Accumulation of pollutants in nestlings of an endangered avian scavenger related to territory urbanization and physiological biomarkers

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Abstract

We monitor pollutant accumulation and investigate associated changes at the physiological level within the population of an obligate avian scavenger, the Egyptian Vulture (*Neophron percnopterus*), from Catalonia (NE Spain). This population is expanding its range, presumably because of the use of human waste disposal sites as food resource. We hypothesized that habitat urbanization, presumably associated with feeding from human wastes, could influence the accumulation of persistent organic pollutants and metals. The aim of this study was to explore the relationship between accumulated pollutants and biochemical parameters in nestling blood. We used the proportion of urban surface within an 8 km radius of each nest as a proxy to study the relationship between anthropic influence and pollutant accumulation. Observed blood levels of metals, organochlorine pesticides, polychlorinated biphenyls (PCBs), per- and polyfluoroalkylated substances (PFAS) and polybrominated diphenyl ethers (PBDEs) were relatively low, as expected for nestling individuals due to short-term exposures. CB-180 and PBDEs were associated with variations in blood biochemistry parameters; hexa-BDEs appeared positively associated with activities of the enzymes aspartate aminotransferase and lactate dehydrogenase, whereas CB-180 accumulation was associated with an increased activity of creatine phosphokinase and elevated glutathione levels. Increased CB-180 levels were also related to decreased blood concentrations of calcium, cholesterol, α -tocopherol and lutein. A proportion of urban surfaces as low as 6.56% within a radius of 8 km around the nest appears related to the accumulation of CB-180, the majority of analysed PFAS and of PBDE congeners 99 and 209, and increased urbanization was also associated with decreased plasma levels of α -tocopherol and carotenoids. These associations suggest that changes in blood profiles of vitamins, carotenoids or other analytes, despite related to increased plasma levels of CB-180, would be consequence of exploitation of artificial food sources rather than of a direct effect of the pollutants.

1. Introduction

Because of their special feeding regime among birds of prey, obligate avian scavengers can be of special interest in the study of pollutant accumulation and its relationship with physiological responses. The currently available data show no patterns of higher or lower pollutant

accumulation by avian scavengers compared to other diurnal raptors (Van Wyk et al., 1993, 2001; Garcia-Fernandez et al., 2005; Shlosberg et al., 2012). However, there is hardly any data for these species on how accumulated pollutants associate with biochemical indicators of fitness.

The Egyptian Vulture (*Neophron percnopterus*) is a globally endangered, obligate scavenger known to exploit a wide range of food resources (Donazar, 2004). The species' population located in Catalonia (NE Iberian Peninsula) is known to be expanding its range and colonizing new territories (Tauler et al., 2015), which is presumably related to the exploitation of novel anthropogenic food resources such as landfills (Tauler-Ametller et al., 2017, 2018). Individuals feeding on these anthropogenic food sources seem to experience lesser food shortages, although associated with a decreased intake of exogenous antioxidants such as vitamins and carotenoids, compared with birds exploiting those resources at a lower frequency (Tauler-Ametller et al., 2019). However, the influence that exploitation of anthropogenic food resources may have on pollutant accumulation patterns remains unknown for raptor birds in general.

We hypothesize that the use of highly available human-derived food sources by Egyptian Vultures could result in an increased exposure to pollutants associated with human activities, which can in turn cause physiological alterations in the exposed organisms. Under the Stockholm Convention, industrial, agricultural and domestic use of most persistent organic pollutants (POPs) is currently discontinued, although their massive utilization in the past together with their low degradation rate make their environmental levels still high enough to accumulate in wildlife (e.g. Gómez-Ramírez et al., 2014, 2017; Espín et al., 2016). Metals are naturally occurring elements in the environment, the Earth's crust being their main reservoir. Some human activities like mining and groundwater extraction, as well as their use in many industrial processes, products and materials (e.g. Mateo-Tomás et al., 2016), have contributed to mobilize metals from the lithosphere, increasing their bioavailability and potential for causing toxic effects (Maiz et al., 2000).

The aims of this study were to analyse how the accumulation of metals and POPs (i.e. organochlorine compounds—OC—, polybrominated diphenyl ethers—PBDEs—, and per- and polyfluoroalkyl substances—PFAS—) was related to biochemical responses in Egyptian Vulture nestlings from an expanding population in Catalonia, and to investigate how the degree of urbanization in vulture territories influenced that relationship. Urbanization of territories, which was measured as the proportion of urban surface within a radius around each nest, was considered as a proxy of the degree of human presence in the feeding area.

