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# Effects of heavy metals on biomarkers for oxidative stress in Griffon vulture (*Gyps fulvus*)

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ABSTRACT

Metals are involved in the formation of reactive oxygen species (ROS) which may result in metal-related oxidative stress that can lead to oxidative damage to lipids, DNA and proteins. It is necessary to understand the mechanisms of metal toxicity in wild birds, and the concentrations that cause effects on oxidative stress biomarkers. The aim of this study is to assess the concentrations of lead (Pb), cadmium (Cd), mercury (Hg), copper (Cu) and zinc (Zn) with regards to oxidative stress in blood samples of 66 Griffon vultures (*Gyps fulvus*) from two areas of the Autonomous Community of Valencia (East of Spain). The two study areas (Alcoy $n=36$ and Cinctorres $n=30$) were selected as random locations of interest that had not yet been studied, and are feeding stations where supplementary food, mainly of pork origin, is provided for vultures. Given that the two study areas are not considered polluted sites, we expected to find low metal concentrations. However, there are no known threshold concentrations at which metals can affect antioxidant systems, and low metal levels may have an effect on antioxidant biomolecules. In this study, since sampling was done at the beginning of the hunting season, the low Pb levels found in most Griffon vultures from Alcoy and Cinctorres (median = 12.37 and 16.26 μg/dl, respectively) are suggestive of background levels usually found in vultures that feed on pork carcasses all year round. The ingestion of game meat with bullet fragments in carcasses or with Pb shots embedded in the flesh could be the cause of the high blood Pb concentrations found in three vultures from Cinctorres (83, 290 and 362 μg/dl). Griffon vultures feeding in Cinctorres had enhanced CAT and GST activities and tGSH concentrations, which may be interpreted as protective response against the higher TBARS levels. This study provides threshold concentrations at which metals affect antioxidant system derived from 66 samples of Griffon vulture. Blood Cd concentrations greater than 0.05 μg/dl produced an induction of 33% in GPx and of 44% in CAT activity in erythrocytes of vultures from Alcoy. Hg concentrations in blood higher than 3 μg/dl produced an induction of 10% in SOD activity. Concentrations of Pb above 15 μg/dl in blood produced an inhibition of 12.5% in GPx and 11.3% in CAT activity, and a TBARS induction of 10.7% in erythrocytes of Griffon vultures.



## 1. Introduction

The presence of some metals in the environment is mainly caused by human activity, and their ubiquity, persistence and accumulation in organisms implies that living beings are continuously exposed to them (García-Fernández et al., 2005a). Although several essential metals play a crucial role in the normal biological functioning of cells (Flora et al., 2008), several reports have been published on metals that induce toxicity in birds, altering their reproductive success, behaviour, immune response, and biochemical processes (Frederick and Jayasena, 2010; Mateo et al., 2003a; Snoeijs et al., 2004). It has been suggested that one of the mechanisms involved in metal toxicity is the induction of reactive oxygen species (ROS) by these elements (Ercal et al., 2001; Stohs and Bagchi, 1993), highly reactive oxygen-containing molecules produced in oxidation–reduction reactions (Dowling and Simmons, 2009). This ROS formation results in metal-related oxidative stress, a state of imbalance between antioxidant defence and ROS production, so that the defence is overcome by radical formation (Halliwell and Gutteridge, 2007). An excess of radicals can cause oxidative damage to membrane lipids, DNA and proteins, and their oxidation may ultimately lead to cellular dysfunction and tissue injury (Hoffman et al., 1998; Valavanidis et al., 2006).

Since experimental studies have shown metal induced oxidative stress in waterfowl species (Mateo and Hoffman, 2001; Mateo et al., 2003a), the levels of antioxidant molecules and activities of

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antioxidant enzymes could be interesting biomarkers of the metal exposure and effect on birds. Nevertheless, there are differences in metal tolerance among waterfowl and raptor species (Hernández-García, 2010). García-Fernández et al. (2008) found very high blood lead levels in healthy Griffon vultures (*Gyps fulvus*), suggesting that this species may be more resistant to lead effects. The Griffon vulture is a large bird of prey from the Accipitridae family. It belongs to the Old World vultures. It is a scavenger that feeds mostly on carcasses of dead domestic livestock and, to a lesser extent, on wild species found dead in the field (Donázar, 1993). The world population of Griffon vultures extends from North Africa, through several South European countries, to Central Asia; and a significant population is concentrated in Spain (Del Moral, 2009). This species is considered sedentary across most of its breeding area, except for the young and immature birds that often disperse or migrate from north to south (Ferguson-Lees and Christie, 2001).

