infected birds significantly required longer treatments than uninfected individuals (mean days of treatment (SD): infected = 28.22 (56.89); uninfected = 9.85 (16.81)) (Figure 1). Also, birds admitted to rehabilitation centres during winter (n = 28) received medical care for longer time periods than birds admitted during spring (n = 25) or autumn (n = 3) (mean days of treatment (SD): winter = 30.43 (51.55); spring = 4.24 (11.98); autumn = 6.50 (11.67)) (Figure 2).

Table 2. Factors explaining variation in the number of days those wild birds admitted to the rehabilitation centre required treatment. A General lineal model was used with body condition, haemosporidian infection, rehabilitation centre (locality), and the season of admission to rehabilitation centres as predictor variables. Sample size was 56 individuals. Significant factors are highlighted in bold.

Variable	Type III SS	d.f.	F	*p*
Body condition	0.017	1	0.038	0.846
Haemosporidian infection	2.172	1	4.747	**0.034**
Rehabilitation centre	0.010	1	0.021	0.884
Season	6.112	1	13.358	**0.001**

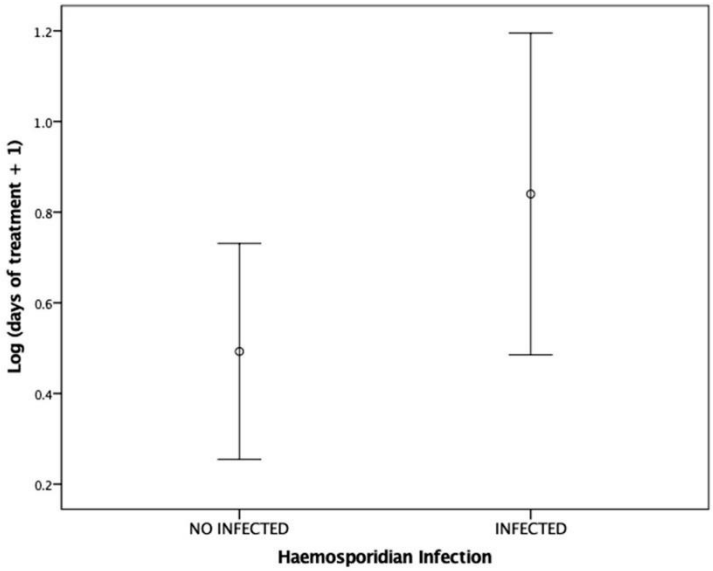

Figure 1. Error bar plots (mean ± 95% CI) showing the number of days (log transformed) that haemosporidian-infected (n = 23) and -uninfected (n = 33) birds admitted to rehabilitation centre required medical treatment.

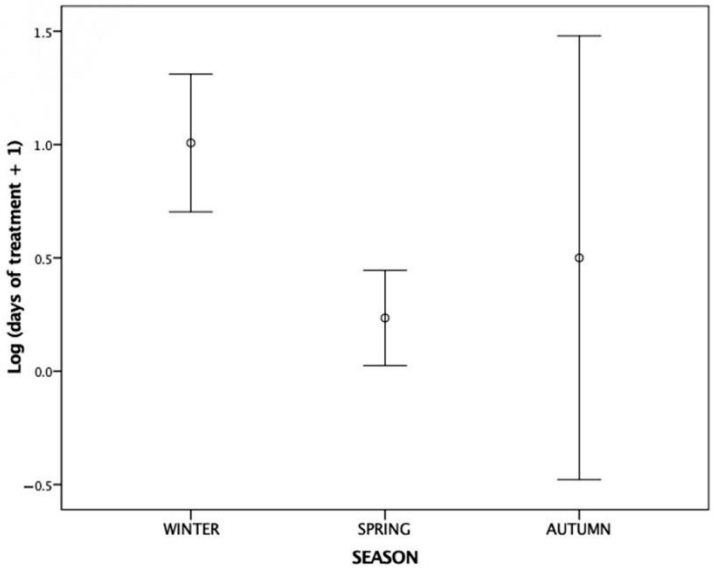

Figure 2. Error bar plots (mean ± 95% CI) showing the number of days (log transformed) requiring medical treatment for birds admitted to rehabilitation centre with respect to the season when they were admitted: winter (n = 28), spring (n = 25), and autumn (n = 3).

3.3. Factors Determining Haemosporidian Infection

We also analysed haemosporidian infection in relation to body condition, avian taxonomic order, the rehabilitation centre where birds were admitted, the season of admission to rehabilitation centres, and the reasons for admission to rehabilitation centres. Avian taxonomic order significantly explained variation in haemosporidian infection (Table 3). Notably, six out of seven (85.7%) of Strigiformes showed haemosporidian infection. Also, 40.6% of Charadriiformes were infected with haemosporidian parasites, whereas only 20% of birds belonging to the order Accipitriformes showed haemosporidian infection (Table 1). Remarkably, the order Strigiformes was also the only order having double infections (*Leucocytozoon* and *Haemoproteus*). In addition, the prevalence of haemosporidian infection also varied with the reasons for admission to rehabilitation centres (Table 3). Birds admitted due to debilitating diseases had a significantly higher prevalence of haemosporidian infection (52.2%, n = 23) compared to cases of admission due to physical trauma (27.6%, n = 31), with the remaining two individuals admitted for undetermined reasons.

Table 3. Factors explaining variation in the probability of haemosporidian infection. A backward stepwise procedure was used in a logistic regression analysis with avian taxonomic order, body condition, rehabilitation centre (locality), the season of admission to rehabilitation centres, and the reasons for admission to rehabilitation centres as predictor variables. Only independent variables selected by the stepwise procedure are listed. Significant factors are highlighted in bold.

Variable	*B*	S. E.	Wald	d.f.	*p*	Exp (B)
Avian taxonomic order	0.180	0.091	0.943	1	**0.047**	1.197
Reason for admission	−1.256	0.524	5.741	1	**0.017**	0.285
Constant	0.442	0.868	0.259	1	0.611	1.555

4. Discussion

Over a third of the approximately 500 bird species living in Europe are threatened or have a poor conservation status [4]. In mainland Portugal, more than 30% of bird populations have been recently categorised as threatened with extinctions [7]. Hence, preserving and restoring avian populations is one of the cornerstones of EU biodiversity policy. Wildlife rehabilitation is an undervalued and potentially useful tool for stabilising some declining populations and could be targeted to support in situ interventions [47]. In this line, rehabilitation centres and other captive breeding facilities play an essential role in species conservation and preservation. Here, we analysed the prevalence and genetic diversity of avian haemosporidians in birds admitted to rehabilitation centres in Portugal to explore the factors explaining variation in haemosporidian infection and to evaluate features influencing the length of required treatment. Our main findings were: (i) about 40% of birds were infected with haemosporidians, also showing a great genetic diversity of haemosporidian lineages and a high number of new host–parasite associations; (ii) birds infected with haemosporidians required longer medical treatments during their stay in the rehabilitation centres; and (iii) the prevalence of avian haemosporidian infection was higher in Strigiformes and in those admitted to rehabilitation centres due to debilitating diseases. Next, we will discuss these results in detail.

4.1. Prevalence and Genetic Diversity of Haemosporidian Parasites

We showed an overall haemosporidian prevalence of 41% in the wild birds sampled. This probability of infection is similar to those found in recent studies using molecular methodologies to analyse haemosporidian infections in birds admitted to rehabilitation centres. For example, Nourani et al. [48] examined the infection with haemosporidian parasites in captive raptors in two rehabilitation facilities in North and Northeast Iran, determining an overall prevalence of 36%. Likewise, Pornpanom et al. [49] reported a haemosporidian prevalence of 34% in 12 owl species in a raptor rehabilitation unit in Thailand. Also, Gomes et al. [35] explored the occurrence of blood parasites in wild birds from a wildlife rehabilitation centre in Central Portugal, revealing that 48% of sampled birds were positive for haemosporidians.