2. Materials and methods

2.1. Sample collection and estimation of human influence

Samples were obtained between 2012 and 2016 from Egyptian Vulture nestlings (N = 77 individuals, see Table S1 in the Supplementary Material for sample size distribution per year) from 21 territories located across central eastern Catalonia (NE Iberian Peninsula), and covering the whole area of distribution of the species in the region. Nests were located and monitored during the breeding season (March–August) with the help of a spotting scope (60×). Once nestlings were around 45–55 days old, experienced climbers accessed the nests to capture the chicks, which were then carefully transported to the top of the cliff for blood collection. A visual examination of animals was performed in order to ensure they were in a good health status. The examination consisted of palpation of the crop, observation of the buccal cavity, and measurement of body mass and body condition. No symptoms of impaired health were observed, and all examined nestlings were included in the study. We extracted 2.2 mL of blood from the brachial vein with a 20-gauge needle and then returned nestlings back to their nests. Blood was stored in heparinized vials at 4 °C. For the analyses described in the present study, we used 2 mL, which were centrifuged, within the 12 h after collection, at 10,000 rcf for 5 min to separate plasma from the cellular fraction (red blood cells, RBCs). Immediately after centrifugation, both samples were frozen in liquid nitrogen and stored at –80 °C until analysis. The analyses described below were conducted during autumn 2016, and no evidence of blood haemolysis in any of the samples was observed after centrifugation or before analysis.

To determine the degree of urbanization of territories, we used the percentage of surface occupied by urban areas within an 8 km radius of each nest, which was estimated from the information contained in the Topographical Map of Catalonia (1:25,000, Institut Cartogràfic de Catalunya) using ARCGIS 10.2 (ESRI). This area is consistent with the home range of breeding Egyptian Vultures from southern Spain (Carrete et al., 2007). The proportion of urban surface in the study area is correlated with other variables indicative of human presence (Tauler-Ametller et al., 2017).

2.2. Chemical analyses

Details on analytical procedures, detection limits and quality controls for the used methods are given in the Supplementary material (Table S2). The list of analysed chemicals is shown in Table 1.

Table 1. Summary of results (geometric means and maximum values) obtained from the analysis of pollutants in blood of Egyptian Vulture nestlings (N = 77) from Catalonia. Percentage of samples where each substance was detected is shown. Substances marked with an asterisk (*) had an inter-quartile range different from zero and were included in further statistical analyses. For these substances, results of the Spearman's correlations with the proportion of urban areas around the nest are shown (N = 76 for all correlations).

Variable with % urban area	% samples detected		Geometric mean			Maximum		Correlation
RS	p							
p,p'-DDT (ng mL ⁻¹)	1.32	< LoD	0.934	–	–			
p,p'-DDE (ng mL ⁻¹)	22.4	0.017	3.522	–	–			
β-HCH (ng mL ⁻¹)	2.63	< LoD	13.83	–	–			
γ-HCH (ng mL ⁻¹)	1.32	< LoD	3.786	–	–			
α-heptachlor epoxide (ng m L ⁻¹)			7.89	< LoD	2.056	–	–	
β-heptachlor epoxide (ng mL ⁻¹)			1.32	< LoD	1.408	–	–	
CB-138 (ng mL ⁻¹)	6.58	< LoD	2.733	–	–			
CB-153 (ng mL ⁻¹)	6.58	< LoD	1.399	–	–			
CB-180 (ng mL ⁻¹)*	58.4	0.084	5.126	0.281	0.014			
PFBA (ng mL ⁻¹)*	60.8	0.133	3.850	0.008	0.942			
PFHxS (ng mL ⁻¹)*	56.8	0.075	4.534	0.222	0.054			
PFOA (ng mL ⁻¹)*	24.3	0.027	819.6	0.197	0.088			
PFNA (ng mL ⁻¹)*	48.7	0.165	62.55	0.043	0.711			
PFOS (ng mL ⁻¹)*	93.2	2.273	55.90	0.268	0.019			
PFDA (ng mL ⁻¹)*	77.0	0.253	8.267	0.207	0.073			
PFUnA (ng mL ⁻¹)*	86.5	0.380	3.151	0.189	0.102			
PFDoA (ng mL ⁻¹)*	64.9	0.141	6.659	0.274	0.017			
PFTriDA (ng mL ⁻¹)*	89.2	0.175	6.447	0.369	0.001			
BDE-47 (ng mL ⁻¹)*	86.5	0.026	0.103	0.128	0.272			
BDE-99 (ng mL ⁻¹)*	82.4	0.025	0.140	0.246	0.032			
BDE-100 (ng mL ⁻¹)*	44.6	0.011	0.113	–0.025	0.832			
BDE-153 (ng mL ⁻¹)*	55.4	0.014	0.103	–0.050	0.669			
BDE-154 (ng mL ⁻¹)*	51.4	0.011	0.089	–0.172	0.137			
BDE-183 (ng mL ⁻¹)*	33.8	< LoD	0.039	–0.040	0.732			

BDE-209 (ng mL ⁻¹)*	83.9	0.023	1.100	0.254	0.027
Pb (µg g ⁻¹ w.w.)	18.2	< LoD	0.215	–	–
Cd (µg g ⁻¹ w.w.)	2.60	< LoD	0.013	–	–
As (µg g ⁻¹ w.w.)	15.6	< LoD	0.779	–	–
Cu (µg g ⁻¹ w.w.)*	64.9	0.279	4.505	0.069	0.556
Zn (µg g ⁻¹ w.w.)*	100	7.864	10.99	–0.042	0.720

Metals were analysed in RBCs. Samples were freeze-dried (Christ Alpha 1e2, Braun Biotech) and weighed before and after freeze-drying (mean $\pm$ SD sample dry weight = 0.119 $\pm$ 0.021 g, wet weight = 0.379 $\pm$ 0.026); all concentrations are given relative to wet weight (w.w.). Samples were analysed for lead (Pb), cadmium (Cd), arsenic (As), copper (Cu) and zinc (Zn) as detailed in Ortiz-Santaliestra et al. (2015).

