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Impact of urbanization on the house sparrow (*Passer domesticus*): Serum proteome and pathogen prevalence

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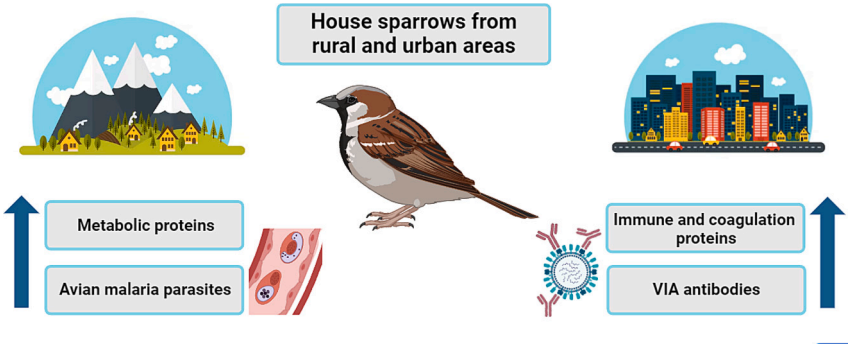
HIGHLIGHTS

- Serum proteome analysis of urban and rural house sparrows reveals physiological effects of urbanization.
- Rural sparrows exhibited significantly higher SMI and avian malaria prevalence, hinting to body condition related higher immune capacity.
- Rural sparrows expressed the most active metabolic proteins, suggesting higher food availability.
- Urban sparrows overexpress immune, coagulation and lipid metabolization proteins, indicating exposure to stress factors.

GRAPHICAL ABSTRACT



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ABSTRACT

The house sparrow (*Passer domesticus*) is a globally distributed species found in rural, urban and other humanised environments. In Europe, sparrow populations have significantly declined in recent decades, especially in urbanised areas. In the present study, we analysed the impact of urbanization on sparrow body condition, pathogen prevalence, and serum proteome changes. Sparrows were captured in four locations with two different urbanization status (rural/urban). Biometric data, blood samples and oral and cloacal swabs were collected. Rural sparrows exhibited significantly better body condition compared to urban sparrows, with no notable differences between sexes. Haemoparasite prevalence was higher in rural sparrows 70.16 % (87/124) than in urban sparrows 50 % (27/54). No avian influenza virus (AIV) or West Nile virus (WNV) genetic material was found, although one urban sparrow (0.58 %) had antibodies to AIV. Serum proteomics revealed that rural sparrows showed an up-regulation of proteins involved in the metabolism, in contrast to proteins of the immune

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system and the coagulation system, which were found to be over-represented in urban sparrows. Thus, we documented a worse body condition and immune system activation in urban sparrows in contrast to a more active metabolism and a higher prevalence of avian malaria in rural sparrows, and at least occasional exposure to AIV in urban habitats. This information suggests exposure to urban environments may alter the host-pathogen relationship. Urbanization in combination with exposure to AIV, could modulate their role in viral spread and transmission.

1. Introduction

In recent decades, due to the increase in human population, urban environments have increased (Rouffaer et al., 2017). This growth in urbanization, is one of the most radical forms of change in land use in terrestrial ecosystems (Alberti, 2015). In wild birds, urbanization leads to highly stressful situations, such as variation in food availability, human presence, new predators, new vegetation and exposure to chemical, light, and noise pollution (Bókony et al., 2010). Only a small number of species can adapt to such environments, taking advantage of new opportunities provided by cities, such as mild weather, reduced predator density and increased availability of food, water, and nesting sites (Fokidis et al., 2008; Saaroni et al., 2000; Sumasgutner et al., 2014). Many avian species have a low tolerance to urbanization, though other species tolerate or apparently thrive in urbanised areas. Highly urban areas generally support lower biodiversity than rural or natural environments (McKinney and Lockwood, 1999; Shochat et al., 2010). Furthermore, urbanization is projected to increase which is projected to reduce urban biodiversity (McDonald et al., 2018, 2020). Urbanization has negative effects on the body condition and tarsal length of birds, resulting in urban birds being characteristically smaller and in poorer body condition than rural birds (Biard et al., 2017), previously observed in the house sparrow (Liker et al., 2008). Both evidence for immune suppression (e.g. Kamiński et al., 2024) and (pathogen-related) immune stimulation in urban birds has been found. Effects of urban environments on immunity in urban birds have been attributed to food quality and abundance, pollution, and stress although conclusive data across species is still lacking (Minias, 2023). Most studies addressing this issue rely on phenotypic evaluation, although more recently transcriptomic approaches have evidenced differences in gene expression between urban and rural birds (Capilla-Lasheras et al., 2017; Watson et al., 2017). Likewise, differences in pathogen exposure have been stated for birds in urbanised environments depending on pathogen transmission routes (direct vs vector based) suggesting on one hand reduced exposure to for example, vector-transmitted haemoparasites due to reduced vector populations (Fokidis et al., 2008; Ferraguti et al., 2016) in contrast to evidence for increased exposure to also vector- transmitted West Nile virus across different bird species (Bradley et al., 2008).

The house sparrow (*Passer domesticus*) is a gregarious, generalist, granivorous passerine bird of the family *Passeridae*. These monogamous birds, which breed in southern Europe from April to August, are an excellent bio-indicator of habitat quality from their first year of life (Anderson, 2006) because of their ubiquitous and extremely sedentary nature. Sparrows have a global distribution associated with highly urbanised environments (Herrera-Dueñas et al., 2017) and are considered a model species of urban "exploiter", as they can coexist with humans and take advantage of their waste for feeding (Anderson, 2006).

The diet of urban sparrows, with low antioxidant availability, leads to higher oxidative stress and higher oxidative enzyme activity compared to more rural habitats (Herrera-Dueñas et al., 2017). Alterations in the ω-6/ω-3, (omega-6/omega-3 fatty acid) ratio have been observed with an increase in ω-6 (pro-inflammatory) and a decrease in ω-3 (anti-inflammatory) in urban birds (Arnold et al., 2015). Furthermore, a diet high in carbohydrates and saturated fats and low in protein, such as that of urban sparrows, results in increased serum glucose and lower uric acid and liver-free glycerol levels, demonstrating that urbanization significantly alters the nutritional physiology of house sparrows (Gadau et al., 2019).

In recent decades, there has been a marked decline in sparrow numbers, most significantly in urban sparrows, while in rural areas the trend appears to have stabilised (Ghosh et al., 2010). Both, pollution, for instance, from unleaded fuels (Dandapat et al., 2010) and heavy metals (Kekkonen et al., 2012), and pathogens, such as malaria parasites (Dadam et al., 2019), have been considered as possible causes for these declines.

