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A 25 year overview of the contaminant exposure and effects in Eurasian Eagle-owl (*Bubo bubo*) from southern Spain

Uma síntese de 25 anos sobre a exposição e efeitos de contaminantes em bufo-real (*Bubo bubo*) no sul de Espanha

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ABSTRACT

The Eurasian Eagle-owl (*Bubo bubo*) meets the requirements of a suitable sentinel species for biomonitoring environmental contaminants in Southeastern Spain (Murcia Region and Alicante Province). In this area, it is an abundant species, showing the highest breeding density within its distribution range. Since 1992, different sample types from free-living nestlings and adults (blood, feathers, unhatched eggs) or from injured individuals admitted in wildlife rehabilitation centres (blood, feathers, liver, kidney, brain and bones) were analysed for a wide range of pollutants (metals, organochlorine pesticides, anticoagulant rodenticides, neonicotinoid insecticides). Specific biomarkers (antioxidant molecules, lipid peroxidation, δ-ALAD and blood clinical parameters) were analysed to perform risk assessment. As main findings, the patterns and concentrations of contaminants in Eurasian Eagle-owl samples reflected the contamination influenced by agriculture, an ancient mining site (AMS) and the use of anticoagulant rodenticides. In general, biomarkers were highly correlated to contaminants (i.e. δ-ALAD inhibition by lead, antioxidant enzymes inhibition by lead and cadmium, lipid peroxidation induction by mercury). Overall, we can confirm the suitability of the Eurasian Eagle-owl to biomonitor contaminants in our study area.

Keywords: anticoagulant rodenticides, *Bubo bubo*, metals, pesticides, flame retardants

RESUMO

O bufo-real (*Bubo bubo*) preenche os requisitos de espécie sentinela para a biomonitorização de contaminantes ambientais no sudoeste de Espanha (região de Múrcia e província de Alicante). Nesta área, é uma espécie abundante, apresentando a maior densidade da sua área de distribuição. Desde 1992, foram recolhidos vários tipos de amostras provenientes de aves juvenis e adultas em liberdade (sangue, penas, ovos não eclodidos), ou admitidas em centros de recuperação (sangue, penas, fígado, rim, cérebro e ossos), tendo sido analisadas para quantificação de uma grande variedade de contaminantes (metais, pesticidas organoclorados, rodenticidas anticoagulantes, inseticidas neonicotinóides). Biomarcadores específicos (moléculas antioxidantes, peroxidação lipídica, δ-ALAD e parâmetros clínicos sanguíneos) foram analisados para avaliação dos riscos. Os padrões e as concentrações de contaminantes em amostras de bufo-real refletem a contaminação influenciada pela agricultura, por uma antiga mina (AMS) e pelo uso de rodenticidas anticoagulantes. Em geral, os biomarcadores apresentaram correlações elevadas com os contaminantes (i.e. inibição de δ-ALAD por chumbo, inibição de enzimas anti-oxidantes por chumbo e cádmio, indução de peroxidação lipídica por mercúrio). De uma forma geral, confirmámos a adequação do bufo-real para biomonitorizar contaminantes na área de estudo.

Palavras-chave: *Bubo bubo*, metais, pesticidas, raticidas anticoagulantes, retardantes de chamas

Introduction

Exposure to environmental pollutants has been linked to detrimental effects on health, both in humans and wildlife (Woodruff, 2011). As a consequence, some European regulations have been set, for instance the REACH directive (European Commission, 2006), or regulations on persistent organic pollutants (European Commission, 2004, 2007), and on pesticides and biocides (European Commission, 2012). To assess the effectiveness of such regulations, as well as spatio-temporal trends and their effects on health, biomonitoring contaminants using birds is considered a suitable tool (Gómez-Ramírez et al., 2014). In fact, several European countries such as Sweden, Norway or United Kingdom have set national biomonitoring programmes using raptors (Gjershaug et al., 2008; Helander et al., 2008; Walker et al., 2008) and a wide range of sample types are collected and used across Europe for wide scale contaminant monitoring using sentinel raptors (Espín et al., 2016).

Among birds, owls and other raptors are specially suitable to biomonitor persistent contaminants due to their high position in the trophic chain (Gómez-Ramírez et al., 2014). Specifically, the Eurasian Eagle-owl (*Bubo bubo*) in our study area (Southeastern Spain) meets the requirements for a suitable sentinel species for monitoring environmental pollutants (NRC, 1991): it is easy to collect samples from, it is exposed to environmental pollutants, and it is relatively common (Martínez & Calvo, 2006; Pérez-García & Sánchez-Zapata, 2015), with the highest breeding density reported within its distribution range (Pérez-García et al., 2012). In addition, this large owl is a long-lived top predator with a very varied diet (Martínez & Calvo, 2001; Lourenço, 2006) and therefore likely to reflect local pollution (Lourenço et al., 2011). For these reasons, different types of samples (mainly blood) from Eurasian Eagle-owl have been analysed since 1992 by the "Toxicology" research group of the University of Murcia, to obtain as much information as possible to assess the exposure and effects of the most relevant pollutants in this population, looking for spatio-temporal trends and local sources of pollution.

Due to ethical and legal reasons, sampling of free-ranging wildlife is limited. Thus, active monitoring of live birds of prey would only allow the non-destructive sampling of blood, biopsies, plucked feathers and preen oil. Other samples taken without contact with a living bird would be moulted feathers, addled or deserted eggs, regurgitated pellets, excrement, and tissues from carcasses from animals found dead in the field or died in rehabilitation centres (Espín et al., 2016).

For sampling live birds of prey, nestlings are often selected, as they are easier to capture and sample than adults (Andersen, 2007) and are more likely to reflect local pollution, as these are usually fed with prey caught close to the nest (Frank and Lutz, 1999).

