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Oxidative stress biomarkers in Eurasian eagle owls (*Bubo bubo*) in three different scenarios of heavy metal exposure ☆

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ABSTRACT

The main aim of the present study is the assessment of oxidative stress related to metals in the Eurasian eagle owl (*Bubo bubo*) from three areas (agricultural and rural area, industrial area, and mining area) of Murcia, Southern Spain. Mean blood metal concentrations were Cd=0.07 ± 0.21, Pb=3.27 ± 5.21, Cu=10.62 ± 4.77, Zn=311.47 ± 67.14, Hg=2.32 ± 3.83 μg/dl wet weight. Although individuals from the mining area had significant higher Pb and Hg concentrations, and significant lower glutathione-S-transferase (GST) and catalase (CAT) activities in red blood cells (RBC); 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. Despite the low levels of metals, several oxidative stress biomarkers were correlated with metal concentrations.

This study provides threshold concentrations at which metals cause effects on the antioxidant system in Eagle owls. Blood Cd concentrations greater than 0.3 μg/dl produced an inhibition in GPx (32%) and CAT (26%) activity in RBC. However, Cd concentrations higher than 0.02 μg/dl were enough to produce an inhibition of these enzymes. Regarding Pb levels, blood concentrations above 2 μg/dl produced an inhibition of 8% and 10.5% in GPx and CAT activities, respectively, in RBC. A depletion of 16% and 4% in tGSH levels was associated with Pb concentrations higher than 15 and 3 μg/dl, respectively, in individuals from the ancient mine site. In addition, Pb concentrations above 2 and 10 μg/dl produced a TBARS induction of 10% and 28%, respectively, in individuals from both the industrial and the mining area. Finally, Hg concentrations greater than 3 and 10 μg/dl resulted in a TBARS induction of 102% and 190%, respectively, in Eurasian eagle owls from the industrial area. Our findings show that Pb may produce effects on oxidative stress biomarkers in Strigiformes at lower concentrations than those typically accepted for considering physiological effects in Falconiformes (20 μg/dl in blood). In addition, we provide new data on Hg and Cd concentrations related to effects in the antioxidant system.

1. Introduction

The ability of metals to induce reactive oxygen species (ROS) has been suggested as one of the mechanisms involved in metal toxicity (Ercal et al., 2001). ROS are unstable and very reactive molecules produced in oxidation–reduction reactions (Dowling and Simmons, 2009), presenting damaging effects on lipids, proteins and DNA (Rotilio et al., 1995). In this sense, redox-active metals such as iron and copper catalyze the Fenton reactions generating reactive hydroxyl radicals (Stohs and Bagchi, 1995). However, the primary route for redox-inactive metals such as lead, cadmium and mercury is to induce oxidative stress indirectly by depleting the major antioxidants of cells, such as glutathione and other thiol-containing antioxidants and protein-bound thiol groups (Stohs and Bagchi, 1995). However, they are also capable of inducing ROS formation indirectly (Lund et al., 1991; Monteiro et al., 1989; Ribarov and Bochev, 1982; Watanabe et al., 2003).

To protect themselves against negative effects of ROS, living beings have developed an important mechanism able to prevent, neutralize and remove harmful toxicants from the body, the

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antioxidant defense (Koivula and Eeva, 2010). It consists of both endogenous and dietary antioxidants such as glutathione (GSH), ascorbate and vitamin E and different antioxidant enzymes, which operate in association with each other forming an integrated antioxidant defense system (Halliwell and Gutteridge, 1999). GSH is an endogenously produced antioxidant with an important role in the protection of cells against oxidative stress, because it participates in binding with ROS and metals through the sulfhydryl (SH) group, and it is also involved in enzymatic detoxification reactions for ROS as a cofactor or a coenzyme for enzymes such as glutathione S-transferase (GST) and glutathione peroxidase (GPx) (Gurer and Ercal, 2000). Other important components of the antioxidant system are enzymes such as superoxide dismutase (SOD), catalase (CAT) and GPx, that can detoxify peroxides, O_2 and H_2O_2, respectively (Gurer and Ercal, 2000). The imbalance between the production of ROS and the antioxidant molecules is defined as oxidative stress, so that the defense is overcome by radical formation (Halliwell and Gutteridge, 1999). An excess of ROS can react with biomolecules and induce lipid, protein and DNA oxidation leading to oxidative damage that may ultimately lead to cellular dysfunction and tissue injury (Valavanidis et al., 2006).

Previous works have proposed that levels of antioxidant molecules and activities of antioxidant enzymes could be useful biomarkers of metal related oxidative stress in birds. Because of the close cooperation of the antioxidant defense system and the interspecific differences in the use of antioxidants against ROS, it is essential to use different biomarkers, enzymatic and non-enzymatic, when measuring oxidative stress (Berglund et al., 2007; Costantini and Verhulst, 2009). Moreover, in order to make inferences about oxidative stress, it is necessary to measure at least a marker of oxidative damage (Costantini and Verhulst, 2009). Although metal related oxidative stress has been demonstrated in experimental studies with birds (Hoffman et al., 2005; Mateo et al., 2003), few studies have been conducted on the effects of heavy metals on oxidative stress biomarkers in free-living birds exposed to metals under natural conditions (Berglund et al., 2007; Espín et al., 2014a; Koivula et al., 2011; Martínez-Haro et al., 2011). Thus, it is necessary to understand the mechanisms of metal toxicity in wild birds, and the concentrations that cause effects on the antioxidant system. The present study has as a main aim the assessment of oxidative stress related to metals in a terrestrial wild bird widely distributed in Southeastern Spain, the Eurasian eagle owl (*Bubo bubo*). Concentrations of lead (Pb), cadmium (Cd), mercury (Hg), copper (Cu) and zinc (Zn) in blood samples obtained from Eurasian eagle owl from three areas located in the province of Murcia have been evaluated. The three different areas selected corresponding, to an agricultural and rural area, an industrial area, and an ancient mining site. It was expected to find low metal concentrations in the agricultural and rural area. However, owls living in the industrial area or in the ancient mine site were expected to have higher metal concentrations. In addition, threshold concentrations at which metals can affect antioxidant systems are not known, and low metal levels may have an effect on antioxidant biomolecules. We analyzed a battery of biomarkers including total GSH content, antioxidant enzymes activities (GPx, SOD, CAT, and GST) and lipid peroxidation in order to evaluate the potential effects that these metals bear on Eurasian eagle owl oxidative stress biomarkers.

2. Material and methods

2.1. Study area and species

The study area is located in the east of the province of Murcia, Southeastern Spain (37°45′N, 0°57′W) (Fig. 1). Since the study area is relatively large and some differences in land use and contamination sources are known, it was divided in three subareas. In the Northern area (mountains Escalona, Altaona, Monte el Valle and Columbares) land is mainly dedicated to citrus and dry farming, and the European rabbit is abundant, accounting for 71% of the prey consumed by Eurasian eagle owls (authors' unpublished data). No contamination sources are known in this zone, and we have called this subarea as “agricultural and rural area”. In the Southern area, the European rabbit is less abundant (35% of the Eurasian eagle owls' diet), and the raptor consumes a similar proportion of rats (*Rattus rattus* and *Rattus norvergicus*) (23% of the diet), apart from pigeons (*Columba spp.*) (14%), partridges (*Alectoris rufa*) (5.26%), hedgehogs (*Erinaceus europeus* and *Atelerix algirus*) (5.26%) and yellow-legged gulls (*Larus michahellis*) (3.16%) (authors' unpublished data). This subarea is delimited by Sierra Minera Cartagena-La Union, La Muela-Cabo Tiñoso and Almenara, and irrigation farming is predominant. In this Southern area 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). We have called this subarea as “industrial area”. More than 50 years ago there were many chemical products manufacturing factories near the city of Cartagena. As a consequence, soils in this area have been affected by industrial wastes, and display a rather high metal pollution level, mainly of copper, zinc, cadmium, lead, and mercury (Faz-Cano et al., 2001). Besides the large amounts of metals and other contaminants present in this environment, renders the geographical and climatic factors to prevent the efficient dispersion of these contaminants (García-Fernández et al., 1995). A study conducted in the mid90s showed that the highest concentrations of metals were found in the blood of wild birds (Eagle owl included) from Cartagena in comparison to that of those from the rest of the province of Murcia (García-Fernández et al., 1995). Finally, given that the southeast of the Iberian Peninsula has a long history of the extraction of its mineral resources, there are still high amounts of waste in the mining areas. These materials are extremely rich in heavy metals, such as lead, copper and zinc, and also evidence of cadmium, arsenic and mercury can be seen (Faz-Cano et al., 2001). In the Southern area there are some nests located close to an ancient mine site called “Sierra Minera Cartagena-La Unión” (Fig. 1). This third subarea is called as “mining area”. This mining district was the main source for lead and zinc in Spain during the nineteenth century (Estevan-Senís, 1967); and it has been an intensive mining activity until 1991; mainly of lead, zinc, copper, tin, iron, manganese and silver (Pavetti et al., 2006). In addition, according to previous studies (Espín et al., 2014b; García-Fernández et al., 1995), the main route of exposure to heavy metals in Eurasian eagle owl from Murcia is through the food chain. In this sense, Espín et al. (2014b) found positive correlations between blood Hg concentrations in Eagle owls and Hg levels in rabbit muscles in Murcia, showing that Hg concentrations in Eagle owls reflect Hg levels in their main prey, and these species are mainly exposed to this metal through food consumption.