Sparrow populations face threats that can have significant ecological impacts such as parasites (avian malaria) and viruses and are also considered potential carriers of the latter (Anderson and May, 1979; Johnson et al., 2015). Parasites such as avian malaria (*Plasmodium* and *Haemoproteus*), transmitted by dipterans, are common in wild birds and can affect sparrows' health and survival (Ferraguti et al., 2021; Martínez-de la Puente et al., 2010; Marzal et al., 2005).

Viruses such as avian influenza virus (AIV) and West Nile virus (WNV) pose additional risks to birds. AIV can cause economic losses in poultry and affect wild birds, including sparrows, which can transmit the virus between wildlife and domestic animals (Brown et al., 2007; Peterson et al., 2008; Spickler et al., 2008). WNV, transmitted mainly by *Culex* mosquitoes, affects wild birds and has been experimentally shown to infect sparrows, which are considered reservoirs especially in urban areas and competent hosts for virus transmission to ornithophilic mosquitoes, posing a risk to human health (Del Amo et al., 2014; Kernbach et al., 2019; López et al., 2008; Nasci et al., 2002).

The physiology of the house sparrow can be affected by a wide range of factors, some of them related to infections of various kinds or to the urbanization status (Gadau et al., 2019). This can lead to a modification of essential functions, resulting in a decrease in the number of individuals, especially at the urban level. To study specifically how the environment and/or pathogens can affect sparrows, we must consider that pathogen-host interactions are multidimensional in nature, as they can alter several components of the host, such as the transcriptome, proteome, mRNA, metabolome and lipidome (Mishra et al., 2017). In recent years, most studies on pathogen-host interactions have focused on the transcriptome, to identify elements important in disease regulation. For instance, the quantification of gene expression in urban and rural great tit (Watson et al., 2017), was found to be related to immune and inflammatory responses, gene regulation, etc. Nevertheless, transcriptomic studies cannot provide information on post-transcriptional regulation, post-translational modifications, or protein-protein interactions (Josset et al., 2013). Proteomics, in contrast, can be used to explore large-scale protein expression, protein-protein interactions, and post-translational modifications (Gingras et al., 2007; Altelaar et al., 2013), and thus deepen the systems-level understanding of the differences in metabolic, immune and even infectious processes of individuals from different environments. This integrates a different 'omic' approach into the investigation of immune adaptations to urban life which is needed to provide a more complete picture of their status (Minias, 2023). Here we provide the first exploration of the serum proteome in urban and rural house sparrows and evaluate its potential relation to pathogen exposure.

Based on the available information our starting hypothesis for this study was that exposure of sparrows to urban, compared to rural environments, impacts metabolic and immune pathways. This affects house sparrow serum proteome composition, which as a consequence affects the susceptibility of urban house sparrows to infection if they are

exposed to pathogens evaluated in thus study.

Thus, the aim of this work is to study the effect of urbanization on house sparrows, both in terms of pathogen prevalence (AIV, flavivirus and avian malaria) and serum protein variation, by comparing individuals from urban and rural populations.

2. Material and methods

2.1. Study area

House sparrows were sampled in two different environments, rural and urban (all areas considered rural are very close to livestock operations) in central and Southern Spain (Fig. 1), characterised by a continental Mediterranean climate with hot and dry summers, and cold and moderately rainy winters, and large variations in diurnal temperatures. Samples were taken at the end of the wintering period and prior to breeding, between January 14 and April 14, 2021.

We classified localities into rural and urban, based on the habitat type and number of inhabitants, (Fig. 1). Rural sparrows were captured at a red-legged partridge farm on the outskirts of Villarubia de los Ojos, Castilla —La Mancha (9902 inhabitants) and a captive breeding centre for endangered birds of prey, Fundación Aquila, in Lagartera (1405 inhabitants) with nearby extensive livestock (sheep and cattle) grazing. Urban sparrows were captured at the Veterinary Faculty teaching farm, Complutense University Madrid (3,323,000 inhabitants), and at the facilities of an urban zoological garden in Jerez, Cádiz (213,105 inhabitants).

2.2. Bird sampling

House sparrows were selected as a model species because of their high abundance, and their sedentary nature (Anderson, 2006). They are a suitable species to assess the impact of urbanization and pathogen infection on individual traits because they are distributed in both urban and rural environments and are sensitive to atmospheric changes, allowing comparative studies (Liker et al., 2008; Parra Ochoa, 2014). Also, considerable data is available on their susceptibility to the pathogens, such as AIV, WNV and haemoparasites, studied here.

Birds were captured at the end of the winter period and before breeding, using mist nets set at dawn and dusk, then transferred to individual cloth collection bags until handling. Each individual was ringed

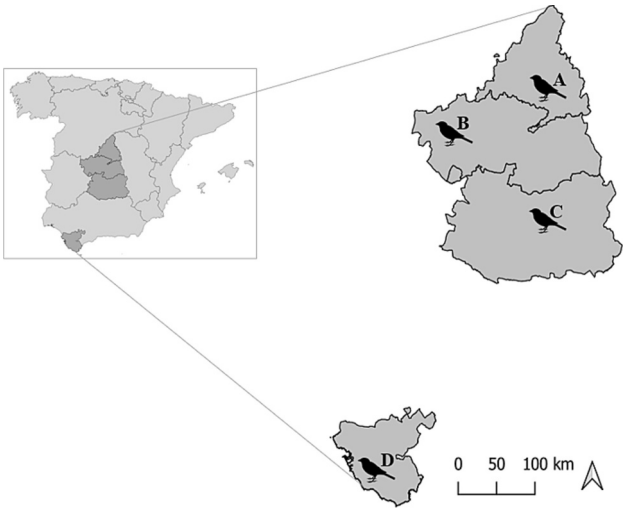

Fig. 1. Sampling locations in South Central Spain. Urban: A (Madrid, 3,323,000 inhabitants; W3°44′25.3″, N40°26′58.2″) and D (Jerez, 213,105 inhabitants; W6°8′14″, N36°41′12″). Rural: B (Lagartera, 1405 inhabitants; W5°12′34.1″, N39°56′11.2″) and C (Villarrubia de los Ojos, 9902 inhabitants; W3°38′25.8″, N39°12′12.6″).

(Euring-scheme), sexed, weighed using a digital scale (± 0.01 g) and tarsus length was measured to the nearest (± 0.01 mm with a digital caliper). We collected cloacal and oropharyngeal swab samples in viral transport medium (Hank's balanced solution containing 10 % glycerol, 200 u/ml penicillin, 200 μg/ml streptomycin, 100 u/ml polymyxin B, 250 μg/ml gentamicin and 50 u/ml nystatin) (Munster et al., 2007). Blood samples were drawn from the jugular vein using sterile syringes with sterile 29G needles impregnated with heparin. The collected blood was immediately transferred to sterile eppendorf tubes, and centrifuged in the field, to separate the cell fraction from serum, and stored on ice. All samples were transferred to −80 °C until further analysis, within 8 h of collection.

