



Oxidative status in relation to blood parasite infections in house sparrows living along an urbanization gradient[☆]

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ABSTRACT

Living organisms are exposed to a wide range of substances - internal and external - which act like reactive oxygen species (ROS). Oxidative damage occurs when the balance between ROS and antioxidant defenses is altered. Urbanization and parasite infection are both important sources of ROS with different harmful effects on wildlife health, but the potential synergies between both factors are poorly known. Here, we analyse the oxidative stress of wild juvenile male house sparrows (*Passer domesticus*) along an urbanization gradient in relation to the infection status by three common blood parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) and bird body condition. We analysed samples from 688 birds captured at 45 localities from southern Spain grouped into triplets including an urban, a rural and a natural habitat, with 15 localities per habitat type. We measured i) thiobarbituric acid reactive substances (TBARS) levels as indicator of the oxidative damage to lipids, and the activity of three antioxidant enzymes ii) glutathione peroxidase (GPx), iii) superoxide dismutase (SOD) and iv) glutathione reductase (GR) as indicators of bird's antioxidant capacity. Birds infected with *Haemoproteus* and urban birds showed significantly and marginally higher levels of TBARS than uninfected and rural birds, respectively. The relationship between TBARS and body condition is different regarding the infection status (significant) and habitat (marginally significant) being negative for *Haemoproteus* infected and urban birds but positive for uninfected and non-urban birds. The antioxidant activity was significantly lower in *Plasmodium* infected birds but marginally higher in *Leucocytozoon* infected birds than in uninfected ones. Individuals with higher body condition had higher GPx and SOD activity in relation to a lower GR activity. Overall, these results suggest that blood parasites infections and urbanization affect the oxidative status of wild birds and highlight the role of bird's body condition on the regulation of the oxidative stress status.

1. Introduction

Reactive oxygen species (ROS) comprehend a series of chemical substances of different origin that can cause oxidative damage to biomolecules such as proteins, lipids and DNA (Li et al., 2016). Organisms have evolved antioxidant systems, based on both endogenous enzyme systems and external non-enzymatic compounds (i.e., vitamins from dietary sources), that prevent, reduce and repair ROS-induced damage

(Costantini and Verhulst, 2009; Li et al., 2016; Sorci and Faivre, 2009). When the balance between antioxidants and pro-oxidants is altered, either due to an increase in ROS or to a decrease of antioxidant activity, oxidative damage to biomolecules may occur, negatively affecting organisms health (Costantini and Verhulst, 2009; Li et al., 2016) (see Table 3).

Along with other substances, ROS are produced internally as by-products of basal metabolism and non-specific physiological responses

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(Costantini, 2019; Isaksson, 2015; Li et al., 2016; Sorci and Faivre, 2009). For example, in the course of an infection, organisms develop inflammatory responses during which some cells of the immune system produce cytotoxic compounds that act like ROS to attack the pathogen but that can also harm the host (Sorci et al., 2013; Sorci and Faivre, 2009). Inflammatory responses can be costly, but individuals unable to produce them are more likely to suffer an increased infection-induced mortality (Sorci and Faivre, 2009). Among others, avian malaria parasites of the genus *Plasmodium* and the related malaria-like genera *Haemoproteus* and *Leucocytozoon* may have harmful effects on the physiological condition and on the life-history traits of birds (Atkinson et al., 1991; Valkiūnas, 2005). Both correlative and experimental studies have confirmed that these parasites are an important source of physiological stress (del Cerro et al., 2010; Martínez-de la Puente et al., 2011; Videvall et al., 2015) with negative impacts on health (Marzal et al., 2008; Merino et al., 2000), survival (Martínez-de la Puente et al., 2010), reproductive success (Cuevas et al., 2021; Marzal et al., 2005; Merino et al., 2000) or hormone modulation (Names et al., 2021).

Environmental and intrinsic factors of the individual could determine its susceptibility to infection, its capacity to modulate physiological responses to fight off infections and the potential costs of these responses (Delhayé et al., 2018). Landscape urbanization has created new habitats where only few species are able to survive (Mckinney, 2002; Sol et al., 2014), often experiencing different detrimental effects on their body size and condition (Jiménez-Peñuela et al., 2019; Liker et al., 2008), reproductive success and survival (Chamberlain et al., 2009; Sepp et al., 2018; Sumasgutner et al., 2014) and telomere attrition rates (Ibáñez-Álamo et al., 2018; Salmón et al., 2017), among others. Additionally, different substances derived from human activities, i.e., burning of fossil fuels, generate toxic chemicals, heavy metal particles or pollutants like NO_x, SO₂ and O₃ that could act as ROS, increasing the oxidative stress in birds living in urban habitats (Isaksson, 2015, Koivula and Eeva, 2010).

