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Blood concentrations of 50 elements in Eagle owl (*Bubo bubo*) at different contamination scenarios and related effects on plasma vitamin levels☆

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ABSTRACT

Some metals and metalloids (e.g. Pb, Hg, Cd and As) are well-known for their bioaccumulation capacity and their toxic effects on birds, but concerns on other minor elements and rare earth elements (ME and REE) are growing due to their intensive use in modern technology and potential toxicity. Vitamins and carotenoids play essential roles in nestling growth and proper development, and are known to be affected by the metals classically considered as toxic. However, we are unaware of any attempts to evaluate the exposure to 50 elements and related effects in plasma vitamins and carotenoids in raptor species. The main goals of this study are: (i) to assess the exposure to 50 elements (i.e. classic toxic elements, trace elements, REE and ME) in nestling Eagle owls (*Bubo bubo*) inhabiting three differently polluted environments (mining, industrial and control areas) in southeastern Spain, and (ii) to evaluate how element exposure affects plasma vitamin and carotenoid levels, hematocrit and body measurements (mass and wing length) of the individuals. Our results show that local contamination in the mining area contributes to increased blood concentrations of Pb, As and Tl in nestlings, while diet differences between control and mining/industrial areas may account for the different levels of Mn, Zn, and Sr in blood, and lutein in plasma. Plasma tocopherol levels were increased in the mining-impacted environment, which may be a mechanism of protection to prevent toxic element-related oxidative stress. Plasma α-tocopherol was enhanced by 20% at blood Pb concentrations ≥8 ng/ml, and nestlings exhibited up to 56% increase in α-tocopherol levels when blood Pb concentrations reached 170 ng/ml. Tocopherol seems to be a sensitive biomarker under an exposure to certain toxic elements (e.g. Pb, As, Tl).

1. Introduction

Raptors are especially suitable and have been widely used as sentinel species in biomonitoring programs worldwide (García-Fernández, 2014; Gómez-Ramírez et al., 2014; Espín et al., 2016a). Such studies can provide early warning of contaminant occurrence and related effects on wildlife and the environment, and are useful to track the success of legislative emission reductions (Espín et al., 2016a; García-Fernández et al., 2020). The scientific community agrees that it is essential to perform biomonitoring studies in raptors in order to evaluate contaminant exposure and related effects (Movalli et al., 2019).

Some metals and metalloids (i.e. Pb, Hg, Cd and As) are well-known for their persistence, bioaccumulation capacity and their

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toxic effects on birds, mainly affecting physiology, immune function, behavior, and reproduction (Eeva et al., 2005; Sánchez-Virosta et al., 2015; Espín et al., 2016b, Espín et al., 2016c; Whitney and Cristol, 2018; Pain et al., 2019; Vallverdú-Coll et al., 2019). Accordingly, these elements are ranked in the first positions of the Substance Priority List elaborated by the Agency of Toxic Substances and Disease Registry (ATSDR, 2019). However, concerns on other minor elements and rare earth elements (ME and REE) are growing due to their use in modern technology, generating emissions to the atmosphere and megatonnes of e-waste (Hussain and Mumtaz, 2014; Tansel, 2017). In spite of this, exposure and related effects of these elements have been rarely evaluated (e.g. in wildlife: Espín et al., 2020b,c, and in humans: González-Antuña et al., 2017; Gaman et al., 2019).

Birds normally show minimal clinical signs of disease, and the evaluation of some biochemical parameters in plasma becomes particularly relevant to evaluate potential metal-related health effects (Harr et al., 2005). In this regard, some authors have provided biochemical reference values in avian species (e.g. Harr, 2002; Casado et al., 2002; Han et al., 2016; Gómez-Ramírez et al., 2016; Agusti Montolio et al., 2018). Vitamins and carotenoids are nutrients extracted from the diet playing different essential roles in nestling growth and proper development. α-Tocopherol is the major form of vitamin E, a lipid-soluble vitamin with different functions: it is an antioxidant protecting membranes against lipid damage, it can be beneficial to bones, it has anti-inflammatory properties, and it stimulates immune response and phagocytic function (Traber and Atkinson, 2007; Chin and Ima-Nirwana, 2014; Rizvi et al., 2014). Retinol is the active antioxidant form of vitamin A, and plays important roles in differentiation and proliferation of cells, in growth, antioxidant protection and immune function, and in the reduction of oxidized tocopherol into the useful form (Wang and Quinn, 1999; Zile, 2001, 2004; Tanumihardjo, 2011). In general, birds have higher plasma α-tocopherol and retinol levels than mammals (Schweigert et al., 1991), and some research has shown higher concentrations of α-tocopherol and retinol in plasma of birds of prey compared to herbivorous birds/mammals (Müller et al., 2011; Ingram et al., 2017). Carotenoids are essential for breeding, immune function, coloration, and some of them are precursors of vitamin A (Britton, 1995; Chew and Park, 2004), while the role of some carotenoids as antioxidants has been questioned and is still under debate (Costantini and Møller, 2008; Koch et al., 2018). The effects of the elements classically considered as toxic (e.g. Pb, Hg, As) on plasma vitamin and carotenoid concentrations have been evaluated in some avian species (Geens et al., 2009; Martínez-Haro et al., 2011; Ortiz-Santaliestra et al., 2015; Ruiz et al., 2016; Sánchez-Virosta et al., 2018). However, we are unaware of any attempts to evaluate the exposure to as many as 50 elements and related effects in plasma vitamins and carotenoids in raptor species, or in any wild animal except for a recent study on Red-necked nightjars (*Caprimulgus ruficollis*) (Espín et al., 2020b,c).