2.3. Ethical statement

This study was authorised by the ethical committee of the University of Castilla-La Mancha (OHPR202214) and the Junta de Comunidades de Castilla-La Mancha (avp22_123) and was conducted in accordance with all applicable institutional and national guidelines for the care and use of animals in experimentation. The capture and banding of the birds were carried out by expert ringers certified either by the Spanish Ornithological Society (SEO), or Aranzadi Science Society.

2.4. Pathogen detection

Pathogen detection was performed using DNA and RNA extraction followed by targeted PCR and real time RT-PCR assays to identify avian influenza virus, flaviviruses, and haemosporidia infections.

For pathogen detection, DNA was extracted from the cell fraction using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. In addition, cloacal swabs were pooled from 5 individuals and RNA was extracted with the High Pure RNA Isolation kit (Roche diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. Pooling swabs is a commonly accepted methodology for screening of avian influenza viruses in avian samples. This approach not only reduces costs but also dilutes potential inhibitors present in cloacal swabs that can hinder the amplification of genetic material, thus improving test efficiency and pathogen detection (Fereidouni et al., 2012; Hepker et al., 2024; Indriani et al., 2010).

Extracted RNA was challenged for Avian influenza virus (AIV) using a real-time RT-PCR described by Ward et al. (2004), modified according to Munster et al. (2007). A generic real-time generic RT-PCR was used for the detection of Flaviviruses following Moureau et al. (2007). All real-time PCRs were performed on an IQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad, Richmond, CA, USA).

All samples were examined for haemosporidia infections by a nested PCR, a modified PCR intended to reduce non-specific binding in products, and thus increase the specificity of the test, using the protocol described by Waldenström et al. (2004), designed to amplify a portion (479 bp) of the mitochondrial cytochrome *b* gene of *Plasmodium* spp., *Haemoproteus* spp., and *Leucocytozoon* spp. parasites. Nested PCRs use a second pair of "internal" primers to amplify a region within the product of the first PCR greatly increasing sensitivity and reducing the number of false positives and unspecific reactions (Green and Sambrook, 2019). PCRs were performed on a C1000 Touch PCR thermal cycler (Bio-Rad, Richmond, CA, USA).

2.5. Sequencing

Haemosporidia identification PCR positive samples as *Plasmodium* spp., *Haemoproteus* spp., and *Leucocytozoon* spp. parasites was confirmed through Sanger sequencing, followed by sequence analysis and comparison with malaria parasite sequences in GenBank.

To confirm species identification of the pathogen, samples that tested positive by nested PCR for haemoparasites were submitted for

purification and Sanger sequencing (SECUGEN SL, Madrid, Spain). The sequences obtained were analysed and edited using Chromas 2 software (Technelysium Pty Ltd., South Brisbane, Australia). Nucleotide BLAST (megablast algorithm) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare our amplified sequences with available sequences of malaria parasites deposited in GenBank.

2.6. Serology and haemagglutination inhibition (HIH) test

Antibodies against AIV, WNV, and cross-reacting flaviviruses were detected using competitive ELISAs, with positive AIV samples further analysed for H5/H7 serotypes by hemagglutination inhibition assays.

For the detection of antibodies against AIV, WNV and cross-reacting Flaviviruses in sparrow serum samples, competitive ELISAs using commercial kits (Ingenasa-Eurofins, Madrid, Spain) were performed following the manufacturer's instructions.

Samples positive for AIV antibodies in the competitive ELISA were subsequently tested by HIH for the presence of antibodies against AIV serotypes H5/H7. Procedures were performed according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (WOAH, 2023), with reference antigens, H5N2 (A/Ost/Den/72420/96) and H7N7 (A/Tky/Eng/647/77), from the Animal and Plant Health Agency laboratory (APHA, Weybridge, UK).

2.7. Statistical analysis

We used two statistical models to analyse the effects of urbanization on body condition (scaled mass index) and prevalence of avian malaria, the only pathogen detected by PCR, as the results for AIV and WNV were negative.

Scaled mass index (SMI), as proposed by Peig and Green (2009), was calculated separately for both sexes and for adult and juvenile individuals, to obtain an estimate of the body condition for each of the 181 individuals sampled.

We analysed the influence of urbanization on body condition and haemoparasites prevalence using R-4.3.0 software (R Core Team, 2020). The results were considered significant at a $p < 0.05$. Two models were performed, a generalised linear model (GzLM) with a binomial distribution and logit link, using the probability of avian malaria infection (positive/negative) as the response variable and two environmental levels (urban, rural), sex (male, female), body condition (SMI) and the interactions with urbanization as predictors in the model.

In addition, the effect of urbanization on individual condition was evaluated by means of general linear model (GLM). The model included body condition (SMI) as response variable and sex, environment (rural/urban), infection status (presence/absence of avian malaria) and the interactions between them as predictors.

2.8. Serum proteomics

To identify and compare serum proteins, sparrows from urban and rural environments were grouped, and serum pools were analysed using tandem mass tag (TMT) labelling and mass spectrometry, enabling the identification and quantification of proteins differentially represented between environments and their associated biological processes.

To identify and compare the serum proteins we classified the sparrows as belonging to two groups, one from rural and one from urban origin and, therefore, exposed to different environmental conditions and created serum sample pools. Combining individuals with the same conditions in groups allows us to identify which proteins are representative for each condition, and in which biological activities they are involved and thus relate them to each other and the potential causes for their modification. For this reason, we created pools of sera that were as homogeneous as possible, in terms of health status, origin and maintaining the sex ratio. Each pool contained samples from 5 individuals in urban and 6 individuals in rural areas and for each condition (urban/rural) we analysed six biological replicates.

The serum pools were analysed at the Proteomics Service of the Centro de Biología Molecular Severo Ochoa (CBMSO), Cantoblanco, Madrid, by tandem mass tag (TMT)-labelling followed by reverse phase liquid chromatography coupled to mass spectrometry (RP-LC-MS/MS). Briefly, protein serum samples were on-gel concentrated and visualised by Coomassie staining. The unseparated proteins bands were excised, cut into cubes (2 × 2 mm) and trypsin digested with sequencing-grade trypsin (Promega, Madison, WI) as previously described (Villar et al., 2015).

A total of 60 μg of peptides from each sample were labelled using the TMT Isobaric Mass Tagging Kit (Thermo Fisher Scientific, MA, USA) as described by manufacturer. Each set of labelled peptides was mixed and fractionated using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific, MA, USA) as described with minor modifications. Each sample re-swollen in 0.1 % TFA (*Trifluoroacetic acid*) and then, loaded onto an equilibrated, high-pH, reversed-phase fractionation spin column. A step gradient of increasing acetonitrile concentrations (5–80 %) in a volatile high-pH (Triethylamine (0.1 %) is then applied to the columns to elute bound peptides into nine different fractions collected by centrifugation. The fractions obtained from high-pH, reversed-phase labelled mixture were dried and stored until analysis by mass spectrometry for quantification.

