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 highly demanding life stages. For instance, migrating birds need to fuel 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 blood parasites during migration. During autumn migration, we mist-netted young male European 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 by haemosporidian parasites with PCR techniques and their parasitaemia with 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). Infected birds had a lower antioxidant capacity of plasma, and individuals with more oxidative damage preferred anthocyanin-enriched food. Our results suggest that parasite infections may increase individuals' antioxidant needs, which could affect migration performance if the urge to find dietary antioxidants reduces fuel consumption rate.

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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). Infected birds had a lower antioxidant capacity of plasma, and individuals with more oxidative damage preferred anthocyanin-enriched food. Our results suggest that parasite infections may increase individuals' antioxidant needs, which could affect migration performance if the urge to find dietary antioxidants reduces fuel consumption rate.

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

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 depending on nutritional requirements (Catoni et al. 2011). Both dietary needs and the availability of food sources necessary 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-Alvarez 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 the physiology of the host could be enhanced when they are experiencing a demanding situation, such as migration (Jenni-Eiermann 2017). One important link between parasite infection and host feeding behaviour may be oxidative stress (Isaksson et al. 2013; Delhaye et al. 2016; Muriel 2020). Oxidative stress results from the imbalance between endogenous or exogenous antioxidants, which protect animals against oxidative damage to DNA, proteins, or lipids, and reactive oxygen species (ROS) that cause such damage (Finkel and Holbrook 2000; Monaghan et al. 2009). When animals can perceive the nutritional content of food and select the most appropriate food choices for their oxidative state (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 of 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 otherwise would help to maintain oxidative balance (Monaghan et al. 2009). Therefore, favouring dietary antioxidants may help parasitised individuals maintain oxidative status by reinforcing antioxidant defences, but 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, such as growth (Hall et al. 2010), reproduction (Guindre-Parker and Rubenstein 2018), or migration (McWilliams et al. 2021), which may exacerbate the influence of parasites on animal behaviour and performance (Chrétien et al. 2023).

The impact of haemosporidan parasites on bird oxidative status may be particularly strong during migration periods. Bird migration involves intense aerobic exercise, which 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 stores are prone to oxidation, and endurance flight effort might increase ROS production that needs to be compensated with up-regulation of endogenous antioxidant defences and/or increasing antioxidant-rich food intake during stopovers (Skrip et al. 2015; Eikenaar et al. 2020; McWilliams et al. 2021). Therefore, parasite infections are expected to alter migrating bird feeding decisions, tipping the balance to favour dietary antioxidants although fattening is a priority (Mancio-Silva et al. 2017; Zuzarte-Luís and Mota 2018).

We conducted a behavioural assay with European 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 combine high antioxidant potency and high availability in bird-consumed fruits may be particularly important to tackle with parasite-induced oxidative stress, and flavonoids such as anthocyanins outstand on both regards (Catoni, Peters, et al. 2008). 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; Garcia-Longoria et al. 2022). Therefore, we predict that the more parasites blackcaps harbour, the greater will be their preference for anthocyanin-enriched food.

Materials and Methods

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 tape-lured blackcaps into mist nets to maximize capture rate. Birds 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). During the mornings, between 9:00 and 15:30 p.m., the birds were kept in individual cotton bags for 30 minutes to reduce stress and increase motivation to eat after mild fasting. After that, we weighed birds to the nearest 0.01 g and placed them in individual cages (cloth mesh, 25 x 25 x 25 cm). We recorded behaviour using a video camera 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 melon (about 2 cm^3) as the substrate for both food choices, thereby avoiding variation in size, shape or other fruit attributes known to influence fruit choice by birds (González-Varo et al. 2022). Each piece of melon had been soaked overnight in extra virgin olive oil (Coosur(r)) or cranberry juice (Granini(r)), 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; 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 (Supporting information).

Migrating blackcaps arrive at stopover sites lean, start to gain mass typically on day 2 post-arrival, and put on fat until reaching maximum body mass at stopover 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 0.01mm precision. At the end of the manipulation of the birds, we took a blood sample ($< 1\%$ body mass) from the jugular vein, used a drop to make a blood smear, and kept the rest refrigerated in heparinised tubes during fieldwork. Birds were fitted with a standard aluminium ring to avoid repetitions and were released unharmed at the capture site.

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 (time end = 1390 s, set by the shortest recording). Because we minimised the time birds spent captive for ethical reasons, not all individuals ate during the test, or pecked the fruit one or a few times. 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. All videos were blindly analysed to the infection status, body mass, or physiological condition of the individuals.