POPs were determined from plasma samples. Quantification of OC, either organochlorine pesticides or polychlorinated biphenyls (PCBs), was performed on an Agilent Technologies 6890 N series gas chromatograph equipped with a 30 m fused silica capillary column of 0.32 mm ID and 0.25 µm of film thickness (HP-5 from J&W Scientific, USA), coupled to an electron capture detector (ECD), in conditions optimized for the analytes and specified in Ortiz-Santaliestra et al. (2015). Target compounds were extracted with n-hexane from 100-µl plasma samples previously spiked with a fixed amount of 100 pg of CB-209 (Dr. Ehrenstorfer, Augsburg, Germany) as internal standard, followed by at least three clean-up repetitions with sulphuric acid as described in Mateo et al. (2012).

The same extracts used for OC analysis were reinjected for determination of PBDEs as described in Zapata et al. (2018). Samples were analysed on an Agilent 6890 gas chromatograph coupled to an Agilent 5973 Network mass spectrometer (Santa Clara, CA, USA) using electron-capture negative ionization (GC-ECNI-MS).

PFAS were determined following the method adapted from Vicente et al. (2012), with modifications, in an Acquity Ultra Performance Liquid Chromatography (UPLC) system connected to a Triple Quadrupole Mass Spectrometry Detector (Waters, USA).

2.3. Plasma biochemical and oxidative stress biomarkers

Plasma samples (200 µL) were used to determine circulating levels of calcium, cholesterol, creatinine, glucose, magnesium, phosphorus, total proteins, triglycerides, urea and uric acid, and the enzymatic activity of alkaline phosphatase (ALP), aspartate aminotransferase (AST),

creatine phosphokinase (CK) and lactate dehydrogenase (LDH). These parameters were determined spectrophotometrically in an automatic analyser A25 using the reaction kits available for each parameter (BioSystems, Barcelona, Spain). A control sample provided by the manufacturer was analysed and all measurements were within the certified range. We determined the levels of free retinol, α -tocopherol and carotenoids (zeaxanthin and lutein) in plasma samples (50 μ L) using HPLC (Agilent Technologies 1100 Series) coupled to a photodiode detector (DAD) and a fluorescence detector (FLD), following the extraction and chromatographic methods described in Rodríguez-Estival et al. (2010). This technique has been extensively used in our laboratory and quality checks (i.e. $\leq 8.6\%$ coefficient of variation within repetitions ($n = 14$) of the same sample) have been published (Lopez-Antia et al., 2015).

We measured oxidative stress biomarkers by spectrophotometry in homogenates of RBCs following the methods described in Reglero et al. (2009). Namely, we quantified total and oxidized glutathione (GSH) concentrations and calculated the molar proportion of total GSH present as oxidized form. We used Ransel and Ransod kits (Randox Laboratories, Crumlin, UK) to measure the activities of glutathione peroxidase (GPX, EC 1.11.1.9) and superoxide dismutase (SOD, EC 1.15.1.1), respectively. Enzyme activities were calculated relative to mg of protein, which were determined in RBC homogenates according to Bradford (1976). As a measure of oxidative damage caused by reactive oxygen substances (ROS) to membrane phospholipids, we quantified malondialdehyde (MDA) in the RBC homogenates by HPLC in an Agilent Technologies 1100 Series coupled to a photodiode detector (DAD) and a fluorescence detector (FLD). Determination of MDA was performed following the method by Agarwal and Chase (2002) with the modifications of Romero-Haro and Alonso-Alvarez (2014). Quality checks of the techniques used in our laboratory to analyse all these oxidative stress biomarkers can be found in Lopez-Antia et al. (2015).

2.4. Statistical analysis

We excluded from further statistical analyses those chemicals that, because of a high proportion of measures below detection limits, had a null interquartile range. Consequently, the chemicals considered for statistical analysis were Cu, Zn, chlorinated biphenyl (CB)-180, and all analysed PFAS and PBDEs (Table 1). All variables considered for statistical analysis were log transformed prior to the analysis to minimize variance heterogeneity. The influence of sampling year on pollutant levels was analysed with Generalized Linear Models using a Tweedie distribution with logarithmic link for the dependent variables. Sampling year effect was not significant in any of the models, and hence was excluded from further tests.