In the light of this uncertainty, the main goals of this study are: (i) to assess the exposure to 50 elements (i.e. ATSDR's list of toxic elements, trace elements, REE and ME) in nestling Eagle owls (*Bubo bubo*) inhabiting three differently polluted environments (mining, industrial and control areas) in southeastern Spain, and (ii) to evaluate how element exposure affects plasma vitamin and carotenoid levels, hematocrit and body measurements of the individuals. Increased blood Pb concentrations are expected in nestlings from the mining-impacted environment based on previous findings (Espín et al., 2015), but the exposure to many other elements and their accumulation capacity are still unknown. Moreover, we hypothesize that exposure to Pb and other toxic elements could alter vitamin levels in plasma (Martínez-Haro et al., 2011; Ruiz et al., 2016).

2. Material and methods

2.1. Species and study area

The Eagle owl is a large nocturnal raptor from the Strigidae family, resident and highly territorial, and with an abundant population in Murcia province (Martínez and Zuberogoitia, 2003; Martínez and Calvo, 2006; León-Ortega et al., 2017). The study zone is located in the east of Murcia province, SE Spain (37°45′ N, 0°57′ W) (Fig. 1), characterized by a Mediterranean semi-arid climate. Different land uses and pollution sources are known in this zone, so it was divided into three areas. The northern zone (hereafter control area) is characterized by citrus and non-irrigation farming, with no known metal contamination sources (Espín et al., 2014b). European rabbit (*Oryctolagus cuniculus*) is plentiful in this area (71% of Eagle owl's prey; own unpublished data). The southern zone is divided into two areas, the industrial and the mining areas. The industrial area has an industrial complex of an international plastics plant (Innovative Plastics, SABIC company) in "La Aljorra" (Cartagena); this company was sanctioned by the Regional Ministry of the Environment "Consejería de Medio Ambiente de Murcia" for the emission of different metals (i.e. As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sb, Ti, Tl, V, Zn) during 2016 and 2017 according to González (2019). The emission limit values are 0.05 mg/m^3 for Cd, Tl and Hg, and 0.5 mg/m^3 for Sb, As, Pb, Cr, Co, Cu, Mn, Ni and V (RD 815/2013). The mining area is an ancient mine site called "Cartagena-La Unión Mining District" with extraction activity since Phoenicians, Carthaginians and Roman times until 1992 (Conesa et al., 2008). However, toxic elements still spread through dispersion of contaminated soil by runoff waters (Conesa and Schulin, 2010). Significant blood levels of Pb, Hg and Cd (García-Fernández et al., 1995; Espín et al., 2014b,c, 2020b) and more recently of As (Espín et al., 2020b) have been reported in wildlife inhabiting this mining area. The southern zone (including both industrial and mining areas) is characterized by irrigation farming, and the rabbit is less abundant (accounting for 35% of Eagle owls' diet). Therefore, owls also consume rats (*Rattus norvergicus* and *Rattus rattus;* 23%), pigeons (*Columba* spp.; 14%), hedgehogs (*Erinaceus europaeus* and *Atelerix algirus*; 5.26%), partridges (*Alectoris rufa*; 5.26%), and yellow-legged gulls (*Larus michahellis*; 3.16%) (own unpublished data).

2.2. Sampling, measurements and analyses

A total of 87 blood samples were collected from Eagle owl nestlings (ca. 35 days old) from 30 nests in the period ranging 16^{th} March 2017 − 8^{th} May 2017 (n = 18 nests/50 nestlings from the control area, 5 nests/14 nestlings from the industrial area and 7 nests/23 nestlings from the mining area; Fig. 1). Best practice guidance was followed according to Espín et al. (2020a). All nestlings were marked with metal rings, and both body mass and wing length were recorded. The health status of the individuals was evaluated by a veterinarian before sample collection, all nestlings being considered clinically healthy (no symptoms were observed in any individual). Blood (ca. 3–5 ml) was collected by venipuncture using a needle (23G) and a syringe, and samples were stored in heparinized tubes and transported under refrigerated conditions until processed in the same day of collection. Hematocrit (% of erythrocytes from total volume) was calculated using a tube reader after blood centrifugation (2200 rcf, 5 min). One tube containing blood was frozen (−80 °C) for toxic element determination, and the second tube was centrifuged (9600 rcf, 5 min) to separate plasma that was also frozen at −80 °C until vitamin and carotenoid analysis. The handling process per individual lasted 10–15 min and nestlings were returned to their nests. The prey remains found in