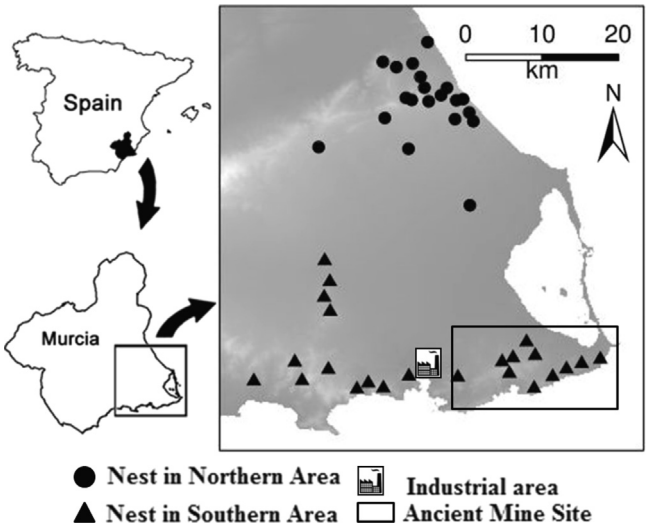

Fig. 1. Map with the location of the study area (Murcia, Spain). Circles and triangles represent nests in Northern (agricultural and rural area) and Southern areas respectively. Industrial area is represented by a factory figure and area under mining influence is framed by a square.

The climate is meso-arid Mediterranean with 275–400 mm of annual rainfall and a high average annual temperature of 19 °C. In our study area, Eurasian eagle owl prefers low to medium cliffs (Martínez and Calvo, 2000). This species is the largest nocturnal raptor in Spain, it is sedentary and highly territorial all year round, and occupies home ranges that vary in size depending on prey availability. Eurasian eagle owl population in the province of Murcia is abundant and estimated to be approximately 240–270 pairs (Martínez and Zuberogoitia, 2003; Martínez and Calvo, 2006).

2.2. Sampling method

A total of 141 blood samples from 2011 ($n=71$) and 2012 ($n=70$) (72 from the agricultural and rural area, 40 from the industrial area and 29 from the mining

area) were analyzed for metal concentrations in Eurasian eagle owl chicks ($n=133$) of approximately 30-days old and adult females ($n=8$) from Murcia (Fig. 1). A total of 140 red blood cell (RBC) samples were analyzed for oxidative stress biomarkers. The sampling was authorized by the General Directorate of Natural Patrimony and Biodiversity from the Autonomous Community of Murcia Region.

Blood samples were collected by puncturing brachial vein with a 23 G needle and a syringe, and stored in heparinised Eppendorf tubes under refrigerated conditions until processed in the laboratory. We collected approximately 3–5 ml of blood, depending upon the stage of development of the nestling. After sampling, the nestlings were returned to their nest. Adults Eurasian eagle owls were captured in their territories with a mist net placed strategically close to the nest. One Eppendorf tube with whole blood was separated and another Eppendorf tube with blood was used to separate plasma and RBC fractions (10,000 rpm, 5 min). Plasma was separated in a new tube and RBC samples were washed with saline solution and centrifuged again at 10,000 rpm during 5 min. Hematocrit was recorder using capillary tubes reader after centrifugation at 5000 rpm during 5 min. Finally, three Eppendorf tubes with whole blood, plasma and RBC were stored at −80 °C until analysis.

The health status of the birds was clinically evaluated by a veterinarian prior to blood sampling. This clinical exploration includes the evaluation of general body conformation, posture, attitude, stimulus response, and character of respiration. Also it includes exploration of the feathers, skin, beak, eyes, ears, cere, nares, oral cavity, bones, muscles (especially breast muscle), wings, feces, abdomen and vent. Besides, a plasma biochemistry analysis was done in every individual to check normal health status and ensure that birds did not suffer any subclinical pathology. An A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain) was used to determine plasma biochemistry with commercial kits from BioSystems S.A. The plasma enzyme activities analyzed were alkaline phosphatase (ALP; Enzyme Commission (EC) no. 3.1.3.1), aspartate aminotransferase (AST; EC 2.6.1.1), butyrylcholinesterase (CHE; EC 3.1.1.8), creatine kinase (CK; EC 2.7.3.2), gamma-glutamyltransferase (g-GT; EC 2.3.2.2), and lactate dehydrogenase (LDH; EC 1.1.1.27). The plasma constituents analyzed were albumin, total protein, cholesterol, glucose, triglycerides, uric acid, calcium and phosphorus.

2.3. Blood metals analysis

Cd, Pb, Cu and Zn levels were analyzed in blood samples following the method described by García-Fernández et al. (1995). A volume of 200 μl of whole blood was placed in a quartz digestion tube, to which 0.5 ml of an acid mixture (nitric: perchloric:sulfuric, 8:8:1) was added. The sample was then submitted to a progressive thermal treatment and, once dried, was left to cool. Tetradistilled purified water was added and transferred to the measuring vessel, adjusting the final volume to 10 ml. Prior to by anodic stripping voltammetry (ASV), 50 μl of hydrochloric acid was added to the measuring vessel as an electrolyte support. The pH of the final solution was between 1 and 2. The anodic stripping voltammeter (VA-757 Computrace Workstation; Metrohm, Switzerland) used was equipped with three standard electrodes: working electrode (hanging Hg drop), reference electrode (Ag/AgCl; KCl, 3 mol/l), and auxiliary electrode (platinum).

We used the differential normal pulse technique with an electrolysis time of 120 s and modulation amplitude of 50 mV. The concentration of each metal in the digested sample was calculated after twice adding dilutions prepared from standard solutions of Cd, Pb, Cu and Zn (Sigma, St. Louis, MO). Mean recoveries, which approached 96%, were calculated analyzing 10 identical samples of reconstituted lyophilized blood (European Union Reference Standards CRM195). Detection limits were 0.05 and 0.1 μg/l for Cd and Pb, respectively, and 0.3 and 0.04 mg/l for Zn and Cu, respectively. All the reagents used were Suprapur quality from Merck (Darmstadt, Germany). The quartz tubes used for the wet digestion were first washed with 2% nitric acid for 48 h and then rinsed twice with tetradistilled water and dried in an oven at 100 °C.

Total Hg was analyzed in a Milestone DMA-80 direct Hg analyzer by atomic absorption spectrophotometry with a detection limit of 0.005 ng. Blood samples (100 μl wet weight) were loaded in a nickel boat and analyzed. A calibration curve was done with 10 points (in duplicate) from 0 to 1004 ng of Hg. Precision and accuracy of the method were tested using a certified reference material (CRM) (Hg Standard for AAS, Fluka, 1000 mg/l Hg in 12% nitric acid, prepared with high purity Hg metal, HNO_3 TraceSELECT® and water TraceSELECT® Ultra). Recovery of total Hg from five replicates of CRM diluted to 1 ppm was 104.2 ± 11.8% (mean ± standard deviation). The coefficient of variation for the repeatability was 11.4%.

