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A multidisciplinary approach to the evaluation of the effects of foraging on landfills on white stork nestlings

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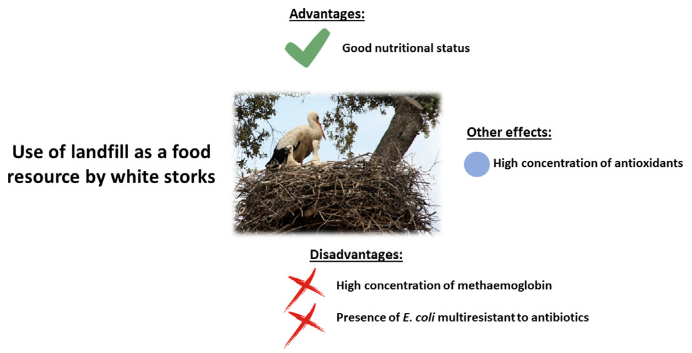
HIGHLIGHTS

- Foraging in landfills implied good nutritional status for white stork nestlings.
- Feeding in landfills increase the antioxidants in blood, possibly due to a hormetic response.
- Methaemoglobin increased in white stork nestlings fed landfill foraged food.
- Foraging in landfills increases the presence of multiresistant *E. coli*.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of landfills as foraging areas by white storks (*Ciconia ciconia*) is a recent well-known behaviour. While several studies have highlighted positive effects at a populational level others suggest that the presence of pollutants, pathogens and the lower presence of antioxidants in the food could pose a health risk for individuals. The objective of this study was to evaluate potential effects of the use of landfills as a food resource on the physiology and health of white stork nestlings, by a multidisciplinary approach based on the analysis of nutritional status, body condition, blood parameters, oxidative stress balance and the presence of pathogens. Results showed better body condition in individuals associated with landfills compared to the ones feeding on natural resources, as well as better nutritional status, as indicated by higher levels of albumin, cholesterol, and triglycerides in plasma. As many pollutants have a pro-oxidant effect, we evaluated oxidative stress balance, with no differences in the indicators of damage except for methaemoglobin (metHb), significantly higher in nestlings associated with landfill-origin food. Regarding antioxidants, GSH was higher in nestlings associated with landfills, which may suggest a hormetic response induced potentially by the presence of pollutants in waste. Nestlings fed food from landfills also had a higher presence of *Escherichia coli* with a multiresistant phenotype to antibiotics. In conclusion, our results show that nestlings fed with a higher proportion of food from landfills present a better nutritional status and body condition than those fed with a higher proportion of natural diet, being the only indicators of negative effects of the use of this food resource the higher percentage of metHb in the peripheral blood and the presence of antibiotic-resistant *E. coli*.

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1. Introduction

In the last decades, urban household waste landfills have become one of the most important Predictable Anthropogenic Food Subsidies (PAFS), due to the large amount of organic material present, which makes them both predictable and virtually unlimited as food resources (Oro et al., 2013). Therefore, landfills are highly productive feeding patches, with the potential to sustain many individuals, having an impact at the population level of numerous species worldwide (Plaza and Lambertucci, 2017), with the white stork (*Ciconia ciconia*) as one of the most iconic.

White storks are a well-studied species, primarily due to their association with anthropised areas (Ciach and Kruszyk, 2010; Flack et al., 2016, del Hoyo et al., 1992). They have a natural diet composed by earthworms, insects, fish, amphibians, and small mammals (Ciach and Kruszyk, 2010). In the 20th century, this species suffered a sharp decline primarily due to habitat destruction by agriculture intensification, as storks are opportunist foragers using large irrigated meadows and grasslands (Carrascal et al., 1993). However, since the end of the ‘80s, the population steadily increased, especially in Europe (BirdLife International, 2015). One of the main thrives for this increase has been related to the adaptation to the use of landfills as a food resource (Tortosa et al., 2002; Gilbert et al., 2016).

Feeding in landfills improves body condition, reproductive fitness, survival and the abundance of individuals in different species (Oro et al., 2013; Plaza and Lambertucci, 2017). In white storks, feeding on landfills has been associated with significantly higher breeding success in populations in the North of Africa, Central and Southern Europe (Djerdali et al., 2016a). In addition, the use of this feeding source has changed the migratory pattern of some individuals and age groups within the species, leading to shorter migration distance and wintering in Europe as an alternative to Africa (Archaux et al., 2004) or even complete loss of migratory behaviour in some other individuals, thus decreasing both daily energy expenditure (Flack et al., 2016) and the risk of mortality (Rotics et al., 2016). Furthermore, residency allows for the occupation of the better nesting locations, consequently providing an earlier onset of breeding phenology, which is related to larger clutches and higher breeding success (Vergara et al., 2007; Gilbert et al., 2016).

While overall the use of landfills appears to have had a positive impact on the species, negative aspects have also been described at both individual and colony levels. On one hand, potential physical damage could result from the presence of glass, metal, wire and plastic (Plaza and Lambertucci, 2017; Jagiello et al., 2020). Physical damage by ingestion of rubber bands, confused with earthworms, can affect the absorption of nutrients, may artificially induce satiety and their accumulation could also produce intestinal obstruction (Henry et al., 2011). Exposure to chemical pollutants, such as metals and organic compounds, can result from the presence of these in waste, mixed with organic material, because of their use in industrial products; i.e. lead in plastics and crystal glass; mercury in tubes and lamps and cadmium in batteries (Smith, 2009). Organic compounds such as bromated flame retardants have already been reported in eggs from storks feeding in landfills (Muñoz-Arnanz et al., 2011). In addition, food obtained from landfills could be deficient in some important dietary components especially organic compounds that rapidly degrade such as vitamins (Tauler-Ametller et al., 2019). Furthermore, in addition to physical damage and the exposure to toxic substances, the acquisition of pathogenic bacteria by birds feeding on waste has also been documented in the last years (Plaza and Lambertucci, 2018; Höfle et al., 2020). This represents a concern both because of the potential of pathogen dispersal by birds during local and migratory movements and due to the potential effects on nestling survival (Ciach and Kruszyk, 2010).

Previous studies demonstrated the effect of the use of this food resource on the dynamics of the population of white storks (Tortosa et al., 2002; Gilbert et al., 2016). However, there is a lack of information about the effect of the use of this resource at individual level. The objective of this study is to determine the different effects of the use of this food resource on the status of nestlings, for which we use a multidisciplinary approach based on the evaluation of nutritional status, blood parameters, oxidative stress balance and the presence of potential pathogen microorganisms. This approach will allow us to have an overview of both positive and negative effects at individual level by the use of this food resource.

2. Material and methods

2.1. Study area

We conducted our study on four white stork colonies located in the province of Ciudad Real in the south-central Iberian Peninsula (with a mean altitude of 629 m.a.s.l.) along a gradient of exposure to landfill foraged food (Fig. 1). Colony “Natural” (“N”) (384579 X, 4349217 Y) is inside a National Park; colony “Semi-Natural” (“SN”) (375153 X, 4306733 Y) is in an open oak forest with extensive sheep farming; colony “Semi-Landfill” (“SL”) (396688 X, 4287548 Y) is located at a landfill site that was sealed in 2007; and colony “Landfill” (“L”) (480827 X, 4362491 Y) is directly associated to an open active landfill site. All four colonies are part of a ringing scheme that has been in place for more than three decades which includes ringing of all nestlings of each colony with metal rings and coded white PVC rings for visual recapture. As a result, some of the adult breeders of each colony can be recognized, as well as each year's fledglings. During the study year and two consecutive years counts and visual recapture of ringed birds was carried out at the two major landfills in the area in ten-day intervals. In addition, two adult birds from each colony were captured during breeding on the nest (when they had recently hatched chicks) and tagged with satellite transmitters. Both, visual recapture from PVC rings and satellite tracking data confirmed that storks were, during the breeding season, spatially linked to their colonies with frequent use of landfills for foraging by birds from the L and SL colonies and local natural habitat foraging in the other two colonies, while during winter landfills were the primary foraging site for many individuals from all colonies (see Bécares et al. (2019) for analysis of the satellite tracking data). Furthermore, it has been established that the distance to the landfills is associated with the intensity of the use of this food resource (Bécares et al., 2019; Djerdali et al., 2016a; Gilbert et al., 2016), being the colony “N” 64.91 km from the nearest landfill; “SN” 60.95 km; “SL” 50.58 km and “L” less than 1 km of their nearest landfills.