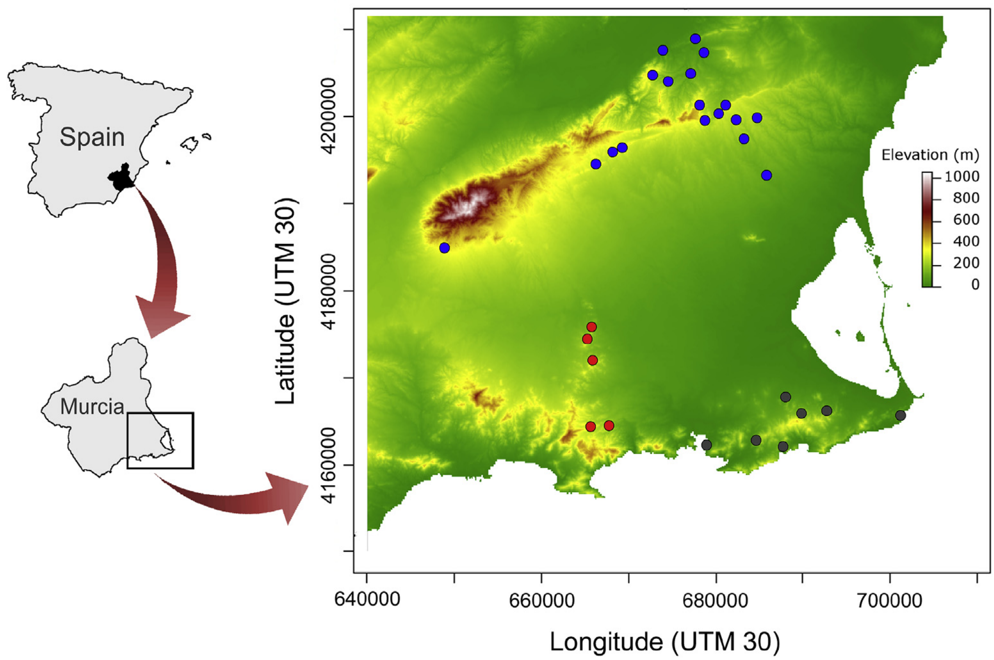

Fig. 1. Geographical location of the studied environments. Blue, red and grey circles represent Eagle owl (*Bubo bubo*) nest sites in the control (n = 18 nests/50 nestlings), industrial (n = 5 nests/14 nestlings), and mining (n = 7 nests/23 nestlings) areas, respectively. Coordinates are indicated as UTM 30S (meters). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the nests were recorded for investigating the diet in the different areas.

The methods to determine 50 elements in blood samples (see list of elements in Table 1; González-Antuña et al., 2017) and retinol, α-tocopherol and lutein in plasma samples (Rodríguez-Estival et al., 2010) have been previously described (Espín et al., 2020b, Espín et al., 2020c) and are detailed in Document S1 and Table S1 (Supplementary Material).

2.3. Statistical procedures

Data analyses were performed using the statistical software R v. 3.6.3 (R Core Team, 2020), which is freely distributed under the GNU General Public License and available at http://www.R-project.org/. Mean ± SD and range values were calculated for the 50 elements analyzed in blood samples (Table 1). Most elements showed a low proportion of values above the limit of quantification (>LOQ) (Table 1). Therefore, for statistical comparison, we selected those 13 elements with medium (38–45%) or high detection rates (97–100%). Values below LOQ were substituted by a random number between 0 and LOQ (Croghan and Egeghy, 2003), using the RANDBETWEEN function in Excel.

For each element, biochemical parameters (hematocrit, retinol, tocopherol and lutein) and body measurements (mass and wing length), we applied linear mixed models (LMMs) using the "nlme" package (Pinheiro et al., 2020), and considering "zone" as a fixed factor and "nest" as a random factor. In a second set of LMMs, we further tested the associations between those 13 elements and the biochemical parameters, where element concentrations were used as explanatory variables and "nest" as random factor in the models. Finally, associations among elements, biochemical parameters and morphological measures were inspected using Pearson correlation (r) test. Variables were $\log_{10}$-transformed prior to analysis to make data conform to normality. Alpha level was 0.05 in the analyses.

3. Results and discussion

3.1. Blood element concentrations in three different scenarios of pollution

Most of the elements analyzed (37 out of 50) showed a low number of concentrations above LOQ (with a percentage of values above LOQ of 26% or lower; Table 1), mainly indicating general low blood concentrations. For those 13 elements with medium (38–45%) or high detection rates (97–100%), Table 2 shows mean ± SD, median and range values in blood of nestling Eagle owls by sampling environment. Concentrations of Pb, As and Tl were significantly increased in nestlings inhabiting the mining area compared to the control area, while Sr, Mn and Zn levels were reduced in owls from the industrial and mining areas compared to the control area (for Zn, differences were found only between mining and control areas) (Table 2, Fig. S1). In this sense, As, Pb and Tl were positively correlated ($r_{As\text{-}Pb} = 0.5$, $r_{As\text{-}Tl} = 0.6$, $r_{Pb\text{-}Tl} = 0.7$; $p < 0.001$, $n = 87$), as well as Mn, Sr and Zn ($r_{Mn\text{-}Sr} = 0.3$, $r_{Mn\text{-}Zn} = 0.4$, $r_{Sr\text{-}Zn} = 0.5$; $p < 0.02$, $n = 87$), while negative correlations were found between these two groups of elements (As-Sr, Pb-Sr, Pb-Mn, Pb-Zn, Tl-Mn, Tl-Sr: $r=(-0.2) - (-0.5)$, $p < 0.03$, $n = 87$) (Table S2 in Supplementary Material).

The increased blood Pb concentrations (ca. 63 times) found in the mining area compared to the control area were expected. Different bird species (including Eagle owl) inhabiting close to this mining site have shown higher blood Pb concentrations along the years (1993–2017) (García-Fernández et al., 1995, 1997; Gómez-Ramírez et al., 2011; Espín et al., 2014b, 2020b) because of the mining activity generated for more than 2500 years until its closure in 1992 (Pavetti et al., 2006; Conesa et al., 2008). Pb concentrations found in this study were similar to those reported in previous years in Eagle owl from the same area and in Black kites (*Milvus migrans*) from Spain (García-Fernández et al., 1995, 1997; Blanco et al., 2003;

Table 1
Element concentrations (ng/ml, w/w) in whole blood of Eagle owl (*Bubo bubo*), n = 87 nestlings.