Oxidative stress is a concept that cannot be quantified itself but that can be estimated measuring different biomarkers including non-enzymatic antioxidants (i.e. vitamins, carotenoids or glutathione), enzymatic antioxidants (i.e. superoxide dismutase – SOD –, catalase – CAT –, glutathione peroxidase – GPx –, glutathione reductase – GR –), substances derived from oxidative damage to biomolecules (i.e., TBARS or MDA for lipid peroxidation; DNA TD or 8-OHdG for DNA damage) or oxidant substances itself (i.e., reactive oxygen metabolites – ROM –). The key is to quantify both the availability of antioxidants and the presence of pro-oxidants or the oxidative damage level to a biomolecule (see Skrip and McWilliams (2016) for further details). Yet, up to date, the results obtained in the different studies regarding the relationship between oxidative stress and urbanization and/or blood parasites infection are contradictory. For example, some studies did not find any effect of infection by avian malaria (understood as the infection by *Plasmodium*, *Haemoproteus* or *Leucocytozoon*) on ROM concentration (Schoenle et al., 2017) or enzymatic antioxidant activity (Razavi et al., 2016). However, other studies found a change in ROM levels (Delhayé et al., 2016; van de Crommenacker et al., 2012) as well as in enzymatic antioxidant activity (De Angeli Dutra et al., 2017; van de Crommenacker et al., 2012) associated to malaria infection. Similarly, Giraudeau et al. (2014) did not find any effect of urbanization in lipid peroxidation of house finches (*Haemorrhous mexicanus*) while Herrera-Dueñas et al. (2017) and Amri et al. (2017) found higher oxidative damage in urban than in non-urban sparrows. Additionally, the activity of antioxidant enzymes varied, being higher, lower or similar in urban and rural birds (Amri et al., 2017; Giraudeau and McGraw, 2014; Herrera-Dueñas et al., 2017; Li et al., 2021; Salmón et al., 2018).

Here, we tested the impact of urbanization, parasite infections and their interaction on the oxidative status of wild birds by assessing the oxidative damage to lipids and the enzymatic antioxidant activity in blood. Given that we previously found that the body condition of birds is affected by both urbanization and malaria infection (Jiménez-Peñuela

et al., 2019), we consider the body condition of individuals as well as its interaction with habitat type and infection status as it could affect bird's physiological functions such as immune responses (Butler et al., 2021; Navarro et al., 2003) or antioxidant capacity (Beaulieu et al., 2011). Birds infected and uninfected by *Plasmodium*, *Haemoproteus* and *Leucocytozoon* sampled in urban, rural, and natural habitats were included. We used the house sparrow (*Passer domesticus*) as study model as this is a widespread, abundant and sedentary urban exploiter species that has lived in contact with humans for centuries frequently feeding on anthropogenic food resources (Anderson, 2006). This species is currently undergoing an important population decline, especially in urban areas likely due to factors such as nutritional constraints, large-scale intensive farming and agriculture, pollution, pesticides and infectious diseases such as avian malaria, among others (Bichet et al., 2013; Dadam et al., 2019; Herrera-Dueñas et al., 2017, 2014; Meillère et al., 2017; Meyrier et al., 2017).

Due to the deleterious effects of both urbanization and parasite infections, we hypothesized that: 1) infected birds and/or birds from urban habitats (especially urban infected birds) would suffer greater oxidative damage than uninfected and/or birds from non-urban habitats; 2) birds with lower body condition would experience higher levels of oxidative damage, especially if they are infected or living in altered environments; 3) infected birds would show higher antioxidant activity to counteract immune and inflammatory responses against parasites, this would be particularly evident in those birds living in less disturbed environments (i.e., rural or natural habitats) and with a higher body condition, as they may benefit from better resources that allow them to improve their physiological response; 4) birds in urban habitats, especially those in low body condition, would show lower antioxidant enzyme activity than birds in less altered environments due to greater exposure to stressors and lower quality resources availability.

2. Material and methods

2.1. Study area and sampling design

Birds were sampled in the province of Huelva, Seville and Cadiz, southern Spain, at 45 localities grouped into 15 geographically close triplets. Each triplet consisted of one urban (defined as a more densely populated area), one rural (characterized by high density of livestock) and one natural habitat (better preserved areas where wildlife predominated over both livestock and human activity), reflecting a gradient of urbanization from high to low altered environments. The categorization of the localities was based on a visual inspection of the habitat (for further information on the sampling design, see Ferraguti et al., 2016). The average distance within localities of the same triplet was 5875.19 m (±S.D. 4584.28 m).

2.2. Bird sampling

From July to October 2013 sparrows were captured using mist-nets and bird call playbacks. Birds were ringed with a numbered metal ring, sexed and aged when possible according to their plumage characteristics and skull ossification (Svensson, 2009). Birds were classified as juveniles (i.e., those born in that calendar year; EURING code 3), adults (i.e., those born before that calendar year; EURING code 4) or of unknown age (i.e., birds for which it was not possible to differentiate between these two categories; EURING code 2). Here we only analysed samples from juvenile birds as they had been exposed to environmental conditions for a short period of time and only could have been infected during the recent nestling or post-fledgling period (Cosgrove et al., 2008). In addition, due to the potential sex differences in the responses to oxidative damage and antioxidant production (Costantini, 2010), only male birds were included. Bird body mass and wing length were measured on an electric balance (digital scale to the nearest 0.1 g) and with a metal ruler (to the nearest 0.5 mm), respectively. Both variables

were used to estimate individual body condition according to Peig and Green (2009). A blood sample was taken from the jugular vein with a sterile syringe (never exceeding 1% of the birds' body mass). The birds were released at the place of capture without any apparent damage. Blood samples were transferred to Eppendorf tubes and stored in cold boxes during the fieldwork and kept at 4 °C until centrifugation the following morning for 10 min at 1700g (4000 rpm) to separate serum and cellular fractions, later frozen at -20 °C until further analyses.