2.4. Biomarker analyses in red blood cells (RBC)

Oxidative stress parameters (total glutathione, glutathione peroxidase, superoxide dismutase, catalase, glutathione-S-transferase and thiobarbituric acid-reactive substances) were analyzed in RBC, after homogenization (1:10 w/v) in a stock buffer (1.15% KCl in 0.01 M PBS (pH 7.4) with 0.02 M EDTA). Lipid peroxidation, estimated as thiobarbituric acid-reactive substances (TBARS), was assessed following the methodology described by Alonso-Álvarez et al., (2008) with a spectrophotometer (UV-1603, Shimadzu). Levels of total glutathione (tGSH) were obtained as described by Reglero et al. (2009) with an automated spectrophotometer A25-Autoanalyzer (BioSystems). The activities of glutathione peroxidase (GPx; EC1.11.1.9) and superoxide dismutase (SOD; EC1.15.1.1) were determined spectrophotometrically (A25-Autoanalyzer, BioSystems) using the Ransel and Ransod kits (Randox Laboratories), respectively, following descriptions of Reglero et al. (2009) with some modifications for RBC. Homogenized samples were diluted by 1:20 and 1:25 (v:v) with Ransel diluting agent and Ransod sample diluents (Randox Laboratories), for GPx and SOD determinations respectively. GPx and SOD results were expressed as units per gram protein.

Catalase (CAT; EC 1.11.1.6) activity was assayed following the methodology described by Clairbone (1985), based on the decomposition of hydrogen peroxide (H_2O_2) in molecular oxygen and water by this enzyme. The rate of enzymatic decomposition of H_2O_2 was determined as absorbance decrements at 240 nm with a spectrophotometer (UV-1603, Shimadzu). The assay mixture consisted of 950 μl of potassium phosphate buffer (0.05 M, pH 7.0), 500 μl of H_2O_2 (0.03 M) and 50 μl of sample. Results were expressed as μmol H_2O_2 consumed per minute per milligram protein.

The activity of glutathione-S-transferase (GST; EC 2.5.1.18) was determined by the method described by Habig et al. (1974), based on the measurement of the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione. This reaction is catalyzed by GST, and it is determined as absorbance increments at 340 nm. The assay mixture consisted of 1850 μl of potassium phosphate buffer (0.2 M, pH 7.9), 50 μl of CDNB solution (8.17 mM) (Sigma) diluted in ethanol:water (1:1.5), 50 μl of GSH solution (8.17 mM) (Sigma) and 50 μl of sample. The results were expressed as nanomoles produced per minute per milligram protein.

As enzyme activities were expressed in relation to grams of protein in the homogenates, total protein contents were measured in the homogenates using a spectrophotometer at 595 nm following the Bradford (1976) method, using bovine serum albumin as standard protein.

2.5. Statistical analysis

All analyses were carried out using the SPSS v.15.0 statistical package. Reported metal concentrations and oxidative stress biomarker values represent the mean ± standard deviation, median and range. The data were tested for normality using a Kolmogorov–Smirnov test and data were normalized using log-transformation. Differences in metal concentrations and biomarker values between areas and age groups were calculated by ANOVA. Correlations between biomarkers were checked using Pearson's correlation coefficient. Simple linear regression was performed to evaluate the effect of each metal on the oxidative stress biomarkers in each studied area (agricultural and rural area, industrial area, and ancient mine site) and with pooled data of all individuals to increase the number of samples. Pearson's correlations between metals and biomarkers were also provided. The level of significance for these tests was set at $\alpha=0.05$. Because of the limited amount of blood from some birds, sample sizes were not the same for all the parameters analyzed. Generalized linear models (GLMs) with a normal distribution and an identity function were performed to study combined effects of metals and their interactions on the biomarkers. Biomarker value was the response variable, metal concentrations were selected as covariates and studied area and age were selected as factors. A backward stepwise procedure was used to select the final models. Predictor variables and interaction terms were retained when they significantly improved model fit ($p<0.05$). We also provide Akaike's information criterion (AIC) and the Akaike weight (likelihood that a given model is the best among all candidate models). The model with the greatest AIC weight and lowest AIC value indicates the closest to unknown reality.

3. Results

3.1. Metal concentrations in blood

Metal concentrations in blood of Eurasian eagle owl and metal levels according to area and age are presented in Table 1. Pb and Hg concentrations in blood of Eurasian eagle owl (mean ± SD) were 3.3 ± 5.2 μg/dl and 2.3 ± 3.8 μg/dl wet weight, respectively (Table 1). Cd concentrations in blood were 0.07 ± 0.21 μg/dl and this metal was detected above the detection limit only in the 26% of the samples. Finally, concentrations of the essential elements in the bloods of Eurasian eagle owls were 311.5 ± 67.1 and 10.6 ± 4.8 μg/dl for Zn and Cu, respectively.

Significant differences were found in Pb ($p<0.001$) and Hg ($p=0.004$) concentrations among areas, with the highest median levels in Eurasian eagle owls from the ancient mine site (Table 1). However, no significant differences were found for Cd, Zn and Cu concentrations according to area (Table 1). For the five metals analyzed, only Cu levels showed significant differences between

Table 1
Metal concentrations in blood samples (μg/dl, wet weight) of Eurasian eagle owl for all individuals and according to area and age.

Metal concentrations in blood of Eurasian eagle owl							
			Cd (μg/dl)	Pb (μg/dl)	Cu (μg/dl)	Zn (μg/dl)	Hg (μg/dl)
All individuals (*N*=141)		Mean ± SD	0.07 ± 0.21	3.27 ± 5.21	10.62 ± 4.77	311.47 ± 67.14	2.32 ± 3.83
		Median (range)	0.006 (0.006–1.94)	1.31 (0.05–31.23)	10.63 (3.36–34.20)	299.20 (62.50–610.52)	1.5 (0.15–38.44)
Area	Agricultural and rural area (*N*=72)	Mean ± SD	0.08 ± 0.26	1.24 ± 1.12[a,b]	10.35 ± 4.19	318.30 ± 78.90	1.61 ± 1.48[a]
		Median	0.006	0.92	10.46	307.22	1.20
	Industrial area (*N*=40)	Mean ± SD	0.08 ± 0.16	3.75 ± 6.07[a,b]	10.80 ± 6.29	318.04 ± 54.42	3.16 ± 6.45
		Median	0.006	1.32	10.53	300.99	1.47
	Mining area (*N*=29)	Mean ± SD	0.05 ± 0.07	7.64 ± 7.11[b]	11.03 ± 3.70	285.47 ± 41.75	2.94 ± 2.65[a]
		Median	0.006	4.93	11.08	284.14	2.16
Age	Nestling (*N*=133)	Mean ± SD	0.07 ± 0.21	3.38 ± 5.33	10.29 ± 4.56[b]	310.38 ± 67.41	2.31 ± 3.93
		Median	0.006	1.36	10.50	297.30	1.44
	Adult (*N*=8)	Mean ± SD	0.006 ± 0.01	1.39 ± 0.95	16.14 ± 5.05[b]	329.59 ± 63.69	2.49 ± 0.74
		Median	0.006	1.19	14.63	316.21	2.31

N=number of samples.
[a] *Note:* Significant differences in metal concentrations according to area: $p < 0.005$.
[b] Significant differences in metal concentrations according to area or age group: $p < 0.001$.

Table 2
Enzyme activities, glutathione levels and lipid peroxidation in red blood cells of Eurasian eagle owls, and values according to area and age.