Simultaneous and direct measurement of contaminants in fluids and tissues can reflect not only the exposure of this species, but also offer information on absorption and accumulation. However, monitoring the biological responses would give more direct information about their adverse effects (Vanparys et al. 2008). These responses, called biomarkers, comprise any alteration at cellular or biochemical components or processes, structures or functions, which are measurable in a biological system or sample (NRC, 1987). Levels of antioxidant molecules, activities of antioxidant enzymes, blood δ-ALAD activity or eggshell thinning are some of the most common biomarkers studied in birds (Gómez-Ramírez et al., 2011; Helander et al., 2002; Koivula & Eeva, 2010).

The main aim of this work is to provide an overview of the contaminant exposure and related effects in a Eurasian Eagle-owl population from Southeastern Spain after 25 years of research.

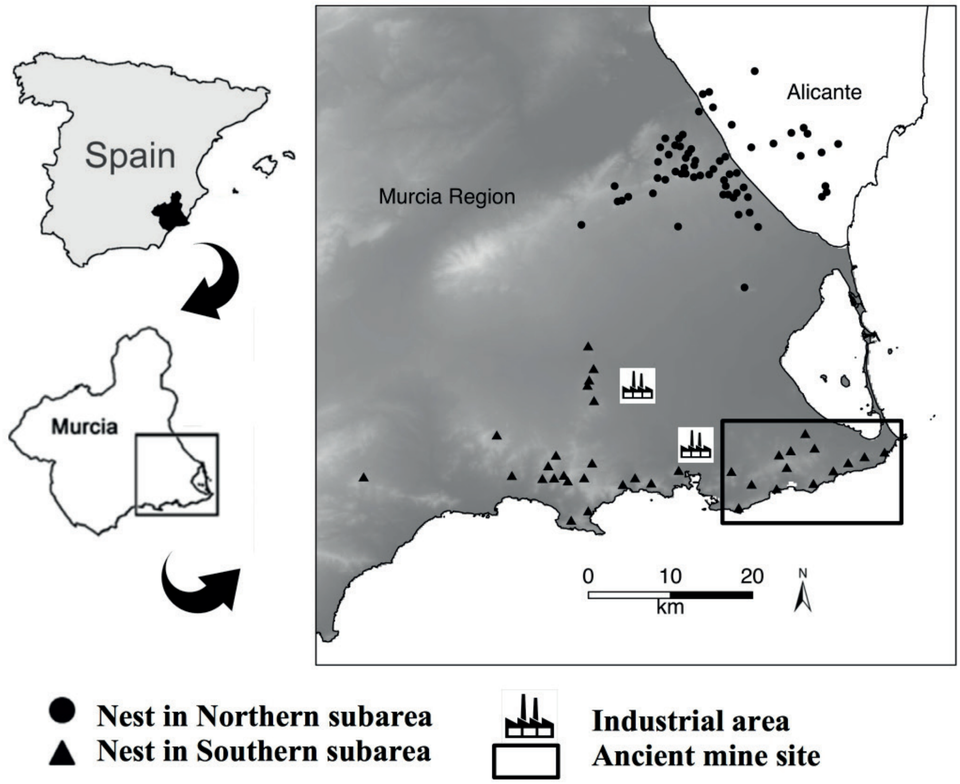
Methods

Study area and species

The study area (Fig. 1) comprises the south of the province of Alicante and Murcia Region, in Southeastern Spain (Escalona, Altaona, Monte el Valle, Columbares, Sierra Minera Cartagena-La Union, La Muela-Cabo Tiñoso and Almenara mountains; 37º 45' N, 0º 57' W). This is a relatively large area, with different land uses and local sources of pollution. Hence, the study area was divided in two subareas: the Northern subarea (Escalona, Altaona, Monte el Valle and Columbares), where main land uses are citrus and dry farming, and the European Rabbit (*Oryctolagus cuniculus*) is very abundant (71% of the prey of Eurasian Eagle-owls; León et al., 2008). In

Figure 1 - Map showing the geographical location of the study area (Province of Alicante and Murcia Region, in Southeastern Spain).

Figura 1 - Localização geográfica da área de estudo (província de Alicante e região de Múrcia, no sudoeste de Espanha).



the Southern subarea, irrigation farming is predominant, with a history of intensive use of pesticides. Some nests were found in an ancient mining site (AMS), in the "Sierra Minera de Cartagena-La Unión" range and most nests are around 16 km from Cartagena, an important industrial city. In addition, in Southern subarea, the European Rabbit is less abundant (35% of the prey of Eurasian Eagle-owls), and the diet is completed with rats (*Rattus rattus* and *R. norvegicus*), pigeons (*Columba* spp.), Red-legged Partridges (*Alectoris rufa*), Hedgehogs (*Erinaceus europaeus* and *Atelerix algirus*) and Yellow-legged Gulls (*Larus michahellis*) (León et al., 2008).

Sampling

Sampling started in 1992. Samples from tissues (liver, kidney, bone, brain) were obtained by necropsy from individuals that died or arrived dead to the Wildlife Rehabilitation Centres in Murcia and Alicante. Samples from free-living individuals were collected by the veterinarians of the "Toxicology" research group in collaboration with the "Mediterranean Ecosystems" research group (University of Murcia, Spain) and the "Area of Ecology" research group (Miguel Hernández University, Spain). Sampling methods for blood, feathers and unhatched eggs coincide with the

EURAPMON sampling and contaminant monitoring protocol for raptors described by Espín et al. (2014a) and further developed by Espín et al. (2021). Except feathers, all the samples were transported in cool conditions and stored frozen at -40º or -80ºC until analysis. The feathers were individually put into sealed plastic bags and they were stored at room temperature in a dark and dry place until analysis. The number of samples analysed for each contaminant is indicated in the corresponding section.