2.3. Molecular analyses

Genomic DNA was extracted from the blood cellular fraction using the Maxwell®16 LEV System Research (Promega, Madison, WI). The sex of individuals that could not be clarified in the field using plumage characteristics was molecularly determined following Griffiths et al. (1998, 1996). The infection status and the identity of the parasite genera were assessed by amplifying a 478bp fragment of the cytochrome *b* gene following Hellgren et al. (2004). Negative samples were repeated to rule out false negatives (McClintock et al., 2010). Both negative controls for PCR reactions (at least one per plate) and DNA extractions (one per 15 samples) were included in the analyses. PCR amplifications were resolved in 1.2% agarose gels and positive amplifications were sequenced unidirectionally (Macrogen Inc.; Amsterdam, The Netherlands). Sequences were edited using Sequencher™ v.4.9 (Gene Codes Corp. © 1991–2009, Ann Arbor, MI 48108, USA) and parasite genera were identified by comparison with sequences deposited in GenBank (National Center for Biotechnology Information) and MalAvi databases (Bensch et al., 2009). Information on birds' parasite infections was analysed in the context of other previous studies (Ferraguti et al., 2018; Jiménez-Peñuela et al., 2019).

2.4. Biochemical analyses

Physiological blood measurements may provide an overall assessment of the organism's oxidative status and its variability, especially when the stress or exposure is low to moderate, and chronic rather than acute (Skríp and McWilliams, 2016). Blood cells were mixed with miliQ water (1:4 proportion) and centrifuged at 14,000 rpm during 30 min at 4 °C to lyse the cells. The supernatant was separated and kept at -80 °C until analyses. Blood samples from one triplet were excluded due to the lack of enough samples with enough blood volume for analyses.

The oxidative damage caused by oxidant agents to lipids (lipid peroxidation) particularly affects unsaturated lipids and produces malondialdehyde metabolites (MDA) (Buege and Aust, 1978; Costantini, 2019). To quantify it, thiobarbituric acid reactive substances (TBARS) were measured (nmol MDA/ml) as they react with MDA producing a red-pink product measured at a wavelength of 535 nm (Buege and Aust, 1978). The antioxidant capacity of birds was measured by quantifying the activity of the three enzymes that take part in the glutathione (GSH) redox system (see Fig.S1): the superoxide dismutase (SOD), the glutathione peroxidase (GPx) and the glutathione reductase (GR), see Appendix A for further information on the role of each enzyme. The SOD activity (U/mg of protein) was determined with the inhibition rate of xanthine oxidase (XOD) as the source of peroxide radicals. The catalysis of XOD produced red products that were measured at a wavelength of 550 nm (Arthur and Boyne, 1985; Suttle, 1986; Suttle and McMurray, 1983; Wooliams et al., 1983). The activities of GPx and GR were quantified by the decrease in colour absorbance at a wavelength of 340 nm due to the oxidation of NADPH to NADP⁺ (Goldberg and Spooner, 1983; Melissinos et al., 1981; Paglia and Valentine, 1967). All methods were adapted to the Cobas Integra 400 autoanalyser and the concentration of each biomarker refers to the total protein content expressed in mg/ml. In all analyses, samples were randomly distributed on the plates and standardized samples were included in each plate to calibrate analyses and control for plate-to-plate variation. In the analyses of enzymes some samples were run in duplicate and, in addition to CV, we

calculated the intra-class correlation coefficient (ICC) using the package *psych* with the function *ICC*. The CV and repeatability as estimated by ICC of enzymes analyses were CV: 7.85% and ICC: 0.99 for GR (sample size = 72); CV: 4.57% and ICC: 0.98 for SOD (sample size = 56); and CV: 4.04% and ICC: 0.96 for GPx (sample size = 68). Due to limitations of available volume of samples, TBARS analyses were run once but measured twice with a coefficient of variance (CV) of 3.5% (sample size = 688).