2.2. Field procedures

In July 2011, at the end of the breeding season, 103 white stork nestling (43 ± 7 days old) were sampled (31 individuals from 11 nests in “N” colony; 30 from 13 nests in the “SN” colony; 31 from 15 nests in the “SL” colony; and 11 from 4 nests in the “L” colony) and equipped with a metal ring and a visual recapture ring (PVC rings). Each bird was weighed using a digital scale (to the nearest 5 g), and tarsus and beak length measured using an electronic calliper (to the nearest 1 mm). Sampling was done early in the morning to eliminate the potential effect of diurnal rhythm changes (Rodríguez et al., 2006). The use of nestlings as sample units ensures through parental food provisioning a representation of feeding sources from the specific habitat within which the colony stands.

Using sterile needles and syringes, blood samples (3 ml) were collected via venipuncture of the brachial vein. Immediately after extraction, two blood smears were prepared, air-dried and kept at room temperature. Approximately 0.01 ml of the sample was transferred to 1 ml absolute alcohol for sexing and the rest of the blood was transferred to sterile containers containing Lithium-heparine as an anticoagulant. With sterile cotton swabs, we collected cloacal swabs in AMIES transport medium (Deltalab, Barcelona, España) for the isolation of enterobacteria. Blood and swabs were refrigerated directly after

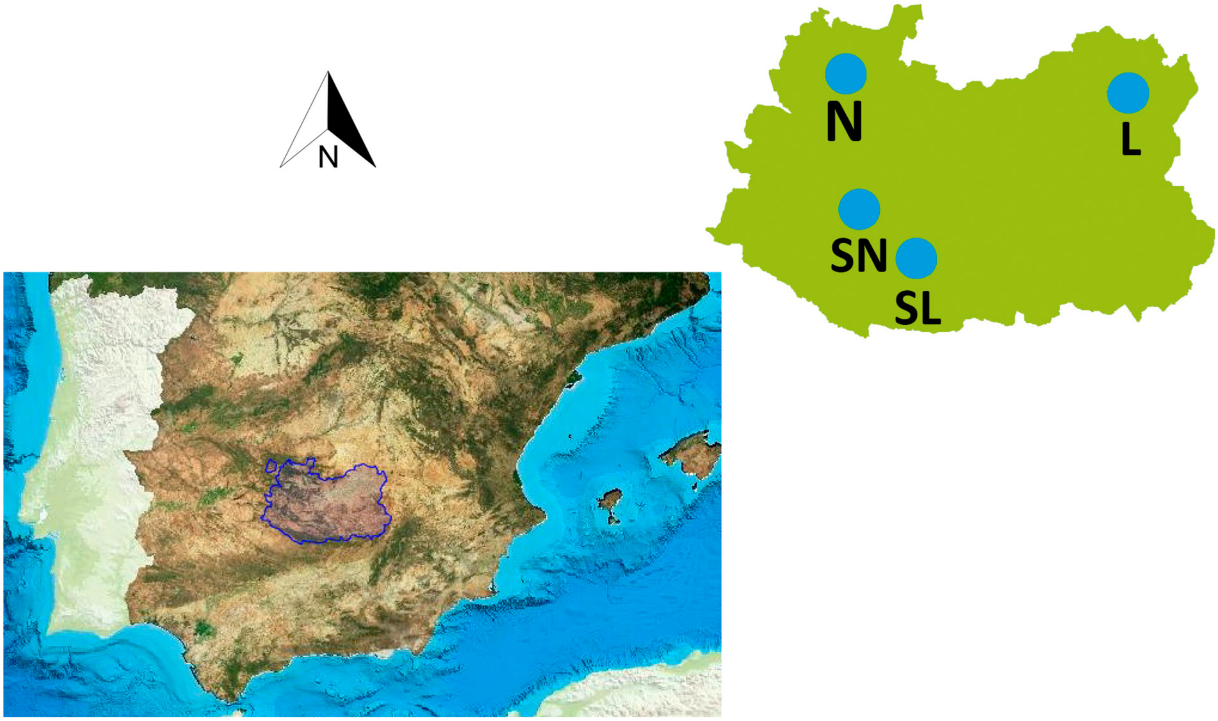

Fig. 1. Geographic location of the study colonies in the Iberian Peninsula.

extraction and were maintained at 4–8 °C until arrival at the laboratory in less than 12 h.

2.3. Laboratory analysis

Upon arrival at the laboratory, 50 μl of the anticoagulated whole blood was separated into cryovials and frozen immediately in liquid nitrogen for metHb analysis. The remaining sample was centrifuged at 10080 ×g for 10 min to separate the plasma and blood cells. The plasma was divided into several aliquots for biochemistry and carotenoid determination and frozen at −80 °C. The red blood cells (RBC) were washed three times in ice-cold physiologic (0.9%) sodium chloride solution and stored at −80 °C until further analysis. One of the blood smears was stained with a commercial Papanicolaou type stain (Hemaquick, Biochemical Sciences, Swedesboro, The Netherlands).

2.4. Sex and body condition

For sex determination of the nestlings, DNA was extracted from blood samples in absolute ethanol by ammonium acetate technique and used in the PCR described by Fridolfsson and Ellegren (1999).

We calculated body condition according to the scaled mass index (SMI) proposed by Peig and Green (2009). The SMI has been calculated according to the equation:

$$SMI = M_i \times \left(\frac{L_0}{L_i}\right)^{b_{SMA}}$$

Where M_i and Li are the body mass and tarsus length of individual i, respectively; L_0 is the arithmetic mean value of tarsus length for the study population; and bSMA is the scaling exponent estimated by the standardized major axis (SMA) regression of ln M on ln L.

2.5. Haematology and blood parameters

In the blood smears an estimated total white cell (TWCC) and the differential count was obtained using a light microscope. The counts were carried out blindly by two researchers and results were averaged between the two observers. The estimated TWCC was calculated after recording the total number of white blood cells in 20 fields at 400× (TWCC) magnification then dividing the total leucocyte count by the number of fields counted (20) and multiplying by 2000 (Fudge, 2000). For the differential count at least 200 cells were identified and counted at 1000× magnification under oil immersion. The heterophil/lymphocyte ratio (H/L Ratio) was calculated to obtain a measure of response to acute stressors and an indirect approximation to first-line constitutive immunity (Maxwell, 1993; Ots et al., 1998; Tella et al., 2000).

We obtained a plasma biochemistry profile that included albumin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) calcium (Ca), bilirubin, cholesterol, creatine kinase (CK), creatinine, ferritin, glucose, magnesium (Mg), phosphorous (P), transferrin, triglycerides, and uric acid. All analyses were carried using an A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain, using the corresponding reaction kit for each determination (BioSystems, Barcelona, Spain) according to the manufacturer's instructions.

2.6. Oxidative stress balance

Using plasma, we evaluated its total antioxidant capacity (TAC) (BAP test, Diacron international, Grossetto, Italy), correcting the value with the uric acid value (Costantini, 2011). Also, in plasma, we evaluate the presence of reactive oxygen metabolites (ROMs; d-ROM kit, Diacron international), and lactate dehydrogenase (LDH). We also determined the plasmatic levels of dietary antioxidants such as α-tocopherol (aka vitamin E), retinol (aka vitamin A), and the carotenoids lutein and zeaxanthin by high-performance liquid chromatography (HPLC, Agilent Technologies 1100 Series) coupled to a photodiode detector and a fluorescence detector (Rodríguez-Estival et al., 2010).