Metal analysis in blood, tissues and feathers

Cadmium and lead were analysed in samples from individuals admitted in "El Valle" Wildlife Rehabilitation Centre between 1993-1994 (whole blood: *n* = 5, kidney: *n* = 26, liver: *n* = 26, brain: *n* = 26 and bone: *n* = 15), using Anodic Stripping Voltammeter (VA-646 processor and VA-647 workstation, Methrohm, Switzerland) after a complete high temperature digestion with a mix of acids to eliminate organic impurities (see García-Fernández et al., 1995, 1997). Following the same digestion method but using a VA-757 Computrace Workstation (Methrohm, Switzerland), cadmium, lead, zinc and copper were analysed in whole blood samples (*n* = 304) from free-living nestlings born between 2004 and 2007 and 2011–2012 (see Gómez-Ramírez et al., 2011; Espín et al., 2014b, 2015).

Total mercury was analysed by atomic absorption spectrophotometry using a Milestone DMA-80 Direct Mercury analyser (Milestone GmbH, Germany) and the method described by Espín et al. (2014c), in whole blood samples from nestlings (*n* = 600) born between 2006-2012 and body feathers (*n* = 229) from nestlings born between 2006-2008. Muscle samples (*n* = 40) from rabbits found in nests as prey in 2009, 2011 and 2012, were also analysed to correlate with levels in nestlings (Espín et al., 2014c).

Organohalogen compounds analysis

Organochlorine insecticides (α-HCH, β-HCH, δ-HCH, lindane, aldrin, dieldrin, endrin, endrin aldehyde, endosulfan I, endosulfan II, endosulfan sulphate, *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE, heptachlor and heptachlor-epoxide) were analysed in liver, brain and fat collected after necropsy from 16 Eurasian Eagle-owls registered in "El Valle" Wildlife Rehabilitation Center (Murcia) between 1994-1996. Samples (liver: *n* = 14, fat: *n* = 6 and brain: *n* = 14) were extracted following the method described by María-Mojica et al. (2000), using organic solvents and solid phase extraction. Analysis were conducted by gas chromatography with an electron capture detector (GC-ECD 17 Shimadzu). Whole blood samples collected between 2003–2007 (*n* = 316) from free-living nestlings were analysed following the same method but slightly modified and adapted by Martínez-López et al. (2009).

Analyses of unhatched eggs collected between 2004–2009 (*n* = 58) were performed at Alterra (Wageningen UR) in Wageningen (The Netherlands) under the supervision of Dr. Nico W. van den Brink following the method described by Gómez-Ramírez et al. (2012a). Briefly, homogenised egg contents were extracted with n-hexane and analysed by GC-MSD (Agilent 6890 Series GC System; 5973 Network Mass Selective Detector, Agilent Technologies, Palo Alto, CA, USA) to detect PCBs (8, 31, 44, 52, 70, 101, 149, 151, 28, 105, 138, 195, 118, 170, 77, 128, 126, 156, 153, 169, 180, 194), α-HCH, β-HCH, γ-HCH, δ-HCH, ε-HCH; heptachlor, heptachlor-epoxide, aldrin, isodrin, dieldrin, endrin, endosulfan, cis and trans chlordane, *o,p'*-DDT, *o,p'*-DDD, *o,p'*-DDE, *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE; hexachlorobenzene (HCB), metoxychlor, mirex and PBDEs (28, 47, 99, 100, 153, 154 and 183). Egg parameters were measured according to EURAPMON sampling and contaminant monitoring protocol for raptors (Espín et al., 2014a).

Anticoagulant rodenticides analysis in blood and liver

Warfarin, coumatetralyl, brodifacoum, bromadiolone, difenacoum, chlorophacinone and diphacinone were analysed in liver samples (n = 18) from adult Eurasian Eagle-owls that arrived dead or died at the Wildlife Rehabilitation Center "Santa Faz" (Alicante) and in whole blood samples (n = 50: 9 adults caught between 2008 and 2010 and 41 nestlings born between 2008 and 2010). The extraction was performed using a modification of QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe), while detection and quantification was carried out with high performance liquid chromatography coupled to mass spectrometry (Agilent 1100 Series ESI/LC/MSD ion Trap VL), as described by Gómez-Ramírez et al. (2012b).

Neonicotinoids analysis in blood

Whole blood samples from nestlings born in 2016 (n = 30) were analysed to assess the exposure to the neonicotinoids authorised in Spain (acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, nitenpyram and thiamethoxam). Two extraction techniques based on QuEChERS were adapted and compared: a) using acetate buffer (AB); and b) using citrate buffer (CB). The AB method was chosen based on the best values of repeatability and suitable recoveries. Detection and quantification was carried out with high performance liquid chromatography HPLC (Agilent Series 1200, Agilent Technologies, Santa Clara, CA, USA) coupled to time of flight mass spectrometry (Agilent 6220 accurate mass TOF, Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operating in the positive ionization mode, as described by Taliansky-Chamudis et al. (2017).

Serum biochemical clinical parameters and haematocrit

Albumin, calcium, inorganic phosphate, aspartate aminotransferase (AST), alkaline phosphatase (AP), cholesterol, triglycerides (TGL), creatine kinase (CK), γ-glutamyltransferase (γ-GT), glucose, lactate dehydrogenase (LDH), total proteins (TP) and uric acid in serum were analysed in 15 adults (6 males and 9 females) and 258 nestlings of free-living Eurasian Eagle-owl using an automated spectrophotometrical analyser (A25, Atom®), as described in Gómez-Ramírez et al. (2016). Haematocrit was studied in 13 adults (2 males and 11 females) and 162 nestlings centrifuging microcapillaries at 2200 g for 5 min and using a microhaematocrit reader.

