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Differences in fatty acids composition between *Plasmodium* infected and uninfected house sparrows along an urbanization gradient

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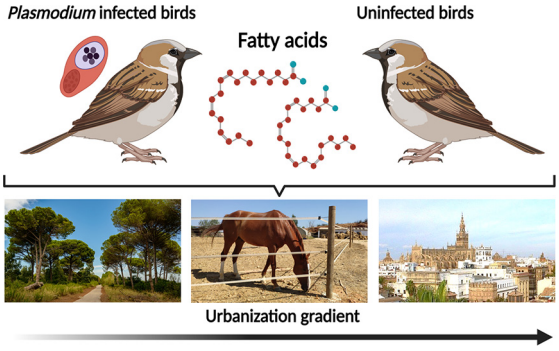
HIGHLIGHTS

- Fatty acids were analysed in birds from habitats along an urbanization gradient.
- ω-3 and ω-6 proportion differed between *Plasmodium* infected and uninfected birds.
- The ω-6/ω-3 ratio was lower in *Plasmodium* infected birds from natural habitats.
- Fatty acids composition in natural habitats may help birds to fight off infections.
- The higher concentration of fatty acids in urban birds suggests a fat-rich diet.

GRAPHICAL ABSTRACT



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ABSTRACT

Anthropogenic activities such as intensification of agriculture, animal husbandry and expansion of cities can negatively impact wildlife through its influence on the availability of high-quality food resources and pathogen transmission. The house sparrow (*Passer domesticus*), an urban exploiter, is undergoing a population decline. Nutritional constrains and infectious diseases has been highlighted as potential causes. Fatty acids (FAs) play an important role in modulating certain immune responses needed to combat parasite infections. FAs are highly influenced by dietary availability and have been shown to vary between urban and rural birds. Habitat anthropization also affects avian malaria epidemiology but little attention has been given to the relationship between blood parasite infection, host FAs composition and anthropization. Here, we analysed 165 juvenile birds either infected by *Plasmodium* or uninfected, captured at 15 localities grouped in triplets containing urban, rural and natural habitats. The total level of FAs was higher in birds from urban than from rural habitats, suggesting a greater availability of fat-rich foods sources. Furthermore, *Plasmodium* infected birds had higher relative levels of ω-3 polyunsaturated fatty acids (PUFAs) but lower of ω-6 PUFAs than uninfected birds. In concordance, the ω-6/ω-3 ratio was also lower in infected than in uninfected birds, but only from natural habitats, likely driven by the slightly higher ω-3 PUFAs in infected birds from natural habitats. Birds from anthropized environments may metabolize the ω-3 PUFAs to promote anti-inflammatory responses against stressors, which would result in lower ω-3 affecting their response against *Plasmodium*. Alternatively, lower ω-6

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PUFAs may influence birds susceptibility to infection due to a weaker pro-inflammatory response. These descriptive results do not allow us to identify the causality of these associations but highlight the need to further investigate the relevance of FAs for birds to fight infectious diseases in habitats with different degree of urbanization.

1. Introduction

The intensification of agriculture, animal husbandry and the expansion of cities have changed the environmental conditions around the globe (Bradley and Altizer, 2007; Grimm et al., 2000). Human activities can reduce local biodiversity and alter interactions between organisms, such as disease transmission (Bradley and Altizer, 2007; Seress and Liker, 2015; Sol et al., 2014). However, some species are able to thrive within anthropogenic environments. Cities can provide favourable conditions for birds such as milder climate during winter (Saaroni et al., 2000), higher availability of nesting sites (Sumasgutner et al., 2014), reduced density of predators (Møller and Díaz, 2018; but see Dulisz et al., 2016) and higher predictability of food and water (Fokidis et al., 2008). Nevertheless, urban-dwelling individuals seem to pay a cost in terms of reduced body size and condition, higher oxidative stress, lower reproductive success and accelerated telomere attrition (Chamberlain et al., 2009; Jiménez-Peñuela et al., 2019; Liker et al., 2008; Salmón et al., 2016; Sepp et al., 2018; Sumasgutner et al., 2014; Watson et al., 2017). The house sparrow (*Passer domesticus*) is a sedentary urban exploiter bird species, that is historically known to thrive in areas with human activities and settlements, taking advantage of food resources and cavities for nesting (Hanson et al., 2020; Mckinney, 2002). However, this species is undergoing a consistent population decline throughout Europe, especially in cities (Anderson, 2006; De Laet and Summers-Smith, 2007; Hanson et al., 2020). Nutritional constrains, intensive animal husbandry and agriculture, pollution, pesticides and infectious diseases caused by blood parasites, have been suggested as potential causes for this population decline (Bichet et al., 2013; Dadam et al., 2019; Herrera-Dueñas et al., 2014, 2017; Meillère et al., 2017; Meyrier et al., 2017; Peach et al., 2018; von Post et al., 2012).

Nutrient rich food is especially important during early life stages to fulfil physiological requirements during development to grow faster, obtain a higher body condition and a better immunocompetence (Chamberlain et al., 2009; Peach et al., 2018; Seress et al., 2020; Twining et al., 2016, 2018). Invertebrates predominate the diet of Passeriformes' chicks, however, anthropized environments often have less suitable prey available and instead the diet is substituted by anthropogenic food (i.e., human waste) (Heiss et al., 2009). These resources are generally rich in carbohydrates and saturated fats but poor in protein content compared to more natural food resources. A high intake of anthropogenic food strongly influence fledgling growth and survival, and thereby, nesting success and population demography (Anderson, 2006; Mckinney, 2002; Meyrier et al., 2017; Peach et al., 2018; Salleh Hudin et al., 2016). The food type consumed determines the intake of different key physiological compounds like polyunsaturated fatty acids (PUFAs), which influence the capacity of animals to mount immune responses (Arnold et al., 2015; Hulbert et al., 2005; Isaksson et al., 2017). Recent studies have shown differences in fatty acid (FA) composition of plasma and yolk between urban and rural birds, but most importantly differences were found for omega-6 (ω-6) and omega-3 (ω-3) PUFAs (Andersson et al., 2015; Isaksson et al., 2017; Toledo et al., 2016). The long-chain PUFAs can modulate both cellular and humoral immune responses by affecting the production of lymphocytes, heterophils and splenocytes as well as IgM and IgG antibodies (Alagawany et al., 2019). Specifically, long-chained ω-6 PUFAs are metabolized to pro-inflammatory prostaglandins, while the long-chained ω-3 PUFAs are metabolized into anti-inflammatory agents, so an alteration in the ω-6/ω-3 ratio has been associated with changes in immune responses and in oxidative stress (Arnold et al., 2015; Hulbert et al., 2005; Simopoulos, 2011). Indeed, under higher exposure to pro-oxidants caused by internal processes (e.g., oxidative burst in response to infection or as a by-product of metabolism) or external factors (e.g., air pollution), unsaturated fatty acids can peroxidize (lipid peroxidation) which affects their structure and thereby their function (Eikenaar et al., 2020; Herrera-Dueñas et al., 2014; Isaksson et al., 2017; Pamplona et al., 2000, 2002; Salmón et al., 2018).