Element	Category*	Mean	SD	Min	Max	% > LOQ	LOQ
Aluminum (Al)	2	124	713	<LOQ	5560	2	38.4
Antimony (Sb)	2	0.1	0.4	<LOQ	2.5	9	0.038
Arsenic (As)	2	10.0	32.8	0.4	214	100	0.008
Barium (Ba)	2	7.8	30.5	<LOQ	269	15	1.016
Beryllium (Be)	2	0.03	0.1	<LOQ	0.8	7	0.013
Bismuth (Bi)	4	0.03	0.1	<LOQ	0.6	13	0.008
Cadmium (Cd)	2	0.04	0.3	<LOQ	2.0	2	0.015
Cerium (Ce)	3	0.1	0.5	<LOQ	3.9	7	0.035
Chromium (Cr)	1	1.2	4.2	<LOQ	23.7	8	0.229
Cobalt (Co)	1	9.1	6.6	2.2	35.6	100	0.011
Copper (Cu)	1	225	48	154	460	100	1.724
Dysprosium (Dy)	3	0.005	0.02	<LOQ	0.2	11	0.001
Erbium (Er)	3	0.003	0.02	<LOQ	0.1	9	0.001
Europium (Eu)	3	0.003	0.02	<LOQ	0.2	10	0.0003
Gadolinium (Gd)	3	0.007	0.03	<LOQ	0.2	10	0.002
Gallium (Ga)	4	0.1	0.1	<LOQ	0.8	23	0.009
Gold (Au)	4	0.2	0.6	<LOQ	4.6	26	0.007
Holmium (Ho)	3	0.003	0.02	<LOQ	0.2	13	0.0002
Indium (In)	4	0.007	0.02	<LOQ	0.1	21	0.001
Iron (Fe)	1	218434	27698	152422	282851	100	24.6
Lanthanum (La)	3	0.04	0.1	<LOQ	0.7	7	0.020
Lead (Pb)	2	21.7	41.8	<LOQ	173	38	0.361
Lutetium (Lu)	3	0.002	0.02	<LOQ	0.1	2	0.0001
Manganese (Mn)	1	24.5	11.6	10.8	71.1	100	0.371
Mercury (Hg)	2	6.9	8.4	<LOQ	46.8	97	0.028
Molybdenum (Mo)	1	17.2	5.8	7.0	38.7	100	0.148
Neodymium (Nd)	3	0.04	0.1	<LOQ	0.6	10	0.010
Nickel (Ni)	1	14.5	80.3	<LOQ	737	2	7.95
Niobium (Nb)	4	0.02	0.1	<LOQ	0.3	15	0.005
Osmium (Os)	4	0.01	0.1	<LOQ	0.6	9	0.002
Palladium (Pd)	2	0.002	0.02	<LOQ	0.2	2	0.001
Platinum (Pt)	4	0.01	0.1	<LOQ	0.6	23	0.001
Praseodymium (Pr)	3	0.01	0.04	<LOQ	0.2	10	0.003
Ruthenium (Ru)	4	0.0	0.0	<LOQ	0.0	0	0.000
Samarium (Sm)	3	0.009	0.03	<LOQ	0.2	11	0.002
Selenium (Se)	1	451	139	252	994	100	0.153
Silver (Ag)	2	1.4	11.6	<LOQ	108	17	0.029
Strontium (Sr)[a]	2	90.7	54.0	24.6	249	100	0.439
Tantalum (Ta)	4	0.02	0.1	<LOQ	0.9	14	0.001
Terbium (Tb)	3	0.003	0.02	<LOQ	0.2	13	0.0003
Thallium (Tl)	2	0.2	0.3	<LOQ	1.8	38	0.008
Thorium (Th)	2	0.008	0.03	<LOQ	0.2	9	0.002
Thulium (Tm)	3	0.002	0.02	<LOQ	0.1	11	0.000
Tin (Sn)	2	1.0	2.8	<LOQ	19.4	15	0.199
Titanium (Ti)	4	5.1	9.8	<LOQ	52.5	23	0.757
Uranium (U)	2	0.01	0.1	<LOQ	0.8	3	0.002
Vanadium (V)	2	0.8	1.4	<LOQ	9.1	45	0.034
Ytterbium (Yb)	3	0.003	0.02	<LOQ	0.1	7	0.001
Yttrium (Y)	3	0.02	0.1	<LOQ	0.2	13	0.005
Zinc (Zn)	1	4200	682	2462	5957	100	51.0

*Category: 1 = Essential trace elements, 2 = ATSDR's list of toxic elements, 3 = Rare earth elements, 4 = Other minor elements. LOQ = Limit of quantification.
[a] Stable Sr is considered of relatively low toxicity (only Strontium-90 is included in the ATSDR's Substance Priority List).