Both in plasma and RBC homogenates, we measured malondialdehyde (MDA), commonly used as an indicator of lipid peroxidation, by high-performance liquid chromatography (HPLC, Agilent Technologies 1100 Series) coupled to a fluorescence detector (Romero-Haro and Alonso-Alvarez, 2014).

Only in RBC homogenates, total (GSHt) and oxidised (GSSG) glutathione levels were measured with an A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain). For antioxidant enzymes, we used Ransod and Ransel kits (Randox Laboratories, Cornella de Llobregat, Spain) to measure the activities of superoxide dismutase (SOD, EC 1.15.1.1) and glutathione peroxidase (GPX, EC 1.11.1.9) in RBC homogenates. Their activities were calculated relative to protein (mg), using the Bradford method to quantify total proteins (Bradford, 1976).

Finally, we used whole blood to evaluate the percentage of metHb using spectrophotometric biochemistry analyser (A25 Random Access Analyser, BioSystems; Martínez-Haro and Mateo, 2008). This oxidised form of the haemoglobin shows a lack of function as effective oxygen-transporting protein, and its proportion in the blood is used as a biomarker of exposure to oxidising toxicants.

2.7. Pathogen detection and serology

Cloacal swab samples in AMIES transport medium were cultured for the detection of *Salmonella* spp. and *Escherichia coli* (*E. coli*) as described previously in Camacho et al. (2016), including testing of *E. coli* strains for the presence of five genes characteristic of avian pathogenic *E. coli* (APEC) (Johnson et al., 2008).

Briefly, for isolation of *E. coli* cloacal swabs were streaked onto MacConkey agar (Scharlab S.L., Barcelona, Spain) and incubated at 37 °C for 24 h. Lactose positive Colonies morphologically compatible with *E. coli* were further tested for phenotypic pattern of antimicrobial resistance. For this, they were cultured in MacConkey agar supplemented with 16 μg/ml gentamicin (Sigma-Aldrich Chemical, Madrid, Spain), 4 μg/ml cefotaxime (Sigma-Aldrich Chemical, Madrid, Spain) or 4 μg/ml enrofloxacin (Sigma-Aldrich Chemical, Madrid, Spain) respectively and incubated overnight. Colonies morphologically compatible with *E. coli* were considered indicative of growth above breakpoint concentrations the tested antimicrobials based on the Clinical and Laboratory Standards Institute (CLSI) document (CLSI, 2020). Three lactose positive colonies of phenotypically cefotaxime resistant *E. coli* were collected into brain heart infusion broth and stored frozen at −80 °C, for molecular characterisation and resistance gene detection (Höfle et al., 2020).

All isolates morphologically identified as *E.coli* were tested by a multiplex PCR for the genes described previously by Johnson et al. (2008) as the minimal predictors of avian pathogenic *E. coli* (APEC), specifically iroN, *ompT*, *hlyF*, *iutA* and *iss*. For this, DNA extracted by boiling from confluent growth on MacConkey agar was subjected to a multiplex PCR in a 25 μl reaction volume containing 2 μl of extracted DNA, 0.3 μM of each of the ten primers, 0.25 mM dNTPs, 4 mM $MgCL_2$, and 1 U DNA Polymerase. The samples were amplified on an applied Biosystems 2720 thermocycler (Applied Biosystems, Foster City, CA, EEUU) using the following protocol: 94 °C, 2 min; 35 cycles at 94 °C, 30 seg., 63 °C, 30 seg., 72 °C, 60 seg and one cycle of 72 °C, 10 min. The products were subjected to electrophoresis in a 4% agarose gel, stained using GelRed™ (Biotium, Hayward, USA) and visualized in a UV transiluminator (UVitec Ltd. Cambridge, UK).

Isolation of *Salmonella* spp. was performed according to the standard ISO 6579 method (2017). For this, samples were pre-enriched in peptone water at 37 °C for 24 h. One ml of this broth was then transferred into 9 ml Rappaport Vassiliadis broth (Scharlab S.L., Barcelona, Spain) and incubated at 42 °C for 24 h. Finally, the sample was plated on XLD agar (Scharlab S.L., Barcelona, España) and incubated at 37 °C for 24 h. Colonies morphologically compatible with *Salmonella* spp. by their morphology were collected in ultrapure water for DNA extraction by boiling and confirmed as *Salmonella* spp. by amplification of the invA gene using the protocol described by Rahn et al. (1992). Amplification was carried out in a reaction mixture of a total volume of 30 μl containing: 2 μl of DNA; 0.2 μl Enzyme (5 U/ μl) (Biotools DNA polymerase, biotools, Madrid, Spain); 3 μl of invA-L (0.01 mM) primer; 3 μl invA-R primer (0.01 mM); 3 μl Buffer (10×); 0.6 μl dNTPs (10 mM); 0.9 μl MgCl2 (50 mM); 17.3 μl ddH2O. The reaction was performed in a Techne thermal cycler TC-512 (Techne Inc. Cambridge, UK) using the following protocol: 94 °C/3 min; 40 cycles of 95 °C/30 s, 55 °C/30 s, 72 °C/30 s; and a final cycle of 72 °C/10 min. The samples were subjected to electrophoresis in a 2% agarose gel. Bands were stained using GelRed™ (Biotium, Hayward, USA) and visualized in a UV transiluminator (UVitec Ltd. Cambridge, UK).

We studied the phenotypic pattern of antimicrobial resistance in isolates confirmed as *Salmonella* spp., by plating colonies on XLD agar supplemented with the same antibiotics and concentrations used in the evaluation of *E. coli* antimicrobial resistance. The plates were incubated overnight at 37 °C and examined for growth of colonies compatible with *Salmonella* spp. The concentrations of antimicrobials used are based on the Clinical and Laboratory Standards Institute (CLSI) document (CLSI, 2020).

2.8. Statistical analysis

We used general linear mixed models fitted with REML (Restricted Maximum Likelihood) to analyse the data. To assess how the degree of association of the colony to landfills affects the different dependent variables, we constructed 29 different models with normal distributed dependent variables (Table 1, Table 2) and 4 models with binomial distributed dependent variables with logit function (Table 3, Table 4). *TWCC* and *H/L* ratio were log-transformed. In all models, *Colonies* (“N”, “SN”, “SL” and “L”) and *Sex* (Male or Female) were included as factors, and Nestlings (number of siblings) is included as a covariate. *Nest* was included as a random factor in all models to avoid pseudo-replication. A detailed description of each variable is included in Table S3.

All models were validated by visual inspection of the residual graphs to verify the assumptions of normality of the residuals and homogeneity of the variances. We used a backward stepwise method to create a final model only with significant variables, but always keeping *Colonies* in the final model as the objective of the study was the evaluation of the differences between colonies. When the factor *Colonies* showed significant differences, pairwise comparison with *p* adjust method “Bonferroni” was done to establish which colonies differed. All analyses were performed in R 3.6.2 (R Core Team, 2019) using the R packages ‘lme4’ (1.1–21), ‘lmerTest’ (3.1–1), ‘car’ (3.0–5) and ‘REdaS’ (0.9.3) (Bates et al., 2015; Kuznetsova et al., 2017; Fox and Weisberg, 2019; Maier, 2015). Significance was set at $p \leq 0.05$ for all analysis.

3. Results

Our results in four white stork colonies along a gradient of exposure to landfill foraged food showed a better nutritional status and body condition, differences in the antioxidant response, increased metHb percentages and acquisition of *E. coli* with a multiresistant phenotype in stork nestlings fed a landfill-foraged diet.