Enzyme activities, glutathione levels and lipid peroxidation in red blood cells								
			GPx[a]	SOD[b]	CAT[c]	GST[d]	tGSH[e]	TBARS[f]
All individuals		*N*	140	139	140	140	139	135
		Mean ± SD	617.12 ± 208.73	686.38 ± 179.26	21.19 ± 7.24	10.28 ± 2.97	8.63 ± 2.07	0.051 ± 0.026
		Median	641.61	697.82	23.70	10.17	8.43	0.047
		(range)	(105.13–1423.43)	(267.59–1092.66)	(8.74–36.99)	(3.77–25.29)	(1.43–14.62)	(0.009–0.213)
Area	Agricultural and rural area	*N*	71	71	71	71	71	68
		Mean ± SD	649.23 ± 203.66	678.42 ± 187.63	21.98 ± 6.91	11.24 ± 3.20[g,h]	8.32 ± 2.20	0.054 ± 0.027
		Median	653.58	670.66	23.83	11.29	8.20	0.047
	Industrial area	*N*	40	39	40	40	40	39
		Mean ± SD	604.15 ± 222.13	705.44 ± 164.18	21.43 ± 7.31	9.81 ± 2.24[g]	8.94 ± 2.07	0.046 ± 0.029
		Median	687.67	734.33	25.54	9.85	8.51	0.044
	Mining area	*N*	29	29	29	29	28	28
		Mean ± SD	556.38 ± 192.82	680.21 ± 181.87	18.93 ± 7.73	8.57 ± 2.33[h]	8.94 ± 1.58	0.054 ± 0.016
		Median	585.06	712.33	14.09	8.96	8.60	0.052
Age	Nestling	*N*	133	132	133	133	131	127
		Mean ± SD	617.44 ± 211.07	674.34 ± 171.56[h]	21.29 ± 7.32	10.19 ± 3.00	8.67 ± 2.08	0.050 ± 0.025[g]
		Median	631.16	692.17	23.83	10.11	8.47	0.047
	Adult	*N*	7	7	7	7	8	8
		Mean ± SD	611.03 ± 170.46	913.39 ± 182.01[h]	19.33 ± 5.44	11.98 ± 1.63	7.82 ± 1.73	0.068 ± 0.028[g]
		Median	689.48	983.44	19.46	12.02	8.00	0.061

N=number of samples.
[a] *Note*: glutathione peroxidase (U/g protein).
[b] Superoxide dismutase (U/g protein).
[c] Catalase (μmol/min/mg protein).
[d] Glutathione-S-transferase (nmol/min/mg protein).
[e] Total glutathione (μmol/g).
[f] Lipid peroxidation, estimated as thiobarbituric acid-reactive substances (μmol/g).
[g] Significant differences in biomarkers values according to area or age group: $p < 0.05$.
[h] Significant differences in biomarker values according to area or age group: $p < 0.001$.

age groups, with the highest concentrations in adult individuals (Table 1).

3.2. Oxidative stress

Enzyme activities, total glutathione levels and lipid peroxidation in RBC of Eurasian eagle owls are presented in Table 2. Table 2 also shows results according to area and age. Both SOD activity and TBARS concentrations showed significant differences between age groups, with higher SOD activity and TBARS levels in adult individuals than in chicks (Table 2).

Significant location-related differences were found only for GST activity, with lower activity in Eurasian eagle owls from the ancient mine site (Table 2). However, when adult individuals were excluded from the analysis, because of the possible effect of age, significant differences were also found for CAT activity ($p=0.05$; CAT in agricultural and rural area ($n=65$)=22.32 ± 6.98; CAT in mining area ($n=29$)=18.93 ± 7.73 μmol/min/mg protein).

We conducted simple linear regression analysis to search for biomarker response on single metal concentrations in Eurasian eagle owl (Table 3). We also developed GLMs to study combined effects of metals and their interactions, studied area and age on the biomarkers (Table 4).

Despite the low levels of metals, several oxidative stress biomarkers correlated with contaminant concentrations. When only Eurasian eagle owls from the agricultural and rural area were selected, it was found positive relationships between CAT activity and Hg concentrations ($r=0.343$, $F=9.23$, $p=0.003$, $n=71$), SOD activity and Hg concentrations ($r=0.316$, $F=7.636$, $p=0.007$, $n=71$) (Fig. 2), and GST activity and Cd levels ($r=0.367$, $F=10.734$, $p=0.002$, $n=71$).

3.2.1. Glutathione peroxidase (GPx)

GPx activity was inversely correlated with Cd concentrations, and a marginally inverse correlation between GPx activity and Pb concentrations was also found (Table 3). When adult individuals were excluded of the analysis because of the possible effect of age, the inverse correlation between GPx activity and Pb concentrations was significant ($r=-0.174$, $F=4.073$, $p=0.046$, $n=133$). When GLMs were performed, the best model for GPx activity was constructed only with Cd (Table 4). The GPx activity and Cd concentration inverse relationship was still true and even stronger when only the individuals from the industrial area ($r=-0.578$, $F=19.082$, $p<0.001$, $n=40$) or mining area ($r=-0.376$, $F=4.459$, $p=0.044$, $n=29$) were selected. In the mining area, a significant positive relationship between Hg concentrations and GPx activity was also found ($r=0.414$, $F=5.575$, $p=0.026$, $n=29$). Cd levels ≥ 0.3 μg/dl in blood produced an inhibition of 32% in GPx activity. However, Cd concentrations ≥ 0.02 μg/dl were enough to produce an inhibition of 13% in the activity of this enzyme. Regarding Pb levels, concentrations ≥ 2 μg/dl in blood produced an inhibition of 8% in GPx activity in RBC of Eurasian eagle owl.

Table 3
Linear regression analysis of biomarker response on single metal concentrations in Eurasian eagle owl.

Biomarker response (*Y*)[a]	Metal (*X*)	Intercept (*a*)	Regression coefficient (*b*)	*F*	*p*	*r*	*n*
log GPx	log Cd	6.047	−0.072	12.712	<0.001	−0.290	140
log GPx	log Pb	6.381	−0.050	3.475	0.064	−0.157	140
log CAT	log Cd	2.622	−0.085	19.949	<0.001	−0.355	140
log CAT	log Pb	3.021	−0.074	8.732	0.004	−0.244	140
log CAT	log Cu	3.634	−0.284	17.454	<0.001	−0.335	140
log TBARS	log Hg	−3.107	0.118	7.535	0.007	0.232	135

Regressions follow the model $Y=a+bX$. r=Pearson's correlation coefficient. n=number of samples.

[a] GPx=glutathione peroxidase, SOD=superoxide dismutase, CAT=catalase, GST=glutathione-S-transferase, and TBARS=thiobarbituric acid-reactive substances.

3.2.2. Superoxide dismutase (SOD) and glutathione-S-transferase (GST)

No relationship was found with any single metal and no significant models were constructed with the studied variables for SOD activity. However, a significant negative correlation was found between SOD and Pb levels in the industrial area ($r=-0.329$, $F=4.483$, $p=0.041$, $n=39$), and a significant positive correlation was found between SOD

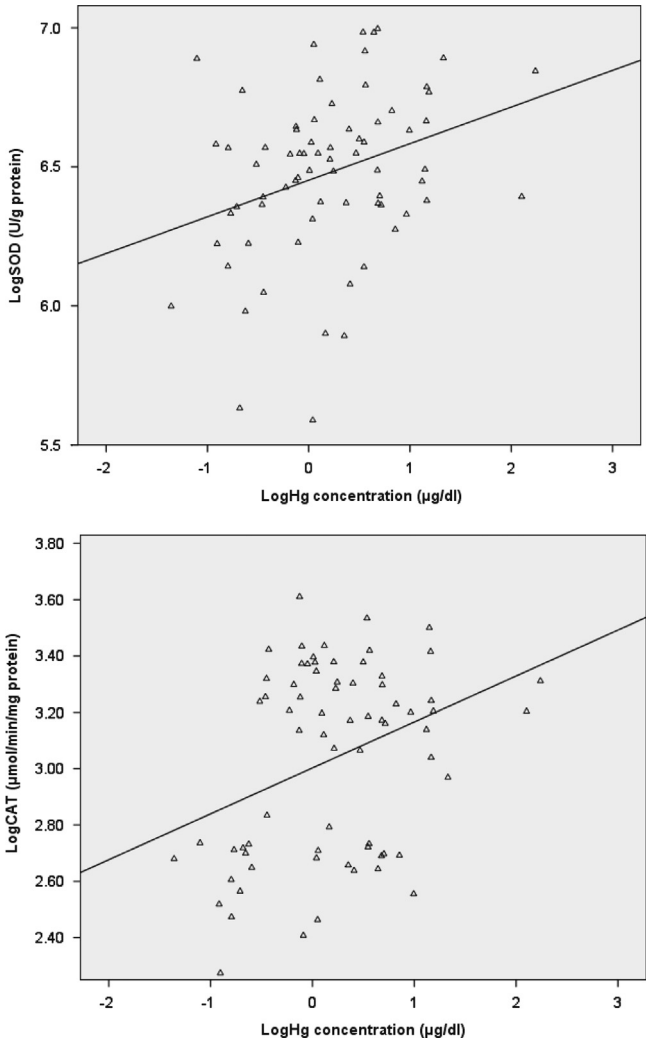

Fig. 2. Relationship between log Hg concentrations (μg/dl) in blood and log SOD activity (U/g protein) in red blood cells ($r=0.316$, $p=0.007$, $n=71$), and between log Hg levels in blood and Log CAT activity (μmol/min/mg protein) in red blood cells ($r=0.343$, $p=0.003$, $n=71$) of Eurasian eagle owls from the agricultural and rural area.

