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 when exposed to certain toxic elements (e.g. Pb, As, Tl).

***Keywords*:** metal exposure; tocopherol; vitamins; lutein; *Bubo bubo*

Capsule: Increased blood toxic elements, plasma α-tocopherol and lutein in nestling Eagle owls inhabiting a mining-impacted environment

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 impacts in wildlife and the environment, and can be used to track the success of the 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 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, c; 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 intensive use in modern technology, generating aerial emissions and tons 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. 2020, 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 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; Martinez-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. 2020a, b).

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 toxic elements, trace elements, REE and ME) in nestling Eagle owls (*Bubo bubo*) inhabiting three different scenarios of pollution (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 (Martinez-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. This species is the largest nocturnal raptor in Spain, resident and highly territorial, and its population in the province of Murcia is abundant (Martínez, J.A.; Zuberogoitia, I. 2003; Martínez, J.E.; Calvo, J.F. 2006; León-Ortega et al. 2017). The study zone is located in the east of the province of Murcia, southeastern Spain (37º45’ N, 0º57’ W) (Figure 1), characterized by a Mediterranean semi-arid climate. Different land uses and contamination sources are known in this zone, so it was divided into three areas. The northern zone (hereafter control area) is mainly dedicated to citrus and non-irrigation farming, with no known metal contamination sources (Espín et al. 2014b). In this area, the European rabbit (*Oryctolagus cuniculus*) is abundant, accounting for 71% of the prey consumed by Eagle owls (authors’ unpublished data). The southern zone is divided into two areas, the industrial and the mining areas. The industrial area has an important industrial complex of an international plastic 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 (González 2019). 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 are still spread by small creeks from headwaters, due to the eroding process of runoff waters, impacting on surrounding ecosystems (Conesa and Schulin 2010). Significant blood levels of Pb, Hg and Cd (García-Fernández et al. 1995; Espín et al. 2014c, b, 2020a) and more recently of As (Espín et al. 2020a) have been reported in wildlife inhabiting this mining area. In the southern zone (including both industrial and mining areas) irrigation farming is predominant, and the European rabbit is less abundant (35% of Eagle owls’ diet), such that the raptor consumes a similar proportion of rats (*Rattus rattus* and *Rattus norvergicus*) (23% of the diet), in addition to pigeons (*Columba* spp.) (14%), partridges (*Alectoris rufa*) (5.26%), hedgehogs (*Erinaceus europaeus* and *Atelerix algirus*) (5.26%) and yellow-legged gulls (*Larus michahellis*) (3.16%) (authors’ unpublished data).

2.2. Sampling and measurements

A total of 87 blood samples were collected from Eagle owl nestlings (ca. 35 days old) from 30 nests in the period ranging 16th March 2017 – 8th 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; Figure 1). All nestlings were individually marked with metal rings, and both body mass and wing length were recorded. The health status of the individuals was clinically evaluated by a veterinarian before blood sampling, all nestlings being considered clinically healthy (no symptoms were observed in any individual).

Blood samples (ca. 3-5 ml) were collected by puncturing brachial veins with a 23G needle and a syringe, and stored in heparinized Eppendorf tubes under refrigerated conditions until processed in the laboratory in the same day of collection. Hematocrit (% of red blood cells from total sample volume) was recorded using a capillary tube reader after blood centrifugation (2200 rcf, 5 min). One Eppendorf tube containing whole blood was frozen at −80°C until element analysis, and other tube with whole blood was centrifuged (9600 rcf, 5 min) to separate plasma that was also frozen at −80°C until vitamin and carotenoid analysis. Duration of the handling process per individual ranged from 10 to 15 minutes and nestlings were returned to their nests. The prey remains found in the nests were recorded for investigating the diet in the different areas.

2.3. Metal analysis

We analyzed blood concentrations of 50 elements (see Table 1) selected according to their toxicity and/or use in electronic products (Hussain and Mumtaz 2014; Tansel 2017). We used an Agilent 7900 ICP-MS equipment (Agilent Technologies, Tokyo, Japan) equipped with standard nickel cones, Ultra High Matrix Introduction (UHMI) system, and a cross-flow nebulizer with a Make-Up Gas Port (X400 Nebulizer, Savillex Corporation, MN, USA). We followed the procedure described by González-Antuña et al. (2017). Briefly, blood (130 µL) was diluted with 1120 µL of ammonia solution (0.05% of EDTA, 0.05% of Triton X-100, and 1% of NH_4OH), and 50 µL of internal standards (ISTD) were added (scandium, germanium, rhodium, and iridium; stock concentration of 20 mg/mL each). Pure standards in acid solution (5% HNO_3, 100 mg/L) were purchased from CPA Chem (Stara Zagora, Bulgaria). Two standard curves (ten points, 0.005 ng/mL – 20 ng/mL) were prepared to avoid interferences between elements: i) one using a commercial multi-element mixture (CPA Chem, 100 mg/L, 5% HNO_3) containing all the essential elements and main toxic metals, and ii) other multi-element mixture tailor-made in our laboratory from individual elements (CPA Chem), which contained the REE and ME. The limits of quantification (LOQs) ranged between 0.005 and 1.0 ng/ml, and the accuracy of measurements were in the range of 79 − 138%, with relative standard deviations (RSD) below 6% (González-Antuña et al. 2017).