Avian malaria of the genus *Plasmodium* are common blood parasites (Atkinson et al., 1991; Valkiūnas, 2005) with detrimental effects on health and survival of birds (Martínez-de la Puente et al., 2010; Marzal et al., 2005; Merino et al., 2000). Their epidemiology has been shown to be altered in anthropized landscapes (Bichet et al., 2013; Bradley and Altizer, 2007; Calegaro-Marques and Amato, 2014; Ferraguti et al., 2018; Geue and Partecke, 2008). Despite the well-known effects of habitat anthropization for both the prevalence of avian malaria infections and FAs composition, there are no studies to date that investigate how these two factors are related in birds living in environments with different degree of anthropization. Here, we investigate the FAs composition, the total FAs quantity (FA_{tot}) and the peroxidation index (PI) of juvenile house sparrows either infected by *Plasmodium* or uninfected. Birds were caught in populations along a gradient of urbanization that included urban, rural and natural habitats. The PI is an estimate of the overall susceptibility to lipid damage when exposed to oxidants such as an oxidative burst to defeat parasitic infections. Thus, individuals with more unsaturated FAs, especially long-chained PUFAs, would have higher PI and, consequently, be more susceptible to suffer negative impacts from increased oxidative stress.

Using a correlative and explorative approach, the main objective of this study is to shed light into how different landscapes affect the relationship between FAs composition and avian malaria infection in wild birds. However, based on previous research we outline three main predictions. First, we predict that urban birds have an overall higher level of FA_{tot} and a low proportion of long-chained PUFAs, such as ω-6 and, specially, ω-3 PUFAs compared to birds from rural and natural habitats (see Andersson et al., 2015; Isaksson et al., 2017; Toledo et al., 2016). This prediction is based on the assumption that urban birds feed on predictable, abundant and fat-rich but PUFA-poor anthropogenic food sources. Second, birds infected by *Plasmodium* may have higher proportion of ω-3 PUFAs to develop an anti-inflammatory immune response against infections, especially under a higher pro-oxidant environment such as anthropogenic areas (Herrera-Dueñas et al., 2014; Salmón et al., 2018), as higher ω-6 PUFAs could increase their oxidative stress and thereby, negatively affect birds' health. Lastly, the ω-6/ω-3 ratio would probably vary both with habitat, due to different diet availability, and with infection status, due to physiological needs to counteract infection. However, following our above forecast, we predict that the ω-6/ω-3 ratio is lower in infected birds, and especially from natural habitats due to differential diet availability (i.e., urban birds would have less ω-3 available).

2. Materials and methods

2.1. Study area and bird sampling

Juvenile house sparrows were caught using mist-nets and a bird call playback recorder at 15 localities in southwestern Spain from July to October of 2013 (Fig. S1). Localities were grouped into 5 triplets, each comprising an urban, a rural and a natural habitat. Habitats were classified according to visual characteristics based on the anthropogenic use of each habitat: (1) Urban habitats correspond to highly populated areas with human infrastructures, i.e., cities or villages; (2) rural habitats were selected based on the presence of higher density of livestock, and where humans were less abundant than in urban areas i.e. agriculture and/or farming; and (3) natural habitats were characterized as higher preserved habitats with a lower density of both livestock or humans than rural and urban areas, respectively, where human activities were absent or very

occasionally present. To further identify differences in the level of anthropization between these habitat types, an urbanization score was obtained for each locality using a semi-automated software (http://keplab.mik.uni-pannon.hu/en/urbanization-index) according to Czúni et al. (2012), Lipovits et al. (2015) and Seress et al. (2014). In each triplet, urban habitats had significantly higher levels of urbanization index, followed by rural habitats, while natural habitats had the lowest levels. See Table S1 for locality-specific values and Appendix A for further details of the statistical analyses.

Each bird was ringed with an individual numbered metal ring, and sexed and aged based on plumage characteristics and skull ossification when possible (Svensson, 2009). The body mass of the birds was measured with an electronic balance (to the nearest 0.1 g). A blood sample was extracted from the jugular vein using a sterile syringe and never exceeding 1% of the bird's body mass. Blood samples were stored in Eppendorf tubes maintained in cold-boxes during field work, and subsequently kept for less than 24 h at 4 °C until centrifugation for 10 min at 1700*g* (4000 rpm) to separate serum and cellular fractions. The cellular fractions were frozen at −20 °C and serum at −80 °C until further molecular and biochemical analysis. Birds were released at the place of capture.

2.2. Molecular analyses

Genomic DNA was extracted using the Maxwell®16 LEV System Research (Promega, Madison, WI, USA). Sex-determination was performed by plumage characteristics when possible, otherwise it was molecularly determined using the primer pair CHD-P2 (5′-TCTGCATCGCTAAATCCTTT-3′) and CHD-P8 (5′-CTCCCAAGGATGAGRAAYTG-3′) following the protocols by Griffiths et al. (1996, 1998). The infection status and parasite identity were determined through the amplification of a 478-bp fragment of the *Plasmodium* mitochondria *cytochrome b* gene (Hellgren et al., 2004). Negative samples were tested twice to avoid false negatives (McClintock et al., 2010) and both negative controls for the PCR reactions (at least one per plate) and DNA extractions (one per 15 samples) were included in the analysis. Positive amplifications were sequenced unidirectionally to reduce the cost of sequencing a large number of amplicons (Dubiec et al., 2016) by Macrogen Inc. (Amsterdam, The Netherlands). Sequences were edited using the software Sequencher™ v 4.9 (Gene Codes Corp. © 1991–2009, Ann Arbor, MI 48108, USA). The parasites genera were identified by comparison with sequences deposited in GenBank (National Center for Biotechnology Information) and MalAvi databases (Bensch et al., 2009). The *Plasmodium* infection status of the birds studied here was previously analysed in Ferraguti et al. (2018) and Jiménez-Peñuela et al. (2019, 2021).