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 479 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. We quantified the intensity of the infection as infected cells in 5000 RBC. Individuals who were PCR positive but had no visible parasites were assigned an intensity of infection of 0 infected cells in 5000 RBC. We used any observation of the presence of more than one parasite lineage in blood (mixed DNA sequence signal, multigenus amplification in multiplex PCR, or detection of different parasites by molecular and microscopy methods) as evidence of multiparasite infection, hereafter multiple infection (Pérez-Tris and Bensch 2005; Valkiūnas et al. 2006; Ciloglu et al. 2019). Otherwise, infected individuals were considered to harbour single-parasite infections.

Physiological Analyses

To assess whether parasites could be causing anaemia, on day of capture we measured haemoglobin concen-

tration 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 Pérez-Rodríguez et al. (2015). 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-Alvarez (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 = 39.46% and intraassay CV = 15.41%) and RBC-MDA ($r_i = 0.76$, $n = 48$, $p < 0.001$; inter-assay CV = 18.30% 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 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%.

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). When outliers were observed, their influence was assessed by repeating the analyses with or without outliers. 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 examine the effects of physiological condition as predictors of food choice.

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). 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 MuMIn package (Barton 2023 Mar 15). 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 ΔAICc [?] 2 to compute the significance of effects (Symonds and Moussalli 2011).

Results

Of 48 birds tested, 28 had single infections, 14 had multiple infections, and 6 were not infected with haemosporidian parasites. Detailed information on the prevalence of each parasite lineage found in this sample is available in Supporting 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.

Food preference and infection

We found significant effects of year, residual mass, and multiple infection on food preference (Table 1). Birds that harboured multiple infections preferred anthocyanin-enriched food (z = 2.75;p_{Tukey} = 0.02), while individuals infected by one parasite and non-infected birds showed no detectable preference (allp_{Tukey} values $>$ 0.31, Fig. 1). Fat preference was higher in 2020 and increase 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).

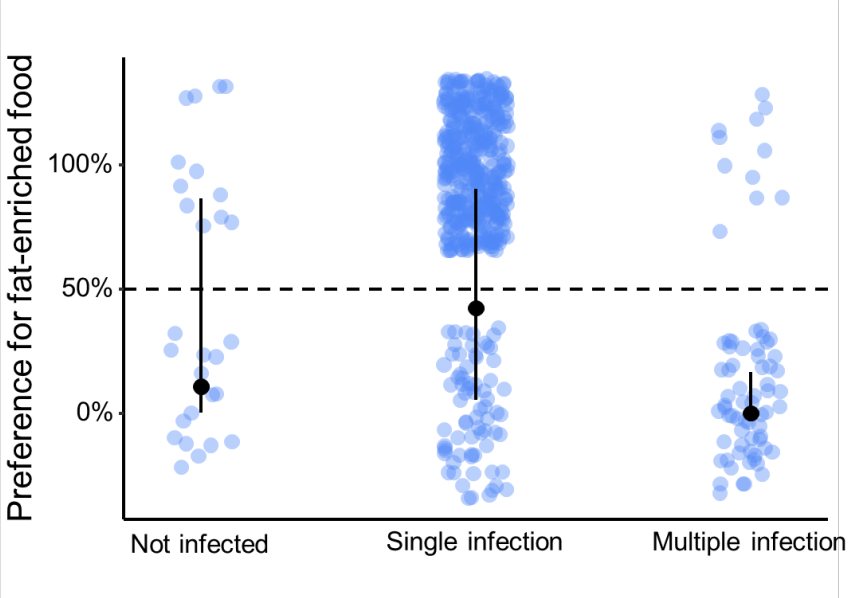

Figure 1. Variation in preference for fat-enriched food (as opposed to preference for anthocyanin-enriched food) between blackcaps with different levels of multiple status of infection by haemosporidian parasites. Preference estimates with 95% confidence intervals represent the probability (expressed as a percentage) that feeding-oriented behaviours are directed to fat-enriched food, controlling for residual body mass and year. The horizontal dashed line set at 50% indicates the absence of preference for either food choice. Dots are observations of single feeding-oriented behaviours, which therefore take values of 0% if directed to anthocyanin-enriched food and 100% if directed to fat-enriched food, jittered to reduce overlapping. The identity of individuals expressing these behaviours has been controlled as a random factor in the model.

Table 1. GLMMs that analyze variation in food preference as a function of ecological condition (residual 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.