Wildlife rehabilitation likely generates millions of animal records annually worldwide [50]. Yet, despite an increasing acknowledgment of the usefulness of wildlife rehabilitation centre data, it remains a prevailing tendency to underutilise this valuable source of information [51]. Moreover, rehabilitation centres provide an excellent opportunity to explore the host range, genetic diversity, and geographic distribution of haemosporidian infections in wild birds which are rarely sampled in nature [52,53]. For example, Gomes et al. [35] have recently described the first occurrence of *Leucocytozoon* sp. in the booted eagle *Hieraaetus pennatus*, the short-toed snake eagle *Circaetus gallicus*, and the European honey buzzard *Pernis apivorus*, and *P. relictum* in the European honey buzzard. Also, Pornpanom et al. [49] reported 17 new lineages of haemosporidian parasites in owls from Southern Asia. Here, we detected 15 haemosporidian lineages infecting a third of bird species sampled. Importantly, we compared our sequences with those in the MalAvi database (Version 2.5.8, October 2023, [13]) and discovered that two out of these 15 detected haemosporidian lineages had not been obtained previously in other studies. Moreover, of these 15 identified haemosporidian lineages, only five (ATNO1, CIAE02, STAL2, GAGLA05, and LINN1) had previously been reported in Portugal, with STRURA03, COCOR02, and STAL3 being also first documented in the Iberian Peninsula, and STAL5 representing the first report outside of Turkey. Furthermore, 30% of the infected bird species had not been found to harbour malaria parasites in preceding studies. Such numbers of newly discovered lineages and the new records of bird hosts infected with blood parasites suggest that the diversity of avian haemosporidians infecting some species of Strigiformes, Charadriiformes, Columbiformes, Pelecaniformes, and Accipitriformes has been insufficiently investigated.

In addition, our analyses also revealed nine new bird–parasite interactions, thus identifying new host records for these haemosporidian parasites. These new bird–haemosporidian associations are made up of the two newly described haemosporidian lineages (ARCIN01 and LARFUS01), plus seven parasite lineages previously identified as infecting alternative hosts. Some of these new host–parasite associations are worthy of being underlined. First, we found a *Leucocytozoon* parasite (GenBank acc. Number OL897562) infecting *Bubo bubo* that has been previously identified in pooled samples of the blackfly *Simulium meridionale* captured throughout Mississippi, USA [54]. Second, *Streptopelia decaocto*, an avian host that had not been reported as infected by haemosporidians in previous studies, was found infected by *Leucocytozoon* ATNO1, a parasite lineage that had been previously recorded exclusively infecting the little owl (*Athene noctua*) (MalAvi database Version 2.5.8, October 2023, [13]). Finally, the other detected haemosporidian lineages had been identified in related hosts in previous studies. For example, the *Haemoproteus* lineage (GenBank acc. Number ON950078) infecting *L. fuscus* and *L. michahellis* was recently described in other larid hosts [55], the *Leucocytozoon* lineage MILVUS02 is commonly found in other Accipitriformes [56,57], and *Plasmodium matutinum* LINN1 is a generalist haemosporidian lineage found infecting species of a wide range of avian orders, including Charadriiformes (MalAvi database Version 2.5.8, October 2023, [13]). The new diversity records on host–parasite interactions provided in this study will be valuable for detecting host range and transmission areas of haemosporidian parasites, and will improve our knowledge of the mechanisms of adaptation of avian haemosporidians to new hosts. However, we should be cautious in the interpretation of these new records because these new host–parasite interactions may not fully determine the competence of these avian hosts supporting development of infective stages (gametocytes in avian hosts) that can reach a new host [58,59]. In this line, some haemosporidian parasites may infect avian hosts without completing their full life cycle, hence leading to abortive development in these dead-end hosts. Because the amplification of parasite DNA by PCR does not distinguish gametocytes from asexual parasite stages [60], further studies examining blood smears to detect the presence of gametocytes circulating in peripheral blood are needed to complement our findings and determine the competence of these avian hosts to transmit these parasites. Moreover, microscopy is a fast and cheap methodology that allows the quantification of parasitemias and to detect mixed infections, which are sometimes difficult to find with molecular screening. Furthermore, although low-intensity avian malaria infections could be difficult to detect solely by microscopic examination of blood smears, the combined use of PCR and traditional microscopy could be especially relevant to connect genetic lineages with morphospecies and describe new species of haemosporidians [60].

4.2. Factors Determining the Length of Medical Treatment

The process of wildlife rescue and rehabilitation encompasses the rescue, treatment, and care of injured, sick, or orphaned native animals, with the goal of their release into their natural habitat or a more suitable environment [31]. However, a prolonged period in captivity can result in loss of survival skills in wildlife [61]. In this line, Cope et al. [62] have recently conducted a global systematic review and meta-analysis evaluating the factors influencing the success of wildlife rehabilitation, concluding that shorter periods of rehabilitation enhance the probabilities of survival of released animals after the treatments. Wild birds admitted to rehabilitation centres worldwide are frequently parasitized by haemosporidians [35,63,64], although whether haemosporidian infection may extend the rehabilitation period in wild birds remains largely unknown. Our findings revealed that birds infected with haemosporidian required longer periods of medical treatment than non-infected birds, which may affect survival up to release or survival post-release [62]. Therefore, the initial diagnosis of haemosporidian infections in wild birds admitted to rehabilitation centres becomes crucial for an early assignation of a

correct anti-malaria treatment that could minimise their length of stay in the centre and thus enhance their survival prospects.

Rehabilitation centres are often self-funded or heavily subsidising their own rehabilitation work [47,65], hence facing constraints due to insufficient funding, staff availability, and access to appropriate veterinary care [66]. Beyond the mentioned increased fitness benefits to the wildlife of shorter rehabilitation periods, a timely assessment and treatment of haemosporidian infection can also have an economic impact on the rehabilitation centre, as it may promote the effective use of limited resources. In this line, the length of medical treatment has been proposed as an indicator of resource usage [67]. The daily cost per animal in a wildlife rehabilitation centre has been estimated at EUR 0.19 [32]. According to the mean values of the length of stay of haemosporidian-infected and non-infected birds from our study (28.22 days and 9.85 days, respectively), the average expenses of rehabilitation of a non-infected bird can be estimated at approximately EUR 1.9, whereas these costs rise to EUR 5.4 for an infected bird, representing an additional cost of EUR 3.5 per animal.

Our results also show a significant increase in the duration of medical treatment of the birds admitted to rehabilitation centres during the winter months. This finding could be attributed to the reluctance to release rescued birds into the wild during winter, when seasonal environmental conditions are less favourable [68], consequently prolonging their stay in the centres. Alternatively, because metabolic disorders are associated with high recovery times in wild birds [32], the extended period of rehabilitation during winter can also be explained by the higher metabolic costs associated with thermogenesis in winter [69,70]. The large variation in days of treatment showed in Figure 2 for autumn can be explained because birds admitted to rehabilitation during that season are released either quickly, before winter, or held over winter [68]. However, these data should be interpreted with caution because of the low number of sampled birds admitted to rehabilitation centres during that season (n = 3).