The fractions were resuspended in 0.1 % formic acid and analysed by RP-LC-MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro hybrid mass spectrometer (Thermo Scientific). The peptides were concentrated (online) by reverse phase chromatography using a 0.1 mm × 20 mm C18 RP precolumn (Thermo Scientific), and then separated using a 0.075 mm × 250 mm C18 RP column (Phenomenex) operating at 0.22 μl/min. Peptides were eluted using a 90-min dual gradient. The gradient profile was set as follows: 5–25 % solvent B for 68 min, 25–40 % solvent B for 22 min, 40–100 % solvent B for 2 min and 100 % solvent B for 18 min (solvent A: 0.1 % formic acid in water, solvent B: 0.1 % formic acid, 80 % acetonitrile in water). ESI ionization was done using a Nano-bore emitters Stainless Steel ID 30 μm (Proxeon) interface at 2.1 kV spray voltage with S-Lens of 60 %.

The instrument method consisted of a data-dependent top-20 experiment with an Orbitrap MS1 scan at a resolution (m/Δm) of 30,000 followed by twenty high energy collision dissociation (HCD) MS/MS mass-analysed in the Orbitrap at 7500 (Δm/m) resolution. MS2 experiments were performed using HCD to generate high-resolution and high mass accuracy MS2 spectra.

The minimum MS signal for triggering MS/MS was set to 500. The lock mass option was enabled for both MS and MS/MS mode and the polydimethylcyclosiloxane ions (protonated (Si(CH3)2O))6; *m/z* 445.120025) were used for internal recalibration of the mass spectra.

Peptides were detected in survey scans from 400 to 1600 amu (1 μscan) using an isolation width of 1.3 u (in mass-to-charge ratio units), normalised collision energy of 40 % for HCD fragmentation, and dynamic exclusion applied during 60 s periods. Charge-state screening was enabled to reject unassigned and singly charged protonated ions.

Peptide identification from raw data (a single search was performed with all nine raws from the fractionation) was carried out using PEAKS Studio Xpro search engine (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada). Database search was performed against uniprot-Passeridae (47,612 entries; UniProt release 07/2022) (decoy-fusion database). The following constraints were used for the searches: tryptic cleavage after Arg and Lys (semispecific), up to two missed cleavage sites, and tolerances of 20 ppm for precursor ions and 0.05 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation, N-ethylmaleimide on cysteines and Cys carbamidomethylation and fixed TMT reagent labelling at the N-terminus and lysine residues. False discovery rates (FDR) for peptide spectrum matches (PSM) was limited to 0.01. Only those proteins with at least two unique peptides being discovered from LC/MS/MS analyses were considered reliably identified and sent to be quantified.

Quantitation of TMT labelled peptides was performed with PEAKS Studio Xpro search engine, selecting "Reporter Ion Quantification iTRAQ/TMT" under the "Quantifications" options (Zhou et al., 2019). We used the auto normalization mode to calculate global ratios from the total intensity of all labels in all quantifiable peptides and perform inter-experiment normalization with spiked channel "MIX". The -10LgP, Quality (5) and Reporter Ion Intensity (1e4) were used for Spectrum filter and Significance (PEAKSQ method) was used for peptide and protein abundance calculation. We evaluated protein groups for peptide uniqueness and used only unique peptides for protein quantification.

Differentially represented proteins were individually searched in the UniProt database (https://www.uniprot.org/) to identify the biological processes and molecular functions of the proteins.

3. Results

We sampled a total of 181 sparrows, 51 females (28.18 %) and 130 males (71.82 %). Of these, 127 (70.17 %) came from rural areas and 54 (29.83 %) from urban areas, all of them adults (Table 1). Since it was not possible to collect enough blood from some individuals, 178 individuals were tested for avian malaria and 172 individuals were tested for AIV and Flavivirus seroprevalence, due to the availability of blood and serum, respectively (Table 1). The purity conditions of the blood serum required for serum protein analysis allowed the inclusion of samples from 66 individuals (36 from rural areas and 30 from urban areas) in the serum proteomic analysis (Table 1).

Regarding the influence of urbanization on body condition and pathogen prevalence it should be noted that when both models were reduced by eliminating the non-significant explanatory variables, the significant result obtained for the "Environment" variable was maintained (Table 2).

The SMI of sparrows differed significantly between the two environments, independent of sex, being higher in sparrows from rural areas (GLM, β =0.4084, SD = 0.5437, Df = 1, $p < 0.001$) than in from urban areas (Fig. 2, Table 2).

All sparrows were negative for AIV and Flavivirus by real-time RT-PCR.

In total, 64.04 % (114/178) of the sparrows from which blood was obtained were infected by avian malaria parasites. Infections were equally probable in both sexes (Fig. 3, Table S1), but the probability of infection is significantly lower in urban (27/54) (GzLM, $\beta = -6.7493$, SD = 6.2336, Df = 1, $p = 0.00882$) than in rural environments (87/124).

We recorded sequences from 107 *Plasmodium* (107/114, 93.86 %) and 7 (7/114, 6.14 %) *Haemoproteus* species, two rural sparrows from Villarrubia de los Ojos were co-infected with both species. According to sequence similarity with reference sequences available in GenBank, house sparrows were infected by *Plasmodium* spp. ($n = 105$) and *Haemoproteus* spp. ($n = 14$).

None of the sparrows tested ($n = 172$) had antibodies to Flavivirus, and only one individual (0.58 %), from an urban environment (Jerez Zoo) had antibodies to AIV. Subsequent analysis by HI ruled out antibodies to an H5 or H7 subtype, suggesting that the individuals' exposure had likely been to a low pathogenic AIV of a subtype other than H5 and H7.

Table 2
Summary of the results of the GzLM (Model 1) and GLM (Model 2) for the effects of the explanatory variables (avian malaria, scaled mass index (SMI)) on the response variables: sex M(male), environment U(urban) and interactions. Significant effects are highlighted in bold.