Gómez-Ramírez et al., 2011; Espín et al., 2014b), and higher than those found in Northern goshawk (*Accipiter gentilis*) and Black kites from Spain and Norway (Baos et al., 2006; Dolan et al., 2017) (Fig. 2). These Pb concentrations have been related to effects on different physiological parameters in Eagle owls in this area (up to 79% decrease in blood δALAD, depletion of antioxidant enzymes and glutathione levels and induction of lipid damage in red blood cells) (Espín et al., 2014b, 2015). However, to the best of our knowledge, blood levels of other toxic elements such as As have never been reported in this owl species, or rarely described in any wild bird species in the case of Tl levels (Espín et al., 2020b). Evaluating As exposure in wild birds is uncommon in spite of its known toxicity (Sánchez-Virosta et al., 2015), and this is particularly important in areas influenced by past or present mining activities where As accumulates in plants growing in contaminated soils, which in turn will be consumed by animals (including prey of Eagle owls) entering the food chain (Martínez-López et al., 2014).

Our results show that local contamination in the mining area also contributes to the higher concentrations of other important toxic elements, since nestlings inhabiting the mining area had mean blood As and Tl concentrations 15 and 17 times higher, respectively, than those found in the control site (Table 2). In addition, the positive correlations found between As, Pb and Tl suggest common origins in the polluted site. Thallium may be released into the atmosphere from both natural and human sources, and increased levels are found in the mining areas, smelters and coal-burning facilities (Karbowska, 2016). This element tends to bioaccumulate in organisms, and blood concentrations higher than 100 ng/ml are considered toxic in humans (Lansdown, 2013; Karbowska, 2016). In spite of the increased Tl levels in the mining-impacted site, concentrations in this study seem to be relatively low (Table 2), mean values in the mining area (0.52 ± 0.43 ng/ml; max.

Table 2
Mean ± SD, median (range) concentrations of elements (ng/ml, w/w) in blood, body measurements, hematocrit and plasma biochemistry in Eagle owl (*Bubo bubo*) at three sampling environments (control, industrial and mining area), n = 87 nestlings.

Element	Control area (N = 50)	Industrial area (N = 14)	Mining area (N = 23)
	Mean ± SD **Median (range)**	**Mean ± SD** **Median (range)**	**Mean ± SD** **Median (range)**
ATSDR's list of toxic elements in whole blood			
Arsenic (As)	2.22 ± 3.04 1.08 (0.48–16.2)	1.04 ± 0.98 0.57 (0.4–3.49)	32.2 ± 59.0* 12.4 (2.31–214)
Lead (Pb)	1.24 ± 2.74 0.19 (<LOQ - 10.7)	2.44 ± 4.50 0.20 (<LOQ - 11.5)	77.9 ± 48.0* 83.1 (12.0–172)
Mercury (Hg)	6.15 ± 8.90 3.05 (<LOQ - 46.7)	7.54 ± 9.34 4.92 (0.71–34.4)	7.94 ± 6.59 5.69 (1.15–24.0)
Strontium (Sr) [a]	120 ± 53.1 121 (32.5–249)	61.8 ± 21.2* 58.8 (30.7–122)	44.6 ± 11.7* 43.7 (24.6–74.2)
Thallium (Tl)	0.03 ± 0.10 <LOQ (<LOQ - 0.51)	0.05 ± 0.07 <LOQ (<LOQ - 0.17)	0.52 ± 0.43* 0.29 (<LOQ - 1.77)
Vanadium (V)	0.82 ± 1.52 <LOQ (<LOQ - 9.12)	0.16 ± 0.57 <LOQ (<LOQ - 2.15)	1.06 ± 1.40 0.65 (<LOQ - 5.24)
Essential trace elements in whole blood			
Cobalt (Co)	9.67 ± 6.29 7.64 (2.36–29.2)	7.89 ± 9.83 4.65 (2.55–35.6)	8.51 ± 4.86 8.39 (2.25–21.7)
Copper (Cu)	226 ± 38.2 227 (153–297)	231 ± 71.9 215 (172–459)	218 ± 51.0 214 (161–411)
Iron (Fe)	221043 ± 28740 219356 (161264–275123)	216888 ± 20237 213024 (194096–256380)	213702 ± 29613 213007 (152422–282850)
Manganese (Mn)	29.1 ± 12.6 27.3 (12.8–71.1)	16.1 ± 5.59* 15.5 (10.8–33.7)	19.4 ± 5.56* 17.7 (11.8–32.0)
Molybdenum (Mo)	18.3 ± 6.73 17.6 (6.99–38.6)	15.4 ± 4.27 14.3 (11.0–27.3)	15.8 ± 3.37 15.0 (11.2–26.8)
Selenium (Se)	477 ± 162 428 (290–993)	472 ± 84.0 473 (340–637)	380 ± 74.7 381 (251–544)
Zinc (Zn)	4409 ± 741 4299 (3261–5957)	4046 ± 387 4029 (3256–4727)	3838 ± 504* 3790 (2461–5059)
Body measurements, hematocrit and plasma biochemistry			
Hematocrit (%)	25.8 ± 4.49 26.0 (15.0–35.0)	26.1 ± 2.12 26.0 (23.0–29.0)	27.4 ± 4.70 27.0 (19.0–36.0)
Body mass (g)	1232 ± 230 1212 (800–1850)	1320 ± 208 1288 (1000–1825)	1290 ± 292 1250 (825–2000)
Wing length (mm)	198 ± 42 195 (115–292)	218 ± 40 210 (135–280)	212 ± 80 180 (112–400)
Retinol (μM)	16.5 ± 2.39 16.2 (11.4–22.3)	15.7 ± 1.13 15.8 (13.6–17.9)	15.8 ± 2.14 15.3 (13.0–22.5)
Tocopherol (μM)	79.8 ± 15.9 78.9 (47.0–112)	87.1 ± 13.1 84.3 (69.2–108)	99.3 ± 18.3* 96.6 (68.9–155)
Lutein (μM)	6.36 ± 3.78 5.48 (1.57–16.3)	9.35 ± 4.39 9.08 (3.81–18.3)	9.42 ± 3.61** 8.82 (4.55–16.8)