Determination of blood δ-ALAD activity

Enzymatic activity was determined in 218 blood samples (22 from AMS and 196 from unpolluted areas) obtained from nestlings born between 2003–2007 (Gómez-Ramírez et al., 2011), and both the enzymatic activity and the ratio between the non-activated and the *in vitro* activated enzymes were determined in 139 blood samples collected in 2011 and 2012 from nestlings (n =131) and adult females (n = 8) (71 from Agricultural and Rural Area, 40 from Industrial Area and 28 from AMS; Espín et al., 2015). δ-ALAD activity was determined using the method described by Scheuhammer (1987a) using a UV spectrophotometer (UV-1603, Shimadzu). Quantification was based on measuring the colour of porphobilinogen (PBG). Activity of δ-ALAD was expressed as µmol PBG/h/l red blood cells (RBC). The activity ratio was calculated by dividing the non-activated enzyme absorbance and the reactivated enzyme absorbance.

Table 1 - Levels of lead and cadmium (mean, range) in blood (µg dL-1) and tissues (µg g-1, w.w.) of Eurasian Eagle-owls admitted in the "El Valle" Rehabilitation Centre between 1993-1994 (García-Fernández et al., 1995, 1997).

Tabela 1 - Níveis de chumbo e de cádmio (média, intervalo) no sangue (µg dL-1) e em tecidos (µg g-1, w.w.) de bufos-reais admitidos no Centro de Reabilitação "El Valle" entre 1993 e 1994 (García-Fernández et al., 1995, 1997).

	LEAD (µg dL^{-1} in blood; µg g^{-1} in tissues, w.w.)	CADMIUM (µg dL^{-1} in blood; µg g^{-1} in tissues, w.w.)
Blood	7.71 3–20 (n = 12)	0.1 0.04–0.28 (n = 5)
Liver	0.48 0.03–0.51 (n = 19)	0.14 0.002–0.380 (n = 7)
Kidney	0.36 0.03–0.67 (n = 19)	0.21 0.01–0.51 (n = 7)
Brain	0.14 0.03–0.22 (n = 19)	0.012 0.001–0.033 (n = 7)
Bone	16.97 0.62–43 (n = 12)	0.004 ND–0.047 (n = 3)

Oxidative stress biomarkers

141 red blood cell (RBC) samples obtained between 2011–2012 were analysed for oxidative stress biomarkers [total glutathione (tGSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and thiobarbituric acid-reactive substances (TBARS)] after RBC homogenization (1:10 w/v) in a stock buffer following the method described by Espín et al. (2014b).

Statistical analyses

Statistical analyses were performed as described in García-Fernández et al. (1995, 1997), Gómez-Ramírez, 2011; Gómez-Ramírez et al. (2011, 2012a, 2012b, 2016), Espín et al. (2013, 2014b, 2014c, 2015) and Taliansky-Chamudis et al. (2017).

Results

Metal levels in blood, tissues and feathers from Eurasian Eagle-owls

Table 1 shows the calculated average levels of lead and cadmium in Eurasian Eagle-owls admitted in the "El Valle" Rehabilitation Centre between 1993–1994 and analysed by García-Fernández et al. (1995, 1997). Bone and blood showed the highest concentrations of lead in tissues while cadmium tends to accumulate in kidney. On the other hand, strong positive correlations were found between tissues for both lead and cadmium ($r > 0.7, p < 0.05$). Detailed description of the results can be found in García-Fernández et al. (1995, 1997).

Samples collected between 1993–1994 (Table 1) included juveniles and adults but samples obtained between 2004–2012 from free-ranging birds were taken mostly from

Table 2 - Levels (mean ± SD, median) of lead, mercury, cadmium, zinc and copper (μg dL^{-1}) in blood of Eurasian Eagle-owls born between 2004–2012 (Espín et al., 2013).
*indicates significant differences between subareas ($p < 0.05$).

Tabela 2 - Níveis (média ± SD, mediana) de chumbo, mercúrio, cádmio, zinco e cobre (μg dL^{-1}) em sangue de bufos-reais nascidos entre 2004 e 2012 (Espín et al., 2013).
*indica diferenças significativas entre subáreas ($p < 0.05$).

	NORTHERN SUBAREA	SOUTHERN SUBAREA
Lead*	2.11 ± 2.65 1.19 (*n* = 191)	5.77 ± 6.40 3.52 (*n* = 113)
Mercury*	3.38 ± 14.89 1.24 (*n* = 435)	4.15 ± 12.83 1.84 (*n* = 188)
Cadmium	0.08 ± 0.20 0.01 (*n* = 191)	0.07 ± 0.13 0.01 (*n* = 113)
Zinc	353.24 ± 185.68 323.93 (*n* = 191)	317.78 ± 107.88 299.20 (*n* = 113)
Copper	34.30 ± 198.71 11.98 (*n* = 191)	21.70 ± 66.09 11.69 (*n* = 113)

nestlings. Only 8 adults were sampled and levels were not statistically different from nestlings, except for copper. However, as shown in table 2, levels of mercury and lead were higher ($p < 0.05$) in birds from the Southern subarea than from the Northern.

As mentioned above, some nests in the Southern subareas are in an AMS, hence about 15% of the individuals sampled were born in that area. When those were compared with the rest of the population, the differences in lead and mercury levels increased (see Gómez-Ramírez et al., 2011; Espín et al., 2014b, 2014c, 2015). Mercury levels in rabbit muscle did not differ significantly between subareas. However, when samples from the AMS were compared with the rest, mean mercury levels in rabbit muscles were significantly higher in rabbits from the AMS (24.98 ± 12.76 vs 12.46 ± 12.38 μg kg^{-1} wet weight (ww); $F = 6.83$; *d.f.* = 1,23; $p = 0.019$; Espín et al., 2014c). In the same study, positive correlations were found between mercury levels in nestlings blood and rabbit muscle ($r > 0.38$, $p < 0.029$). "Year" was the most important factor influencing blood mercury concentrations in Eurasian Eagle-owl (Espín et al., 2014c).