2.4. Vitamin and carotenoid analysis

Retinol and α-tocopherol in plasma were analyzed by high-pressure liquid chromatography coupled to diode array and fluorescence detection (HPLC-DAD-FLD) according to Rodríguez-Estival et al. (2010). Samples (ca. 100 µL plasma) were mixed with 200 µL of water and 150 µL of ethanol in an Eppendorf tube, to which ISTD were added (50 µL of retinyl acetate - 58 mM - and α-tocopheryl acetate - 1.04 mM - in ethanol). The head-space of the tube was flushed with N_2 and immediately capped to avoid vitamin oxidation during the extraction process. Samples were then vortexed (5 min), sonicated (1 min), and extracted twice with 1 mL of hexane using vortex mixing (15 min each time). Hexane phases were recovered after centrifuging (14,000 rcf, 5 min, 4 ºC) and evaporated to dryness with N_2 flow. Residues were redissolved in methanol (200 µL) and injected into the HPLC-DAD-FLD system (Agilent 1200 Series). Vitamins were separated using an Agilent ECLIPSE XDB-C18 4.6mm x 150mm 5um. Samples were eluted isocratically using 80% acetonitrile (Hipersolv Chromanorm HPLC LC-MS grade, Prolabo), 19% methanol (Hipersolv Chromanorm, Gradient Grade, Prolabo) and 1% water. This starting proportion was maintained for 15 min; the acetonitrile was then increased to 100% during a 15 min period, was held at this level for 1 min, and then returned to initial conditions over 2 min. The flow rate was 1 mL/min and the injection volume was 20 µL. Data were collected using DAD and FLD simultaneously. The DAD wavelength used for free retinol was 325 nm; in FLD, the excitation and emission wavelengths for α-tocopherol were 295 nm and 325 nm, respectively. Calibration curves were prepared with standards of free retinol and α-tocopherol (Sigma). The percentage of recovery was 90% for both vitamins.

2.5. 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%). For these elements we substituted <LOQ values by a random number between 0 and LOQ.

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 them better conform normal distribution. Alpha level was set to 0.05 in all 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 rate of values 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%), concentrations in whole blood of nestling Eagle owls by sampling environment are shown in Table 2. Concentrations of As, Pb and Tl were significantly increased in blood of individuals captured at 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 area) (Table 2). 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 S1 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, 2020a) due to the intensive mining activity generated for more than 2500 years until its closure in 1992 (Pavetti et al. 2006; Conesa et al. 2008). The 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; 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) (Figure 2). These Pb concentrations have been related with effects on different physiological parameters in Eagle owls in this area (up to 79% decrease in blood δALAD, inhibition of antioxidant enzymes, depletion of 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. 2020a). 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 is also contributing 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 origin in the polluted site. Thallium may be released into the biosphere from natural and anthropogenic sources, and increased levels are found in the vicinity of 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. 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 purposes, blood As levels in other raptor species were compiled in Figure 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 similar to those found in Black kites from Spain and Portugal (Blanco et al. 2003; Carneiro et al. 2018) (Figure 2). Black kites sampled in Doñana (Spain) in 1999 after the Aznalcóllar mine spill showed remarkable 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 when it comes to 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 as 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. 2014b, a, 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 be contributing to the 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 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.

As explained before, the control area is home to abundant European rabbits, accounting for 71% of the prey consumed by Eagle owls, while in the southern zones (including both the industrial and mining areas) this prey is less abundant (35% of Eagle owl's diet), and birds consume a similar proportion of rats (23% of the diet), including also in their diet pigeons, partridges, hedgehogs and yellow-legged gulls (authors' 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, this should be further evaluated by analyzing element concentrations in prey remains in future studies. Moreover, Mn, Zn and Sr were positively correlated, which could reflect common origin through dietary intake and/or homeostatic regulation controlling absorption and body trace element levels (Espín et al. 2020a).