Body condition, expressed as SMI, was higher in nestlings from colony “L”, followed by the ones in colony “SL”, being lowest in individuals from colonies “SN” and “N” ($F_{38.39,} = 14.48$, $p < 0.01$) (Tables 1 and 2, Fig. 2).

The plasmatic concentrations of albumin ($F_{34.68} = 5.162$, $p < 0.01$), glucose ($F_{38.51} = 7.37$, $p < 0.01$), cholesterol ($F_{39.2} = 3.107$, $p = 0.037$) and triglycerides ($F_{37.53} = 5.078$, $p < 0.01$) was lowest in nestlings from the “N” colony (Tables 1 and 2, Fig. 2). Magnesium concentration was significantly lower in nestling from colony “L” ($F_{34.04} = 6.329$, $p < 0.01$) (Tables 1 and 2, Fig. 2).

Aspartate (AST; $F_{35.34} = 11.86$, $p < 0.01$) and alanine aminotransaminase (ALT; $F_{35.71} = 9.343$, $p < 0.01$) activities present lowest values in “L” colony nestlings, and, despite the highest values are in “SN” colony, the values appear in a general pattern, increase as the degree of exposure to landfill foraged food decreases (Tables 1 and

Table 1

Results of the final models performed to assess nutritional status, health status, oxidative damage, antioxidant defence and immune system status on nestling white stork in a gradient of use of landfill as a food resource. Significant factors ($p \le 0.05$) have been highlighted in **bold**. Random term values are in *italic*.

	Fixed terms	Rejected terms	F	*P*	Random terms	Variance
Nutritional status						
Albumin	**Colonies**	Sex, Nestlings	**5.162 (3, 34.68)**	**<0.01**	*Nest*	*1.994*
					Residual	*1.308*
Calcium	Colonies	Nestlings	2.459 (3, 37.47)	0.078	*Nest*	*0.598*
	Sex		**20.44 (1, 67.22)**	**<0.01**	*Residual*	*0.207*
Cholesterol	**Colonies**	Nestlings	**3.107 (3, 39.2)**	**0.037**	*Nest*	*736*
	Sex		**17.51 (1, 65.82)**	**<0.01**	*Residual*	*229*
Glucose	**Colonies**	Sex, Nestlings	**7.37 (3, 38.51)**	**<0.01**	*Nest*	*270*
					Residual	*347*
Magnesium	**Colonies**	Nestlings	**6.329 (3, 34.04)**	**<0.01**	*Nest*	*0.038*
	Sex		**7.712 (1, 82.61)**	**<0.01**	*Residual*	*0.045*
Phosphorus	Colonies	Sex, Nestlings	2.13 (3, 34.48)	0.114	*Nest*	*0.735*
					Residual	*0.345*
Triglycerides	**Colonies**	Sex, Nestlings	**5.078 (3, 37.53)**	**<0.01**	*Nest*	*508.8*
					Residual	*473.7*
Health status						
ALP	Colonies	Sex	2.678 (3, 35.62)	0.062	*Nest*	*20,946*
	Nestlings		**6.94 (1, 36.66)**	**0.012**	*Residual*	*7998*
ALT	**Colonies**	Nestlings	**9.343 (3, 35.71)**	**<0.01**	*Nest*	*104*
	Sex		**5.712 (1, 73.63)**	**0.019**	*Residual*	*69.13*
AST	**Colonies**	Sex	**11.86 (3, 35.34)**	**<0.01**	*Nest*	*1063*
	Nestlings		**4.229 (1, 36.16)**	**0.047**	*Residual*	*298*
Bilirubin	**Colonies**	Sex, Nestlings	**7.72 (3, 31.61)**	**<0.01**	*Nest*	*0.582*
					Residual	*0.388*
CK	Colonies	Sex, Nestlings	2.034 (3, 36.26)	0.126	*Nest*	*34,778*
					Residual	*16,449*
Creatinine	Colonies	Sex, Nestlings	1.928 (3, 28.91)	0.147	*Nest*	*0.002*
					Residual	*0.005*
SMI	**Colonies**	Sex, Nestlings	**14.48 (3, 38.39)**	**<0.01**	*Nest*	*123,233*
					Residual	*17,113*
Oxidative damage						
LDH	**Colonies**	Sex, Nestlings	**5.11 (3, 30.08)**	**<0.01**	*Nest*	*21,472*
					Residual	*28,192*
MDA (plasmatic)	Colonies	Sex, Nestlings	1.752 (3, 34.59)	0.175	*Nest*	*12.3*
					Residual	*9.869*
MDA (RBCs)	Colonies	Sex, Nestlings	2.128 (3, 30.11)	0.117	*Nest*	*1302*
					Residual	*2690*
MetHb	**Colonies**	Sex, Nestlings	**22.07 (3, 30.22)**	**<0.01**	*Nest*	*2.485*
					Residual	*3.362*
ROMs	**Colonies**	Sex, Nestlings	**7.878 (3, 25.97)**	**<0.01**	*Nest*	*242*
					Residual	*158*
Antioxidant defence						
Ferritin	Colonies	Sex, Nestlings	0.48 (3, 82)	0.697	*Nest*	*0*
					Residual	*2.67*
GPx	Colonies	Sex, Nestlings	0.623 (3, 38.76)	0.605	*Nest*	*0.014*
					Residual	*0.003*
GSH	**Colonies**	Sex, Nestlings	**9.325 (3, 34.08)**	**<0.01**	*Nest*	*0.115*
					Residual	*0.133*
GSSG	Colonies	Nestlings	1.145 (3, 36.84)	0.344	*Nest*	*0.187*
	Sex		**4.177 (1, 74.22)**	**0.044**	*Residual*	*0.075*
Lutein	Colonies	Nestlings	2.854 (3, 36.71)	0.05	*Nest*	*2.428*
	Sex		**7.725 (1, 54.23)**	**<0.01**	*Residual*	*0.27*
Retinol (Vitamin A)	**Colonies**	Sex, Nestlings	**5.757 (3, 32.26)**	**<0.01**	*Nest*	*20.66*
					Residual	*10.42*
SOD	Colonies	Sex, Nestlings	0.273 (3, 36.06)	0.845	*Nest*	*0.154*
					Residual	*0.067*
TAC	Colonies	Sex, Nestlings	1.344 (3, 24.18)	0.284	*Nest*	*27,278*
					Residual	*10,271*
Tocopherol (Vitamin E)	Colonies	Sex, Nestlings	1.78 (3, 37.42)	0.168	*Nest*	*12.14*
					Residual	*4.39*
Transferrin	**Colonies**		**7.465 (3, 17.82)**	**<0.01**	*Nest*	*0.182*
	Sex		**9.536 (1, 69.02)**	**<0.01**	*Residual*	*3.177*
	Nestlings		**5.898 (1, 17.98)**	**0.026**		
Zeaxanthin	Colonies	Nestlings	2.396 (3, 38.36)	0.083	*Nest*	*3.105*
	Sex		**11.33 (1, 60.92)**	**<0.01**	*Residual*	*0.705*
Immune system						
Ratio H/L	**Colonies**	Sex, Nestlings	**18.1 (3, 37.8)**	**<0.01**	*Nest*	*0.083*
					Residual	*0.332*
TWCC	**Colonies**	Sex, Nestlings	**3.298 (3, 36.03)**	**0.031**	*Nest*	*3664*
					Residual	*4030*

ALP: Alkaline phosphatase; AST: Aspartate transaminase; ALT: Alanine transaminase; CK: Creatine Kinase; SMI: Scaled Mass Index; LDH: Lactate dehydrogenase; MDA: Malondialdehyde; MetHb: Methaemoglobin; ROMs: Radical Oxygen Metabolites; GPx: Glutathione peroxidase; GSH: Reduced glutathione; GSSG: Oxidized glutathione; SOD: Superoxide dismutase; TAC: Total Antioxidant Capacity (BAP corrected by uric acid concentration); TWCC: Total White Cell Count.