Because the majority of pollutant variables did not follow a normal distribution, we used non-parametric statistics for their analysis. Thus, to analyse how human presence influenced the pollutant accumulation levels in Egyptian Vulture nestlings, we run Spearman's bivariate correlations between chemicals and the proportion of urban area around the nest. In order to

estimate the approximate values of proportion of urban surface that could influence pollutant accumulation, the proportion of urban surface was categorized in four groups corresponding to its quartiles, i.e. delimited by the 25th, 50th and 75th percentiles (0.93, 1.61 and 6.56%, respectively). Median values of each significant chemical variable were compared among urban area quartiles using non-parametric, Kruskal-Wallis (for overall comparison) or Mann-Whitney's U (for pairwise comparison) tests. Because physiological parameters did not follow normal distributions, their relationship with the proportion of urban area was tested through Pearson's bivariate correlations. Average values per urban area quartile of the significantly correlated physiological parameters were compared using one-way analyses of the variance (ANOVAs) followed by Tukey's tests for pairwise comparisons.

To analyse the influence of the set of pollutants on physiological parameters we run a redundancy analysis (RDA). In order to reduce the redundancy among variables prior to RDA, we explored the correlation matrices within both the chemical and the physiological variables (Table S3). Among the latter, total proteins (high collinearity with cholesterol), oxidized GSH (high collinearity with % oxidized GSH), α -tocopherol and zeaxanthin (high collinearity with lutein) were removed from the analysis. For chemical variables, we detected a high degree of collinearity within PFAS and PBDEs. To eliminate this collinearity, we run a principal component analysis (PCA) including all chemical variables considered for statistical purposes ($N = 18$). The four first axes (PCs) of the PCA explained 60.7% of the system variance. All chemicals but CB-180 and BDE-209 showed a correlation coefficient >0.5 with any of these four PCs (Table S4). Consequently, the scores of the four extracted PCs together with plasma levels of CB-180 and BDE-209 were used as explanatory variables in the RDA, whereas blood parameters, once excluded the variables with high collinearity, were used as response variables. To estimate the influence that the degree of urbanization could have on the relationship between chemicals and physiological responses, we introduced the proportion of urban areas around the nest as a covariate in the RDA and calculated variance partitioning between chemicals and proportion of urbanized lands.

We run the RDA using the correlation matrix (ter Braak, 1994). The most significant explanatory variables influencing the pool of response variables in the RDA were determined using a forward selection according to a Monte Carlo permutation test (999 permutations). In order to identify which specific responses were influenced by the selected chemical components, we run bivariate correlations of every response variable with the explanatory variables significantly selected by the Monte Carlo test (Pearson's correlations if a PC was selected, or, because of a high proportion of values below limits of detection, Spearman's correlation if CB-180 or BDE-209 were selected). When a response was significantly correlated with any of the chemicals or PCs selected in the RDA, we determined the influence of the degree of urbanization on that correlation through a multiple linear regression, using the selected chemical (or group of relevant chemicals if a PC was selected) and the proportion of urban surface as independent variables. The most influencing variables in each case were selected using a backwards method.

3. Results

A summary of pollutant accumulation in Egyptian Vulture nestlings from Catalonia is shown in Table 1. Among OCs, only CB-180 was detected in more than half of the analysed samples and was, together with p,p'-DDE, the only OC for which a mean value above limits of detection could be calculated. PBDEs and PFAS were commonly detected in a higher proportion of analysed samples, with PFOS as the most abundant and ubiquitous compound. With the exceptions of Cu and Zn, metals had, in general, a low concentration in the analysed blood samples.

The proportion of urban areas around the nests was significantly and positively correlated with plasma levels of CB-180, PFOS, PFTriDA and PBDE congeners 99 and 209 (Table 1). In addition, PFHxS, PFOA and PFDA showed a close to significant ($p < 0.1$), positive correlation with the proportion of urban areas around the nests. Median values per category (quartile) or urbanization were also compared in these close-to-significant variables. Increased plasma levels of these POPs appeared in nestlings from nests that would fall into the third (1.61–6.56%) or the fourth (>6.56%) quartiles of area urbanization (Fig. 1). Only for PFHxS, increased levels were already detected in individuals from nests included in the second quartile (0.93–1.61% of area urbanization). With regards to the physiological parameters, the proportion of urban areas around the nest was positively correlated with activities of ALP and CK, and with levels of Mg and GSH, and negatively correlated with plasma levels of cholesterol, triglycerides, total proteins, α -tocopherol, retinyl palmitate and the two analysed carotenoids (Table 2). Comparisons of average values of these parameters across urban area quartiles reflected that α -tocopherol and the two carotenoids showed decreased levels in individuals from nests corresponding to the fourth quartile compared to the rest of individuals (Fig. 2). For CK and cholesterol, differences between urbanization categories appeared when comparing nestlings from the first and fourth quartiles, whereas for Mg, triglycerides and retinyl palmitate (Fig. 2) the differences across quartiles did not follow a sequential pattern. In the case of ALP, proteins and GSH, Tukey tests failed to detect significant differences between any pair of urbanization categories (Fig. S1).