4.3. Factors Determining Haemosporidian Infection

Our findings showed differences in the prevalence of infections among avian orders in birds brought to wildlife centres in Portugal, where birds from the orders Strigiformes and Charadriiformes showed the highest probabilities of being infected. Several factors have been proposed to explain why some bird species are prone to becoming infected with haemosporidians. For example, colonial bird species (such as most species of Charadriiformes), or those with larger body sizes or prolonged stays of their nestlings on the nests, usually show a high prevalence of infections [16]. Also, these differences in haemosporidian prevalence have been suggested to be determined by vector preferences [71] or host behaviour characteristics [27,71,72]. For example, the higher prevalence of haemosporidians in owls compared to diurnal birds of prey has been well documented [73–76], and it has been attributed to two primary factors. First, Strigiformes have nocturnal behaviour, which coincides with the crepuscular or nocturnal hours in which mosquito vector species perform host-seeking behaviour [74,77]. Second, their preference for concealed and shaded perches during the day, as well as their nesting sites, may expose Strigiformes more frequently to a variety of haemosporidian vector species [78].

Finally, we showed that the prevalence of infection was higher in birds admitted to the rehabilitation centres due to debilitating diseases than in birds admitted for other causes. Several experimental studies have demonstrated that haemosporidian parasites may impair the physiology of their avian hosts, provoking anaemia [79], the blockage of brain capillaries [80], a diminished body condition, a decrease in fat reserves and atrophy of pectoral muscles [81], and reduced haematocrit [82]. All these negative effects may explain the observed association between infection and debilitating disease in birds. Alternatively, a poor body condition, inadequate nutritional status, or heightened stress levels in birds may compromise their immune system [83,84], and thus increase the likelihood of haemosporidian infection of these debilitated birds [16].

5. Conclusions

This study assessed the prevalence and genetic diversity of haemosporidian parasites in birds at wildlife rehabilitation centres in mainland Portugal, also analysing their effect on the required rehabilitation period and the factors explaining their infection. We have revealed newly discovered parasite lineages and new records of bird hosts infected with blood parasites, thus confirming that the diversity of avian haemosporidians is still insufficiently investigated in some avian species. In addition, these findings are also relevant because host-switching of blood parasites is relatively frequent among birds housed in zoos and rehabilitation centres, provoking fatal infections [85,86]. We have also identified that Strigiformes and birds admitted to rehabilitation due to debilitating disease showed the highest probabilities of being infected with haemosporidians, highlighting the reciprocal relationship between debilitating state and blood parasite infection. Moreover, our study sheds light on the largely unknown impact of avian haemosporidian infections on the length of stay in the rehabilitation centres. Haemosporidian-infected individuals required nearly three times more days in veterinary care compared to non-infected counterparts, impairing their survival prospects and exacerbating resource constraints in wildlife rehabilitation centres. These findings emphasise the need for integrating analyses of haemosporidian infection into diagnostic and treatment protocols, also highlighting the importance of blood sampling the same day of admittance to the rehabilitation centre. Moreover, the seasonal variations observed in the length of veterinary treatment needed, particularly during winter months, stress the importance of adaptive management strategies that account for seasonal fluctuations in rehabilitation demands. In light of our results, some additional recommendations are indicated for further studies exploring factors influencing the haemosporidian infection, such as larger sample sizes and incorporating data from summer infections. Also, repeated blood sampling of individuals beyond the day of admittance to the rehabilitation centre would allow assessment of the effectiveness of treatment and monitoring of whether birds are infected during their stay in the centre. Because the study of blood parasites is also relevant to control parasite infections in birds before translocation or liberation [87], the insights gained from this study have significant implications for avian conservation and wildlife rehabilitation efforts. By highlighting the challenges posed by haemosporidian infections in avian conservation and rehabilitation, this study emphasises the importance of future research endeavours aimed at enhancing our understanding of avian health and guiding conservation strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani14081216/s1, Table S1: MalAvi parasite lineages, Parasite, genus (H *Haemoproteus*, P *Plasmodium*, L *Leucocytozoon*), GenBank accession numbers, recorded host in this study (Host), and alternative hosts and alternative location in which parasite lineages were previously recorded.

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Appendix XIV. Article co-authored published in Animals

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Article

The Impact of Avian Haemosporidian Infection on Feather Quality and Feather Growth Rate of Migratory Passerines

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Simple Summary: Feathers are essential for a bird's flight, insulation, communication, and camouflage. They degrade over time, so birds must moult regularly. This study examined how avian haemosporidian infection and the size of the uropygial gland affect feather quality and growth rate in two migratory bird species in southwestern Spain—the house martin (*Delichon urbicum*) and the sand martin (*Riparia riparia*). We found that house martins had the highest haemosporidian infection rates, likely due to their large colony size. Infection only decreased feather quality in house martins and did not affect the feather growth rate in any of the two hirundinids. Additionally, feather growth rate was positively linked to feather quality, but only in house martins. Finally, we found no connection between the uropygial gland size and feather quality or feather growth rate. These results show, for the first time, that avian haemosporidian parasites can negatively impact the feather quality of migratory birds, thus potentially affecting their flight and survival. Further research is needed to fully understand these relationships.

Abstract: Bird feathers have several functions, including flight, insulation, communication, and camouflage. Since feathers degrade over time, birds need to moult regularly to maintain these functions. However, environmental factors like food scarcity, stress, and parasite infections can affect feather quality and moult speed. This study examined the impact of avian haemosporidian infection and uropygial gland volume, as well as feather quality and feather growth rate in two migratory hirundine species captured in southwestern Spain—the house martin (*Delichon urbicum*) and sand martin (*Riparia riparia*). Our findings showed that the prevalence of infection varied among species, with house martins having the highest rates, possibly due to their larger colony size. Moreover, haemosporidian infection had a different impact on each species; infected house martins exhibited lower feather quality than healthy individuals, although this outcome was not observed in sand martins. Furthermore, no effect of infection on feather growth rate was observed in both hirundinids. Additionally, feather growth rate only correlated positively with feather quality in house martins. Finally, no link was observed between uropygial gland volume and feather quality or feather growth rate in any of the species in this study. These findings highlight the effect of haemosporidian infections on the plumage of migratory birds, marking, for the first time, how avian haemosporidian infection is shown to adversely impact feather quality. Even so, further research is needed to explore these relationships more deeply.

Keywords: feather growth rate; feather quality; *Haemoproteus*; haemosporidian parasites; *Leucocytozoon*; moult; *Plasmodium*; uropygial gland

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1. Introduction

Feathers are essential for birds, greatly assisting them in flight by covering the wings with aerodynamically efficient structures and forming an aerofoil-shaped body [1]. The flight-oriented feathers of the wings are called remiges, which are divided into primaries, secondaries, and tertiaries; those that cover the gaps at the base of remiges are termed coverts or tectrices. Also, tail feathers are typically flight-oriented as well and are called rectrices [1]. Besides locomotion, feathers also perform other critical functions in birds (see review in [2]). For example, the colour and shape of plumage largely determine the appearance of birds, which is intimately linked to camouflage and to both intra- and interspecific communication [3]. In addition, modifications on the structure of feathers aid in buoyancy and waterproofing, hence allowing many groups of birds to inhabit aquatic and marine environments [4,5]. Moreover, some specialized feathers in facial discs can help some Strigiformes to amplify and localize sounds in total darkness, improving their hunting capabilities [6].

However, feathers are lost or continuously damaged due to mechanical abrasion, alterations of the physical structure of keratin during sunlight exposure, ectoparasites consuming feather material, or keratin-degrading bacteria [7–9]. With the aim of keeping the plumage in optimal condition and maintaining its functionality, worn feathers need to be periodically replaced, a process termed moulting. Because new feathers are fully developed and functional long after old feathers are shed, birds unavoidably experience a temporary reduction in plumage function during the moulting period [1], which may impair their survival due to increase predation vulnerability [10], reduction in aerobic scope [11], and/or a decrease in thermoregulatory capabilities [7]. Hence, the fast replacement of feathers would minimize the time of reduced plumage functionality. As moult duration is linked to the feather growth rate [12], selection might therefore favour birds with increased feather growth rate. This could be especially relevant in bird species showing time constraints in their annual cycle, such as long-distance migrants, which may accelerate the moult by increasing the growth rate of individual feathers [1].