Table 2
Estimates and standard error of each significant model (the values are in comparison with N colony). Random terms values are in *italic*. Arrow indicates the direction of the increase of each parameter.

	Estimate (standard error)		
	Colony SN	**Colony SL**	**Colony L**
		Nutritional status	
Albumin	2.317 (0.685)	2.293 (0.654)	1.936 (0.945)
		→	
Cholesterol	29.26 (12.04)	30.96 (11.61)	36.08 (17.05)
		→	
Glucose	26.17 (8.825)	37.61 (8.444)	37.5 (12.08)
		→	
Magnesium	0.037 (0.101)	-0.163 (0.098)	-0.524 (0.142)
		←	
Triglycerides	23.92 (11.17)	40.33 (10.81)	38.87 (15.65)
		→	
		Blood parameters	
ALT	8.557 (4.948)	-9.984 (4.718)	-22.3 (6.867)
		←	
AST	56.83 (14.71)	3.288 (14.45)	-52.59 (20.28)
		←	
Bilirubin	0.369 (0.36)	0.429 (0.348)	-1.854 (0.51)
		←	
SMI	-191 (152)	487 (144)	949 (212)
		→	
		Oxidative damage	
LDH	2.424 (79)	-14.42 (75.34)	-383.8 (107.9)
		←	
MetHb	0.299 (0.857)	1.078 (0.845)	9.107 (1.203)
		→	
ROMs	22.74 (8.69)	-2.369 (8.444)	-26.47 (10.95)
		←	
		Antioxidant defence	
GSH	0.327 (0.172)	0.831 (0.167)	0.759 (0.251)
		→	
Retinol (Vitamin A)	1.83 (2.102)	7.215 (2.058)	8.249 (2.95)
		→	
Transferrin	-0.003 (0.536)	2.316 (0.567)	0.268 (0.744)
		→	
		Immune system	
Ratio H/L	-1.205 (0.199)	-1.25 (0.194)	-1.23 (0.272)
		←	
TWCC	-63.12 (30.62)	-89.28 (29.86)	-22.1 (43.11)
		←	

2, Fig. 3). Bilirubin concentration was significantly lower in nestlings from colony “L” ($F_{31.61} = 7.72$, $p < 0.01$) (Tables 1 and 2, Fig. 3).

Lactate dehydrogenase (LDH) activity was significantly lower in nestlings from colony “L” ($F_{30.08} = 5.11$, $p < 0.01$) (Tables 1 and 2, Fig. 4). The same happens with ROMs, which showed the lowest concentration in the plasma of nestlings from colony “L”, followed by individuals in colony “SL” ($F_{25.97} = 7.878$, $p < 0.01$) (Tables 1 and 2, Fig. 4). However, metHb percentages were significantly higher in the blood of nestlings from colony “L” ($F_{30.22} = 22.07$, $p < 0.01$) (Tables 1 and 2, Fig. 4).

GSHt concentration was significantly higher in RBC of nestlings from colonies “L” and “SL” ($F_{34.08} = 9.325$, $p < 0.01$) (Tables 1 and 2, Fig. 5). The same pattern was found regarding the plasmatic concentration of retinol ($F_{32.26} = 5.757$, $p < 0.01$) (Tables 1 and 2, Fig. 5). The concentration of transferrin was higher in plasma of nestlings from colony “SL” than in the nestlings from colonies “SN” and “N” ($F_{17.82} = 7.465$, $p < 0.01$; Tables 1 and 2, Fig. 5).

Higher TWCC was observed in nestlings sampled in the colony “N” in comparison with the ones sampled in the colonies “SN” and “SL” ($F_{36.03} = 3.298$, $p = 0.031$). In addition, nestlings from the colony “N” had a significantly higher H/L ratio than those from the other three colonies ($F_{37.8} = 18.1$, $p < 0.01$; Tables 1 and 2, Fig. 6).

Three nestlings tested positive for *Salmonella* sp., while *E. coli* was isolated from nearly all nestlings. Phenotypic resistance of *E. coli* isolates against enrofloxacin (Chi = 15.5, $p < 0.01$), gentamicin (Chi = 10.65, $p = 0.014$), or both together (Chi = 10.35, $p = 0.016$) were more frequent in isolates from nestlings from colonies “L” and “SL” (Tables 3 and 4). Prevalence of phenotypically cefotaxime resistant *E. coli* did not vary significantly between the different colonies (Table 3).

We have found no differences between colonies in the rest of the parameters evaluated.

4. Discussion

We observed that the use of landfills as food resource had a positive effect on the nutritional status of white stork nestlings, with differences in the response of the oxidative stress balance among colonies. However, the higher prevalence of *E. coli* with multiresistant phenotypes suggested an impact on the level of resistance of the bacterial

Table 3
Values of the models of each dependent variable with a binomial distribution. Significant factors (p ≤ 0.05) have been highlighted in **bold**. Random terms values are in *italic*.

	Fixed terms	Rejected terms	Chi-square (d.F.)	p
APEC	Colonies	Sex, Nestlings	4.87 (3)	0.182
Resistance to Enrofloxacin	**Colonies**	**Sex, Nestlings**	**15.5 (3)**	**<0.01**
Resistance to Gentamicin	**Colonies**	**Sex, Nestlings**	**10.65 (3)**	**0.014**
Resistance to Enro/Genta	**Colonies**	**Sex, Nestlings**	**10.35 (3)**	**0.016**
Resistance to Cefotaxime	Colonies	Sex, Nestlings	5.033 (3)	0.169

APEC: Avian pathogenic *Escherichia coli*.

Table 4
Estimate and standard error of each significant model with a dependent variable with a binomial distribution (the values are in comparison with Colony N). Significant factors ($p \leq 0.05$) have been highlighted in **bold**. Arrow indicates the direction of the increase of each parameter.

	Estimate (standard error)		
	Colony SN	**Colony SL**	**Colony L**
Enrofloxacin resistant E. coli	1.828 (5.665)	19.29 (6.57)	21.6 (8.29)
		→	
Gentamicin resistant E. coli	3.269 (1.302)	4.391 (1.593)	6.995 (2.299)
		→	
Resistance to both Enro/Genta	1.749 (4.606)	16.58 (6.48)	19.37 (7.05)
		→	

microbiota or acquisition of antibiotic resistant bacteria through landfill foraged food.

White storks have associated for centuries with anthropised habitats, due to the preference of this species to establish near water bodies (Kruszyk and Ciach, 2010; Ciach and Kruszyk, 2010). Changes in agricultural practices have dramatically reduced foraging areas for white storks in all of Europe. However, this trend has been reversed in the recent past, primarily due to the adaptation of storks to using landfills as a continuous predictable food source (Tortosa et al., 2002; Ciach and Kruszyk, 2010; Gilbert et al., 2016). In these areas, they change their traditional preys (earthworms, insects, fish, amphibians, and small mammals) by chicken, meat, offal and eggs (Plaza and Lambertucci, 2017).