2.3. Fatty acid analyses

The FAs from serum were extracted following the protocol described in Andersson et al. (2015). Briefly, 50 μl of chloroform:methanol (2:1 v/v) with internal standard (methyl (Z)-10-heptadecenoate, 33.3 ng/μl) was added to 5 μl of defrosted serum and left for 1 h at room temperature. The internal standard allows us to estimate the absolute concentration of FAs. The organic phase was then evaporated to dryness under a gentle N_2 flow followed by adding of 100 μl 0.5 M KOH solution in methanol. The mix was left in the oven at 40 °C for 1 h to convert the FAs into Methyl Esters (FAMEs). After methanolysis, 100 μl of 0.5 M HCl in methanol followed by 300 μl of heptane were added. The mix separates into two phases, the bottom methanol phase was removed, and the FAME extract in the heptane phase was washed with 200 μl of water twice. Finally, to completely remove all the water, anhydrous sodium sulphate salt (Na_2SO_4) was added to the FAME extract. The supernatant was then transferred to a glass vial for analysis by Gas Chromatography/Mass Spectrometry (GC/MS). Specifically, we used an Agilent 5975 MS coupled to an Agilent 6890 GC equipped with an HP-INNOWax capillary column (30 m, 0.25 mm id, df 0.25 μm; Agilent, CA, USA). The oven temperature was set to 80 °C for 1 min, then increased by 10 °C/min to 230 °C and was held at 230 °C for 20 min. Helium was used as carrier gas at a constant flow of 1 ml/min. The FAMEs present in serum were identified by comparing their mass spectra and retention times with those of synthetic standards (Supelco 37-Component FAME Mix, Sigma-Aldrich, Stockholm, Sweden), and quantified by comparing the peak area of the measurement with the peak area of the internal standard of known concentration. Nine samples were extracted twice for assay repeatability. The intraclass correlation coefficient (ICC), one for each functional group of three representative FAs, was calculated with the function *ICC* from the package *pysch*. The coefficient of variance (CV) for SFA (16:0) was 1.033 and the ICC 0.99. For MUFA (18:1n), the CV was 0.565 and the ICC 1. Lastly, for PUFA (18:2n-6), the CV was 2.023 and the ICC 1. Intra-assay CVs below 10 are considered good estimates.

2.4. Sample selection

Initially a large number of birds were caught in the context of a different study, see Ferraguti et al. (2018) and Jiménez-Peñuela et al. (2019, 2021). For the present study, a subset of individuals was selected for FAs analyses based on the following criteria: first, only juvenile birds were considered, as they better reflect local habitat characteristics such as food quality and availability, as well as the abundance and richness of local parasite circulation during the nestling and post-fledging phase (Cosgrove et al., 2008; Valkiūnas, 2005). Second, to test the relationship between malaria infection and FAs composition, only birds infected exclusively by *Plasmodium*, or uninfected birds were included. This allows us to avoid any confounding effects produced by other blood-parasites (e.g., *Haemoproteus* and/or *Leucocytozoon*) or co-infections. To address possible differences between habitats of the same type, samples from 15 localities grouped into 5 triplets were included to obtain replicates. The number of individuals included in the analyses from each locality varied according to the availability of the samples, ranging from 7 to 12 individuals (mean = 11; S.D. = 1.73, per locality). These samples were selected to balance the number of individuals per site, the number of males and females, and according to their infection status by avian *Plasmodium*, i.e., infected or uninfected. Overall, FAs were analysed in 58, 54 and 53 house sparrows from urban, rural, and natural habitats, respectively. Of them, 88 were males and 77 females, corresponding to 81 *Plasmodium* infected and 84 uninfected individuals (see Table S1).

2.5. Statistical analysis

The individual FAs were grouped into their chemical class (Table 1): saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), ω-6 PUFAs, ω-3 PUFAs, and all PUFAs together ($PUFA_{tot}$ = ω-6 PUFAs + ω-3 PUFAs + ω-9 PUFAs). All groups are presented as relative proportions (%) to the total FAs concentration. The FA_{tot} concentration per individual (ng/μl) was used to get an indication of the total fat intake. Moreover, we quantified two additional biomarkers: the ω-6/ω-3 ratio and the PI. The latter was estimated by adding the peroxidation potential of all individual unsaturated FAs (i.e., the number of double bonds multiplied by its relative abundance). Thus, PI was calculated as PI = ((% monoenoic × 0.025) + (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 4) + (% pentaenoic × 6) + (% hexaenoic × 8)) following Pamplona et al. (1998, 2000). For the statistical analyses, the proportion of FAs groups were logit-transformed (log(y/(1-y))); (Warton et al., 2011), and the ω-6/ω-3 ratio and the FA_{tot} were logarithmically transformed to normalize its distribution.

Linear Mixed-Effects Models (LMM) fitted by maximum likelihood were used to test the effects of habitat category and *Plasmodium* infection on the serum FAs composition of wild birds. Different models were fitted for each group (SFAs, MUFAs, ω-6 PUFAs, ω-3 PUFAs and $PUFA_{tot}$), the ω-6/ω-3 ratio, the PI and the FA_{tot}. Habitat category (urban, rural, and natural; categorical), infection status by *Plasmodium* (infected and uninfected; categorical), and their two-way interaction were included as independent variables. Hour of capture (continuous), day of capture (continuous), and body mass (continuous) were included as covariates. Locality (categorical) nested in triplet (categorical) was included as a random factor to account

Table 1
Relative proportion of serum fatty acids (% of total concentration) in urban, rural and natural habitats and from *Plasmodium* infected and uninfected juvenile house sparrows. Table shows mean values ± standard error. C:Dn-x, number of carbon atoms:double bonds-position; LA: Linoleic Acid, AA: Arachidonic acid; EPA: Eicosapentanoic acid; DPA: Docosapentanoic acid; DHA: Docosahexaenoic acid.