Feather quality is also critical for bird fitness, due to its close relationship with feather abrasion resistance [13], which is crucial for intensive locomotor activities such as long-distance migration. In this sense, it has been proposed that long-distance migrants probably suffer more feather wear, particularly due to sunlight [1]. It has been demonstrated that feathers of suboptimal quality often led to reduced efficiency in flight, foraging, and mating [14–17]. Moreover, the quality of feathers, including their strength, flexibility, and resistance to wear, is pivotal for the survival and reproductive success of birds [18]. Furthermore, poor-quality feathers may show a weakened feather structure that can lead to feather breakage [19], which may impair efficient escape from predators [20]. Also, [21] revealed that the flight performance of the trans-Saharan migrant barn swallows (*Hirundo rustica*) is negatively associated with the number of holes in the wings' flight feathers, which is one of the main indicators of the functional quality of these feathers [22].

Bird malaria and related haemosporidian parasites (genera *Plasmodium*, *Haemoproteus,* and *Leucocytozoon*) are a diverse group of protozoans with widespread global distribution that infect bird species from a wide range of taxonomic orders [23,24]. Their life cycles are obligately heteroxenous, comprising sexual reproduction stages within the dipteran vector, whereas asexual reproduction takes place on a vertebrate host [23,25]. Because of their diversity, abundance, and wide geographical distribution, empirical and experimental studies on avian malaria and related haemosporidians nowadays provide a distinctive animal model for comprehending the ecology and evolution of vector-borne diseases [26]. An infection with haemosporidian parasites provokes detrimental effects on several traits of their avian hosts, resulting in reduced survival [27–30], impaired body condition [31,32], and decreased reproductive success [33,34], among others. However, the negative impact of avian haemosporidians on some other host traits has not yet been addressed. For example, although some studies have analysed whether parasites may provoke adverse effects on bird plumage [35–37], the number of studies exploring the effects of haemosporidian

infection on the feather growth rate and feather quality of birds is still limited to a reduced number or species, and the results remain inconclusive [38–41]. For instance, Fithian [38] explored the relationship between haemosporidian infection and feather reflectance in adult prothonotary warblers (*Protonotaria citrea*). While no significant correlation was found between infection status and visible light reflectance or hue, haemosporidian-infected birds tended to exhibit lower levels of UV reflectance. Also, Marzal et al. [39] revealed a detrimental association between avian haemosporidian infection and the inferred growth rate of the tail feathers of house martins (*Delichon urbicum*), showing a lower feather growth rate in haemosporidian-infected birds. Moreover, Marzal et al. [40] also found that house martins harbouring co-infections with two haemosporidian lineages exhibited the lowest inferred growth rate in their tail feathers when compared to uninfected and single-infected individuals, although no effect of haemosporidian co-infection was observed on the feather quality of birds. Additionally, they found a negative correlation between feather quality and feather growth rate, suggesting a trade-off between both traits. Furthermore, Coon et al. [41] inoculated *Plasmodium* in house sparrows (*Passer domesticus*), experimentally demonstrating that haemosporidian infection reduced the feather growth rate.

The uropygial gland (also named *preen gland*) is a holocrine gland located in the integument above the posterior free caudal vertebrae of most bird species [42]. Uropygial gland secretion is mainly composed of a wide variety of substances, such as waxes, alcohols, terpenes, and fatty acids [43]. Among other functionalities, it has been proposed that its secretion plays an important role in maintaining feather integrity and plumage maintenance (see review in Moreno-Rueda, [44]). For example, Moreno-Rueda [45] showed a negative correlation between uropygial gland size and the number of feather holes caused by chewing lice in house sparrows. Similarly, Fülöp et al. [46] reported that the number of feather holes was negatively related to uropygial gland size during the breeding season in both male and female house sparrows. Since the number of feather holes is negatively correlated with feather quality [47], these results may suggest that gland secretion could promote feather quality by affording resistance against these ectoparasites. Also, other studies have revealed an antimicrobial capacity against feather-degrading bacteria in the uropygial secretion of some bird species such as hoopoe (*Upupa epops*) [48] and spotless starling (*Sturnus unicolor*) [49]. More recently, Bodawatta et al. [50] tested the potential defensive properties of uropygial gland bacteria from great tits (*Parus major*), showing that some of the bacterial isolates restricted the growth of feather-degrading bacteria. Since feather bacteria may provoke plumage degradation [51], the antimicrobial properties of uropygial secretion may improve feather quality. Nevertheless, studies directly analysing the relationships between uropygial gland volume and feather quality would be desirable. Moreover, it has been suggested that the uropygial gland may affect moult speed. In this sense, Moreno-Rueda [52] examined moult performance and uropygial gland size in house sparrows, showing that individuals with smaller uropygial glands had more feather holes, and those with more feather holes moulted later and faster. However, the number of studies exploring the relationship between uropygial gland secretion and moult duration is still scarce.

Here we first aim to determine whether avian haemosporidian infection influences feather quality and feather growth rate in two species of migratory passerines during the breeding season—the house martin (*Delichon urbicum*) and the sand martin (*Riparia riparia*). If avian haemosporidian infection negatively impacts both avian parameters, then we can expect that haemosporidian-infected birds should have lower feather quality and/or a lower feather growth rate than non-infected individuals. We also explored the role of the uropygial gland on the feather growth rate and feather quality in the two species of hirundines. If uropygial gland secretion positively influences plumage quality and moult performance, then we predict that individuals with smaller uropygial glands should have lower values of plumage quality and feather growth rate.

2. Material and Methods

2.1. Study Species

The house martin is a migratory passerine bird that breeds in the Palearctic region, from western Europe and North Africa to eastern Asia, and winters in Ethiopian region, across tropical and southern Africa to the sub-Saharan area, with a fairly large range of occurrence [53]. This is an insectivorous species closely associated with human environments, as it has an affinity for building its nests and forming numerous colonies in structures of anthropic origins such as buildings, bridges, and dams [54].

The sand martin is also a migratory species in the swallow family. It is widely distributed worldwide, breeding throughout temperate, boreal, and arctic latitudes of the Nearctic (North America) and Palearctic regions, from western Europe to the northern half of Asia and northern Japan. This insectivorous species winters in Ethiopian (eastern and southern Africa), Neotropical (across South America), and Oriental regions (Indian Subcontinent) [53]. Its seasonality in the Iberian Peninsula coincides with that of the house martin, although it is more specialized in building its nests, constructing tunnels in slopes resulting from fluvial erosion, but it has also been able to adapt to artificial substrates resulting from anthropic activities, such as gravel pits and quarries [55].

2.2. Study Area and Bird Sampling

In late May and mid-June 2020, we used mist nets to capture 219 adult hirundinids in the province of Badajoz (Extremadura), southwestern Spain (Figure 1). House martins (N = 123) were captured on 12 June in one breeding colony located under a water tank (38°53′10.3″ N 6°55′32.3″ W). Sand martins (N = 96) were mist-trapped on 31 May at their nesting site in a sandy cliff of a sand pit close to the Guadiana River (38°51′28.3″ N 7°01′41.8″ W). Each bird was ringed with a numbered metal ring, and its age and sex were determined when possible, according to their plumage characteristics and skull ossification [56]. For each captured individual, we assessed body mass using a digital balance accurate to 0.1 g. Tarsus length was measured using a digital calliper with a precision of 0.01 mm. Subsequently, we estimated the scaled body mass index, a reliable metric for evaluating the physical condition of birds [57,58]. Additionally, we collected the second right outer rectrix feather from each individual and stored it in a plastic bag for subsequent estimations of feather growth rate and feather quality. During sampling, we did not collect feathers from individuals whose plumage were not fully developed or whose tails were in poor condition. Also, a blood sample was extracted from the jugular vein of each bird using sterile syringes and stored until molecular analysis. The volume of blood extracted from each individual was according to its body size and never exceeded 1% of its body mass. After manipulation, each bird was promptly released unharmed at its site of capture.

