UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

Implicaciones ecológicas de la infección por parásitos sanguíneos en aves: dieta, fisiología y comportamiento

Ecological implications of avian blood parasite infections: diet, physiology and behaviour

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Lucía Gloria Jiménez Gallardo

Directores

Carolina Remacha Sebastián
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"One must still have chaos in oneself to be able to give birth to a dancing star"

Friedrich Nietzsche

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- General introduction: Pablo Quiles
- Chapter 1: Daniel A. Truchado
- Chapter 2: Carmen Ureña-Lara
- Chapter 3: Ana Jiménez-Gallardo
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Abstract

Ecological implications of avian blood parasite infections: diet, physiology and behaviour

The study of parasites in wild animals is essential to disentangle the host-parasite relationships in nature, and to examine future zoonotic reservoirs. Prevalence of infection depends on exposure to parasites and host competence, which differ between individuals and species. The exposure to parasites is determined by the abundance and diversity of parasites, the encounter rate between parasites and competent hosts, and the time of exposure to parasites. Host competence depends on coping mechanisms, such as the strategies of resistance and tolerance of each host. Haemosporidian parasites are blood parasites from the phylum Apicomplexa that infect a high variety of vertebrate hosts, including birds, which are mostly infected with parasites from the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*. These infections have costs that could affect the reproductive success, performance or survival of birds. Since haemosporidian parasites are transmitted by dipteran vectors, one important key to understand the exposure to parasites is the differential biting preference of each vector. Biting probabilities of vectors could depend on the detectability of each bird (CO_2 cues, body size) or traits such as the amount of exposed surface of their body. Moreover, the behaviour of the birds could also determine the exposure to vectors, since it could shape their encounter rates with vectors. Both among and within species, there is also variation in the capacities of each bird to cope with infections. The first step to fight against haemosporidians is the activation of the humoral response, which can lead to tissue damage due to the production of reactive oxygen and nitrogen species (RONS). When this oxidative damage is not controlled by

endogenous or exogenous antioxidants, parasitic infections can induce oxidative stress. One way to control the oxidative damage caused by haemosporidians is the intake of antioxidants through the diet, mostly from plant origin.

The aim of this thesis is to discover the role of the exposure to vector bites (at species level) and the infection coping mechanisms mediated by oxidative stress and diet (at species and individual level) in the distribution and costs of haemosporidian parasites in birds. To do so, we structured this thesis in three chapters. In Chapter 1, we explored the importance of exposure to vector bites and the proportion of plant-based matter included in the diet of a local community of birds in the Neotropics. Studying the prevalence of haemosporidian infections in resident understory birds from lowland primary rainforests in French Guiana, where all infections were locally acquired and there is a huge diversity of potential hosts, and using phylogenetic techniques, we found that exposure to vector bites and plant-based diet were positively correlated with the local prevalence in passerines. In this chapter, we could also describe the community of parasites residing in this underexplored region. Chapter 2 focuses on the individual capacity of Eurasian blackcaps (*Sylvia atricapilla*) from different sexes and ages to cope with blood parasites during autumn migration, which imposes physiological challenges *per se*. We discovered that, during stopovers, first-year blackcaps from both sexes suffer from more parasitic infections than older individuals. The costs of infections in terms of oxidative stress were noticeable when birds harboured coinfections, which are considered to be more virulent, especially when they involve *Plasmodium* parasites. We also corroborated that migration might alter the oxidative balance of blackcaps. Finally, in Chapter 3 we tested within the same ecological context whether young male blackcaps can choose their diet according to their needs, favouring dietary antioxidants to cope with

oxidative damage caused by blood parasites and migration. During stopover, we offered antioxidant-enriched food and fat-enriched food to each individual to measure their election and we found that infected birds had less antioxidant capacity, which could be dealt with through diet since birds infected with more than one different parasite preferred antioxidant-enriched food. Moreover, we found that birds with lower fat stores also chose antioxidant-enriched food, perhaps to cope with oxidative stress derived from flight effort.

We discovered that exposure to vectors and coping mechanism through diet may be key factors shaping haemosporidian prevalence, at species and individual level. We also found evidence of plant-based diets playing a role in host-parasite interactions, maybe with the implication of oxidative stress. We also demonstrated the importance of studying tropical environments to discover new parasites and parasite reservoirs. There are also individual differences in the capacity to fight against infections, where younger individuals might be more likely to harbour coinfections. Studying the costs of infections during physiologically challenging periods such as migration might be essential for uncovering such costs in natural, non-experimental settings. The general conclusion of this thesis is that not all birds are equally susceptible and competent hosts for haemosporidian parasites due to the interplay between (1) ecological traits of species influencing on host exposure and coping abilities, (2) trade-offs between costly activities (such as fighting parasite infections and aerobic exercise during migration), and (3) individual behavioural decisions that may determine the physiological outcomes of all the above.

Resumen

Implicaciones ecológicas de la infección por parásitos sanguíneos en aves: dieta, fisiología y comportamiento

El estudio de parásitos en animales silvestres es esencial para desentrañar las relaciones parásito-hospedador y para determinar posibles reservorios de zoonosis. Las prevalencias de infección dependen de la exposición a los parásitos y de la competencia hospedadora, que difieren entre individuos y especies. La exposición a parásitos está determinada por la abundancia y diversidad de parásitos, la tasa de encuentro entre parásitos y hospedadores competentes, y el tiempo de exposición a parásitos. La competencia hospedadora depende de los mecanismos para lidiar contra infecciones, como las estrategias de resistencia y tolerancia de cada hospedador. Los parásitos hemosporidios son parásitos sanguíneos del filo Apicomplexa que infectan a una gran variedad de hospedadores vertebrados, incluyendo aves, que son infectadas mayoritariamente por parásitos de los géneros *Plasmodium*, *Haemoproteus* y *Leucocytozoon*. Estas infecciones tienen costes que afectan al éxito reproductivo, el desempeño o la supervivencia de las aves. Dado que los parásitos hemosporidios de las aves se transmiten a través de dípteros, las diferentes preferencias de alimentación de cada vector son clave para comprender la exposición a parásitos. Las probabilidades de picaduras por vectores pueden depender de la detectabilidad de cada pájaro (señales de CO_2, tamaño corporal) o rasgos tales como la cantidad de superficie corporal expuesta. Además, el comportamiento de las aves también podría determinar la exposición a vectores si modula su tasa de encuentro con vectores. Tanto entre las especies de hospedadores como dentro de cada una de ellas, hay variación en la capacidad de cada ave para lidiar con las infecciones. El primer paso para luchar contra los hemosporidios

es la activación de la respuesta humoral, lo que podría conllevar daño en los tejidos debido a la producción de especies reactivas de oxígeno y de nitrógeno (RONS). Cuando no se controla el daño oxidativo mediante antioxidantes endógenos o exógenos, las infecciones parasitarias pueden desembocar en estrés oxidativo. Una forma de controlar el daño oxidativo causado por hemosporidios es el consumo de antioxidantes a través de la dieta, mayoritariamente de origen vegetal.

El objetivo de esta tesis es descubrir el papel de la exposición a picaduras de vectores (a nivel interespecífico) y de los mecanismos para lidiar con infecciones mediados por estrés oxidativo y dieta (inter e intraespecífico) en la distribución y los costes en aves de parásitos hemosporidios. Para ello, estructuramos la tesis en tres capítulos. En el Capítulo 1, exploramos la importancia de la exposición a picaduras de vectores y la proporción de materia vegetal en la dieta de una comunidad local de aves del neotrópico. Al estudiar la prevalencia de infecciones de hemosporidios en aves residentes de sotobosque de una zona baja de bosque primario de Guayana Francesa, donde todas las infecciones son locales y hay una gran diversidad de hospedadores potenciales, y usando técnicas filogenéticas, encontramos que la exposición a picaduras de vectores y la importancia de la materia vegetal en la dieta estaban positivamente relacionadas con la prevalencia local en paseriformes. En este capítulo, también pudimos describir la comunidad de parásitos de esta región poco explorada. El Capítulo 2 se centra en la capacidad individual de currucas capirotadas (*Sylvia atricapilla*) de diferentes edades y sexos para lidiar con parásitos sanguíneos durante la migración otoñal, que impone desafíos fisiológicos *per se*. Descubrimos que durante las paradas migratorias, las currucas de ambos sexos de primer año sufrían más infecciones parasitarias que los individuos más viejos. Los costes de las infecciones en términos de estrés oxidativo

fueron notorios cuando las aves tenían coinfecciones, que pueden ser más virulentas especialmente cuando intervienen parásitos del género *Plasmodium*. También corroboramos que la migración podría alterar el balance oxidativo de las currucas. Finalmente, en el Capítulo 3 examinamos en el mismo contexto ecológico si los machos jóvenes de curruca pueden elegir su dieta dependiendo de sus necesidades, favoreciendo los antioxidantes para lidiar con el daño oxidativo causado por los parásitos sanguíneos y la migración. Durante la parada migratoria, ofrecimos a cada individuo comida enriquecida con antioxidantes o con grasas y medimos su elección, encontrando que las aves infectadas tenían menos capacidad antioxidante, lo que podría compensarse mediante la dieta, ya que las aves infectadas con más de un parásito diferente preferían la comida enriquecida con antioxidantes. Además, descubrimos que las aves con menores reservas de grasa elegían comida enriquecida con antioxidantes, posiblemente para lidiar con el estrés oxidativo derivado del esfuerzo del vuelo.

Hemos descubierto que la exposición a vectores y los mecanismos para lidiar con parásitos a través de la dieta pueden ser determinantes en la prevalencia de hemosporidios, a nivel de especie e individual. Encontramos evidencias de que las dietas vegetales juegan un papel importante en las interacciones parásito-hospedador, posiblemente con implicación del estrés oxidativo. También demostramos la importancia de estudiar ambientes tropicales para descubrir nuevos parásitos y reservorios de estos. Hay diferencias individuales en la capacidad de luchar contra infecciones, siendo los individuos más jóvenes más susceptibles a las coinfecciones. Estudiar los costes de las infecciones en momentos exigentes fisiológicamente, tales como la migración, es esencial para desvelar los costes en condiciones naturales, no experimentales. La conclusión general es que no todos los pájaros son igualmente susceptibles y competentes frente a

parásitos haemosporidios debido a la relación entre (1) los rasgos ecológicos de las especies que tienen que ver con la exposición y las estrategias de defensa contra parásitos, (2) compromisos entre actividades costosas (como la lucha contra parásitos y el ejercicio aeróbico durante la migración) y (3) comportamientos individuales que pueden determinar los resultados fisiológicos de todo lo anterior.

List of abbreviations

ABTS	2,2 ′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
AICc	Corrected Akaike information criterion
BSA	Bovine serum albumin
Cyt-b	Cytochrome *b*
DNA	Deoxyribonucleic acid
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid tetrasodium salt dehydrate
GLMM	Generalised linear mixed models
HWI	Hand-wing index
LM	Linear models
MAB	Mechanical advantage of the beak
MDA	Malondialdehyde
ML	Maximum likelihood
NADPH	Nicotinamide adenine dinucleotide phosphate
PCA	Principal component analysis
PCR	Polymerase chain reaction

PGLS	Phylogenetic general least squares models
RBC	Red blood cells
RONS	Reactive oxygen and nitrogen species
RTL	Relative tarsus lenght
TAS	Total antioxidant status
TBA	Thiobarbituric acid
tGSH	Total glutathione
VIF	Variance inflation factor

General introduction

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Parasitic infections are widely studied under controlled circumstances and mostly trying to interpret their impact on human or livestock health (Corwin 1997; Pullan and Brooker 2008; Sabbatani et al. 2010). However, the use of wild animals to understand host-parasite interactions has exponentially grown (Atkinson et al. 2008; Hawley and Altizer 2011; García-Longoria et al. 2016). This approach enables the discovery of reservoirs of zoonotic pathogens (Johnson et al. 2015; García-Longoria et al. 2016), potentially damaging wildlife and humans.

All parasites induce costs to their hosts, burdening their performance, reproductive success or survival (Krams et al. 2013; Delhaye et al. 2016; Chrétien et al. 2022). The costs depend on parasite traits such as parasite load, activity or growth rate; and on host traits such as tolerance or resistance strategies (Råberg et al. 2007; Muriel 2020). The competence of a host is defined as the extent to which a host can successfully transmit a parasite, which varies depending on its susceptibility to invading parasites and its suitability for parasite growth and transmission once parasites have entered their body (Stewart Merrill and Johnson 2020; Huang et al. 2023). In the case of vector-borne parasites, the transmission of parasites is also strongly influenced by the exposure to vectors (which may depend on host traits and vector preferences; Medeiros et al. 2013): if host susceptibility and suitability remain constant, hosts that are more often bitten by vectors amplify parasite transmission (Yan et al. 2021). Host susceptibility and suitability determine the fate of an infection since parasite invasion (Stewart Merrill and Johnson 2020; Huang et al. 2023), which may depend to a great extent on coping mechanisms that allow the host to fight against or live with parasites (Dietsch 2007; Hawley et al. 2011; Langenhof and Komdeur 2018). The competence of a host could be studied from different

perspectives. Hosts species might have coevolved with a parasite community, with adaptations against infections (Gandon et al. 2002; Møller et al. 2005). Individuals from the same species could also exhibit different functional traits for parasite management (Huang et al. 2023). Hence, the importance of studying host competence and parasitic exposure at different levels to understand the distribution and repercussions of infections.

The probability of transmission of a parasite between hosts depends on the exposure to parasites, which in turn depends on (1) the abundance and diversity of parasites and vectors (in vector-borne parasites) present in the home range of the host (Fecchio et al. 2017), (2) the encounter rate with parasites (that also depends on the abundance of other competent hosts and vectors, and avoidance behaviours from the host; Hawley and Altizer 2011; Isaksson et al. 2013; Barron et al. 2015), and (3) the time of exposure to parasites, incrementing the probability of transmission (Reece et al. 2017). Once infected, hosts may have coping mechanisms to deal with infections: resistance or tolerance (Råberg et al. 2007; Sorci 2013; Arriero et al. 2018). When the costs of infection exceed the costs of an immune response, hosts use their resistance mechanisms to reduce parasite load or eliminate parasites, incurring in energetic and physiological burden of the activation of the immune system (Delhaye, Jenkins, et al. 2018; Muriel 2020). On the other hand, when the costs of living with the parasites are not too high, hosts may opt for tolerance mechanisms that help counteract the possible impact of parasites in their health (Råberg et al. 2009). Coping mechanisms also include behavioural adjustments (sickness syndromes; Barber and Dingemanse 2010; Ashley and Wingfield 2012; Hite et al. 2019) that could enhance the immune response (Hawley and Altizer 2011; Barron et al. 2015); prevent further spread of parasites (Hawley and Altizer 2011; Barron et al. 2015) or

enhance the physiological status of the bird. Adaptive feeding may be one of such mechanisms (de Roode et al. 2013; Masello et al. 2018; Hite et al. 2019).

Haemosporidian parasites (Apicomplexa: order Haemosporida) are a group of protozoans that parasitize a wide range of vertebrates using blood-sucking dipterans as transmission vectors (Valkiūnas 2005). Some of these parasites cause human malaria and related animal diseases, relevant for public and animal health (Phillips and Pasvol 1992; Cumnock et al. 2018). Initially, bird haemosporidians were thoroughly investigated as surrogate models for human malaria parasites (Rivero and Gandon 2018; Santiago-Alarcón and Marzal 2020), but currently their study in natural populations is important for conservation purposes, since they have a great invasive potential and can have a serious impact when colonising new ecosystems (van Riper et al. 1986). The use of molecular techniques for the identification of the different lineages and species has been very helpful, since their morphological identification is extremely complicated and requires the help of specialists in the field (Hellgren et al. 2004; Bensch et al. 2009; Fecchio, Chagas, et al. 2020). Bird haemosporidians are included in the genera *Plasmodium* (which causes avian malaria)*, Haemoproteus* (which is divided into two subgenera, *Haemoproteus* and *Parahaemoproteus*)*, Fallisia* (never found in passerines and, therefore, not investigated in this thesis), and *Leucytozoon* (Valkiūnas 2005; Fecchio, Chagas, et al. 2020; Santiago-Alarcón and Marzal 2020).

The life cycle of these parasites is rather complicated, with differences between groups. In general, the sexual stage occurs inside the vectors (definitive hosts), leaving the birds as intermediate hosts where they reproduce asexually. The vectors inoculate sporozoites through their feeding process that move from the blood and accumulate in the tissues of the bird as exoerythrocytic meronts. Meronts reproduce asexually (merogony),

producing merozoites that spread within the bird. In the acute phase, short after the infection, the intensity of infection increases via induction of merogony of sexual stages in blood cells, creating gametocytes. The gametocytes usually live within blood cells and can generate microgametes (various male microgametes arise from each microgametocyte through exflagellation) or macrogametes (macrogametocytes differentiate into female gametes). When a vector bites a bird with an active infection in the blood, it could take gametocytes with the blood meal (Valkiūnas 2005; Fecchio, Chagas, et al. 2020; Nourani et al. 2020). Then, gametocytes enter the midgut, start the gametogenesis, and get out of the bird's blood cells. The process of fertilization occurs extracellularly, producing a zygote, which transforms in an ookinete that exits the midgut and transforms in an oocyst. The oocyst produces sporozoites (sporogony) that enter the haemocele and salivary glands, where they could be transferred to a new bird (Valkiūnas 2005; Fecchio, Chagas, et al. 2020; Nourani et al. 2020). The different genera of parasites are transmitted by different vectors, which could impact their distribution between hosts since each dipteran vector has different feeding preferences. *Plasmodium* parasites are transmitted by mosquitos from the family Culicidae; *Haemoproteus* by hippoboscid and ceratopogonid flies; and *Leucocytozoon* by simuliid and ceratopogonid flies, although there is a paucity of specific research that leaves this question far from resolved (Santiago-Alarcón et al. 2012).

It is in the blood of birds that haemosporidian parasites are best studied, since they can be easily collected and visually identified without harming the individual with highly invasive procedures. Then, we could summarise the process of infection on birds in different stages: prepatent, when parasites are in the tissues of the bird; acute, when the parasites are in the blood and there is an increase in parasitemia; crisis, when the

parasitemia is the highest; chronic, and latent, when the parasitemia decreases controlled by the immune response of the host. It is usual for the infection to persist in birds, with periodical relapses of parasitemia in stressful periods (Valkiūnas 2005).

Avian malaria is proven to come at a cost for their hosts (Rivero and Gandon 2018; Videvall 2019). There is a lot of interest on the physiological effects of the infections (Isaksson et al. 2013; Badás et al. 2015), as such effects can lead to long-term costs on fitness (Asghar et al. 2011; Krams et al. 2013; Muriel 2020; Remacha et al. 2023). But not all individuals suffer the same amount of costs, as the latter vary depending on traits such as sex, age, or physiological condition (Muriel 2020). There is evidence of differences in immune function between females and males, leading to higher risks of infection to males since testosterone may impair the immune system functioning (mostly the humoral response; Roved et al. 2017; Vincze et al. 2022) or alter their behaviour (e.g., territorial behaviour), sometimes shifting their exposure to vectors (McCurdy et al. 1998; Hawley et al. 2011). Younger birds that are developing (Santiago-Alarcón et al. 2012; Muriel 2020), or senescent birds that are losing the capacity to deal with infections (Finkel and Holbrook 2000; Monaghan et al. 2009) could also suffer greater infection costs.

The costs of haemosporidians not only vary between individuals, but also between life-stages that impose energetic or physiological challenges. During challenging moments, birds might need to invest energy and resources on self-maintenance, being unable to properly cope with infections (Hawley and Altizer 2011; Van de Crommenacker et al. 2012). There are many studies that link the reproductive effort with an increase in parasitemia and long-term costs on fitness (Asghar et al. 2011; Badás et al. 2015; Pigeault et al. 2018). But there are fewer studies involving other costly activities, such as migratory effort (Soares et al. 2020; de Angeli Dutra et al. 2021).

Moreover, the parasitemia and the virulence of the parasites imply different costs for the bird. There is evidence that *Plasmodium* parasites, and some parasite lineages (Rivero and Gandon 2018), are more virulent and have stronger consequences in birds (Santiago-Alarcón et al. 2012; Videvall 2019). In some hosts, it is usual to find more than one parasite lineage (from the same or different genus) at the same time; those coinfections are commonly more virulent than single infections (Alizon and van Baalen 2008; Marzal et al. 2008; Palinauskas et al. 2018; Pigeault et al. 2018). Also, the same parasite lineage could have different effects depending on their host species (Ortiz-Catedral et al. 2019). Then, the combination of bird and parasite traits influence the costs of the interaction (Palinauskas et al. 2008).

Birds differ in their exposure to parasites and vectors, and in their host competence through coping capacity (Hawley et al. 2011). Then, one of the main focus when studying differential prevalence of infection is the exposure to parasites and, since haemosporidian parasites are transmitted by dipteran vectors, it is connected with the probability of being bitten by an infective (competent) vector (Cator et al. 2012; Gutiérrez-López et al. 2019). Although indispensable, exposure to vectors has not been exhaustively studied yet (but see Santiago-Alarcón et al. 2012; Chakarov et al. 2020; Nourani et al. 2020). These parasites can change the feeding preferences of vectors (Cator et al. 2014; Pigeault et al. 2015; Santiago-Alarcón and Marzal 2020) and the hormones of the birds to increase the biting rate on competent hosts, enhancing the probability of successful transmission (Dhondt and Dobson 2017). The detectability of the birds for vectors depends on traits such as the size of the bird, cues of CO_2 emissions, or the proportion of exposed surface without feathers (around beak and eyes, and legs; Martínez-de la Puente et al. 2015; Fecchio, Chagas, et al. 2020). Birds can also exhibit avoidance behaviours: foraging in

places with low density of vectors, decreasing their encounter with other competent hosts, sickness behaviours, etc. (Pigeault et al. 2015; Nourani et al. 2020). Then, there are several behaviours that can modulate the probability of transmission of haemosporidian parasites that could be species-specific, such as foraging site, incubation time, migration, social behaviour, flocking behaviour, diet, or nesting behaviour (González et al. 2014; Matthews et al. 2016; Fecchio et al. 2022; Aguiar de Souza Penha et al. 2023). The diversity of these traits (together with the diversity of host species) could be important to disentangle host exposure to parasites. Since tropical regions are very rich in biodiversity (and abundance) of birds and dipteran vectors, they have recently been the focus of several studies accounting for exposure to haemosporidian infections (Fecchio, Chagas, et al. 2020; Santiago-Alarcón and Marzal 2020). These areas are therefore excellent scenarios to understand evolution of host-parasite interactions, and possible sources of disease spreading.

There are also differences in the capacity of each host to cope with haemosporidian infections since not all birds have the same physiological condition (Isaksson et al. 2013). When birds face excessive exploitation due to parasite multiplication, their immune system responds producing oxidant substances, reactive oxygen and nitrogen species (RONS; Bichet et al. 2012; Delhaye et al. 2016; Muriel 2020), that not only attack the parasites but also the tissues of the host, producing oxidative damage and also having a signalling role in the inflammatory response (Costantini and Møller 2009; Costantini 2010a). The imbalance between the antioxidant defences of an organism and the prooxidants is called oxidative stress (Costantini 2010a). To compensate the damage caused by infections and the immune response, birds can up-regulate their endogenous antioxidant defences, or they could increase the intake of

antioxidant-rich food (Costantini 2014). In nature, most antioxidant food comes from plants (Lehane and Saliba 2008; Rudrapal and Chetia 2017), so birds that are able to perceive the nutritional content of food sources and adaptively change their diet to compensate their needs (Catoni, Schaefer, et al. 2008; Beaulieu and Schaefer 2014) would be able to better cope with parasitic costs (Beaulieu and Schaefer 2013; de Roode et al. 2013).

Nevertheless, oxidative stress and other consequences of haemosporidian infections are difficult to assess in the wild. When sampling birds, we take a small portion of the avian population, leading to bias in our findings since really sick birds could be under-sampled due to their higher mortality (Rivero and Gandon 2018; Muriel 2020). The sampled birds would then be the ones that are able to cope with parasites and maintain activity levels closer to normal (Atkinson et al. 2008). Then, studying infections during challenging moments for the birds is key to uncover the hidden costs of parasites (Dhondt and Dobson 2017; Andersson et al. 2018), since they have to trade-off between investing resources in those specific requirements or to deal with infections (Sheldon and Verhulst 1996; Costantini 2010a). Some of those challenges can be development, reproduction or migration, which impose energetic and physiological demands (Christe et al. 2012; Delhaye et al. 2016; Eikenaar, Hegemann, et al. 2020; de Angeli Dutra et al. 2021). However, the relation between migration and haemosporidian infections is poorly studied.

The understanding of how birds have differential competence and exposure towards haemosporidian infections is essential to disentangle the distribution of prevalence and diversity of parasites and their repercussions in each individual and species. This thesis addresses these problems using approaches at different scales, from

the distribution of parasites at the community level to the impact of infections on individuals.

Aims and hypothesis

With this thesis, we aim to uncover how variation in exposure to parasites and diet (as a determinant of host competence) may shape the distribution of haemosporidian parasites among hosts, how the impact of parasites interferes with the capacity of individuals to deal with oxidative stress, and to what extent parasite impacts may be attenuated through adaptively feeding on dietary antioxidants. To do so, we focused on the exposure to vector bites (Chapter 1) necessary to acquire infections, and the coping ability related to diet (Chapters 1 and 3) and oxidative stress (Chapters 2 and 3), which may be determinant of birds' suitability as parasite reservoirs (Huang et al. 2023). The thesis is structured as follows:

- Chapter 1. We approached the study of the parasite exposure and the coping capacity of a community of understory birds from French Guiana, using phylogenetic comparative methods. This area is a hotspot of avian biodiversity and all sampled birds are residents, which creates a great environment to study haemosporidian parasites that are locally transmitted and have a huge range of potential host species. We also described the community of circulating parasites in this understudied region. We expected that bird species with a higher exposure to vector bites (either because of their own morphology or their behaviour) would have higher prevalence. Also, species whose diet were based mostly on plant matter would have lower prevalence, because they are expected to gain easier access to compounds that may help them to better control the infections.

- Chapter 2. We conducted a study at the individual level focusing on the capacity of Eurasian blackcaps (*Sylvia atricapilla*) to cope with blood parasites during a physiologically demanding period (autumn migration), differentiating sex and age classes. We thought that, during stopover, birds would show physiological stress caused by infections and migration, and that more virulent infections, those with multiple parasites and/or *Plasmodium* parasites, would have stronger consequences. Also, we expected that younger individuals would suffer more the physiological costs of parasites and migration, since they did not have previous migratory experience and their infections were recently acquired. We also thought that there might be difference between sexes in their capacity to deal with oxidative stress due to the differential effect of sexual hormones (with testosterone being immunosuppressive and estrogen immunoenhancing), being males the ones with worst capacity to cope with infection.
- Chapter 3. We tested the capacity of young male blackcaps, in the same context than in Chapter 2, to deal with oxidative stress caused by infections and migration through diet choices. Offering different food choices during stopover, we expected that birds with worse oxidative status would prefer antioxidant-enriched food items, while birds that had a better oxidative status would choose to put on fats, which are very necessary to continue the next migratory stage.

Chapter 1: Differences in exposure to vector bites and plant-based diet explain among-species variation in prevalence of locally transmitted Neotropical avian malaria parasites

This chapter reproduces entirely the manuscript: Lucía Jiménez-Gallardo, Daniel A. Truchado, Laura Benítez, Marta Correas-Araus, Guillermo Fandos, Borja Milá, Michaël A. J. Moens, Álvaro Ramirez, Alvar Veiga-Schmitz, Javier Pérez-Tris and Carolina Remacha. Differences in exposure to vector bites and plant-based diet explain among-species variation in prevalence of locally transmitted Neotropical avian malaria parasites. In preparation.

Differences in exposure to vector bites and plant-based diet explain among-species variation in prevalence of locally transmitted Neotropical avian malaria parasites

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Abstract

Parasites have a heterogeneous distribution among host species, but the determinants of this variation in ecological communities, where major reservoirs coexist with incompetent hosts, remain unclear. We studied the species' traits correlated with prevalence of haemosporidian blood parasites in a community of resident understory birds from the primary rainforest of French Guiana. In this area, all infections are locally acquired making prevalence descriptive of variation in local exposure to parasites or species' abilities to cope with infections. We screened *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infections in a sample of 822 birds of 91 species, with molecular methods that allowed the identification of parasites using DNA barcodes. Focusing on the best sampled passerine species, we conducted a comparative analysis of ecological correlates of parasite prevalence based on records of behaviour and morphology. We tested the

influence of two phenotypic gradients that measured the degree of exposure to vector bites and the importance of vegetal food in the diet as a surrogate of the availability of nutrients that may boost immune function. Controlling for bird phylogeny, exposure to vector bites and dependence on plant-based diet were positively correlated with prevalence. We found a positive effect of territoriality, but no effect of body size on prevalence. In this community of passerines, where between-species differences in prevalence cannot be confounded by geographical or temporal variation in activity or community composition of parasites, our results indicate that species differ in host competence and exposure to parasites independently of their evolutionary ancestry. Such variation is correlated with species' traits associated with exposure to vectors and abilities to cope with infections, highlighting the importance of both factors in bird-haemosporidian interaction outcomes.

Keywords: birds, ecological traits, haemosporidian parasites, tropical rainforest, host exposure.

1. Introduction

Parasites play a key role in ecosystems by reducing the fitness of their hosts (Poulin 2007; Schmid-Hempel 2021). However, the impact of parasites as a selective agent is not equally shared by all candidate host species, which vary in their exposure to parasite invasion (Barrow et al. 2019) or in the way they cope with parasite exploitation through resistance or tolerance mechanisms (host competence; Råberg et al. 2007). Variation among species in exposure to parasites and coping abilities determine the realised distribution of parasite burdens in host communities, where major reservoirs coexist with incompetent hosts (Kilpatrick et al. 2006; Paull et al. 2012; Fenton et al. 2015). Comparative studies of species' traits associated with host competence (the extent to which a species can successfully transmit a parasite) and exposure to parasites are key to uncover the ecological processes that boost or restrain parasite acquisition and persistence within communities (Barron et al. 2015; Stewart Merrill and Johnson 2020; Huang et al. 2023). However, tracing parasite exchange among species is often impracticable, and therefore studies aimed at finding what shapes host-parasites interactions are usually based on parasite prevalence (Paull et al. 2012).

Avian haemosporidians (Apicomplexa: Haemosporida) are parasitic protozoans of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, which can be detected in the blood of birds using PCR-based methods that target universal genetic markers (Hellgren et al. 2004; Ciloglu et al. 2019; but see Valkiūnas et al. 2006). These practicalities, along with the large diversity of both birds and haemosporidians, have deemed this relationship a favourite model in the study of host-parasite interactions, which has produced a unique knowledge of their distribution among bird species worldwide (Bensch et al. 2009; Fecchio, Chagas, et al. 2020). The ecological significance

of this interaction is substantiated by negative impacts of haemosporidian infections on body condition, survival and breeding success of birds (Asghar et al. 2015; Pigeault et al. 2018). Haemosporidians are vector-borne parasites, which makes their presence in particular hosts greatly dependent on species' exposure to the bite of competent vectors. The latter include different families of blood-sucking dipterans that vary in their competence for transmitting each avian haemosporidian genera, their distribution, and their bird preference (Valkiūnas 2005; Hellgren et al. 2008; Santiago-Alarcón et al. 2012; Gutiérrez-López et al. 2019; Ferreira et al. 2020; Ferreira et al. 2020).

Various studies have analysed which host features best explain the distribution of avian haemosporidians among host species (Ellis, Fecchio, et al. 2020), including global approaches (Fecchio, Clark, et al. 2021), regional studies (Fecchio, Bell, et al. 2020; Gupta et al. 2020; Fecchio et al. 2022), and analyses of local communities (Ricklefs et al. 2005; Ellis, Huang, et al. 2020). These studies have found variable outcomes regarding the influence of host traits on haemosporidian prevalence (Ellis, Fecchio, et al. 2020), which may partly be explained because variation in prevalence not always reliably reflect species' roles in the dynamics of transmission of local parasites. For example, migratory species sampled in a given area may carry parasites acquired elsewhere, which may not be effectively brought in the community of locally transmitted parasites if competent vectors are locally absent (Hellgren et al. 2007; Ricklefs et al. 2017). In other cases, comparative studies involve samples obtained during a long time or at various locations for logistic reasons, where species identity may be confounded with geographic location, habitat type or sampling period (Latta and Ricklefs 2010; Filion et al. 2020; Fecchio, Clark, et al. 2021). These sources of variance make it difficult to reveal how variation

among species in exposure to vector bites or host competence shape the structuring of local host-parasite networks.

Lowland primary Neotropical rainforests are excellent scenarios to study which species' traits best explain the distribution of haemosporidian parasites at community level. The Neotropics are among the most diverse regions worldwide, both for birds and avian haemosporidians (Clark et al. 2014; Moens and Pérez-Tris 2016; Fecchio et al. 2019; Harvey et al. 2020). Consequently, vectors and parasites encounter a high variety of potential hosts in this region, which greatly differ in their evolutionary ancestry, ecological niche and morphological traits, all of which may influence on their exposure to parasites and competence as parasite hosts (Fecchio, Chagas, et al. 2020). Importantly, Neotropical lowland primary rainforests are rarely visited by migratory birds (Chesser 1994; Jahn et al. 2020), and their understory species are among the most extreme residents in the bird community, to the point that their populations often become structured by soft barriers such as rivers (Hayes and Sewlal 2004). Therefore, species' differences in haemosporidian prevalence of understory species can be safely assumed to be the outcome of local host-parasite dynamics, where parasite encounter rates and coping abilities of each species may be key.

We conducted a comparative analysis of variation in haemosporidian prevalence among understory birds of the Nouragues nature reserve (French Guiana), one of best-preserved primary rainforests in the Neotropics (Bongers et al. 2001). Inland rainforests of the Guiana shield are fairly disconnected from both Amazonian lowlands and major avian migratory routes in South America, and not a single migrant was included among 6658 individual birds of 248 species recorded during a 4-year survey conducted in Nouragues (Thiollay 1994). This paucity of migrant birds, exceptional in the Neotropical

region (Robinson et al. 2021), makes this area unique for the analysis of local determinants of among-species variation in parasite prevalence, as it allows to safely assume the absence of parasites being regularly imported from other areas. Previous research has revealed divergent host roles in this bird community, where some species concentrate infections while others seem to escape them, scoring lower prevalence than expected from random transmission within the community (Truchado et al. 2020).