2.5. Statistical analyses

We independently analysed TBARS with respect to SOD, GPx and GR as they reflect different aspects of the oxidative status of birds. TBARS is a biomarker that measures the oxidant reactions of ROS with lipids and their consequent oxidative damage while SOD, GPx and GR reflect the enzymatic antioxidant capacity of birds due to their implication in the GSH redox system functioning (Fig. S1 and Appendix A). Thus, a principal component analysis (PCA) was performed using the correlation matrix to summarize the antioxidant activity of SOD, GPx and GR enzymes into two principal components (PC1 and PC2, see Results section). Data of SOD, GPx and GR were logarithmically transformed, centred and scaled prior to the PCA. The effects of habitat type, parasite infections, bird's body condition and their interactions on birds' oxidative status (both in lipid peroxidation and in antioxidant enzyme activity) were tested by fitting Linear Mixed-Effects Models (LMM). TBARS levels, PC1 and PC2 (continuous) were included as dependent variables in different models. Time and date of sampling (continuous), body condition (continuous), habitat type (categorical: urban, rural, and natural), *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infection status (categorical: infected/uninfected by each parasite), and the two-way interactions between body condition and habitat, body condition and infection status and habitat and infection status by each parasite were included as independent variables. Sampling locality nested in the triplet was included as a random factor to control for the geographical stratification of the study design. A backward stepwise selection procedure was applied by removing either the variable or the interaction with the highest *p* values. This procedure was used until a simplified model including only variables with $p \leq 0.10$ was obtained. When the interaction was retained in the model, both variables included in the interaction were maintained. The differences between the three factor levels of the habitat category were tested with Tukey's post-hoc tests when necessary. For each model, the conditional (R_c^2 , considering both the fixed and random factors) and the marginal (R_m^2 , considering only fixed factors) variance explained was calculated (Nakagawa and Schielzeth, 2013). Collinearity between independent variables was tested with the variance inflation factor (VIF) (Zuur et al., 2010) and no evidence of collinearity (all values were <4) was found. The normality of continuous variables and the residuals of all the LMMs were tested by checking normality *qq*-plots. Models were run with the higher sample size available for each analysis. All statistical analyses were conducted in R (version 4.0.4 "Lost Library Book"; R Foundation for Statistical Computing, 2021) using the packages: *arm*, *car*, *cowplot*, *emmeans*, *ggplot2*, *Hmisc*, *lattice*, *lme4*, *lmerTest*, *lsmeans*, *MASS*, *Matrix*, *multcomp*, *MuMIn*, *nlme*, *phia*, *psych*, *Rcpp*, *Eigen* and *stats*.

3. Results

Initially, 688 individuals were included in the analyses, but due to low volume of blood available for some individuals, the sample size for TBARS ($n = 600$) differed from those of GPx, GR and SOD ($n = 488$).

For the TBARS analyses (Table 1 we found that birds infected by *Haemoproteus* showed significantly higher TBARS levels than uninfected birds (Fig. 1). The relationship between TBARS and body condition was significantly different for *Haemoproteus* infected and uninfected birds, being negative for infected birds but positive for uninfected ones (Fig. 2). TBARS levels of birds and the relationship of TBARS and body

Table 1

Results of the LMMs of the effect of urbanization, avian malaria infection and bird's body condition on the levels of oxidative damage to lipids (TBARS). Estimates, standard errors (S.E.), χ^2 , degree freedom (d.f.) and p -value (p) from the model are shown. Significant associations ($p < 0.05$) are shown in bold while marginally significant associations ($p < 0.10$) are shown in italic. Conditional and marginal (in brackets) R^2 values of the model are reported.

	Estimate \pm S.E.	χ^2	d. f.	p
Intercept	1.17 (0.09)	194.16	1	<0.001
Body Condition	-0.002 (0.003)	0.26	1	0.609
Habitat - Urban	0.00 ^a	5.51	2	0.064
Habitat - Natural	-0.10 (0.12)			
Habitat - Rural	-0.29 (0.13)			
Uninfected (by <i>Haemoproteus</i>)	0.00 ^a	3.89	1	0.049
<i>Haemoproteus</i> infected	0.30 (0.15)			
Body Condition * Habitat - Urban	0.00 ^a	5.47	2	0.065
Body Condition * Habitat - Natural	0.004 (0.005)			
Body Condition * Habitat - Rural	0.01 (0.005)			
Body Condition * Uninfected	0.00 ^a	4.00	1	0.045
Body Condition * <i>Haemoproteus</i> infected	-0.012 (0.006)			
R^2	0.30 (0.02)			

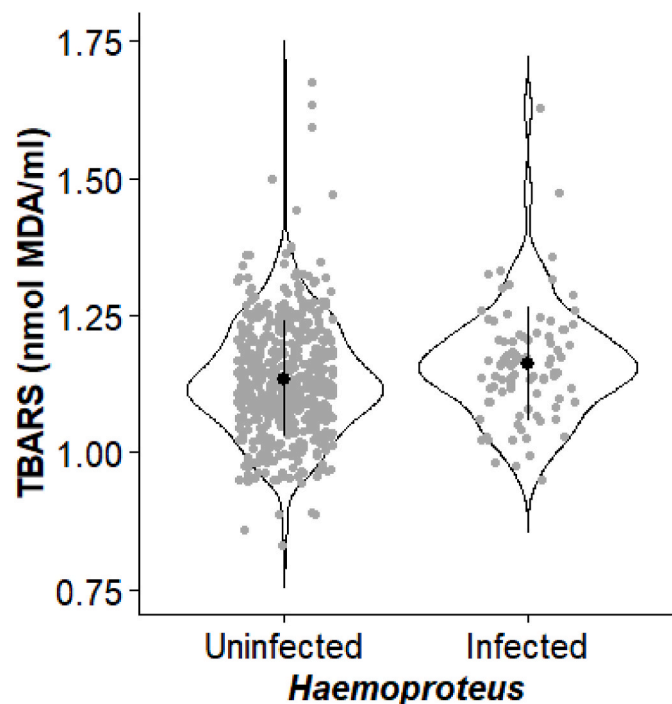


Fig. 1. Mean values (\pm S.D.) of TBARS for *Haemoproteus* uninfected and infected house sparrows ($N_{\text{Uninfected}} = 511$; $N_{\text{Infected}} = 89$). Grey points represent raw data distribution. Density curves represents the frequency of data points in each region of y axis.