Model 1. GzLM. Avian malaria					
		β (±SD)	SD	Df	p
Sex	M	5.9473	5.4737	1	0.50051
Environment	U	−6.7493	6.2336	1	**0.00882**
SMI		0.1675	6.0256	1	0.46181
Sex: SMI	M	−0.2149	0.2019	1	0.34583
Environment: SMI	U	0.2122	0.2292	1	0.34049
Sex: Environment	M:U	0.2549	0.7975	1	0.74881
Model 2. GLM. SMI					
Sex	M	0.4091	0.5391	1	0.87119
Environment	U	−0.8778	0.6069	1	**0.00073**
Malaria		0.4084	0.5437	1	0.47158
Sex: Malaria	M	−0.5059	0.5987	1	0.39937
Environment: Malaria	U	0.4411	0.5674	1	0.43795
Sex: Environment	M:U	−0.4617	0.6311	1	0.46544

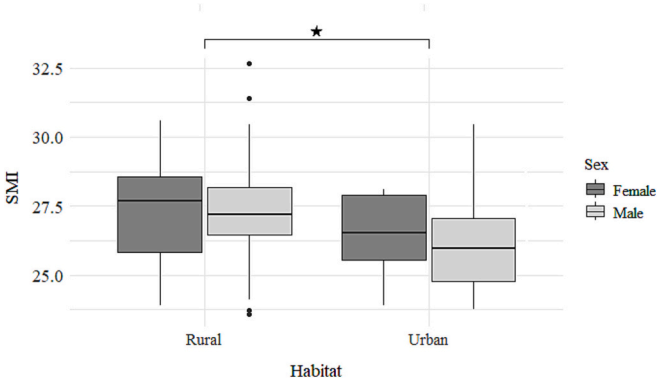

Fig. 2. Boxplot of SMI (scaled mass index) of house sparrows sampled as a function of environment (rural/urban) and sex (female/male). Dots above the boxes denote outliers in each group and significant differences are represented by an asterisk. (GLM, β =0.4084, SD = 0.5437, Df = 1, $p < 0.001$).

A total of 61 proteins were identified in serum samples, of which 16 proteins showed significant differences after TMT quantitative analysis, with a probability of error of 1 % and a significance of 20. The proteins differentially represented among the samples are mostly involved in immune response, cellular metabolism, and coagulation (Fig. 4, Table S2). Within the set of modified proteins related to the immune response, proteins belonging to the C5 component (A0A3L8S2M0) of the complement system, the immunoglobulins complex (A0A3L8Q4U9), Ig J (A0A7K5R338), the A2M protein (A0A3L8Q8M4) of the TED domain of the C3, C4 and C5 components and pentraxin (PXT) (A0A7L3AGT3) stand out. We observed a consistent over-representation of proteins related to the immune response in urban sparrows (Fig. 5, Table S2).

Table 1
Summary of data obtained from house sparrows sampled according to environments (rural/urban) and sex (female/male) and number of samples analysed for the different pathogens. Percentage of sampled or analysed individuals (number of analysed or sampled individuals/total of individuals). n (number of individual), F (female), M (Male).

Environment	Sex	n	n/n_{total}	Avian malaria		Serology		Proteome	
				n	$n_{total/}$ environm	n	$n_{total/}$ environm	n	$n_{total/}$ environm
Rural	F	36	70.17 %	34	69.66 %	33	69.19 %	8	54.55 %
	M	91	(127/181)	90	(124/178)	86	(119/172)	28	(36/66)
Urban	F	15	29.83 %	15	30.34 %	15	30.81 %	8	45.45 %
	M	39	(54/181)	39	(54/178)	38	(53/172)	22	(30/66)

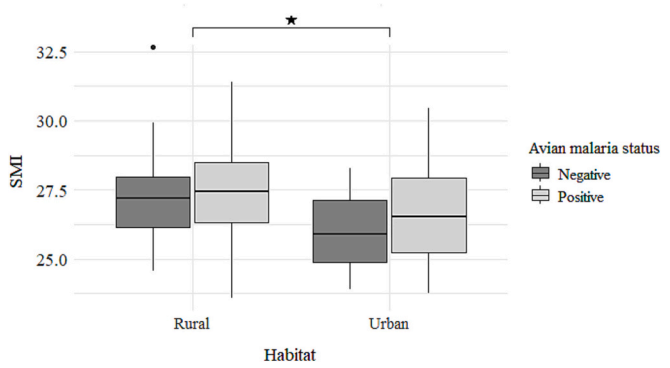

Fig. 3. Boxplot of avian malaria parasite prevalence differentiated by environment (rural/urban) . Dots above the boxes denote outliers in each group and significant differences are represented by an asterisk. (GzLM, $\beta = -6.7493$, SD $= 6.2336$, Df $= 1$, $p = 0.00882$).

In the set of metabolic proteins, Betaine-Homocysteine Methyltransferase (BHMT)(A0A7K5R218), the Glycoside Hydrolase family 31 (GH31) (A0A7K5R0Z9), Citrate Synthase (CS) (A0A3L8RYU5), Lecithin Cholesterol Acyl Transferase (LCAT) (A0A7K5R7U4) and Malate Dehydrogenase (MDH) (A0A3L8SRF3) stand out for their deregulation. In contrast to immune system related proteins, we detected a greater variety among the different proteins, with a tendency to increase in rural sparrows, except for the LCAT family proteins, which increase in urban sparrows and remain unchanged in rural sparrows (Fig. 6, Table S2).

Finally, coagulation factor II (FII), or Prothrombin (TP), (A0A3L8SWJ0), a protein related to the coagulation process shows increased expression in urban sparrows (Fig. 7, Table S2).

4. Discussion

To the best of our knowledge, our study is the first to evaluate the serum proteome of urban and rural house sparrows. In line with previous work, we compared the physical condition and prevalence of certain pathogens in sparrows from environments with different urbanization status . This allows us to relate proteomic changes, body condition and malaria parasite status in house sparrows to the urbanization status and evaluate the possible consequences that the environment may have on these birds.

The proteomic profile shows differences between urban and rural sparrows, indicating variations in the expression levels of certain proteins. Proteome analysis revealed the activation of certain metabolic pathways in rural sparrows, while in urban sparrows the proteomic modifications were those associated with immune and coagulation processes.

In this study, house sparrows in urban environments in central and southern Spain have poorer body condition than house sparrows in rural environments, which is consistent with results obtained in previous studies (Herrera-Dueñas et al., 2017; Liker et al., 2008; Bókony et al., 2010). However, in contrast to Liker et al. (2008), who found a better body condition in males, we did not detect significant differences between sexes. This may relate to the timing of our study, with captures at the end of winter and before breeding, when the conditions to which both sexes are subjected are more similar than at other times of the year such as the breeding or post-breeding period.

Differences in SMI between urban and rural sparrows can be explained by the resources available in each habitat. Sparrows, are omnivorous and opportunistic birds that adapt their diet according to the environment. In rural areas, during the juvenile and breeding phase, their diet is based mainly on insects and other protein-rich invertebrates, while in adulthood it becomes mainly granivorous (Murgui and Macias, 2010). In contrast, in urban environments, their diet consists of bread, birdseed and, to a lesser extent, arthropods (Galbraith et al., 2014), which negatively affects their growth (Heiss et al., 2009). This less nutritious diet has been shown to reduce the number of fledglings and chick survival (Kekkonen et al., 2012; Peach et al., 2008). Urban birds are smaller and have poorer body condition than rural birds, consistent with our results (Biard et al., 2017; Liker et al., 2008).