Landfills are nowadays important foraging areas during the non-breeding season leading to reduced migration time-lapse and distance and thus reduced mortality and favouring early occupation of breeding sites (Tortosa et al., 2002; Archaux et al., 2004; Vergara et al., 2007). Studies have also shown that the proximity of landfills near wintering and breeding areas has a positive effect on clutch size, egg volume, hatching and breeding success (Djerdali et al., 2016b). Such effects are likely to be mediated by a higher food intake by the parents (with the consequent higher spare energy to spend in the care of nestlings) and in consequence a higher and more constant feeding rate by nestlings, as reflects their higher concentration of glucose in blood. The better body condition and significantly higher concentrations of albumin, cholesterol, and triglycerides in the blood of stork nestlings from landfill-associated colonies found in our results, seems to confirm this assumption. Higher body weight and seemingly increased body condition have also been documented in other species feeding on landfills such as

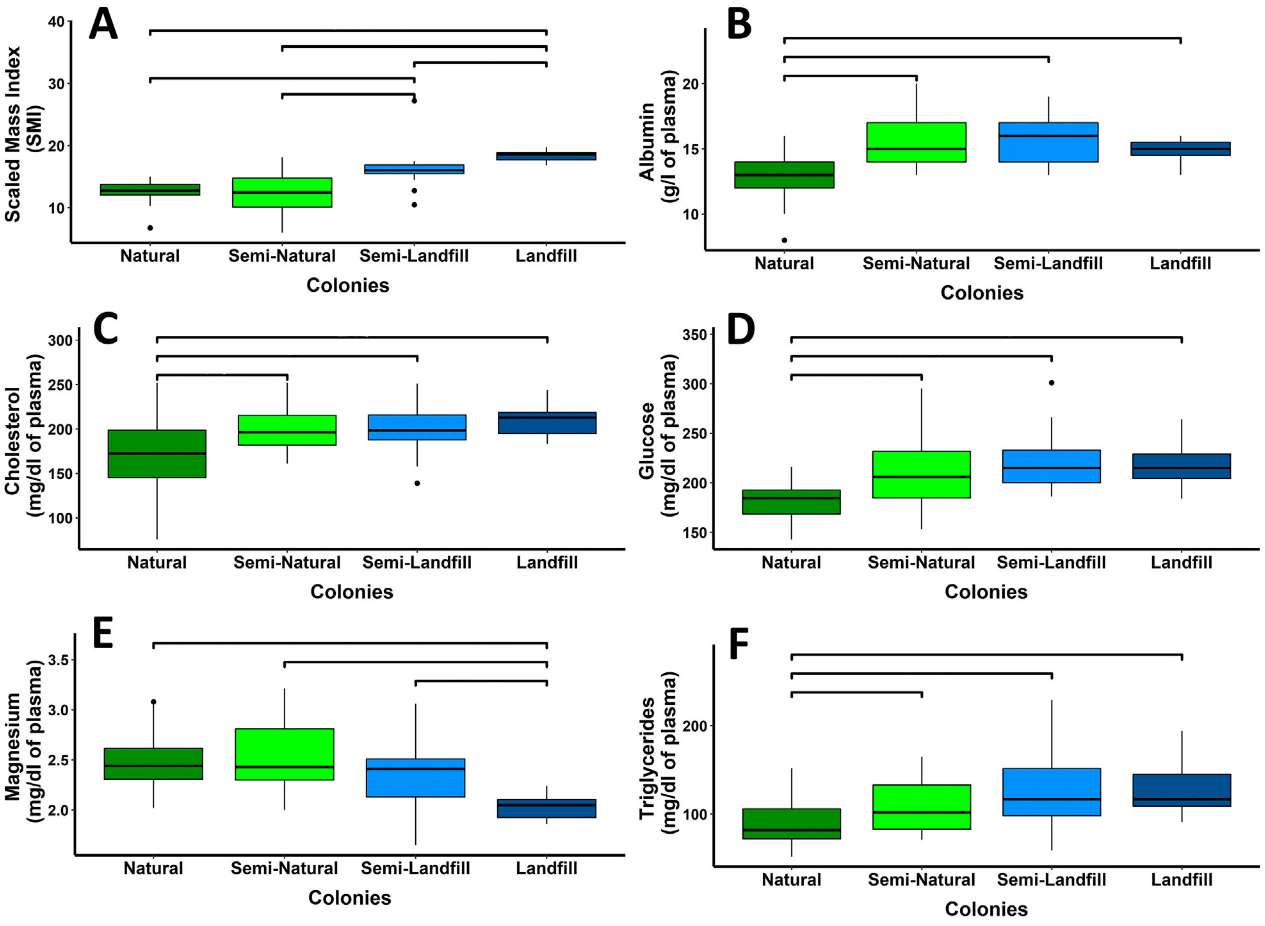

Fig. 2. A) SMI; concentration of **B) albumin**, **C) cholesterol**, **D) glucose** and **E) magnesium** and **F) triglycerides** in plasma of white stork nestlings in a gradient of use of landfill as a food source. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Keys indicate significant differences between groups.

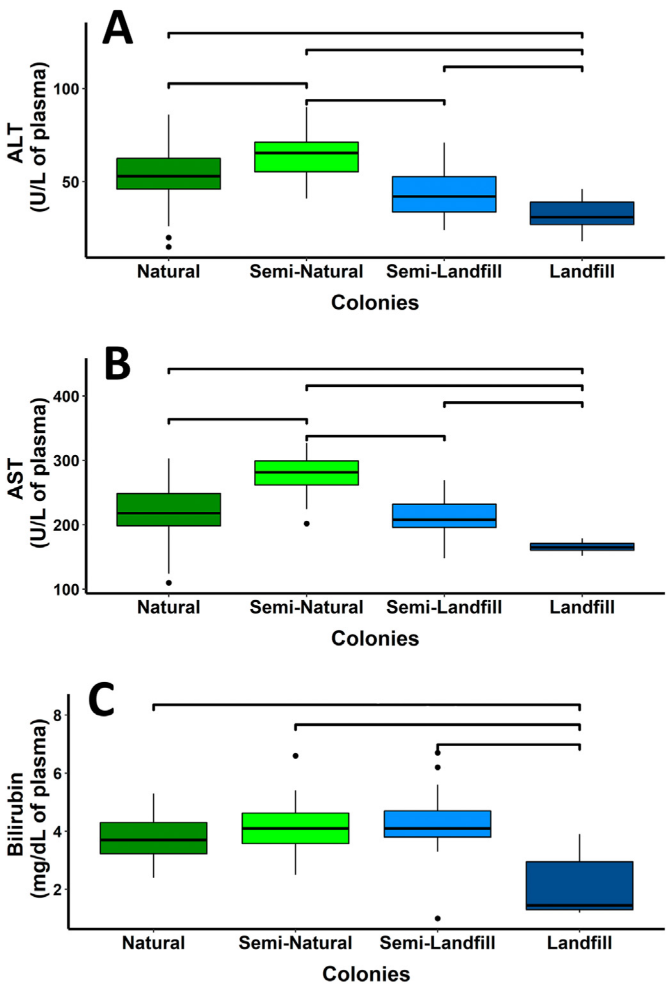

Fig. 3. Plasmatic activities of **A) ALT**, **B) AST** and plasmatic concentration of **C) Bilirubin** in plasma of white stork nestlings in a gradient of use of landfill as a food resource. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Keys indicate significant differences between groups.

aegyptian vulture (*Neophron percnopterus*; Tauler-Ametller et al., 2019) and American black vulture (*Coragyps atratus*; Plaza and Lambertucci, 2018). However, in contrast to natural prey, food resources foraged at landfills could be expected be scarce in antioxidants (Tauler-Ametller et al., 2019). We have found no differences in the concentration of phosphorus or calcium, but nestlings from landfill associated colonies had significantly less magnesium in their blood than nestlings from natural habitats. Egyptian vultures, Tauler-Ametller et al. (2019) have shown a negative effect on vitamin and carotenoid concentrations in the blood of nestlings fed on a diet-related to landfills. However, we did not detect any difference in tocopherol concentration in the blood of stork nestlings between colonies; furthermore, retinol was present in higher concentrations in plasma of nestlings from the anthropized habitats.