condition tended, but not significantly, to differ between habitats. Specifically, birds from rural areas showed lower TBARS levels than birds from urban habitats (estimate \pm S.E. = -0.30 ± 0.13 , $z = -2.33$, $p = 0.05$) but no significant differences were found between birds from rural and natural habitats (estimate \pm S.E. = -0.20 ± 0.13 , $z = -1.47$, $p = 0.30$) or between birds from natural and urban habitats (estimate \pm S.E. = -0.10 ± 0.13 , $z = 0.80$, $p = 0.70$). The relationship between TBARS and body condition was positive in natural and rural habitats but negative in urban habitats (Fig. S2).

From the PCA performed to summarize the antioxidant activity of the three enzymes we obtained two principal components which explained



Fig. 2. Relationship between TBARS levels and the body condition of uninfected (grey straight line and circles) and infected (black dotted line and triangles) birds by *Haemoproteus*. The 95% confidence level interval is shown in grey.

the 82.68% of the variance of the variables GPx, GR and SOD. The PC1 eigenvalue was 1.6 explaining 53.21% of the variance, while the PC2 eigenvalue was 0.9 and explained 29.5% of the variance. The contribution of the variables GPx, GR and SOD to the principal components were 39.05, 17.47 and 43.48 to PC1 and 16.44, 80.2 and 3.36 to PC2. The loadings for GPx, GR and SOD were 0.62, 0.42 and 0.66 to PC1 and were -0.41 , 0.89 and -0.18 to PC2 (Fig. 3). Thus, PC1 reflected the overall enzymatic activity of GPx, GR and SOD while PC2 was higher when GR levels were high and/or GPx and SOD were low.

PC1 ($R_c^2 = 0.43$, $R_m^2 = 0.04$; Table 2) was significantly lower in birds infected by *Plasmodium* (estimate \pm S.E. = -0.21 ± 0.10 , $\chi^2 = 4.69$, d.f. = 1, $p = 0.03$) and tended, but not significantly, to be higher in individuals infected by *Leucocytozoon* (estimate \pm S.E. = 0.20 ± 0.11 , $\chi^2 = 3.21$, d.f. = 1, $p = 0.07$) compared to uninfected birds (Fig. 4). PC2 ($R_c^2 = 0.26$, $R_m^2 = 0.07$; Table 3) was positively associated with the hour of capture (estimate \pm S.E. = 1.05 ± 0.32 , $\chi^2 = 10.48$, d.f. = 1, $p = 0.001$) but negatively associated with the date of capture (estimate \pm S.E. = -0.01 ± 0.02 , $\chi^2 = 11.24$, d.f. = 1, $p = 0.001$) and the body condition of birds (estimate \pm S.E. = -0.04 ± 0.02 , $\chi^2 = 3.94$, d.f. = 1, $p = 0.047$)

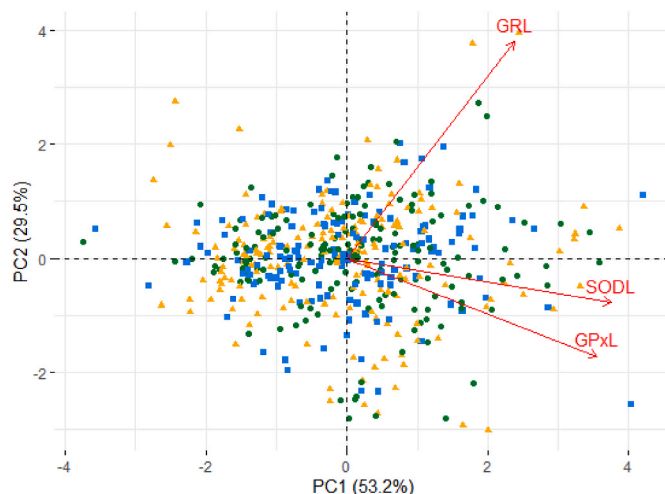


Fig. 3. Principal Components Analyses for GPx, GR and SOD activities of juvenile male house sparrows from natural (green circles), rural (yellow triangles) and urban (blue squares) habitats. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2

Results of the LMMs on the effect avian malaria infection on enzymatic antioxidant activity (PC1). Estimates, standard errors (S.E.), χ^2 , degree freedom (d.f.) and *p*-value (*p*) from the model are shown. Significant associations ($p < 0.05$) are shown in bold while marginally significant associations ($p < 0.10$) are shown in italic. Conditional and marginal (in brackets) R^2 values of the model are reported.