House sparrows were sampled because this species is considered a potential bridge in avian influenza (AIV) transmission (Forrest et al., 2010) and a competent reservoir and host for West Nile virus (WNV) (Del Amo et al., 2014; Kernbach et al., 2019). These birds are especially relevant in urban settings, as they coexist with humans and can be an important reservoir for these zoonoses (Mostafa et al., 2018; Rouffaer et al., 2017). However, sampling was performed in a non-optimal period for the detection of these pathogens, which could explain the negative results obtained by PCR. This inadequate timing is because it was performed before the optimal period of Flavivirus vector activity, which in southern Europe occurs from May to October (Nasci et al., 2002; ECDC, 2024), outside the classical season for AIV circulation (winter) and before H5N1 highly pathogenic AIV circulation in Spain in 2022. Furthermore, the lack of detection of these pathogens in sparrows could also be explained by the short duration of viremia and excretion in natural infections (Brown et al., 2007; Pérez-Ramírez et al., 2014).

In contrast to the viral pathogens examined, we detected a high prevalence of haemoparasites. Previous studies show that these parasites, especially *P. relictum,* are frequent in house sparrows (Bichet et al., 2013) and can have a significant impact on the health and survival of sparrow populations (Dadam et al., 2019; Martínez-de la Puente et al.,

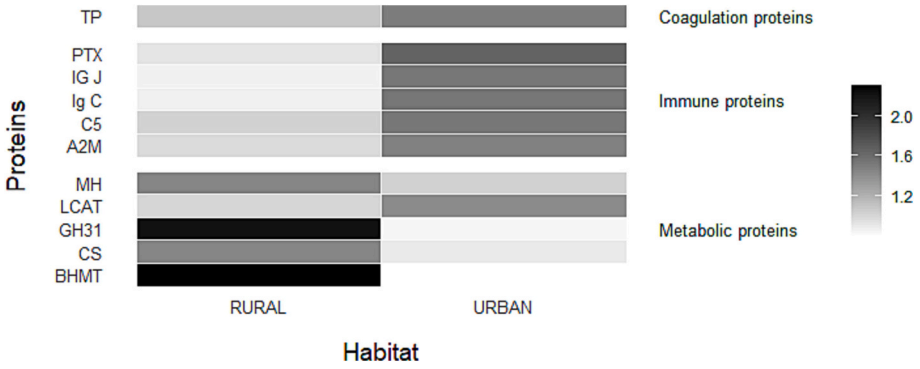

Fig. 4. Serum proteins related to immune response, metabolism and coagulation with modified detection according to rural and urban environments. Coagulation protein: Prothrombin (TP). Immune proteins: Pentraxin (PTX) (A0A7L3AGT3), C5 component (C5) (A0A3L8S2M0), Immunoglobulins complex (Igs) (A0A3L8Q4U9), Immunoglobulin J (Ig J) (A0A7K5R338), A2M protein (A2M) (A0A3L8Q8M4) of the TED domain of the C3, C4 and C5 components. Metabolic proteins: Malate Dehydrogenase (MDH) (A0A3L8SRF3), Lecithin Cholesterol Acyl Transferase (LCAT) (A0A7K5R7U4), Glycoside Hydrolase family 31 (GH31) (A0A7K5R0Z9), Citrate Synthase (CS) (A0A3L8RYU5) and Betaine-Homocysteine Methyltransferase (BHMT) (A0A7K5R218).

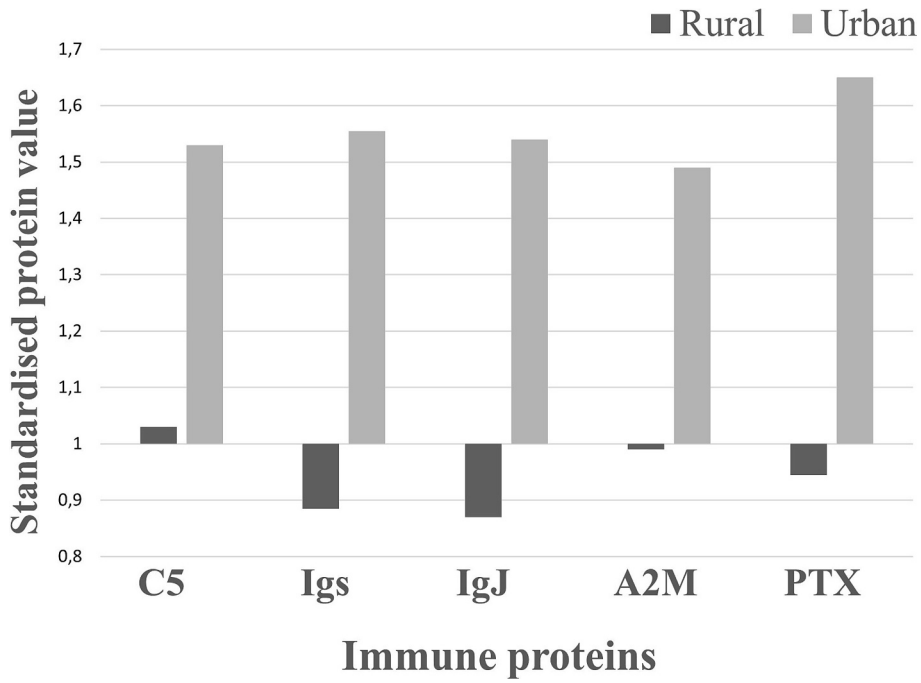

Fig. 5. Proteins involved in the immune response with modified presence in the different groups (urban and rural sparrows). Pentraxin (PTX) (A0A7L3AGT3), C5 component (C5) (A0A3L8S2M0), Immunoglobulins complex (Igs) (A0A3L8Q4U9), Immunoglobulin J (Ig J) (A0A7K5R338), A2M protein (A2M) (A0A3L8Q8M4) of the TED domain of the C3, C4 and C5 components.