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 zone (Table 2). 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 S2 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 S2). Regarding Pearson correlations, tests showed that plasma α-tocopherol levels were positively correlated with blood As, Pb and Tl ($r = 0.22\text{-}0.36$, $p < 0.039$, $n= 87$) and negatively correlated with blood Sr levels ($r = -0.27$, $p = 0.012$, $n= 87$) (Table S1). 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 S1).

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. 2020a, b). Previous field and experimental studies have found a similar response in different avian species exposed to toxic elements in Spain, Hungary and Finland (Martinez-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, b; 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, b). 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 the process of 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 concentrations of Pb, As and Tl in blood of 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 when exposed 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. 2020b), further studies should evaluate the potential combined effects of Pb, As and Tl on mineralization-related parameters in nestling Eagle owls experiencing an active growing process.

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institutional and/or national guidelines for the care and use of animals were followed. The authors declare that they have no conflict of interest.

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Figures

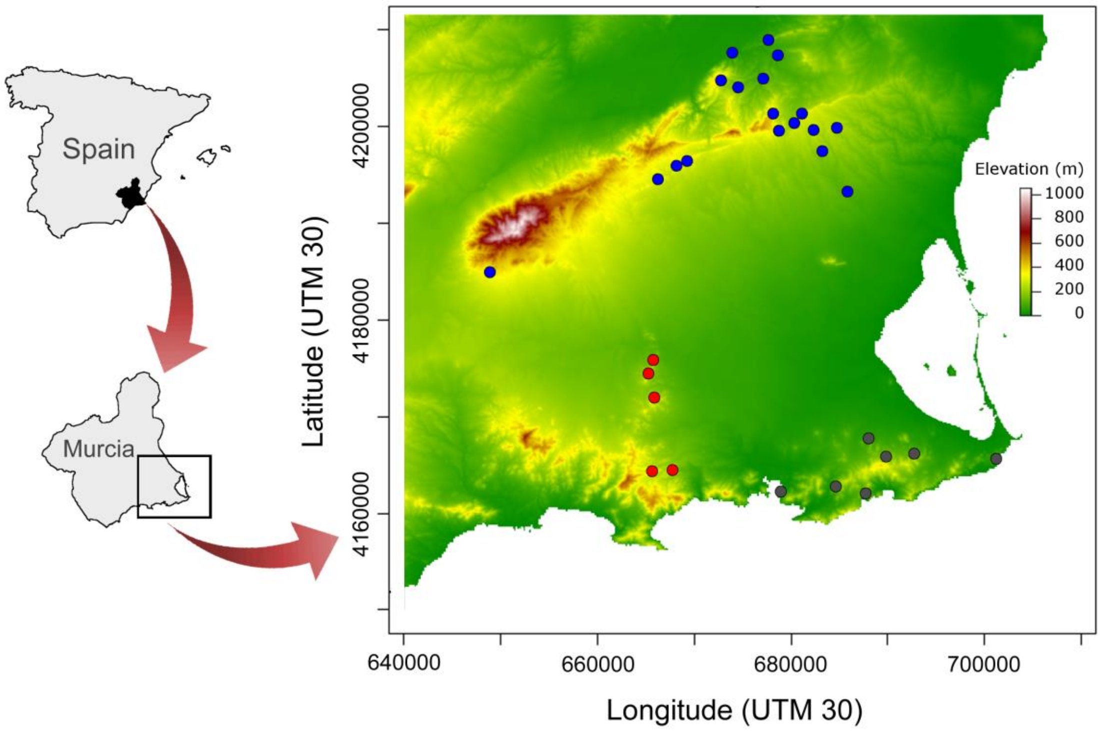


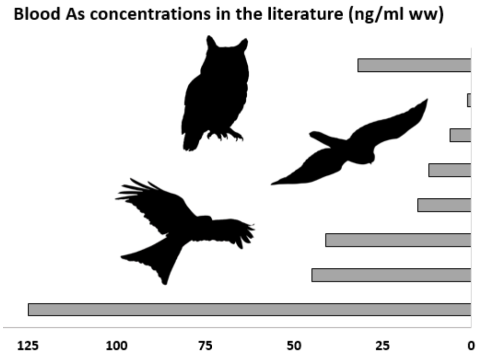
Figure 1. Map showing the geographical location of the studied areas. Blue, red and grey circles represent Eagle owl (*Bubo bubo*) nest sites in the control (n=18 nests/50 nestlings), industrial (n=5 nests/14 nestlings), and mining (n=7 nests/23 nestlings) areas, respectively.