Table 4
Generalized linear models (GLMs) evaluating combined effects of metals on the oxidative stress biomarkers response.

Biomarker response[a]	Model[b]	AIC[c]	ΔAIC[d]	Akaike weight[e]	X^2	*p*	*n*
log GPx	log Cd (<0.001)	122.733	0.00	0.31	12.337	<0.001	140
log CAT	log Pb (0.047)+log Cd (0.004)+log Cu (0.004)	95.737	0.00	0.39	31.295	<0.001	140
log GST	Area (<0.001)+log Pb (0.03)	36.045	0.00	0.32	25.343	<0.001	140
log TBARS	Age (0.044)+log Hg (0.013)	142.330	0.922	0.24	11.437	0.003	135

[a] GPx=glutathione peroxidase, CAT=catalase, and TBARS=thiobarbituric acid-reactive substances.

[b] Model indicates the most influential explanatory variables (partial significance of each variable in the model) in the response variable. We select the best variables in a model according to *p*-value criteria.

[c] AIC (Akaike's information criterion) value.

[d] $\Delta AIC=AIC_{min}-AIC_i$.

[e] The Akaike weight is the likelihood that a given model is the best among all candidate models. The model with the greatest AIC weight and lowest AIC value indicates the closest to unknown reality.

and Pb concentrations in the mining area ($r=0.371$, $F=4.298$, $p=0.048$, $n=29$). Regarding GST activity, no relationship was found with any single metal. However, when GLMs were performed, the best explanatory variables for GST model were area and Pb concentrations (Table 4).

3.2.3. Catalase (CAT)

Regarding CAT activity, it was inversely related with concentrations of the single metals Cd, Pb and Cu (Table 3). The best explanatory variables when GLMs were constructed were Cd, Pb and Cu concentrations (Table 4). In the Southern area, CAT activity was negatively related with both Cd ($r=-0.570$, $F=18.255$, $p<0.001$, $n=40$ in the industrial area and $r=-0.424$, $F=5.920$, $p=0.022$, $n=29$ in the mining area) and Cu ($r=-0.708$, $F=27.132$, $p<0.001$, $n=29$ in the mining area) concentrations. However, in the agricultural and rural area, CAT activity was related with Pb levels ($r=-0.284$, $F=6.052$, $p=0.016$, $n=71$). In the present study, Cd levels ≥ 0.3 μg/dl in blood produced an inhibition of 26% in CAT activity in RBC of Eurasian eagle owls. However, Cd concentrations ≥ 0.02 μg/dl were enough to produce an inhibition of 20% in the activity of CAT. Regarding Pb levels, concentrations ≥ 2 μg/dl in blood produced an inhibition of 10.5% in CAT activity in RBC of Eurasian eagle owl.

3.2.4. Total glutathione (tGSH)

Regarding tGSH levels, no relationship was found with any single metal and no significant models were constructed with the studied variables. However, linear regression analysis was performed selecting only the individuals living in the ancient mine site ($n=29$, Fig. 1) in order to elucidate if the significant higher levels of Pb in this subarea (7.64 ± 7.11 μg/dl) in comparison with the rest of the population could have an effect on tGSH concentrations. In this sense, a significant negative relationship was found between blood Pb concentrations and tGSH levels in RBC of Eagle owls from the mining area ($r=-0.396$, $F=4.847$, $p=0.037$, $n=28$; Fig. 3). A depletion of 16% and 4% in tGSH levels were associated with Pb concentrations ≥ 15 and 3 μg/dl, respectively, in Eurasian eagle owls from the ancient mine site.

3.2.5. Lipid peroxidation

TBARS levels were positively related with Hg concentrations (Table 3). The explanatory variables in the best model were age and Hg concentrations (Table 4). When only Eurasian eagle owls from the industrial area were selected, the relationship between TBARS and Hg levels was stronger ($r=0.593$, $F=20.119$, $p<0.001$, $n=39$) (Fig. 4). In addition, when Eurasian eagle owls from both the industrial and the mining area were selected, a significant relationship between TBARS levels and Pb concentrations was found ($r=0.259$, $F=4.687$, $p=0.03$, $n=67$) (Fig. 4). Pb concentrations ≥ 2 and 10 μg/dl produced a TBARS induction of 10% and 28%, respectively, in individuals from both the industrial and the mining area. Regarding Hg, concentrations ≥ 3 and 10 μg/dl resulted in a TBARS induction of 102% and 190%, respectively, in Eurasian eagle owls from the industrial area.

3.2.6. Correlations among oxidative stress biomarkers

Correlations among oxidative stress biomarkers were also conducted, and some of them were correlated with each other. We found positive correlation between GPx and CAT enzymes ($r=0.641$, $p<0.001$, $n=140$), SOD and CAT ($r=0.209$, $p=0.014$, $n=139$), GST and GPx ($r=0.316$, $p<0.001$, $n=140$), GST and CAT ($r=0.268$, $p=0.001$, $n=140$), and tGSH and TBARS levels ($r=0.264$, $p<0.001$, $n=135$). A negative correlation was found between CAT and TBARS ($r=-0.242$, $p=0.004$, $n=134$), and SOD and TBARS ($r=-0.181$, $p=0.037$, $n=133$).

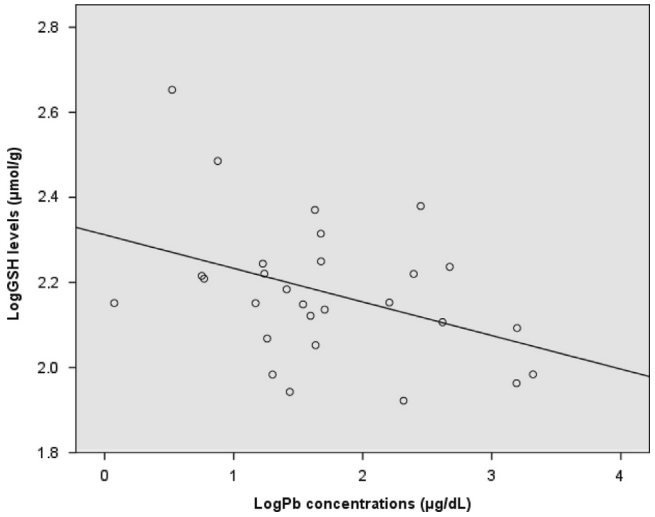

Fig. 3. Relationship between log Pb concentrations (μg/dl) in blood and log total glutahione levels (μmol/g) in red blood cells of Eurasian eagle owls from an ancient mine site ($r=-0.396$, $p=0.037$, $n=28$).