Fatty acid name	C:Dn-x	FAs class	All		Natural		Rural		Urban	
			Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected
Myristic acid	14:0	SFAs	0.43 ± 0.02	0.43 ± 0.03	0.35 ± 0.04	0.28 ± 0.03	0.50 ± 0.04	0.50 ± 0.04	0.44 ± 0.05	0.48 ± 0.04
Palmitic acid	16:0		24.20 ± 2.67	24.72 ± 2.70	22.39 ± 4.23	21.23 ± 4.25	25.98 ± 5.20	26.61 ± 4.94	24.41 ± 4.61	25.82 ± 4.71
Margaric acid	17:0		0.12 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	0.14 ± 0.02	0.11 ± 0.02	0.10 ± 0.02	0.12 ± 0.01	0.10 ± 0.01
Stearic acid	18:0		16.03 ± 0.37	15.88 ± 0.42	17.52 ± 0.74	18.52 ± 0.71	15.04 ± 0.57	14.67 ± 0.60	15.42 ± 0.51	14.84 ± 0.65
Arachidic acid	20:0		0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.05 ± 0.01	0.09 ± 0.01	0.10 ± 0.02
Palmitoleic acid	16:1n-7	MUFAs	1.72 ± 0.14	1.95 ± 0.16	1.46 ± 0.26	1.04 ± 0.20	1.92 ± 0.20	2.50 ± 0.27	1.79 ± 0.26	2.18 ± 0.26
Oleic acid	18:1n-9		25.39 ± 0.90	24.80 ± 0.84	22.51 ± 1.62	19.51 ± 1.58	25.39 ± 1.13	26.45 ± 1.13	28.27 ± 1.64	27.61 ± 1.25
cis-Vaccenic acid	18:1n-7		1.21 ± 0.05	1.23 ± 0.04	1.14 ± 0.10	1.09 ± 0.07	1.28 ± 0.07	1.31 ± 0.08	1.19 ± 0.07	1.26 ± 0.07
Eicosenoic acid	20:1n-7		0.11 ± 0.01	0.10 ± 0.02	0.09 ± 0.02	0.05 ± 0.02	0.11 ± 0.02	0.10 ± 0.02	0.12 ± 0.03	0.15 ± 0.03
Mead acid	20:3n-9	ω-9 PUFA	0.82 ± 0.04	0.86 ± 0.04	0.94 ± 0.08	0.98 ± 0.07	0.80 ± 0.06	0.79 ± 0.05	0.72 ± 0.05	0.84 ± 0.06
LA	18:2n-6	ω-6 PUFAs	16.52 ± 0.91	16.25 ± 0.85	17.67 ± 1.65	20.21 ± 1.53	16.38 ± 1.1	13.96 ± 1.19	15.49 ± 1.82	15.18 ± 1.46
γ-Linolenic acid	18:3n-6		0.59 ± 0.10	0.50 ± 0.09	0.81 ± 0.23	0.64 ± 0.25	0.58 ± 0.19	0.41 ± 0.12	0.37 ± 0.10	0.46 ± 0.09
Eicosadineoic acid	20:2n-6		0.01 ± 0.004	0.01 ± 0.005	0.016 ± 0.008	0.005 ± 0.005	0.01 ± 0.007	0.01 ± 0.007	0.009 ± 0.006	0.02 ± 0.01
Dihomo-γ-linolenic acid	20:3n-6		0.20 ± 0.03	0.19 ± 0.02	0.18 ± 0.06	0.08 ± 0.03	0.18 ± 0.04	0.23 ± 0.04	0.24 ± 0.05	0.25 ± 0.04
AA	20:4n-6		10.08 ± 0.38	10.40 ± 0.47	11.78 ± 0.66	13.31 ± 0.79	9.32 ± 0.63	10.06 ± 0.80	9.06 ± 0.54	8.29 ± 0.56
Adrenic acid	22:4n-6		0.37 ± 0.03	0.39 ± 0.04	0.31 ± 0.04	0.40 ± 0.06	0.41 ± 0.05	0.35 ± 0.04	0.38 ± 0.04	0.42 ± 0.08
DPA	22:5n-6		0.48 ± 0.04	0.53 ± 0.05	0.43 ± 0.07	0.61 ± 0.09	0.52 ± 0.08	0.49 ± 0.08	0.49 ± 0.07	0.51 ± 0.08
α-Linolenic acid	18:3n-3	ω-3 PUFAs	0.35 ± 0.05	0.35 ± 0.05	0.46 ± 0.10	0.57 ± 0.13	0.32 ± 0.08	0.22 ± 0.05	0.27 ± 0.05	0.28 ± 0.03
EPA	20:5n-3		0.30 ± 0.06	0.21 ± 0.04	0.54 ± 0.14	0.23 ± 0.09	0.19 ± 0.07	0.15 ± 0.05	0.16 ± 0.05	0.26 ± 0.08
DPA	22:5n-3		0.09 ± 0.02	0.08 ± 0.02	0.13 ± 0.04	0.09 ± 0.03	0.08 ± 0.02	0.05 ± 0.02	0.06 ± 0.02	0.11 ± 0.03
DHA	22:6n-3		0.92 ± 0.07	0.92 ± 0.07	1.08 ± 0.13	0.94 ± 0.13	0.79 ± 0.08	0.98 ± 0.11	0.87 ± 0.12	0.85 ± 0.10

for the geographical stratification of the sampling design (Kuznetsova et al., 2017).

Tukey's post hoc test was used to test for differences between pairs of habitats when the factor "habitat" was statistically significant. Contrasts of factor interactions, performed with the package *phia*, were used to test for differences between levels of the interaction between habitat category and *Plasmodium* infection (Martínez, 2015). The sum of squares was calculated using an ANOVA type III. The marginal (considering only fixed factors) and conditional (considering fixed and random factors) variance explained by each model was determined by a *pseudo-R^2* (Nakagawa and Schielzeth, 2013), estimated with the package *MuMin* (Bartón, 2019). For each LMM, the collinearity between independent variables was tested by calculating the Variance Inflation Factor (VIF) (Zuur et al., 2010). Residuals from each LMM, and all dependent variables, were checked for normality by using *qq-plots*. Statistical analyses were conducted in R (4.0.5 "Shake and Throw"; R Foundation for Statistical Computing) using the packages: *arm*, *car*, *cowplot*, *ggplot2*, *lattice*, *lme4*, *lmerTest*, *lsmeans*, *MASS*, *Matrix*, *multcomp*, *MuMIn*, *nlme*, *phia* and *Rcpp*.

3. Results

Overall, 21 different FAs were identified in the serum of juvenile house sparrows (Table 1). Oleic acid (MUFA), palmitic acid (MUFA), stearic acid (SFA), linolenic acid (LA, ω-6 PUFA), and arachidonic acid (AA, ω-6 PUFA) accounted for the highest proportions of FAs, and range between 8% and 27% of the total FAs concentration (Table 1). Table 2 shows the mean proportion of serum FAs for the different groups (% of total FAs concentration), the FA_{tot} (ng/μl) along with the ω-6/ω-3 ratio and the PI for each habitat category and *Plasmodium* infection status.