Fig. 1

Fig. 1. Box plots showing the distribution of blood levels of persistent organic pollutants in Egyptian Vulture nestlings as a function of the quartiles of the proportion of urban areas around their nests. Q1: less than 0.93% urbanized; Q2: 0.93–1.61% urbanized; Q3: 1.61–6.56% urbanized; Q4: more than 6.56% urbanized. Boxes represent the interquartile range, including the median value (black line), external bars represent the limit for values to not be considered outliers (distanced 1.5 times the interquartile range from either 25th or 75th percentile), circles represent outliers and asterisks represent far out values (distanced at more than 3 times the interquartile range from either 25th or 75th percentile). Different letters indicate

differences between categories as resulting from Mann-Whitney's U tests for pairwise comparison of median values ($p < 0.05$).

Table 2. Mean values (and standard deviations) of plasma biochemistry, vitamins and carotenoids, as well as oxidative stress biomarkers in RBCs measured in Egyptian Vulture nestlings from Catalonia. Results of the Pearson's correlations of these physiological parameters with the proportion of urban areas around the nest are shown (N = 76 for all correlations).

Parameter	Abbr.	Mean	SD	Correlation with % urban area			
Pearson' R	p						
Alkaline phosphatase (U L ⁻¹)	ALP	903.6	188.2	0.264	0.021		
Aspartate aminotransferase (U L ⁻¹)	AST	144.8	29.39	-0.047	0.685		
Calcium (mg dL ⁻¹)	Ca	12.32	2.401	-0.164	0.156		
Cholesterol (mg dL ⁻¹)		252.2	65.17	-0.370	0.001		
Creatine phosphokinase (U L ⁻¹)	CK	4954	1209	0.404	<0.001		
Creatinine (mg dL ⁻¹)		0.514	0.202	0.078	0.501		
Glucose (mg dL ⁻¹)		310.9	97.06	-0.039	0.736		
Lactate dehydrogenase (U L ⁻¹)	LDH	1824	510.6	0.170	0.142		
Magnesium (mg dL ⁻¹)	Mg	2.015	0.916	0.243	0.034		
Phosphorus (mg dL ⁻¹)	P	8.959	10.06	0.017	0.887		
Total plasma proteins (g L ⁻¹)		34.44	5.656	-0.331	0.004		
Triglycerides (mg dL ⁻¹)		153.8	69.23	-0.304	0.008		
Urea (mg dL ⁻¹)		10.23	4.088	0.014	0.905		
Uric acid (mg dL ⁻¹)		11.30	5.156	-0.051	0.660		
Retinol (μM)		52.13	13.91	0.160	0.167		
α-Tocopherol (μM)		79.95	22.97	-0.488	<0.001		
Retinyl palmitate (μM)		0.246	0.275	-0.301	0.008		
Lutein (μM)		30.12	17.30	-0.445	<0.001		

Zeaxanthin (μM)		7.840	4.281	-0.414	<0.001		
Glutathione peroxidase (U mg^{-1} protein)	GPx			0.351	0.120	0.183	0.113
Superoxide dismutase (U mg^{-1} protein)	SOD			1.145	0.345	0.123	0.291
Total glutathione ($\mu\text{mol g}^{-1}$)	GSH	3.961	1.411	0.267	0.020		
% oxidized glutathione %oxGSH		32.10	9.385	-0.017	0.887		
Malondialdehyde ($\mu\text{M g}^{-1}$)	MDA	0.075	0.020	0.069	0.553		

Fig. 2

Fig. 2. Box plots showing the distribution of blood levels of biochemical responses in Egyptian Vulture nestlings as a function of the quartiles of the proportion of urban areas around their nests. For details on plot elements and description of categories, see caption of Fig. 1. Different letters indicate differences between categories as resulting from Tukey post-hoc tests following one-way ANOVAs ($p < 0.05$).

The use of the proportion of urban surface as a covariate in the RDA reflected the importance of this variable to explain the variance in the entire set of physiological responses. The comparison of eigenvalues between the full model (0.143) and the model with covariate (0.094) showed that a 34.3% of the total variance explained in the full model could be attributed to the effect of the proportion of urban surface (Table S5). Once eliminated the effect of the urbanization, the RDA selected the CB-180 and the PC2 (indicative of increasing plasma levels of PBDE congeners 100, 154, 153 and 183) as significantly influencing the full set of variables representing biochemical and oxidative stress biomarkers measured in nestlings (Fig. 3). The two canonical axes considered in the RDA explained a low cumulative percentage of the variance of the responses (6.4%) (Table S5), but there was a strong relationship between the pollutants and physiological parameters, with a responses-predictors correlation of 0.685 in the first axis.

Fig. 3

Fig. 3. Graphic representation of the results of the RDA and Monte Carlo permutation test to analyse the effect of selected pollutant-related variables (black arrows), on physiological parameters (grey arrows).