We aimed to distinguish between exposure to vector bites and species' abilities to cope with infections as drivers of such variation in Nouragues. We predicted higher exposure-mediated prevalence of parasites for species that dwell in dense understory where vectors rest, as well as for birds with greater surface of bare parts exposed to vector bites (Martínez-de la Puente et al. 2015; Fecchio, Chagas, et al. 2020). To assess species' competence, we quantified the importance of plant matter in the diet as a measure of the access to nutrients that may boost the immune system (Beaulieu and Schaefer 2013; de Roode et al. 2013), or to vegetal compounds with antimalarial effects, such as flavonoid antioxidants (Lehane and Saliba 2008; Rudrapal and Chetia 2017; Masello et al. 2018). Therefore, we predicted a coping-mediated negative correlation between proportion of vegetal diet and prevalence of haemosporidians (Aguiar de Souza Penha et al. 2023). In our analyses, we controlled possible effects of territoriality, which may be negatively associated with exposure to vectors if territory spacing reduces parasite transmission rates, or positively if patrolling movements to defend the territory increase vector encounters (Herrera and Nunn 2019). We also controlled for body size as a possible determinant of species' exposure to parasites and coping abilities (Kamiya et al. 2014; Ruhs et al. 2020).

2. Materials and methods

2.1.Study area and sample collection

The samples were collected in a remote lowland tropical rainforest in northern South America, in The Nouragues Nature Reserve, French Guiana, in two camps located approximately 6 km apart, Pararé (4°1.9'N, 52°40.8'W, elevation 70 m a.s.l.) and Inselberg (4°5.01'N, 52°40.8'W, 112 m a.s.l.; Fig. 1). The area is characterized by an Equatorial climate influenced by the Inter-tropical Convergence Zone, where annual temperature averages 26 ºC approximately and annual precipitation is higher than 3,000 mm, with a dry season between August and November. The Inselberg camp, located on the foothills of a 400-m high rocky outcrop, is dominated by Caesalpiniaceae and Sapotaceae species. The forest at Pararé, on the banks of the Arataye River, is dominated by Burseraceae species. The canopy height can reach 40-50 m but different forest understory structures dotted with vines and small forest gaps from tree falls can be observed (Thiollay 1994; Bongers et al. 2001).

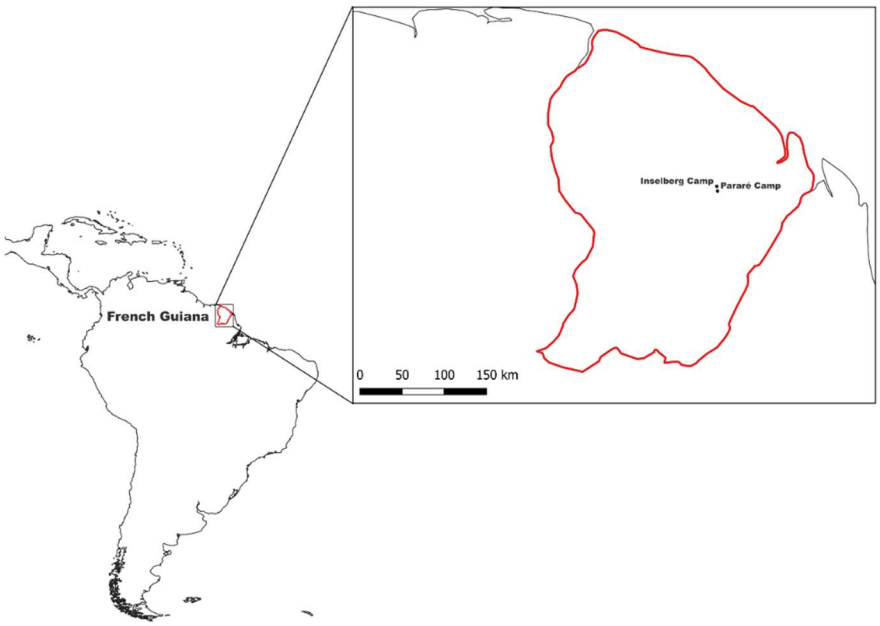

Figure 1. Location of the two camps in Nouragues Natural Reserve in French Guiana used as sampling sites.

We mist-netted understory birds, during October-November of 2016 and November-December of 2017 (38 sampling days at Pararé, 23 sampling days at Inselberg). Within each camp we sampled different areas, changing sampling spot every 1-2 days to avoid bird habituation, and setting a variable number of mist nets depending on habitat configuration and weather (total mist-net length per day ranged 48-189 m), starting at sunrise and ending when bird activity decreased at mid-day. All birds were measured and taken a blood sample by venepuncture (< 1 % body mass; Carpenter and Campbell 1995). After processing, birds were fitted a numbered ring and released unharmed at the site of capture. Blood samples were kept in absolute ethanol at ambient temperature in the field and stored at -20 ºC at the end of each sampling session. Our sample included 822 individuals of 91 species, spanning 8 orders and 23 families

(Supplementary Table S1). Based on local bird monitoring programs (Thiollay 1994), all 91 species sampled were resident.

2.2.Parasite screening

We extracted total DNA from blood samples with a standard ammonium acetate protocol (Green et al. 2012) or using the SpeedTools DNA extraction kit (Biotools), and we included at least one tube with water as blank in each extraction batch. We quantified DNA concentration and diluted the samples to 25 ng/µl. We first tested all samples with a sexing protocol (Fridolfsson and Ellegren 1999), or variants of this method when required to sex some species (e.g., Han et al. 2009 for hummingbirds), which was used as a control of sample quality for PCR and to obtain the sex of individuals (not used in further analyses). We then screened samples and extraction blanks for parasite DNA presence with a multiplex PCR that simultaneously targets DNA fragments of different size of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* parasites (Ciloglu et al. 2019). The final reaction volume was 10 µl, with 5 µl of 2× Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.2 µl of each primer (10 µM; PMF/R for *Plasmodium,* HMF/R for *Haemoproteus*, and LMF/R for *Leucocytozoon*), 1.8 µl of ddH_2O, and 2 µl of DNA diluted template. The PCR started with a hot start at 95 °C for 15 min, followed by 35 cycles at 94 °C for 30 s, 59 °C for 90 s and 72 °C for 30 s, and an extension at 72 °C for 10 min. All reactions included negative controls (ddH_2O) and a triple positive control consisting of DNA of all three parasite genera obtained by mixing different bird samples. We visualized the result by running 4 µl of the PCR product in 3 % agarose gels stained with 100× GelRed®.

Positive samples in the multiplex PCR screening were amplified with a nested PCR (Hellgren et al. 2004) that targets the MalAvi DNA barcode, a 478-bp fragment of the

parasite *Cyt b* gene broadly used to determine the identity of parasites infecting each bird (Bensch et al. 2009). A pre-amplification step targeted all three genera (primers HAEMNFI and HAEMNR3), which was followed by specific amplifications of either *Plasmodium* and *Haemoproteus* (primers HAEMF and HAEMR2) or *Leucocytozoon* (primers HAEMFL and HAEMR2L). Reactions were set at 25 µl total volumes, including 0.5 units AmpliTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 1 µl of each 10 µM primer, 2.5 µl of a mix of 1.25 mM of each dNTP, 1.1 µl of 25 mM of $MgCl_2$, 2.5 µl of buffer 10×, 14.8 µl of ddH_2O and 2 µl of DNA template. The thermal profile included a denaturation step at 94 °C for 3 min; 20 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, and an extension of 10 min at 72 °C. We used 1 µl of the PCR product as template for one or both specific amplification steps, according to the results of the multiplex PCR screening, with the same PCR conditions and temperature profile as the pre-amplification step, but running 35 cycles and using 15.8 µl of ddH_2O. All reactions included negative (ddH_2O) and triple positive controls. We sequenced nested PCR products using an Applied Biosystems™ 3730xl DNA Analyzer and manually edited with Bioedit v 7.7 software (Hall 1999). Sequences that differed by at least 1 bp in the MalAvi barcode were deemed as different lineages (Bensch et al. 2009). Newly discovered linages were sequenced from both sides and coinfections were identified by multiple products in the multiplex PCR and/or by mixed sequence signal in sequencing electropherograms (Pérez-Tris and Bensch 2005a). Some samples that tested negative in nested PCR were re-tested with the multiplex PCR screening conditions but using specific uniplex PCR protocols, with the primers that target the genera for which a fragment of the expected size had been amplified by multiplex PCR. This step allowed to distinguish true infections from unspecific amplifications that occasionally produced bands of

expected sizes as visualised on agarose gels. Positive results that could not be confirmed by sequencing or reproduced by specific PCR were deemed negative.

We calculated the prevalence of infection for each species as the proportion of sampled individuals that were infected. We computed total parasite prevalence (without distinguishing parasite genera) and prevalence of each parasite genus. We discarded all species with less than 10 sampled individuals to avoid low-precision prevalence estimates (Ricklefs et al. 2005; Jovani and Tella 2006). Our sample was dominated by passerines and hummingbirds, which together accounted for 90.1 % of species and 97.7 % of individuals. Other bird orders were poorly represented in the sample (Supplementary Table S1) and were therefore excluded from between-species comparisons. Various hummingbird species were frequently sampled, but the singular lifestyle of hummingbirds introduced undesirable heterogeneity for comparison purposes. For instance, the morphological traits we considered are hardly interpreted as having the same function in hummingbirds and passerines. As an example, we used beak slenderness as a proxy for insectivorous diet, and wing roundness as indicative of high manoeuvrability in dense vegetation (see below), two interpretations valid for passerines but not for hummingbirds. Therefore, although we report all parasite interactions found in our study for descriptive purposes, we excluded hummingbirds from the comparative analyses of correlations between species' traits and parasite prevalence, which therefore focused on well-sampled passerine species.

2.3.Host traits

We used published data from all species included in the comparative analysis ($n \geq 10$) to assess which species attributes were associated with variation in haemosporidian prevalence. We focused on host functional traits associated with the

probability of vector bite or with the importance of plant matter in the diet. We assumed that the ability to cope with infections is boosted by protective nutrients of vegetal origin (Cornet et al. 2013; Delhaye, Glaizot, et al. 2018). We obtained morphological data from the AVONET database, a resource that includes measurements of various bird dimensions obtained with at least 0.1-mm precision from at least 4 live birds or museum specimens (Tobias et al. 2022). Behavioural data were obtained from the EltonTraits 1.0 database (Wilman et al. 2014), which reports the percentage of use of foraging strata and of different elements present in the diet with 10 % precision.

To compute a size-independent index of exposure to vector bites, we conducted a principal components analysis (PCA) of morphological correlates of microhabitat use within the forest and observations of species microhabitat distribution. The hand-wing index (HWI) was used as a measure of flying capabilities, where more aerial birds have high HWI and more manoeuvrable species have low HWI (Savile 1957; Desrochers 2010; Tobias et al. 2022). Among understory birds of tropical rainforests, improved manoeuvrability (low HWI) is typical of species dwelling in dense vegetation, where most dipteran vectors rest and parasite encounter rate increases consequently (Snow 1955; Service 1971; Burkett-Cadena et al. 2013; van Hoesel et al. 2019). In addition, we used relative tarsus length (RTL, the residuals of tarsus length on log-transformed body mass) as a measure of the surface of bare skin exposed to vector bites (Yan et al. 2017; Gutiérrez-López et al. 2019). Finally, we used the amount of time spent within the understory as a measure of microhabitat preference that increases the probability of encounter with vectors (Fecchio et al. 2022). We used the percentage of time spent foraging below 2 m as a measure of preference for dense understory microhabitat. The PC1 accounted for 66.7 % of the variance (factor loadings: HWI = -0.82; RTL = 0.90;

foraging below 2 m = 0.72), and we interpreted PC1 scores (hereafter exposure to vectors) as indicative of high rate of parasite encounters.

We conducted another PCA to obtain an index of the importance of plant-based diet for each species. We used beak depth divided by beak length as an index of the mechanical advantage of the beak (MAB), a functional trait that balances bite force transmission and jaw closing speed during biting and is highly correlated with the importance of hard-processing plant matter in avian diet (Navalón et al. 2019). Therefore, MAB was used as a morphological correlate of plant-based diet. This relationship between beak shape and feeding ecology is straightforward for passerines, with insectivores and granivores on opposite extremes of the gradient (Navalón et al. 2019), but bill shape may also depend on nondietary factors (Bright et al. 2016). Because of this reason, we also considered the proportion of plant matter in the diet of each species, which we computed by summing up all dietary categories including plant food in the EltonTraits 1.0 database (Wilman et al. 2014). Species with equal proportion of plant matter in their diet differed in MAB, so we conducted the PCA with beak morphology and diet data as sources of information on species' feeding ecology. The PC1 accounted for 87.4 % of the variance (factor loadings for MAB and proportion of plant matter in diet = 0.93), and we interpreted PC1 scores (hereafter plant-based diet) as indicative of greater importance of plant matter in the diet of the species (Navalón et al. 2019).

We controlled the possible confounding effects of body size and territoriality in our analyses of the relationships between parasite prevalence and exposure to vectors or plant-based diet. Body mass was used as a measure of body size of each species, which is often associated with variation in parasite abundance among species (Kamiya et al. 2014; Downs et al. 2019), including some studies of avian haemosporidians (Scheuerlein

and Ricklefs 2004; Ricklefs et al. 2005). We obtained data on territoriality of each species from Tobias and Pigot (2019), distinguishing between strong territoriality (species that maintained territories all year round) and weak or no territoriality (when territories were defended seasonally or were restricted to the nest surroundings).

2.4.Data analyses

We conducted comparative analyses controlling the phylogeny of bird species, for which we obtained a consensus tree from 1,000 Erikson trees computed by Birdtree (Jetz et al. 2012). We used the R package caper version 1.0.3 (Orme et al. 2023) to fit phylogenetic general least squares models (PGLS) of species' prevalence as a function of exposure to vectors and plant-based diet, controlling for possible confounding effects of body size and territoriality. We estimated the phylogenetic signal of the model residuals by optimizing Pagel's λ (Orme et al. 2023). We tested the same model structure for total prevalence (regardless of parasite genus) and for *Plasmodium* prevalence. We did not analyse *Haemoproteus* or *Leucocytozoon* infections because of smaller sample sizes of these parasites (see results). In all analyses, we transformed continuous variables whenever needed to meet normality, and z-standardized them to compute comparable model coefficients. We also checked that residuals were distributed according to model assumptions. We used R 4.2.2 (R Core Team 2022) in all statistical analysis.

3. Results

Out of 822 individuals tested, we found 81 individuals infected by one or more parasites. Parasite detectability was boosted by the multiplex PCR screening, as 41.9 % of all parasite infections found by this method were not reproduced by nested PCR. We

retrieved 47 parasite DNA sequences from 43 birds of 22 species. We found 22 different parasite linages, 7 of which were new to the MalAvi database (Bensch et al. 2009), including 5 *Haemoproteus* and 17 *Plasmodium* (none of the *Leucocytozoon* infections could be sequenced; Supplementary Table S1). We detected coinfections in 8 birds, and in 3 of them we could not retrieve fully resolved parasite sequences (based on electropherogram peaks, 3 of the parasites involved in these cases might add to the list of newly discovered lineages). Newly discovered lineages were often close relatives of parasites previously found in the Neotropics (Fig. 2). The most abundant linage was THACAE08 (27.7 %) present in seven species of antbirds (Thamnophilidae) and one manakin (Pipridae). Detailed information on the distribution of parasite lineages among species are found in Supplementary Table S1.

Community-level prevalence (9.9 %) was heterogeneously distributed among parasites of the genera *Plasmodium* (65 infections / prevalence 7.9 %), *Haemoproteus* (17 / 2.1 %), and *Leucocytozoon* (4 / 0.5 %). There were 18 passerine species with $n \geq 10$ individuals sampled (with parasite prevalence ranging 0 – 53.9 %), which were selected for comparative analyses. These species spanned a broad phylogenetic diversity, including 6 families (Furnariidae, Pipridae, Thamnophilidae, Thraupidae, Turdidae and Tyrannidae; Fig. 3). Antbirds (Thamnophilidae) were the best represented family with nine species including the species with the highest prevalence, *Isleria guttata* with 53.9 % of individuals infected. The mean total prevalence of well sampled species was 17.0 % ± 16.4: *Plasmodium* 14.0 % ± 15.4, *Haemoproteus* 2.5 % ± 4.0 and *Leucocytozoon* 0.5 % ± 1.2.

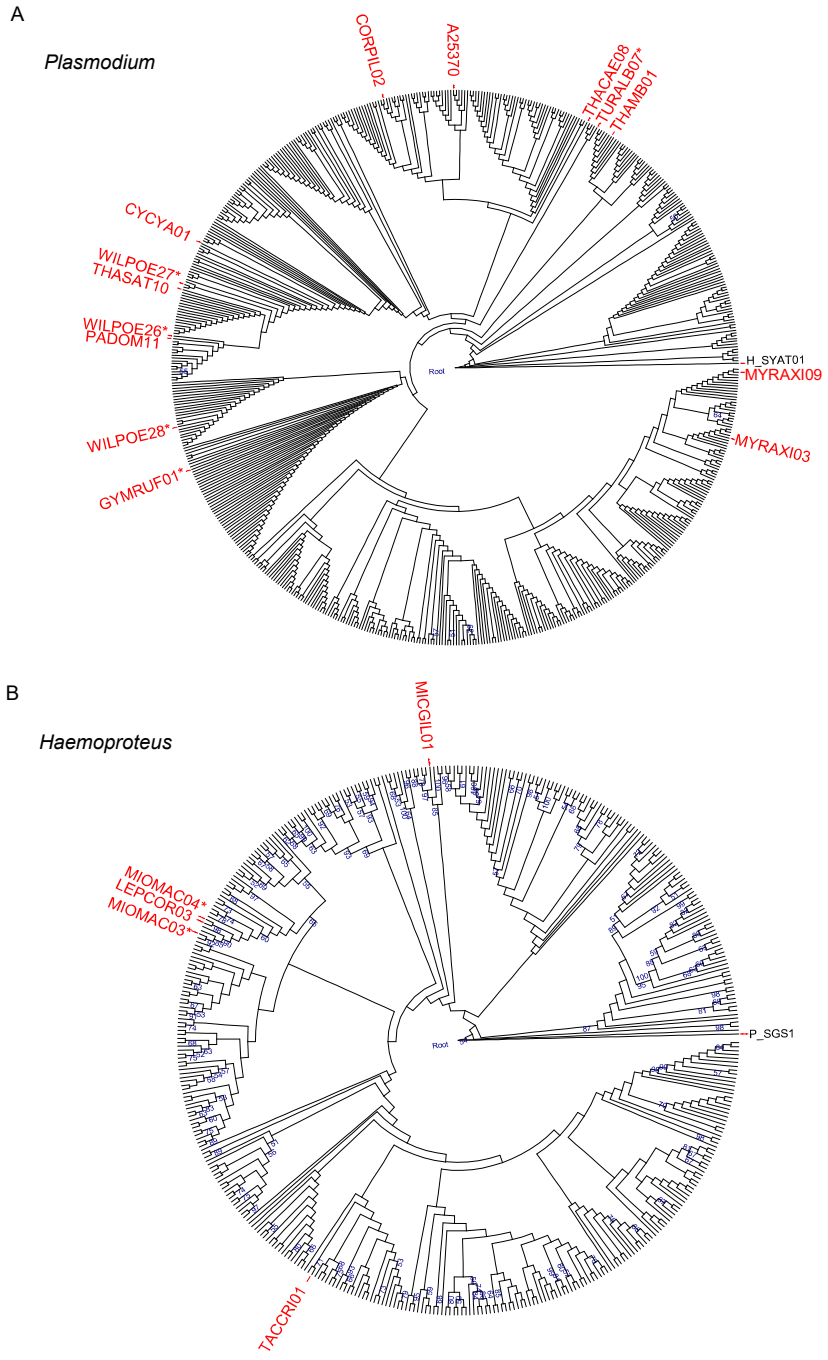

Figure 2. Consensus tree after 100 bootstrap replicates with sequences of 479 bp of cytochrome *b* of *Plasmodium* (A) and *Haemoproteus* (B) parasite lineages found in South American birds. Data were extracted from the MalAvi database (Bensch et al. 2009). Lineage

names are shown for parasites found in our study (newly discovered lineages are marked with stars). Bootstrap support values for nodes above 50 are shown. The *Haemoproteus* tree has been rooted using a *Plasmodium* lineage (SGS1), and *vice versa* (SYAT01 roots the *Plasmodium* tree).

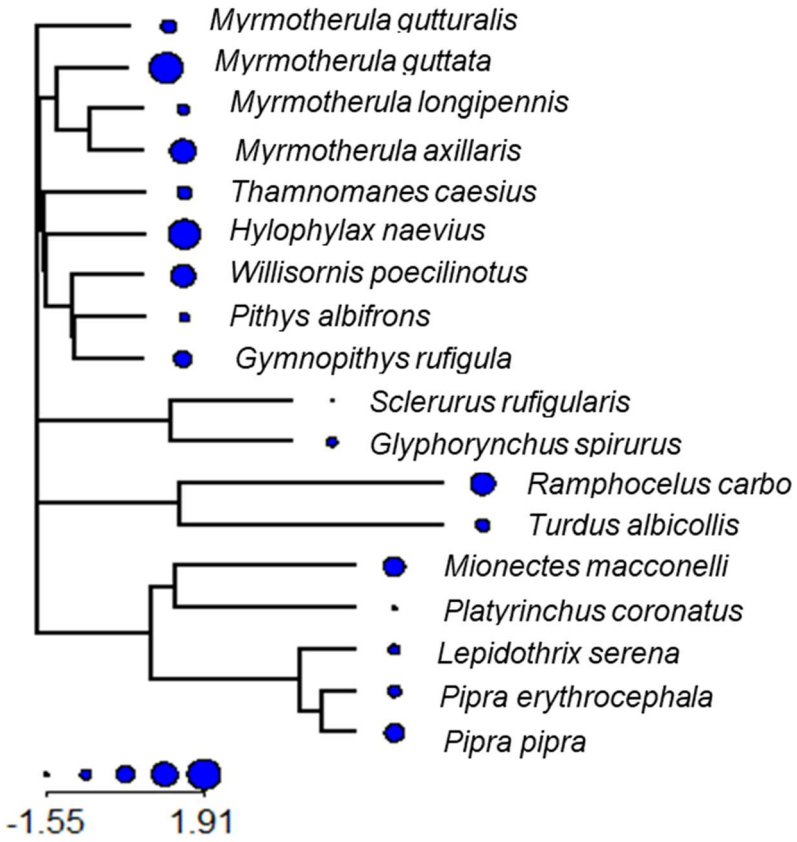

Figure 3. Phylogeny of 18 passerine species with at least 10 individuals sampled. The tree was obtained by the majority rule consensus from 1,000 Ericson trees produced by Birdtree (Jetz et al. 2012). Blue dots represent the standardized prevalence of infection of each species.

In the PGLS model of total parasite prevalence as a function of exposure to vector bites, plant-based diet, body mass and territoriality, phylogenetic signal was not significantly different from either zero ($P = 1$) or 1 ($P = 0.09$), and the model run with a ML-estimated $\lambda = 0$ (undetectable phylogenetic signal). We found positive effects of both exposure to vectors and plant-based diet on parasite prevalence (Table 1, Fig. 4A). Body mass was not significantly correlated with prevalence, but strong territoriality showed a significant positive correlation with prevalence. When we modelled the variation in prevalence of *Plasmodium* as a function of the same species' traits, phylogenetic signal was not significantly different from either zero ($P = 0.46$) or 1 ($P = 1$), and the model run with a ML-estimated $\lambda = 1$ (Brownian evolution). We found the same associations between prevalence of *Plasmodium* and species' traits observed for total parasite prevalence (Table 1; Fig. 4B).

Table 1. Phylogenetic general least squares models analysing variation in parasite prevalence (total or *Plasmodium*) as a function of two phenotypic gradients obtained by PCA of morphological and behavioural variables (exposure to vector bites and plant-based diet), body mass and territoriality (strong *vs* weaker modes of territorial spacing). The phylogenetic signal of each model (maximum likelihood estimate of λ) is also shown.

	Parasite prevalence (λ = 0)			*Plasmodium* prevalence (λ = 1)		
	Estimate ± se	$F_{1,13}$	*P*	Estimate ± se	$F_{1,13}$	*P*
Exposure to vector bites	1.15 ± 0.27	18.26	< 0.001	1.09 ± 0.26	18.03	< 0.001
Plant-based diet	1.20 ± 0.39	9.63	0.008	1.13 ± 0.39	8.21	0.013
Body mass	-0.16 ± 0.24	0.44	0.517	-0.04 ± 0.26	0.02	0.888
Territoriality (strong)	0.67 ± 0.27	6.43	0.025	0.81 ± 0.27	9.03	0.010

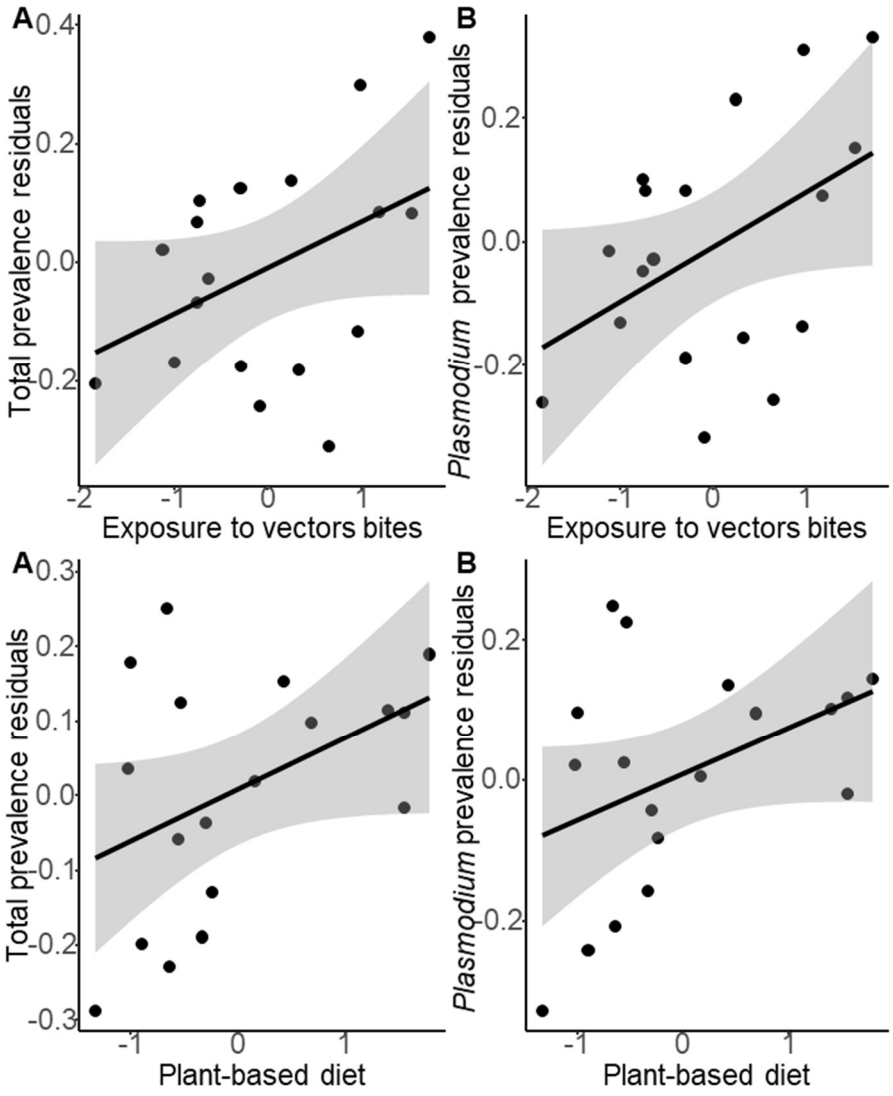

Figure 4. Residual effects in a phylogenetic general least squares model of the exposure to vectors bites (upper plots) and plant-based diet (lower plots) on total prevalence (A) and *Plasmodium* prevalence (B), controlling for exposure to vector bites (only for plant-based diet), plant-based diet (only for exposure to vector bites), body mass and territoriality (strong *vs* weaker modes of territorial spacing). The dots are species' estimates, and grey areas represent 95 % confidence intervals.

4. Discussion

In this community of Neotropical resident passerines, exposure to vectors and plant-based diet were positively correlated with prevalence of haemosporidian parasites. Controlling for phylogenetic relationships among species, understory birds that dwell in dense vegetation and have more skin exposed to vector bites scored higher parasite prevalence. In addition, species that depend more strongly on plant matter attained higher parasite prevalence. Besides, strong territoriality was correlated with higher rates of parasitism. Importantly, these relationships are interpretable as the outcome of differences among species in their susceptibility and suitability as hosts for a community of locally transmitted parasites.

In our study, parasite diversity was as high (22 distinct lineages in 47 parasites sequenced) as previously reported for Amazonian lowlands (245 linages in 520 sequences in Fecchio et al. 2019). The parasite community was clearly dominated by the genus *Plasmodium*, which represented 77 % of sequenced parasite lineages (similar to 81 % in Amazonian forests reported by Fecchio et al. 2019) using Nested PCR as the screening method. The genus *Haemoproteus* had low prevalence and diversity, and *Leucocytozoon* occurred at very low frequency (only 4 infections found in different species: Silver-beaked Tanager *Ramphocelus carbo*, Wedge-billed Woodcreeper *Glyphorynchus spirurus*, Musician Wren *Cyphorhinus arada* and White-crowned Manakin *Pseudopipra pipra*). Similar results have been found for the latter parasite genus in Amazonian lowlands (PCR-based prevalence < 1 %; Fecchio et al. 2018). All *Leucocytozoon* infections in our study were detected by multiplex PCR screening and later confirmed by uniplex PCR with specific primers, but we failed to amplify MalAvi barcodes by nested

PCR and therefore could not identify genetic lineages, probably due to low intensity infection as the multiplex method is more sensitive (Ciloglu et al. 2019). In fact, the combination of multiplex PCR screening and nested PCR greatly increased parasite detectability in our study, in which nearly half (41.9 %) of infections would have been missed by the nested PCR methods currently used in most comparative studies of these parasites, thereby biasing prevalence estimates. Therefore, our study further recommends using highly sensitive methods in analyses of prevalence of avian haemosporidians (Ciloglu et al. 2019), even if detected parasites are hard to sequence. On the other side, double checking of the multiplex PCR screening with specific primers and sequencing of uniplex PCR products when in doubt proves advisable to avoid inflating the rate of false positives by deeming unspecific amplifications as parasite detections.

The composition of the parasite community could be the outcome of variation in the abundance or activity of different groups of dipteran vectors, with mosquitos of the family Culicidae (which transmit *Plasmodium*) dominating over vectors of *Haemoproteus* (all lineages found in our study belong to subgenus *Parahaemoproteus*, transmitted by biting midges of the family Ceratopogonidae) and *Leucocytozoon* (blackflies of the family Simuliidae; Ferreira et al. 2020). However, our results provide strong evidence for local transmission of all three parasite genera in the primary rainforest of the Guiana shield, which means that competent vectors are present although perhaps not in the same abundance. Therefore, *Haemoproteus* and *Leucocytozoon* might be less efficiently transmitted than *Plasmodium* in this area, for example if unknown environmental constraints affected parasite development in the vectors, a possibility that has been postulated for *Leucocytozoon* in Amazonian lowlands (Fecchio et al. 2018; Fecchio, Bell, et al. 2020).

Although vector activity may play a role in structuring parasite communities, there is evidence that host-parasite compatibility rather than vector activity determines distribution of prevalence in bird communities (Medeiros et al. 2013). Moreover, some *Haemoproteus* parasites have problems to develop gametocytes in the blood of Neotropical passerines (Moens et al. 2016), meaning that for some parasites low prevalence could be the outcome of their interaction with hosts with immune abilities that restrain their blood circulation. Rainforest bird communities are dominated by long-lived species with slow-paced life-histories (Wiersma et al. 2007), which are associated with more robust constitutive innate immunity, reduced inflammatory responses and enhanced anti-inflammatory mechanisms (Tieleman et al. 2005; Sears et al. 2011). Different immune abilities of tropical and temperate birds could contribute to structuring parasite communities in each region, with *Haemoproteus* and *Leucocytozoon* attaining low prevalence due to strong host regulation of their parasitaemia. Compared to these two genera, *Plasmodium* undergoes merogony in blood and cannot hide in other tissues (Valkiūnas 2005), which may increase the cost of mounting an immune response against this genus (Cornet et al. 2013; Delhaye, Jenkins, et al. 2018), promoting strong resistance against *Plasmodium* parasites at initial phases of parasitaemia but tolerance during chronic infection, as it has been observed for these parasites in experimental settings (Sorci 2013).

Parasite infections were heterogeneously distributed among bird species. For example, hummingbirds were abundant in our sample (187 individuals tested of 11 species), but we only found one Fork-tailed Woodnymph *Thalurania furcata* infected, with a *Plasmodium* parasite that could not be sequenced. The paucity of haemosporidian infections in hummingbirds contrasts with prevalence records ranging from 14.3 % to

80 % in Andean forests (Moens et al. 2016), a difference which may be explained by the apparent absence in our study site of *Haemoproteus witti*, the most abundant hummingbird parasite in the Andes (Moens et al. 2016). This observation supports the idea that local transmission determines species' host roles in our study.

Our comparative analysis of 18 resident passerine species with reliable prevalence estimates provided evidence that exposure to vector bites was an important driver of parasite prevalence. Bird species with long legs and round wings that forage in dense understory might acquire more parasites by facing more frequent vector bites. The epitome of this trait combination are antbirds (Thamnophilidae), a family of understory dwellers that contained the species with the highest prevalence in our sample (Supplementary Table S1; see also Truchado et al. 2020), as it has been observed in other studies in Amazonia (Fecchio et al. 2017), although the existence of other relevant hosts kept the phylogenetic signal low in our models. Increased transmission in dense understory may principally involve *Plasmodium* parasites, the genus which drives the patterns of variation in prevalence in this system, because female mosquitos rest and find blood meals in cluttered understory vegetation (Service 1971; Burkett-Cadena et al. 2013; van Hoesel et al. 2019). However, it is unclear whether dense understory species are more exposed to vector bites due to habitat use (Fecchio et al. 2022) rather than to the morphological correlates of their lifestyle.

We predicted that bird species that include abundant plant matter in their diet would have lower prevalence, as it has been observed for example in Psittaciformes (Masello et al. 2018). However, we found a significant correlation with the opposite sign. This result could be explained if high reliance on plants were confounded with dwelling in low forest strata because fruits, a major source of protective nutrients of plant origin

(Lehane and Saliba 2008; Beaulieu and Schaefer 2013; Rudrapal and Chetia 2017), abound in the Neotropical forest understory (Schaefer et al. 2002). However, exposure to vector bites and plant-based diet were negatively associated in our sample (r = -0.66, $p < 0.01$). Therefore, we interpret the positive correlation between plant-based diet and prevalence as evidence of increased parasite tolerance in plant eaters. Assisted by the protective effects of dietary antiparasitic compounds, plant eaters could afford elevated intensity of chronic infections (Sorci 2013), which would increase parasite detectability as a consequence, particularly so for *Plasmodium* parasites (Hellgren et al. 2004). If this interpretation is correct, elevated parasite load would increase both the attractiveness to mosquitos of infected plant-feeding birds (Yan et al. 2018) and the rate of parasite transmission to mosquitos feeding upon their blood (Cornet et al. 2014), boosting the role of plant-feeding species as avian malaria reservoirs in Neotropical rainforests.