	Estimate ± S.E.	χ^2	d.f.	<i>p</i>
Intercept	0.03 (0.14)	0.04	1	0.84
Uninfected (by <i>Plasmodium</i>)	0.00 ^a	4.69	1	0.03
<i>Plasmodium</i> infected	-0.21 (0.10)			
Uninfected (by <i>Leucocytozoon</i>)	0.00 ^a	3.21	1	<i>0.07</i>
<i>Leucocytozoon</i> infected	0.20 (0.11)			
R^2	0.43 (0.01)			

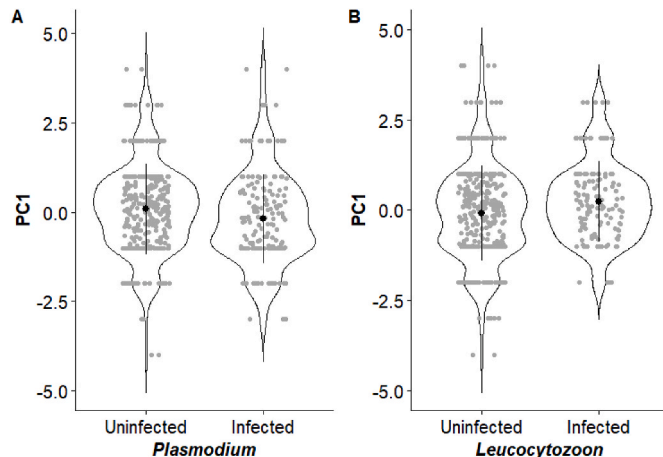


Fig. 4. Mean values (\pm S.D.) of PC1 with respect to bird infection status by (A) *Plasmodium* ($N_{\text{Uninfected}} = 392$; $N_{\text{Infected}} = 208$) and (B) *Leucocytozoon* ($N_{\text{Uninfected}} = 444$; $N_{\text{Infected}} = 156$). Significant differences were only found for *Plasmodium*. Grey point represents raw data distribution. Density curves represents the frequency of data points in each region of *y* axis.

Table 3

Results of the LMMs on the effect body condition has on enzymatic antioxidant activity (PC2). Estimates, standard errors (S.E.), χ^2 , degree freedom (d.f.) and *p*-value (*p*) from the model are shown. Significant associations ($p < 0.05$) are shown in bold, marginal associations ($p < 0.10$) are shown in italic. Conditional and marginal (in brackets) R^2 values of the model are shown.

	Estimate ± S.E.	χ^2	d.f.	<i>P</i>
Intercept	2.07 (0.60)	11.98	1	<0.001
Time	1.05 (0.32)	10.48	1	0.001
Date	-0.01 (0.02)	11.24	1	<0.001
Body Condition	-0.04 (0.02)	3.94	1	0.047
R^2	0.26 (0.07)			

(Fig. 5). The variable habitat and its interaction with bird body condition or infection status were not retained in any of the models related with the enzymatic antioxidant activity of birds.

4. Discussion

Here we tested the potential effects of urbanization and parasite infections on the oxidative stress status of birds considering the role of bird’s body condition. We measured four different oxidative stress-related biomarkers in juvenile male house sparrows: TBARS, reflecting the oxidative damage to lipids and GPx, SOD and GR reflecting the enzymatic antioxidant activity. We found that parasite infections significantly affected the oxidative status of birds, influencing both lipid oxidative damage and the enzymatic antioxidant activity. Urbanization

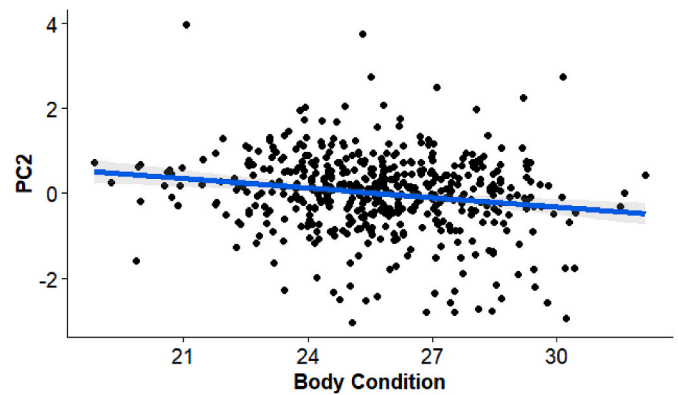


Fig. 5. Relationship between PC2 and the body condition of juvenile male house sparrows. The 95% confidence level interval is shown in grey.

only marginally affected the oxidative damage to lipids. The body condition of birds significantly related to the enzymatic antioxidant activity and also influenced the TBARS levels associated to parasite infections (significantly) and urbanization (marginally). We further discuss the relationships found here in the following sections.

4.1. Effects of parasite infections on the oxidative status of birds

Significantly higher levels of TBARS were found in birds infected with *Haemoproteus* compared to uninfected ones although no difference was recorded for *Plasmodium* and *Leucocytozoon* infected birds. Moreover, PC1, which reflects the overall enzymatic activity of GPx, SOD and GR, was significantly lower in *Plasmodium* infected birds, tended (but not significantly) to be higher in *Leucocytozoon* infected birds and did not vary in *Haemoproteus* infected birds in comparison with uninfected individuals.