Increased plasma levels of CB-180 were correlated with increased blood activities of CK and concentrations of Mg and total GSH ($R_s \geq 0.232$, $p \leq 0.042$, $N = 77$), and with decreased levels of Ca, cholesterol, α -tocopherol and lutein ($R_s \leq -0.228$, $p \leq 0.046$, $N = 77$). The stepwise regression models kept both CB-180 and proportion of urban surface as variables significantly explaining changes in blood levels of cholesterol and GSH, being the proportion of urbanization

the most determinant variable in both cases (Table S6). For the rest of the physiological parameters, the regression kept only one of the two explanatory variables, either CB-180 (for Mg and Ca) or the proportion of urban surface (for CK, α -tocopherol and lutein). In these cases, the removed parameter did not significantly explain the residual variability left by the selected one.

PC2 was positively correlated with plasma levels of AST ($R = 0.250$, $p = 0.028$, $N = 77$), LDH ($R = 0.305$, $p = 0.007$, $N = 77$) and uric acid ($R = 0.222$, $p = 0.049$, $N = 77$) measured in nestlings. Regression models revealed that AST was negatively associated with BDE-100 and positively with BDE-154, that LDH was positively associated with BDE-153, and that uric acid was positively associated with BDE-183 (Table S6). None of these models kept the proportion of urban area around the nest as a variable significantly explaining the variations in the analysed parameters.

4. Discussion

4.1. Pollutant accumulation in Egyptian Vulture nestlings

PBDEs and PFAS were the analysed chemicals that most frequently occurred in blood samples collected from the Egyptian Vulture nestlings. The present study provides the first published data of accumulation of brominated and perfluoroalkyl POPs in this species. Considering other vulture species, PFAS levels measured in Egyptian Vultures from Catalonia, with the exception of some individuals who showed outlying high values, seem relatively low. Shlosberg et al. (2012) measured mean blood values of 5.8 ng PFOS mL⁻¹ and 2.4 ng PFOA mL⁻¹ in juvenile Griffon Vultures (*Gyps fulvus*) from Israel, with maximum values of 7.4 ng PFOS mL⁻¹ and 3.5 ng PFOA mL⁻¹. Regarding other raptor species, Bustnes et al. (2013) quantified PFOS in plasma of nestlings of Northern Goshawk (*Accipiter gentilis*) and Marine White-Tailed Eagle (*Haliaeetus albicilla*) from northern Norway and found mean values between 12.1 and 22.0 ng g⁻¹ plasma for Goshawks and between 25.4 and 38.7 ng g⁻¹ for Eagles. Assuming a plasma density ~ 1 , these values reflect much higher concentrations than those found in Egyptian Vulture nestlings from Catalonia. Reference values for PFOS toxicity have been proposed for fish-eating birds at levels much higher than those found in the present study (i.e. 2900 and 240 ng mL⁻¹ serum for adult males and females, respectively; Newsted et al., 2005). However, data on PFAS accumulation in birds reflect a strong dependence on the species, geographical origin, feeding regime, age or sampling time for the majority of compounds (Bustnes et al., 2013, 2015; Munoz et al., 2017; Løseth et al., 2019). Therefore, without additional data specifically obtained from Egyptian Vultures, the reasons for the comparatively low values of PFOS and PFOA found in the present study are difficult to estimate.

The majority of studies reporting blood levels of PBDEs in raptors have been conducted in North America (Venier et al., 2010; Fernie et al., 2017; Guo et al., 2018), where these POPs were discontinued from the market several years after they were in Europe (Van den Steen et

al., 2007). Within a European context, high BDE-209 accumulation in raptors compared to values observed in the present study were reported by Eulaers et al. (2014) in White-Tailed Eagle nestlings from central Norway. BDE-209 and the lower brominated BDE-47 and BDE-99 were the most frequently found congeners in our study. BDE-209 has been one of the most commercially used congeners (Birnbaum and Staskal, 2004), and therefore is one of the most abundant in the environment. However, because of its low bioavailability and of the potential for environmental biodegradation (Stapleton et al., 2006), concentrations of BDE-209 accumulated in wildlife specimens relative to other congeners are very variable. The appearance of BDE-47 and BDE-99 as highly abundant PBDEs in wildlife monitoring is also frequent (e.g. Fernie et al., 2017), and is surely related to the high bioavailability of these congeners. Toxicity reference values of PBDEs in raptor blood are scarce. Henny et al. (2009) and Nordlf et al. (2010) reported no reproductive effects of PBDEs in Osprey (*Pandion haliaetus*) and White-Tailed Eagles, respectively, at egg concentrations that would relate, according to the comparisons among sample types made by Jaspers et al. (2013), to blood levels lower than those measured in the present study.