Asterisks denote significant differences between industrial or mining area and control area (*p < 0.01, **p < 0.05) as observed in the linear mixed models ("zone" used as fixed factor and "nest" used as random factor; response variables were log_{10}-transformed prior to analysis). LOQ = Limit of quantification.
[a] Stable Sr is considered of relatively low toxicity (only Strontium-90 is included in the ATSDR's Substance Priority List).

1.77 ng/ml) being below the levels considered normal in blood of animals or humans (<1 ng/ml and <2 ng/ml; Mulkey and Oehme, 1993; Lansdown, 2013).

In regards to As, concentrations reached in nestlings may be of special concern in the mining area. For comparison, blood As levels in other raptor species were compiled in Fig. 2. In general, nestling Eagle owls showed higher As levels than those reported in Northern goshawk and Common buzzard (*Buteo buteo*) from Spain, Norway and Portugal (Carneiro et al., 2014; Dolan et al., 2017), and were similar to those found in Black kites from Spain and Portugal (Blanco et al., 2003; Carneiro et al., 2018) (Fig. 2). Black kites sampled in Doñana (Spain) in 1999 after the Aznalcóllar mine spill showed remarkably higher As levels (125 ng/ml) than those found in nestling Eagle owls, which was related to the toxic spill and the foraging habits of the species in that sampling site (marine fish were found as prey remains in the nests) (Baos et al., 2006). In this study, few individuals reached As blood levels higher than 100 ng/ml (up to 214 ng/ml, Table 2). This metalloid is not well-documented in birds, and the threshold blood values related to sublethal adverse effects have not been properly established in avian species (Sánchez-Virosta et al., 2015). Different authors refer to blood As levels below 20 ng/ml as a suggested reference baseline value for birds in unpolluted areas (Benito et al., 1999; Ortiz-Santaliestra et al., 2015; Rodríguez-Estival et al., 2019). However, recent studies have shown that, for other elements classically considered toxic (i.e. Pb, Cd, Hg), blood levels below the threshold value commonly accepted for physiological effects in raptors are able to produce effects on the antioxidant system in Eagle owls and other bird species (Espín et al., 2014a,b, 2016b). Therefore, potential As-related effects on physiology in Eagle owls inhabiting mining-impacted areas cannot be discarded, even more if we consider that nestlings may be unable to regulate the As (and metals) body burden as efficiently as adults (Burger and Gochfeld, 1997).

On the other hand, pollutant-related indirect effects (e.g. lower food quality and quantity or changes in diet due to resource limitations) may contribute to lower essential element (Mn, Zn) and Sr concentrations in the mining-impacted and industrial sites compared to the control area. Strontium is classically considered a non-essential element, because it does not cause death when absent (Pors Nielsen, 2004), but different studies show that this