3.1. The effect of bird infection status and habitat on fatty acid composition

Results from the LMMs testing the effects of *Plasmodium* infection and habitat on the proportion of serum FAs groups are summarized in Table 3. *Plasmodium* infected birds had a significantly lower proportion of ω-6 PUFAs ($\chi^2 = 4.16$, d.f. $= 1$, $p = 0.04$; Fig. 1) and a higher proportion of ω-3 PUFAs ($\chi^2 = 5.80$, d.f. $= 1$, $p = 0.02$; Fig. 1) than uninfected individuals. Additionally, infected birds tended to have a higher proportion of MUFAs and a lower proportion of $PUFA_{tot}$ than uninfected ones, but these differences did not reach significance (MUFAs: $\chi^2 = 3.01$, d.f. $= 1$, $p = 0.08$; $PUFA_{tot}$: $\chi^2 = 2.97$, d.f. $= 1$, $p = 0.08$; Fig. 1). Infection status was not associated to SFAs ($\chi^2 = 0.14$, d.f. $= 1$, $p = 0.70$; Fig. 1). Moreover, a marginally significant trend was found for the interaction between infection status and habitat for the proportion of ω-3 PUFAs ($\chi^2 = 5.68$, d.f. $= 2$, $p = 0.06$), with infected birds showing a significantly higher proportion of ω-3 PUFAs than uninfected birds in natural ($\chi^2 = 5.8$, d.f. $= 1$, $p = 0.02$; Fig. 2), but not in urban ($\chi^2 = 0.12$, d.f. $= 1$, $p = 0.74$; Fig. 2) nor in rural habitats ($\chi^2 = 0.32$, d.f. $= 1$, $p = 0.57$; Fig. 2). The proportions of the other FAs groups were not related to habitat or the interaction between infection status and habitat (Table 3). Results for LMMs analyses testing the

Table 2
Relative proportion of serum fatty acids groups (% of total fatty acid concentration), two biomarkers and the total fatty acid concentration (FA_{tot}; ng/μl) in urban, rural and natural habits, and from *Plasmodium* infected and uninfected juvenile house sparrows. Table shows mean values ± standard error. SFAs: Saturated Fatty Acids; MUFAs: Monounsaturated Fatty Acids; $PUFA_{tot}$: Total Polyunsaturated Fatty Acids; PI: Peroxidation Index; FA_{tot}: Total Fatty Acid Concentration (ng/μl).

Fatty acid class group/biomarker	All habitats		Natural		Rural		Urban	
	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected
SFAs	40.85 ± 0.46	41.21 ± 0.45	40.45 ± 0.84	40.24 ± 0.7	41.71 ± 0.6	41.93 ± 0.7	40.4 ± 0.87	41.33 ± 0.87
MUFAs	28.42 ± 1.03	28.08 ± 0.99	25.20 ± 1.92	21.68 ± 1.83	28.71 ± 1.35	30.37 ± 1.4	31.38 ± 1.83	31.20 ± 1.48
ω-6 PUFAs	28.63 ± 1.13	28.79 ± 1.13	31.60 ± 2.03	36.09 ± 1.95	27.76 ± 1.55	25.87 ± 1.55	26.43 ± 2.11	25.54 ± 1.76
ω-3 PUFAs	1.90 ± 0.21	1.72 ± 0.17	2.57 ± 0.45	1.91 ± 0.42	1.65 ± 0.32	1.59 ± 0.22	1.47 ± 0.24	1.69 ± 0.25
$PUFA_{tot}$	30.73 ± 1.10	30.71 ± 1.11	34.35 ± 1.97	38.08 ± 1.89	29.59 ± 1.45	27.70 ± 1.55	28.14 ± 2.02	27.47 ± 1.69
ω-6/ω-3	29.17 ± 3.79	30.74 ± 3.41	22.23 ± 4.01	38.23 ± 5.94	29.84 ± 4.75	21.60 ± 2.95	35.50 ± 9.25	33.33 ± 7.49
PI	0.75 ± 0.02	0.76 ± 0.03	0.87 ± 0.04	0.93 ± 0.04	0.71 ± 0.04	0.72 ± 0.04	0.69 ± 0.04	0.67 ± 0.04
FA_{tot}	24.71 ± 0.77	24.81 ± 1.49	23.38 ± 1.09	22.33 ± 1.66	22.96 ± 1.26	22.15 ± 1.11	27.59 ± 1.48	29.44 ± 3.69

Table 3
Results of the Linear Mixed Models (LMM) analysing the effect of time (hour of capture), date (day of capture), body mass, *Plasmodium* infection status (Pla Inf: infected and uninfected birds), habitat category (Habitat: urban, rural, and natural) and the interaction between infection status by *Plasmodium* and habitat (Pla Inf * Hab) on the proportion of the different groups of fatty acids. Significant relationships ($p \leq 0.05$) are highlighted in bold. Conditional (and marginal) R^2 are shown. SFAs: Saturated Fatty Acids, MUFAs: Monounsaturated Fatty Acids, ω-6 PUFAs: Omega 6 Polyunsaturated Fatty Acids, ω-3 PUFAs: Omega 3 Polyunsaturated Fatty Acids, and $PUFA_{tot}$: Polyunsaturated Fatty Acids together.

Independent variables	SFAs			MUFAs			ω-6 PUFAs			ω-3 PUFAs			$PUFA_{tot}$		
	χ^2	d.f.	*p*	χ^2	d.f.	*p*	χ^2	d.f.	*p*	χ^2	d.f.	*p*	χ^2	d.f.	*p*
Intercept	0.89	1	0.35	0.07	1	0.79	14.17	1	**<0.001**	15.73	1	**<0.001**	13.17	1	**<0.001**
Time	6.35	1	**0.01**	2.27	1	0.13	5.63	1	**0.02**	0.52	1	0.47	6.52	1	**0.01**
Date	0.04	1	0.84	4.44	1	**0.03**	6.68	1	**0.01**	0.03	1	0.85	5.76	1	**0.02**
Body mass	2.01	1	0.16	0.69	1	0.41	1.98	1	0.16	0.10	1	0.75	2.38	1	0.12
Pla Inf	0.14	1	0.70	3.01	1	0.08	4.16	1	**0.04**	5.80	1	**0.02**	2.97	1	0.08
Habitat	0.99	2	0.61	4.24	2	0.12	2.58	2	0.28	0.30	2	0.86	3.26	2	0.20
Pla Inf *Hab	0.29	2	0.87	2.99	2	0.22	4.28	2	0.12	5.68	2	0.06	3.93	2	0.14
R^2	0.30 (0.08)			0.43 (0.17)			0.52 (0.18)			0.35 (0.05)			0.50 (0.2)		

effects of *Plasmodium* infection and habitat on the ω-6/ω-3 ratio are summarized in Table 4. The ω-6/ω-3 ratio differed significantly with the infection status of birds ($\chi^2 = 8.7$, d.f. $= 1$, $p = 0.003$) and the interaction between the infection status and habitat category ($\chi^2 = 8.28$, d.f. $= 2$, $p = 0.02$). *Plasmodium* infected birds had lower ω-6/ω-3 ratio levels than uninfected individuals in natural habitats ($\chi^2 = 8.69$, d.f. $= 1$, $p = 0.003$; Fig. 3), but there were no significant differences in birds from rural ($\chi^2 = 0.70$, d.f. $= 1$, $p = 0.40$; Fig. 3) nor urban habitats ($\chi^2 = 0.01$, d.f. $= 1$, $p = 0.93$; Fig. 3).