2.3. Measurement of Feather Growth Rate and Determination of Feather Quality Index

Feathers collected from all individuals were used to measure the feather growth rate and feather quality. Bird feathers exhibit a series of light and dark bands perpendicular to the feather rachis. Each light and dark band combination represents a growth bar, equivalent to approximately 24 h of growth [59–62]. Therefore, the number of dark bands indicates the number of days spent moulting these feathers. The number of growth bars and the length of the feather rectrix were measured using a gel documentation system (Bio-Rad Gel Doc XR + System), following the methodology outlined by Shawkey et al. [63]. Briefly, feathers were placed in a light cabinet to visualize the growth bars, with a ruler (0.1 mm accuracy) located nearby as a scale guide. Once optimal contrast and brightness conditions were achieved, digital images of the feathers were captured. These images were then post-processed using ImageJ software (Version 1.53e 2020) [64] to enhance lighting conditions for a clearer visualization of the growth bars. Using this software, the number of growth bars and the length of the rectrix were measured. Feather growth rate is reported as the average length of growth per day (mm/day) [39–41]. Feather mass was estimated with an analytical balance (Shimazdu AP225WD) to the nearest 0.0001 g. The ratio between

feather mass and feather length served as an index of feather quality at an intraspecific level [36,40], as it reflects the density of structural elements and, hence, indicates feather durability [65].

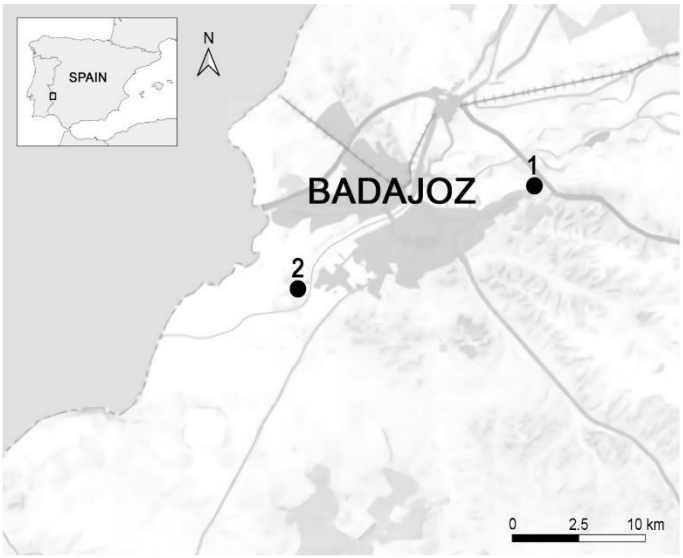

Figure 1. Distribution of the bird colonies of the study's species. 1: house martins; 2: sand martins.

2.4. Uropygial Gland Volume

A digital calliper with a precision of 0.01 mm was used to measure the length, height, and width of the uropygial gland. The volume of the uropygial gland was calculated by multiplying its length, height, and width [66], as this is known to be positively correlated with the volume of uropygial gland secretions [67,68]. Since the uropygial gland is a soft tissue [67,69], we conducted three measurements for each of its dimensions to assess repeatability [52,67,70].

2.5. Molecular Detection of Haemosporidian Infection

DNA from blood samples were extracted using the MAGMAX PATHOGEN RNA/DNA KIT (Applied Biosystems™, reference: 4462359). Diluted genomic DNA (25 ng/µL) was used as a template in a nested polymerase chain reaction (nested-PCR) to determine the presence or absence of haemosporidian infections following protocols described by Hellgren et al. [71]. Briefly, we used specific primer HaemNF1 (5′-CATATATTAAGAGAAITATGGAG-3′) and HaemNR3 (5′-ATAGAAAGATAAGAAATACCATTC-3′) in the first PCR, followed by two nested PCRs (Applied Biosystems™ SimpliAmp™ Thermal Cycler) to amplify *Haemoproteus* and *Plasmodium* genera using the primer pair HaemF (5′-ATGGTGCTTTCGATATATGCATG-3′) and HaemR2 (5′-GCATTATCTGGATGTGATAATGGT-3′), as well as amplify *Leucocytozoon*, using primers HaemFL (5′-ATGGTGTTTTAGATACTTACATT-3′) and HaemR2L (5′-CATTATCTGGATGAGATAATGGGC-3′). The amplification was evaluated by running 2.5 µL of the final PCR product on a 2% agarose gel. All PCR experiments contained one negative control (ddH_2O) for every 8 samples and one positive control for *Haemoproteus*/*Plasmodium* and another one for *Leucocytozoon* for every 24 samples.

2.6. Statistical Analysis

We conducted Shapiro–Wilk tests to evaluate the normality of the data distribution of all continuous variables used in statistic models. A Chi-squared test was conducted to examine potential differences in haemosporidian prevalence among the two bird species. General linear models (GLMs) were employed to investigate the factors contributing to variation in the feather quality index for each bird species, separately. Predictor variables included scaled body mass index, haemosporidian infection status (uninfected or infected), sex, uropygial gland volume, and feather growth rate. Additionally, GLMs were used to investigate the effect of sex, scaled body mass index, infection status (uninfected or infected), feather quality rate, and uropygial gland volume on the feather growth rate for each bird species separately. There were no significant correlations among the predictor variables (Pearson correlation, all $p > 0.05$); hence, they were included in the models as independent variables. Given the normality of the data, the Gaussian family was selected. Additionally, the adequacy of the models was assessed by examining their explained variances. To evaluate correlation between feather growth rate and feather quality, we calculated the Pearson correlation coefficient for each bird species, stratifying by infection status. All statistical analyses were carried out with R software version 4.2.2 [72].

3. Results

3.1. Haemosporidian Prevalence

Out of the 219 adult birds captured, 40 individuals showed avian haemosporidian infection, of which 32 (14.61%) corresponded to *Haemoproteus/Plasmodium* infections and 12 (5.48%) corresponded to *Leucocytozoon* infections (overall prevalence = 18.26%, 95% C.I. = 0.137–0.239). Specifically, out of the 123 house martins (75 males, 48 females), 25 (20.33%) were infected with *Haemoproteus/Plasmodium*, and 11 (8.94%) were infected with *Leucocytozoon*; while out of the 96 sand martins (43 males, 53 females), 7 (7.29%) were infected with *Haemoproteus/Plasmodium*, and only 1 (1.04%) was infected with *Leucocytozoon*. The haemosporidian prevalence differed among bird species (Chi-square test: $\chi^2 = 10.14$, d.f. = 1, $p < 0.05$). Thus, the prevalence of infection was higher in house martins ($N = 123$; prevalence = 26.02%; 95% C.I. = 0.004–0.333) than in sand martins ($N = 96$; prevalence = 8.33%; 95% C.I. = 0.043–0.156). Of the total number of birds, 1.83% (95% C.I. = 0.007–0.046) were coinfected with *Haemoproteus* or *Plasmodium* and *Leucocytozoon* parasites; these coinfections were only found in 4 house martins.