Mercury was significantly higher in feathers than in blood (mean ± SD: 328.88 ± 447.15 μg kg^{-1} vs 36.83 ± 145.58 μg L^{-1} ww; $F = 1590.61$; *d.f.* = 1228; $p < 0.001$). On the other hand, a positive correlation between blood and feather mercury concentrations was found ($r = 0.339$, $p = 0.001$, $n = 229$). A predictive equation was estimated by simple linear regression: log (mercury) in blood (μg L^{-1}, ww) = −0.588 + 0.617 × log (mercury) in feathers (μg kg^{-1}) (Espín et al., 2014c).

Organohalogen compounds

All the organochlorine insecticides except *pp*'- DDD, *pp*'- DDT, aldrin and dieldrin were detected in the tissues sampled between 1993–1994. δ-HCH followed by endrin aldehyde and endosulfan I were the most frequent. Fat showed the highest levels of total organochlorine, followed by brain and liver. The highest concentrations were found for *p,p'*-DDE in fat (mean = 726.7 ng g^{-1}). Detailed description of results can be found in María-Mojica (1998).

In blood, frequency of detection of the insecticides was in general low (< 20%), being *pp'*-DDE, lindane and Σendosulfan I and II the most frequent. Dieldrin, Σendosulfan I and II, lindane and diphenyl aliphatics were detected at the highest levels. "Year" was considered the most influential variable and linear mixed models showed significant differences among years for all the compounds, except δ-HCH. Like most insecticides, levels of aldrin, dieldrin and Σdiphenylaliphatics in blood tended to decrease along the study period. Detailed description is presented in Gómez-Ramírez (2011).

Regarding levels in unhatched eggs, HCB, *p,p'*-DDE, trans-chlordane and dieldrin were the most common, while dieldrin and *p,p'*-DDE, together with heptachlor-epoxide and endosulfan, were detected in the highest concentrations. On the contrary, cis-chlordane, *o,p'*-DDD, isodrin and its epoxide endrin, were not detected, while *p,p'*-DDE, predominated in all samples. When general linear models were applied, significant interactions between subareas and years were found for *pp'*-DDE, β-HCH, HCB, ∑PCBs and ∑PBDEs. While concentrations in the Southern subarea tended to increase, levels in the Northern tended to decrease, except ∑PCBs and HCB, which remained stable (see Gómez-Ramírez et al., 2012a).

Anticoagulant rodenticides in blood and liver

No traces of anticoagulant rodenticides were detected in blood samples (Gómez-Ramírez et al., 2012b), while about 83% of the liver samples (*n* = 18) presented residues, being difenacoum and brodifacoum, followed by bromadiolone, the most frequent (82%, 64% and 17%, respectively; Gómez-Ramírez, 2011).

Neonicotinoids in blood of Eurasian Eagle-owl nestlings

Imidacloprid was the only compound detected, in a single blood sample (3.28 ng mL^{-1}). The sample was obtained from a nestling born in a dry land farming area (Taliansky-Chamudis et al., 2017).

Serum clinical biochemical parameters and haematocrit

Most blood clinical parameters were higher in nestlings than in adults, but only significantly for AP, inorganic phosphate and uric acid. On the contrary, γ-GT was significantly lower in nestlings. In adults, sex differences were not significant. Haematocrit levels were significantly higher in adults than in nestlings. Results of this section are described in detail in Gómez-Ramírez et al. (2016).

Blood δ-ALAD activity as biomarker of lead exposure and effect

Significant negative correlations were found between δ-ALAD - Log blood lead levels in Eurasian Eagle-owls studied by Gómez-Ramírez et al. (2011) and Espín et al. (2015). In the first study, the correlation (P = -0.137, p = 0.044) was stronger and more significant when blood lead levels were > 4 µg dL^{-1} (P = -0.341, p = 0.006). This suggested an inhibition of the enzyme activity, which reaches a 55% when blood lead concentrations were > 15 µg dL^{-1}. In the case of birds studied by Espín et al. (2015), the significant negative relationship between δ-ALAD ratio or δ-ALAD activity and Log blood lead levels were r = -0.471 and r = -0.292, respectively, p < 0.001. δALAD activity was inhibited by 50% at blood lead concentrations > 10 µg dL^{-1}, and by 79% when lead levels exceeded 19 µg dL^{-1}.

Oxidative stress biomarkers of metal exposure and effect

SOD activity and TBARS levels were higher in adults than in nestlings. Significant location-related differences were found only for GST activity, with lower activity in Eurasian Eagle-owls from the AMS. However, when adult individuals were excluded, significant higher activity of CAT in the agricultural area than in the AMS was also found ($p = 0.05$, Espín et al., 2014b).

Several oxidative stress biomarkers were inversely correlated with metal concentrations (Espín et al., 2014b). That study provided threshold concentrations at which metals cause effects on the antioxidant system of Eurasian Eagle-owl: Cadmium > 0.3 and 0.02 µg dL^{-1} in blood caused an inhibition of 32% and 8% in GPx activity, respectively, and an inhibition of 26% and 20% in CAT activity, respectively. Lead > 2 µg dL^{-1} in blood also inhibited 8 and 10.5% GPx and CAT activities, respectively, while lead > 15 and 3 µg dL^{-1} caused a depletion of 16% and 4% in tGSH in individuals from the AMS. TBARS were induced by lead (> 2 and 10 µg dL^{-1} produced a TBARS induction of 10% and 28%, respectively) in individuals from both the industrial and mining area. TBARS were especially induced by mercury (> 3 and 10 µg dL^{-1} resulted in a TBARS induction of 102% and 190%, respectively) in Eurasian Eagle-owls from the industrial area.