A)

Species	Mean (ng/ml)	Country	Reference
Bubo bubo	78 ± 48[a]	Spain	This study
Accipiter gentilis	5[b] (2-30)[c]	Spain	Dolan et al., 2017
Accipiter gentilis	5[b] (1-20)[c]	Norway	Dolan et al., 2017
Accipiter gentilis	4[b] (0.8-20)[c]	Norway	Dolan et al., 2017
Bubo bubo	83 ± 67[a]	Spain	García-Fernández et al., 1995
Bubo bubo	76 ± 20[d]	Spain	García-Fernández et al., 1997
Bubo bubo	86 ± 68[a]	Spain	Gómez-Ramírez et al., 2011
Bubo bubo	76 ± 71[a]	Spain	Espín et al., 2014b
Milvus migrans	47 ± 38[a]	Spain	Baos et al., 2006
Milvus migrans	84 ± 52[d]	Spain	Blanco et al., 2003
Buteo buteo	147 ± 652[a] (59)[b]	Portugal	Carneiro et al., 2014
Milvus migrans	194 ± 293[a] (104)[b]	Portugal	Carneiro et al., 2018

[a]Standard deviation, [b]Median, [c]Range, [d]Standard error

B)



Species	Mean (ng/ml)	Country	Reference
Bubo bubo	32 ± 59[a]	Spain	This study
Accipiter gentilis	1[b] (0.1-2)[c]	Spain	Dolan et al., 2017
Accipiter gentilis	6[b] (2-40)[c]	Norway	Dolan et al., 2017
Accipiter gentilis	12[b] (1-100)[c]	Norway	Dolan et al., 2017
Buteo buteo	15 ± 15[a] (14)[b]	Portugal	Carneiro et al., 2014
Milvus migrans	41 ± 64[d]	Spain	Blanco et al., 2003
Milvus migrans	45 ± 57[a] (24)[b]	Portugal	Carneiro et al., 2018
Milvus migrans	125 ± 260[a]	Spain	Baos et al., 2006

[a]Standard deviation, [b]Median, [c]Range, [d]Standard error

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

Tables

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	0.0	0.1	<LOQ	0.8	7	0.013
Bismuth (Bi)	4	0.0	0.1	<LOQ	0.6	13	0.008
Cadmium (Cd)	2	0.0	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	0.0	0.0	<LOQ	0.2	11	0.001
Erbium (Er)	3	0.0	0.0	<LOQ	0.1	9	0.001
Europium (Eu)	3	0.0	0.0	<LOQ	0.2	10	0.000
Gadolinium (Gd)	3	0.0	0.0	<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	0.0	0.0	<LOQ	0.2	13	0.000
Indium (In)	4	0.0	0.0	<LOQ	0.1	21	0.001
Iron (Fe)	1	218434	27698	152422	282851	100	24.6
Lanthanum (La)	3	0.0	0.1	<LOQ	0.7	7	0.020
Lead (Pb)	2	21.7	41.8	<LOQ	173	38	0.361
Lutetium (Lu)	3	0.0	0.0	<LOQ	0.1	2	0.000
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	0.0	0.1	<LOQ	0.6	10	0.010
Nickel (Ni)	1	14.5	80.3	<LOQ	737	2	7.95
Niobium (Nb)	4	0.0	0.1	<LOQ	0.3	15	0.005
Osmium (Os)	4	0.0	0.1	<LOQ	0.6	9	0.002
Palladium (Pd)	2	0.0	0.0	<LOQ	0.2	2	0.001
Platinum (Pt)	4	0.0	0.1	<LOQ	0.6	23	0.001
Praseodymium (Pr)	3	0.0	0.0	<LOQ	0.2	10	0.003
Ruthenium (Ru)	4	0.0	0.0	<LOQ	0.0	0	0.000
Samarium (Sm)	3	0.0	0.0	<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)	2	90.7	54.0	24.6	249	100	0.439
Tantalum (Ta)	4	0.0	0.1	<LOQ	0.9	14	0.001
Terbium (Tb)	3	0.0	0.0	<LOQ	0.2	13	0.000
Thallium (Tl)	2	0.2	0.3	<LOQ	1.8	38	0.008
Thorium (Th)	2	0.0	0.0	<LOQ	0.2	9	0.002
Thulium (Tm)	3	0.0	0.0	<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	0.0	0.1	<LOQ	0.8	3	0.002
Vanadium (V)	2	0.8	1.4	<LOQ	9.1	45	0.034
Ytterbium (Yb)	3	0.0	0.0	<LOQ	0.1	7	0.001
Yttrium (Y)	3	0.0	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 toxic elements, 3 = Rare earth elements, 4 = Other minor elements.