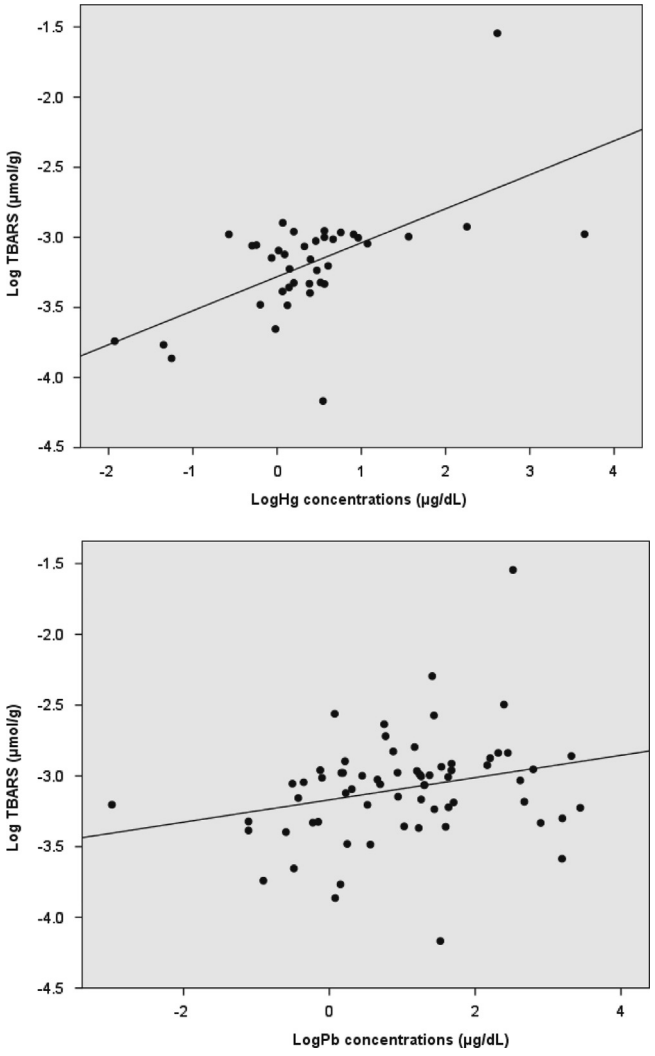

Fig. 4. Relationship between log TBARS (μmol/g) in red blood cells and log Hg concentrations (μg/dl) in blood ($r=0.593$, $p<0.001$, $n=39$) of Eurasian eagle owls from the industrial area, and between log TBARS (μmol/g) in red blood cells and log Pb concentrations (μg/dl) in blood ($r=0.259$, $p=0.034$, $n=67$) of Eurasian eagle owls from the Southern area (industrial and mining areas).

3.2.7. Relationship between oxidative stress biomarkers and size of the brood

Finally, we checked if the size of the brood could have an effect on the different biomarkers. We found negative relationships between the number of nestlings in each nest and GPx activity ($r=-0.316$, $p<0.001$, $n=133$) or CAT activity ($r=-0.201$, $p=0.021$, $n=133$), and a positive relationship between the brood size and GSH levels ($r=0.205$, $p=0.019$, $n=133$) (Fig. 5).

4. Discussion

In general, plasma biochemistry parameters were similar to those described as baseline data in Eagle owls by several authors (García-Rodríguez et al., 1987; Gómez-Ramírez, 2011; Jennings, 1996), and are indicative of normal heath status.

4.1. Metal concentrations in blood

Mean Pb levels were lower than those found by García-Fernández et al. (1995) (mean$=8.3\pm6.7$ μg/dl) and García-Fernández et al. (1997) (mean$=7.6$ μg/dl, median$=6.5\pm2$ μg/dl) in Eagle owls from the province of Murcia, and similar to those found by Gómez-Ramírez et al. (2011) (3.7 ± 4.2 μg/dl) in nestling Eagle owls from the same studied area. Studies in Southeastern Spain show that Pb exposure has decreased in the last 10 years (García-Fernández et al., 2008). Our study confirms a decrease in Pb exposure in the last 20 years in Eurasian eagle owls from the province of Murcia, which could be explained by the closure of mines in 1991 and the ban on leaded petrol in 2001.

In general, Pb concentrations were similar to those found in raptor species from non-polluted areas (Henny et al., 1994; Martínez-López et al., 2004). In fact, García-Fernández et al. (1995) suggested that raptors in the province of Murcia were chronically exposed to rather low Pb levels, mainly through food consumption. These authors reported that only four individuals presented Pb concentrations higher than 20 μg/dl, which was the minimum blood level for considering physiological effects in Falconiformes according to Franson (1996). However, more recent studies have found that Pb concentrations below 15 μg/dl in blood are enough to cause sublethal effects, such as inhibition of aminolevulinic acid dehydratase (ALAD) activity in raptors in the field (Gómez-Ramírez et al., 2011; Martínez-López et al., 2004).

Local contamination sources have probably contributed to the higher Pb concentrations detected in Eurasian eagle owl from the two areas in the South comparing with Pb levels in owls from the agricultural and rural area. In the Southern area, 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). Large amounts of metals and other contaminants were present in this environment, and geographical and climatic factors avoided the efficient dispersion of these pollutants (García-Fernández et al., 1995). In this sense, García-Fernández et al. (1995) found higher concentrations of Pb and Cd in tissues of wild birds (Eagle owl included) from Cartagena in comparison with the rest of the province of Murcia. Moreover, in the Southern area there are some nests located close to an ancient mine site called "Sierra Minera Cartagena-La Unión" (Fig. 1). In fact, the highest mean Pb concentrations (7.64 ± 7.11 μg/dl) were found in individuals from this mining area, and these levels were close to those described 15 years ago by García-Fernández et al. (1997). The mining district of Cartagena-La Unión has been an intensive mining activity until 1991; mainly of lead, zinc, copper, tin, iron, manganese and silver; for more than 2500 years (Pavetti et al., 2006). This area was the main source for Pb and Zn in Spain during the nineteenth century (Estevan-Senís, 1967). Gómez-Ramírez et al. (2011) also found that Eagle owl chicks from the mining area "Sierra Minera Cartagena-La Unión" or their surroundings had higher Pb concentrations than the rest of the population in the