Positive correlations ($r > 0.21$, $p < 0.01$) were found among oxidative stress biomarkers: GPx-CAT, SOD–CAT, GST-GPx, GST-CAT, and tGSH-TBARS levels. Negative weak correlations ($r = -0.2$, $p < 0.04$) were found between CAT-TBARS, SOD-TBARS and δ-ALAD-Log CAT. Brood size was negatively correlated with GPx and CAT activity while a positive relationship between the brood size and tGSH levels was found. Results of this section are described in detail by Espín et al. (2014b).

Discussion

Metal levels in blood, tissues and feathers from Eurasian Eagle-owls

The results found by García-Fernández et al. (1995, 1997) confirmed that bone was the main organ of lead accumulation, followed by kidney and liver, and finally, by brain and blood. This distribution model agrees with studies in other bird species where bone lead concentrations were considered useful for monitoring chronic exposure (Hutton & Goodman, 1980; Stendell, 1980; Scheuhammer, 1987b; Honda, et al., 1990). In addition, and based on the strong positive correlations also found by García-Fernández et al. (1995, 1997), blood was considered a useful indicator of lead and cadmium exposure in wild birds, raptors included.

Our results show a decreasing trend in blood lead levels in the Eurasian Eagle-owl for the last 25 years in Murcia Region (García-Fernández et al., 1995, 1997; Gómez-Ramírez et al., 2011; Espín et al., 2014b). The end of mining activities in 1991 and the ban on leaded petrol in 2001 could be the cause of this decrease. Particularly, the influence of the latter was demonstrated by García-Fernández et al. (2005a) in tissues of Common Kestrels (*Falco tinnunculus*). Lead exposure was in general similar to birds of prey from non-polluted areas (Henny et al., 1994; Martínez-López et al., 2004). However, some local contamination sources could be the cause for the highest lead concentrations detected in the Southern subarea. There is an important industrial zone near the city of Cartagena (Fig. 1), including electric power plants, and explosives and shipbuilding factories (García-Fernández et al., 1995). In addition, in the AMS, lead, zinc, copper, tin, iron, manganese and silver was extracted for more than 2500 years (Pavetti et al., 2006). As a matter of fact, this was the main source for lead and zinc in Spain during the nineteenth century (Estevan-Senís, 1967).

Cadmium concentrations were considered low and, based on the lack of differences among areas, no important cadmium emissions were identified. Hence, we can suggest that Eurasian Eagle-owls in Southeastern Spain have been exposed to chronic low doses through the diet in the last 25 years (García-Fernández et al., 1995, 1997; Gómez-Ramírez, 2011; Espín et al., 2014b).

The information about blood mercury levels in Eurasian Eagle-owls is scarce (Espín et al., 2014c). However, concentrations were much lower than those reported for fish-eating raptors (Jagoe et al., 2002; Langner et al., 2012). Although the study area is not considered mercury polluted, spatial differences seem to be mostly related to the AMS. The temporal variations in blood mercury concentrations may be related to rainfalls during the seven years of study (Espín et al., 2014c). In this sense, rainfalls may contribute to a higher mercury removal from the atmosphere and local wet deposition. The positive correlations found between mercury concentrations in blood of nestlings and in muscles of their prey suggest that mercury levels are greatly influenced by mercury ingested through rabbit consumption (Espín et al., 2014c).

In general, zinc and copper concentrations were within the range of physiologic levels in several healthy bird species, including birds of prey (García-Fernández et al., 2005b).

Organohalogen compounds

Compared to other studies in birds collected in the 90's, levels of organochlorines in tissues were considered low (María-Mojica, 1998). However, the high frequency of detection is in agreement with the agricultural use during the study period, since they were still allowed and recommended (Decision 2000/801/EC, Regulation (EC) No 850/2004, Decision 2005/864/EC). Based on the accumulation pattern, fat was considered an ideal tissue for monitoring organochlorine insecticides.

The high frequency of detection of lindane, endosulfan and *p,p'*-DDE in blood of Eurasian Eagle-owl nestlings coincides with previous studies in Booted Eagle (*Aquila pennata*) nestlings from Murcia Region (Martínez-López et al., 2009). Although lindane was banned for agriculture in 2000 (Decision 2000/801/EC), industrial, domestic and forestry use were allowed until 2007 in Europe (Regulation (EC) No 850/2004). Dieldrin levels in some samples were similar to other raptor nestlings born between 1999-2003 in Southeastern Spain (Martínez-López, 2005) and even higher than in Bald Eagles (*Haliaeetus leucocephalus*) caught in 1977 in USA (Henny et al., 1981). Despite its low persistence in the organisms (Wiemeyer, 1996), the detection of endosulfan in our samples was expected, due to the recent ban in the study area (Decision 2005/864/EC). The most abundant and frequent compound was *p,p'*-DDE, similarly to scavenger raptors from Africa (van Wyk et al., 2001) and Spain (Gómara et al., 2004). The concentrations also fell in the range found in blood of Eurasian Buzzard (*Buteo buteo*), Northern Goshawk (*Accipiter gentilis*) and Booted Eagle nestlings born between 1999-2003 in Murcia Region (Martínez-López, 2005; Martínez-López et al., 2009). These concentrations were below the mean described by Donaldson et al. (1999) in plasma of Bald Eagle nestlings without reproductive impairment.