Parasite infections harm their hosts directly by draining their resources and indirectly through the costs of producing inflammatory and immune responses against infections that increase the presence of ROS (Bertrand et al., 2006; Costantini and Møller, 2009). In line with our predictions, we found higher oxidative damage to lipids (TBARS) in *Haemoproteus* infected than in uninfected birds. In other studies, no differences were found between avian malaria-infected and uninfected birds regarding oxidative damage to lipids (Delhaye et al., 2016; Messina et al., 2021; Razavi et al., 2016), although an increase in pro-oxidants was associated with *Haemoproteus* and *Plasmodium* infections (Delhaye et al., 2016; Isaksson et al., 2013; van de Crommenacker et al., 2012), probably leading to oxidative damage to different molecules, including lipids (Li et al., 2016).

Contrary to our predictions, PC1 was lower in *Plasmodium* infected than in uninfected birds. Similarly, van de Crommenacker et al. (2012) reported decreased antioxidant capacity in infected warblers with *Haemoproteus* from Seychelles, and Isaksson et al. (2013) found low oxidized GSH (GSSG) in great tits infected by *Plasmodium* from Sweden, probably derived from a downregulation of GPx activity (see Fig.S1). By contrast and in line with our predictions, birds infected with *Leucocytozoon* tended to have a higher PC1 than uninfected birds. Higher GPx activity in fledged great tits infected by blood parasites (either with *Plasmodium*, *Haemoproteus* or *Leucocytozoon*) was also reported in comparison with uninfected birds (De Angeli Dutra et al., 2017), likely indicating protection against oxidative damage. Lastly, birds infected with *Haemoproteus* showed no significant differences in their enzymatic antioxidant activity compared to uninfected individuals. Razavi et al. (2016) neither found significant differences in the activity of GPx or SOD in *Haemoproteus* infected and uninfected pigeons. The contradictory patterns found between each genus regarding the antioxidant enzyme activity could be due to the different infection patterns of each parasite. For example, both *Plasmodium* and *Haemoproteus* develop inside red blood

cells, while *Leucocytozoon* infects white blood cells (Valkiūnas, 2005). Thus, the physiological pathways involved in avian responses to infections in different target cells together with the virulence of each genus (Valkiūnas, 2005) could explain our results. Indeed, virulence can also vary even within different parasite lineages of the same genus and differently affect the oxidative stress of birds (Isaksson et al., 2013; Lachish et al., 2011), and even may explain apparently contradictory results between studies.

An increase in the expression of the genes involved in both immune and antioxidant responses was found in birds experimentally infected with *Plasmodium* (Videvall et al., 2015). Increased antioxidant activity probably helps to counteract the inflammatory responses and ROS production against parasites likely avoiding oxidative damage (Sorci et al., 2013; Sorci and Faivre, 2009). Indeed, for birds infected with *Haemoproteus* we found oxidative damage to lipids but no disruption of antioxidant enzymes activity, whereas for *Plasmodium* and *Leucocytozoon*, we found no oxidative damage to lipid but a decrease and increase antioxidant enzyme activity, respectively. Immune responses to pathogens may be energetically costly (Martin et al., 2003), thus, under natural conditions where available resources are limited, a trade-off may exist between developing antioxidant or immune responses (Morales et al., 2006), especially if non-enzymatic antioxidant substances are limited in the environment (Hörak et al., 2004). Nevertheless, the relationship found here between avian malaria infections and bird's oxidative stress were independent from the habitat type as none of the interactions of infection status and habitat type were significant.

4.2. Effects of habitat anthropization on the oxidative status of birds

Urbanization processes are severely transforming the landscape with implications for the life-history traits of different avian species (Bradley and Altizer, 2007; Grimm et al., 2000; Sepp et al., 2018; Sol et al., 2014). Salmón et al. (2018) showed that the urbanization index highly correlates with NO_x levels, one of the main pro-oxidants and stressors substances in urban areas. Pollutants such as toxic chemicals, heavy metal particles and other substances derived from human activities have negative consequences on urban-dwelling wildlife and affect their health by increasing the oxidative damage to molecules (Herrera-Dueñas et al., 2014; Isaksson, 2015; Koivula et al., 2011). Indeed, our results indicated that birds from urban areas showed marginally higher TBARS levels, suggesting that the pro-oxidants had exceeded the antioxidant capacity of the organism and individuals from urban habitats are suffering higher oxidative damage to lipids than birds from rural habitats. Previous studies also described higher lipid peroxidation in birds from urban habitats than in birds from less anthropized environments (Amri et al., 2017; Berglund et al., 2007; Herrera-Dueñas et al., 2017). Pérez-Rodríguez et al. (2015) found that the oxidative damage to lipids (measure through MDA) was positively associated with blood concentrations of plasma triglycerides (the main form of storage and transport of polyunsaturated fatty acids or PUFA) in different bird species and ages, which could bias the interpretation of our results. The fatty acids composition of a subsample of 66 individuals included in this study was previously analysed in Jiménez-Peñuela et al. (2022). Although the total fatty acids concentration was higher in the blood of bird's from urban habitats compared to birds from rural and natural habitats, no difference between habitats was found on the relative proportion of any specific fatty acids group, not even Omega-3 and Omega-6 PUFA (Jiménez-Peñuela et al., 2022) which suggest that the results regarding the TBARS levels reported here are not bias by differences in lipid concentration between habitats.