Controlling for the major effects of exposure to vector bites and plant-based diet, strong territoriality was positively associated with parasite prevalence. Previous studies found higher prevalence of blood parasites in group-living species (Tella 2002; Fecchio et al. 2011). However, studies conducted so far compared solitary and colonial species, while our analyses of understory passerines did not include social birds (except for manakins that gather in leks). Therefore, it remains an open question whether strong territoriality is associated with higher vector encounter rates if defensive patrolling movements bring birds into vector-rich microhabitats (Herrera and Nunn 2019), or it rather increases susceptibility. In fact, territorial behaviour increases testosterone levels (Wingfield et al. 1987; Hau et al. 2000), which has an immunosuppressive effect (Folstad and Karter 1992) and may induce persistent susceptibility to *Plasmodium* as observed in mammals (Benten et al. 1997).

Host body size was not correlated with parasite prevalence in our study. Most comparative analyses of prevalence of avian haemosporidians have tested for this correlation, and very few have detected it (Matthews et al. 2016; Ellis, Fecchio, et al. 2020). Finding an effect of body size may be difficult in studies that combine species at different trophic levels or from different habitats (Kamiya et al. 2014), but such influences were irrelevant in our study. Although variation in body size is clearly associated with parasite abundance in mammals and fish, the relationship is generally weak and largely mediated by phylogenetic effects in birds (Poulin 1995), likely due to the smaller range of body size variation in birds compared to other vertebrates. Homogeneously small body size may further obscure any relationships between host body size and prevalence in studies of small passerine species captured in mist nets (the body mass of species included in our comparative analyses ranged 8.1 to 54 g). On the other hand, although body size has been pointed out as a universal predictor of parasite abundance across taxa (see a metanalysis in Kamiya et al. 2014), it remains to be investigated whether the same conclusion applies to vector-borne parasites.

Haemosporidian prevalence is known to be correlated with factors other than the ones included in the phenotypic gradients analysed here. For example, behavioural traits such as nest type and location in the forest, and life-history traits such as development time, clutch size or lifetime expectancy have been found to be correlated with prevalence in comparative analyses of Neotropical birds (Ellis, Fecchio, et al. 2020). Small per species sample size recommended not to test many predictors in our models, therefore we focused on ecological traits associated with exposure to vector bites and diet as predictors of parasite prevalence, omitting other relevant ecological traits. Nevertheless, many of these traits are largely captured by the phenotypic gradients analysed in our study, for

example nest height is correlated with foraging height, and body size captures much variation in life-history parameters. Besides, our study of passerine species limits variation in life-histories and behavioural traits compared to analyses conducted at broader phylogenetic scale (but see an example of correlation between life-history traits and prevalence within a passerine family in Aguiar de Souza Penha et al. 2023). Prevalence may greatly vary seasonally or in relation to migratory behaviour, but these factors were irrelevant in our study as we sampled resident birds during the same season (we are aware that different species may have different seasonal patterns of variation in prevalence, although seasonal patterns tend to be weaker in Neotropical communities; Chapa-Vargas et al. 2020). Finally, we did not study the relationship between species' traits and prevalence of *Haemoproteus* nor *Leucocytozoon* in this community, which are usually more host-specialised and might follow different transmission dynamics compared to *Plasmodium*, the genus that drives the observed patterns (Ellis, Fecchio, et al. 2020; Lima and Pérez-Tris 2020). Future studies with greater databases may uncover other relationships between species' traits and parasite prevalence in this community of resident Neotropical birds, broadening the understanding of ecological determinants of heterogeneous host competence directly linked to local transmission dynamics.

Supplementary information

Supplementary Table S1

Table S1. Taxonomic composition of the sample of birds analysed in this study, with information on the number of individuals tested, number of infected by *Haemoproteus* (H), *Plasmodium* (P) and *Leucocytozoon* (L), and identity of the parasites detected (genus and genetic lineage according to the MalAvi database; Bensch et al. 2009). The number of sequences found of each parasite lineage is indicated. Parasites that were identified to the genus level by specific uniplex PCR but could not be sequenced the MalAvi barcode are named "screening". Some sequences could not be fully resolved but provided enough data to be treated as new lineages (their name includes "Undetermined"). Bird species that were included in the comparative analysis of species' traits correlated with parasite prevalence are highlighted in bold.

Orden	Family	Species name	n tested	infection (H/P/L)	Genus	Linage in MALAVI	n sequences
Accipitriformes	Accipitridae	*Pseudastur albicollis*	1	(0/0/0)			
Caprimulgiformes	Trochilidae	*Campylopterus largipennis*	33	(0/0/0)			
		Florisuga mellivora	1	(0/0/0)			
		Glaucis hirsutus	10	(0/0/0)			
		Heliothryx auritus	1	(0/0/0)			
		Phaethornis bourcieri	21	(0/0/0)			
		Phaethornis malaris	15	(0/0/0)			

		Phaethornis ruber	2	(0/0/0)			
		Phaethornis superciliosus	55	(0/0/0)			
		Thalurania furcata	47	(0/1/0)	*Plasmodium*	screening	
		Threnetes niger	1	(0/0/0)			
		Topaza pella	1	(0/0/0)			
Columbiformes	Columbidae	*Geotrygon montana*	1	(0/0/0)			
		Leptotila rufaxilla	6	(0/0/0)			
Coraciiformes	Alcedinidae	*Chloroceryle aenea*	3	(0/0/0)			
		Chloroceryle inda	2	(0/0/0)			
Coraciiformes	Momotidae	*Momotus momota*	1	(0/0/0)			
Falconiformes	Falconidae	*Micrastur ruficollis*	1	(1/0/0)	*Haemoproteus*	H_MICGIL01	1
Passeriformes	Cardinalidae	*Cyanoloxia cyanoides*	5	(0/3/0)	*Plasmodium*	P_PADOM11	1
	Conopophagidae	*Conopophaga aurita*	2	(0/0/0)			
	Cotingidae	*Cotinga cayana*	1	(0/0/0)			
		Lipaugus vociferans	3	(0/0/0)			
		Phoenicircus carnifex	1	(0/0/0)			
	Formicariidae	*Formicarius analis*	3	(0/1/0)	*Plasmodium*	screening	
		Formicarius colma	6	(0/3/0)	*Plasmodium*	screening	
	Furnariidae	*Automolus ochrolaemus*	7	(0/0/0)			
		Campylorhamphus procurvoides	1	(0/0/0)			
		Deconychura longicauda	1	(0/0/0)			
		Dendrocincla fuliginosa	1	(0/0/0)			
		Glyphorynchus spirurus	**63**	**(1/0/1)**	***Haemoproteus***	**screening**	
					Leucocytozoon	**screening**	
		Philydor erythrocercum	4	(0/0/0)			

		Philydor pyrrhodes	3	(0/0/0)			
		Sclerurus caudacutus	3	(0/0/0)			
		Sclerurus rufigularis	**10**	**(0/0/0)**			
		Xenops minutus	4	(0/0/0)			
		Xiphorhynchus pardalotus	8	(1/0/0)	*Haemoproteus*	H_MIOMAC03 (new)	1
	Hirundinidae	*Progne chalybea*	1	(0/0/0)			
	Pipridae	***Ceratopipra erythrocephala***	**16**	**(1/1/0)**	***Haemoproteus***	**H_MIOMAC04 (new)**	**1**
					Plasmodium	**P_Undetermined_2 (new)**	**1**
		Corapipo gutturalis	5	(0/0/0)			
		Lepidothrix serena	**24**	**(0/1/0)**	***Plasmodium***	**screening**	
		Manacus manacus	9	(0/0/0)			
		Pseudopipra pipra	**45**	**(3/4/1)**	***Haemoproteus***	**H_MIOMAC04 (new)**	**1**
					Plasmodium	**P_THACAE08**	**2**
					Haemoproteus	**H_LEPCOR03**	**1**
					Plasmodium	**P_Undetermined_3 (new)**	**1**
	Polioptilidae	*Microbates collaris*	2	(0/1/0)	*Plasmodium*	P_CYCYA01	1
		Ramphocaenus melanurus	2	(0/0/0)			
	Thamnophilidae	*Cercomacra nigricans*	2	(0/0/0)			
		Cercomacroides tyrannina	1	(0/0/0)			
		Cymbilaimus lineatus	1	(0/1/0)	*Plasmodium*	P_THAMB01	1

Epinecrophylla gutturalis	**21**	**(0/2/0)**	***Plasmodium***	**P_CYCYA01**	**1**
Gymnopithys rufigula	**14**	**(0/2/0)**	***Plasmodium***	**P_GYMRUF01 (new)**	**1**
			Plasmodium	P_THACAE08	1
Hylophylax naevius	**10**	**(0/5/0)**	***Plasmodium***	**A25370**	**1**
			Plasmodium	**P_THACAE08**	**4**
Hypocnemis cantator	8	(0/1/0)	*Plasmodium*	P_THACAE08	1
Isleria guttata	**13**	**(1/6/0)**	***Plasmodium***	**A25370**	**1**
			Plasmodium	**P_THACAE08**	**2**
			Haemoproteus	**screening**	
Microrhopias quixensis	2	(0/0/0)			
Myrmoderus ferrugineus	3	(0/0/0)			
Myrmornis torquata	2	(0/0/0)			
Myrmotherula axillaris	**15**	**(0/5/0)**	***Plasmodium***	**P_MIRAXI03**	**1**
			Plasmodium	**A25370**	**2**
			Plasmodium	**P_MYRAXI09**	**1**
Myrmotherula longipennis	**19**	**(0/1/0)**	***Plasmodium***	**P_THACAE08**	**1**
Myrmotherula menetriesii	4	(0/0/0)			
Myrmotherula surinamensis	3	(0/0/0)			
Percnostola rufifrons	4	(0/1/0)	*Plasmodium*	screening	
Pithys albifrons	**62**	(1/1/0)	*Haemoproteus*	*low trace values*	
			Plasmodium	*low trace values*	

		Thamnomanes ardesiacus	9	(0/2/0)	*Plasmodium*	P_THACAE08	1
		Thamnomanes caesius	**21**	**(0/2/0)**	***Plasmodium***	**P_THACAE08**	**1**
		Thamnophilus murinus	2	(0/0/0)			
		Willisornis poecilinotus	**31**	**(0/9/0)**	***Plasmodium***	**P_WILPOE26 (new)**	**3**
					Plasmodium	**P_THASAT10**	**2**
					Plasmodium	**P_WILPOE27 (new)**	**1**
					Plasmodium	**P_Undetermined_ 1**	**1**
					Plasmodium	**P_WILPOE28 (new)**	**1**
	Thraupidae	*Cyanerpes caeruleus*	1	(0/0/0)			
		Islerothraupis luctuosa	1	(0/0/0)			
		Lanio fulvus	2	(0/0/0)			
		Maschalethraupis surinama	8	(1/1/0)	*Haemoproteus*	H_TACCRI01	1
		Ramphocelus carbo	**23**	**(2/4/1)**	***Plasmodium***	**P_CORPIL02**	**1**
					Haemoproteus	**H_TACCRI01**	**1**
		Saltator maximus	2	(0/0/0)			
		Sporophila angolensis	1	(0/0/0)			
	Tityridae	*Myiobius barbatus*	7	(1/0/0)	*Haemoproteus*	H_MIOMAC04 (new)	1
		Onychorhynchus coronatus	2	(0/0/0)			
		Schiffornis turdina	4	(0/0/0)			
	Troglodytidae	*Cyphorhinus arada*	6	(0/1/1)	*Plasmodium*	screening	

					Leucocytozoon	screening	
		Microcerculus bambla	2	(0/0/0)			
		Pheugopedius coraya	3	(0/1/0)	*Plasmodium*	screening	
	Turdidae	***Turdus albicollis***	**13**	**(0/1/0)**	***Plasmodium***	**P_TURALB07 (new)**	**1**
	Tyrannidae	*Attila spadiceus*	2	(0/0/0)			
		Corythopis torquatus	4	(0/0/0)			
		Mionectes macconnelli	**32**	**(4/3/0)**	***Haemoproteus***	**H_MIOMAC04 (new)**	**1**
					Haemoproteus	**H_MIOMAC03 (new)**	**2**
					Plasmodium	**screening**	
		Myiozetetes cayanensis	1	(0/0/0)			
		Platyrinchus coronatus	**11**	(0/0/0)			
		Platyrinchus saturatus	4	(0/1/0)	*Plasmodium*	screening	
		Rhynchocyclus olivaceus	2	(0/0/0)			
		Tolmomyias poliocephalus	2	(0/0/0)			
		Tyrannus melancholicus	2	(0/0/0)			
	Vireonidae	*Tunchiornis ochraceiceps*	4	(0/0/0)			
Piciformes	Galbulidae	*Galbula albirostris*	3	(0/0/0)			
	Picidae	*Celeus elegans*	1	(0/0/0)			

Chapter 2: Blackcaps *Sylvia atricapilla* with blood parasites have impaired oxidative status during autumn migration

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Blackcaps *Sylvia atricapilla* with blood parasites have impaired oxidative status during autumn migration

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Abstract

Avian haemosporidian parasites decrease bird fitness, and their impact is expected to increase when their hosts face difficulties to maintain the balance between antioxidant defences and prooxidants (oxidative balance). During physiologically challenging life stages such as migration, blood parasite infections may impair performance of individuals that are less prepared to tackle oxidative stress and disease. We tested whether infection status and oxidative balance during autumn migration differed between young blackcaps, which are on their first migratory journey and have recently acquired parasite infections, and older ones, which have more experience in migration and parasite defence. We also analysed variation between males and females, which may deal with infections or oxidative stress differently due to differences in sexual hormones and their implication in the immune system. We captured blackcaps in a stopover area and measured body condition, the number of blood parasites linages as determined from DNA sequences, and physiological condition using different parameters descriptive of birds' oxidative status. Blackcaps with large body mass, high haemoglobin and triglyceride content, and lower

uric acid content (which we interpreted as being physiologically more prepared to depart from stopover) had lower levels of antioxidant capacity of plasma, probably because of physiological adjustments prior to the next flight stage. Young individuals had higher frequency of coinfections (different parasites in blood) than adults, and *Plasmodium* parasites occurred only in coinfections. The antioxidant capacity of plasma was lower in coinfected birds than in not infected birds, and individuals coinfected with *Plasmodium* had more oxidative damage to lipids in their red blood cells. Our results support the idea that haemosporidian infections impair the oxidative balance of blackcaps during autumn migration and may be especially harmful to young individuals, an impact which could impair individual migration performance and ultimately fitness.

Keywords: oxidative stress, young blackcaps, ecophysiology, haemosporidian parasites, migration stopover, *Sylvia atricapilla*.

1. Introduction

Parasite infections impair host performance and ultimately fitness (Poulin 2007; Krams et al. 2013; Chrétien et al. 2022). Avian haemosporidian parasites (Apicomplexa) are vector-borne blood parasites of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* that invade the blood and other tissues of a wide range of bird species (Valkiūnas 2005). They have negative impacts on development, reproduction, and survival of their hosts (Asghar et al. 2015; Pigeault et al. 2015; Muriel 2020). Birds infected with haemosporidian parasites rely on resistance mechanisms to reduce intensity of infection, which may induce a strong humoral response with energetic and physiological consequences; or they rather express tolerance mechanisms to live with the infection minimizing damage (Sorci 2013; Muriel 2020). Whatever strategy is chosen, it requires an investment of resources that become unavailable for other vital functions, which entails long-term costs (Tomás et al. 2007; Van de Crommenacker et al. 2012).

During their life cycle, haemosporidian parasites activate the immune system of their hosts (Delhaye, Jenkins, et al. 2018) and break red blood cells (RBC) and other invaded cells, inducing cell malfunction, anaemia and lower haemoglobin concentration (Phillips and Pasvol 1992), an impact that is most important during the acute phase of infection, short after parasites are acquired (Krams et al. 2013; Muriel 2020). Parasite metabolism together with the immune response of the host produce reactive oxygen and nitrogen species (RONS; Bichet et al. 2012; Bryla et al. 2022), which could damage tissues if they accumulate in the organism (Sorci and Faivre 2009). Oxidative stress results from the imbalance between reactive species and antioxidant defences, which can be endogenous or exogenous (Monaghan et al. 2009; Costantini 2010a). Then, when confronting an oxidant situation, animals could either increase the production of

endogenous antioxidants or promote the consumption of dietary antioxidants (Monaghan et al. 2009; Costantini 2010a; Chapter 3).

There are several stressful situations when birds may incur in oxidative damage such as development, senescence, reproduction, or migration (Finkel and Holbrook 2000; Guindre-Parker and Rubenstein 2018; Salmón et al. 2018). During migration, intense aerobic exercise and physiological adjustments during refuelling (including putting on fats prone to oxidation) make birds specially prone to oxidative stress (Marasco et al. 2021), which promotes up-regulation of endogenous defences or increased intake of dietary antioxidants during stopovers (McWilliams et al. 2021). During those challenging moments, parasitic effects are expected to be stronger, since limited antioxidant defences may strengthen the trade-off between immune defence and oxidative repair (Eikenaar et al. 2018).

When dealing with parasitic infections and oxidative stress, individual performance may be influenced by traits like sex, age, or body condition (Monaghan et al. 2009; Christe et al. 2012; Isaksson et al. 2013; Muriel 2020). The immunosuppressive effect of testosterone to the humoral response (Folstad and Karter 1992; Roved et al. 2017; Vincze et al. 2022) may increase *Plasmodium* parasitemia (as it has been observed in mice; Benten et al. 1997), which could increase prevalence and the cost of infections in males (Jenkins et al. 2015), making it imperative to measure the relationships between parasites and oxidative stress differentiating between males and females (Roved et al. 2017). Host age is also important since youngs and adults differ in the degree of development of their immune system, their capacity to tackle oxidative stress (Costantini et al. 2006; Alonso-Álvarez et al. 2010; Romero-Haro and Alonso-Álvarez 2014), their experience to overcome migration challenges (Rotics et al. 2016), or how long they have

harboured parasite infections (Huang et al. 2020). Therefore, comparing young and adult birds is essential to fully understand how birds cope with parasites during migration.

We studied individual differences in the capacity of Eurasian blackcaps (*Sylvia atricapilla*) to maintain oxidative balance when infected by haemosporidian parasites during autumn migration. We differentiated between single infections and coinfections, considering the different parasite genera and lineages present in blood (Bensch et al. 2009; Ciloglu et al. 2019), and investigated with special detail the infections of *Plasmodium* parasites, which are known to be more virulent than the other genera of parasites (García-Longoria et al. 2022; Messina et al. 2022). We predicted a negative relationship between parasite infection status and oxidative status measured by indices of oxidative damage and antioxidant capacity, an effect which we expected to be stronger for males due to the immunosuppressive effect of testosterone and the better immune protection against parasites in females (Vincze et al. 2022), and especially for young blackcaps, since they are developing physiological and immunological mechanisms to cope with oxidative stress and parasites.

2. Materials and Methods

2.1. Field sampling

We conducted our sampling during the peak of autumn migration of blackcaps (Cantos 1995) in October 2019 and 2020 (during 4 sampling days each year, 4-11 October in 2019 and 14-26 October in 2020), in a stopover site located in central Spain (40°20'08"N 3°24'27"W, 780 m a.s.l). We captured individuals from 9:30 until 15:30 aproximately with mist-nets, using blackcap song records to lure them. We captured 154

blackcaps in total, 89 in 2019 and 65 in 2020. Following Svensson (1992), we determined the sex and age of individuals, differentiating between young birds born in the previous spring and older individuals (adults). Our sample included 66 females and 88 males; and 51 adults and 103 young birds. A sample of 49 males (48 of them young) were kept in cages during ca. 30 min before processing, as part of a collateral study on feeding preferences of young individuals (Chapter 3). The rest of birds were kept in cotton bags until processed as quickly as possible. We weighed birds to the nearest 0.01 g and measured tarsus length with 0.01 mm precision. Finally, we took a blood sample (< 1% body mass) from the jugular vein. We used standard aluminium rings to individualise birds avoiding pseudorreplication and then released them. We kept blood samples refrigerated in heparinised tubes until the afternoon, when samples were transported to the laboratory and processed. Samples were centrifuged to separate plasma from RBC and each subsample was stored at -80 °C until further analysis.

Blackcaps arrive lean at stopovers, where they put on fats until departure (Langslow 1976; Arizaga et al. 2008). Then, to measure body condition as a proxy of stored fat, we used the residuals of the linear regression of log-transformed body mass on tarsus length.

2.2.Haemosporidian parasite infections

We extracted total DNA from blood with the SpeedTools Tissue DNA extraction kit (Biotools, Madrid, Spain) and diluted the samples to 25 ng/µl. Then, all the samples were screened for haemosporidian parasites following a multiplex PCR that distinguished between *Haemoproteus*, *Plasmodium,* and *Leucocytozoon* parasites (Ciloglu et al. 2019). Total volumes were set at 10 µl, including 5 µl of 2× Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.2 µl of each primer (10 µM; PMF/R for *Plasmodium,*

HMF/R for *Haemoproteus* and LMF/R for *Leucocytozoon*), 1.8 µl of ddH_2O, and 2 µl of DNA template. The protocol included a hot start at 95 °C for 15 min, 35 cycles at 94 °C for 30 s, 59 °C for 90 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. All reactions included a negative control (ddH_2O) and a DNA sample that was positive for all three genera of parasites. We run 4 µl of the PCR products on 2 % agarose gels stained with 100× GelRed®.

All samples found positive in the multiplex PCR were amplified with a nested PCR (Hellgren et al. 2004). This PCR had a first amplification targeting the three genera with primers HAEMNFI and HAEMNR3. Then, a specific amplification targeted *Plasmodium* and *Haemoproteus* (HAEMF and HAEMR2), or *Leucocytozoon* (HAEMFL and HAEMR2L). The first step was set at 25 µl and included 0.5 units of AmpliTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 1 µl of each 10 µM primer, 2.5 µl of a mix of 1.25 mM of each dNTP, 1.1 µl of 25 mM of $MgCl_2$, 2.5 µl of Buffer 10×, 14.8 µl of ddH_2O, and 2 µl of DNA template. The reaction started with a step at 94 °C for 3 min; followed by 20 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s, and ended with an extension of 10 min at 72 °C. Then, 1 µl of the product of the first step was used as the template in the second one, with different primers depending on the results of the screening multiplex PCR. This second step had 35 cycles following the same temperature profile and PCR conditions as described above, but compensating the volume difference with ddH_2O. We always included a negative and a triple positive control. The final products of amplification were used to sequence a fragment of 478 bp of *Cyt-b* gene, which is used to identify avian malaria lineages (Bensch et al. 2009), using an Applied Biosystems™ 3730xl DNA analyser. We considered as different parasite lineages the sequences that differed by one or more base pairs (Bensch et al. 2009).

If the presence of several parasite lineages was confirmed either by the multiplex PCR or by double peaks in sequencing electropherograms, we considered the sample as coinfected (Pérez-Tris and Bensch 2005a; Valkiūnas et al. 2006; Ciloglu et al. 2019). If only one parasite lineage could be confirmed, we considered them as single-infected. Therefore, we classified the infection status of individuals using a factor with three levels: not infected, infected by one parasite, and coinfected by two or more parasites (only 5 individuals had 3 different parasites). Most infections involved *Haemoproteus* parasites, while *Leucocytozoon* were much less common, and *Plasmodium* always occurred in coinfection with parasites of other genera (see results). Therefore, we conducted additional analyses focusing on *Plasmodium* coinfections, which we deemed more virulent due to the synergistic effect of coinfection and the presence of a parasite that is usually more virulent to birds (Marzal et al. 2008; García-Longoria et al. 2022; Messina et al. 2022).

2.3.Oxidative status and physiological markers

Before separating plasma from RBC, we measured haemoglobin concentration with Drabkin reaction (Spinreact, Girona, Spain), following manufacturer's instructions. Haemoglobin levels could be related to parasite activity if haemosporidians cause anaemia, and is also known to vary during migration stopovers (Piersma et al. 1996). We run each sample, calibrator and blank (ddH_2O) in duplicates on 96-well plates. The absorbance was measured in a Biotek Epoch microplate spectrophotometer (BioTek Instruments, Inc.) at 540 nm. The repeatability was $r_i = 0.89$ ($n = 154$; $p < 0.001$), with an inter-assay CV of 12.61 % and an intra-assay CV of 1.78 %. As described before, we centrifuged the remaining blood sample for 10 min at 12000 rpm to separate plasma from

RBC. The same observer determined the degree of haemolysis on plasma distinguishing 3 levels of redness (0: clear sample, 1: pale red stain, 2: red sample).

To standardize measurements on RBC (see below), we measured protein content of RBC using Bradford assay (Sigma-Aldrich, St. Louis, MO). We run each sample in duplicates on 96-well plates, including protein standard (BSA) ranging from 0.1 to 1.4 mg/ml and a blank (ddH2O). All measures followed manufacturer's instructions, taking absorbance at 595 nm using a Synergy HT MultiMode Microplate199 Reader (BioTek Instruments, Inc.; this spectrophotometer was used in all subsequent analyses). Repeatability was $r_i = 0.58$ ($n = 154$; $p < 0.001$), inter-assay CV between the samples was 17.21 % and the CV intra the samples was 6.50 %.

We measured an endogenous antioxidant indicator in RBC using intracellular total glutathione levels (tGSH) following López-Arrabé et al. (2014). Briefly, all samples were diluted (1:20 w/v), homogenised in stock buffer (0.01 M phosphate buffered saline and 0.02 M EDTA) and mixed with an equal volume of 10 % trichloroacetic acid. After that, samples were vortexed during 5 s three times in 10 min and then centrifuged at 2000 g for 10 min at 6 °C. We separated the supernatant and added firstly NADPH and DTNB, and GSH reductase after 15 s. We run all samples in 96-well plates in duplicates, with a blank (buffer), and a standard curve (serial dilution of GSH from 0.5 to 0.031 mM). We measured one 12-well row at a time, measuring absorbance at 405 nm after 15 and 45 s. The tGSH was determined comparing the output of the change in absorbance with the standard curve. The tGSH levels were standardised to the total protein content in the sample by dividing tGSH by protein values. The repeatability was $r_i = 0.92$ ($n = 153$; $p < 0.001$), inter-assay CV was 12.60 % and the intra-assay CV was 8.31 %.

As a measure of oxidative damage to cell membranes we used a by-product of lipid peroxidation, malondialdehyde (MDA). Blood parasites could cause peroxidation of phospholipids in RBC because of the activation of the immune system and their own metabolism (Percário et al. 2012). Also, during migration, the integrity of cell membranes is key to the performance of the pectoral muscle (Weber 2009). Then, we measured MDA in plasma (plasma-MDA) and in RBC (RBC-MDA), as two indices of general lipid peroxidation and damage to RBC membranes, respectively. We followed Romero-Haro and Alonso-Álvarez (2014), including a blank and a standard curve per batch diluted from 1,1,3,3 tetraethoxypropane with 40 % ethanol. We added butylated hydroxytoluene, phosphoric acid, and thiobarbituric acid (TBA) solutions to each sample and incubated them for 1 h in a dry bath at 100 °C. MDA-TBA adducts were formed, and we added pure n-butanol, vortexed and centrifuged. We collected the upper phase and transferred it into an HPLC vial. We injected all samples in duplicates when possible (139 out of 149 samples of plasma-MDA, and 152 out of 154 samples of RBC-MDA) into an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA), and measured them with a fluorescence detector (ref. G1321A, Agilent Technologies). Repeatability of plasma-MDA was $r_i = 0.91$ ($n = 139$; $p < 0.001$), with an inter-assay CV = 27.44 % and an intra-assay CV = 17.22 %. The repeatability of RBC-MDA was $r_i = 0.76$ ($n = 152$, $p < 0.001$), inter-assay CV = 20.58 % and intra-assay CV = 15.81 %. We standardised the levels of RBC-MDA dividing by the total protein content.

We quantified the antioxidant capacity of plasma as a marker of the level of circulating antioxidant defences, such as vitamins C or E and carotenoids. We measured total antioxidant status (TAS) following the protocol proposed by Miller et al. (1993) and modified by López-Arrabé et al. (2014). We run samples, blank and calibrator (Trolox,

an α-tocopherol derivative) in duplicates whenever there was enough plasma (132 out of 148 samples), in 96-well plates. We run just one row at a time to control the reaction delay. Briefly, we added metmyoglobin (an equilibrated mixture of myoglobin and potassium ferricyanate), a chromogen (2,2 ′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid or ABTS), and H_2O_2 to start the reaction in each well. We recorded absorbance at 660 nm, each 10 s at 37 °C. The repeatability was $r_i = 0.96$ ($n = 132$; $p < 0.001$), inter-assay CV was 14.19 % and the intra-assay CV 5.44 %.

Uric acid concentration is usually positively correlated to TAS values, and it is also an endogenous antioxidant and a product of the catabolism of amino acids in birds (Cohen et al. 2007; Pérez-Rodríguez et al. 2008; Costantini 2010b). Therefore, we measured uric acid in plasma to correct TAS values using a commercial kit (Biosystems, Barcelona, Spain). The test was run according to manufacturer's instructions. We used 96 well plates incubating them at 37 °C for 10 min and measuring absorbance at 520 nm. We run each sample in duplicates whenever possible (140 out of 151 samples). Repeatability was $r_i = 0.95$ ($n = 140$, $p < 0.001$), inter-assay CV was 4.07 % and the intra-assay CV was 5.76 %.

Finally, we measured plasma triglycerides, as an indicator of the fattening status of the birds during stopover (Jenni and Jenni-Eiermann 1998), using a commercial kit (Biosystems, Barcelona, Spain) according to manufacturer's instructions. Furthermore, triglycerides can be related to MDA levels and therefore may need to be controlled for in the analysis of this variable (Pérez-Rodríguez, Romero-Haro et al. 2015). We measured absorbance at 500 nm. The repeatability of duplicates (138 out of 148) was $r_i = 0.98$ ($n = 138$; $p < 0.001$), inter-assay CV was 1.72 % and the intra-assay CV was 5.76 %.

2.4.Statistical analyses

We used the R package MASS v 7.3-60 (Venables and Ripley 2002) to fit an ordinal logistic regression model testing whether individuals of each sex or age class differed in their odds of bearing more parasites, using the multiple infection variable as a factor with three ordered levels, from not infected to coinfected. We used the Brant's test implemented in the brant package v 0.3-0 (Schlegel and Steenbergen 2020) to check the proportional odds (parallel regression) assumption of the model. We fitted a logistic regression (with binomial error and logit link function) to analyse differences between sexes and age classes in the probability of harbouring *Plasmodium* infections.

We checked the influence of haemolysis level and plate identity to each measurement and corrected them when needed. Since TAS and uric acid levels were strongly correlated (r = 0.78), we extracted the residuals of that relation as a TAS corrected measure. We checked the relation between all the variables that could be involved directly in the migratory metabolism of blackcaps, namely body condition as a measure of fat content, haemoglobin, uric acid, and triglycerides, and performed a Principal Component Analysis (PCA) with them. The PC1 accounted for 34.0 % of variance in the correlation matrix (factor loadings: body condition = 0.61, haemoglobin = 0.63, uric acid = -0.58, and triglycerides = 0.50), and we interpreted PC1 scores as an index of individuals' readiness to continue the migratory journey (Jenni et al. 2006). Although the correlation between plasma-MDA and triglycerides was weak (r = 0.15, $F_{1,142}$ = 3.40, p = 0.07), we considered more appropriated to correct plasma-MDA values using the residuals of their regression on triglycerides following previous studies (Pérez-Rodríguez, Romero-Haro et al. 2015).

To assess the effect of infections, sex, and age on the physiological condition of individuals, we fitted lineal models (LM) with physiological parameters as dependent variables. We tested separately the effect of the multiple infection variable (not infected, single infected, or coinfected) and the effects of *Plasmodium* coinfections (present or absent), including age, year, and sex as covariates. We also included readiness to depart (PC1 scores) in the analyses where none of the variables included in this PCA was the dependent variable. When an oxidative damage biomarker was analysed as a dependent variable, we included all antioxidant biomarkers as covariates and viceversa. We also included in the analysis the two-way interactions between infection and age, sex, and the physiological parameters included as covariates, when the corresponding interactions were significant. We tested which variables and interactions best predicted the response variable using the Akaike information criterion (AIC; Burnham and Anderson 2002) using the dredge function with the MuMIn package v 1.47.5 (Bartoń 2023). Based on AIC values corrected for small sample size (AICc), we focused in all models with ΔAICc ≤ 2 as the best models. When the best models included the null model, we concluded that none of the effects tested was significant. Otherwise, we averaged the best models to compute the significance of effects (Symonds and Moussalli 2011). Whenever infection (the focal factor in our study) was included in any of the best models, we used the option fixed in MuMin to recalculate model-averaged estimates only with models including infection. All estimates were full averaged across models. Finally, when the multiple infection variable was retained in the model averaging step, we used the relevel option to estimate all pairwise between-level differences. All continuous variables were checked for normality, transformed when advisable (square root-transformed uric acid, tGSH, and TAS; log-transformed plasma-MDA and triglycerides) and z-standardised. Residuals of

the models were distributed according to model assumptions. We conducted all statistical analyses using R 4.3.2 (R Core Team 2023).

3. Results

We tested 154 blackcaps, of which 130 were infected with haemosporidian parasites, 43 harboured more than one parasite, and 22 were coinfected by *Plasmodium* parasites (see Supplementary Table 1 for further information of parasite lineages). Out of 103 young blackcaps tested, 91 (88.3 %) were infected, and 36.3 % of these harboured coinfections, compared to 39 (76.5 %) adults infected, 25.6 % with coinfections. There were 10 (15.2 %) females not infected, 37 single-infected (56.1 %), and 19 coinfected (28.8 %); while males were 14 not infected (15.9 %), 50 single-infected (56.8 %), and 24 coinfected (27.3 %). For young birds, the odds of bearing higher levels of the multiple infection variable, i.e., being infected or coinfected versus not infected, was 2.22 times that of adults (ordinal logistic regression estimate for young = 0.80; se = 0.35; 95 % CI [0.12, 1.50]; $t_{152} = 2.27$; $p = 0.023$; Fig. 1), holding constant the effect of sex, which was not significant (estimate for females = 0.24; se = 0.33; $t_{152} = 0.73$; $p = 0.47$). The probability of harbouring *Plasmodium* was slightly higher for females than for males, although the difference was not significant (estimate for females = 0.86; se = 0.50; z = 1.80; $p = 0.072$). Controlling for sex in the logistic regression, both age classes showed similar probabilities of having *Plasmodium* infections (estimate for young = 0.51; se = 0.53; z = 0.97; $p = 0.33$).