3.2. The effect of bird infection status and habitat on the PI and FA_{tot} levels

Results for LMMs analyses testing the effects of *Plasmodium* infection and habitat on the PI and FA_{tot} are summarized in Table 4. The FA_{tot} was only affected by habitat ($\chi^2 = 7.12$, d.f. $= 2$, $p = 0.03$), with urban individuals showing a higher amount of FA_{tot} concentration than rural birds (Est. $= 0.09$, S.E. $= 0.04$, $z = 2.59$; $p = 0.03$; Fig. 4), but there were no significant differences between urban and natural habitats (Est. $= 0.07$, S.E. $= 0.04$, $z = 1.79$; $p = 0.17$) nor between rural and natural habitats (Est. $= -0.02$, S.E. $= 0.04$, $z = -0.64$; $p = 0.79$). In addition, we found a marginally significant trend for a difference in PI between the different habitats ($\chi^2 = 5.32$, d.f. $= 2$, $p = 0.07$). However, none of the post hoc tests between habitats were significant (rural-natural: Est. $= -0.16$, S.E. $= 0.07$, $z = -2.15$, $p = 0.08$; urban-natural: Est. $= -0.14$, S.E. $= 0.07$, $z = -1.85$, $p = 0.15$; and urban-rural: Est. $= 0.02$, S.E. $= 0.07$, $z = 0.28$, $p = 0.96$; Fig. S2). Similarly, a marginally significant trend was revealed for PI between the infected and uninfected birds, where the PI tended to be lower in *Plasmodium* infected than in uninfected birds ($\chi^2 = 2.98$, d.f. $= 1$, $p = 0.08$; Fig. S2).

4. Discussion

In the present study, we investigated how the serum composition of FAs in juvenile house sparrows was related to habitat anthropization and avian malaria infection. The main results revealed that urban birds had higher levels of FA_{tot} in their bloodstream than birds from rural areas, although the proportion of FAs groups in birds from different habitats did not differ. Additionally, in accordance with our prediction, *Plasmodium* infected birds

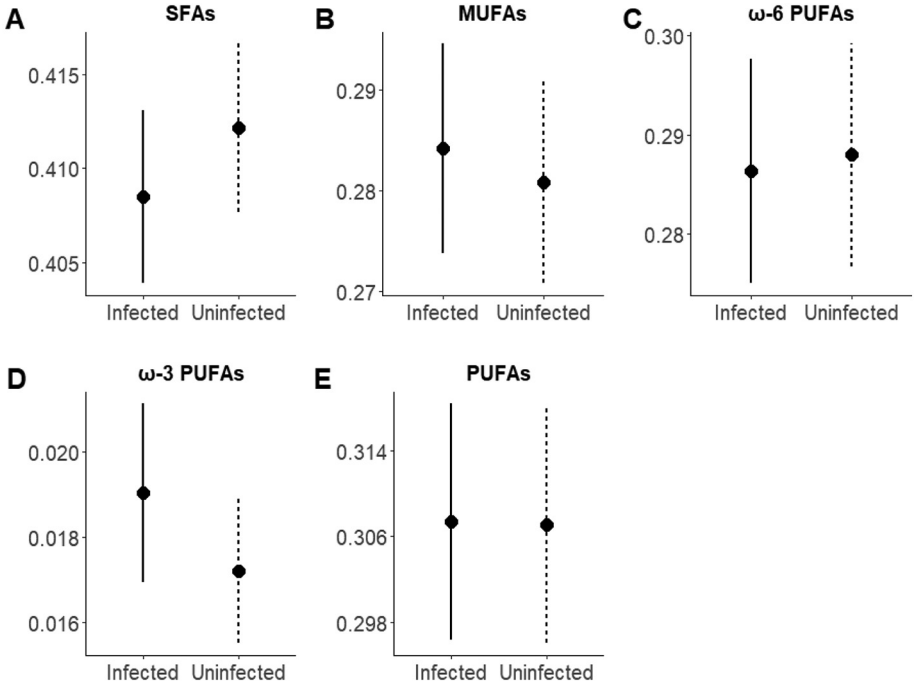

Fig. 1. Mean relative proportion ± standard error of (A) saturated fatty acids (SFAs), (B) monounsaturated fatty acids (MUFAs), (C) omega-6 polyunsaturated fatty acids (ω-6 PUFAs), (D) omega-3 polyunsaturated fatty acids (ω-3 PUFAs) and (D) polyunsaturated fatty acids ($PUFA_{tot}$) for juvenile house sparrows either infected (solid line) or uninfected (dashed line) by *Plasmodium*.

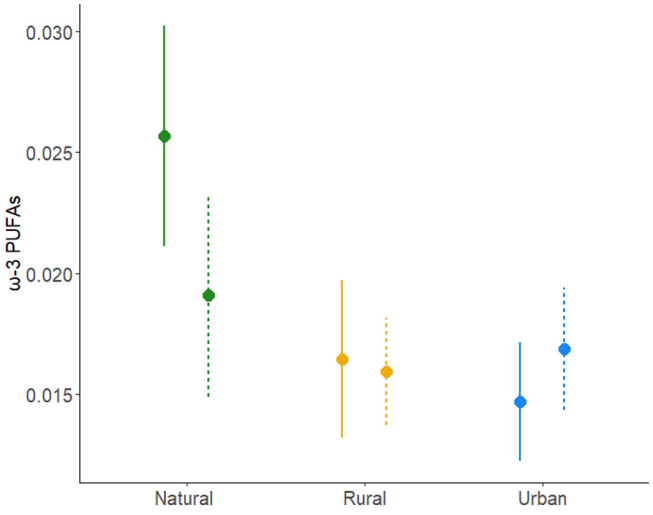

Fig. 2. Mean proportion ± standard error of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) of *Plasmodium* infected (solid line) and uninfected birds (dashed line) in natural (green), rural (yellow), and urban (blue) habitats.