Contrary to our prediction, we did not find significant differences in the activity of antioxidant enzymes (GR, GPx or SOD) between birds from different habitats. These results were in concordance with those found for SOD by Herrera-Dueñas et al. (2017) and Salmón et al. (2018) and for GR by Giraudeau et al. (2014). Similarly, Goodchild et al. (2022) found that circulating glutathione (total, reduced, and oxidized) did not

vary between urban and non-urban birds. By contrast, differences in the activity of antioxidant enzymes were found between habitats, i.e., higher levels of GPx (e.g., Herrera-Dueñas et al., 2017; Koivula et al., 2011), SOD and CAT (Amri et al., 2017) and GR and CAT (Berglund et al., 2007) in urban than in rural birds, while the opposite pattern for SOD was reported by Li et al. (2021). It is important to note that other non-enzymatic antioxidant substances not discussed here such as thiols, carotenoids and vitamins A, C and E obtained from diet may also be involved in neutralising ROS and controlling the antioxidant balance in the organism (Costantini and Verhulst, 2009; Finke et al., 2020; Li et al., 2016; Sorci and Faivre, 2009).

4.3. The role of body condition on birds' oxidative status

According to our results, PC2 was negatively related to bird's body condition. In other words, birds with higher body condition had lower GR and/or higher GPx and SOD activity. The GPx and SOD interact directly with molecules acting as ROS (Fig. S1), thus a higher activity of these enzymes (in birds with higher body condition) could be related to a lower possibility of oxidative damage and, therefore, to lower levels of TBARS.

Furthermore, we found that the relationship between the levels of TBARS with the infection status and the habitat type are affected by birds' body condition, although in the case of habitat the associations were only marginally significant. Specifically, we found that the oxidative damage to lipids increased with body condition in uninfected birds and in those living in rural and natural habitats but decreased in birds infected by *Haemoproteus* and in those from urban habitats. Thus, it seems that birds under stressful conditions (i.e., when birds are infected or living in urban habitats) and with high body condition (i.e., with access to optimal resources) likely increase GPx and SOD activity to reduce ROM and thus, decrease oxidative damage. Nevertheless, the causality of this effect could not be assessed using our correlative results. Hence, further experimental and longitudinal studies could help to improve our understanding of the role of body condition in the capacity of urban-dwelling species to deal with stressors of anthropic origin and parasite infections while keeping the oxidative equilibrium.

5. Conclusions

To sum up, the biomarkers discussed here are part of a series of highly integrated physiological responses in organisms that vary across the tissues affected, host life-stages and life history, making them difficult to interpret in nature (Costantini and Verhulst, 2009; Isaksson, 2020). Our study provides, to our knowledge, the first evidence of the combined effects of urbanization and blood parasite infections on the physiological stress responses of birds. Our results support that the oxidative status in the widespread house sparrows was affected by both urbanization and parasite infections and that these last effects may vary according to blood parasite genus. Additionally, we show a link between body condition, oxidative damage and the activity of enzymes involved in the redox system.

The results regarding parasite infections put in manifest the importance of considering different parasites genus when evaluating physiological responses to infection as we could obtain contradictory patterns. Moreover, future studies should consider the intensity of infection as well as do experimental infections to further evaluate the physiological implications to the host of the different phases of the infection and parasite loads. In light of the increase of harmful environmental stressors, reducing the negative effects of urbanization on the health of birds should become a goal of conservation biology. Our results indicate that living in more anthropic environments implies higher oxidative damage to lipids, thus, reducing the levels of oxidative substances such as toxic chemicals or heavy metal particles derived from human activities could improve the health of urban-dwelling wildlife (and likely our own health). Lastly, we highlight the key role of the bird's body

condition to understand how birds lead with common blood parasite infections and environmental stressors while regulating their oxidative stress status. Thus, although we found no direct significant effect of the combination of urbanization and avian malaria infection, maybe this effect could be indirect (i.e., urbanization reducing bird body condition), so future studies should continue to take this effect into account to understand the host-parasite interaction under the scenario of global change.

Credit author statement

Jéssica Jiménez-Peñuela: Conceptualization, Investigation, Data curation, Formal analysis, Writing – Original draft, Writing – Review & Editing, Visualization; **Martina Ferraguti:** Conceptualization, Investigation, Data curation, Formal analysis, Writing – Original draft, Writing – Review & Editing, Visualization, Funding acquisition; **Josué Martínez-de la Puente:** Conceptualization, Resources, Writing – Review & Editing, Supervision; **Ramón C. Soriguer:** Resources, Writing – Review & Editing, Funding acquisition; **Jordi Figuerola:** Conceptualization, Resources, Writing – Review & Editing, Supervision, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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