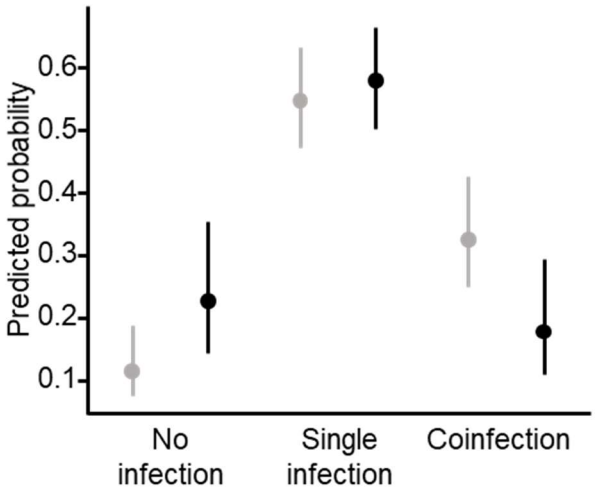

Figure 1. Estimated probability of bearing different levels of parasite infection (no infection, single infection, or coinfection) estimated for young (grey) and adult (black) blackcaps in an ordinal logistic regression model with age and sex (not significant) as predictors of infection status. Bars denote 95 % confidence intervals.

Models with $\Delta AIC \leq 2$ analysing variation in physiological parameters included the null model for haemoglobin, uric acid, plasma-MDA (in the case of total infections, but not in *Plasmodium* infections), and triglycerides (Supplementary Table 2 and 3). We found a positive relation between tGSH and RBC-MDA (Fig. 2 and 5), and birds closer to departure (with higher readiness PC1 scores) had lower levels of antioxidant capacity of plasma (TAS; Fig. 3). Moreover, when testing for the different levels of the multiple infection parameter (fixed for model averaging), we found that birds infected with multiple parasites had lower TAS compared to not-infected individuals (Fig. 3). Finally, birds infected by *Plasmodium* parasites had higher values of plasma-MDA (Fig. 4).

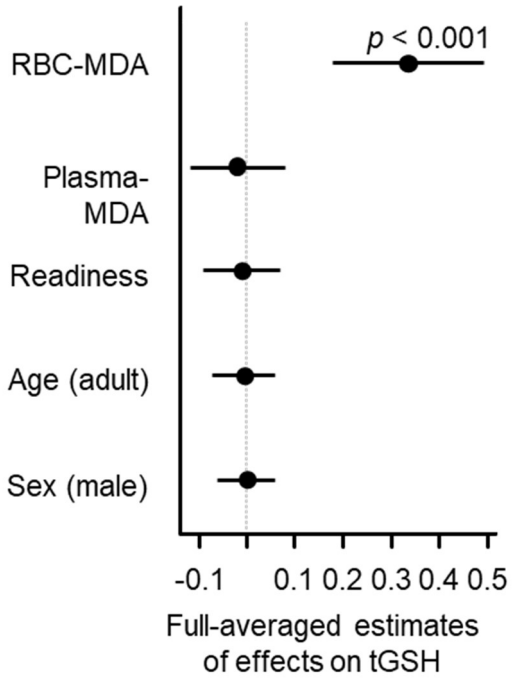

Figure 2. Full model averaging estimates with 95 % confidence intervals of the effects of variables included in the best models for variation in tGSH (square root transformed) of blackcaps. None of the best models included multiple infection status or *Plasmodium* infection as predictors. The *p*-values of statistically significant effects are shown. Plasma-MDA was log-transformed.

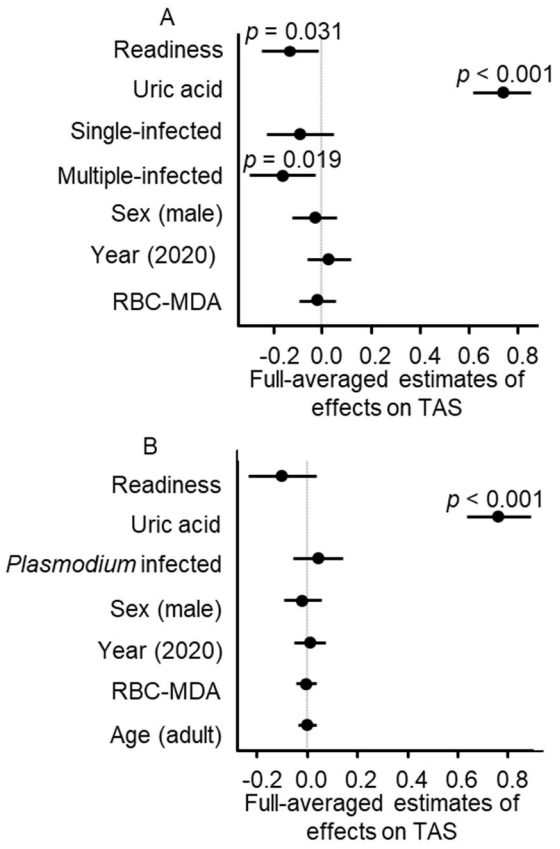

Figure 3. Full model averaging estimates with 95 % confidence intervals of the effects of variables included in the best models for variation in TAS (square root transformed) of blackcaps, when these were tested using multiple infection status (A) or *Plasmodium* infection (B) as parasite-related predictors. The *p*-values of statistically significant effects are shown. The effect of multiple infection status is shown with not infected as the reference level. Changing the reference to single-infected: 0.064 ± 0.051, $p = 0.208$ not-infected; -0.082 ± 0.050, $p = 0.106$ multiple-infected. Uric acid was square root transformed.

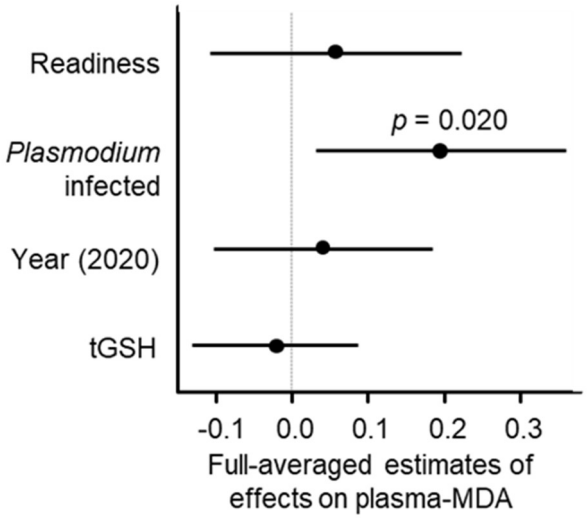

Figure 4. Full model averaging estimates with 95 % confidence intervals of the effects of variables included in the best models for variation in plasma-MDA (log transformed) of blackcaps, when these were tested using *Plasmodium* infection as the parasite-related predictor (the null model was included among the best models when we used this variable to test parasite influences). The *p*-values of statistically significant effects are shown. Square root transformation was applied to tGSH.

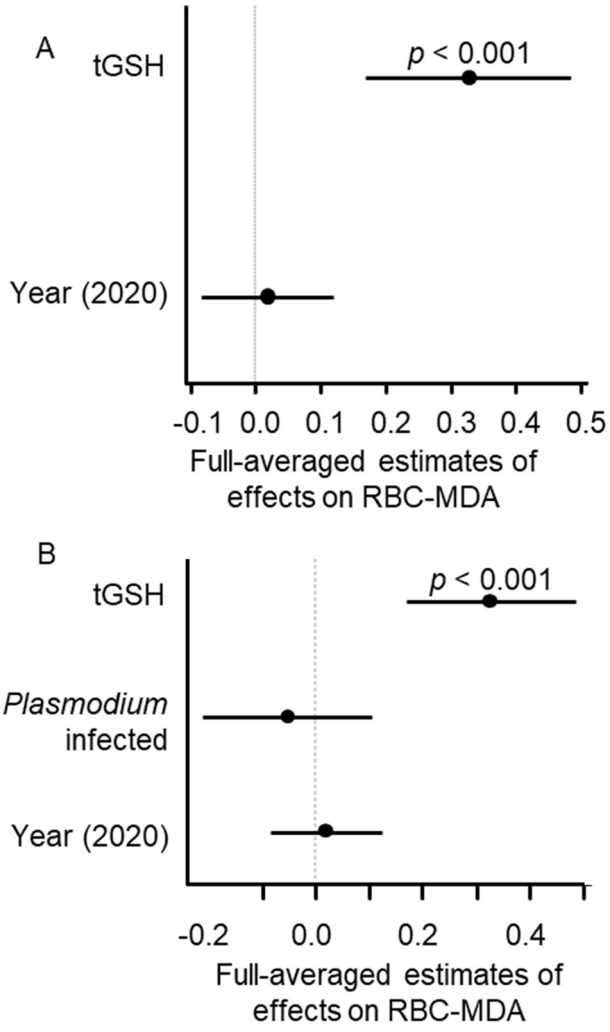

Figure 5. Full model averaging estimates with 95 % confidence intervals of the effects of variables included in the best models for variation in RBC-MDA of blackcaps, when these were tested using multiple infection status (A) or *Plasmodium* infection (B) as parasite-related predictors (none of the best models included multiple infection status as predictor when we tested the effect of parasites with this variable). The *p*-values of statistically significant effects are shown. The effect of multiple infection status is shown with not infected as the reference level. Square root transformation was applied to tGSH.

4. Discussion

We found that young blackcaps during autumn migration presented more coinfections than adults, which were more likely to harbour single parasites when they were infected. We also found that *Plasmodium* parasites were always present in coinfection with other parasites, something we would not have been able to detect without combining several techniques for parasite detection (Hellgren et al. 2004; Ciloglu et al. 2019). Globally, infected birds showed signs of poorer capacity to maintain oxidative balance during migration, as shown lower levels of TAS in coinfected individuals compared to not infected, and higher oxidative damage to lipids in blackcaps infected by *Plasmodium* parasites. We did not find any effect of age or sex on the physiological status nor the body mass of migrating blackcaps. Nevertheless, since *Plasmodium* parasites were always found in coinfection, and coinfections can be more virulent (Alizon and van Baalen 2008; Pigeault et al. 2018; García-Longoria et al. 2022) and concentrated in young blackcaps, our results support the idea that blackcaps in autumn migration face the strongest parasite burden during their first year of life.

Birds infected by *Plasmodium* parasites in our population had higher levels of damage to lipids in their plasma as measured by MDA values. Moreover, we found a gradual decrease in the antioxidant capacity of plasma with increasing number of parasites in blood, which led to a significant difference between not infected and coinfected individuals. The small size of this effect was probably due to high variability of TAS measurements among birds that are (1) analysed in an observational study and (2) undergoing profound changes of their oxidative balance associated with migration, which combines the physiological consequences of flight effort with metabolic and dietary adjustments during stopovers (Beaulieu and Schaefer 2013; Chapter 3). Taken

together, these effects probably inflate the error term in our analyses, so that the observed effect and its progressivity from not infected to coinfected individuals may be evidence of a relevant influence of parasites on the oxidative balance of migrating blackcaps (Van de Crommenacker et al. 2012).

Our results suggest that multiple haemosporidian infections and *Plasmodium* infections could be especially harmful to blackcaps, as they might increase the cost of migration by impairing oxidative status (Butler et al. 2021; Messina et al. 2022). Other studies have also found an increased virulence of coinfections of avian haemosporidians (Marzal et al. 2008; Pigeault et al. 2018), and our study contributes to unveil the physiological link between haemosporidian coinfection and impaired bird fitness. Coinfections may severely diminish the fitness of blackcaps if they decrease their chances of successfully completing the first migration cycle and, consequently, joining the breeding population as recruits. This selective filter may partly explain the lower prevalence of coinfections (which often included *Plasmodium* parasites) among adult blackcaps, although this effect could also be explained if adults had cleared most virulent infections acquired earlier in their life, and tolerate less aggressive parasites in chronic infections (Belo et al. 2011; Ellis et al. 2014). These two possible causes of age-related differences in the frequency of most virulent infections are not mutually exclusive.

We found a syndrome of physiological variables (greater fat deposits, high haemoglobin level, high triglyceride content and lower uric acid level) indicative of blackcaps' readiness for departure from the stopover site (Jenni et al. 2006). During stopovers, birds replenish their fat stores, stop using proteins to obtain energy (having less protein catabolic subproducts such as uric acid as a consequence; Romero-Haro and Alonso-Álvarez 2014; Pérez-Rodríguez, Romero-Haro et al. 2015), and compensate the

haemoglobin concentration (Piersma et al. 1996). However, we are careful with this interpretation since the link between triglyceride content and the duration of the stopover might not always be straitforward, although it seems to be negatively linked to the departure time, being the birds with more triglycerids the ones that spend less time in the stopover (Williams et al. 2007; DeSimone et al. 2023). Contrary to what we expected, birds that seemed to be physiologically prepared for departure had also lower levels of antioxidant capacity of plasma. The total antioxidant capacity of plasma might be depleting during stopover since in this period birds need to compensate the oxidative damage caused during flight through hyperphagia, which is linked to a decrement in the metabolic rate to increment the fattening (McWilliams et al. 2021). Antioxidant capacity might also be lower at the end of the stopover because of the accumulation and usage of polyunsaturated fatty acids that are prone to oxidation (McWilliams et al. 2021). Also, there was a positive correlation between tGSH levels and damage to lipids in RBC. Although tGSH levels not only measure endogenous antioxidant defences (Romero-Haro and Alonso-Álvarez 2015), our results clearly align with previous studies that have found a up-regulation of glutathione peroxidase in migrating birds, which was interpreted as an adaptation to maintain a balanced oxidative status when exercise increases the production of pro-oxidants (Jenni-Eiermann et al. 2014). Therefore, our results could be interpreted as a hormetic response to a constrained overproduction of RONS during migration (Costantini 2010a; Jenni-Eiermann et al. 2014; McWilliams et al. 2021). In RBC, the lipid damage during stopover could be dealt with through the upregulation of endogenous antioxidants (Leeuwenburgh and Heinecke 2001). Therefore, blackcaps might be suffering with oxidative stress from migration that needs to be compensated during stopovers. In turn, the complex physiology of the migrating bird may make the links between parasites and oxidative stress difficult to uncover in natural settings. For

example, we did not detect differences in the physiological status of male and female blackcaps, which were expected based on previous research (Roved et al. 2017; Vincze et al. 2022), a result which may parly be explained because the modulating effect of sex hormones on the immune system may be attenuated before sexual maturity is attained, and infections were concentrated in young blackcaps during migration.

Our results suggest a combined effect of migration and haemosporidian parasites on the oxidative balance of young and adult blackcaps. Previous studies also found individual differences in how haemosporidian parasites were associated with oxidative status of breeding birds (Isaksson et al. 2013). Migration may be an important challenge, and future longitudinal studies may be key to interpret the lifetime costs of carrying parasites during seasonal migration in the same and other species with different migratory patterns. While our data point towards a greater impact of parasites early in life, we could not uncover how parasites change their prevalence and impact as individuals age (Finkel and Holbrook 2000; Balbontín et al. 2009). Moreover, in our study, most of the infections have probably already become chronic, having fewer effects on the immune system and oxidative status of their hosts (Ellis et al. 2014). From this perspective, parasites acquired during migration may have a stronger impact if the physiological cost of infection increases during acute parasitaemia (Palinauskas et al. 2008), a cost which might promote the selection of stopover habitat far from the reach of parasite vectors. Supporting this idea, many species of migratory forest birds shift habitat preferences during migration. Birds often increase searching behaviour when arriving lean in stopovers compared to when they are already fat prior to departure (Moore and Aborn 2000), and frequently select edge-dominated and early successional forests during autumn migration (Rodewald and Brittingham 2004). These behaviours are compatible with birds searching for habitats

where fleshy fruits that contain protective antioxidants are more abundant (Kollmann and Schneider 1999; Tellería et al. 2008; Chapter 3), but also where encounters with parasite vectors may be more easily avoided (Snow 1955; Service 1971; Burkett-Cadena et al. 2013; van Hoesel et al. 2019; Chapter 1). All in all, the oxidative costs of haemosporidian parasite infections need to be considered during the different life-stages of the hosts, since these costs may contribute to establish the physiological nexus between parasite infections and bird fitness.

Supplementary information

Supplementary Table S1

Table S1. Lineages of haemosporidian parasites from 130 infected blackcaps, distinguishing between adults and young individuals. Parasite lineages differ from other lineages by at least one base pair of the MalAvi barcode, 478-bp of the *Cyt b* gene (Bensch et al. 2009). Infections that could not be sequenced are included as "Unknown". The total number of parasite occurrences is higher than the number of infections due to multiple infections (the table shows the number of single infections of each parasite followed by the number of multiple infections in which it was involved in parentheses). Twenty-four individuals were not infected. Prevalence is calculated based on the total number of individuals tested from each age.

Hosts' age	Genus	Lineage	Species	n	Prevalence
Adult	*Haemoproteus*	SYAT01	*H. parabelopolskyi*	5 (7)	23.52 %
		SYAT02	*H. parabelopolskyi*	2 (2)	7.84 %
		SYAT03	*H. pallidulus*	1	1.96 %
		SYAT07	*H. parabelopolskyi*	10 (3)	25.49 %
		SYAT13	Undetermined	1	1.96 %
		Unknown *Haemoproteus*	Undetermined	11 (6)	
		Unknown			
	Plasmodium	*Plasmodium*	Undetermined	1 (4)	
	Leucocytozoon	BT2	Undetermined	1	1.96 %
		SFC8	Undetermined	0 (3)	5.88 %
		SYAT22	Undetermined	0 (1)	1.96 %
		Unknown *Leucocytozoon*	Undetermined	0 (1)	
Hosts' age	Genus	Lineage	Species	n	Prevalence
Young	*Haemoproteus*	SYAT01	*H. parabelopolskyi*	24 (14)	36.89 %
		SYAT02	*H. parabelopolskyi*	14 (8)	21.36 %
		SYAT03	*H. pallidulus*	1	0.97 %
		SYAT07	*H. parabelopolskyi*	8 (4)	11.65 %
		SYAT10	Undetermined	1	0.97 %
		SYAT13	Undetermined	5 (1)	5.83 %
		WW2	*H. majoris*	1	0.97 %
		Unknown *Haemoproteus*	Undetermined	17 (4)	

Plasmodium	SYAT05	*P. vaughani*	1 (1)	1.94 %
	Unknown *Plasmodium*	Undetermined	0 (6)	
Leucocytozoon	BT2	Undetermined	1 (1)	1.94 %
	SFC8	Undetermined	1 (2)	2.91 %
	SYAT22	Undetermined	1	0.97 %
	Unknown *Leucocytozoon*	Undetermined	2 (1)	

Supplementary Table S2

Table S2. Selection of the best models including the multiple infection status to explain variation in each physiological variable, according to the Akaike Information Criterion corrected by sample size (models with ΔAICc ≤ 2 compared to the best). When the null model was one of the best, we only report other models with lower AICc. Variables that were significant in the averaged model are in bold.

Model	df	AICc	ΔAICc	Weight
Haemoglobin				
1. year	3	439.6	0.00	0.19
2. Null model	2	440.1	0.49	0.15
tGSH (sqrt)				
1. **RBC-MDA**	3	395.2	0.00	0.15
2. plasma-MDA (log) + **RBC-MDA**	4	396.2	1.02	0.09
3. readiness + **RBC-MDA**	4	396.7	1.44	0.07
4. age + **RBC-MDA**	4	397.1	1.84	0.06
5. sex + **RBC-MDA**	4	397.1	1.84	0.06
TAS (sqrt)				
1. **coinfection** + **readiness** + **uric acid (sqrt)**	6	251.1	0.00	0.06
2. **coinfection** + **readiness** + sex + **uric acid (sqrt)**	7	251.3	0.20	0.05
3. **coinfection** + **readiness** + **uric acid (sqrt)** + year	7	251.6	0.47	0.04
4. **coinfection** + **readiness** + sex + **uric acid (sqrt)** + year	8	251.9	0.72	0.04
5. **coinfection** + **readiness** + RBC-MDA + **uric acid (sqrt)**	7	252.3	1.19	0.03
6. **readiness** + **uric acid (sqrt)**	4	252.5	1.32	0.03
7. **coinfection** + **readiness** + sex + RBC-MDA + **uric acid (sqrt)**	8	252.5	1.36	0.03
8. **coinfection** + **readiness** + RBC-MDA + **uric acid (sqrt)** + year	8	252.6	1.49	0.03
9. **readiness** + sex + **uric acid (sqrt)**	5	252.7	1.56	0.03
10. **coinfection** + **readiness** + sex + RBC-MDA + **uric acid (sqrt)** + year	9	252.8	1.71	0.02
Uric acid (sqrt)				
1. coinfection	4	431.0	0.00	0.18
2. Null model	2	431.6	0.64	0.13
Plasma-MDA (log)				
1. readiness	3	404.1	0.00	0.05
2. Null model	2	404.2	0.07	0.05
RBC-MDA				
1. **tGSH (sqrt)**	3	394.8	0.00	0.15
2. **tGSH (sqrt)** + year	4	396.4	1.59	0.07
Triglycerides (log)				

1. age	3	421.2	0.00	0.17
2. age + year	4	421.2	0.07	0.16
3. sex + year	4	422.0	0.87	0.11
4. age + sex	4	422.0	0.88	0.10
5. age + sex + year	5	422.1	0.93	0.10
6. year	3	422.1	0.94	0.07
7. sex	3	422.9	1.73	0.06
8. Null model	2	423.1	1.93	0.02

Supplementary Table S3

Table S3. Selection of the best models including *Plasmodium* infections to explain variation in each physiological variable, according to the Akaike Information Criterion corrected by sample size (models with ΔAICc ≤ 2 compared to the best). When the null model was one of the best, we only report other models with lower AICc. Variables that were significant in the averaged model are in bold.

Model	df	AICc	ΔAICc	Weight
Haemoglobin				
1. year	3	439.6	0.00	0.16
2. Null model	2	440.1	0.49	0.13
tGSH (sqrt)				
1.**RBC-MDA**	3	395.2	0.00	0.12
2. plasma-MDA (log) + **RBC-MDA**	4	396.2	1.02	0.07
3. readiness + **RBC-MDA**	4	396.7	1.44	0.06
4. age + **RBC-MDA**	4	397.1	1.84	0.05
5. sex + **RBC-MDA**	4	397.1	1.84	0.05
TAS (sqrt)				
1. readiness + **uric acid (sqrt)**	4	252.5	0.00	0.06
2. readiness + sex + **uric acid (sqrt)**	5	252.7	0.24	0.05
3. readiness + **uric acid (sqrt)** + year	5	253.5	1.03	0.03
4. *Plasmodium* + readiness + **uric acid (sqrt)**	5	253.6	1.16	0.03
5. readiness + sex + **uric acid (sqrt)** + year	6	253.8	1.32	0.03
6. readiness + RBC-MDA + **uric acid (sqrt)**	5	253.9	1.43	0.03
7. **uric acid (sqrt)**	3	254.1	1.60	0.03
8. readiness + sex + RBC-MDA + **uric acid (sqrt)**	6	254.1	1.67	0.03
9. *Plasmodium* + readiness + sex + **uric acid (sqrt)**	6	254.3	1.81	0.02
10. age + readiness + **uric acid (sqrt)**	5	254.3	1.84	0.02
Uric acid (sqrt)				
1. *Plasmodium*	3	430.5	0.00	0.17
2. *Plasmodium* + sex	4	430.7	0.19	0.16
3. Null model	2	431.6	1.05	0.10
Plasma-MDA (log)				
1. ***Plasmodium*** + readiness	4	400.5	0.00	0.07
2. ***Plasmodium***	3	400.7	0.20	0.06
3. ***Plasmodium*** + readiness + year	5	401.1	0.59	0.05
4. ***Plasmodium*** + year	4	401.1	0.63	0.05
5. ***Plasmodium*** + tGSH (sqrt)	4	402.0	1.46	0.03
6. ***Plasmodium*** + readiness + tGSH (sqrt)	5	402.0	1.47	0.03
7. ***Plasmodium*** + tGSH (sqrt) + year	5	402.4	1.91	0.03
RBC-MDA				

1. **tGSH (sqrt)**	3	394.8	0.00	0.14
2. **tGSH (sqrt)** + year	4	396.4	1.59	0.06
3. *Plasmodium* + **tGSH (sqrt)**	4	396.5	1.69	0.06
Triglycerides (log)				
1. age	3	421.2	0.00	0.13
2. age + year	4	421.2	0.07	0.12
3. sex + year	4	422.0	0.87	0.08
4. age + sex	4	422.0	0.88	0.08
5. age + sex + year	5	422.1	0.93	0.08
6. year	3	422.1	0.94	0.08
7. age + *Plasmodium*	4	422.3	1.12	0.07
8. age + *Plasmodium* + year	5	422.5	1.33	0.06
9. sex	3	422.9	1.73	0.05
10. Null model	2	423.1	1.93	0.05

Chapter 3: Young male blackcaps with blood parasite coinfections cope with oxidative stress favouring anthocyanin-rich food during migratory fattening

This chapter reproduces entirely the manuscript: Lucía Jiménez-Gallardo, Jimena López-Arrabé, Javier Pérez-Tris and Carolina Remacha (2023). Young male blackcaps with blood parasite coinfections cope with oxidative stress favouring anthocyanin-rich food during migratory fattening. *Authorea.* 2023. DOI: 10.22541/au.169422122.23514803/v1. Currently in second revision in *Journal of Avian Biology*.

Young male blackcaps with blood parasite coinfections cope with oxidative stress favouring anthocyanin-rich food during migratory fattening

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Abstract

Parasites may alter host physiology, which may promote behavioural adaptations to counteract their effect. Adaptive feeding may help individuals to cope with infection, especially during physiologically demanding life stages. For instance, migrating birds need fuel for long-distance flights and repair oxidative damage caused by intense aerobic exercise, and parasites may influence on how individuals balance these needs. Infected birds may face increased oxidative challenges, which could induce them to favour antioxidant defences over other needs, such as fattening. We tested whether migrating birds can adaptively choose food according to their needs, favouring dietary antioxidants to cope with oxidative stress caused by haemosporidian blood parasites during migration. During autumn migration, we mist-netted young male Eurasian blackcaps (*Sylvia atricapilla*) stopping over in central Spain. We placed the birds in cages where they were offered fat and anthocyanin-enriched food alternatives. We measured preference for each food offer. We tested their infections with haemosporidian parasites by PCR techniques

and their parasitaemia by blood smear inspection. We also measured physiological variables that account for nutritional and oxidative status in red blood cells and plasma. We found that birds with multiple infections favoured anthocyanin-enriched food controlling for an effect of body mass on food preference (lean blackcaps preferred anthocyanins, likely because they are urged to repair oxidative damage upon arrival on stopover with depleted energy reserves). Haemosporidian-infected birds had a lower antioxidant capacity of plasma, although no effect of infections on oxidative damage was detected, and individuals with more oxidative damage preferred anthocyanin-enriched food. Our results suggest that haemosporidian infections may increase individuals' antioxidant needs, which could affect migratory performance if the urge to find dietary antioxidants reduces the rate of fuel consumption.

Keywords: adaptive food choice, dietary antioxidants, ecophysiology, haemosporidian parasites, migration stopover, *Sylvia atricapilla*.

1. Introduction

Animals face different physiological and energetic challenges during their life cycle, which entail trade-offs that usually promote behavioural adaptations (Ricklefs and Wikelski 2002). One such adaptation is the food choice according to nutritional requirements (Catoni et al. 2011). Both dietary needs and the availability of food sources needed to meet them vary among life cycle stages, forcing individuals to adaptively allocate limited resources among different activities, such as growth (Romero-Haro and Alonso-Álvarez 2014), reproduction (Seress et al. 2020), daily activity (Beaulieu and Schaefer 2014) or migration (Jenni-Eiermann 2017). Thus, the idea that 'you are what you eat' may be expanded because 'you need to be different things at different times'.

The effects of parasites on the physiology and behaviour of their hosts have been long recognised (Moore 2002; Poulin 2007), and accounting for parasites may be key in studies of animal performance (Chrétien et al. 2023). By depleting host resources and eliciting the activation of the immune system, parasites may influence on nutrient requirements and resource allocation of their hosts (Cornet et al. 2014; Nwaogu et al. 2020), which may promote dietary shifts as an adaptive response to cope with parasite infections. All these effects on host physiology could be enhanced in high-energetic demanding situations, such as migration (Jenni-Eiermann 2017; Hegemann et al. 2018). One important link between parasite infection and host feeding behaviour may be oxidative stress (Isaksson et al. 2013; Delhaye et al. 2016; Muriel 2020). Reactive oxygen species (ROS) are produced during metabolic activity or during the activation of the immune system, and they may damage DNA, proteins, or lipids. Endogenous and exogenous antioxidants represent an important defence mechanism against ROS-induced oxidative stress (Finkel and Holbrook 2000; Monaghan et al. 2009). When animals can perceive the nutritional

content of food and select antioxidants when they face oxidative damage (Schaefer et al. 2008; Schaefer et al. 2014), self-medication arises as a possible way to maintain oxidative balance (Beaulieu and Schaefer 2013; de Roode et al. 2013).

Avian haemosporidians are the protozoans that cause malaria and similar diseases in birds (Valkiūnas 2005; Atkinson et al. 2008). These parasites have great impact on bird populations by decreasing host reproductive success and survival (Merino et al. 2000; Asghar et al. 2015). Haemosporidian infections induce oxidative stress through activation of the humoral immune system (Delhaye et al. 2018; Muriel 2020) and breakage of red blood cells (RBC; Pigeault et al. 2015). Consequently, parasitised individuals must allocate resources to immunity (Sheldon and Verhulst 1996; Tschirren and Richner 2006), which may deplete antioxidant defences that would otherwise help to maintain oxidative balance (Monaghan et al. 2009). Therefore, favouring dietary antioxidants may help parasitised individuals maintain a balanced oxidative status by reinforcing antioxidant defences, and directly fighting against infections, since some dietary antioxidants such as anthocyanins, the most important class of flavonoids derived from plants usually consumed by birds, are known to have an important antimalarial role (Lehane and Saliba 2008; Rudrapal and Chetia 2017; Masello et al. 2018; Akinnusi et al. 2023). However, this feeding behaviour could compromise the consumption of other important nutrients (Beaulieu and Schaefer 2014) or increase the oxidative imbalance if antioxidants become excessive relative to the amount of ROS (Beaulieu and Schaefer 2013). These trade-offs may be most important during life stages when maintaining oxidative balance is difficult (Hall et al. 2010; Guindre-Parker and Rubenstein 2018; McWilliams et al. 2021), which may exacerbate the negative influence of parasites on animal behaviour and performance (Chrétien et al. 2023).

The impact of haemosporidian parasites on bird oxidative status may be particularly strong during migration periods (Hegemann et al. 2018), when birds engage in intense aerobic exercise that increases both energy requirements and oxidative challenges (McWilliams et al. 2021). Migrating birds put on fat to fuel long-distance flight (Araújo et al. 2019), but unsaturated fatty acids used as energy reserves are prone to oxidation, and endurance flight effort might increase ROS production that needs to be compensated by up-regulation of endogenous antioxidant defences and/or increasing during stopovers the intake of antioxidant-rich food (Skrip et al. 2015; Eikenaar et al. 2020; McWilliams et al. 2021), such as fruits with more anthocyanins (Bolser et al. 2013). Therefore, parasite infections are expected to alter migrating bird feeding decisions, favouring dietary antioxidants rather than more energetic food although fattening is a priority (Beaulieu and Schaefer 2013; Zuzarte-Luís and Mota 2018).

We conducted a behavioural assay with young male Eurasian blackcaps *Sylvia atricapilla* to assess if individuals infected by haemosporidian parasites differ from their uninfected counterparts in their preference for major dietary antioxidants or fat when faced with this choice during migration, and whether oxidative status could mediate the relationships between parasite infection and individual feeding preferences during this period of high exposure to oxidative challenges. Blackcaps are a model of research on migratory behaviour and bird-haemosporidian relationships, and provide an excellent opportunity for this analysis because they consume both antioxidant-rich and fat-rich fruits on migration stopovers and can assess the relative content of these nutrients based on fruit colour (Schaefer et al. 2008; Schaefer et al. 2014). Blackcaps may accrue both energetic and defensive benefits from the same fruits; for example, wild or cultivated olives may be the

primary source of fat for blackcaps to restore energy reserves on Mediterranean stopover sites (Rey et al. 1996; Jordano 2013), but also contain phenolic compounds, α-tocopherol and β-carotene with antioxidant properties (Visioli et al. 2002; Catoni, Peters, et al. 2008). However, dietary antioxidants that have high antioxidant potency and are abundant in bird-consumed fruits, among which anthocyanins outstand from both regards, may be particularly important to cope with parasite-induced oxidative stress (Catoni, Peters, et al. 2008). Anthocyanins have been found to have anti-parasitic properties (Rudrapal and Chetia 2017; Masello et al. 2018; Akinnusi et al. 2023), anthocyanin-rich diets are known to boost the immune system (Bakuradze et al. 2019), and blackcaps that select fruits with high anthocyanin content enhance their humoral immune response (Catoni, Schaefer, et al. 2008). Therefore, we predict that parasitised blackcaps captured during migration will favour anthocyanin-enriched over fat-enriched food to control an increased risk of oxidative damage. If preference for anthocyanins is a defensive behaviour against oxidative stress, we also predict that infected birds will have poorer oxidative status measured through physiological indices of oxidative damage or antioxidant capacity, and that variation in these physiological indices will mediate individual food preferences. In addition, the physiological impact of haemosporidian infections may depend on the proportion of RBC that are infected (intensity of infection) or on the synergistic effects of multiple infections, where different parasites coexisting in the blood increase the cost of the disease (Pigeault et al. 2018; García-Longoria et al. 2022). Therefore, we predict that the more parasites blackcaps harbour, the greater will be their preference for anthocyanin-enriched food.

2. Materials and Methods

2.1. Study Area and Field Methods

We mist-netted migrating blackcaps in a stopover site located near Campo Real, central Spain (40°20'08"N 3°24'27"W, 780 m a.s.l), during October 2019 and 2020. The area is dominated by olive groves (*Olea europaea*) crossed by a stream where fruiting shrubs abound, especially daneworts (*Sambucus ebulus*) and brambles (*Rubus fruticosus*). Therefore, blackcaps stopping over in this area during autumn migration can choose between fat-rich olives and anthocyanin-rich berries typical of their diet during the autumn-winter period (Herrera 1987; Schaefer et al. 2008; Jordano 2013).

We tried to narrow the sampling period within capture sessions and years to avoid the effects of daily and phenological variation on the physiology and behaviour of blackcaps (due to circadian routines, different origin of individuals, changing prevailing weather conditions, or other temporal influences). To this end, we captured blackcaps between 9:00 and 15:30 CET during four sampling days each year (sampling dates ranged 4-11 October in 2019 and 14-26 October in 2020), within the peak of passage of the species in this region (Cantos 1995). To maximize capture rates, we tape-lured birds into mist nets using male song recordings. To minimise the time elapsed from capture to handling, we watched the nets (one or two operating simultaneously) with binoculars and took birds as they hit the net whenever this was possible, or otherwise visiting the nets every 10 minutes. Birds were kept in individual cotton bags for 30 minutes to homogenise stress levels (Huber et al. 2021) and to increase motivation to eat after mild fasting. They were sexed and aged according to plumage, distinguishing between young (individuals born in the previous spring) and older birds (Svensson 1992). We wanted to minimise

sample size for ethical reasons, and thus focused on young male birds to reduce the number of factors in the statistical design. Males were chosen instead of females because our sample was sex-biased (63 % males); therefore, our study is relevant to the young male fraction of the population. Young blackcaps are ideal for studying the ecological correlates of food preference during migration because they are facing first parasite infections, which reduces the behavioural variation associated with a previous infection history (Remacha et al. 2023). In addition, they are visiting the stopover site for the first time in their lives and therefore have the same experience.