Regarding the potential negative effects of the use of this food resource, very few studies have evaluated the impact of exposure to chemical pollutants on the physiology of the exposed animals (Plaza and Lambertucci, 2017; Tauler-Ametller et al., 2019). Previous studies have demonstrated that the use of landfills by white storks produced higher concentrations of brominated flame retardants (BFRs) in addled eggs (Muñoz-Arnanz et al., 2011); and higher blood concentrations of different metals such as lead, arsenic and mercury (de la Casa-Resino et al., 2015). We evaluated those potential negative effects on health using a combination of blood and oxidative stress parameters. Blood parameters that have been previously analysed in white stork nestlings included triglycerides, cholesterol, AST and ALT (Puerta et al., 1989, Jerzak et al., 2010). The means we obtained for these parameters are all within the 2.5 to 97.5 percentile ranges of the values obtained by Jerzak et al. (2010): 68–443 (mg/dl), 137–273 mg/dl), 155–284 (U/l) and 17–57 (U/l) for triglycerides, cholesterol, AST and ALT; being our means 113 (mg/dl), 193 (mg/dl), 218 (U/l) and 48 (U/l), respectively. Blood parameters associated to liver functionality (ALT and AST) and the increase of which could indicate liver damage, were significantly lower in nestlings more exposed to food foraged on landfills, while no differences were detected for creatinine or creatin kinase (CK). These results suggest that in short term the use of this food resource entrails no direct effect on organ function of the individuals.

We found significantly higher values for calcium, cholesterol, magnesium, ALT, GSSG, lutein, transferrin and zeaxanthin in males. To date there is no clear explanation of the reason why some blood parameters differ between sexes in stork nestlings. One hypothesis could be differences in sexual hormones; however, this hypothesis is based on studies on the domestic chicken (*Gallus gallus domesticus*), and white storks differ in their longer development to adulthood and the lack of sexual differences in external morphological traits (Jerzak et al., 2010). In any case, the explanation could vary between variables, as some of them are related to nutrition (triglycerides and cholesterol) while others such as AST could be affected by numerous processes (Jerzak et al., 2010). Sex differences in some blood parameters were found in other species such as red-legged partridges (*Alectoris rufa)* (Rodríguez et al., 2004), stone curlews (*Burhinus oedicnemus*) (Lierz and Hafez, 2005) and the Japanese quail (*Coturnix coturnix japonica*) (Scholtz et al.,

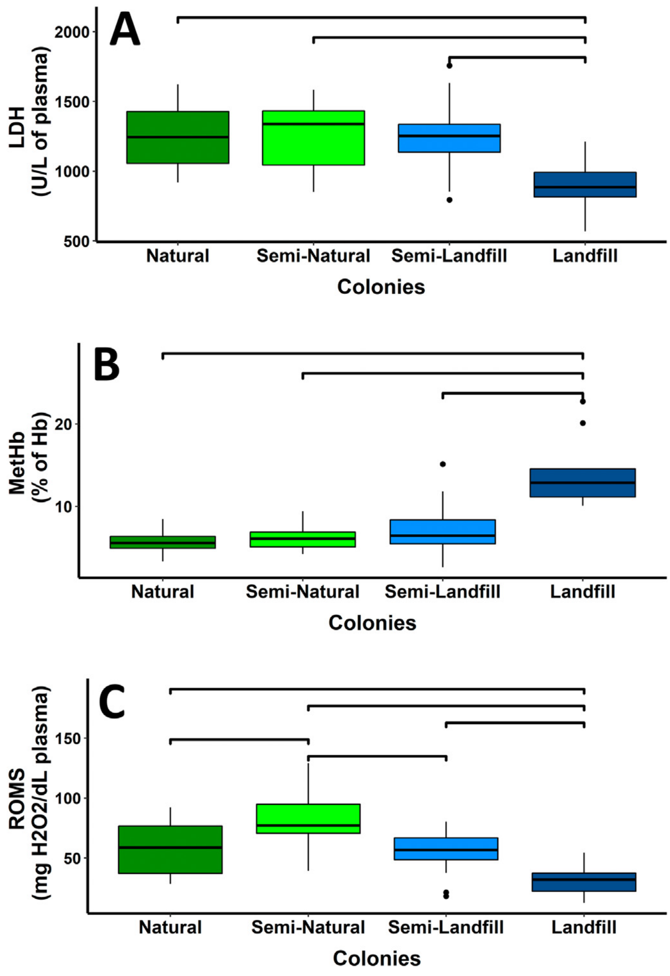

Fig. 4. Plasmatic activity of **A) LDH**, percentage of **B) MetHb** in blood and of **C) ROMs** in plasma of white stork nestlings in a gradient of use of landfill as a food resource and concentration. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Keys indicate significant differences between groups.

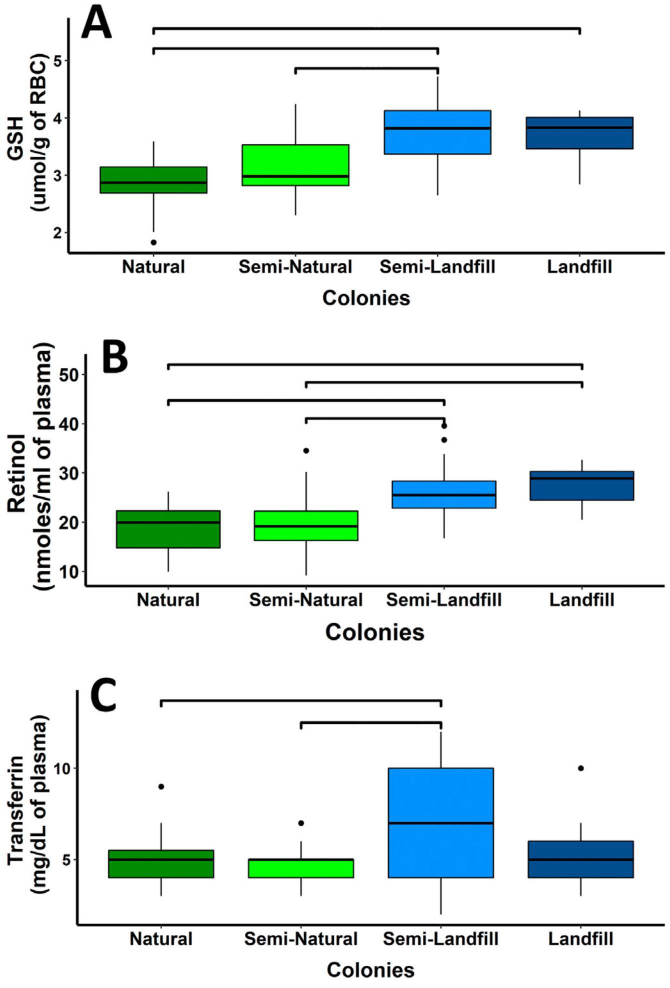

Fig. 5. Concentration of **A) GSH** in RBC and **B) retinol** and **C) transferrin** in plasma of white stork nestlings in a gradient of use of landfill as a food resource. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Keys indicate significant differences between groups.

2009). However, the parameters affected by sex are not consistent between species.

Many pollutants are active pro-oxidants, or in other cases could inhibit gene expression of antioxidants (Isaksson, 2010). In contrast to studies on white stork nestlings exposed to metal pollution from mining activities (Kamiński et al., 2009; Tkachenko and Kurhaluk, 2012, 2013) we did not find any increase in oxidative damage indicators in nestlings of colonies associated to landfills, with no differences in the concentration of MDA (as an indicator of lipid peroxidation) neither in RBC nor in plasma. In addition, we found a lower presence of ROS and lower activity of LDH in the plasma of these nestlings. A potential explanation could be a lower degree of pollution to which nestlings were exposed related to those from the other studies, as these were exposed to pollution derived from mining activities and copper manufacture (Kamiński et al., 2009; Tkachenko and Kurhaluk, 2012, 2013).