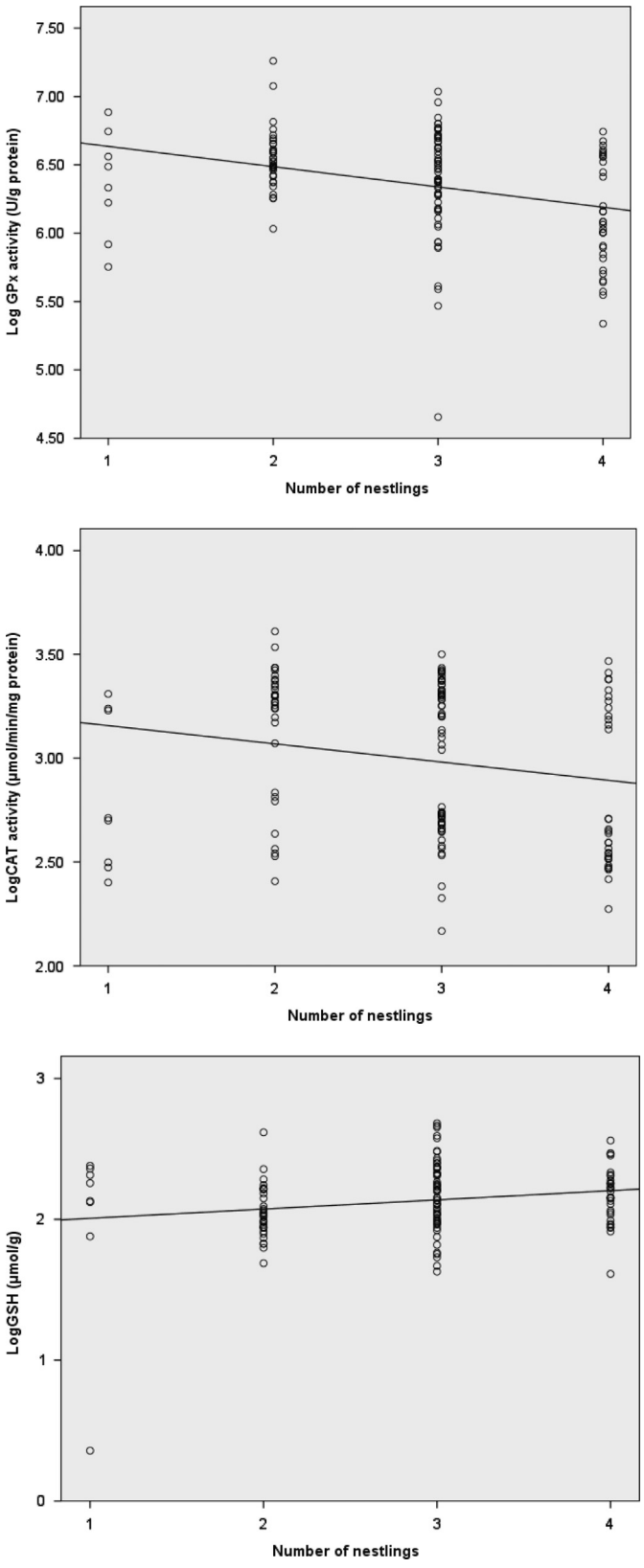

Fig. 5. Relationship between the number of Eurasian eagle owl nestlings and log glutathione peroxidase activity (U/g protein) ($r=-0.324$, $p<0.001$, $n=133$), log catalase activity (μmol/min/mg protein) ($r=-0.201$, $p=0.021$, $n=133$), and log total glutahione levels (μmol/g) ($r=0.205$, $p=0.019$, $n=133$).

period from 2003 to 2007. The low concentrations found in the agricultural and rural area in the present study were expected, since nests are located far from potential sources for heavy metals.

Although no significant differences were found between age groups, adult individuals had lower Pb concentrations than nestlings (Table 1). However, it may be noted that seven of the eight adult individuals were captured in the agricultural and rural area, and these individuals presented Pb concentrations between 0.30 and 1.62 μg/dl, while the only adult individual from the industrial area had Pb levels of 3.53 μg/dl.

Regarding Hg concentrations, there is few data available about Hg levels in blood of Eagle owls (Espín et al., 2014b). However, concentrations found were much lower than those reported for fish-eating raptors (Jagoe et al., 2002; Langner et al., 2012). A previous study in the same studied area (Espín et al., 2014b) analyzed Hg concentrations in blood and feathers of Eurasian eagle owl and in muscle of their main prey, the European rabbit, from 2006 to 2012. As explained by Espín et al. (2014b), the region is not considered Hg polluted. However, spatial differences in Hg concentrations in Eagle owls appear to be mostly related to local contamination (ancient mine site), and probably diet composition plays a role of less extent. Besides, Espín et al. (2014b) found significant differences in blood Hg concentrations between years in Eagle owls from the province of Murcia. This result may be affected by the rainfalls during the seven years of study, which may contribute to a higher Hg removal from the atmosphere and local wet deposition in years with the highest rainfalls (Espín et al., 2014b).

Cd concentrations were similar to those found by other authors (García-Fernández et al., 1995; Gómez-Ramírez, 2011) (0.1 μg/dl), and they were within the range considered as low exposure levels in birds (0.01–0.28 μg/dl) (García-Fernández et al., 1996). Taking into account these low Cd levels in Eagle owls from Murcia in the last 20 years (García-Fernández et al., 1995; Gómez-Ramírez, 2011), it could be suggested that exposure is to low dosages over time, and most probably as a consequence of dietary Cd ingestion (García-Fernández et al., 1996). The lack of differences in Cd concentrations in owls among areas probably shows that there are no important Cd emissions in the studied areas.

Finally, concentrations of the essential metals Zn and Cu were similar to those found by Gómez-Ramírez (2011) (median levels of 328 and 14 μg/dl for Zn and Cu, respectively). In general, Zn and Cu concentrations were within the range of physiologic levels in several health bird species (163–495 μg/dl for Zn and 13–120 μg/dl for Cu), including different raptor species (García-Fernández et al., 2005).

4.2. Oxidative stress

4.2.1. Oxidative stress biomarkers in Eagle owls from three areas of Murcia (Southeastern Spain)

It has been suggested that oxidative damage increases with age (Koivula and Eeva, 2010), which has been related with an increased production and susceptibility to ROS at older age (Hulbert et al., 2007). This could explain the higher TBARS concentrations found in adult birds in comparison with chicks in the present study (Table 2). In addition, Oropesa et al. (2013) also described the highest SOD activity in adult White storks (*Ciconia ciconia*).

Studies published so far show that metals can induce oxidative stress, but the response varies very much depending on the concentration of exposed metals, duration of exposure, and species studied (Hoffman et al., 2000a; Ji et al., 2006; Mateo and Hoffman, 2001). Several authors have found an enhanced activity of antioxidant enzymes, and higher levels of GSH and lipid peroxidation in birds from polluted areas compared with reference sites (Berglund et al., 2007; Espín et al., 2014a; Ji et al., 2006; Kamiński et al., 2009). In the present study, we found lower GST and CAT activity in Eurasian eagle owls from the ancient mine site. GST is an enzyme used to catalyze the conjugation of GSH with cytotoxic aldehydes produced during lipid peroxidation (Halliwell and Gutteridge, 1999) and also with pollutants, and moreover, some GST isozymes posess non-Se-dependent GPx activity (Prohaska and Ganther, 1977). CAT enzyme catalyzes the transformation of H_2O_2 to H_2O and O_2, and it has a heme molecule as the prosthetic group (ExPASy, 2012). An experimental study with Mallards (*Anas platyrhynchos*) has shown a reduction in GST and GPx activities in Pb-treated individuals (Mateo et al., 2003). In addition, it is known that Pb reduces the absorption of iron and inhibits the heme biosynthesis (Gurer and Ercal, 2000), reducing CAT activity in Pb-exposed animals (Sandhir and Gill, 1995; Sandhir et al., 1994). In this sense, it is possible that the higher concentrations of Pb and Hg in owls from the mining area in comparison to the agricultural and rural area could induce a depletion of GST and CAT in this population. No differences were found in TBARS concentrations in RBC of Eurasian eagle owl among areas (Table 2). Although individuals from the mining area had significant higher Pb and Hg concentrations, and significant lower GST and CAT activities; 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.

4.2.2. Effect of metal concentrations in oxidative stress biomarkers

In the present study, we found positive relationships between Hg concentrations and CAT and SOD activity (Fig. 2), and between Cd levels and GST activity in Eurasian eagle owls from the agricultural and rural area. These results suggest a possible protective response by an increase of antioxidant enzymes activities in individuals from this area under exposure of low Cd and Hg levels (Martínez-Haro et al., 2011). In this sense, Hussain et al. (1999) reported increased CAT activity in mice exposed to Hg, what was explained as a possible compensatory mechanism to scavenge ROS levels produced as a result of Hg accumulation. Moreover, Hg stimulates the activity of copper–zincSOD (Gurer and Ercal, 2000), probably as a protective effect. In this sense, changes in active scavengers of free radicals such as SOD enzyme are dependent on exposure time and level of Hg (Ji et al., 2006), thus low-dose Hg exposure would result in increased levels of SOD as a counteractive response of the redox-defense system (Elia et al., 2003). Espín et al. (2014a) also found that SOD activity depends mostly on Hg concentrations in Griffon vultures (*Gyps fulvus*). Besides, rats treated with Cd had an increase of 17% in liver GST activity (Jurczuk et al., 2006). As suggested by Jurczuk et al. (2006), GST may be induced by this metal, since GST catalyzes the conjugation of Cd with GSH.

4.2.2.1. Glutathione peroxidase (GPx). Several studies have found that rats treated with Cd showed decreased GPx activity (Gambhir and Nath, 1992; Jamall and Smith, 1985; Omaye and Tappel, 1975), and increased dietary selenium (Se) prevented the inhibition of GPx activity (Jamall and Smith, 1985). Se supplementation in rats treated with Cd resulted in a high accumulation of Se in liver and kidney, indicating the formation of Cd–Se complex (Gambhir and Nath, 1992). In this sense, GPx activity showed a 47% decrease in liver of starlings (*Sturnus vulgaris*) treated with Cd, due to inhibition of Se-dependent fraction of the enzyme (Congiu et al., 2000). GPx enzyme reduces peroxides in cells, such as the transformation of H_2O_2 to H_2O by oxidizing GSH (Koivula and Eeva, 2010), and requires Se as a cofactor (ExPASy, 2012). Thus the formation of Cd–Se complex as a protective effect of Se against Cd toxicity could be the reason of the negative relationship found between GPx activity and Cd levels in the present study. Regarding

the negative correlation found between GPx activity and Pb concentrations in Eurasian eagle owl chicks, an antagonistic effect between Pb and Se has also been described (Schrauzer, 1987), resulting in reduced Se uptake that may affect GPx activity. Several authors have found an inhibition of GPx activity in experimentally and naturally Pb exposed birds (Espín et al., 2014a; Mateo et al., 2003; Somashekaraiah et al., 1992).