A total of 48 young male blackcaps were included in the study (25 captured in 2019 and 23 in 2020). We weighed birds to the nearest 0.01 g with a digital scale and placed them in individual cages (cloth mesh, 25 x 25 x 25 cm). We recorded behaviour using a video camera (GoPro Hero 9 Black) placed in front of the cage, covering all other sides and the top of the cage with filter paper to minimise external stimuli. The cages were fitted with a perch near the side facing the camera, with two food choices presented on each side nailed to a semi-vertical twig.

We designed a meaningful nutrient-preference test presenting birds with two food choices that were equal in all respects except for the dietary treatment. We used a piece of toad skin melon (about 2 cm^3) as the substrate for both food choices. By using large pieces of fruit with high water content, we made sure that the birds found the food (Remacha et al. 2023) and avoided variation in size, shape or fruit attributes known to influence fruit choice by birds (González-Varo et al. 2022), other than the changes caused by our treatment. Each piece of melon had been soaked overnight in extra virgin olive oil (Coosur®) or cranberry juice (Granini®), hereafter named fat-enriched or anthocyanin-enriched food, respectively. Blackcaps consume abundant wild and cultivated olives,

whose principal nutritional reward is energy from fats (Herrera 1987; Jordano 2013). Olive oil is highly energetic and contains a high proportion of unsaturated fatty acids that help migrant birds to build fat reserves very efficiently (Pierce et al. 2005). Importantly, olive oil is also rich in polyunsaturated fatty acids such as omega-3 and omega-6 (Zarrouk et al. 2019), which enhance the aerobic capacity of migrating birds but must be obtained from dietary sources (Price and Guglielmo 2009; Weber 2009). Conversely, cranberry juice provides little energy rewards but has a high anthocyanin content (Bakuradze et al. 2019). Migratory birds in autumn migration show preference for fruits with high anthocyanin content, which has been interpreted as a protective behaviour against oxidative stress associated with endurance flight (Bolser et al. 2013). Although olives contain other dietary antioxidants such as phenolic compounds, α-tocopherol and β-carotene (Visioli et al. 2002; Catoni, Peters, et al. 2008), compared to these antioxidant compounds anthocyanins are various orders of magnitude more available in fruits consumed by blackcaps, show much higher antioxidant potency, and are known to be important antioxidants in the fight against haemosporidian parasites (Catoni, Peters, et al. 2008; Lehane and Saliba 2008; Akinnusi et al. 2023). Fat- and anthocyanin-enriched melon pieces acquired distinctive golden and purple-reddish tinges, respectively, which we deemed useful for blackcaps to assess the nutritional rewards of either choice (Schaefer et al. 2008; Schaefer et al. 2014). The two food choices swapped cage location between individuals (Supplementary information).

Migrating blackcaps arrive on the stopover sites lean, start to gain mass typically on day 2 post-arrival, and put on fat until reaching maximum body mass at departure time (Langslow 1976), increasing approximately 0.2 g day^{-1} in Iberian locations (Arizaga et al. 2010). In our sample, the heaviest individual (21.4 g) was 46.6 % heavier than the

lightest (14.6 g). Therefore, we used the residuals of the linear regression of body mass on tarsus length, both log transformed (residual mass hereafter) as a measure of body condition of individuals, which was indicative of both their metabolic efficiency and the time they had spent on stopover. Once the video-recording finished, we measured the length of the tarsus with 0.01 mm precision using a digital calliper. At the end of the manipulation of the birds, we took a blood sample (< 1 % body mass) from the jugular vein. We used a drop to make a blood smear, which was air-dried and fixed with absolute ethanol for 3 minutes. The rest of the blood was kept refrigerated in heparinised tubes during fieldwork. Birds were fitted with a standard aluminium ring (SEO/Birdlife Spanish ringing scheme) to avoid repetitions and were released unharmed at the capture site.

2.2. Behavioural analyses

We visualised the video recordings using the VLC media player (3.0.8 Vetinari). We analysed the feeding behaviours observed between time 0, when the researcher disappeared from the image after leaving the bird in the cage, and the first time when the researcher reappeared to retrieve the bird. To standardize individual observations, we stopped recording behaviours at time = 1390 s, set by the shortest recording. Not all individuals ate during the test, or pecked the fruit one or a few times, probably because the time birds spent captive (which was short for ethical reasons) was not enough for all individuals to decide to eat on a new food source (cube-shaped pieces of melon). To overcome this limitation, we considered three feeding-oriented behaviours that could be unequivocally recognised in our video recordings: pecking the fruit, pecking attempts (when the bird approached its beak to the fruit without pecking), and showing interest (when the bird directed its head towards one fruit and kept eye contact for more than 2 seconds without attempting to peck). Considering the proportion of occasions in which

individuals directed each feeding-oriented behaviour to one food offer as indicative of their inclination for that choice, we found that the propensity to peck, to attempt pecking or to show interest for the same food choice were all positively correlated (pairwise Pearson correlations between proportions of each type of behaviour directed to fat-enriched food as the reference: pecking - pecking attempts r = 0.87, n = 17; pecking - showing interest r = 0.95, n = 17; pecking attempts - showing interest r = 0.88, n = 15; all three correlations with $p < 0.001$), meaning that all three feeding-oriented behaviours conveyed the same information on individual food preferences. Then, we used each individual behaviour as a dichotomous decision between food choices. All videos were blindly analysed to the infection status, body mass, or physiological condition of the individuals.

2.3. Infection status

We assessed the infection status of blackcaps by combining PCR techniques and microscopy. We extracted total DNA from blood with the SpeedTools DNA extraction kit, following the manufacturer's protocol, and diluted the samples to a final concentration of 25 ng/µl. We first tested all samples with a multiplex PCR that simultaneously targets DNA fragments of different sizes of *Haemoproteus*, *Plasmodium,* and *Leucocytozoon* parasites (Ciloglu et al. 2019). Reactions were set in total volumes of 10 µl, including 5 µl of 2× Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.2 µl of each of six primers (10 µM; PMF/R for *Plasmodium,* HMF/R for *Haemoproteus* and LMF/R for *Leucocytozoon*), 1.8 µl of ddH_2O, and 2 µl of DNA template. The temperature profile included a hot start at 95 °C for 15 min, followed by 35 cycles at 94 °C for 30 s, 59 °C for 90 s, and 72 °C for 30 s, and an extension at 72 °C for 10 min. The PCR reactions included a negative control (ddH_2O) and a triple positive control (DNA positive for all

three genera of parasites). We visualised 4 µl of the PCR product on 2 % agarose gels stained with 100× GelRed®.

To identify the parasite lineages infecting each bird, positive samples for the previous multiplex PCR were amplified with a nested PCR (Hellgren et al. 2004), which targets 478 bp of the parasite *Cyt-b* gene broadly used as a DNA barcode for avian haemosporidians (Bensch et al. 2009). Nested PCR consisted of a preamplification step that targeted the three genera (with primers HAEMNFI and HAEMNR3) followed by specific amplifications targeting either *Plasmodium* and *Haemoproteus* (with primers HAEMF and HAEMR2) or *Leucocytozoon* (primers HAEMFL and HAEMR2L). We set pre-amplification reactions at 25 µl total volume, including 0.5 units of AmpliTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 1 µl of each 10 µM primer, 1.25 mM of each dNTP, 1.5 mM of $MgCl_2$, 2.5 µl of Buffer 10×, 14.8 µl of ddH_2O, and 2 µl of DNA template. The thermal profile included an initial step at 94 °C for 3 min; 20 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s, and an extension of 10 min at 72 °C. We used 1 µl of the product for one or both specific amplification steps depending on the result of multiplex PCR. Specific reactions involved 35 cycles with the same temperature profile and PCR conditions as pre-amplification, but including 15.8 µl of ddH_2O. All reactions included negative and triple positive controls passed from pre-amplification to the specific amplification step. We sequenced the final PCR products using an Applied Biosystems™ 3730xl DNA analyzer, and considered parasite lineages that differed in one or more base pairs in the MalAvi DNA barcode as distinct lineages (Bensch et al. 2009).

We stained blood smears with Giemsa (pH 7.2) for 1 h and inspected them with a light microscope (LEICA DM2500, Leica Microsystems, Wetzlar, Germany), with a

sampling effort of at least 5000 red blood cells (RBC) observed at 1000× magnification. Infected individuals were quantified the intensity of the infection as the number of infected cells in 5000 RBC. Individuals that were PCR positive but had no visible parasites were thus estimated an intensity of infection of 0 infected cells in 5000 RBC (no structural zeros were included as only infected birds were analysed). We considered any possible observation of the presence of more than one parasite lineage in blood (multi-genus amplification by multiplex PCR, mixed sequence signal in DNA barcodes obtained by nested PCR, or detection of different parasites by molecular and microscopy methods) as evidence of multi-parasite infection, hereafter multiple infection, including infections with two or more different lineages from the same or different genus (Pérez-Tris and Bensch 2005; Valkiūnas et al. 2006; Ciloglu et al. 2019). Otherwise, infected individuals were considered to harbour single-parasite infections. The lineage composition of multiple infections could not be determined in many cases, for example when not all parasite genera detected by multiplex PCR could be retrieved by nested PCR. Because of that reason, and due to sample size limitations, we did not test the effect of the exact number of parasite lineages coinfecting the bird, or the effect of particular parasite lineages or parasite combinations, on the physiology and behaviour of blackcaps.

2.4. *Physiological Analyses*

To assess whether parasites could be causing anaemia, after fieldwork on each day of capture we measured haemoglobin concentration in blood with Drabkin reaction (Spinreact, Girona, Spain), following the manufacturer's instructions. We set each sample in duplicates on 96-well plates, including a calibrator and blank (ddH_2O). We measured absorbance at 540 nm using a Biotek Epoch microplate spectrophotometer (BioTek Instruments, Inc.). Repeatability of the assay was high ($r_i = 0.97$; $n = 48$; $p < 0.001$), inter-

assay CV was 12.61 % and intra-assay CV was 1.03 %. Immediately after the analysis, we centrifuged the rest of the blood for 10 min at 12000 rpm to separate plasma from RBC. We visually determined the degree of hemolysis using a three-level scale based on the redness of plasma (0: clear sample, 1: pale red stain, 2: red sample). Plasma and red blood cell (RBC) fractions were stored at -80 °C until further analysis.

We measured the protein content of the RBC samples to standardize other measurements (see below) using the Bradford assay (Sigma-Aldrich, St. Louis, MO), testing them in duplicate on 96-well plates, following the manufacturer's instructions. In each plate, we added protein standard (BSA) ranging from 0.1 to 1.4 mg/ml, blank (ddH2O), or RBC. We measured absorbance at 595 nm using a Synergy HT MultiMode Microplate199 Reader (BioTek Instruments, Inc.; this spectrophotometer was used in all subsequent analyzes). The repeatability of the protein measurements was high (r_i = 0.73; n = 48; p < 0.001), inter-assay CV between the samples was 17.21 % and the CV intra the samples was 5.26 %.

We determined intracellular total glutathione levels in RBC (tGSH) as an endogenous antioxidant indicator in RBC following López-Arrabé et al. (2014). The samples were diluted (1 : 20 w/v) and homogenised in stock buffer (0.01 M phosphate buffered saline and 0.02 M EDTA) and mixed with an equal volume of 10 % trichloroacetic acid. We vortexed the mixture three times during 5 s in 10 min and then centrifuged it at 2000 g for 10 min at 6 °C, to separate the supernatant. After that, we added NADPH and DTNB in the first step and GSH reductase in the second step after 15 s. We used 96-well plates including duplicate samples, a blank (buffer), and a standard curve (serial dilution of GSH from 0.5 to 0.031 mM). Only one 12-well row from the plate was used at a time. We measured absorbance at 405 nm after 15 and 45 s, and the

change in absorbance was used to determine the intracellular tGSH concentration by comparing the output with the standard curve. Levels were standardised to the total protein content in the sample by dividing tGSH by protein values. The repeatability of the standardised tGSH calculated in duplicates was high (r_i = 0.93; n = 48; $p < 0.001$), inter-assay CV was 12.60 % and the intra-assay CV was 8.17 %.

We used malondialdehyde (MDA) to measure lipid peroxidation as an index of oxidative damage to cell membranes. The integrity of membrane phospholipids is key for the metabolic performance of pectoral muscle during endurance flight (Weber 2009), and parasite invasion may cause peroxidation of phospholipids of RBC membranes due to the combined effects of parasite metabolism and host immune responses (Percário et al. 2012). Therefore, we quantified the concentration of MDA both in plasma (plasma-MDA, as a measure of general lipid peroxidation faced by the individual) and RBC (RBC-MDA, a measure of oxidative damage to the RBC membrane), following Romero-Haro and Alonso-Álvarez (2014). We included a blank and a standard curve per batch diluted from 1,1,3,3-tetraethoxypropane with 40 % ethanol. We added butylated hydroxytoluene, phosphoric acid, and thiobarbituric acid (TBA) solutions to each sample (standard or blank) and incubated them for 1 h in a dry bath at 100 °C. MDA-TBA adducts were formed, and pure n-butanol was added, vortexed, centrifuged, and the upper phase was collected to transfer it into an HPLC vial. We extracted the samples to inject them in duplicate into an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA), and measured them with a fluorescence detector (ref. G1321A, Agilent Technologies). Repeatability was high for both plasma-MDA (r_i = 0.87; n = 45; $p < 0.001$; inter-assay CV = 27.44 % and intra-assay CV = 15.41 %) and RBC-MDA (r_i = 0.76,

$n = 48$, $p < 0.001$; inter-assay CV = 20.58 % and intra-assay CV = 14.45 %). We standardised the levels of RBC-MDA dividing by the total protein content.

To obtain a biomarker of the general level of circulating antioxidant defences of the individual, as vitamin C, vitamin E and carotenoids that are frequently obtained with the diet, we measured the antioxidant capacity of plasma quantifying total antioxidant status (TAS) following Miller et al. (1993) with modifications described in López-Arrabé et al. (2014). We used an α-tocopherol derivative (Trolox) as a calibrator. The samples were run in duplicates whenever there was enough plasma (44 out of 46 samples), using 96-well plates with a calibrator and blank. We ran a row per time to control the reaction delay. We added metmyoglobin (an equilibrated mixture of myoglobin and potassium ferricyanate) to each sample, blank, or control. We also added a chromogen (2,2 ′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid or ABTS) and H_2O_2 to start the reaction. We recorded absorbance at 660 nm, each 10 s at 37 °C. The repeatability calculated in duplicates was high ($r_i = 0.95$; $n = 43$; $p < 0.001$), inter-assay CV was 14.19 % and the intra-assay CV 5.83 %.

Uric acid is the main form of nitrogen excretion due to catabolism of amino acids in birds. Furthermore, it is also an endogenous antioxidant whose concentration is frequently positively related to TAS values (Cohen et al. 2007; Pérez-Rodríguez et al. 2008), potentially confounding the interpretation of this biomarker (Cohen et al. 2007; Costantini 2011). Therefore, we measured uric acid in plasma to correct TAS values and as a measure of nutritional status, using a commercial kit (Biosystems, Barcelona, Spain). The test details were implemented according to the manufacturer's instructions, using 96 well plates that we incubated at 37 °C for 10 min and measured absorbance at 520 nm.

All duplicates had high repeatability (r_i = 0.99, n = 46, p < 0.001), inter-assay CV was 4.07 % and the intra-assay CV was 3.86 %.

Finally, we measured plasma triglycerides, as an indicator of the nutritional status of the birds and to standardise MDA measurements if necessary, using a commercial kit (Biosystems, Barcelona, Spain) according to the manufacturer's instructions. Triglyceride concentrations can be an index of lipid absorption a few hours before sampling and therefore reflect the individual's state of fattening (Jenni and Jenni-Eiermann 1998). Furthermore, triglyceride levels can be related to MDA levels, either because MDA is also present in food or because MDA can also be influenced by the amount of circulating lipids (Pérez-Rodríguez, Romero-Haro et al. 2015). We tested 45 out of 48 samples in duplicate and measured their absorbance at 500 nm. The repeatability of duplicates was high (r_i = 0.97; n = 44; p < 0.001), inter-assay CV was 1.72 % and the intra-assay CV was 3.98 %.

2.5. *Data Analysis*

All analyses were performed using R 4.2.2 (R Core Team 2022). Continuous variables were checked for normality, transformed when advisable, and z-standardised for comparison of effect estimates. We checked that residuals were distributed according to model assumptions with DHARMa R package (Hartig 2022) and specific diagnostic plots in each model. First, we tested our specific hypothesis, whether there is an effect of infection on food preference. Then, we analysed the relationships between infections in each physiological condition variable indicative of oxidative status. Finally, we examined the effects of physiological condition as predictors of food choice. When the multiple

infection status had a significant effect, we conducted Tukey's post-hoc comparisons of factor levels using the emmeans R package (Lenth 2022).

To analyse the effect of infection on food preference, we fitted generalised linear mixed effects models (GLMM) with binomial error and logit link function using the lme4 package R (Bates et al. 2015). Individual food preferences estimated as the proportion of feeding-oriented behaviours directed to either food choice had variable reliability depending on the number of behaviours performed by the individual (Douma and Weedon 2019). Therefore, we use each behaviour as an observation of the binomial response variable 'preference', with values 0 (preference for anthocyanin-enriched food) or 1 (preference for fat-enriched food). The 19 birds that did not show feeding-oriented behaviours were excluded from the analysis of food choice. We include the identity of the individuals as a random factor in our models to control the information available to estimate the preference of the individuals. Residual mass and infection were included as fixed predictors, along with year to reduce unexplained variance. We fitted two types of models to assess the nature of the effect of haemosporidian infections, each considering a different measure of infection: (1) whether the bird had no parasites, harboured a single-parasite infection, or had a multiple infection, and (2) the intensity of infection.

We controlled that haemolysis did not significantly affect physiological parameters (all differences between haemolysis levels: $p > 0.08$). Uric acid was controlled in the analysis of TAS, as both variables were positively correlated ($r = 0.86$). RBC-MDA was positively correlated with tGSH ($r = 0.58$), which we interpreted as a hormetic response (Costantini 2014) of migrating birds at stopover regulating antioxidant defences according to oxidative status. To correctly score the oxidative status of individuals captured at different stages along this gradient, we performed a Principal Component

Analysis (PCA) with RBC-MDA and tGSH, where PC1 measured hormesis (both variables with factor loading = 0.89, 79.1 % of variance explained, RBC hormesis hereafter), and PC2 measured exposure to lipid peroxidation relative to enzymatic antioxidant defences of RBC (factor loadings: tGSH = -0.46 and RBC-MDA = 0.46, 20.9 % of variance explained, RBC oxidative stress hereafter).

To assess the relationships between infection, and physiological condition of individuals, we fitted lineal models (LM) with physiological parameters as dependent variables. We assessed two types of models in relation to infection or intensity of infection (as described above) and included the year and residual mass as covariables. When an oxidative damage biomarker was analysed as a dependent variable, we included an antioxidant biomarker as a covariate and vice versa.

Finally, we evaluated the relationships between physiological variables indicative of oxidative status and bird food preference. We assessed which physiological variables best predicted food preference using the Akaike information criterion (AIC) (Burnham and Anderson 2002) with the dredge function in the MuMIn package (Bartoń 2023). We used GLMM with preference as a binomial response and individual identity as a random factor. The saturated model included the fixed effects of year, three biomarkers of oxidative status (plasma-MDA, RBC hormesis and RBC oxidative stress), a biomarker of antioxidant capacity (TAS), and triglycerides and uric acid to correctly interpret the effects of plasma-MDA and TAS, respectively (note that uric acid also has antioxidant properties). We calculated multicollinearity with the variance inflation factor (VIF). We selected the best model based on AIC values corrected by small sample size (AICc), and averaged all models with $\Delta AICc \leq 2$ to compute the significance of effects (Symonds and Moussalli 2011).

3. Results

Of 48 birds tested, 6 (4 in 2019 / 2 in 2020) were not infected with haemosporidian parasites, 28 (15/13) had single infections, and 14 (6/8) had multiple infections, two of which involved more than two parasite lineages. Detailed information on the prevalence of each parasite lineage found in this sample is available in Supplementary information. The infected birds had a very low intensity of infection (mean = 3.30 parasites in 5000 RBC, sd = 4.33). Twenty-nine blackcaps performed feeding-oriented behaviours, of which 4 were not infected, 19 had single infections and 6 had multiple infections.

3.1. Food preference and infection

We found significant effects of year, residual body mass, and multiple infection on food preference (Table 1). Birds that harboured multiple infections showed a stronger preference for anthocyanin-enriched food than single-infected birds (z = 2.75; p_{Tukey} = 0.016), but we failed to detect different food preferences between non-infected birds and individuals of the two infected groups (all p_{Tukey} values > 0.31), although the sample size of non-infected individuals showing feeding-oriented behaviours was small (Fig. 1). Fat preference was higher in 2020 and increased as blackcaps were fatter (Table 1). The intensity of infection did not influence food preference after controlling for the significant effect of residual mass and year (Table 1).

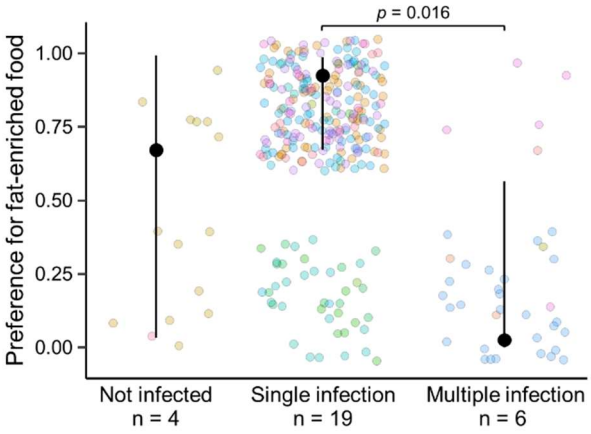

Figure 1. Variation in preference for fat-enriched food (as opposed to preference for anthocyanin-enriched food) between blackcaps with different levels of multiple status of infection by haemosporidian parasites (sample sizes in each group are indicated). Preference estimates (marginal means) with 95% confidence intervals represent the probability that feeding-oriented behaviours are directed to fat-enriched food, controlling for residual body mass and year. The *p-value* is shown for comparisons that were statistically significant in a Tukey's post-hoc test. Dots are observations of single feeding-oriented behaviours, which therefore take values of 0 if directed to anthocyanin-enriched food and 1 if directed to fat-enriched food, jittered to reduce overlapping. The identity of individuals expressing these behaviours (represented with dots of different colour) has been controlled as a random factor in the model.

Table 1. GLMMs analysing variation in food preference as a function of ecological condition (residual body mass and parasite infection), controlling for the fixed effect of year, and including individual differences as a random factor. Significant results are highlighted in bold. Different models (in columns) with parasite infection quantified as the multiple infection status or the intensity of the infection were tested. Significance was assessed with likelihood ratio tests (LRT). Tukey's post-hoc results for pairwise comparisons between levels of multiple status of infection (estimate ± se): not-infected *vs* single-infected = -1.79 ± 2.31, $p = 0.72$; not-infected *vs* multiple-infected = 4.33 ± 2.96, $p = 0.32$; single-infected *vs* multiple-infected = 6.12 ± 2.22, $p = 0.02$.

	Multiple status of infection			**Intensity of infection (log)**		
	Estimate ± se	***LRT* (df)**	***p***	**Estimate ± se**	***LRT* (df)**	***p***
Parasite infection		**8.02 (2)**	**0.02**	-0.70 ± 1.11	0.38 (1)	0.54
Residual body mass	**1.92 ± 1.03**	**4.16 (1)**	**0.04**	**2.08 ± 0.95**	**5.99 (1)**	**0.01**
Year (2019)	**-2.82 ± 0.99**	**8.64 (1)**	**0.003**	**-3.10 ± 1.34**	**7.52 (1)**	**0.006**

3.2. Physiology and infection

The multiple status of infection of the individuals had a marginal effect on the antioxidant capacity of plasma ($F_{2,37} = 2.62$; $p = 0.09$), although the plotted data (Fig. 2) suggested that single- and multiple-infected birds had very similar TAS values corrected by uric acid levels (not-infected: mean ± *SE* = 0.43 ± 0.51; single-infected: -0.05 ± 0.58; multiple-infected: -0.05 ± 0.26). Taking single- and multiple-infected birds together, TAS values of infected birds were significantly lower than those of non-infected birds (contrast between infected and non-infected individuals: $F_{1,37} = 4.87$; $p = 0.034$). We did not find any significant relationship between residual mass or infection status and haemoglobin, triglycerides, uric acid, plasma-MDA, RBC hormesis, or RBC oxidative stress (Supplementary information).

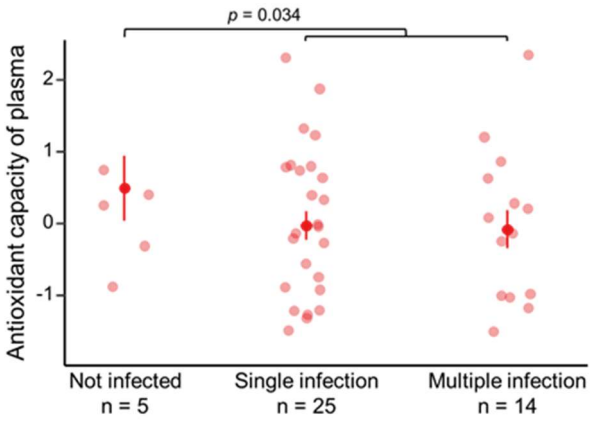

Figure 2. Variation in plasma antioxidant capacity (z-standardised TAS values) between blackcaps with different levels of multiple status of infection by haemosporidian parasites (sample sizes in each group are indicated). Estimates (dark circles) and 95 % confidence intervals were computed from a model controlling for uric acid content, residual body mass, year and plasma MDA. Pale dots are individual estimates, jittered horizontally to reduce overlapping. The p-value corresponds to the difference between non-infected birds and the two groups of infected individuals (single- and multiple-infected birds together) when the model was fitted with this contrast for the levels of the multiple status of infection.

3.3. *Food preference and physiology*

We obtained six models with $\Delta AIC \leq 2$ (Table 2), which were deemed equally good to explain food preference. The model with lowest AICc value included RBC oxidative stress, plasma-MDA (log-transformed), uric acid and year. Given that the best model was not strongly weighted against competitor models, we used a full model averaging of the six models to obtain parameter estimates following (Symonds and Moussalli 2011). Plasma-MDA was the only physiological variable with a significant effect on food preference: individuals with lower log-transformed plasma-MDA preferred fat-enriched food (full model estimate ± adjusted SE = -2.08 ± 1.04, $P = 0.047$) controlling for an

effect of the year (estimate ± adjusted SE = -1.99 ± 1.01, $P = 0.049$, all other effects with $P > 0.07$).

Table 2. Selection of the best models to explain food preference as a function of physiological parameters involved in oxidative status. The models are arranged according to the Akaike Information Criterion corrected by sample size (AICc), where the best model (number 1) is the one with lowest AICc. Models with ΔAICc ≤ 2 are shown (6 best models out of 128 candidates), indicating their AICc value, the increase in AICc compared to the best model, the model weight and the number of parameters. The same information is shown for the null model (ranked 95) at the end of the table.

Model	AICc	ΔAICc	Weight	k
1.RBC stress + plasma MDA (log) + uric acid + year	210.67	0.00	0.07	6
2. plasma MDA (log) + uric acid + year	211.12	0.45	0.05	5
3.RBC stress + plasma MDA (log) + uric acid + TAS + year	211.92	1.25	0.04	7
4.RBC stress + plasma MDA (log) + year	212.01	1.34	0.03	5
5. RBC hormesis + RBC stress + plasma MDA (log) + uric acid + year	212.39	1.72	0.03	7
6.RBC stress + plasma MDA (log) + TAS + year	212.58	1.91	0.03	6
95. Null model	217.55	6.88	0.00	2

4. Discussion

Our behavioural test with young male blackcaps captured on migration showed that birds preferred anthocyanin- or fat-enriched food alternatives depending on their physiological condition. The results supported a scenario in which feeding decisions may help birds to cope with the oxidative challenges they face as they recover from and prepare to the intense aerobic exercise during endurance flight. In this scenario, haemosporidian parasites could have an impact on host physiology by exacerbating oxidative challenges through the depletion of the antioxidant capacity. This impact may

have influenced on individual food preferences making birds with infections by multiple parasites more likely to choose antioxidant-enriched food, which supports the idea that increasing the intake of dietary antioxidants may be a self-medication mechanism to cope either with elevated risk of oxidative stress due to infection (Zuzarte-Luís and Mota 2018), or with the infection itself (Lehane and Saliba 2008; Masello et al. 2018). Our observations of infected birds having lower total antioxidant capacity, and individuals with higher levels of oxidative damage to lipids having preference for anthocyanin-enriched food, are compatible with this interpretation. However, further analyses with larger samples especially of non-infected birds, and preferably under more controlled conditions would be required to substantiate this conclusion.

The small sample size for non-infected birds showing feeding-oriented behaviours did not allow drawing reliable conclusions regarding the feeding preferences of this group, and therefore we focus our discussion on the two groups of infected individuals. Co-infected blackcaps showed stronger preference for anthocyanin-enriched food than single-infected ones. Consumption of antioxidants such as anthocyanins could be a means of self-medicating against haemosporidians (Akinnusi et al. 2023) if it reduced oxidative damage caused by the parasite or by activation of the immune system during the infection (Beaulieu and Schaefer 2013; de Roode et al. 2013; Muriel 2020). This effect of parasitism was only significant in multiple infections, which could increase parasite virulence or the cost of the host's immune response (Pigeault et al. 2018; Garcia-Longoria et al. 2022), although the small sample size of uninfected birds forces us to take this result with caution. In this context, the consumption of antioxidants could be detrimental to the energy intake essential for migration performance (Beaulieu and Schaefer 2014), or it could compromise the active search of alternative essential nutrients, such as

polyunsaturated fatty acids that may enhance physiological performance during migration (Weber 2009). However, the consumption of anthocyanin-enriched food by multiple-infected birds could be a means to alleviate the potential oxidative stress caused by virulent infections.

We found a small correlation between infection and the oxidative balance, since infected birds showed lower values of plasma antioxidant capacity. Antioxidant depletion associated with immune activation (Costantini and Møller 2009) or production of free radicals as a result of parasite exploitation (Percário et al. 2012) may induce an increase in ROS in infected birds (Delhaye et al. 2016), that should be compensated through diet. Similar results of infections have been found in other stressful situations such as during reproduction (Badás et al. 2015), or in low-quality habitat (Messina et al. 2022). However, we did not detect any correlation between infection status and other biomarkers of oxidative status. These correlations could have been concealed by a hormetic response, which is supported by a positive correlation between tGSH and RBC-MDA. Faced with predictable oxidative stress, migrating birds could up-regulate antioxidant defences before suffering too much oxidative damage (Costantini 2010; McWilliams et al. 2021), thus buffering the relationships between oxidative parameters and the sources of stress.

We did not find any relationship between parasitemia and biomarkers of oxidative stress, although other studies have found it in other species such as great tits *Parus major* (Isaksson et al. 2013; Delhaye et al. 2016). However, blackcaps showed very low parasitemia in our study, probably as a consequence of parasite retreat to internal tissues after the summer (Valkiūnas et al. 2004; Pérez-Rodríguez, de la Hera et al. 2015), although mortality during acute infection in younger individuals may contribute to explain the pattern (Garcia-Longoria et al. 2022). This result indicates that blackcaps

during autumn migration have mostly chronic infections with few parasites in the bloodstream (possibly derived from infections acquired during the breeding season; Pérez-Rodríguez, de la Hera et al. 2015), yet such low-intensity infections may have an impact on oxidative status during migration, which could contribute to the mortality cost of haemosporidian infections observed in other migratory bird species (Asghar et al. 2015).

We found a positive correlation between relative body mass and preference for fat-enriched food, which we attribute to the fact that body mass of migrating blackcaps captured on stopover is most dependent on the time they have spent at the stopover site, as birds gain body mass from arrival to departure (Langslow 1976; Arizaga et al. 2010). Thus, leaner birds may prefer anthocyanin-enriched food if dietary antioxidants help them cope with oxidative damage associated to the aerobic effort of the preceding flight stage (Costantini 2008; Eikenaar et al. 2020). Conversely, fatter birds closer to departure may focus on putting on fats to fuel the next flight stage, thereby preferring fat-enriched food. Preference for olive oil could be further increased close to departure if birds benefit from acquiring so-called "natural doping" polyunsaturated fatty acids present in olive oil as these may increase metabolic efficiency during endurance flight (Weber 2009). Our interpretation of the relationship between body mass and food preferences observed in blackcaps is supported by the fact that blackcaps lose weight on arrival at stopover sites (Langslow 1976), which may be indicative of a period when tissue repair is being prioritized over fat deposition, dependent on individual condition at arrival. Furthermore, birds with less oxidative damage in plasma (as measured by MDA) selected fats rather than anthocyanins, supporting the idea that individuals with a good oxidative status could focus on rebuilding fat stores or prioritise the intake of alternative essential nutrients that

may give them a physiological advantage during endurance flight (Weber 2009; Costantini 2010; Beaulieu and Schaefer 2014).

Understanding the intricate relationships between body condition, oxidative status and dietary preferences during migration has long been at the centre of research on the physiological performance of migrating birds (Weber 2009). Although the small sample size (especially of non-infected birds) and the influence of natural variation in our study force us to draw conclusions with caution, our results support the idea that haemosporidian infections have an influence on these relationships by exacerbating the oxidative challenges faced by birds during this critical life cycle stage, thereby providing a physiological mechanism to explain the negative impact of chronic avian malaria infections on survival observed in migrating birds (Asghar et al. 2015), and emphasising the importance of accounting for parasite infections in studies of animal performance (Chrétien et al. 2023).

Supplementary information

Supplementary Figure S1

Figure S1. Posterior view of the cage as displayed for the blackcaps. They could see the outside through the front, where the camera was installed. All other surfaces were covered. The cage had a perch in the front and two semi-vertical perches where the two food choices were presented, in this case anthocyanin-enriched food is to the left of the image and fat-enriched food to the right. Note that the two food choices are readily distinguishable by their colour, which blackcaps may use as a reliable clue of nutrient content of fruits (Schaefer et al. 2008).

Supplementary Table S1

Table S1. Identity and prevalence (n = 48) of the haemosporidian parasites found in the study. Distinct parasite lineages differ from other lineages by at least one base pair of the MalAvi barcode (478 bp of the Cytochrome *b* gene), and are named referred to according toby their names in that database (Bensch et al., 2009). Lineages have been identified to genus level based on DNA sequence similarity with known parasites, and those that belong to known species are named accordingly. Unknown parasites are those for which the genetic barcode could not be sequenced. The total number of parasite occurrences is higher than the number of infections due to multiple infections (the table shows the number of single infections of each parasite followed by the number of multiple infections in which it was involved in parentheses). Six individuals were not infected.