3.2. Factors Explaining Variation in Feather Quality Index

All variables included in the GLMs showed normal distribution ($p > 0.05$). We found that only haemosporidian infection and feather growth rate significantly explained variation in feather quality in house martins (Table 1). Specifically, haemosporidian-infected birds exhibited lower feather quality values than uninfected individuals (mean feather quality index (SD): uninfected = 0.180 (0.024) mg/mm; infected = 0.164 (0.016) mg/mm) (Table 1, Figure 2). In addition, we found a significant positive correlation between the quality of the feather and the feather growth rate for both infected (Pearson correlation, r = 0.570, $p < 0.05$) and uninfected (Pearson correlation, r = 0.316, $p < 0.05$) house martins (Figure 3). By contrast, none of the predictors significantly explained variation in feather quality in sand martins (Supplementary Table S1, all $p > 0.05$).

Table 1. Results from the GLM explaining variation in feather quality (mg/mm) for house martins (N = 123). Haemosporidian infection, sex, scaled body mass index, uropygial gland volume (mm^3), and feather growth rate (mm/day) were included as predictor variables. Significant factors are highlighted in bold.

Independent Variables	Estimate	Std. Error	t	p
Haemosporidian infection (infected)	−0.043	0.014	−3.060	**0.003**
Sex	0.006	0.011	0.495	0.622
Scaled body mass index	−0.269	0.197	−1.363	0.176
Uropygial gland volume	<0.001	<0.001	0.137	0.891
Feather growth rate	0.262	0.087	3.004	**0.004**

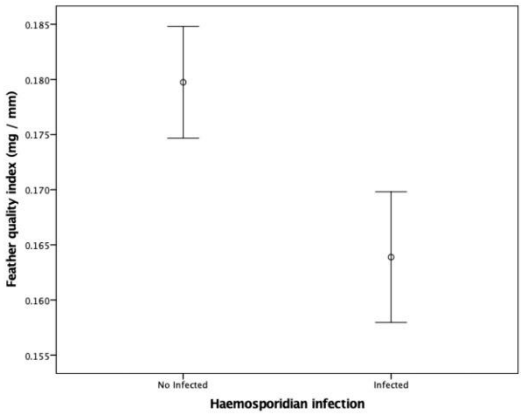

Figure 2. Feather quality index (mg/mm) for uninfected (N = 91) and infected (N = 32) house martins. Error bar plots show means ±95% confidence interval.

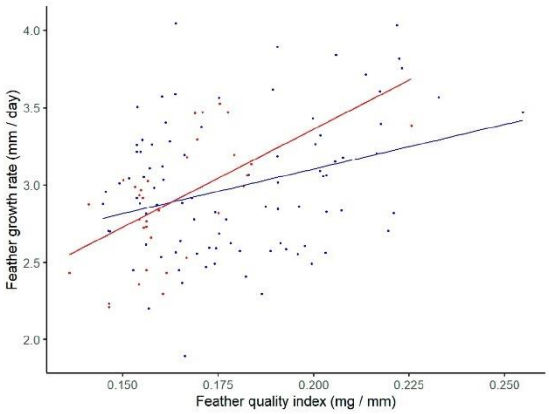

Figure 3. Scatter plot showing the relationship between the feather growth rate (mm/day) and feather quality (mg/mm) for uninfected (blue circle, N = 91) and infected house martins (red circle, N = 32).

3.3. Factors Explaining Variation in Feather Growth Rate

We found that only feather quality significantly explained variation in the feather growth rate in house martins (Table 2, Figure 3). Additionally, we observed a non-significant positive trend in the relationship between feather growth rate and feather quality for both infected (Pearson correlation, r = 0.478, p = 0.231) and uninfected (Pearson correlation, r = 0.159, p = 0.171) sand martins (Supplementary Figure S1). None of the other predictors (avian haemosporidian infection, sex, scaled body mass index, and uropygial gland volume) significantly influenced feather growth rate in sand martins (all $p > 0.05$) (Supplementary Table S2).

Table 2. Results from the GLM explaining variation in feather growth rate (mm/day) for house martins (N = 123). Haemosporidian infection, sex, scaled body mass index, uropygial gland volume (mm^3), and feather quality (mg/mm) were included in the analysis as predictor variables. Significant factors are highlighted in bold.

Independent Variables	Estimate	Std. Error	t	p
Haemosporidian infection (infected)	0.004	0.018	0.219	0.827
Sex	−0.014	0.013	−1.011	0.315
Scaled body mass index	0.359	0.234	1.535	0.129
Uropygial gland volume	−0.001	0.001	−1.627	0.107
Feather quality	0.370	0.123	3.004	**0.004**

4. Discussion

Feathers serve birds not only as a means of flight but also by fulfilling other vital functions such as providing a protective barrier, insulation, aiding in communication among conspecifics, and camouflage [1]. Throughout the annual cycle, the continuous degradation of plumage requires birds to regularly replace worn feathers to maintain these functions and improve their fitness. However, environmental conditions experienced during moulting, including factors such as food availability, stressors, and pathogen infection, may impair feather quality and the growth rate of newly produced feathers [7]. In addition, it has been proposed that uropygial gland secretion may improve feather quality and reduce moult duration [46,52]. Here, we investigated whether avian haemosporidian infection and uropygial gland volume influence the feather quality and feather growth rate of the second outermost tail feathers in three species of migratory hirundinids during the breeding season in southwest Europe. Our main findings showed that (i) the prevalence of avian haemosporidian infection varied significantly among bird species; (ii) house martins infected with haemosporidian parasites exhibited lower feather quality; (iii) feather quality was positively correlated with feather growth rate in house martins; (iv) avian haemosporidian infection did not affect feather growth rate in any of the analysed bird species; and (v) no relationship was found between uropygial gland volume and feather quality or feather growth rate in any of the studied species.

4.1. Differences in Haemosporidian Prevalence between Bird Species

Our findings revealed significant variations in avian haemosporidian infection among the two hirundine species studied. This aligns with previous research across different avian taxonomic orders, which also reported differences in haemosporidian prevalence among closely related bird species. For instance, Inumaru et al. [73] found variations in the prevalence of infection with *Plasmodium* and *Haemoproteus* across five species of *Gallinago* snipes in Japan. Similarly, Dubiec et al. [74] observed differential prevalence rates of haemosporidian parasites (i.e., *Plasmodium* and *Haemoproteus*) in nest-box breeding populations of great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) in Southern Gotland, Sweden, with great tits exhibiting higher infection rates than blue tits. Moreover, Ellis et al. [75] analysed the haemosporidian prevalence in Neotropical birds, showing variations among taxonomic families, genera, and even species within the same genus. Recently, Bukauskaité et al. [76] examined the prevalence of haemosporidian parasites in

two sympatrically breeding species of the order Accipitriformes from temperate forests of central–eastern Europe, reporting significantly lower infection rates in the white-tailed eagle *Haliaeetus albicilla* compared to the lesser spotted eagle *Clanga pomarina*.

Our study revealed a higher prevalence of infection in house martins compared to sand martins. Such interspecific variation in haemosporidian parasite prevalence is often attributed to differences in vector exposure [77,78]. For example, open-nesting bird species are more susceptible to haemosporidian infections than closed-nesting species or birds nesting in cavities, probably due to increased vector detection [79–81]. However, the inter-individual transmission of pathogens is assumed to increase linearly with host density [82], potentially explaining the higher prevalence observed in house martins due to their larger colony size [83,84]. In our study, the number of breeding pairs in the house martin colony was higher than the number of pairs nesting in the sand martin colony, which may explain the higher haemosporidian prevalence observed in house martins. Although sand martins also breed in substantial numbers, their nesting behaviour, with eggs laid in tunnels up to a meter long [85], may limit vector exposure [86]. In fact, the abundance and diversity of mosquitoes in the area of the sand martin colony are notably higher than in the area of the house martin colony [87], which seems to support this latter idea.