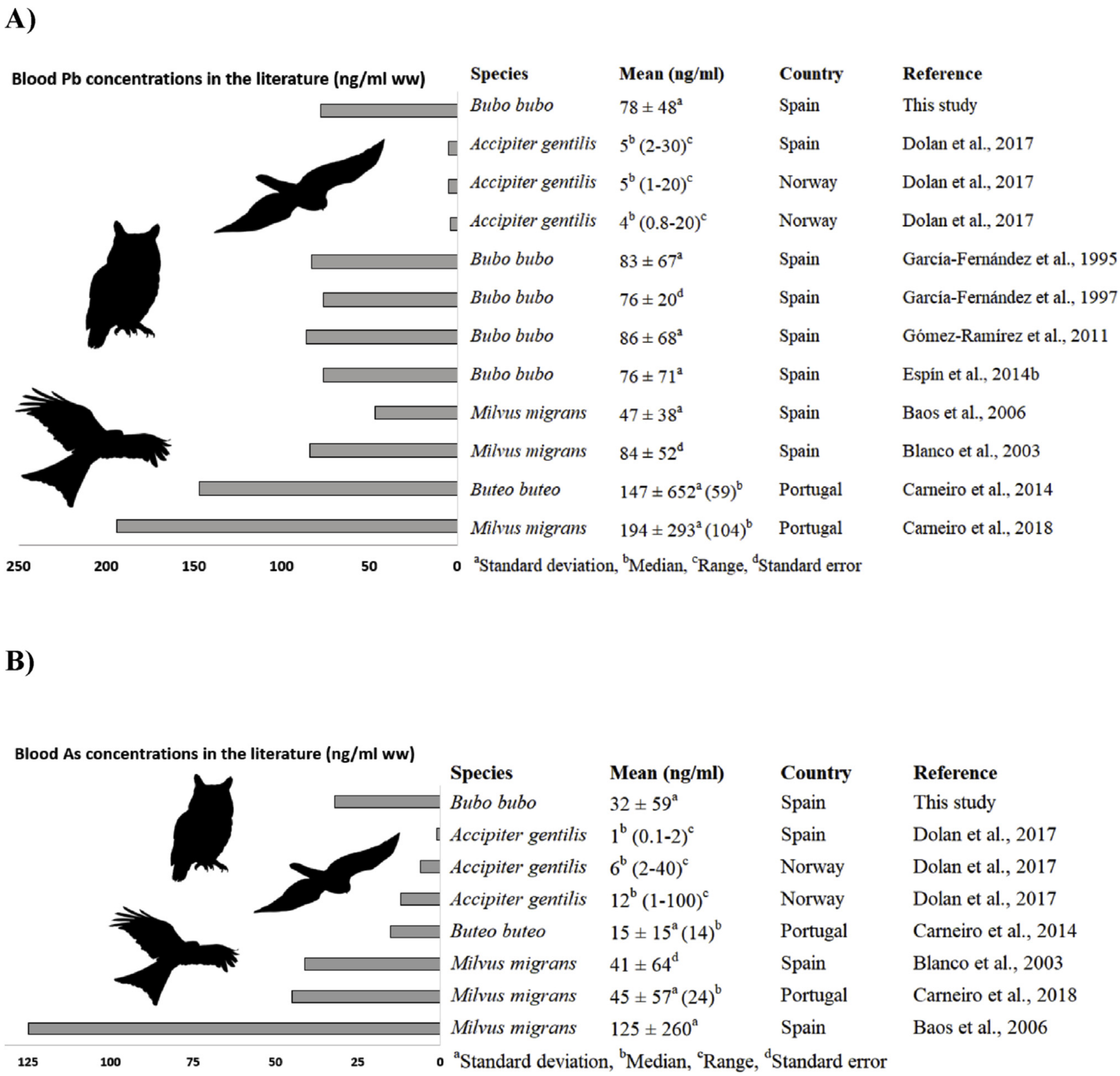

Fig. 2. Blood Pb (A) and As (B) concentrations (ng/ml, w/w) in raptor species inhabiting polluted and urban environments in the literature.

element is taken up at the bone, its supplementation increases calcified bone volume and limits bone resorption, preventing from bone mass loss, so it has been suggested that it may have a role in bone development (Marie et al., 1993; Sila-Asna et al., 2007; Pemmer et al., 2013; Maciejewska et al., 2014). However, further studies are needed to better understand the essentiality of this element.

European rabbits are abundant in the control area (71% of Eagle owl's prey), while in the southern zones (including both the industrial and mining areas) this prey is less abundant (35% of the diet), and owls also consume rats (23% of the diet), pigeons, partridges, hedgehogs and yellow-legged gulls (own unpublished data). In this study, similar results were observed when recording the prey remains found in the nests (Table 3). In the control area, rabbits represented 70% of the diet, while 30% was represented by other prey types. However, in the mining site, rabbits represented 50% of the diet, and Eagle owls also consumed partridges (20%), pigeons, rats, and hedgehogs (10% each). In the industrial area, rabbits represented 100% of the prey found in nests. However, it should be noted that there were only 4 nests with 1 rabbit each (Table 3). These diet differences may account for the different input of essential elements and Sr between control and mining and industrial areas. However, further studies are needed to evaluate element concentrations in prey remains. Moreover, Mn, Zn and Sr were positively correlated, which could reflect common origins through dietary intake and/or homeostatic regulation controlling absorption and body trace element levels (Espín et al., 2020b).

3.2. Effects of toxic elements on body measurements, hematocrit, plasma vitamin and lutein levels

Nestlings in the mining area showed increased plasma α-tocopherol and lutein concentrations, while the other parameters (hematocrit, retinol and body measurements) were not affected by

Table 3
Diet items found in 30 nests of Eagle owl (*Bubo bubo*) at three sampling environments (control, industrial and mining area) from Murcia, Spain, in 2017. Both the number of each prey item and the percentage of the total number of prey are provided.

Diet item	Control area (18 nests)		Industrial area (5 nests)		Mining area (7 nests)	
	Total number	%	Total number	%	Total number	%
European rabbit (*Oryctolagus cuniculus*)	16	69.6	4	100	5	50
Pigeon (*Columba* spp.)	2	8.7	0	0	1	10
Rats (*Rattus rattus* and *Rattus norvergicus*)	1	4.3	0	0	1	10
Mallard (*Anas platyrhynchos*)	1	4.3	0	0	0	0
European hedgehogs (*Erinaceus europaeus*)	1	4.3	0	0	1	10
Partridge (*Alectoris rufa*)	2	8.7	0	0	2	20
Nests with no prey	**4**		**1**		**2**	

zone (Table 2, Fig. S1). Results from LMMs showed significant positive associations between blood Pb levels and plasma α-tocopherol ($F = 9.53$, $p = 0.003$), and blood Pb levels and plasma lutein ($F = 5.44$, $p = 0.023$), while negative associations between blood Mo ($F = 13.39$, $p < 0.001$), Co ($F = 7.65$, $p = 0.008$) and Sr ($F = 4.28$, $p = 0.043$) and plasma lutein were observed (Table S3 in Supplementary Material). No element-related effects were observed in hematocrit nor retinol, and few associations were found between elements and body measurements: negative for blood Mo and body mass ($F = 4.15$, $p = 0.046$) and positive for blood Fe ($F = 4.72$, $p = 0.034$) and Se ($F = 6.75$, $p = 0.012$) and wing length (Table S3). Plasma α-tocopherol levels were positively correlated with blood As, Pb and Tl ($r = 0.22–0.36$, $p < 0.039$, $n = 87$) and negatively correlated with blood Sr levels ($r = -0.27$, $p = 0.012$, $n = 87$) (Table S2). Plasma lutein levels were negatively correlated with Mo, Co and Sr ($r = (-0.35) - (-0.37)$, $p < 0.005$, $n = 87$) and positively correlated with Pb ($r = 0.22$, $p = 0.042$, $n = 87$). Finally, plasma α-tocopherol and lutein levels were also positively correlated ($r = 0.38$, $p < 0.001$, $n = 87$) (Table S2).