Genus	Lineage	Species	Occurrences	Prevalence
Haemoproteus	SYAT01	*H. parabelopolskyi*	12 (5)	35.4 %
	SYAT02	*H. parabelopolskyi*	1 (8)	18.8 %
	SYAT03	*H. pallidulus*	1	2.1 %
	SYAT07	*H. parabelopolskyi*	1 (6)	14.6 %
	SYAT10	Undetermined	1	2.1 %
	SYAT13	Undetermined	2 (0)	4.2 %
	WW2	*H. majoris*	1	2.1 %
	Unknown *Haemoproteus*	Undetermined	7 (1)	
Plasmodium	SYAT05	*P. vaughani*	0 (1)	2.1 %
	Unknown *Plasmodium*	Undetermined	2	
Leucocytozoon	BT2	Undetermined	1	2.1 %
	SYAT22	Undetermined	1	2.1 %
	Unknown *Leucocytozoon*	Undetermined	2 (1)	

Supplementary Table S2

Table S2. Linear models analysing the relationships between physiological parameters and parasites, controlling by year and residual body mass. All significant results are highlighted in bold. We used parasites as a global term to refer to the multiple infection status and the intensity of infection. TAS11. TAS measurements are corrected with uric acid when included as covariate.

		Multiple infection status			**Intensity of infection (log)**		
		df	**F**	***p***	***df***	**F**	***p***
Haemoglobin	Parasite variable	2,43	0.74	0.48	1,38	2.52	0.12
	Residual mass	1,43	0.06	0.8	1,38	1.41	0.24
	Year	1,43	1.23	0.27	1,38	0.55	0.46
TAS	Parasite variable	2,37	2.62	0.09	1,33	0.69	0.41
	Residual mass	1,37	0.12	0.74	1,33	0.67	0.41
	Year	1,37	5.15	**0.03**	1,33	3.38	0.07
	Uric acid	1,37	137.6	**<0.001**	1,33	130	**<0.001**
	Plasma-MDA (log)	1,37	0.01	0.92	1,33	0.05	0.82
Uric acid							
	Parasite variable	2,42	0.74	0.49	1,37	1.88	0.18
	Residual mass	1,42	0.31	0.58	1,37	0.9	0.35
	Year	1,42	0.25	0.62	1,37	0.22	0.64
Plasma-MDA	Parasite variable	2,38	0.40	0.68	1,34	0.56	0.46
	Residual mass	1,38	3.13	0.09	1,34	1.67	0.21
	Year	1,38	0.58	0.45	1,34	0.74	0.40
	TAS[1]	1,38	<0.01	0.95	1,34	0.07	0.80
RBC hormesis	Parasite variable	2,43	0.93	0.40	1,38	0.19	0.66
	Residual mass	1,43	0.25	0.61	1,38	0.79	0.38
	Year	1,43	0.41	0.53	1,38	0.66	0.42
RBC oxidative stress	Parasite variable	2,43	0.02	0.98	1,38	0.43	0.52
	Residual mass	1,43	0.96	0.33	1,38	0.61	0.44
	Year	1,43	0.24	0.63	1,38	0.53	0.47
Triglycerides	Parasite variable	2,42	0.15	0.86	1,37	0.06	0.81
	Residual mass	1,42	0.09	0.76	1,37	0.05	0.83
	Year	1,42	1.95	0.17	1,37	1.56	0.22

[1]TAS values were corrected by uric acid when TAS was included as a covariate.


ElenaTena

General discussion

In this thesis we have applied a multilevel approach to unveil the differences in exposure to vectors and competence of birds as hosts for haemosporidian parasites, at the among-species level in a community of Neotropical birds, and at the within-species level in a population of migrating blackcaps. We found that, at the community level, there are some ecological traits of species that shape parasite prevalence, such as exposure to vectors and coping behaviours that may determine host competence in tropical areas (Chapter 1). Chapters 2 and 3 delve into the differences in host competence among individual birds from the same species by investigating behavioural and physiological mechanisms that help them cope with parasite infections. In Chapter 2, the distribution of parasite infections in the population, and variation in physiological parameters of individuals of different sex and age classes in relation to infection levels, are coherent with the existence of trade-offs between coping with infection and other physiological demands (in this case associated with migration). Finally, we show that birds could have behavioural adaptations (i.e., feeding choice) to better cope with parasites during physiologically demanding periods, which may help them to reduce the physiological cost of infection (Chapter 3).

As shown in this thesis, the coping capacity of birds against haemosporidian parasites may differ between species and individuals. We found that behavioural responses to infections are key in the distribution of prevalence of infection. Our results suggest that host feeding behaviour may be important in host-parasite interactions. We found evidence of dietary shifts as a behavioural response of individuals to infections, and, according to our comparative analysis, dietary differences among species may play a key role in shaping the distribution of prevalence at the community level. In contrast

with most studies regarding diet and infection, we have paid a lot of attention to the formulation of the feeding behaviour and have developed an observational trial to test the capacity of birds to select their food to meet their needs. We found an important role of plant-based diets most of which could be rich in antioxidants and antimalarial compounds (Lehane and Saliba 2008; Rudrapal and Chetia 2017). These dietary antioxidants could be compensating the oxidative damage generated by the metabolism of the parasite (Costantini and Møller 2009; Sorci and Faivre 2009) or the activation of the immune system (Costantini 2010a); or enhancing the immune system itself (Sorci and Faivre 2009). Then, the effects of feeding behaviour could vary depending on the coping mechanism activated. This dietary mechanism is highly important in the case of migratory birds that rely on plant-based diets during stopovers; however, the effect of other kinds of diets and movements are still to discover. In the case of the tropical community, the bird species that presented the highest prevalence were the ones consuming most plant-based diets (as detected by beak slenderness and the actual proportion of plant matter in their diets; Bright et al. 2016; Navalón et al. 2019), which could be compatible with defensive mechanisms activated through diet (Beaulieu and Schaefer 2013; Masello et al. 2018). On the other hand, migrating blackcaps showed a preference for antioxidant-rich diets when presenting coinfections that previous studies have shown to be generally more virulent than single infections (Marzal et al. 2008; García-Longoria et al. 2022), which could be a mechanism to reduce the costs of infections or to clear them (Costantini and Møller 2009; Percário et al. 2012; Costantini 2014). If feeding on (antioxidant-rich) plants helps birds live with parasites without reducing fitness (i.e., plant-based diet favours tolerance), our results support the idea that plant-based diets make more competent bird hosts for haemosporidian parasites.

We highlight the importance to carry out studies that contemplate individual and species differences in relation to parasitic infections to uncover the differential competence of hosts. At individual level, we found that first-year blackcaps suffer more haemosporidian coinfections during autumn migration. These blackcaps are still developing their immune system and physiology, and carry infections for the first time, therefore the impact of infections might be especially strong for them, since we found a concentration of coinfections that might be more virulent (Palinauskas et al. 2018). We did not find any sex difference in the effects of haemosporidian parasites, maybe due to the concentration of infections in young individuals, which are not sexually mature and hence may have an attenuated influence of sex hormones modultating their immune system (Roved et al. 2017; Vincze et al. 2022). Parasite prevalence of different passerine species from French Guiana was correlated with ecological traits indicative of their exposure to vector bites and their plant-based diet, which could increase parasite acquisition and host competence. Although we found evidence that differences in parasite prevalence among Neotropical species depended on exposure to vectors and coping mechanisms that may boost host competence, we did not find any phylogenetic tendency. This could mean that it is the ecological traits of birds and not just their ancestry what is leading parasitic prevalence. However, we found that antbirds (Thamnophilidae), which were the best represented group in our sample, are accumulating infections in this community, being reservoirs for haemosporidinas in tropical regions (Fecchio et al. 2017). This group of birds presents a morphology and life-style that increment the exposure to vector bites (long legs and a foraging strategy that implyies species dwelling in dense vegetation, where most dipteran vectors rest; Snow 1955; Service 1971; Burkett-Cadena et al. 2013; van Hoesel et al. 2019), mostly to mosquitoes transmitting *Plasmodium* parasites (Service 1971; Burkett-Cadena et al. 2013; van Hoesel et al. 2019;

Santiago-Alarcón and Marzal 2020). We did not find any relationship between the body size of Neotropical birds and parasite prevalence (Ellis, Huang, et al. 2020). Although there is evidence of some effect of body size on the abundance of parasites (Scheuerlein and Ricklefs 2004; Ricklefs et al. 2005), this trait has not been found relevant in many other studies (Matthews et al. 2016; Ellis, Fecchio, et al. 2020), possibly because of phylogenetic influences as body size is correlated with many life-history traits that may be more important in determining variation in prevalence (Poulin 1995). We did not find any relationship between body size and prevalence, although the homogeneity of body sizes in our passerine sample could have made such an effect especially difficult to detect.

The role of exposure to vectors in the transmission of haemosporidian parasites has been studied at the community level. We included not only morphological traits of species that could increase the probability of being bitten by a dipteran vector, such as the proportion of skin not covered by feathers. But also behavioural and life-history traits that could modulate the rate of vector bites, such as the foraging time spent within the understory (Fecchio et al. 2022). We found that species with strongest territorial behaviour harboured higher prevalence, possibly increasing the encounter rate with vectors due to patrolling movements (Herrera and Nunn 2019), or because of weaker immune system due to increased testosterone levels (Wingfield et al. 1987; Folstad and Karter 1992; Hau et al. 2000).

One of the main results of this thesis is the importance of trade-offs to discover parasitic costs and the capacity of each host to deal with them. Migration is known to have physiological and energetic costs (Skrip et al. 2015; Eikenaar, Winslott, et al. 2020) which helped us find that, in migrating blackcaps dealing with costly haemosporidian parasites, oxidative stress may arise through an increase in the oxidative damage (Delhaye

et al. 2016) and a consumption of antioxidants (Costantini and Møller 2009). Stopovers are used to rebuild fat stores of migrating birds (Araújo et al. 2019) and cope with oxidative damage from long flights (Eikenaar, Winslott, et al. 2020). Then, when birds are about to continue their journey, they have to focus on putting on fats (Jenni and Jenni-Eiermann 1998; McWilliams et al. 2021). If birds need to deal with oxidative costs of infections, they would need to invest time and resources on this, choosing antioxidant-rich diets instead of more energetic ones (Mancio-Silva et al. 2017; Zuzarte-Luís and Mota 2018). This means that, during migration, blackcaps could self-medicate against oxidative damage when they present virulent infections (Beaulieu and Schaefer 2013; de Roode et al. 2013; Muriel 2020). From this perspective, feeding on protective foods would be a mechanism of tolerance rather than one of resistance, an interpretation which aligns with the observation of higher prevalence on species that rely more strongly on plant food in our comparative study of rainforest passerines from French Guiana. Nevertheless, interpretations about the oxidative status of birds should be carefully considered since most oxidative stress markers could interact with one another and be influenced by different factors, which often makes it difficult to correctly interpret them in natural settings.

In our studies, we found that consequences of haemosporidian infections are detectable only when the infections could be more virulent, i.e., in infections with several parasites and coinfections with *Plasmodium*. Since the parasitemia in all our studies was relatively low, perhaps because most infections were chronic, this factor did not play an important role. However, there is evidence that *Plasmodium* infections and coinfections with multiple parasite lineages are more virulent (Alizon and van Baalen 2008; Marzal et al. 2008; Palinauskas et al. 2018; Pigeault et al. 2018; Rivero and Gandon 2018), causing

more costs to their hosts in some cases (Santiago-Alarcón et al. 2012; Videvall 2019; García-Longoria et al. 2022). This increased virulence could explain the increasing oxidative stress in our blackcap population.

We described the community of parasites (and host-parasite interactions) in birds from French Guiana. Tropical regions are hot-spots for diversity of birds, vectors and parasites (Clark et al. 2014; Moens and Pérez-Tris 2016; Harvey et al. 2020; Santiago-Alarcón and Marzal 2020), making them the perfect scenario to look for haemosporidian reservoirs. The prevalence of infections concentrated in one of our well-sampled groups, passerines. Hummingbirds were also extensively sampled, however, we found only one infection out of 187 birds, that we were unable to sequence, possibly due to the absence of key parasites in hummingbirds, like *Haemoproteus witti* (Moens et al. 2016). We found low prevalence in general (Harrigan et al. 2014), being *Plasmodium* the most abundant genus, followed by *Haemoproteus* and, with very little presence, *Leucocytozoon.* The composition of the parasite community is similar to the observed in other tropical areas (Fecchio et al. 2018), possibly due to the scarcity of competent vectors for *Haemoproteus* (Fecchio et al. 2018; Fecchio, Bell, et al. 2020), or the failed development of some *Leucocytozoon* parasites in the Neotropics (Fecchio et al. 2018; Fecchio, Bell, et al. 2020). Other important aspect of our sampling site in French Guiana, is that all the infections are locally acquired, since all birds were resident (Thiollay 1994), making it possible to unambiguously allocate correlations between ecological traits and local transmission, which was much more difficult for most previous studies (Ricklefs et al. 2005; Ellis, Huang, et al. 2020; Ellis, Huang, et al. 2020; Fecchio, Chagas, et al. 2020; Gupta et al. 2020; Fecchio, Lima, et al. 2021; Fecchio et al. 2022).

We also described the community of parasites present in the blackcap population, which has been widely studied (Pérez-Tris and Bensch 2005b; Pérez-Tris et al. 2007; Pérez-Rodríguez et al. 2013), but not so much during autumn migration and differentiating sex and age classes in the population. Perhaps the different infection status found between first-year birds and adults could be due to a filter of more virulent parasites that decrease the chances of younger individuals to complete their first migration, or the capacity of adults to clear virulent coinfections (Belo et al. 2011; Ellis et al. 2014).

We implemented in all chapters a new methodology to detect haemosporidian infections (Ciloglu et al. 2019). This new multiplex PCR has been useful to detect more infections and multiple infections than the traditional nested PCR (Hellgren et al. 2004), since the multiplex PCR is more sensitive to lower intensities of infection and amplifies the three genera of parasites, making it possible to identify multi-genus coinfections (the traditional nested PCR amplifies *Plasmodium* and *Haemoproteus* with the same primers). In the blackcap population the nested technique only detected 5 out 22 coinfections with *Plasmodium*. We enhanced the parasite detectability with the multiplex PCR (in French Guiana, 41.9 % of infections would have not been detected with just the nested technique). In some samples from French Guiana, we found evidence of interferences of avian genome with the PCR techniques applied. We conducted our determination of the infection status of these birds carefully, with a first screening step with the multiplex PCR, followed by a nested PCR to determine parasite lineages, and ending with an uniplex PCR (using one pair of primers from the multiplex technique) in the cases that were not clear (even sequencing its products). This way, we could unambiguously determine the infection status of Neotropical birds. Then, we recommend using this new multiplex PCR technique (Ciloglu et al. 2019) in all samples since it allows to identify

more coinfections, as well as infections that may have low detectability with classic methods. However, it is advisable to take some precautions especially in the cases of samples that have not being thoroughly tested for this technique, since there might be some problems with the amplification of unspecific products that sometimes may recommend re-testing before confirming a positive. For this matter, we proposed a modification of the multiplex PCR, using a uniplex approach as described above to double check positive infections and sequencing the PCR products when deemed necessary.

Future investigations following this thesis could, in the case of the community of French Guiana, include sampling from more species and incrementing the number of sampled individuals for each species. Doing so could enable us to to expand the set of ecological traits implied in haemosporidian prevalence in a more complete and diverse bird community. A future perspective would the including some intra-species traits such as age, sex or morphological attributes, which was impossible for us because of the small sample size available. The increase in sample size could enhance our statistical power to find new drivers of host competence. Finally, for the blackcap population, future studies should be carried out in controlled conditions, to better test the effect of haemosporidians in diet choice throught longitudinal tests. We could control both the infection status and the food offer and confirm whether infected birds would preferentially choose antioxidant-enriched diets, improving their oxidative status and decreasing their parasitic load. Moreover, studing food choice in other migrating bird species with different stopover regimen and diet shift could make it possible to generalise our findings.

Conclusions

1. In our studies, avian haemosporidian prevalence is partially explained by birds' traits associated with exposure to parasite infections and with their competence as parasite hosts. Feeding behaviour emerges as an important trait, which may play a similar role in anti-parasite defence at species and individual levels.
2. Plant-based diets may mediate in the coping capacity of birds dealing with oxidative stress derived from haemosporidian parasites. From this perspective, our study supports the idea that birds can self-medicate against oxidative stress when infected by haemosporidian parasites.
3. More virulent haemosporidian infections (coinfections and *Plasmodium* infections in our case), are associated with impaired oxidative balance in migrating birds. This observation supports the view that haemosporidian coinfections come at greater cost for the host than single infections.
4. The study of parasites during challenging situations, such as migration, is helpful to unveil the costs of infection. Migrating is physiologically costly, and more so for infected individuals.
5. Young birds might have more difficulties dealing with haemosporidians during challenging situations: in our study, the most virulent parasite infections, which were correlated with impaired oxidative balance, concentrated in young individuals.
6. Behavioural adaptations involving feeding decisions may influence the physiological status of infected birds. Oxidative stress provides a physiological link between parasite infection and host behaviour.

7. The study of tropical environments enables the discovery of new parasites and parasite reservoirs.
8. Combining screening methods that promote high detectability and correct identification of parasites proves essential to produce sound estimates of parasite prevalence, lowering the rate of false negatives and increasing the capacity to detect coinfections. These practicalities may be key in comparative analyses of parasite prevalence or in studies aiming to identify the physiological costs of parasite infections.

References

Aguiar de Souza Penha V, Maia Chaves Bicalho Domingos F, Fecchio A, Bell JA, Weckstein JD, Ricklefs RE, Braga EM, de Abreu Moreira P, Soares L, Latta S, et al. 2023. Host life-history traits predict haemosporidian parasite prevalence in tanagers (Aves: Thraupidae). Parasitol. 150(1):1–10. doi:10.1017/S0031182022001469.

Akinnusi PA, Olubode SO, Adebesin AO, Osadipe TJ, Nwankwo DO, Adebisi AD, Titilayo I B-A, Alo YM, Owoloye A, Oyebola KM. 2023. Structure-based scoring of anthocyanins and molecular modeling of PfLDH, PfDHODH, and PfDHFR reveal novel potential *P. falciparum* inhibitors. IMU. 38:101206. doi:10.1016/j.imu.2023.101206.

Alizon S, van Baalen M. 2008. Multiple infections, immune dynamics, and the evolution of virulence. Am Nat. 172(4):E150–E168. doi:10.1086/590958.

Alonso-Álvarez C, Pérez-Rodríguez L, García JT, Viñuela J, Mateo R. 2010. Age and breeding effort as sources of individual variability in oxidative stress markers in a bird species. Physiol Biochem Zool. 83(1):110–118. doi:10.1086/605395.

Andersson MN, Nilsson J, Nilsson J-Å, Isaksson C. 2018. Diet and ambient temperature interact to shape plasma fatty acid composition, basal metabolic rate and oxidative stress in great tits. J Exp Biol. 221(24):jeb186759. doi:10.1242/jeb.186759.

de Angeli Dutra D, Fecchio A, Martins Braga É, Poulin R. 2021. Migratory birds have higher prevalence and richness of avian haemosporidian parasites than residents. Int J Parasitol. 51(10):877–882. doi:10.1016/j.ijpara.2021.03.001.

Araújo PM, Viegas I, Rocha AD, Villegas A, Jones JG, Mendonça L, Ramos JA, Masero JA, Alves JA. 2019. Understanding how birds rebuild fat stores during migration: insights from an experimental study. Sci Rep. 9(1):10065. doi:10.1038/s41598-019-46487-z.

Arizaga J, Barba E, Belda EJ. 2008. Fuel management and stopover duration of Blackcaps *Sylvia atricapilla* stopping over in northern Spain during autumn migration period. Bird Study. 55(1):124–134. doi:10.1080/00063650809461513.

Arriero E, Pérez-Tris J, Ramírez A, Remacha C. 2018. Trade-off between tolerance and resistance to infections: an experimental approach with malaria parasites in a passerine bird. Oecologia. 188(4):1001–1010. doi:10.1007/s00442-018-4290-4.

Asghar M, Hasselquist D, Bensch S. 2011. Are chronic avian haemosporidian infections costly in wild birds? J Avian Biol. 42(6):530–537. doi:10.1111/j.1600-048X.2011.05281.x.

Asghar M, Hasselquist D, Hansson B, Zehtindjiev P, Westerdahl H, Bensch S. 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. Science. 347(6220):436–438. doi:10.1126/science.1261121.

Ashley GE, Wingfield JC. 2012. Sickness behavior in vertebrates. *Allostasis, life-history modulation, and hormonal regulation*. In: Ecoimmunology. Oxford University Press. http://www.ncbi.nlm.nih.gov/pubmed/11444392.

Atkinson CT, Thomas NJ, Hunter DB, editors. 2008. Parasitic diseases of wild birds. Ames, Iowa: Wiley-Blackwell.

Badás EP, Martínez J, Rivero de Aguilar Cachafeiro J, Miranda F, Figuerola J, Merino S. 2015. Ageing and reproduction: antioxidant supplementation alleviates telomere loss in wild birds. J Evol Biol. 28(4):896–905. doi:10.1111/jeb.12615.

Bakuradze T, Tausend A, Galan J, Groh IAM, Berry D, Tur JA, Marko D, Richling E. 2019. Antioxidative activity and health benefits of anthocyanin-rich fruit juice in healthy volunteers. Free Radic Res. 53(sup1):1045–1055. doi:10.1080/10715762.2019.1618851.

Balbontín J, Møller AP, Hermosell IG, Marzal A, Reviriego M, de Lope F. 2009. Individual responses in spring arrival date to ecological conditions during winter and migration in a migratory bird. J Anim Ecol. 78(5):981–989. doi:10.1111/j.1365-2656.2009.01573.x.

Barber I, Dingemanse NJ. 2010. Parasitism and the evolutionary ecology of animal personality. Phil Trans R Soc B. 365(1560):4077–4088. doi:10.1098/rstb.2010.0182.

Barron DG, Gervasi SS, Pruitt JN, Martin LB. 2015. Behavioral competence: how host behaviors can interact to influence parasite transmission risk. Opin Behav Sci. 6:35–40. doi:10.1016/j.cobeha.2015.08.002.

Barrow LN, McNew SM, Mitchell N, Galen SC, Lutz HL, Skeen H, Valqui T, Weckstein JD, Witt CC. 2019. Deeply conserved susceptibility in a multi-host, multi-parasite system. Ecol Lett. 22(6):987–998. doi:10.1111/ele.13263.

Bartoń K. 2023. MuMIn: Multi-model inference. R package version 1.47.1. https://CRAN.R-project.org/package=MuMIn.

Bates D, Mächler M, Bolker BM, Walker SC. 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software. 67(1):1–48. doi:10.18637/jss.v067.i01.

Beaulieu M, Schaefer HM. 2013. Rethinking the role of dietary antioxidants through the lens of self-medication. Anim Behav. 86(1):17–24. doi:10.1016/j.anbehav.2013.05.022.

Beaulieu M, Schaefer HM. 2014. The proper time for antioxidant consumption. Physiol Behav. 128:54–59. doi:10.1016/j.physbeh.2014.01.035.

Belo NO, Pinheiro RT, Reis ES, Ricklefs RE, Braga ÉM. 2011. Prevalence and lineage diversity of avian haemosporidians from three distinct cerrado habitats in Brazil. PLoS ONE. 6(3):e17654. doi:10.1371/journal.pone.0017654.

Bensch S, Hellgren O, Pérez-Tris J. 2009. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol Ecol Resour. 9(5):1353–1358. doi:10.1111/j.1755-0998.2009.02692.x.

Benten WP, Ulrich P, Kühn-Velten WN, Vohr HW, Wunderlich F. 1997. Testosterone-induced susceptibility to *Plasmodium chabaudi* malaria: persistence after withdrawal of testosterone. J Endocrinol. 153(2):275–281. doi:10.1677/joe.0.1530275.

Bichet C, Cornet S, Larcombe S, Sorci G. 2012. Experimental inhibition of nitric oxide increases *Plasmodium relictum* (lineage SGS1) parasitaemia. Exp Parasitol. 132(4):417–423. doi:10.1016/j.exppara.2012.09.008.

Bolser JA, Alan RR, Smith AD, Li L, Seeram NP, McWilliams SR. 2013. Birds select fruits with more anthocyanins and phenolic compounds during autumn migration. Wilson J Ornithol. 125(1):97–108. doi:10.1676/12-057.1.

Bongers F, Charles-Dominique P, Forget P-M, Théry M, editors. 2001. Nouragues. Dynamics and plant-animal interactions in a Neotropical rainforest. Dordrecht: Springer Netherlands (Dumont HJ, Werger MJA, editors. Monographiae Biologicae). http://link.springer.com/10.1007/978-94-015-9821-7.

Bright JA, Marugán-Lobón J, Cobb SN, Rayfield EJ. 2016. The shapes of bird beaks are highly controlled by nondietary factors. PNAS. 113(19):5352–5357. doi:10.1073/pnas.1602683113.

Bryla A, Zagkle E, Sadowska ET, Cichoń M, Bauchinger U. 2022. Measurements of body temperature and oxidative stress reveal differential costs associated with humoral immune function in a passerine bird. J Exp Biol. 225(23):jeb244897. doi:10.1242/jeb.244897.

Burkett-Cadena N, Graham SP, Giovanetto LA. 2013. Resting environments of some Costa Rican mosquitoes. J Vector Ecol. 38(1):12–19. doi:10.1111/j.1948-7134.2013.12004.x.

Burnham KP, Anderson DR. 2002. Model selection and multimodel inference: a practical information-theoretic approach. 2nd ed. New York: Springer.

Butler MW, Stierhoff EN, Carpenetti JM, Bertone MA, Addesso AM, Knutie SA. 2021. Oxidative damage increases with degree of simulated bacterial infection, but not ectoparasitism, in tree swallow nestlings. J Exp Biol. 224(18):jeb243116. doi:10.1242/jeb.243116.

Carpenter JW, Campbell TW. 1995. Avian hematology and cytology. 2nd ed. Ames, Iowa: Iowa State University Press.

Cantos, FJ. 1995. Migración e invernada de la curruca capirotada (*Sylvia atricapilla*) en la península Ibérica. Ecología. 9: 425–433.

Catoni C, Metzger B, Schaefer HM, Bairlein F. 2011. Garden Warbler, *Sylvia borin*, detect carotenoids in food but differ strongly in individual food choice. J Ornithol. 152(1):153–159. doi:10.1007/s10336-010-0560-6.

Catoni C, Peters A, Schaefer HM. 2008. Life history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. Anim Behav. 76(4):1107–1119. doi:10.1016/j.anbehav.2008.05.027.

Catoni C, Schaefer HM, Peters A. 2008. Fruit for health: The effect of flavonoids on humoral immune response and food selection in a frugivorous bird. Funct Ecol. 22:649–654. doi:10.1111/j.1365-2435.2007.0.

Cator LJ, Lynch PA, Read AF, Thomas MB. 2012. Do malaria parasites manipulate mosquitoes? Trends Parasitol. 28(11):466–470. doi:10.1016/j.pt.2012.08.004.

Cator LJ, Lynch PA, Thomas MB, Read AF. 2014. Alterations in mosquito behaviour by malaria parasites: Potential impact on force of infection. Malar J. 13(1):1–11. doi:10.1186/1475-2875-13-164.

Chakarov N, Kampen H, Wiegmann A, Werner D, Bensch S. 2020. Blood parasites in vectors reveal a united blackfly community in the upper canopy. Parasites Vectors. 13(1):309. doi:10.1186/s13071-020-04177-0.

Chapa-Vargas L, Matta NE, Merino S. 2020. Effects of ecological gradients on tropical avian hemoparasites. In: Santiago-Alarcon D, Marzal A, editors. Avian Malaria and Related Parasites in the Tropics. Cham: Springer International Publishing. p. 349–378. [accessed 2020 Dec 2]. http://link.springer.com/10.1007/978-3-030-51633-8_4.

Chesser RT. 1994. Migration in South America: an overview of the austral system. Bird Conserv Int. 4(2–3):91–107. doi:10.1017/S0959270900002690.

Chrétien E, De Bonville J, Guitard J, Binning SA, Melis É, Kack A, Côté A, Gradito M, Papillon A, Thelamon V, et al. 2022. Few studies of wild animal performance account for parasite infections: A systematic review. J Anim Ecol. 92(4):794–806. doi:10.1111/1365-2656.13864.

Christe P, Glaizot O, Strepparava N, Devevey G, Fumagalli L. 2012. Twofold cost of reproduction: An increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. Proc R Soc B. 279(1731):1142–1149. doi:10.1098/rspb.2011.1546.

Ciloglu A, Ellis VA, Bernotienė R, Valkiūnas G, Bensch S. 2019. A new one-step multiplex PCR assay for simultaneous detection and identification of avian haemosporidian parasites. Parasitol Res. 118(1):191–201. doi:10.1007/s00436-018-6153-7.

Clark NJ, Clegg SM, Lima MR. 2014. A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporida): New insights from molecular data. Int J Parasitol. 44(5):329–338. doi:10.1016/J.IJPARA.2014.01.004.

Cohen A, Klasing K, Ricklefs R. 2007. Measuring circulating antioxidants in wild birds. Comp Biochem Physiol B, Biochem Mol Biol. 147(1):110–121. doi:10.1016/j.cbpb.2006.12.015.

Cornet S, Bichet C, Larcombe S, Faivre B, Sorci G. 2013. Impact of host nutritional status on infection dynamics and parasite virulence in a bird-malaria system. Ardia D, editor. J Anim Ecol. 83(1):256–265. doi:10.1111/1365-2656.12113.

Cornet S, Nicot A, Rivero A, Gandon S. 2014. Evolution of plastic transmission strategies in avian malaria. PLoS Pathog. 10(9):e1004308. doi:10.1371/journal.ppat.1004308.

Corwin RM. 1997. Economics of gastrointestinal parasitism of cattle. Vet Parasitol. 72(3–4):451–460. doi:10.1016/S0304-4017(97)00110-6.

Costantini D. 2008. Oxidative stress in ecology and evolution: Lessons from avian studies. Ecol Lett. 11(11):1238–1251. doi:10.1111/j.1461-0248.2008.01246.x.

Costantini D. 2010a. Redox physiology in animal function: The struggle of living in an oxidant environment. Curr Zool. 56(6):687–702. doi:10.1093/czoolo/56.6.687.

Costantini D. 2010b. On the measurement of circulating antioxidant capacity and the nightmare of uric acid. MEE. 2(3):321–325. doi:10.1111/j.2041-210X.2010.00080.x.

Costantini D. 2014. Oxidative stress and hormesis in evolutionary ecology and physiology. *A marriage between mechanistic and evolutionary approaches*. Berlin, Heidelberg: Springer Berlin Heidelberg. http://link.springer.com/10.1007/978-3-642-54663-1.

Costantini D, Casagrande S, de Filippis S, Brambilla G, Fanfani A, Tagliavini J, dell'Omo G. 2006. Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). J Comp Physiol B. 176(4):329–337. doi:10.1007/s00360-005-0055-6.

Costantini D, Møller AP. 2009. Does immune response cause oxidative stress in birds? A meta-analysis. Comp Biochem Physiol Part A Mol Integr Physiol. 153(3):339–344. doi:10.1016/j.cbpa.2009.03.010.

Cumnock K, Gupta AS, Lissner M, Chevee V, Davis NM, Schneider DS. 2018. Host energy source is important for disease tolerance to malaria. Curr Biol. 28(10):1635-1642.e3. doi:10.1016/j.cub.2018.04.009.

Delhaye J, Glaizot O, Christe P. 2018. The effect of dietary antioxidant supplementation in a vertebrate host on the infection dynamics and transmission of avian malaria to the vector. Parasitol Res. 117(7):2043–2052. doi:10.1007/s00436-018-5869-8.

Delhaye J, Jenkins T, Christe P. 2016. *Plasmodium* infection and oxidative status in breeding great tits, *Parus major*. Malar J. 15(1):531. doi:10.1186/s12936-016-1579-9.

Delhaye J, Jenkins T, Glaizot O, Christe P. 2018. Avian malaria and bird humoral immune response. Malar J. 17(1):77. doi:10.1186/s12936-018-2219-3.

DeSimone JG, Domschot BS, Fylling MA, Blake WM, Breuner CW. 2023. Body mass and triglycerides predict departure of free-living nomadic pine siskins. Funct Ecol. 37:372–382. doi:10.1111/1365-2435.14238.

Desrochers A. 2010. Morphological response of songbirds to 100 years of landscape change in North America. Ecol. 91(6):1577–1582. doi:10.1890/09-2202.1.

Dhondt AA, Dobson AP. 2017. Stress hormones bring birds, pathogens and mosquitoes together. Trends Parasitol. 33(5):339–341. doi:10.1016/j.pt.2017.01.001.

Dietsch TV. 2007. A relationship between avian foraging behavior and infestation by trombiculid larvae (Acari) in Chiapas, Mexico. Biotropica. 40(2):196–202. doi:10.1111/j.1744-7429.2007.00370.x.

Douma JC, Weedon JT. 2019. Analysing continuous proportions in ecology and evolution: A practical introduction to beta and Dirichlet regression. Methods Ecol Evol. 10(9):1412–1430. doi:10.1111/2041-210X.13234.

Downs CJ, Schoenle LA, Han BA, Harrison JF, Martin LB. 2019. Scaling of host competence. Trends Parasitol. 35(3):182–192. doi:10.1016/j.pt.2018.12.002.

Eikenaar C, Hegemann A, Packmor F, Kleudgen I, Isaksson C. 2020. Not just fuel: Energy stores are correlated with immune function and oxidative damage in a long-distance migrant. Curr Zool. 66(1):21–28. doi:10.1093/cz/zoz009.

Eikenaar C, Isaksson C, Hegemann A. 2018. A hidden cost of migration? Innate immune function versus antioxidant defense. Ecol Evol. 8(5):2721–2728. doi:10.1002/ece3.3756.

Eikenaar C, Winslott E, Hessler S, Isaksson C. 2020. Oxidative damage to lipids is rapidly reduced during migratory stopovers. Funct Ecol. 34(6):1215–1222. doi:10.1111/1365-2435.13540.

Ellis VA, Fecchio A, Ricklefs RE. 2020. Haemosporidian parasites of Neotropical birds: Causes and consequences of infection. Auk. 137(4):ukaa055. doi:10.1093/auk/ukaa055.

Ellis VA, Huang X, Westerdahl H, Jönsson J, Hasselquist D, Neto JM, Nilsson J-Å, Nilsson J, Hegemann A, Hellgren O, et al. 2020. Explaining prevalence, diversity, and host specificity in a community of avian haemosporidian parasites. Oikos. 129(9):1314–1329. doi:10.1111/oik.07280.

Ellis VA, Kunkel MR, Ricklefs RE. 2014. The ecology of host immune responses to chronic avian haemosporidian infection. Oecologia. 176(3):729–737. doi:10.1007/s00442-014-3048-x.