Alternatively, environmental conditions at wintering and stopover sites may also influence the likelihood of migratory species becoming infected with haemosporidians. For example, it is known that populations of sand martins from western Europe (Great Britain, Spain, and Portugal) migrate to wintering areas with a sub-Saharan desert climate located in the Senegal River Delta [88], while house martin populations from southwest Spain winter in higher rainfall habitats such as the west African broadleaf forests [89], potentially exposing them to higher vector densities [90]. This, in turn, can increase their probability of acquiring haemosporidian infections [91]. Finally, the interplay between host immune defences and parasite exploitation strategies may further influence haemosporidian prevalence in bird communities [92].

4.2. Factors Influencing Feather Quality Index

Several studies have shown that, among other stressors, parasites can impact the feather quality of birds. For example, Pap et al. [37] observed that house sparrows receiving anticoccidial treatment developed larger and heavier primaries with increased vane area and thicker rachis compared to untreated conspecifics, thus revealing that coccidian infestation reduces the quality of the flight feathers. Similarly, Pérez-Tris et al. [36] reported that feather quality significantly decreased with mite infestation intensity in fledgling blackcaps (*Sylvia atricapilla*). According to our predictions, we observed reduced feather quality in house martins infected with haemosporidians. To our knowledge, this is the first study revealing the negative impact of haemosporidian infection on the feather quality of bird hosts. We propose two non-mutually exclusive hypotheses to explain these results.

First, parasites may compete with their bird hosts for resources. Studies have shown that haemosporidian parasites are unable to de novo synthetize certain amino acids required for their growth and development, such as isoleucine and methionine, which must be acquired from their hosts [93]. Since both methionine and isoleucine are involved in synthesizing feather keratin and are crucial for the feather growth [94,95], a deficiency in these critical feather constituents resulting from pathogen consumption may impair the production of high-quality feathers.

Second, given that both the activation of an immune response to face the pathogen challenge (e.g., haemosporidian infection) and the production of high-quality feathers are energetically and nutritional demanding processes [7,96], a trade-off in resource allocation between these two traits is expected. Supporting this notion, Ben-Hamo et al. [97] reported reduced quality in newly grown feathers after an immune challenge in house sparrows, suggesting that the allocation of resources to mounting an immune response may compete with feather growth. However, haemosporidian infection did not affect feather quality in sand martins. Because of the low number of haemosporidian-infected birds sampled

of this species, further studies with larger sample sizes are needed to assess whether haemosporidian parasites have any effect on feather quality in this hirundine. Also, due to the low number of infected birds, we grouped the infections of all haemosporidian genera to test for the effect of overall infection on feather quality and feather growth rate. As different haemosporidian genera may show different effects on their hosts [98,99], further studies with larger sample sizes are required to separately investigate the effects of distinct parasite genera.

Some authors have pointed out that rapid feather growth may led to poor feather quality [40,100], thus suggesting a trade-off between feather growth rate and feather quality but resulting in the contradiction of high-quality individuals producing feathers of poor quality, which could lead to decreased fitness [7]. However, an increasing body of literature has revealed that, within populations, individuals with high feather growth rates tend to exhibit higher overall quality [101–104]. In addition, feather quality has been linked to good individual body conditions in great tits [16] and house sparrows [105], further supporting the idea that individuals in good conditions generally produce feathers of a higher quality at a faster rate [7].

Our results align with this perspective, showing a positive correlation between feather quality and feather growth rate in house martins. Similar patterns have been observed in other species, such as great tits, where a positive relationship between feather growth rate and feather mass has been documented [106]. Nonetheless, this association was not as straightforward in sand martins. Studies have also identified differences in the relation between feather growth rate and feather quality among species [107], or even within populations of the same species [101]. These differences are often attributed to differences in resource availability during the feather renewal period or time constraints imposed by the potential overlap of annual cycle activities [7,101].

It has been proposed that uropygial secretion plays a role in protecting and maintaining the plumage by shielding it from various external agents, including solar radiation, abrasion, and ectoparasites, among others [44–46]. Therefore, given that larger uropygial glands produce more secretion [69], a positive relationship between plumage quality and uropygial gland volume was to be expected. Yet, no relationship was found between feather quality and uropygial gland volume in either of the two hirundine species. This discrepancy could be attributed to variations in the quantity and composition of uropygial secretion, which can vary not only among different bird species [108] but also seasonally within the same species [68,69,109]. Therefore, both inter- and intraspecific variations in the quantity and composition of uropygial secretion, as well as seasonality, may influence its capacity to protect the plumage and maintain its quality. Despite these observations, further research efforts are needed to understand the role of the uropygial gland and its secretion in the production of plumage and maintenance of feathers of optimal quality.

4.3. Factors Influencing Feather Growth Rate

While numerous studies have explored factors potentially influencing the feather regrowth rate by experimentally plucking feathers, limited data exist on environmental effects on the naturally moulting feather growth rate (see review in [7]). Here, we assessed the association between the growth rate of naturally moulted feathers and haemosporidian infection in two hirundine species. Contrary to our predictions, the feather growth rate was not related to haemosporidian infection in any of the studied bird species. These results contrast with observational and experimental studies analysing the feather growth rate and haemosporidian infection in house martins [39,40] and house sparrows [41] from the same geographical area in previous study years. Similarly, previous studies have also shown contrasting results between study years on the effect of parasite infections on feather quality and feather growth rate. For example, Pap et al. [37] conducted experimental research on house sparrows in two consecutive moults, showing that coccidian infection significantly reduced the stiffness of the feather grown after the first moult but not in the case of feathers grown after the second moult. Also, Dunn et al. [110] investigated the

potential for haemosporidian parasites to impact the feather growth of yellowhammers (*Emberiza citrinella*), revealing that birds infected with haemosporidians had a shorter feather growth rate in the winter of 2007/2008 but not in the winter of 2008/2009. Overall, these inter-annual differences suggest a year-dependent association between parasite infection and reduced feather quality and/or feather growth rate, indicating that the effects of haemosporidian infection on these parameters are still poorly explored and deserve more attention in further studies.

Moreover, other previous studies have also failed to detect the negative effect of haemosporidians on the feather growth rate. For example, Romano et al. [111] investigated haemosporidian infection in adult barn swallows and its consequences on the feather growth rate, showing a negative effect of infection by *Plasmodium* on the feather growth rate in older individuals but not in yearlings. While all the sampled individuals in our analyses were adults, we unfortunately did not categorize them into different age classes. Further studies would now be required to disentangle the potential age-dependent differences in the effect of haemosporidian infections on the feather growth rate.

Also, Henschen et al. [112] analysed plumage ornaments and parasite infection in common yellowthroat (*Geothlypis trichas)* males, revealing that neither the presence nor the intensity of haemosporidian infection was related to the feather growth rate. In this latter study, most haemosporidian infections were chronic, low-intensity infections (the percent of red blood cells infected was less than 2%) rather than acute infections, which usually show few or no measurable harmful effects on their hosts [98,113–115]. Because the negative effects of haemosporidians on the host phenotype usually occur when parasitaemia reaches higher levels (higher than 2% of infected erythrocytes), typically during a short initial primary infection or in relapses [23,25], experimental infections with haemosporidians inducing initial acute parasitaemia would be fundamental to reliably show the potential negative effects of haemosporidian infection on the feather growth rate of their avian hosts.