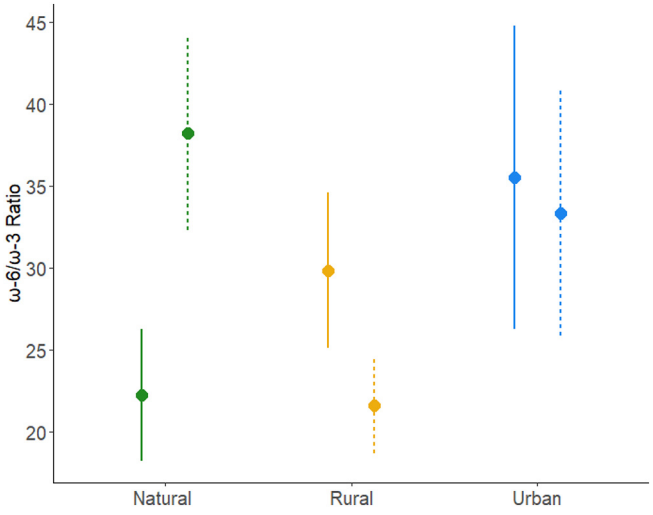

Fig. 3. Mean ratio of ω-6 and ω-3 polyunsaturated fatty acids (PUFAs) ± standard error of *Plasmodium* infected (solid line) and uninfected birds (dashed line) in natural (green), rural (yellow), and urban (blue) habitats.

had a higher proportion of ω-3 PUFAs, but also a lower proportion of ω-6 PUFAs than uninfected birds. This resulted in a lower ω-6/ω-3 ratio in infected birds than in uninfected ones, but only in natural habitats.

In accordance with our prediction, the FA_{tot} was significantly higher in urban than in rural birds, suggesting that the availability of fat-rich food for juvenile urban sparrows was greater than for those in rural habitats. Interestingly, higher levels of FA_{tot} did not result into higher body mass in urban birds. When parent birds feed their offspring, the food demand is high and, if insects are scarce, adults use human refuse or other anthropogenic foods, that are usually high in carbohydrates and fats but low in protein content, to ensure short-term survival (Mckinney, 2002; Meyrier et al., 2017). This may explain the higher levels of FA_{tot} found in juvenile birds from urban areas. If this pattern reflects a nutritionally suboptimal diet, it can be expected that the diet of urban birds negatively affects their growth (Heiss et al., 2009) and the development of their immune system and, consequently, their capacity to fight infections (Klasing, 2007).

Contrary to our predictions, there were no overall differences in any of the proportions of the FAs groups in birds from different habitats, only when infection status was taken into account. This was a surprising result given that previous studies have shown large variation in FAs composition in house sparrows from urban and rural habitats in Sweden (Andersson et al., 2015; Isaksson et al., 2017). Possibly, Spanish house sparrows have a greater variety of food types across the landscape compared to habitats of Sweden, whether anthropogenically altered or not. Alternatively, it is possible that the wide range of urbanization scores of the localities included in the different triplets (Table S1) could dilute the potential effects of urbanization on the overall composition of FAs driven by environmental differences. Nevertheless, the nested structure in triplets allows for comparison of differences between habitats within each triplet, considering these potential geographical discrepancies. In future studies, it could be interesting to investigate the diet composition of birds in the different environments to further understand the use of anthropogenic or natural food resources in each habitat, including also different countries or climates.

Several studies have documented negative effects on fitness or fitness-related traits when infected with avian malaria parasites (Asghar et al., 2015; Atkinson et al., 1991; Jiménez-Peñuela et al., 2019; Lachish et al., 2011; Martínez-de la Puente et al., 2010; Marzal et al., 2005, 2008). Yet, links between FAs composition and avian malaria infection have not been

Table 4
Results of the Linear Mixed Models (LMM) analysing the effect of time (hour of capture), date (day of capture), body mass, infection status by *Plasmodium* (Pla Inf: infected and uninfected birds), habitat category (Habitat: urban, rural and natural) and the interaction between infection status by *Plasmodium* and habitat (Pla Inf * Hab) on different biomarkers of fatty acids. Significant relationships ($p \leq 0.05$) are highlighted in bold. Conditional (and marginal) R^2 are shown. PI: Peroxidation Index, FA_{tot}: Total Fatty Acid concentration.

Independent variables	PI			ω-6/ω-3 ratio			FA_{tot}		
	χ^2	d.f.	*p*	χ^2	d.f.	*p*	χ^2	d.f.	*p*
Intercept	9.24	1	**<0.001**	3.08	1	0.08	724.77	1	**<0.001**
Time	32.59	1	**<0.001**	0.02	1	0.89	12.53	1	**<0.001**
Date	1.51	1	0.22	2.97	1	0.08	0.15	1	0.69
Body mass	1.35	1	0.25	0.01	1	0.919	1.05	1	0.30
Pla Inf	2.98	1	0.08	8.70	1	**0.003**	0.88	1	0.35
Habitat	5.32	2	0.07	0.67	2	0.72	7.12	2	**0.03**
Pla Inf * Hab	2.16	2	0.34	8.28	2	**0.02**	0.63	2	0.73
R^2	0.48 (0.35)			0.49 (0.05)			0.19 (0.17)		

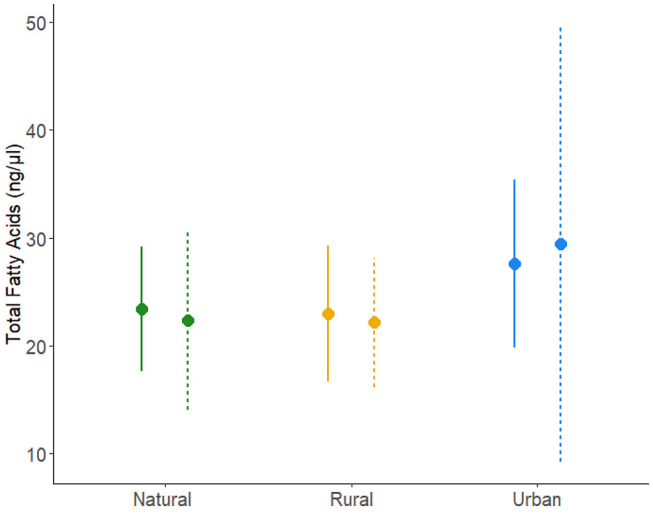

Fig. 4. Mean total fatty acid concentration (ng/µl) ± standard error, in *Plasmodium* infected (solid line) or uninfected (dashed line) house sparrows captured in natural (green), rural (yellow), and urban (blue) habitats.