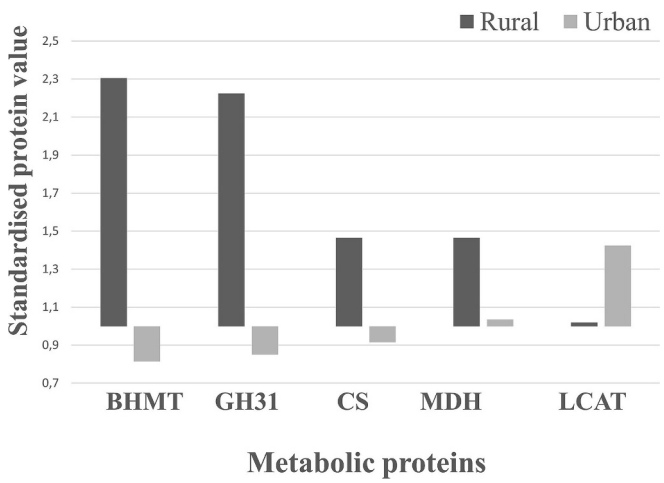

Fig. 6. Proteins involved in metabolism with modified presence in the different groups (urban and rural sparrows). Malate Dehydrogenase (MDH) (A0A3L8SRF3), Lecithin Cholesterol Acyl Transferase (LCAT) (A0A7K5R7U4), Glycoside Hydrolase family 31 (GH31) (A0A7K5R0Z9), Citrate Synthase (CS) (A0A3L8RYU5) and Betaine-Homocysteine Methyltransferase (BHMT) (A0A7K5R218).

2010; Marzal et al., 2005). Prevalence of avian malaria was significantly higher in sparrows from rural compared to urban environments. Sparrows from rural habitats may be more frequently exposed to hematophagous vectors than sparrows from more urbanised environments, such as Jerez Zoo and the UCM teaching farm, where mosquito control protocols are in place (Quevedo, pers. comm).

In our study, birds with a higher SMI had a higher prevalence of malaria. In addition, we observed an indirect relationship between body condition and avian malaria parasite prevalence. It has been shown that immune responses to infections are energetically costly, so individuals with better body condition may develop more effective responses (Navarro et al., 2003), as energy trade-offs between physiological compartments may compromise the energy devoted to the immune system (Olsson et al., 2005; Milinski et al., 2010). In this study, rural sparrows have a significantly higher prevalence of avian malaria parasites and a higher SMI, which may suggest that rural individuals can better tolerate parasitaemia due to better body condition (Minias, 2023). Nevertheless, our observations may also relate to other additional factors (such as pollutants or vector exposure) that we have not been able to measure in this study. In our study, we try to harness the potential of proteomics to explore the mechanisms associated with certain observed traits, such as avian malaria parasitaemia or body condition associated with environment (urban/rural) and as a consequence the likely differences in diet (Gadau et al., 2019). However, we would like to emphasize that our study does not establish direct causal relationships between these variables, but rather identifies patterns of proteomic differentiation that can be correlated with other observed factors, thanks to previous studies that have revealed these correlations, based on analysis other than proteomics.

It is important to understand that the complexity of organisms seems to lie in proteins, as the same gene can give rise to different protein forms. The proteome can be defined as the complete set of proteins produced or modified by an organism or system (Wilkins et al., 1996). Unlike the genome, the proteome is highly dynamic and varies over time and with the requirements and conditions of the organism. For this reason, the study and comparison of the proteome in different metabolic, pathological or environmental situations makes it possible to identify those proteins whose presence, absence or alteration correlates with certain physiological stages (Issaq et al., 2002; Lubec et al., 2003).

In this context, our proteomic study provides information on the status of urban and rural sparrow populations studied at a specific time under specific conditions. However, as a relatively recent technique, no reference ranges and few previous studies in wild birds are available. In fact, this work represents the first comparative serum proteomics study in urban and rural house sparrows.

To minimize potential sources of bias and ensure reliable results, sparrows were grouped into homogeneous pools in terms of sex and environmental exposure. This approach allows us to identify which proteins are unchanged between groups and which are differentially

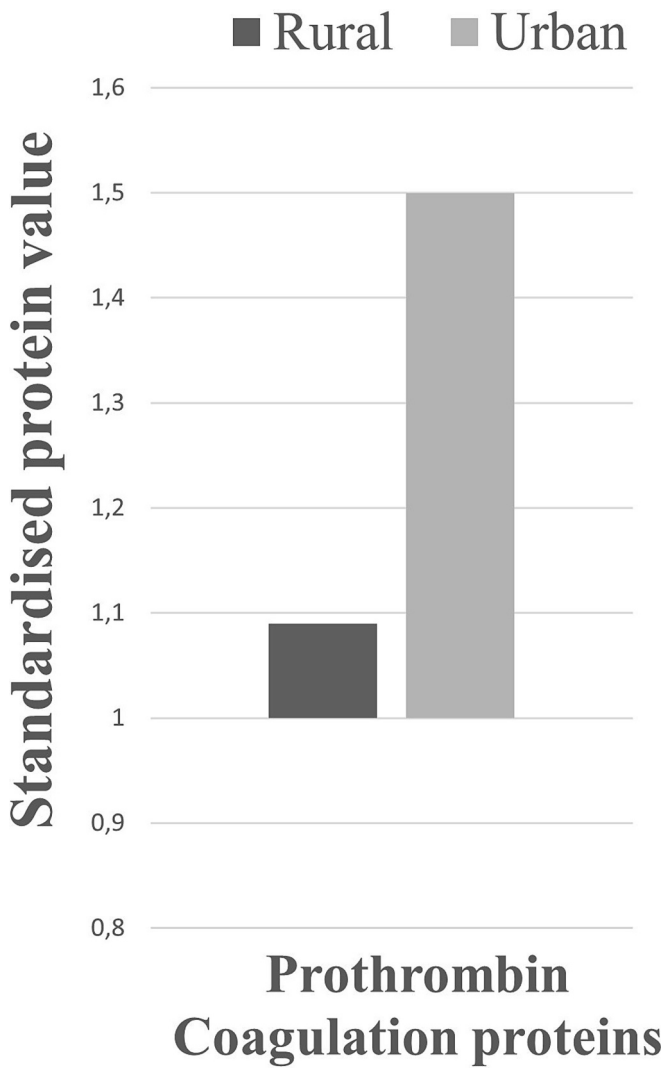

Fig. 7. Prothrombin, a protein involved in coagulation with modified presence in the different groups (urban sparrows and rural sparrows).

represented between rural and urban environments. These associations reflect observational differences, and we do not intend to imply direct causality. Instead, we provide a framework for the discussion of possible causes and biological activities involved in the observed modifications, building on previous literature and suggesting future directions for further investigation of these associations.

Within the set of proteins, the consistent over-representation of proteins related to the immune response in urban sparrows stands out. Within this set of proteins, the most markedly modified proteins include the complement activation immunoglobulins complex (A0A3L8Q4U9) and Ig J (A0A7K5R338) and the A2M proteins (A0A3L8Q8M4) of the TED domain with hydrolase activity. The C5 complement component (A0A3L8S2M0) is a glycoprotein involved in the immune response, promoting bacterial lysis, inflammation, chemotaxis, histamine release, and cytotoxic oxygen radical production. The presence of pentraxins (A0A7L3AGT3), which are implicated in acute immune responses, mediating agglutination, complement activation, and opsonization, was also found to be altered.