Gmara et al. (2004) monitored OC accumulation in serum of five Egyptian Vulture populations across Spain and reported mean levels of DDE ranging from 0.82 to 6 ng mL⁻¹ (maximum 1–38 ng mL⁻¹) and mean DDT levels ranging from 0.22 to 1.9 ng mL⁻¹ (maximum 0.46–5 ng mL⁻¹). Dhananjayan et al. (2011) reported average plasma levels of p,p'-DDT and p,p'-DDE of 5.4 and 8.8 ng mL⁻¹, respectively, for Egyptian Vultures captured in India, with maximum concentrations of 11.3 ng p,p'-DDT mL⁻¹ and 25.6 ng p,p'-DDE mL⁻¹. Regarding plasma levels of PCBs, the concentration of CB-180, the most abundant congener found in the present study, is lower than what was measured by Gmara et al. (2004) in other Spanish populations (i.e. 0.4–2.8 ng mL⁻¹), although maximum levels detected in our study were consistent with those reported by Gmara et al. (2004) (0.78–18 ng mL⁻¹). Gmara et al. (2004) also reported mean levels of CB-138 and CB-153 of 0.26–1.70 and 0.54–4.10 ng mL⁻¹, respectively, while average levels of these congeners in the present study were <0.01 ng mL⁻¹. This generally low OC accumulation in Egyptian Vultures from Catalonia compared to other populations must be taken with caution as we monitored OC accumulation in nestlings, whereas both Dhananjayan et al. (2011) and Gmara et al. (2004) probably worked with adults. Accumulation of OC in raptors is known to increase with age due to prolonged exposures (Harmata, 2011; Shlosberg et al., 2012). Besides this possible influence of age, the lower concentrations of DDT and DDE found in Catalanian vultures relative to Indian ones could also be influenced by the fact that DDT is still in use for certain purposes in India (van den Berg et al., 2017). As for the comparison with other Spanish populations, Gmara et al. (2004) took their samples between 1999 and 2001 and, although DDT and PCB use was banned in Spain in mid-1990s, their high persistence can make environmental levels of these OCs still relevant and the time lapse between studies could partially account for the observed differences.

Blood levels of analysed metals and metalloids were below detection limits in all cases but for Cu and Zn. Because these are essential elements, their blood levels are subject to variations originated from homeostatic control that do not necessarily reflect changes in environmental availability (Wenzel et al., 1996; Rattner et al., 2008; Ortiz-Santaliestra et al., 2015). Donázar et al. (2002) reported mean and maximum metal levels in Egyptian Vultures (with fledging and adult individuals analysed together) from the Canary Islands of 3.615 and 4.467 $\mu\text{g Zn L}^{-1}$ and 0.0005 and 0.0008 $\mu\text{g Cu mL}^{-1}$, respectively. Although we did not measure metal levels referred to whole blood volumes, we can estimate them if assumed that the majority of the analysed elements occur in RBCs. Considering the average haematocrit value of the samples (30.32%, range 21.49–43.56) and an approximate density of 1.1 g mL^{-1} of the blood pellet, blood concentrations of Zn (mean 2.167 $\mu\text{g mL}^{-1}$, maximum 3.029 $\mu\text{g mL}^{-1}$) measured in individuals from Catalonia were slightly lower than those reported by Donázar et al. (2002), whereas Cu levels (mean 0.077 $\mu\text{g mL}^{-1}$, maximum 1.241 $\mu\text{g mL}^{-1}$) would be considerably higher than those reported in Canarian individuals. Despite these Cu concentrations, the levels of both elements found in Catalanian individuals would be well below those shown by birds living in contaminated sites (Benito et al., 1999).

4.2. Influence of urbanization on pollutant accumulation and physiological responses

A proportion of urban surfaces as low as 6.56% (corresponding to the 75th percentile within our samples) within a radius of 8 km around the nest appears related to the accumulation in Egyptian Vulture nestlings of CB-180, two PBDE congeners including BDE-209, and the majority of PFAS. For some of these chemicals, like PFOS, trends relating increased urbanization and accumulation were found even in territories with only 1.6% of urbanization. Positive associations of the degree of urbanization, or of the human population density, with pollutant accumulation in raptor birds have been reported for some POPs, including PCBs (Elliott et al., 2015; Brogan et al., 2017) and PBDEs (Newsome et al., 2010; Elliott et al., 2015). The majority of these studies monitor pollutant accumulation across urbanization ranges that include densely populated or urbanized areas (e.g. >50% of the surface occupied by residential areas, Brogan et al., 2017), whereas the relationship of urbanization with POP accumulation observed in the present study has been found within a range of areas with a very low degree of urbanization (maximum 8.78% of the surface). Although increased environmental levels of some PFAS have been related to urbanization (Gewurtz et al., 2013), the present study reports, to the best of our knowledge, the first quantitative evidence of an association between the degree of habitat urbanization and PFAS accumulation in birds.

The expansion of Egyptian Vulture populations in NE Spain could be associated with increased food availability because of human waste disposal sites (Tauler-Ametller et al., 2017). We used the degree of urbanization as a proxy for the availability of human wastes. This is based on the system existing in the study area for human waste management; rather than small, local disposal sites, each group of neighbour municipalities has a unique site collecting wastes from all nearby settlements. Therefore, the size of those disposal sites, and hence the availability of food therein, is correlated to the population density in the region. The negative association of

urbanization and plasma levels of cholesterol or triglycerides suggests that human waste disposal sites, which are expected to be more available as the degree of urbanization grows, would provide a secure food source, as the increased circulating levels of these analyses have been associated with food shortage (García-Rodríguez et al., 1987). This result supports what we have recently observed in the studied population when comparing the availability of human waste disposal sites with plasma concentrations of cholesterol and triglycerides in vultures (Tauler-Ametlller et al., 2019). However, in that study we also found that this improvement in food quantity could be associated with an impoverishment in quality, especially in what refers to some vitamins and carotenoids.