4.2.2.2. Superoxide dismutase (SOD) and glutathione-S-transferase (GST). Curiously, opposed results were observed among the industrial and the mining area regarding the relationship between Pb concentrations and SOD activity. Lead concentrations were significantly higher in Eurasian eagle owls from the ancient mine site than those from the industrial area (Table 1). A low Pb exposure in the industrial area resulted in an inhibition of SOD activity. In this sense, SOD requires Cu for its activity, and Pb may have an indirect inhibitory effect on SOD due to the Pb-induced Cu deficiency (Gurer and Ercal, 2000). In contrast, a higher Pb exposure in the ancient mine site resulted in an induction of SOD activity. Despite this, mean SOD activities were very similar between individuals from these two areas (Table 2). Thus, this result seems as an equilibrium response of the redox-defense system, showing that the response of the antioxidant biomarkers depends on the exposure levels.

Regarding GST activity, the best explanatory variables for GST model were area and Pb concentrations (Table 4). Berglund et al. (2007) also found that GST activity was influenced by Pb concentrations. However, in general, Pb has not been shown to increase GST activities in previous studies (Mateo and Hoffman, 2001; Mateo et al., 2003).

4.2.2.3. Catalase (CAT). Both Cd and Pb concentrations were inversely related with CAT activity in the present study. CAT enzyme catalyzes H_2O_2 to H_2O and molecular oxygen (Koivula and Eeva, 2010). CAT activity has been inhibited following both in vivo and in vitro exposure to Cd in rats and several fish species (Koizumi and Li, 1992; Palace et al., 1993; Pruell and Engelhardt, 1980; Roméo et al., 2000). Palace et al. (1993) suggested a direct structural alteration of the enzyme and depression of CAT synthesis by Cd. Moreover, CAT enzyme has heme as the prosthetic group (ExPASy, 2012), and Pb reduces the absorption of iron in the gastrointestinal tract and inhibits the heme biosynthesis (Gurer and Ercal, 2000). Several authors have found inhibition of CAT activity in Pb-exposed animals (Sandhir and Gill, 1995; Sandhir et al., 1994).

4.2.2.4. Total glutathione (tGSH). Several studies have found a reduction in GSH concentrations in Pb-exposed rats (Korsrud and Meldrum, 1988) and birds (Mateo et al., 2003; Somashekaraiah et al., 1992). In the present study, we found a significant negative relationship between blood Pb concentrations and tGSH levels in RBC of Eurasian eagle owls from the mining area, which may be explained by GSH role in the excretion of this metal through Pb binding to GSH because of its affinity for sulfhydryl groups (Sharma et al., 2011).

4.2.2.5. Lipid peroxidation. An increase in lipid peroxidation after Pb (Hoffman et al., 2000a, 2000b; Mateo and Hoffman, 2001; Mateo et al., 2003; Somashekaraiah et al., 1992) and Hg exposure (Hoffman et al., 2005; Huang et al., 1996) has been found in birds and rats in several studies. As in the present study, Espín et al. (2014a) also found a correlation between Pb and Hg concentrations and TBARS levels in Griffon vultures. The correlations found are indicative of an effect of Hg and Pb on lipid peroxidation, particularly in the industrial and mining area. In this sense, several mechanisms may be responsible for lipid peroxidation by these metals. Both Hg and Pb may induce generation of ROS, associated with lipid peroxidation in membranes (Lund et al., 1991; Monteiro et al., 1989; Ribarov and Bochev, 1982; Verity et al., 1975). Moreover, as explained above, both metals can alter levels of GSH (Flora et al., 2008) and the activity of antioxidant enzymes (Gstraunthaler et al., 1983; Sandhir and Gill, 1995; Schrauzer, 1987; Zalups and Lash, 1996), which interfere in the protection against lipid peroxidation.

4.2.3. Correlations among oxidative stress biomarkers

The positive correlation found between GPx and CAT enzymes was most probably due to their collaboration in the decomposition of H_2O_2 (Halliwell and Gutteridge, 1999). We also found positive correlations between GST with GPx, and GST with CAT, which may be interpreted as a collaboration of GST with these enzymes since GST also removes H_2O_2 from the cells through the GSH oxidation (Koivula et al., 2011). The negative correlations found between CAT and SOD with TBARS suggest that these enzymes may play a role in the protection of the cell against the lipid peroxides, as has been suggested by other authors for GST enzyme (Isaksson et al., 2009). Moreover, one of the functions of GSH is the reduction of lipid peroxides by GPx (Kidd, 1997), which may explain the positive correlation found between tGSH and TBARS levels.

4.2.4. Relationship between oxidative stress biomarkers and size of the brood

Koivula et al. (2011) found a positive relationship between GST activity and the size of the brood. The higher GST activity in large broods in Great tit (*Parus major*) was explained by an increase within the brood competition for food and space (Koivula et al., 2011). This competition may cause increased oxidative stress among nestlings in larger broods (Koivula et al., 2011). Some studies have found increased oxidative stress in nestlings from larger broods in Common kestrels (*Falco tinnunculus*) and Common starlings (*S. vulgaris*) (Costantini et al., 2010, 2006). In the same manner, Common starlings nestlings raised in experimentally enlarged broods had lower total antioxidant capacity (Bourgeon et al., 2011). In the present study we found negative relationships between the number of nestlings in each nest and GPx or CAT activity (Fig. 5), showing the chicks from larger broods lower enzyme activities. In addition, we found that chicks from larger broods had higher total GSH levels (Fig. 5), probably as a response to the lower antioxidant enzyme activities. Our results suggest that 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.

5. Conclusions

The present study provides information about oxidative stress and blood concentrations of mercury, lead, cadmium, zinc and copper in Eurasian eagle owls related to the exposure to heavy metals in three different scenarios. Although individuals from the mining area had significant higher Pb and Hg concentrations, and significant lower GST and CAT activities; 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.

Despite the low levels of metals, several oxidative stress biomarkers were correlated with contaminant concentrations. Negative relationships between Cd and Pb levels and GPx or CAT activities were found. TBARS was positively correlated with Hg and, in the industrial and mining area, with Pb concentrations.

When only Eurasian eagle owls from the mining area were selected, a negative relationship between Pb concentrations and tGSH levels was found. Finally, results suggested a possible protective response in Eurasian eagle owls from the agricultural and rural area, since the low exposure levels of Hg resulted in increased activities of the antioxidant enzymes CAT and SOD.

Moreover, this study provides threshold concentrations at which metals cause effects on the antioxidant system in Eurasian eagle owls. Our findings show that blood lead concentrations lower than 20 μg/dl which is the threshold level commonly accepted for considering physiological effects in Falconiformes, may produce effects on oxidative stress biomarkers in Strigiformes. In this sense, blood Pb concentrations above 2 μg/dl produced an inhibition of GPx (8%) and CAT (10.5%) activities in RBC of Eurasian eagle owl, and a TBARS induction (10%) in individuals from both the industrial and the mining area. Moreover, a depletion of tGSH levels (4%) was associated with Pb concentrations higher than 3 μg/dl in individuals from the ancient mine site. In addition, we provide new data on Cd and Hg concentrations related to effects in the antioxidant system. Regarding Cd levels, blood concentrations greater than 0.3 μg/dl produced an inhibition of GPx (32%) and CAT (26%) activity in RBC of Eurasian eagle owls. Finally, blood Hg concentrations above 3 μg/dl resulted in a TBARS induction of 102% in individuals from the industrial area.

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