In terms of antioxidant defences, GSHt, the main soluble endogenous antioxidant inside the cells, shows the same pattern, with higher values in nestlings from anthropised colonies. In accordance, Total Antioxidant Capacity (TAC) of plasma, although without significant difference, shows the same trend, with increasing values as the use of landfill as food resource increases. These results suggest that some of the substances contained in the waste where landfill-associated storks forage trigger an effect over oxidative balance, increasing antioxidant defence in a hormetic response (Mattson, 2008; Rattan, 2008). This represents a dose-response phenomenon characterised by a low dose stimulation, high dose inhibition; in which the consideration of a dose as low or high depends on the species and the parameter of study (Mattson, 2008; Rattan, 2008). This phenomenon is a characteristic response to the presence of metals, which have been demonstrated to be present in higher concentrations in white stork individuals that use landfills as a food resource (de la Casa-Resino et al., 2015). Our results agree with previous studies in white storks, where the use of landfills increases the concentration of metals and GSH, without any variation of MDA (de la Casa-Resino et al., 2015). Something similar happens in other studies where the origin of the metal contamination was mining activities (Tkachenko and Kurhaluk, 2012, 2013), having the individuals from the colony near a polluted area higher lipid peroxidation and GPx activity, bur lower activity of SOD. These results illustrate that in our case we could likely be observing a hormetic response. This is in accordance with our previous comment about the lack impact on organ function. Hence, as oxidative stress balance is more sensitive to change, and several pollutants (e.g., metals) exert their toxic effect by inducing oxidative stress in cells (Ercal et al., 2001), if there is no negative effect of the use of this food resource on oxidative stress balance, we do not expect damage to the entire organism in the short term.

The only parameter associated with oxidative damage that increased in nestlings from landfill associated colonies is metHb. MetHb is haemoglobin in which haematic iron is oxidised to iron (III) and therefore cannot function as effective oxygen-transporting protein, leading to tissue hypoxia and possible death (Patton et al., 2016). If the percentage of metHb in blood increases this can cause skin discoloration, frequent urination, and restlessness, even leading to seizures, comas and death (Patton et al., 2016). The percentage of metHb in the blood is used as a biomarker of exposure to oxidising toxicants such as nitrates, nitrites and N-nitroso compounds (Martínez-Haro and Mateo, 2008). Nitrite and nitrate are commonly used food preservatives in meat products (e.g., E249 and E252) that stabilise red meat colour, delay oxidative rancidity, inhibit some anaerobic microorganisms' growth and may contribute to product flavour characterisation (Lammarino and Di Taranto, 2012). These preservatives cannot be considered innocuous for humans (Lammarino and Di Taranto, 2012), and possibly also for birds, as in our study, other than the increase in the concentration of metHb that potentially indicates exposure to such oxidising compounds, we were not able

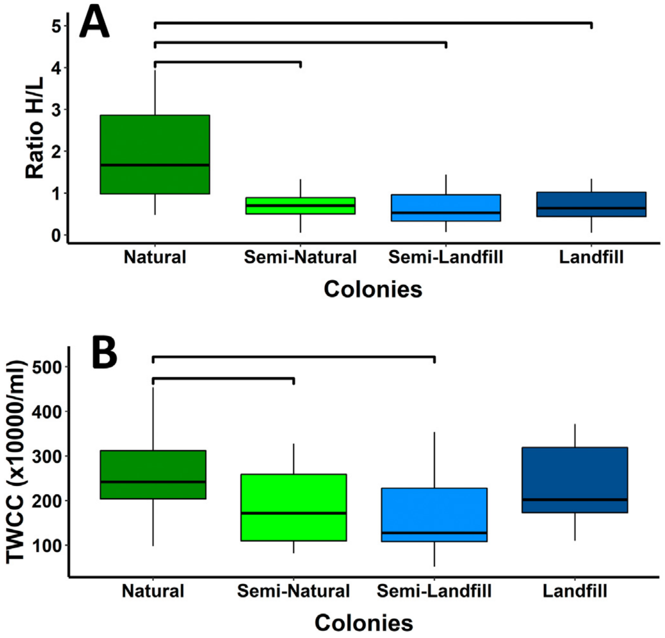

Fig. 6. Total White Cell Counts (TWCC) and **H/L Ratio** of white stork nestlings. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Keys indicate significant differences between groups.

to characterize any additional negative impact on the stork's oxidative balance, as happens in most cases in humans (Mansouri and Lurie, 1993).

Pathogen exposure due to the consumption of waste or refuse has been documented (Plaza and Lambertucci, 2018; Migura-García et al., 2020) but its degree and impact are still largely unknown. Exposure of white stork nestlings to *Salmonella* or APEC in our study seemed not to be related to the use of landfills. However, the higher prevalence of *E. coli* with multiresistant phenotypes in nestlings more exposed to food from landfills suggests an impact on the level of resistance of the intestinal microbiota of these birds. Acquisition of multiresistant bacteria while feeding on waste had been previously reported (Gómez et al., 2016; Camacho et al., 2016; Plaza and Lambertucci, 2017; Migura-García et al., 2020) and could be mediated by the acquisition of resistant strains or resistance genes through contaminated food items or inter and intra-species aggregation, but also due to selection of more resistant phenotypes in the intestinal microbiota by exposure to antibiotic residues (Borquaye et al., 2019). The presence of antibiotics in landfills could be due to their use in the treatment of bacterial infections, where its overuse and uncontrolled disposal lead to their presence in the landfills (Wang et al., 2020), as well as due to the disposal of sludge from wastewater treatment facilities (Ahlstrom et al., 2019). The presence of antibiotics in landfills has produced an increase in the prevalence of ABR (Borquaye et al., 2019), to the point to be identified as one of the primary global health threats and research need (Chung et al., 2018). Another mechanism of acquisition is the co-selection of resistance genes in bacteria exposed to heavy metals, in parallel to the development of resistance against these metals (Xiangyang Li et al., 2019), which could be a potential situation in landfills due to the higher presence of heavy metals. The increase in ABR is a threat to public health, and their relationship with avian species is particularly dangerous, due to the potential role of birds in their dissemination, in particular in the case of white stork because of their close relation with humanised and natural habitat (Gómez et al., 2016).

In turn, the presence of potentially pathogenic microorganisms or a different potentially more aggressive microbiota could lead to an increased antioxidative response of the organism as the one observed here, also suggested in the case of Egyptian vultures (Tauler-Ametller et al., 2019). Dietary deficiencies or pollutant and pathogen exposure through the exploitation of landfills as a food resource could also, both directly and indirectly, impact the immune function of individuals and more specifically nestlings that are in the course of fully maturing their immune capacity. Here we show a lower H/L ratio and TWCC for nestlings exposed to resources from landfills, suggesting such a tendency, however, this needs more exploration using more specific techniques to conclude the mechanisms involved in this context.

In conclusion, our results show that the nestlings fed with food foraged from landfills were apparently healthy and even in better body condition than those fed a diet foraged in a natural environment. Oxidative stress disbalance is very sensitive to the potentially present pollutants in landfill food. However, nestlings exposed to food from landfills seem to present a hormetic response with an increase in their antioxidant defence, being the only indication of a negative effect the higher percentage of metHb, which may be due to the elevated use of nitrite and nitrate in meat products. In addition, exposure to food foraged in landfills significantly increases antibiotic-resistant phenotypes in commensal *E. coli* meaning that birds using landfills could participate in the spread of antimicrobial resistance. The evaluation of nutritional status, oxidative stress balance and the detection of APEC, *Salmonella* and *E. coli* with antibiotic resistant phenotypes seem to allow us to obtain a general vision of the potential effects of the use of this food resource. Although, in order to understand especially potential effects on immune function, additional more specific information such as in vivo or in vitro immune stimulation tests or the characterisation of immune cell subpopulations in peripheral blood, would be necessary.

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CRediT authorship contribution statement

Authors' contributions. U.H. conceived the ideas and designed methodology; J.P.-P., Y.R., A.H.-D., M.M.-H., J.M.H., J.I.A. and U.H. collected samples and data; J.P.-P. and U.H. analysed the data; J.P.-P. led the writing of the manuscript with input from the entire author team.

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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