CB-180, one of the pollutants whose accumulation in Egyptian Vultures was clearly associated with increased urbanization, was also associated with decreased blood levels of some vitamins and carotenoids (i.e. α -tocopherol and lutein). Decreased levels of these compounds are common in organisms exposed to pollutants generating oxidative stress, which occurs because of an excessive generation of ROS from activation of metabolizing CYP 450 enzymes (e.g. Fernie et al., 2005). Both carotenoids and some free vitamin forms like α -tocopherol counteract the excess of ROS by scavenging electrons from these substances (Di Mascio et al., 1991). In this context, we have found a positive relationship between CB-180 and total GSH levels, which was also observed in Bonelli's Eagles (*Aquila fasciata*) relative to total PCB levels (Ortiz-Santaliestra et al., 2015). GSH is the major intracellular antioxidant molecule that helps neutralizing free radicals (Apel and Hirt, 2004), and therefore it is common that its circulating levels drop at the first instance as a response to pollutant exposure. However, upon a sustained exposure to ROS generated by pollutant metabolism, organisms may react by synthesizing GSH de novo as a compensatory response (DeLeve and Kaplowitz, 1991).

Besides vitamins and carotenoids, other blood parameters possibly indicative of nutritional status, like calcium, were negatively associated with CB-180 accumulation. Although it is not possible to elucidate the relative influence of urbanization and/or CB-180 accumulation on this impoverished nutritional status, it could have generated a loss of muscular tissue in nestlings, which may explain the increased levels of CK in individuals from urbanized areas and in individuals showing higher CB-180 accumulation. Plaza and Lambertucci (2018) observed higher CK levels in Black Vultures (*Coragyps atratus*) from Patagonia that were trapped in rubbish dumps than in those caught in the steppe. This hypothesis of CB-180 exposure affecting muscular tissue must, however, be taken cautiously because other parameters like AST or LDH that can also respond to effects on muscle did not vary as a function of changes in plasma levels of CB-180.

Hexa-BDEs appeared positively associated with either AST or LDH. These enzymes are present in a variety of cell and tissue types in the organism, and thereby their altered circulating levels may reflect damage to several organs like liver, kidney or muscle (see review in Maceda-Veiga et al., 2015). Among potentially altered systems that are known to be affected by PBDE

exposure in birds, Sonne et al. (2010, 2012) found positive correlations between PBDE exposure in White-Tailed Eagles, Northern Goshawks and Golden Eagles (*Aquila chrysaetos*) from northern Norway and some biochemical indicators of liver damage, although neither AST nor LDH were analysed in those studies. In the present study, however, we did not find any association between plasma levels of PBDEs and indicators such as ALP that are more specific of liver damage than AST or LDH. Sonne et al. (2013) found signs, in one of the colonies of North Atlantic Great Skua (*Stercorarius skua*) that they studied, of altered kidney function associated with increased PBDE concentrations. However, such signs consisted of altered levels of urea in blood, whereas in the Egyptian Vultures analysed in the present study urea and PBDE levels were not correlated. Thus, further studies are needed to determine whether the reasons for correlations between increased AST or LDH and hexa-BDE accumulation could be reflecting damage to other systems like the respiratory, cardiac or endocrine ones.

5. Conclusion

Pollutant accumulation in Egyptian Vultures from Catalonia reflects, most likely because of the use of nestlings for biomonitoring, low levels compared to what has been measured in other raptor populations from Spain (van Drooge et al., 2008). Considering that dietary uptake of contaminated prey is the main exposure route of raptors to POPs, vultures in general could be less susceptible than other species to accumulate pollutants because of a comparatively high consumption of herbivore prey (and therefore a reduced susceptibility to biomagnification) and/or farmed animals, in which a certain control to minimize pollutant accumulation may exist.

The main physiological correlate of pollutant exposure was the association between exposure to CB-180 and an altered nutritional status reflected in changes in parameters like α -tocopherol, lutein, cholesterol or calcium. However, most of the covariation of CB-180 and physiological parameters can be explained by the degree of urbanization around the nests. This suggests that changes in blood profiles of vitamins, carotenoids or other analytes in parallel with variation in plasma PCB levels would be consequence of the exploitation of artificial food sources rather than of a direct effect of the pollutants on the organism physiology. Ecological factors such as food availability and/or diet composition, both of which are susceptible to be influenced by the presence of human settlements in the immediate surroundings of the nesting sites, must be explored in order to improve our understanding of the relationships between pollutants and biochemical responses in Egyptian Vultures.