Fecchio A, Bell JA, Bosholn M, Vaughan JA, Tkach VV, Lutz HL, Cueto VR, Gorosito CA, González-Acuña D, Stromlund C, et al. 2020. An inverse latitudinal gradient in infection probability and phylogenetic diversity for *Leucocytozoon* blood parasites in New World birds. J Anim Ecol. 89(2):423–435. doi:10.1111/1365-2656.13117.

Fecchio A, Bell JA, Pinheiro RBP, Cueto VR, Gorosito CA, Lutz HL, Gaiotti MG, Paiva LV, França LF, Toledo-Lima G, et al. 2019. Avian host composition, local speciation and dispersal drive the regional assembly of avian malaria parasites in South American birds. Mol Ecol. 28(10):2681–2693. doi:10.1111/mec.15094.

Fecchio A, Chagas CRF, Bell JA, Kirchgatter K. 2020. Evolutionary ecology, taxonomy, and systematics of avian malaria and related parasites. Acta Trop. 204:105364. doi:10.1016/j.actatropica.2020.105364.

Fecchio A, Clark NJ, Bell JA, Skeen HR, Lutz HL, de la Torre GM, Vaughan JA, Tkach VV, Schunck F, Ferreira FC, et al. 2021. Global drivers of avian haemosporidian infections vary across zoogeographical regions. Global Ecol Biogeogr. 30(12):2393–2406. doi:10.1111/geb.13390.

Fecchio A, Dias RI, Ferreira TV, Reyes AO, Dispoto JH, Weckstein JD, Bell JA, Tkach VV, Pinho JB. 2022. Host foraging behavior and nest type influence prevalence of avian

haemosporidian parasites in the Pantanal. Parasitol Res. 121(5):1407–1417. doi:10.1007/s00436-022-07453-3.

Fecchio A, Ellis VA, Bell JA, Andretti CB, d'Horta FM, Silva AM, Tkach VV, Weckstein JD. 2017. Avian malaria, ecological host traits and mosquito abundance in southeastern Amazonia. Parasitol. 144(8):1117–1132. doi:10.1017/S003118201700035X.

Fecchio A, Lima MR, Bell JA, Schunck F, Corrêa AH, Beco R, Jahn AE, Fontana CS, da Silva TW, Repenning M, et al. 2021. Loss of forest cover and host functional diversity increases prevalence of avian malaria parasites in the Atlantic Forest. Int J Parasitol. 51(9):719–728. doi:10.1016/j.ijpara.2021.01.001.

Fecchio A, Lima MR, Silveira P, Braga ÉM, Marini MÂ. 2011. High prevalence of blood parasites in social birds from a Neotropical savanna in Brazil. Emu. 111(2):132–138. doi:10.1071/MU10063.

Fecchio A, Silveira P, Weckstein JD, Dispoto JH, Anciães M, Bosholn M, Tkach VV, Bell JA. 2018. First Record of *Leucocytozoon* (Haemosporida: Leucocytozoidae) in Amazonia: Evidence for Rarity in Neotropical Lowlands or Lack of Sampling for This Parasite Genus? J Parasitol. 104(2):168–172. doi:10.1645/17-182.

Fenton A, Streicker DG, Petchey OL, Pedersen AB. 2015. Are all hosts created equal? Partitioning host species contributions to parasite persistence in multihost communities. Am Nat. 186(5):610–622. doi:10.1086/683173.

Ferreira FC, Santiago-Alarcon D, Braga ÉM. 2020. Diptera vectors of avian haemosporidians: with emphasis on tropical regions. In: Santiago-Alarcon D, Marzal A, editors. Avian malaria and related parasites in the tropics. Springer, Cham. p. 185–250. http://link.springer.com/10.1007/978-3-030-51633-8_6.

Filion A, Eriksson A, Jorge F, Niebuhr CN, Poulin R. 2020. Large-scale disease patterns explained by climatic seasonality and host traits. Oecologia. 194(4):723–733. doi:10.1007/s00442-020-04782-x.

Finkel T, Holbrook NJ. 2000. Oxidants, oxidative stress and the biology of ageing. Nature. 408(6809):239–247. doi:10.1038/35041687.

Folstad I, Karter AJ. 1992. Parasites, bright males, and the immunocompetence handicap. Am Nat. 139(3):603–622. doi:10.1086/285346.

Fridolfsson A-K, Ellegren H. 1999. A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol. 30(1):116–121. doi:10.2307/3677252.

Gandon S, Agnew P, Michalakis Y. 2002. Coevolution between parasite virulence and host life-history traits. Am Nat. 160(3):15.

García-Longoria L, Magallanes S, González-Blázquez M, Refollo Y, de Lope F, Marzal A. 2016. New approaches for an old disease: Studies on avian malaria parasites for the twenty-first century challenges. In: Current topics in malaria. InTech.

García-Longoria L, Magallanes S, Huang X, Drews A, Råberg L, Marzal A, Bensch S, Westerdahl H. 2022. Reciprocal positive effects on parasitemia between coinfecting haemosporidian parasites in house sparrows. BMC Ecol Evo. 22(73):73. doi:10.1186/s12862-022-02026-5.

González AD, Matta NE, Ellis VA, Miller ET, Ricklefs RE, Gutiérrez HR. 2014. Mixed species flock, nest height, and elevation partially explain avian haemoparasite prevalence in Colombia. Braga ÉM, editor. PLoS ONE. 9(6):e100695. doi:10.1371/journal.pone.0100695.

González-Varo JP, Onrubia A, Pérez-Méndez N, Tarifa R, Illera JC. 2022. Fruit abundance and trait matching determine diet type and body condition across frugivorous bird populations. Oikos. 2022(2):oik.08106. doi:10.1111/oik.08106.

Green MR, Hughes H, Sambrook J, MacCallum P. 2012. Molecular cloning: a laboratory manual. In: Molecular cloning: a laboratory manual. 4th ed. CSH Press, UK. p. 1890–1890.

Guindre-Parker S, Rubenstein DR. 2018. The oxidative costs of parental care in cooperative and pair-breeding African starlings. Oecologia. 188(1):53–63. doi:10.1007/s00442-018-4178-3.

Gupta P, Vishnudas CK, Robin VV, Dharmarajan G. 2020. Host phylogeny matters: Examining sources of variation in infection risk by blood parasites across a tropical montane bird community in India. Parasites Vectors. 13(1):536. doi:10.1186/s13071-020-04404-8.

Gutiérrez-López R, Martínez-de la Puente J, Gangoso L, Soriguer R, Figuerola J. 2019. Effects of host sex, body mass and infection by avian *Plasmodium* on the biting rate of two mosquito species with different feeding preferences. Parasites Vectors. 12(1):87. doi:10.1186/s13071-019-3342-x.

Hall ME, Blount JD, Forbes S, Royle NJ. 2010. Does oxidative stress mediate the trade-off between growth and self-maintenance in structured families?: Growth, oxidative stress and family structure. Functional Ecology. 24(2):365–373. doi:10.1111/j.1365-2435.2009.01635.x.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser. 41:95–98.

Han J-I, Kim J-H, Kim S, Park S-R, Na K-J. 2009. A simple and improved DNA test for avian sex determination. Auk. 126(4):779–783. doi:10.1525/auk.2009.08203.

Harrigan RJ, Sedano R, Chasar AC, Chaves JA, Nguyen JT, Whitaker A, Smith TB. 2014. New host and lineage diversity of avian haemosporidia in the northern Andes. Evol Appl. 7(7):799–811. doi:10.1111/eva.12176.

Hartig F. 2022. DHARMa: Residual diagnostics for hierarchical (multi-level / mixed) regression models. R package version 0.4.6. https://CRAN.R-project.org/package=DHARMa.

Harvey MG, Bravo GA, Claramunt S, Cuervo AM, Derryberry GE, Battilana J, Seeholzer GF, McKay JS, O'Meara BC, Faircloth BC, et al. 2020. The evolution of a tropical biodiversity hotspot. Science. 370(6522):1343–1348. doi:10.1126/science.aaz6970.

Hau M, Wikelski M, Soma KK, Wingfield JC. 2000. Testosterone and year-round territorial aggression in a tropical bird. Gen Comp Endocrinol. 117(1):20–33. doi:10.1006/gcen.1999.7390.

Hawley DM, Altizer SM. 2011. Disease ecology meets ecological immunology: Understanding the links between organismal immunity and infection dynamics in natural populations. Funct Ecol. 25(1):48–60. doi:10.1111/j.1365-2435.2010.01753.x.

Hawley DM, Etienne RS, Ezenwa VO, Jolles AE. 2011. Does animal behavior underlie covariation between hosts' exposure to infectious agents and susceptibility to infection? Implications for disease dynamics. ICB. 51(4):528–539. doi:10.1093/icb/icr062.

Hayes FE, Sewlal J-AN. 2004. The Amazon River as a dispersal barrier to passerine birds: Effects of river width, habitat and taxonomy. J Biogeogr. 31(11):1809–1818. doi:10.1111/j.1365-2699.2004.01139.x.

Hegemann A, Alcalde Abril P, Muheim R, Sjöberg S, Alerstam T, Nilsson J-Å, Hasselquist D. 2018. Immune function and blood parasite infections impact stopover ecology in passerine birds. Oecologia 188:1011–1024. doi: 10.1007/s00442-018-4291-3.

Hellgren O, Bensch S, Malmqvist B. 2008. Bird hosts, blood parasites and their vectors - associations uncovered by molecular analyses of blackfly blood meals. Mol Ecol. 17(6):1605–1613. doi:10.1111/j.1365-294X.2007.03680.x.

Hellgren O, Waldenström J, Bensch S. 2004. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. J Parasitol. 90(4):797–802. doi:10.1645/GE-184R1.

Hellgren O, Waldenström J, Pérez-Tris J, Szöllősi E, Hasselquist D, Križanauskienė A, Ottoson U, Bensch S. 2007. Detecting shifts of transmission areas in avian blood parasites - a phylogenetic approach. Molecular Ecology. 16(6):1281–1290. doi:10.1111/j.1365-294X.2007.03227.x.

Herrera CM. 1987. Vertebrate-dispersed plants of the Iberian Peninsula: A study of fruit characteristics. Ecol Monogr. 57(4):305–331. doi:10.2307/2937089.

Herrera J, Nunn CL. 2019. Behavioural ecology and infectious disease: Implications for conservation of biodiversity. Philos Trans R Soc Lond B Biol Sci. 374(1781):20180054. doi:10.1098/rstb.2018.0054.

Hite JL, Pfenning AC, Cressler CE. 2019. Starving the enemy? Feeding behavior shapes host-parasite interactions. Trends Ecol Evol. 35(1):68–80. doi:10.1016/j.tree.2019.08.004.

Huber N, Mahr K, Tóth Z, Szarka EZ, Çınar YU, Salmón P, Lendvai ÁZ. 2021. The stressed bird in the hand: Influence of sampling design on the physiological stress

response in a free-living songbird. Physiol Behav. 238: 113488.doi: 10.1016/j.physbeh.2021.113488.

van Hoesel W, Marzal A, Magallanes S, Santiago-Alarcon D, Ibáñez-Bernal S, Renner SC. 2019. Management of ecosystems alters vector dynamics and haemosporidian infections. Sci Rep. 9(8779). doi:10.1038/s41598-019-45068-4. https://www.nature.com/articles/s41598-019-45068-4.

Huang X, Jönsson J, Bensch S. 2020. Persistence of avian haemosporidians in the wild: a case study to illustrate seasonal infection patterns in relation to host life stages. International Journal for Parasitology. 50(8):611–619. doi:10.1016/j.ijpara.2020.05.006.

Huang ZYX, Halliday FW, Becker DJ. 2023. Host functional traits as the nexus for multilevel infection patterns. Trends Ecol Evol. 38(12):1125–1128. doi:10.1016/j.tree.2023.08.011.

Isaksson C, Sepil I, Baramidze V, Sheldon BC. 2013. Explaining variance of avian malaria infection in the wild: the importance of host density, habitat, individual life-history and oxidative stress. BMC Ecol. 13(1):15. doi:10.1186/1472-6785-13-15.

Jahn AE, Cueto VR, Fontana CS, Guaraldo AC, Levey DJ, Marra PP, Ryder TB. 2020. Bird migration within the Neotropics. Auk. 137(4):ukaa033. doi:10.1093/auk/ukaa033.

Jenkins T, Delhaye J, Christe P. 2015. Testing local adaptation in a natural great tit-malaria system: An experimental approach. PLoS ONE. 10(11):e0141391. doi:10.1371/journal.pone.0141391.

Jenni L, Jenni-Eiermann S. 1998. Fuel supply and metabolic constraints in migrating birds. J Avian Biol. 29(4):521–528. doi:10.2307/3677171.

Jenni L, Müller S, Spina F, Kvist A, Lindström Å. 2006. Effect of endurance flight on haematocrit in migrating birds. J Ornithol. 147(4):531. doi:10.1007/s10336-006-0076-2.

Jenni-Eiermann S. 2017. Energy metabolism during endurance fight and the post-fight recovery phase. J Comp Physiol A. 203:431–438. doi:10.1007/s00359-017-1150-3.

Jenni-Eiermann S, Jenni L, Smith S, Costantini D. 2014. Oxidative stress in endurance flight: An unconsidered factor in bird migration. McGraw K, editor. PLoS ONE. 9(5):e97650. doi:10.1371/journal.pone.0097650.

Jetz W, Thomas GH, Joy JB, Hartmann K, Mooers AO. 2012. The global diversity of birds in space and time. Nature. 491(7424):444–448. doi:10.1038/nature11631.

Johnson PTJ, de Roode JC, Fenton A. 2015. Why infectious disease research needs community ecology. Science. 349(6252):1259504–1259504. doi:10.1126/science.1259504.

Jordano P. 1995. Angiosperm fleshy fruits and seed dispersers: A comparative analysis of adaptation and constraints in plant-animal interactions. Am Nat. 145(2):163–191.

Jovani R, Tella JL. 2006. Parasite prevalence and sample size: Misconceptions and solutions. Trends Parasitol. 22(5):214–218. doi:10.1016/j.pt.2006.02.011.

Kamiya T, O'Dwyer K, Nakagawa S, Poulin R. 2014. What determines species richness of parasitic organisms? A meta-analysis across animal, plant and fungal hosts. Biol Rev Camb Philos Soc. 89(1):123–134. doi:10.1111/brv.12046.

Kilpatrick AM, Daszak P, Jones MJ, Marra PP, Kramer LD. 2006. Host heterogeneity dominates West Nile virus transmission. Proc R Soc B. 273(1599):2327–2333. doi:10.1098/rspb.2006.3575.

Kollmann J, Schneider B. 1999. Landscape structure and diversity of fleshy-fruited species at forest edges. Plant Ecol. 144(1):37–48.

Krams IA, Suraka V, Rantala MJ, Sepp T, Mierauskas P, Vrublevska J, Krama T. 2013. Acute infection of avian malaria impairs concentration of haemoglobin and survival in juvenile altricial birds. J Zool. 291(1):34–41. doi:10.1111/jzo.12043.

Langenhof MR, Komdeur J. 2018. Why and how the early-life environment affects development of coping behaviours. Behav Ecol Sociobiol. 72(3):34. doi:10.1007/s00265-018-2452-3.

Langslow DR. 1976. Weights of blackcaps on migration. Ring Migr. 1(2):78–91. doi:10.1080/03078698.1976.9673706.

Latta SC, Ricklefs RE. 2010. Prevalence patterns of avian haemosporida on Hispaniola. J Avian Biol. 41(1):25–33. doi:10.1111/j.1600-048X.2009.04685.x.

Leeuwenburgh C, Heinecke J. 2001. Oxidative stress and antioxidants in exercise. CMC. 8(7):829–838. doi:10.2174/0929867013372896.

Lehane AM, Saliba KJ. 2008. Common dietary flavonoids inhibit the growth of the intraerythrocytic malaria parasite. BMC Res Notes. 1:26. doi:10.1186/1756-0500-1-26.

Lima MR, Pérez-Tris J. 2020. Host specialization and dispersal in avian haemosporidians. In: Santiago-Alarcon D, Marzal A, editors. Avian malaria and related parasites in the tropics. Cham: Springer Nature Switzerland. p. 379–400. https://doi.org/10.1007/978-3-030-51633-8_11.

López-Arrabé J, Cantarero A, Pérez-Rodríguez L, Palma A, Moreno J. 2014. Plumage ornaments and reproductive investment in relation to oxidative status in the Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae*). Can J Zool. 92(12):1019–1027. doi:10.1139/cjz-2014-0199.

Mancio-Silva L, Slavic K, Grilo Ruivo MT, Grosso AR, Modrzynska KK, Vera IM, Sales-Dias J, Gomes AR, Macpherson CR, Crozet P, et al. 2017. Nutrient sensing modulates malaria parasite virulence. Nature. 547(7662):213–216. doi:10.1038/nature23009.

Marasco V, Sebastiano M, Costantini D, Pola G, Fusani L. 2021. Controlled expression of the migratory phenotype affects oxidative status in birds. J Exp Biol. 224(6):jeb233486. doi:10.1242/jeb.233486.

Martínez-de la Puente J, Figuerola J, Soriguer R. 2015. Fur or feather? Feeding preferences of species of Culicoides biting midges in Europe. Trends Parasitol. 31(1):16–22. doi:10.1016/j.pt.2014.11.002.

Marzal A, Bensch S, Reviriego M, Balbontin J, de Lope F. 2008. Effects of malaria double infection in birds: One plus one is not two. J Evol Biol. 21(4):979–987. doi:10.1111/j.1420-9101.2008.01545.x.

Masello JF, Martínez J, Calderón L, Wink M, Quillfeldt P, Sanz V, Theuerkauf J, Ortiz-Catedral L, Berkunsky I, Brunton D, et al. 2018. Can the intake of antiparasitic secondary metabolites explain the low prevalence of hemoparasites among wild Psittaciformes? Parasites Vectors. 11:357. doi:10.1186/s13071-018-2940-3.

Matthews AE, Ellis VA, Hanson AA, Roberts JR, Ricklefs RE, Collins MD. 2016. Avian haemosporidian prevalence and its relationship to host life histories in eastern Tennessee. J Ornithol. 157(2):533–548. doi:10.1007/s10336-015-1298-y.

McCurdy DG, Shutler D, Mullie A, Forbes MR. 1998. Sex-biased parasitism of avian hosts: relations to blood parasite taxon and mating system. Oikos. 82(2):303–312. doi:10.2307/3546970.

McWilliams S, Carter W, Cooper-Mullin C, DeMoranville K, Frawley A, Pierce B, Skrip M. 2021. How birds during migration maintain (oxidative) balance. Front Ecol Evol. 9:742642. doi:10.3389/fevo.2021.742642.

Medeiros MCII, Hamer GL, Ricklefs RE. 2013. Host compatibility rather than vector-host-encounter rate determines the host range of avian *Plasmodium* parasites. Proc R Soc B. 280(1760):20122947. doi:10.1098/rspb.2012.2947.

Merino S, Moreno J, Sanz JJ, Arriero E. 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). Proc Royal Soc B. 267(1461):2507–2510. doi:10.1098/rspb.2000.1312.

Messina S, Edwards DP, van Houtte N, Tomassi S, Benedick S, Eens M, Costantini D. 2022. Impacts of selective logging on haemosporidian infection and physiological correlates in tropical birds. Int J Parasitol. 52(1):87–96. doi:10.1016/j.ijpara.2021.07.003.

Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci. 84(4):407–412. doi:10.1042/cs0840407.

Moens MAJ, Pérez-Tris J. 2016. Discovering potential sources of emerging pathogens: South America is a reservoir of generalist avian blood parasites. Int J Parasitol. 46(1):41–49. doi:10.1016/j.ijpara.2015.08.001.

Moens MAJ, Valkiūnas G, Paca A, Bonaccorso E, Aguirre N, Pérez-Tris J. 2016. Parasite specialization in a unique habitat: Hummingbirds as reservoirs of generalist blood

parasites of Andean birds. J Anim Ecol. 85(5):1234–1245. doi:10.1111/1365-2656.12550.

Møller AP, Christe P, Garamszegi LZ. 2005. Coevolutionary arms races: Increased host immune defense promotes specialization by avian fleas. J Evol Biol. 18(1):46–59. doi:10.1111/j.1420-9101.2004.00774.x.

Monaghan P, Metcalfe NB, Torres R. 2009. Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. Ecol Lett. 12(1):75–92. doi:10.1111/j.1461-0248.2008.01258.x.

Moore FR, Aborn D. 2000. Mechanisms of *en route* habitat selection: How do migrants make habitat decisions during stopover? Stud Avian Biol. 20:34–42.

Moore J. 2002. Parasites and the behavior of animals. Oxford: Oxford Univ. Press (Oxford series in ecology and evolution).

Muriel J. 2020. Ecophysiological assessment of blood haemosporidian infections in birds. Ecosistemas. 29(2):1979. doi:10.7818/ECOS.1979.

Navalón G, Bright JA, Marugán-Lobón J, Rayfield EJ. 2019. The evolutionary relationship among beak shape, mechanical advantage, and feeding ecology in modern birds. Evolution. 73(3):422–435. doi:10.1111/evo.13655.

Nourani L, Zakeri S, Dinparast Djadid N. 2020. Dynamics of prevalence and distribution pattern of avian *Plasmodium* species and its vectors in diverse zoogeographical areas - A review. Infect Genet Evol. 81:104244. doi:10.1016/j.meegid.2020.104244.

Nwaogu CJ, Galema A, Cresswell W, Dietz MW, Tieleman BI. 2020. A fruit diet rather than invertebrate diet maintains a robust innate immunity in an omnivorous tropical songbird. J Anim Ecol. 89(3):867–883. doi:10.1111/1365-2656.13152.

Orme D, Freckleton R, Thomas G, Petzoldt T, Fritz S, Isaac N, Pearse W. 2023. Caper: Comparative analyses of phylogenetics and evolution in R. R package version 1.0.3. https://CRAN.R-project.org/package=caper.

Ortiz-Catedral L, Brunton D, Stidworthy MF, Elsheikha HM, Pennycott T, Schulze C, Braun M, Wink M, Gerlach H, Pendl H, et al. 2019. *Haemoproteus minutus* is highly virulent for Australasian and South American parrots. Parasites Vectors. 12(1):40. doi:10.1186/s13071-018-3255-0.

Palinauskas V, Valkiūnas G, Bolshakov CV, Bensch S. 2008. *Plasmodium relictum* (lineage P-SGS1): Effects on experimentally infected passerine birds. Exp Parasitol. 120(4):372–380. doi:10.1016/j.exppara.2008.09.001.

Palinauskas V, Žiegytė R, Šengaut J, Bernotienė R. 2018. Different paths – the same virulence: Experimental study on avian single and co-infections with *Plasmodium relictum* and *Plasmodium elongatum*. Int J Parasitol. 48(14):1089–1096. doi:10.1016/j.ijpara.2018.08.003.

Paull SH, Song S, McClure KM, Sackett LC, Kilpatrick AM, Johnson PT. 2012. From superspreaders to disease hotspots: Linking transmission across hosts and space. Front Ecol Environ. 10(2):75–82. doi:10.1890/110111.

Percário S, Moreira D, Gomes B, Ferreira M, Gonçalves A, Laurindo P, Vilhena T, Dolabela M, Green M. 2012. Oxidative stress in malaria. Int J Mol Sci. 13(12):16346–16372. doi:10.3390/ijms131216346.

Pérez-Rodríguez A, Ramírez Á, Richardson DS, Pérez-Tris J. 2013. Evolution of parasite island syndromes without long-term host population isolation: parasite dynamics in Macaronesian blackcaps *Sylvia atricapilla*. Global Ecology and Biogeography. 22(12):1272–1281. doi:10.1111/geb.12084.

Pérez-Rodríguez L, Alonso-Álvarez C, Martínez-Haro M, Viñuela J. 2008. Variation in plasma biochemical parameters in captive adult red-legged partridges (*Alectoris rufa*) during daylight hours. Eur J Wildl Res. 54(1):21–26. doi:10.1007/s10344-007-0102-6.

Pérez-Rodríguez A, de la Hera I, Bensch S, Pérez-Tris J. 2015. Evolution of seasonal transmission patterns in avian blood-borne parasites. Int J Parasitol. 45(9–10): 605–611. doi: 10.1016/j.ijpara.2015.03.008.

Pérez-Rodríguez L, Romero-Haro AA, Sternalski A, Muriel J, Mougeot F, Gil D, Alonso-Álvarez C. 2015. Measuring oxidative stress: The confounding effect of lipid concentration in measures of lipid peroxidation. Physiol Biochem Zool. 88(3):345–351. doi:10.1086/680688.

Pérez-Tris J, Bensch S. 2005a. Diagnosing genetically diverse avian malarial infections using mixed-sequence analysis and TA-cloning. Parasitol. 131(1):15–23. doi:10.1017/S003118200500733X.

Pérez-Tris J, Bensch S. 2005b. Dispersal increases local transmission of avian malarial parasites. Ecol Lett. 8(8):838–845. doi:10.1111/j.1461-0248.2005.00788.x.

Pérez-Tris J, Hellgren O, Križanauskienė A, Waldenström J, Secondi J, Bonneaud C, Fjeldså J, Hasselquist D, Bensch S. 2007. Within-host speciation of malaria parasites. PLoS ONE. 2(2):e235. doi:10.1371/journal.pone.0000235.

Phillips RE, Pasvol G. 1992. 3-Anaemia of *Plasmodium falciparum* malaria. Baillière's Clinical Haematology. 5(2):315–330. doi:10.1016/S0950-3536(11)80022-3.

Pierce BJ, McWilliams SR, O'Connor TP, Place AR, Guglielmo CG. 2005. Effect of dietary fatty acid composition on depot fat and exercise performance in a migrating songbird, the red-eyed vireo. J Exp Biol. 208(7):1277–1285. doi:10.1242/jeb.01493.

Piersma T, Everaarts JM, Jukema J. 1996. Build-up of red blood cells in refuelling bar-tailed godwits in relation to individual migratory quality. The Condor. 98(2):363–370. doi:10.2307/1369154.

Pigeault R, Cozzarolo C-S, Choquet R, Strehler M, Jenkins T, Delhaye J, Bovet L, Wassef J, Glaizot O, Christe P. 2018. Haemosporidian infection and co-infection affect host

survival and reproduction in wild populations of great tits. Int J Parasitol. 48(14):1079–1087. doi:10.1016/j.ijpara.2018.06.007.

Pigeault R, Vézilier J, Cornet S, Zélé F, Nicot A, Perret P, Gandon S, Rivero A. 2015. Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of *Plasmodium*. Phil Trans R Soc B. 370(1675):20140300. doi:10.1098/rstb.2014.0300.

Poulin R. 1995. Phylogeny, ecology, and the richness of parasite communities in vertebrates. Ecological Monographs. 65(3):283–302. doi:10.2307/2937061.

Poulin R. 2007. Evolutionary ecology of parasites. 2. ed. Princeton, NJ: Princeton University Press.

Price ER, Guglielmo CG. 2009. The effect of muscle phospholipid fatty acid composition on exercise performance: A direct test in the migratory white-throated sparrow (*Zonotrichia albicollis*). Am J Physiol Regul Inter Comp Physiol. 297(3):R775–R782. doi:10.1152/ajpregu.00150.2009.

Pullan R, Brooker S. 2008. The health impact of polyparasitism in humans: Are we under-estimating the burden of parasitic diseases? Parasitol. 135(7):783–794. doi:10.1017/S0031182008000346.

R Core Team. 2022. R: A language and environment for statistical computing. https://www.R-project.org/.

Råberg L, Graham AL, Read AF. 2009. Decomposing health: Tolerance and resistance to parasites in animals. Philos Trans R Soc Lond B Biol Sci. 364(1513):37–49. doi:10.1098/rstb.2008.0184.

Råberg L, Sim D, Read AF. 2007. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. Science. 318(5851):812–814. doi:10.1126/science.1148526.

Reece SE, Prior KF, Mideo N. 2017. The life and times of parasites: Rhythms in strategies for within-host survival and between-host transmission. J Biol Rhythms. 32(6):516–533. doi:10.1177/0748730417718904.

Remacha C, Ramirez Á, Arriero E, Pérez-Tris J. 2023. Haemosporidian infections influence risk-taking behaviours in young male blackcaps, *Sylvia atricapilla*. Anim Behav. 196:113–126. doi:10.1016/j.anbehav.2022.12.001.

Rey PJ, Alcántara JM, Sánchez-Lafuente AM. 1996. Temporal variation in food availability and diet of Blackcaps in olive orchards. J Field Ornithol. 67(4):592–603.

Ricklefs RE, Medeiros M, Ellis VA, Svensson-Coelho M, Blake JG, Loiselle BA, Soares L, Fecchio A, Outlaw D, Marra PP, et al. 2017. Avian migration and the distribution of malaria parasites in New World passerine birds. J Biogeogr. 44(5):1113–1123. doi:10.1111/jbi.12928.

Ricklefs RE, Swanson BL, Fallon SM, Martínez-Abraín A, Scheuerlein A, Gray J, Latta SC. 2005. Community relationships of avian malaria parasites in southern Missouri. Ecol Monogr. 75(4):543–559. doi:10.1890/04-1820.

Ricklefs RE, Wikelski M. 2002. The physiology/life-history nexus. Trends Ecol Evol. 17(10):462–468. doi:10.1016/S0169-5347(02)02578-8.

van Riper CI, van Riper SG, Goff ML, Laird M. 1986. The epizootiology and ecological significance of malaria in Hawaiian land birds. Ecol Monogr. 56(4):327–344. doi:10.2307/1942550.

Rivero A, Gandon S. 2018. Evolutionary ecology of avian malaria: Past to present. Trends Parasitol. 34(8):712–726. doi:10.1016/j.pt.2018.06.002.

Robinson WD, Errichetti D, Pollock HS, Martinez A, Stouffer PC, Shen F-Y, Blake JG. 2021. Big bird plots: Benchmarking Neotropical bird communities to address questions in ecology and conservation in an era of rapid change. Front Eco Evol. 9. doi:10.3389/fevo.2021.697511.

Rodewald PG, Brittingham MC. 2004. Stopover habitats of landbirds during fall: Use of edge-dominated and early-successional forests. Auk. 121(4):1040–1055. doi:10.1093/auk/121.4.1040.

Romero-Haro AA, Alonso-Álvarez C. 2014. Covariation in oxidative stress markers in the blood of nestling and adult birds. Physiol Biochem Zool. 87(2):353–362. doi:10.1086/674432.

Romero-Haro AA, Alonso-Álvarez C. 2015. The level of an intracelular antioxidant during development determines the adult phenotype in a bird species: A potential organizar role for Glutathione. Am Nat. 185(3):390-405. Doi: 10.1086/679613.

de Roode JC, Lefèvre T, Hunter MD. 2013. Self-medication in animals. Science. 340(6129):150–151. doi:10.1126/science.1235824.

Rotics S, Kaatz M, Resheff YS, Turjeman SF, Zurell D, Sapir N, Eggers U, Flack A, Fiedler W, Jeltsch F, et al. 2016. The challenges of the first migration: movement and behaviour of juvenile vs. adult white storks with insights regarding juvenile mortality. Both C, editor. J Anim Ecol. 85(4):938–947. doi:10.1111/1365-2656.12525.

Roved J, Westerdahl H, Hasselquist D. 2017. Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. Horm Behav. 88:95–105. doi:10.1016/j.yhbeh.2016.11.017.

Rudrapal M, Chetia D. 2017. Plant flavonoids as potential source of future antimalarial leads. Sys Rev Pharm. 8(1):13–18. doi:10.5530/srp.2017.1.4.

Ruhs EC, Martin LB, Downs CJ. 2020. The impacts of body mass on immune cell concentrations in birds. Proc R Soc B. 287(1934):20200655. doi:10.1098/rspb.2020.0655.

Sabbatani S, Fiorino S, Manfredi R. 2010. The emerging of the fifth malaria parasite (*Plasmodium knowlesi*). A public health concern? Braz J Infect Dis. 14(3):299–309. doi:10.1590/S1413-86702010000300019.

Salmón P, Watson H, Nord A, Isaksson C. 2018. Effects of the urban environment on oxidative stress in early life: Insights from a cross-fostering experiment. ICB. 58(5):986–994. doi:10.1093/icb/icy099.

Santiago-Alarcón D, Marzal A, editors. 2020. Avian malaria and related parasites in the tropics - Ecology, evolution and systematics. Cham: Springer. https://www.springer.com/gp/book/9783030516321.

Santiago-Alarcón D, Palinauskas V, Schaefer HM. 2012. Diptera vectors of avian Haemosporidian parasites: Untangling parasite life cycles and their taxonomy. Biol Rev. 87(4):928–964. doi:10.1111/j.1469-185X.2012.00234.x.

Savile DBO. 1957. Adaptive evolution in the avian wing. Evol. 11(2):212–224. doi:10.2307/2406051.

Schaefer HM, McGraw K, Catoni C. 2008. Birds use fruit colour as honest signal of dietary antioxidant rewards. Funct Ecol. 22(2):303–310. doi:10.1111/j.1365-2435.2007.01363.x.

Schaefer HM, Schmidt V, Wesenberg J. 2002. Vertical stratification and caloric content of the standing fruit crop in a tropical lowland forest. Biotropica. 34(2):244–253. doi:10.1111/j.1744-7429.2002.tb00535.x.

Schaefer HM, Valido A, Jordano P. 2014. Birds see the true colours of fruits to live off the fat of the land. Proc R Soc B. 281(1777):20132516–20132516. doi:10.1098/rspb.2013.2516.

Scheuerlein A, Ricklefs RE. 2004. Prevalence of blood parasites in European passeriform birds. Proc Royal Soc B. 271(1546):1363–1370. doi:10.1098/rspb.2004.2726.

Schlegel B, Steenbergen M. 2020. brant: Test for parallel regression assumption. R Package version 0.3-0. https://CRAN.R-project.org/package=brant.

Schmid-Hempel P. 2021. Evolutionary parasitology: The integrated study of infections, immunology, ecology, and genetics. 2nd ed. Oxford, United Kingdom: Oxford university press.

Sears BF, Rohr JR, Allen JE, Martin LB. 2011. The economy of inflammation: When is less more? Trends Parasitol. 27(9):382–387. doi:10.1016/j.pt.2011.05.004.

Seress G, Sándor K, Evans KL, Liker A. 2020. Food availability limits avian reproduction in the city: An experimental study on great tits *Parus major*. J Anim Ecol. 89(7):1570–1580. doi:10.1111/1365-2656.13211.

Service MW. 1971. The daytime distribution of mosquitoes resting in vegetation. J Med Entomol. 8(3):271–278. doi:10.1093/jmedent/8.3.271.

Sheldon BC, Verhulst S. 1996. Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol Evol. 11(8):317–321. doi:10.1016/0169-5347(96)10039-2.

Skrip MM, Bauchinger U, Goymann W, Fusani L, Cardinale M, Alan RR, McWilliams SR. 2015. Migrating songbirds on stopover prepare for, and recover from, oxidative challenges posed by long-distance flight. Ecol Evol. 5(15):3198–3209. doi:10.1002/ece3.1601.

Snow WE. 1955. Feeding activities of some blood-sucking Diptera with reference to vertical distribution in bottomland forest. Ann Entomol Soc Am. 48(6):512–521. doi:10.1093/aesa/48.6.512.