	Multiple infection status	**Multiple infection status**	**Multiple infection status**	**Inte**
	Estimate ± se	*LRT* **(df)**	p	**Esti**
Parasite infection:		**8.02 (2)**	**0.02**	-0.70
Not infected – single	-1.79 ± 2.31			
Not infected – multiple	4.33 ± 2.96			
Single – multiple	6.12 ± 2.22			
Residual body mass	**1.92 ± 1.03**	**4.16 (1)**	**0.04**	**2.08**
Year (2019)	**-2.82 ± 0.99**	**8.64 (1)**	**0.003**	**-3.1**

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 the infected single and multiple birds had very similar TAS values, which together were significantly lower than the non-infected bird values (difference between infected and non-infected individuals:$estimate \pm SE = 0.27 \pm 0.12$; $F_{1,38} = 5.28$;$p = 0.03$). 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 (Supporting information).

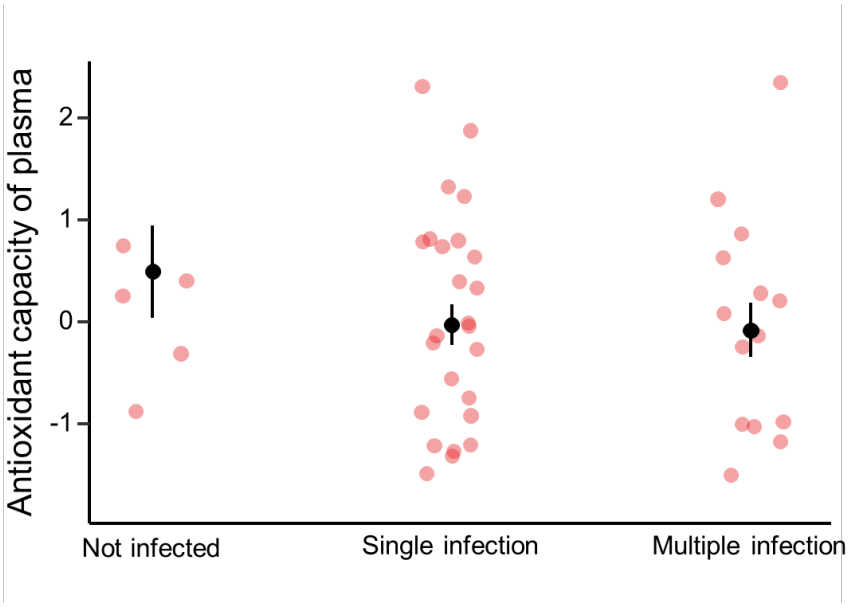

Figure 2. Variation in plasma antioxidant capacity (z-standardised TAS values) between blackcaps with different levels of multiple status of infection by haemosporidian parasites. Estimates and 95% confidence intervals were computed from a model controlling for uric acid content, residual body mass, year and plasma MDA. The dots are individual estimates, jittered horizontally to reduce overlapping.

Food preference and physiology

We obtained six models with ΔAIC [?] 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, 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	210,671	0,000	0,067	6
2. plasma MDA (log) + uric acid + year	211,118	0,447	0,054	5
3.RBC stress + plasma MDA (log) + uric acid + TAS + year	211,916	1,245	0,036	7
4.RBC stress + plasma MDA (log) + year	212,008	1,337	0,034	5
5. RBC hormesis + RBC stress + plasma MDA (log) + uric acid + year	212,392	1,721	0,028	7
6.RBC stress + plasma MDA (log) + TAS + year	212,584	1,913	0,026	6
95. Null model	217,546	6,875	0,002	2

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 helped 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 had an impact on host physiology by exacerbating oxidative challenges, which influenced on individual food preferences supporting the idea that increasing the intake of dietary antioxidants may be a self-medication mechanism to cope with elevated risk of oxidative stress due to infection (Mancio-Silva et al. 2017; Zuzarte-Luís and Mota 2018). This interpretation was supported by our observation of infected birds having lower total antioxidant capacity, and individuals with higher levels of oxidative damage to lipids having more preference for anthocyanin-enriched food.

Co-infected blackcaps preferred anthocyanin-enriched food. Consumption of antioxidants such as anthocyanins could be a means of self-medicating 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). 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).

We found a 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). Similar results of infections have been found in other stressful situations such as during reproduction (Badás et al. 2015), or in poor 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. This result indicates that blackcaps during autumn migration have mostly chronic infections with few parasites in the bloodstream, 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 of tissue repair during which birds do not put on any fat. 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). Our results support the idea that haemosporidian infections 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).

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