The consistent over-representation of immune response-related proteins in urban sparrows highlights a possible difference in the physiological responses of these birds compared to rural birds. Although it might initially seem contradictory to the perception that rural sparrows have a greater immune capacity, previous studies have reported similar findings. For example, Minias (2023) summarizes studies that have found pathogen-induced immune stimulation in urban birds and immunosuppressive effects associated with factors such as poor-quality diet, stress, and pollution in these environments.

In addition, previous research has noted the presence of proinflammatory components in urban sparrows versus predominantly anti-inflammatory components in rural sparrows (Arnold et al., 2015). In this study, we observed a lower representation of immune proteins in rural sparrows at the time of sampling. This lower level could be associated to the type of environment but notably also with the immunomodulatory effects attributed to malaria parasites, such as antigenic variation that may contribute to a decrease in immune activation in infected birds, documented in previous studies (Gomes et al., 2016; Knowles et al., 2010; Kurup et al., 2019; Martínez-de la Puente et al., 2010).

Unfortunately, in this context, our ability to assess the malaria-specific influence on these observed differences is limited, as we do not have comparative proteomic samples between malaria-positive and malaria-negative individuals in the different settings. However, our results provide a valuable starting point to further explore how environmental factors and infections affect the immune response in urban and rural bird populations.

Dietary differences in birds inhabiting urban and rural habitats, are a widely studied topic and have been suggested to, in addition to pollution, underlie many of the impacts of urban environments on body condition and immune capacity (Gadau et al., 2019; Galbraith et al., 2014; Heiss et al., 2009; Herrera-Dueñas et al., 2017). Interestingly we also observed considerable differences in the expression of metabolic proteins between our two study groups. Expression of the set of proteins involved in metabolic processes, is generally higher in rural sparrows, compared to the normalised value, than in urban sparrows. Especially BHMT (A0A7K5R218), which is involved in homocysteine metabolism, stands out for its modified detection. This is regulated by several nutrients, including folate (vitamin B and folic acid) and choline (a component of vitamin B), and reduced expression of BHMT has been associated with osmotic stress. Krebs cycle-related proteins such as MDH and CS (A0A3L8RYU5) involved in fatty acid biosynthesis are also altered. The GH31 family (A0A7K5R0Z9), are enzymes involved in carbohydrate catabolism and LCAT (A0A7K5R7U4) is involved in the extracellular metabolism of serum lipoproteins, including cholesterol. We can observe an increasing trend in rural sparrows, except for the LCAT family proteins, which increases in urban sparrows and remains practically unchanged in rural sparrows. The rural sparrows in the study come from a partridge farm and an eagle conservation centre, both surrounded by pastures and livestock operations. This implies that rural sparrows have virtually unlimited food and insects at their disposal, unlike urban sparrows that have limited access to a balanced diet that meets their needs (Gadau et al., 2019). Albeit, in this study, urban sparrows come from areas housing large numbers of captive animals (the UCM teaching farm and Jerez Zoo) that could facilitate the availability of insects, such as flies. However, the presence of pollutants with insecticidal effects and the existence of pest control protocols have been described in previous studies as potentially decreasing the availability of insects and other invertebrates, resulting in less nutritious diets (Galbraith et al., 2014; Heiss et al., 2009). Therefore, we can hypothesize the general increase, concerning the normalised value, in metabolic proteins in rural sparrows to be due to a greater amount of available nutrients and food, leading to more active metabolic pathways. Notably, the increase in LCAT in urban sparrows, an exception among proteins involved in metabolism, may be due to the activation of fat metabolism due to the high amount of saturated fat that has been reported in the diet consumed by urban sparrows (Heiss et al., 2009; Meyrier et al., 2017).

Other proteins with differentiated expression include an increase in prothrombin (A0A3L8SWJ0) a coagulation related protein in urban house sparrows. Prothrombin plays a role in blood homeostasis, inflammation, and wound healing.

In the sparrows in this study, the over-expression of prothrombin

may be due to activation of the coagulation cascade triggered after jugular puncture to obtain the corresponding blood sample. In humans, thrombocytopenia has been shown to occur in 60–80 % of malaria-positive patients (Fox et al., 2020), associated with a state of imbalance between coagulation and fibrinolytic factors. We can observe that in rural sparrows, the increase in prothrombin values is less marked, which may be related to the high prevalence of malaria, as this may decrease and/or imbalance the activation of the coagulation cascade.

In this study, we were limited by the number of samples from urban environments, due to the difficulty in obtaining them with the same sampling effort. In addition, due to the limited amount of blood that can be extracted from a sparrow, we have also been unable to perform certain complementary tests to support the results obtained. However, we demonstrated exposure to pollutants and changes in oxidative stress balance in urban and rural house sparrows in a previous study with individuals from similar origins (Alarcos et al., 2023). Despite this, the combination of proteomics, PCR and serology provides a comprehensive view of immunity and pathogen prevalence in sparrows. To expand our results, it would be advisable to increase the sample size and include analyses of pollutants and other environmental factors that may influence bird health. A broader, multidisciplinary approach would improve our understanding of the observed changes in sparrows and their environmental risks. To the best of our knowledge, this study provides data from the serum proteome in house sparrows for the first time, showing that it can reveal differences in the impact of anthropisation on sparrow responses, though we must emphasize that these changes in the proteome of this species occurred at a specific time point under specific conditions.

5. Conclusions

Biometry, habitat, and analysis of pathogen prevalence in relation to the serum proteome allow us to obtain a more complete picture of the health status of sparrows in different urban and rural environments. The serum proteome reflects variations in metabolic, immune and coagulation pathways between rural and urban sparrows at the time of sampling. We observed greater activation of proteins related to immune response and coagulation in urban sparrows, which could be associated with environmental stress and the more challenging conditions of these habitats. In contrast, rural sparrows had a higher representation of metabolic proteins, which could be related to a more varied diet due to the availability of foods typical of rural areas, in relation to the presence of crops and livestock.

These findings suggest the importance of further research on the effects of urbanization on the health of wild birds, particularly in those species in close contact with human environments, such as sparrows. It is essential to address these issues both from a species conservation perspective and in relation to potential risks to public and livestock health, taking an integrated One Health approach.

CRediT authorship contribution statement

Sara Minayo Martín: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Margarita Villar:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization. **Alberto Sánchez-Cano:** Writing – review & editing, Methodology, Investigation. **Catarina Fontoura-Gonçalves:** Writing – review & editing, Methodology, Investigation. **José Manuel Hernández:** Writing – review & editing, Resources, Methodology. **Richard A.J. Williams:** Writing – review & editing, Resources, Methodology. **Miguel Ángel Quevedo:** Writing – review & editing, Resources, Methodology. **Ursula Höfle:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.178920.

Data availability

Data will be made available on request.

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