The greater agricultural activity with common use of DDT in the past (Sánchez-Gelabert et al., 2008) was probably the cause of the higher *p,p'*-DDE mean levels in eggs of Eurasian Eagle-owls in our study area compared to eggs of European birds of prey collected during the same decade (Henny et al., 2003; Mañosa et al., 2003; Jaspers et al., 2005; Bustnes et al., 2007; Martínez-López et al., 2007). Dieldrin is very persistent (Martijn et al., 1993). However, unexpectedly, our mean concentrations were as high as in owl eggs collected more than 25 years ago in the

US (Henny et al., 1984). Similarly to blood, the high frequency and concentrations of endosulfan was probably due to its recent restriction (Decision 2005/864/EC). Also coinciding with other raptor eggs (Henny et al., 2003; Mañosa et al., 2003), β-HCH, was the most common and abundant HCHs. Our levels were higher than in owl eggs from other European countries (Jaspers et al., 2005; Bustnes et al., 2007), especially in the eggs from a nest surrounded by intensive agricultural fields in the Northern subarea. Like *p,p'*-DDE, β-HCH has been related to intensive or moderate use of insecticides (Jakszyn et al., 2009). The low frequency and levels of γ-HCH in our samples seem to be related to the total ban of lindane for agricultural use in 2000 (Decision 2000/801/EC) and its rapid metabolism and excretion in birds. Compared to other raptors, exposure to PCBs can be considered intermediate (Wiesmüller et al., 1999; Kubistova et al., 2003; Jaspers et al., 2005; Bustnes et al., 2007). Coinciding with these studies, PCB 138, 153 and 180 were the most abundant. On the contrary, levels of PBDEs were very low compared to other eggs of birds of prey (Jaspers et al., 2005; Chen & Hale, 2010) but similar to Tawny Owl (*Strix aluco*) eggs from Central Norway collected between 2001-2004 (Bustnes et al., 2007). The profile is characteristic of terrestrial birds (Chen & Hale, 2010), and coincides with Little Owl (*Athene noctua*) eggs from Belgium (Jaspers et al., 2005), where BDE 99, 47 and 100 also dominated.

Dietary shifts with a greater ingestion of birds but also the proximity to Cartagena may explain the increasing trend in the Southern subarea. About 36% of the eggs exceeded the No observed adverse effect concentration (NOAEC) of Total toxic equivalents (TEQs) established for Great Horned Owls (*Bubo virginianus*) and 17% of the samples exceeded 400 pg g^{-1} ww, the lowest observed adverse effect concentration (LOAEC) for Total TEQs (Strause et al., 2007). Moreover, the negative correlation between TEQ concentrations and the metabolizable fraction of PCBs (*Fprob* = 0.0018) when TEQs values were above 10 pg g^{-1} ww could indicate hepatic enzymes induction. These females could be suffering from Ah-receptor-related toxic effects, some of which have been related to altered bird reproduction. Finally, a significant negative correlation between *p,p'*-DDE levels and eggshell thickness (r = -0.469, p < 0.001) was observed, with about 17% of eggshell thinning for *p,p'*-DDE levels > 100 μg g^{-1} lipid weight, similarly to previous experimental studies in owls (McLane & Hall, 1972; Newton & Bogan, 1974; Mendenhall et al., 1983). The persistence of this degree of thinning over a period of time has been related to population declines in other birds of prey (Blus, 2011).

Anticoagulant rodenticides in blood and liver

Information regarding the use of rodenticides in the study area is scarce. However, the most frequently detected compounds in our liver samples were also marketed in the highest number of products by the time of sampling in Spain (MARM, 2011), which suggests a higher frequency of use. On the other hand, the lack of detection in blood could be due to the fast transport to the liver, where they can persist up to a year (Erickson & Urban, 2004).

Levels of anticoagulant rodenticides related to toxic effects in owls have been reported between 100–200 ng g^{-1} in liver (Newton et al., 1998, 1999), although concentrations as low as 10 ng g^{-1} of brodifacoum have been related to subcutaneous haemorrhage in Great Horned Owls (Stone et al., 1999). Sublethal haemorrhages may alter locomotion or cause lethargy, predisposing animals to predation, accidental trauma or reduced food intake (Stone et al., 1999). Electrocution and trauma were the main cause of admission of the owls studied. Because 72% of these individuals presented hepatic levels above 10 ng g^{-1}, we could suggest that these compounds could be involved in the cause of death.

Neonicotinoids in blood of Eurasian Eagle-owl nestlings

In this first assessment of neonicotinoids in blood of free-ranging birds of prey, the very low frequency of detection can be due to several reasons (Taliansky-Chamudis et al., 2017). In order to protect pollinators, there is a European Regulation that restricts the use of imidacloprid, clothianidin and thiamethoxam during blooming in the study area (Commission Implementing Regulation (EU) No 485/2013). Sampling season of Eurasian Eagle-owls coincides with the blooming of most plants and trees grown in the irrigation farming areas (peach, apricot, melon, watermelon, etc.). On the contrary, trees from the dry land farming area usually bloom earlier (almond trees) or later (olive trees). Therefore, the probability of finding residues would be higher in the dry land farming areas. In addition, imidacloprid has longer persistence in the environment than the other neonicotinoids (Miranda et al., 2011). Also, it is the most widely used neonicotinoid in Murcia Region (Sanz-Navarro, 2008) and registered in the highest number of products available on the market in Spain, both for agricultural and veterinary use (AEMPS, 2016; MAGRAMA, 2015).

On the other hand, the apparent lack of exposure could indicate a lower suitability of this species as sentinel in neonicotinoid biomonitoring studies. Thus, further knowledge is needed about the bioavailability of neonicotinoids on birds at the top of the food chain, on the analytical method, including the main metabolites of each compound, and on the use of other matrices and experimental studies of exposure to understand the kinetics in birds.