Finally, it has been shown that individuals in better conditions exhibit faster feather growth [41,103]. Because birds with better body conditions normally produce larger uropygial glands [45,70,116,117], a positive relationship between uropygial gland size and feather growth rate could be predicted. However, we did not observe any relationship between the feather growth rate and the size of the uropygial gland. Moreover, Møller and Laursen [118] found that eiders (*Somateria mollisima*) with small uropygial glands grew their feathers at a faster rate. In this regard, the uropygial gland may not be related to high feather quality and higher feather growth rates. Instead, larger glands might be necessary when feathers are subjected to increased damage or when optimal growth is limited. Therefore, uropygial secretion may not suffice to compensate for detrimental effects, which could lead to the observation of individuals with large glands but suboptimal feathers. Further investigations are needed to confirm whether the size of the uropygial gland might affect the feather growth rate.

5. Conclusions

This study appraised the prevalence of avian haemosporidian infection and its impact on the plumage of two species of hirundinids in southwestern Europe. The prevalence of haemosporidian parasites varied among species, with the highest rate observed in house martins, possibly due to the size of their colonies. Additionally, avian haemosporidian infection was found to have adverse effects on the feather quality of this hirundine, representing the first evidence of this effect of haemosporidian parasites on this parameter. However, the infection did not appear to influence the feather quality of sand martins. Likewise, there was no observed relationship between haemosporidian infection and the feather growth rate, although future studies with larger sample sizes could provide more conclusive evidence of these effects. Moreover, a positive correlation was observed between feather quality and moulting speed in house martins, supporting the notion that high-quality or well-conditioned individuals tend to produce high-quality feathers and moult them more quickly. Finally, we found no relationship between the volume of the uropygial

gland and the feather quality or feather growth rate in any species. These findings contribute to our understanding of the impact of haemosporidian infections on the plumage of migratory birds, although further research is required to explore these relationships more comprehensively.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ani14121772/s1. Figure S1: Scatter plot showing the relationship between the feather growth rate (mm/day) and feather quality (mg/mm) for uninfected (blue symbol, N = 88) and infected sand martins (red symbol, N = 8); Table S1: Results from the GLM explaining variation in feather quality (mg/mm) for sand martins (N = 96). Haemosporidian infection, sex, scaled body mass index, uropygial gland volume (mm3) and feather growth rate (mm/day) were included in the analysis as predictor variables; Table S2: Results from the GLM explaining variation in feather growth rate (mm/day) for sand martins (N = 96). Haemosporidian infection, sex, scaled body mass index, uropygial gland volume (mm3) and feather quality (mg/mm) were included in the analysis as predictor variables.

Author Contributions: Conceptualization, C.M.-R., M.F., S.M. and F.d.L.; Methodology, C.M.-R., M.F., S.M., J.T.C. and A.M.; Software, C.M.-R., L.G.-L. and A.M.; Validation, A.M.; Formal Analysis, C.M.-R., L.G.-L. and A.M.; Investigation, C.M.-R., L.G.-L., M.F., S.M. and A.M.; Resources, F.d.L. and A.M.; Data Curation, C.M.-R., L.G.-L. and A.M.; Writing—Original Draft Preparation, C.M.-R., L.G.-L. and A.M.; Writing—Review and Editing, C.M.-R., A.M., L.G.-L., M.F., S.M., J.T.C. and A.M.; Visualization, C.M.-R., L.G.-L. and A.M.; Supervision, F.d.L. and A.M.; Project Administration, F.d.L. and A.M.; Funding Acquisition, C.M.-R., M.F., F.d.L. and A.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Bird trapping was carried out with all the necessary permits in accordance with the current regional and national laws of Spain. Bird sampling on private land and in private residential areas was conducted with all the necessary permits and consent from the owners. Methods were evaluated and approved by the institutional Commission of Bioethics of University of Extremadura (CBUE 49/2019) and by Junta de Extremadura Local Government, (87/2019) and (CN0055/19/ACA).

Informed Consent Statement: Not applicable.

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article and in Supplementary Tables S1 and S2.

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Appendix XV. 1st WIMANET workshop certificate of attendance

CERTIFICATE of ATTENDANCE

Awarded to

João T. Cruz

For the participation in the

1st WIMANET-COST CA22108 workshop

6th-8th December 2023

Online

UNIVERSITY OF LINCOLN

WIMANET-COST Action Chair

Dr. Jenny Dunn

Appendix XVI. 2nd WIMANET workshop certificate of attendance

CERTIFICATE of ATTENDANCE

Awarded to

João T. Cruz

From *Faculty of Veterinary Medicine of the University of Lisbon*

For the participation in the

2nd WIMANET-COST CA22108 workshop

20th-23th February 2024

Cluj-Napoca

UNIVERSITY OF LINCOLN

WIMANET-COST Action Chair

Dr. Jenny Dunn

Appendix XVII. Collection and shipment protocol sent to the Wildlife Rehabilitation Centres and Veterinary Clinics

Projeto de mestrado:
"Prevalence and genetic diversity of avian malaria and related haemoparasites of wild birds from rehabilitation centers in mainland Portugal"

Protocolo de colheita e envio de amostras:

❶ Colheita da amostra

- Colher apenas sangue de aves mas sem preferência de espécie, idealmente fazer uma amostragem o mais ampla possível em termos de espécies, géneros, idades, causas de ingresso, condição corporal, sinais clínicos etc.
- Colocação de 2 gotas de sangue total e sem aditivos, utilizando uma seringa de 1mL, equivalendo a 50-70µL de sangue, no tubo fornecido contendo já o SET buffer para conservação da amostra.

❷ Identificação da amostra

- Identificação da amostra no local em branco no tubo ou no local fosco dependendo do tubo fornecido.
- Colocação do número da amostra seguida da sigla do centro que fez a recolha (Rias- R; CERAS- CE; CRASSA-CRS). EX: 1R; 35CE, 20CRS. Como na figura.
- Colocação de fita cola por cima da identificação de forma a que não se apague.

❸ Preenchimento do Google Forms

- Preenchimento do Google Forms para cada amostra recolhida com os dados da ave em que foi recolhida com a maior quantidade de informação possível, fazendo scan do seguinte QR code:

- Ou a partir do link: https://docs.google.com/forms/d/e/1FAIpQLSdsBdZn6nvfAYrw8bLhWIpTjrm1twd0r0HPIGULRZV9WGxfSA/viewform?usp=sf_link

❹ Armazenamento das amostras

- Armazenar num frigorifico convencional, idealmente a 4ºC até ao envio.
- Nunca congelar.

5 Envio das amostras

- Peço, por favor, que faça o envio das amostras no início da semana (idealmente, segunda ou terça-feira), de forma a evitar que as amostras cheguem às instalações durante o fim-de-semana.
- As amostras devem ser acondicionadas e enviadas da seguinte forma:

1. Coloque os tubos dentro do saco com asas. De seguida, dê um nó no saco, tal como demonstrado na figura. Aconselho a não apertar muito o nó e a pressionar primeiro o saco com as amostras para retirar o ar acumulado dentro do saco e, só depois, a apertar o nó.

2. Coloque o saco com asas com as amostras dentro do saco com fecho ZIP como na Figura. Aconselho a colocar primeiro o saco no envelope de correio verde almofadado antes de fechar o saco com o fecho ZIP, pois entra sempre algum ar que depois dificulta a colocação do saco dentro do envelope.

3. Preencher e colocar primeiro a ficha da categoria das amostras dentro do envelope de correio verde almofadado. Por fim, coloque as amostras já dentro do saco com fecho ZIP dentro do envelope como na Figura. Feche o envelope. Sugiro, se tiver possibilidade, que coloque fita-cola sobre a abertura do envelope de forma a reforçar o seu fecho e evitar que se abra durante o transporte.

- Morada para envio das amostras (já escrita no envelope):
  A/C Professor Luís Madeira de Carvalho
  Laboratório de Parasitologia e Doenças Parasitárias
  Faculdade de Medicina Veterinária
  Pólo Universitário do Alto da Ajuda
  Avenida da Universidade Técnica
  1300-477 Lisboa