α-Tocopherol is the most common form of vitamin E, a potent antioxidant that neutralizes lipid peroxyl radicals preventing from lipid peroxidation in the cell membrane (Traber and Atkinson, 2007). Therefore, the elevated plasma α-tocopherol concentrations in those individuals from the mining area facing increased blood toxic elements, together with the positive association of α-tocopherol with blood As, Pb and Tl, can be interpreted as a protective response that helps them cope with metal-induced oxidative stress and lipid peroxidation (Koivula and Eeva, 2010) in such a way that the antioxidant defense is strengthened. Along the same lines, red-necked nightjars inhabiting the same mining site showed increased blood element levels (i.e. Pb, As and Cd) compared to the control area, and they were also associated with increased α-tocopherol in plasma (Espín et al., 2020b,c). Previous field and experimental studies have found a similar response in different avian species exposed to toxic elements in Spain, Hungary and Finland (Martínez-Haro et al., 2011; Hargitai et al., 2016; Ruiz et al., 2016). In this study, Eagle owls with blood Pb levels ≥8 ng/ml showed a 20% increase in plasma α-tocopherol with regards to the mean α-tocopherol concentration in the control area; and α-tocopherol was enhanced by 31% and 56% at blood Pb concentrations ≥80 ng/ml and 170 ng/ml, respectively. In view of the results found in this and previous studies, α-tocopherol seems to be a very sensitive biomarker when exposed to certain toxic elements (e.g. Pb, As, Tl).

Lutein is the most abundant carotenoid in birds of prey (Ingram et al., 2017). Different factors may affect carotenoid levels, including metal exposure but also food availability and type of diet (Eeva et al., 2008; Dauwe and Eens, 2008; Cohen et al., 2009; Vallverdú-Coll et al., 2016a, Vallverdú-Coll et al., 2016b; Sumasgutner et al., 2018; Pacyna et al., 2018). Blood Pb concentrations were positively associated with lutein levels in this study, as previously reported in other avian species both in experimental and biomonitoring studies (Vallverdú-Coll et al., 2016a, Vallverdú-Coll et al., 2016b). It is well known that Pb, as well as many other metals, can induce oxidative stress in birds (Koivula and Eeva, 2010), and lutein could be increased to counteract this Pb-related oxidative imbalance in the mining area. Although it has been suggested that lutein is not as effective in antioxidant defense as some other carotenoids (see review by Koivula and Eeva, 2010), it may still have antioxidant properties by protecting phospholipids in cell membranes or by participating in recycling vitamin E (Costantini, 2008; Koivula and Eeva, 2010). In this sense, Eagle owl nestlings showed a positive association between plasma α-tocopherol and lutein levels.

However, plasma lutein concentrations in nestlings from the industrial area (showing equivalent element levels to those found in the control site) were similar to lutein levels found in the mining area (Table 2). Therefore, the increased lutein levels in the mining-impacted environment compared to the control site could be mainly related to a higher diet diversity in the south of our study area. Lutein is an abundant carotenoid in the diet and blood of birds, and birds in general contain more carotenoids than mammals (Urich, 1994; McGraw, 2006), thus, the greater consumption of avian prey (pigeons, partridges, gulls) may lead to higher plasma lutein concentrations in Eagle owl inhabiting the southern zone.

4. Conclusions

Our results show that local contamination in the mining area contributes to increased blood concentrations of Pb, As and Tl in nestling Eagle owls, while diet differences between control and mining/industrial areas may account for the different levels of blood Mn, Zn, and Sr, and plasma lutein.

Increased levels of α-tocopherol in plasma of Eagle owls in the mining-impacted environment may prevent toxic element-related oxidative stress, thereby providing a mechanism of protection. This study shows that nestlings with blood Pb levels ≥8 ng/ml showed a 20% increase in plasma α-tocopherol levels. α-Tocopherol seems to be a very sensitive biomarker under an exposure to certain toxic elements (e.g. Pb, As, Tl).

Based on previous findings in other avian species inhabiting the same mining-impacted environment (Espín et al., 2020c), further studies should evaluate the potential combined effects of Pb, As and Tl on mineralization-related parameters in nestling Eagle owls experiencing active growth.

CRediT authorship contribution statement

Pablo Sánchez-Virosta: Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Mario León-Ortega:** Methodology, Writing - review & editing. **José F. Calvo:** Formal analysis, Writing - review & editing. **Pablo R. Camarero:**

Methodology, Writing - review & editing. **Rafael Mateo:** Methodology, Writing - review & editing. **Manuel Zumbado:** Methodology, Writing - review & editing. **Octavio P. Luzardo:** Methodology, Writing - review & editing. **Tapio Eeva:** Formal analysis, Writing - review & editing. **Antonio J. García-Fernández:** Formal analysis, Writing - review & editing. **Silvia Espín:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115012.

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