Serum clinical biochemical parameters and haematocrit

Due to the lack of studies on clinical biochemical parameters and haematocrit on free-ranging Eurasian Eagle-owls, our data have been compared with other species of birds of prey (see Gómez-Ramírez et al., 2016). Most biochemical parameters in our nestlings were in the same range as in the studies mentioned in Gómez-Ramírez et al. (2016). The differences between nestlings and adults had also been found previously in birds of prey: the highest levels of AP in nestlings seem physiological, since this enzyme is synthesised by osteoblasts (Dobado-Berrios & Ferrer, 1997). Phosphorus usually decreases with age, as it is involved in bone and muscle development (Wolf et al., 1985) and was also higher in nestlings than in adults of Spanish Imperial Eagle (*Aquila adalberti*; Dobado-Berrios & Ferrer, 1997), and the same in Red Kites (*Milvus milvus*) and Black Kites (*Milvus migrans*; Viñuela et al., 1991). Another consequence of growing is an increase in protein synthesis, which increases uric acid (Hochleithner, 1994). However, higher levels of uric acid can also be related to the larger daily protein intake of nestlings (Griminger & Scanes, 1986), since it is the main nitrogen waste in birds (e.g. Singer, 2003). γ-GT is a biomarker of liver disease in mammals, but its role in birds is still under discussion. Nevertheless, levels in birds of prey are usually lower in nestlings which coincides with our results (Gómez-Ramírez et al., 2016).

Haematocrits are usually lower in young than in adults in several taxa, including birds of prey (Lanzarot et al., 2001). This could be due to the higher oxygen affinity of foetal haemoglobin, which decreases during development (Bartels et al., 1966). Our haematocrit values, both in adults and nestlings, were in the same range as in other birds of prey, including owls (Ferrer et al., 1987; Jennings, 1996). Thus, we can consider our values as reference for the Eurasian Eagle-owl.

Blood δ-ALAD activity as biomarker of lead exposure and effect

The inhibition of δ-ALAD, an enzyme for the synthesis of the haeme group of haemoglobine, is considered a biomarker of exposure and effect for lead also in birds (Scheuhammer, 1987a; Finkelstein et al., 2012). In our studies, this inhibition was evidenced, even at lower concentrations than in other species, including birds of prey (Henny et al., 1994; Martínez-López et al., 2004). In general, it is considered that blood lead concentrations > 20 µg dL^{-1} inhibit δ-ALAD activity by 50% in birds. Although concentrations as low as 1 µg dL^{-1} showed a 10% decrease in δ-ALAD activity, 4–5 µg dL^{-1} were established as threshold (Gómez-Ramírez et al., 2011; Espín et al., 2015).

Effects of δ-ALAD inhibition in birds may be more severe than in mammals, due to the higher metabolism of the nucleated red blood cells of birds (Allen, 1971; Brace & Altland, 1956). Laboratory and field studies showed that a 45–59% decrease in δ-ALAD can cause anaemia in American Kestrel (*Falco sparverius*) nestlings (Hoffman et al., 1985; Henny et al., 1994). In our samples, the negative correlation between δ-ALAD activity and haematocrit (r = -0.439, p < 0.001, n = 139) could be related to a compensatory response associated with a decrease in δ-ALAD enzyme (Espín et al., 2015).

Oxidative stress biomarkers of metal exposure and effect

Although, as mentioned above, exposure to metals can be considered in general low, several oxidative stress biomarkers were correlated with metal levels (see Espín et al., 2014b for details). Our findings show that lead may alter oxidative stress biomarkers in Strigiformes at lower concentrations than those typically accepted in birds of prey (8–20 µg dL^{-1} in blood; García-Fernández, 2017). Individuals from the AMS had significant higher lead and mercury concentrations, and significant lower GST and CAT activities. However, the lack of differences in oxidative damage to membrane lipids (TBARS) among areas suggests that the antioxidant capacity of the different populations is able to deal with oxidant species and maintain TBARS levels in the same amount (Espín et al., 2014b).

The positive correlations among the different oxidative stress biomarkers are indicative of their close collaboration as part of the antioxidant system. Given the function of CAT in catalyzing H_2O_2 to H_2O, the negative correlation δ-ALAD-CAT activity in Eurasian Eagle-owls may be related to a protective response against reactive oxygen species (Espín et al., 2015).

Finally, larger broods may have a negative effect on the antioxidant capacity of Eurasian Eagle-owl nestlings, and other ecological parameters should be considered when interpreting metal-related oxidative stress (Espín et al., 2014b).

Conclusions

After 25 years studying Eurasian Eagle-owls in Southeastern Spain, we can confirm the suitability of the Eurasian Eagle-owl as sentinel species to biomonitor contaminants in our study area. In this sense, we can conclude that the overview of the biomonitoring studies shows that patterns and concentrations of contaminants in samples from this species reflected the contamination influenced by the anthropogenic activities, such as agriculture, past mining activities and the use of anticoagulant rodenticides to control pests. With respect to specific contaminants we can conclude:

1. Declining lead levels seem to be related

to the end of mining activities and the ban on leaded petrol.

2. Mercury levels in the Eurasian Eagle-owl may be most influenced by weather conditions.

3. Although most organohalogen compounds had been banned long time ago, the relatively high frequency and levels of some compounds (i.e. *pp'*-DDE and dieldrin) are related to the past intensive agricultural practices in the area.

4. Although further research is needed, our first results of anticoagulant rodenticides analysis are in agreement with its market in Spain.

5. Our preliminary results of the exposure to neonicotinoids in Eurasian Eagle-owls must be confirmed, and further research is needed due to recent UE restrictions in the use of these compounds (Butler, 2018).

6. In general, biomarkers were highly correlated to the exposure to contaminants and they must be taken into account in future biomonitoring studies.

Finally, these studies have contributed to a broad dissemination of science and preparation of researchers, more specifically: 11 research papers, 23 congress contributions, 2 MSc thesis and 4 PhD thesis were generated. The collaboration between volunteers and researchers of different disciplines is especially recognised in this overview. Further studies should be addressed in the Eurasian Eagle-owl from Southeastern Spain to evaluate future spatio-temporal trends of contaminants and possible adverse effects.

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