Soares L, Latta SC, Ricklefs RE. 2020. Neotropical migratory and resident birds occurring in sympatry during winter have distinct haemosporidian parasite assemblages. J Biogeogr. 47(3):748–759. doi:10.1111/jbi.13760.

Sorci G. 2013. Immunity, resistance and tolerance in bird-parasite interactions. Parasite Immunol. 35(11):350–361. doi:10.1111/pim.12047.

Sorci G, Faivre B. 2009. Inflammation and oxidative stress in vertebrate host–parasite systems. Phil Trans R Soc B. 364(1513):71–83. doi:10.1098/rstb.2008.0151.

Stewart Merrill TE, Johnson PTJ. 2020. Towards a mechanistic understanding of competence: a missing link in diversity–disease research. Parasitol. 147(11):1159–1170. doi:10.1017/S0031182020000943.

Svensson L. 1992. Identification guide to European passerines. 4th ed. Stockholm: British Trust for Ornithology.

Symonds MRE, Moussalli A. 2011. A brief guide to model selection, multimodel inference and model averaging in behavioural ecology using Akaike's information criterion. Behav Ecol Sociobiol. 65(1):13–21. doi:10.1007/s00265-010-1037-6.

Tella JL. 2002. The evolutionary transition to coloniality promotes higher blood parasitism in birds. J Evol Biol. 15(1):32–41. doi:10.1046/j.1420-9101.2002.00375.x.

Tellería JL, Ramirez Á, Pérez-Tris J. 2008. Fruit tracking between sites and years by birds in Mediterranean wintering grounds. Ecography. 31(3):381–388. doi:10.1111/j.0906-7590.2008.05283.x.

Thiollay J-M. 1994. Structure, density and rarity in an Amazonian rainforest bird community. J Trop Ecol. 10(4):449–481. doi:10.1017/S0266467400008154.

Tieleman BI, Williams JB, Ricklefs RE, Klasing KC. 2005. Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. Proc R Soc B. 272(1573):1715–1720. doi:10.1098/rspb.2005.3155.

Tobias JA, Pigot AL. 2019. Integrating behaviour and ecology into global biodiversity conservation strategies. Phil Trans R Soc B. 374(1781):20190012. doi:10.1098/rstb.2019.0012.

Tobias JA, Sheard C, Pigot AL, Devenish AJM, Yang J, Sayol F, Neate-Clegg MHC, Alioravainen N, Weeks TL, Barber RA, et al. 2022. AVONET: morphological, ecological and geographical data for all birds. Ecol Lett. 25(3):581–597. doi:10.1111/ele.13898.

Tomás G, Merino S, Moreno J, Morales J, Martínez-de la Puente J. 2007. Impact of blood parasites on immunoglobulin level and parental effort: A medication field experiment on a wild passerine. Funct Ecol. 21(1):125–133. doi:10.1111/j.1365-2435.2006.01214.x.

Truchado DA, Remacha C, Jiménez-Gallardo L, Milá B, Moens MAJ, Benítez L, Pérez-Tris J. 2020. Identifying divergent host roles for avian malaria in a community of Neotropical understory passerine birds. In: Diversity of pathogens in Neotropical wild birds: discovery strategies and identification of host species roles. PhD Thesis. Madrid: Universidad Complutense de Madrid.

Tschirren B, Richner H. 2006. Parasites shape the optimal investment in immunity. Proc Biol Sci B. 273(1595):1773–1777. doi:10.1098/rspb.2006.3524.

Valkiūnas G. 2005. Avian malaria parasites and other Haemosporidia. CRC Press.

Valkiūnas G, Bairlein F, Iezhova TA, Dolnik OV. 2004. Factors affecting the relapse of *Haemoproteus belopolskyi* infections and the parasitaemia of *Trypanosoma* spp. In a naturally infected European songbird, the blackcap, *Sylvia atricapilla*. Parasitol Res. 93(3): 218–222. doi: 10.1007/s00436-004-1071-2.

Valkiūnas G, Bensch S, Iezhova TA, Križanauskienė A, Hellgren O, Bolshakov CV. 2006. Nested Cytochrome *B* polymerase chain reaction diagnostics underestimate mixed infections of avian blood haemosporidian parasites: Microscopy is still essential. J Parasitol. 92(2):418–422. doi:10.1645/GE-3547RN.1.

Van de Crommenacker J, Richardson DS, Koltz AM, Hutchings K, Komdeur J. 2012. Parasitic infection and oxidative status are associated and vary with breeding activity in the Seychelles warbler. Proc R Soc B. 279(1733):1466–1476. doi:10.1098/rspb.2011.1865.

Venables WN, Ripley BD. 2002. Modern applied statistics with S. 4th ed. New York: Springer (Statistics and computing).

Videvall E. 2019. Genomic advances in avian malaria research. Trends Parasitol. 35(3):254–266. doi:10.1016/j.pt.2018.12.005.

Vincze O, Vágási CI, Pénzes J, Szabó K, Magonyi NM, Czirják GÁ, Pap PL. 2022. Sexual dimorphism in immune function and oxidative physiology across birds: The role of sexual selection. Gaillard J, editor. Ecol Lett. 25(4):958–970. doi:10.1111/ele.13973.

Visioli F, Poli A, Gall C. 2002. Antioxidant and other biological activities of phenols from olives and olive oil. Med Res Rev. 22(1):65–75. doi:10.1002/med.1028.

Weber J-M. 2009. The physiology of long-distance migration: Extending the limits of endurance metabolism. J Exp Biol. 212(5):593–597. doi:10.1242/jeb.015024.

Wiersma P, Muñoz-Garcia A, Walker A, Williams JB. 2007. Tropical birds have a slow pace of life. PNAS. 104(22):9340–9345. doi:10.1073/pnas.0702212104.

Williams TD, Warnock N, Takekawa JY, Bishop MA. 2007. Flyway-scale variation in plasma triglyceride levels as an index of refueling rate in spring-migrating western sandpipers (*Calidris mauri*). Auk. 124(3):886–897.

Wilman H, Belmaker J, Simpson J, de la Rosa C, Rivadeneira MM, Jetz W. 2014. EltonTraits 1.0: Species-level foraging attributes of the world's birds and mammals. Ecology. 95(7):2027. doi:10.1890/13-1917.1.

Wingfield JC, Ball GF, Dufty AM, Hegner RE, Ramenofsky M. 1987. Testosterone and aggression in birds. Am Sci. 75(6):602–608.

Yan J, Gangoso L, Martínez-de la Puente J, Soriguer R, Figuerola J. 2017. Avian phenotypic traits related to feeding preferences in two *Culex* mosquitoes. Sci Nat. 104:76. doi:10.1007/s00114-017-1497-x.

Yan J, Gangoso L, Ruiz S, Soriguer R, Figuerola J, Martínez-de la Puente J. 2021. Understanding host utilization by mosquitoes: determinants, challenges and future directions. Biological Reviews. 96(4):1367–1385. doi:10.1111/brv.12706.

Yan J, Martínez-de la Puente J, Gangoso L, Gutiérrez-López R, Soriguer R, Figuerola J. 2018. Avian malaria infection intensity influences mosquito feeding patterns. Int J Parasitol. 48(3–4):257–264. doi:10.1016/j.ijpara.2017.09.005.

Zarrouk A, Martine L, Grégoire S, Nury T, Meddeb W, Camus E, Badreddine A, Durand P, Namsi A, Yammine A, et al. 2019. Profile of fatty acids, tocopherols, phytosterols and polyphenols in mediterranean oils (argan oils, olive oils, milk thistle seed oils and nigella seed oil) and evaluation of their antioxidant and cytoprotective activities. Curr Pharm Des. 25(15):1791–1805. doi:10.2174/1381612825666190705192902.

Zuzarte-Luís V, Mota MM. 2018. Parasite sensing of host nutrients and environmental cues. Cell Host & Microbe. 23(6):749–758. doi:10.1016/j.chom.2018.05.018.

Appendix

Open science statement

All data, R code and laboratory protocols necessary to reproduce the results of this thesis are available upon request. Data and code will be made publicly available in open access repositories along with the publications derived from the corresponding thesis chapters.

Statistical packages

For all statistical analysis we used R version 4.2.2 (R Core Team 2022a) and the following R packages extracted from grateful package version 0.2.4 (Rodriguez-Sanchez, 2023) report: ape v. 5.7.1 (Paradis and Schliep 2019), betareg v. 3.1.4 (Cribari-Neto and Zeileis 2010; Grün, Kosmidis, and Zeileis 2012), blmeco v. 1.4 (Korner-Nievergelt et al. 2015), brant v. 0.3.0 (Schlegel and Steenbergen 2020), brms v. 2.18.0 (Bürkner 2017, 2018, 2021), caper v. 1.0.2 (Orme et al. 2023), car v. 3.1.1 (Fox and Weisberg 2019a), coda v. 0.19.4 (Plummer et al. 2006), corrplot v. 0.92 (Wei and Simko 2021), DHARMa v. 0.4.6 (Hartig 2022), DirichletReg v. 0.7.1 (Maier 2014, 2021), DT v. 0.26 (Xie, Cheng, and Tan 2022), effects v. 4.2.2 (Fox 2003; Fox and Hong 2009; Fox and Weisberg 2018, 2019b), emmeans v. 1.8.3 (Lenth 2022), geiger v. 2.0.11 (Harmon et al. 2008; Alfaro et al. 2009; Eastman et al. 2011; Slater et al. 2012; Pennell et al. 2014), ggdist v. 3.2.0 (Kay 2022), gghalves v. 0.1.4 (Tiedemann 2022), ggstance v. 0.3.6 (Henry, Wickham, and Chang 2022), ggtern v. 3.4.1 (Hamilton and Ferry 2018), glmmTMB v. 1.1.5 (Brooks et al. 2017), glue v. 1.6.2 (Hester and Bryan 2022), GPArotation v. 2022.10.2 (Bernaards and I.Jennrich 2005), gridExtra v. 2.3 (Auguie 2017), gtools v. 3.9.4 (Bolker, Warnes, and Lumley 2022), here v. 1.0.1 (Müller 2020), huxtable v. 5.5.2 (Hugh-Jones 2022), jtools v. 2.2.1 (Long 2022), kableExtra v. 1.3.4 (Zhu 2021), knitr v. 1.41 (Xie 2014, 2015,

2022), lattice v. 0.20.45 (Sarkar 2008), lawstat v. 3.5 (Gastwirth et al. 2022), lme4 v. 1.1.31 (Bates et al. 2015), LMERConvenienceFunctions v. 3.0 (Tremblay et al. 2020), lmerTest v. 3.1.3 (Kuznetsova, Brockhoff, and Christensen 2017), lmtest v. 0.9.40 (Zeileis and Hothorn 2002), MASS v. 7.3.58.1 (Venables and Ripley 2002), mblm v. 0.12.1 (Komsta 2019), MCMCglmm v. 2.35 (Hadfield 2010), moments v. 0.14.1 (Komsta and Novomestky 2022), multcomp v. 1.4.20 (Hothorn, Bretz, and Westfall 2008), MuMIn v. 1.47.1 (Bartoń 2022), nFactors v. 2.4.1.1 (Raiche and Magis 2022), nlme v. 3.1.161 (J. C. Pinheiro and Bates 2000; J. Pinheiro, Bates, and R Core Team 2022), officer v. 0.5.0 (Gohel 2022a), ordinal v. 2022.11.16 (Christensen 2022), parallel v. 4.2.2 (R Core Team 2022b), pbkrtest v. 0.5.1 (Halekoh and Højsgaard 2014), PerformanceAnalytics v. 2.0.4 (Peterson and Carl 2020), phylolm v. 2.6.2 (Ho and Ane 2014), phyr v. 1.1.0 (Ives et al. 2020), plot3D v. 1.4 (Soetaert 2021), psych v. 2.2.9 (Revelle 2022), reshape v. 0.8.9 (Wickham 2007a), reshape2 v. 1.4.4 (Wickham 2007b), Rfit v. 0.24.2 (Kloke and McKean 2012), rgl v. 0.110.2 (Murdoch and Adler 2022), rmarkdown v. 2.19 (Xie, Allaire, and Grolemund 2018; Xie, Dervieux, and Riederer 2020; Allaire et al. 2022), ROCR v. 1.0.11 (Sing et al. 2005), rstan v. 2.21.7 (Stan Development Team 2022), rstatix v. 0.7.2 (Kassambara 2023), RVAideMemoire v. 0.9.81.2 (Hervé 2022), rvg v. 0.3.1 (Gohel 2022b), sandwich v. 3.0.2 (Zeileis 2004, 2006; Zeileis, Köll, and Graham 2020), sjPlot v. 2.8.12 (Lüdecke 2022), tidyverse v. 1.3.2 (Wickham et al. 2019), viridis v. 0.6.2 (Garnier et al. 2021), visreg v. 2.7.0 (Breheny and Burchett 2017), writexl v. 1.4.2 (Ooms 2023).

Package	**Version**	**Citation**
ape	5.7.1	Paradis and Schliep (2019)
base	4.2.2	R Core Team (2022a)

Package	Version	Citation
betareg	3.1.4	Cribari-Neto and Zeileis (2010); Grün, Kosmidis, and Zeileis (2012)
blmeco	1.4	Korner-Nievergelt et al. (2015)
brant	0.3.0	Schlegel and Steenbergen (2020)
brms	2.18.0	Bürkner (2017); Bürkner (2018); Bürkner (2021)
caper	1.0.2	Orme et al. (2023)
car	3.1.1	Fox and Weisberg (2019a)
coda	0.19.4	Plummer et al. (2006)
corrplot	0.92	Wei and Simko (2021)
DHARMa	0.4.6	Hartig (2022)
DirichletReg	0.7.1	Maier (2014); Maier (2021)
DT	0.26	Xie, Cheng, and Tan (2022)
effects	4.2.2	Fox (2003); Fox and Hong (2009); Fox and Weisberg (2018); Fox and Weisberg (2019b)
emmeans	1.8.3	Lenth (2022)
geiger	2.0.11	Harmon et al. (2008); Alfaro et al. (2009); Eastman et al. (2011); Slater et al. (2012); Pennell et al. (2014)
ggdist	3.2.0	Kay (2022)
gghalves	0.1.4	Tiedemann (2022)
ggstance	0.3.6	Henry, Wickham, and Chang (2022)
ggtern	3.4.1	Hamilton and Ferry (2018)
glmmTMB	1.1.5	Brooks et al. (2017)
glue	1.6.2	Hester and Bryan (2022)

Package	Version	Citation
GPArotation	2022.10.2	Bernaards and I.Jennrich (2005)
gridExtra	2.3	Auguie (2017)
gtools	3.9.4	Bolker, Warnes, and Lumley (2022)
here	1.0.1	Müller (2020)
huxtable	5.5.2	Hugh-Jones (2022)
jtools	2.2.1	Long (2022)
kableExtra	1.3.4	Zhu (2021)
knitr	1.41	Xie (2014); Xie (2015); Xie (2022)
lattice	0.20.45	Sarkar (2008)
lawstat	3.5	Gastwirth et al. (2022)
lme4	1.1.31	Bates et al. (2015)
LMERConvenienceFunctions	3.0	Tremblay et al. (2020)
lmerTest	3.1.3	Kuznetsova, Brockhoff, and Christensen (2017)
lmtest	0.9.40	Zeileis and Hothorn (2002)
MASS	7.3.58.1	Venables and Ripley (2002)
mblm	0.12.1	Komsta (2019)
MCMCglmm	2.35	Hadfield (2010)
moments	0.14.1	Komsta and Novomestky (2022)
multcomp	1.4.20	Hothorn, Bretz, and Westfall (2008)
MuMIn	1.47.1	Bartoń (2022)
nFactors	2.4.1.1	Raiche and Magis (2022)

Package	Version	Citation
nlme	3.1.161	J. C. Pinheiro and Bates (2000); J. Pinheiro, Bates, and R Core Team (2022)
officer	0.5.0	Gohel (2022a)
ordinal	2022.11.16	Christensen (2022)
parallel	4.2.2	R Core Team (2022b)
pbkrtest	0.5.1	Halekoh and Højsgaard (2014)
PerformanceAnalytics	2.0.4	Peterson and Carl (2020)
phylolm	2.6.2	Ho and Ane (2014)
phyr	1.1.0	Ives et al. (2020)
plot3D	1.4	Soetaert (2021)
psych	2.2.9	Revelle (2022)
reshape	0.8.9	Wickham (2007a)
reshape2	1.4.4	Wickham (2007b)
Rfit	0.24.2	Kloke and McKean (2012)
rgl	0.110.2	Murdoch and Adler (2022)
rmarkdown	2.19	Xie, Allaire, and Grolemund (2018); Xie, Dervieux, and Riederer (2020); Allaire et al. (2022)
ROCR	1.0.11	Sing et al. (2005)
rstan	2.21.7	Stan Development Team (2022)
rstatix	0.7.2	Kassambara (2023)
RVAideMemoire	0.9.81.2	Hervé (2022)
rvg	0.3.1	Gohel (2022b)

Package	Version	Citation
sandwich	3.0.2	Zeileis (2004); Zeileis (2006); Zeileis, Köll, and Graham (2020)
sjPlot	2.8.12	Lüdecke (2022)
tidyverse	1.3.2	Wickham et al. (2019)
viridis	0.6.2	Garnier et al. (2021)
visreg	2.7.0	Breheny and Burchett (2017)
writexl	1.4.2	Ooms (2023)

Package citations

Alfaro, M. E., F. Santini, C. Brock, H. Alamillo, A. Dornburg, D. L. Rabosky, G. Carnevale, and L. J. Harmon. 2009. "Nine Exceptional Radiations Plus High Turnover Explain Species Diversity in Jawed Vertebrates." *PNAS* 106: 13410–14. https://doi.org/10.1073/pnas.0811087106.

Allaire, JJ, Yihui Xie, Jonathan McPherson, Javier Luraschi, Kevin Ushey, Aron Atkins, Hadley Wickham, Joe Cheng, Winston Chang, and Richard Iannone. 2022. *rmarkdown: Dynamic Documents for r*. https://github.com/rstudio/rmarkdown.

Auguie, Baptiste. 2017. *gridExtra: Miscellaneous Functions for "Grid" Graphics*. https://CRAN.R-project.org/package=gridExtra.

Bartoń, Kamil. 2022. *MuMIn: Multi-Model Inference*. https://CRAN.R-project.org/package=MuMIn.

Bates, Douglas, Martin Mächler, Ben Bolker, and Steve Walker. 2015. "Fitting Linear Mixed-Effects Models Using lme4." *Journal of Statistical Software* 67 (1): 1–48. https://doi.org/10.18637/jss.v067.i01.

Bernaards, Coen A., and Robert I.Jennrich. 2005. "Gradient Projection Algorithms and Software for Arbitrary Rotation Criteria in Factor Analysis." *Educational and Psychological Measurement* 65: 676–96.

Bolker, Ben, Gregory R. Warnes, and Thomas Lumley. 2022. *gtools: Various r Programming Tools*. https://CRAN.R-project.org/package=gtools.

Breheny, Patrick, and Woodrow Burchett. 2017. "Visualization of Regression Models Using Visreg." *The R Journal* 9 (2): 56–71.

Brooks, Mollie E., Kasper Kristensen, Koen J. van Benthem, Arni Magnusson, Casper W. Berg, Anders Nielsen, Hans J. Skaug, Martin Maechler, and Benjamin M. Bolker. 2017. "glmmTMB Balances Speed and Flexibility Among Packages for Zero-Inflated Generalized Linear Mixed Modeling." *The R Journal* 9 (2): 378–400. https://doi.org/10.32614/RJ-2017-066.

Bürkner, Paul-Christian. 2017. "brms: An R Package for Bayesian Multilevel Models Using Stan." *Journal of Statistical Software* 80 (1): 1–28. https://doi.org/10.18637/jss.v080.i01.

———. 2018. "Advanced Bayesian Multilevel Modeling with the R Package brms." *The R Journal* 10 (1): 395–411. https://doi.org/10.32614/RJ-2018-017.

———. 2021. "Bayesian Item Response Modeling in R with brms and Stan." *Journal of Statistical Software* 100 (5): 1–54. https://doi.org/10.18637/jss.v100.i05.

Christensen, R. H. B. 2022. "Ordinal—Regression Models for Ordinal Data."

Cribari-Neto, Francisco, and Achim Zeileis. 2010. "Beta Regression in R." *Journal of Statistical Software* 34 (2): 1–24. https://doi.org/10.18637/jss.v034.i02.

Eastman, J. M., M. E Alfaro, P. Joyce, A. L. Hipp, and L. J. Harmon. 2011. "A Novel Comparative Method for Identifying Shifts in the Rate of Character Evolution on Trees." *Evolution* 65: 3578–89. https://doi.org/10.1111/j.1558-5646.2011.01401.x.

Fox, John. 2003. "Effect Displays in R for Generalised Linear Models." *Journal of Statistical Software* 8 (15): 1–27. https://doi.org/10.18637/jss.v008.i15.

Fox, John, and Jangman Hong. 2009. "Effect Displays in R for Multinomial and Proportional-Odds Logit Models: Extensions to the effects Package." *Journal of Statistical Software* 32 (1): 1–24. https://doi.org/10.18637/jss.v032.i01.

Fox, John, and Sanford Weisberg. 2018. "Visualizing Fit and Lack of Fit in Complex Regression Models with Predictor Effect Plots and Partial Residuals." *Journal of Statistical Software* 87 (9): 1–27. https://doi.org/10.18637/jss.v087.i09.

———. 2019a. *An R Companion to Applied Regression*. Third. Thousand Oaks CA: Sage. https://socialsciences.mcmaster.ca/jfox/Books/Companion/.

———. 2019b. *An r Companion to Applied Regression*. 3rd ed. Thousand Oaks CA: Sage. https://socialsciences.mcmaster.ca/jfox/Books/Companion/index.html.

Garnier, Simon, Ross, Noam, Rudis, Robert, Camargo, et al. 2021. *viridis - Colorblind-Friendly Color Maps for r*. https://doi.org/10.5281/zenodo.4679424.

Gastwirth, Joseph L., Yulia R. Gel, W. L. Wallace Hui, Vyacheslav Lyubchich, Weiwen Miao, and Kimihiro Noguchi. 2022. *lawstat: Tools for Biostatistics, Public Policy, and Law*. https://CRAN.R-project.org/package=lawstat.

Gohel, David. 2022a. *officer: Manipulation of Microsoft Word and PowerPoint Documents*. https://CRAN.R-project.org/package=officer.

———. 2022b. *rvg: R Graphics Devices for "Office" Vector Graphics Output*. https://CRAN.R-project.org/package=rvg.

Grün, Bettina, Ioannis Kosmidis, and Achim Zeileis. 2012. "Extended Beta Regression in R: Shaken, Stirred, Mixed, and Partitioned." *Journal of Statistical Software* 48 (11): 1–25. https://doi.org/10.18637/jss.v048.i11.

Hadfield, Jarrod D. 2010. "MCMC Methods for Multi-Response Generalized Linear Mixed Models: The MCMCglmm R Package." *Journal of Statistical Software* 33 (2): 1–22. https://www.jstatsoft.org/v33/i02/.

Halekoh, Ulrich, and Søren Højsgaard. 2014. "A Kenward-Roger Approximation and Parametric Bootstrap Methods for Tests in Linear Mixed Models – the R Package pbkrtest." *Journal of Statistical Software* 59 (9): 1–30. https://www.jstatsoft.org/v59/i09/.

Hamilton, Nicholas E., and Michael Ferry. 2018. "ggtern: Ternary Diagrams Using ggplot2." *Journal of Statistical Software, Code Snippets* 87 (3): 1–17. https://doi.org/10.18637/jss.v087.c03.

Harmon, L. J., J. T. Weir, C. D. Brock, R. E. Glor, and W. Challenger. 2008. "GEIGER: Investigating Evolutionary Radiations." *Bioinformatics* 24: 129–31. https://doi.org/10.1093/bioinformatics/btm538.

Hartig, Florian. 2022. *DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models*. https://CRAN.R-project.org/package=DHARMa.

Henry, Lionel, Hadley Wickham, and Winston Chang. 2022. *ggstance: Horizontal "ggplot2" Components*. https://CRAN.R-project.org/package=ggstance.

Hervé, Maxime. 2022. *RVAideMemoire: Testing and Plotting Procedures for Biostatistics*. https://CRAN.R-project.org/package=RVAideMemoire.

Hester, Jim, and Jennifer Bryan. 2022. *glue: Interpreted String Literals*. https://CRAN.R-project.org/package=glue.

Ho, Lam Si Tung, and Cecile Ane. 2014. "A Linear-Time Algorithm for Gaussian and Non-Gaussian Trait Evolution Models." *Systematic Biology* 63: 397–408.

Hothorn, Torsten, Frank Bretz, and Peter Westfall. 2008. "Simultaneous Inference in General Parametric Models." *Biometrical Journal* 50 (3): 346–63.

Hugh-Jones, David. 2022. *huxtable: Easily Create and Style Tables for LaTeX, HTML and Other Formats*. https://CRAN.R-project.org/package=huxtable.

Ives, Anthony, Russell Dinnage, Lucas A. Nell, Matthew Helmus, and Daijiang Li. 2020. *phyr: Model Based Phylogenetic Analysis*. https://CRAN.R-project.org/package=phyr.

Kassambara, Alboukadel. 2023. *rstatix: Pipe-Friendly Framework for Basic Statistical Tests*. https://CRAN.R-project.org/package=rstatix.

Kay, Matthew. 2022. *ggdist: Visualizations of Distributions and Uncertainty*. https://doi.org/10.5281/zenodo.3879620.

Kloke, John D., and Joseph W. McKean. 2012. "Rfit: Rank-Based Estimation for Linear Models," *The R Journal* 4 (2): 57–64.

Komsta, Lukasz. 2019. *mblm: Median-Based Linear Models*. https://CRAN.R-project.org/package=mblm.

Komsta, Lukasz, and Frederick Novomestky. 2022. *moments: Moments, Cumulants, Skewness, Kurtosis and Related Tests*. https://CRAN.R-project.org/package=moments.

Korner-Nievergelt, Fraenzi, Tobias Roth, Stefanie von Felten, Jerome Guelat, Bettina Almasi, and Pius Korner-Nievergelt. 2015. *Bayesian Data Analysis in Ecology Using Linear Models with r, BUGS and Stan*. Elsevier.

Kuznetsova, Alexandra, Per B. Brockhoff, and Rune H. B. Christensen. 2017. "lmerTest Package: Tests in Linear Mixed Effects Models." *Journal of Statistical Software* 82 (13): 1–26. https://doi.org/10.18637/jss.v082.i13.

Lenth, Russell V. 2022. *emmeans: Estimated Marginal Means, Aka Least-Squares Means*. https://CRAN.R-project.org/package=emmeans.

Long, Jacob A. 2022. *jtools: Analysis and Presentation of Social Scientific Data*. https://cran.r-project.org/package=jtools.

Lüdecke, Daniel. 2022. *sjPlot: Data Visualization for Statistics in Social Science*. https://CRAN.R-project.org/package=sjPlot.

Maier, Marco J. 2014. "DirichletReg: Dirichlet Regression for Compositional Data in r." {Research Report Series{\slash}Department of Statistics and Mathematics} 125. Vienna: WU Vienna University of Economics and Business. https://epub.wu.ac.at/4077/.

———. 2021. *DirichletReg: Dirichlet Regression*. https://github.com/maiermarco/DirichletReg.

Müller, Kirill. 2020. *here: A Simpler Way to Find Your Files*. https://CRAN.R-project.org/package=here.

Murdoch, Duncan, and Daniel Adler. 2022. *rgl: 3D Visualization Using OpenGL*. https://CRAN.R-project.org/package=rgl.

Ooms, Jeroen. 2023. *writexl: Export Data Frames to Excel "xlsx" Format*. https://CRAN.R-project.org/package=writexl.

Orme, David, Rob Freckleton, Gavin Thomas, Thomas Petzoldt, Susanne Fritz, Nick Isaac, and Will Pearse. 2023. *caper: Comparative Analyses of Phylogenetics and Evolution in r*. https://CRAN.R-project.org/package=caper.

Paradis, Emmanuel, and Klaus Schliep. 2019. “Ape 5.0: An Environment for Modern Phylogenetics and Evolutionary Analyses in R.” *Bioinformatics* 35: 526–28. https://doi.org/10.1093/bioinformatics/bty633.

Pennell, M. W., J. M. Eastman, G. J. Slater, J. W. Brown, J. C. Uyeda, R. G. Fitzjohn, M. E. Alfaro, and L. J. Harmon. 2014. “Geiger V2.0: An Expanded Suite of Methods for Fitting Macroevolutionary Models to Phylogenetic Trees.” *Bioinformatics* 30: 2216–18. https://doi.org/10.1093/bioinformatics/btu181.

Peterson, Brian G., and Peter Carl. 2020. *PerformanceAnalytics: Econometric Tools for Performance and Risk Analysis*. https://CRAN.R-project.org/package=PerformanceAnalytics.

Pinheiro, José C., and Douglas M. Bates. 2000. *Mixed-Effects Models in s and s-PLUS*. New York: Springer. https://doi.org/10.1007/b98882.

Pinheiro, José, Douglas Bates, and R Core Team. 2022. *nlme: Linear and Nonlinear Mixed Effects Models*. https://CRAN.R-project.org/package=nlme.

Plummer, Martyn, Nicky Best, Kate Cowles, and Karen Vines. 2006. “CODA: Convergence Diagnosis and Output Analysis for MCMC.” *R News* 6 (1): 7–11. https://journal.r-project.org/archive/.

R Core Team. 2022a. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

———. 2022b. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

Raiche, Gilles, and David Magis. 2022. *nFactors: Parallel Analysis and Other Non Graphical Solutions to the Cattell Scree Test*. https://CRAN.R-project.org/package=nFactors.

Revelle, William. 2022. *psych: Procedures for Psychological, Psychometric, and Personality Research*. Evanston, Illinois: Northwestern University. https://CRAN.R-project.org/package=psych.

Rodriguez-Sanchez, F. (2023). *Grateful: Facilitate citation of R packages*. R package version 0.2.4. https://CRAN.R-project.org/package=grateful

Sarkar, Deepayan. 2008. *Lattice: Multivariate Data Visualization with r*. New York: Springer. http://lmdvr.r-forge.r-project.org.

Schlegel, Benjamin, and Marco Steenbergen. 2020. *brant: Test for Parallel Regression Assumption*. https://CRAN.R-project.org/package=brant.

Sing, T., O. Sander, N. Beerenwinkel, and T. Lengauer. 2005. “ROCR: Visualizing Classifier Performance in r.” *Bioinformatics* 21 (20): 7881. http://rocr.bioinf.mpi-sb.mpg.de.

Slater, G. J., L. J. Harmon, D. Wegmann, P Joyce, L. J. Revell, and M. E. Alfaro. 2012. "Fitting Models of Continuous Trait Evolution to Incompletely Sampled Comparative Data Using Approximate Bayesian Computation." *Evolution* 66: 752–62. https://doi.org/10.1111/j.1558-5646.2011.01474.x.

Soetaert, Karline. 2021. *plot3D: Plotting Multi-Dimensional Data*. https://CRAN.R-project.org/package=plot3D.

Stan Development Team. 2022. "RStan: The R Interface to Stan." https://mc-stan.org/.

Tiedemann, Frederik. 2022. *gghalves: Compose Half-Half Plots Using Your Favourite Geoms*. https://CRAN.R-project.org/package=gghalves.

Tremblay, Antoine, Statistics Canada, Johannes Ransijn, and University of Copenhagen. 2020. *LMERConvenienceFunctions: Model Selection and Post-Hoc Analysis for (g)LMER Models*. https://CRAN.R-project.org/package=LMERConvenienceFunctions.

Venables, W. N., and B. D. Ripley. 2002. *Modern Applied Statistics with s*. Fourth. New York: Springer. https://www.stats.ox.ac.uk/pub/MASS4/.

Wei, Taiyun, and Viliam Simko. 2021. *R Package "corrplot": Visualization of a Correlation Matrix*. https://github.com/taiyun/corrplot.

Wickham, Hadley. 2007a. "Reshaping Data with the Reshape Package." *Journal of Statistical Software* 21 (12). https://www.jstatsoft.org/v21/i12/.

———. 2007b. "Reshaping Data with the reshape Package." *Journal of Statistical Software* 21 (12): 1–20. http://www.jstatsoft.org/v21/i12/.

Wickham, Hadley, Mara Averick, Jennifer Bryan, Winston Chang, Lucy D'Agostino McGowan, Romain François, Garrett Grolemund, et al. 2019. "Welcome to the tidyverse." *Journal of Open Source Software* 4 (43): 1686. https://doi.org/10.21105/joss.01686.

Xie, Yihui. 2014. "knitr: A Comprehensive Tool for Reproducible Research in R." In *Implementing Reproducible Computational Research*, edited by Victoria Stodden, Friedrich Leisch, and Roger D. Peng. Chapman; Hall/CRC. http://www.crcpress.com/product/isbn/9781466561595.

———. 2015. *Dynamic Documents with R and Knitr*. 2nd ed. Boca Raton, Florida: Chapman; Hall/CRC. https://yihui.org/knitr/.

———. 2022. *knitr: A General-Purpose Package for Dynamic Report Generation in r*. https://yihui.org/knitr/.

Xie, Yihui, J. J. Allaire, and Garrett Grolemund. 2018. *R Markdown: The Definitive Guide*. Boca Raton, Florida: Chapman; Hall/CRC. https://bookdown.org/yihui/rmarkdown.

Xie, Yihui, Joe Cheng, and Xianying Tan. 2022. *DT: A Wrapper of the JavaScript Library "DataTables"*. https://CRAN.R-project.org/package=DT.

Xie, Yihui, Christophe Dervieux, and Emily Riederer. 2020. *R Markdown Cookbook*. Boca Raton, Florida: Chapman; Hall/CRC. https://bookdown.org/yihui/rmarkdown-cookbook.

Zeileis, Achim. 2004. "Econometric Computing with HC and HAC Covariance Matrix Estimators." *Journal of Statistical Software* 11 (10): 1–17. https://doi.org/10.18637/jss.v011.i10.

———. 2006. "Object-Oriented Computation of Sandwich Estimators." *Journal of Statistical Software* 16 (9): 1–16. https://doi.org/10.18637/jss.v016.i09.

Zeileis, Achim, and Torsten Hothorn. 2002. "Diagnostic Checking in Regression Relationships." *R News* 2 (3): 7–10. https://CRAN.R-project.org/doc/Rnews/.

Zeileis, Achim, Susanne Köll, and Nathaniel Graham. 2020. "Various Versatile Variances: An Object-Oriented Implementation of Clustered Covariances in R." *Journal of Statistical Software* 95 (1): 1–36. https://doi.org/10.18637/jss.v095.i01.

Zhu, Hao. 2021. *kableExtra: Construct Complex Table with "kable" and Pipe Syntax*. https://CRAN.R-project.org/package=kableExtra.

"All outstanding work, in art as well as in science, results from immense zeal applied to a great idea"

Santiago Ramón y Cajal