Table 2. Mean ± SD, median (range) element concentrations (ng/ml, w.w.) in whole 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	Mean ± SD	Mean ± SD
	Median (range)	Median (range)	Median (range)
ATSDR's list toxic elements in whole blood			
Arsenic (As)	2.22 ± 3.04	1.04 ± 0.98	32.2 ± 59.0*
	1.08 (0.48 - 16.2)	0.57 (0.4 - 3.49)	12.4 (2.31 - 214)
Lead (Pb)	1.24 ± 2.74	2.44 ± 4.50	77.9 ± 48.0*
	0.19 (<LOQ - 10.7)	0.20 (<LOQ - 11.5)	83.1 (12.0 - 172)
Mercury (Hg)	6.15 ± 8.90	7.54 ± 9.34	7.94 ± 6.59
	3.05 (<LOQ - 46.7)	4.92 (0.71 - 34.4)	5.69 (1.15 - 24.0)
Strontium (Sr)	120 ± 53.1	61.8 ± 21.2*	44.6 ± 11.7*
	121 (32.5 - 249)	58.8 (30.7 - 122)	43.7 (24.6 - 74.2)
Thallium (Tl)	0.03 ± 0.10	0.05 ± 0.07	0.52 ± 0.43*
	<LOQ (<LOQ - 0.51)	<LOQ (<LOQ - 0.17)	0.29 (<LOQ - 1.77)
Vanadium (V)	0.82 ± 1.52	0.16 ± 0.57	1.06 ± 1.40
	<LOQ (<LOQ - 9.12)	<LOQ (<LOQ - 2.15)	0.65 (<LOQ - 5.24)
Essential trace elements in whole blood			
Cobalt (Co)	9.67 ± 6.29	7.89 ± 9.83	8.51 ± 4.86
	7.64 (2.36 - 29.2)	4.65 (2.55 - 35.6)	8.39 (2.25 - 21.7)
Copper (Cu)	226 ± 38.2	231 ± 71.9	218 ± 51.0
	227 (153 - 297)	215 (172 - 459)	214 (161 - 411)
Iron (Fe)	221043 ± 28740	216888 ± 20237	213702 ± 29613
	219356 (161264 - 275123)	213024 (194096 - 256380)	213007 (152422 - 282850)
Manganese (Mn)	29.1 ± 12.6	16.1 ± 5.59*	19.4 ± 5.56*
	27.3 (12.8 - 71.1)	15.5 (10.8 - 33.7)	17.7 (11.8 - 32.0)
Molybdenum (Mo)	18.3 ± 6.73	15.4 ± 4.27	15.8 ± 3.37
	17.6 (6.99 - 38.6)	14.3 (11.0 - 27.3)	15.0 (11.2 - 26.8)
Selenium (Se)	477 ± 162	472 ± 84.0	380 ± 74.7
	428 (290 - 993)	473 (340 - 637)	381 (251 - 544)
Zinc (Zn)	4409 ± 741	4046 ± 387	3838 ± 504*
	4299 (3261 - 5957)	4029 (3256 - 4727)	3790 (2461 - 5059)
Body measurements, hematocrit and plasma biochemistry			
Hematocrit (%)	25.8 ± 4.49	26.1 ± 2.12	27.4 ± 4.70
	26.0 (15.0 – 35.0)	26.0 (23.0 – 29.0)	27.0 (19.0 – 36.0)
Body mass (g)	1232 ± 230	1320 ± 208	1290 ± 292
	1212 (800 − 1850)	1288 (1000 − 1825)	1250 (825 − 2000)
Wing lenth (mm)	198 ± 42	218 ± 40	212 ± 80
	195 (115 − 292)	210 (135 − 280)	180 (112 − 400)
Retinol (μM)	16.5 ± 2.39	15.7 ± 1.13	15.8 ± 2.14
	16.2 (11.4 – 22.3)	15.8 (13.6 – 17.9)	15.3 (13.0 – 22.5)
Tocopherol (μM)	79.8 ± 15.9	87.1 ± 13.1	99.3 ± 18.3*
	78.9 (47.0 – 112)	84.3 (69.2 – 108)	96.6 (68.9 – 155)
Lutein (μM)	6.36 ± 3.78	9.35 ± 4.39	9.42 ± 3.61**
	5.48 (1.57 – 16.3)	9.08 (3.81 – 18.3)	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).

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.

	Control area (18 nests)		Industrial area (5 nests)		Mining area (7 nests)	
Diet item	**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 preys	**4**		**1**		**2**	

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