explored so far, despite the known effects of PUFAs on immune responses, their environmental dependence and fitness effects especially in juveniles (Twining et al., 2018, 2021). Here, we show that ω-6 PUFAs were relatively lower and ω-3 PUFAs relatively higher in infected juvenile birds compared to uninfected ones. This is interesting given the two different immune pathways in which these PUFAs are involved in. During infection or other inflammatory processes, ω-6 PUFAs are metabolized into pro-inflammatory prostaglandins, whereas the downstream immune components of the ω-3 PUFAs pathway are anti-inflammatory (Alagawany et al., 2019; Chang et al., 2018; Hulbert et al., 2005; Simopoulos, 2011). Since the present study is correlative, we cannot determine whether infection status is the result of the relative abundance of the two PUFAs groups in the different habitats or whether the result is a differential response to parasite infections across different habitats. Thus, at least two alternative explanations are plausible for our findings. First, considering the different immune responses originated from the two different groups of PUFAs, a higher proportion of ω-3 PUFAs and a lower proportion of ω-6 PUFAs could indicate that these birds were more susceptible to infection due to a weaker pro-inflammatory response, i.e., less pro-oxidants and T-cells were produced/activated to attack parasites (Klasing, 2007). Alternatively, if the PUFAs composition was a response to infection rather than vice versa, lower ω-6 PUFAs and higher ω-3 PUFAs could be the result of a preferential dietary intake and/or mobilization from storage to promote an anti-inflammatory immune response towards *Plasmodium* infections. Indeed, the consumption of food rich in ω-3 PUFAs has been shown to enhance the immune response during active inflammation without increasing the oxidative stress (Alagawany et al., 2019). Nevertheless, these results may have consequences for birds, as the ω-3 PUFAs decrease the development of strong inflammatory responses in birds but also the morbidity in response to pathogen infection (Klasing, 2007).

Significant differences were also found in the ω-6/ω-3 ratio with infected individuals showing lower values than uninfected birds, but interestingly, only in birds from natural habitats. This result could either reflect a low proportion of ω-6 PUFAs, high proportion of ω-3 PUFAs, or both, in infected than in uninfected birds in this habitat. However, since we found a trend for a higher proportion ω-3 PUFAs in infected than uninfected birds in natural habitats, it is likely that this variable plays a key role in explaining differences in the ω-6/ω-3 ratio. Anyhow, we can only speculate about the reasons for this habitat difference for infected birds. One explanation could be that birds from more anthropized environments have already metabolized the ω-3 PUFAs to immune components for other reasons (e.g., environmental stressors such as exposure to chemical and/or light pollution) leaving lower circulating ω-3 PUFAs for other important functions. Also, consistent with the previously outlined scenarios, the low ω-6/ω-3 ratio of *Plasmodium* infected birds could suggest that either sparrows from natural habitats were particularly susceptible to infection, or that these infected birds were in a better nutritional state (i.e., consumption of food rich in ω-3 PUFAs) than infected birds from urban and rural habitats (Andersson et al., 2015; Isaksson et al., 2017). That could potentially reduce their long-term costs to overcome the infection (Delhaye et al., 2018; Pamplona et al., 2002; Sorci and Faivre, 2009). Thus, growing up in anthropized habitats and being infected could have implied suffering greater costs due to the exposure to multiple stressors and limited resources, as previously suggested (Jiménez-Peñuela et al., 2019). Lastly, we cannot rule out the possibility that the pattern reported here is due to a low proportion of ω-6 PUFAs in infected individuals, which could mean a higher use of ω-6 PUFAs in the immune system (such as arachidonic acid, AA), rather than a lower intake.

Additionally, *Plasmodium* infected birds tended to have lower PI values than uninfected birds. This could be caused by an active response to infection resulting in FAs damage when infected birds further limit the potential costs associated with increased oxidative stress during an active infection, hence we found less long-chained PUFAs circulating (Pamplona et al., 2000, 2002). Furthermore, birds from natural habitats tended to have higher PI compared with their urban and rural conspecifics. Contrary to this pattern, Isaksson et al. (2017) found that both urban house sparrows and tree sparrows (*Passer montanus*) showed a higher PI than rural birds, but also higher degree of damaged lipids (lipid peroxidation). Although lipid peroxidation was not measured here, we do not expect that birds from natural habitats are exposed to environmentally induced oxidative conditions, thus we do not expect a similar cost of the slightly higher PI here. In the same way, this result may be originated by differential availability of food rich in long-chained FA in natural habitats in comparison with urban and rural (i.e., ω-3 PUFAs), even though we have not found differences in the abundance of FA groups caused by habitat.

5. Conclusion

The main results of the present study are that urban birds have a higher FA_{tot}, that infected birds have a higher proportion of ω-3 and lower proportion of ω-6, and that infected birds from natural habitats have a lower ω-6/ω-3 ratio than uninfected birds, probably due to a higher relative proportion of ω-3 in infected birds from this habitat. These interesting associations between FAs composition, malaria infection and anthropization in wild birds warrant further investigations. Given our intriguing associations between PUFAs and infection status, future studies should perform experimental dietary supplementations to investigate the implications of the ω-6/ω-3 ratio during infections and whether increased ω-3 PUFAs helps to mount a less costly immune response or increase susceptibility to avian malaria infections. Moreover, the diet composition of birds in different environments should be investigated to better understand the use of anthropogenic and/or natural food resources, their quality and implications for wild birds' health. This could potentially shed some mechanistic light on how different environments can influence the immune system to fight off infectious diseases, affecting the health and demography of wild bird populations living in highly urbanized habitats.

Ethics statement

Bird trapping was carried out with all the necessary permits from Consejería de Medio Ambiente, and Consejería de Agricultura, Pesca y Desarrollo Rural (Junta de Andalucía) and bird sampling on private land and in private residential areas were conducted with all the necessary permits and consent of owners. The CSIC Ethics Committee approved the experimental procedures on 9 March 2012.

CRediT authorship contribution statement

Jéssica Jiménez-Peñuela: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Martina Ferraguti:** Conceptualization, Resources, Writing – review & editing, Supervision. **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. **Caroline Isaksson:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, 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.

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Availability of data and materials

The data supporting the conclusions of this article are included within the article. The datasets used and/or analysed during the present study will be made available by the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.152664.

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