The measurements of metal concentration in blood is a good indicator of recent exposure, and there are some published papers about metal concentrations in vultures (Gangoso et al., 2009; García-Fernández et al., 2005a; Hernández and Margalida, 2009; Shlosberg et al., 2012). However, 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; Custer et al., 2006; Hoffman et al., 1998, 2009, 2011; Koivula et al., 2011; Martínez-Haro et al., 2011), and the differences among various bird species are still unclear (Koivula and Eeva, 2010). Thus, it is necessary to understand the mechanisms of metal toxicity in wild birds, and the concentrations that cause effects on oxidative stress biomarkers. The aim of this study was to assess the concentrations of lead (Pb), cadmium (Cd), mercury (Hg), copper (Cu) and zinc (Zn) with regards to oxidative stress in blood samples obtained from two populations of Griffon vultures. We selected two different areas that serve as two feeding station in the Autonomous Community of Valencia, because they have not yet been studied. Given that the two study areas are not considered polluted sites, we expected to find low metal concentrations. However, there are no known threshold concentrations at which metals can affect antioxidant systems, and low metal levels may have an effect on antioxidant biomolecules. Multiple mechanisms may be responsible for the metal-induced oxidative stress: direct or indirect generation of ROS, depletion of glutathione (GSH) and other thiol-containing antioxidants; and inhibition of antioxidant enzymes are well-known for all redox active (iron and copper) and inactive (lead, cadmium and mercury) metals (Ercal et al., 2001; Koivula and Eeva, 2010). The tripeptide GSH (L-gamma-glutamyl-L-cysteinylglycine) is one of the most abundant sulfhydryl (SH)-containing compounds in most organisms, and plays an important role in binding with ROS and in eliminating metals (Klaassen et al., 1985). The antioxidant enzymes catalyse the breakdown of free radicals (glutathione peroxidase, GPx; superoxide dismutase, SOD; catalase, CAT) and indirectly support the antioxidant defence system by catalysing the conjugation of pollutants with GSH (glutathione-S-transferase, GST) (Gurer and Ercal, 2000). Because several antioxidants are needed to protect against ROS and antioxidant defence may respond differently depending on species, it is essential to use several biomarkers to detect oxidative stress (Berglund et al., 2007; Halliwell and Gutteridge, 1999; Koivula and Eeva, 2010). In order to infer on oxidative stress, it is necessary to measure an antioxidant capacity biomarker and at least an oxidative damage biomarker (Costantini and Verhulst, 2009). We analysed a battery of biomarkers including total GSH content, antioxidant enzymes activities (GPx, SOD, CAT, GST) and lipid peroxidation to evaluate the potential effects that these metals bear on Griffon vulture oxidative stress biomarkers.

## 2. Material and methods

### 2.1. Species and study area

Sixty-six Griffon vultures were caught in baited cage traps at two different feeding stations located in the Community of Valencia, in the East of Spain (Fig. 1). These two feeding stations are places where supplementary food of mainly pork origin from agricultural sources, is provided for vultures. The two study areas were selected as random locations of interest that have not yet been studied. The first sampling was conducted at the feeding station of Cinctorres ($n=30$), in the province of Castellón (40°35′N, 0°12′W), on 27th September and 3rd October 2011. The vulture population has grown in Castellón since 1972, when only three pairs were found. In 2008, 236 breeding pairs were found in this area (93% of the breeding pairs in the Community of Valencia) (Del Moral, 2009). In Cinctorres, food is only provided for approximately 6 weeks every year, for the trapping. However, there are other feeding stations in Castellón (Zorita del Maestrazgo and Vallibona) where food is provided once a week throughout the year. The second sampling was conducted at the feeding station of Alcoy ($n=36$) in the province of Alicante (38°42′N, 0°28′W) on 13th November 2011, where food is provided normally once a week throughout the year. Griffon vultures have been breeding in Alicante since 2005 as a result of a reintroduction programme conducted by the FAPAS-Alcoi NGO in 2000 (Projecte Canyet) (Del Moral, 2009). In 2008, 19 breeding pairs were found in the north of Alicante (Del Moral, 2009). We assume a similar diet composition in both areas (Cinctorres and Alcoy). The individuals sampled in both areas were adults.

### 2.2. Sampling method

Blood samples were collected by puncturing brachial veins with 23G needle and syringe, and stored in heparinised Eppendorf tubes under refrigerated conditions until processed in the laboratory. One tube with whole blood was separated and another tube with blood was centrifuged at 10000 rpm for 5 min to separate plasma and red blood cell (RBC) fractions. Plasma was separated in a new tube and RBC samples were washed with saline solution and centrifuged again at 10000 rpm for 5 min. Hematocrit was recorded using capillary tube reader after centrifugation at 5000 rpm for 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, 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 the 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 analysed were alkaline phosphatase (ALP; Enzyme Commission (EC) number 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 analysed were albumin, total protein, cholesterol, glucose, triglycerides, uric acid, calcium and phosphorus.


**Fig. 1.** Map showing the geographical location of the areas studied, Cinctorres (Castellón) and Alcoy (Alicante), in the Autonomous Community of Valencia (Spain).

### 2.3. Blood metals analysis

Total mercury was analysed in a Milestone DMA-80 direct Hg analyser by atomic absorption spectrophotometry following the method described by Espín et al. (2012). Blood samples (100 μl wet weight) were loaded in a nickel boat and analysed. Calibration curve was done with ten points (in duplicate) from 0 to 1004 ng of Hg. Precision and accuracy of the method were tested using 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 107.06 ± 13.23% (mean ± standard deviation). The coefficient of variation for repeatability was 12.36%. The detection limit was 0.005 ng.

Cd, Pb, Cu and Zn levels were analysed in blood samples following the method described by García-Fernández et al. (1995), in which a volume of 200 μl of whole blood was placed in a quartz digestion tube, and 0.5 ml of acid mixture (nitric: perchloric:sulfuric, 8:8:1) added. The sample was then submitted to a progressive thermal treatment and, once dried, was left to cool. Tetra-distilled purified water was added and transferred to the measuring vessel, adjusting the final volume to 10 ml. Then 50 μl of hydrochloric acid was added to the measuring vessel as an electrolyte support, prior to anodic stripping voltammetry (ASV). The pH of the final solution was between 1 and 2. The anodic stripping voltammeter (VA-757 Computrace Workstation, Methrohm, 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 electrolytic time of 120 s and modulation amplitude of 50 mV. We calculated each metal concentration in the digested sample after twice adding dilutions prepared from standard solutions of Cd, Pb, Cu and Zn, respectively (Sigma, St. Louis, MO). We calculated the mean recoveries, which approached 96%, analysing 10 identical samples of reconstituted lyophilised blood (European Union Reference Standards CRM195) (García-Fernández, 1994). 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. The reagents used were all of 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.

### 2.4. Biomarker analyses in red blood cells (RBC)

We analysed several oxidative stress parameters (total glutathione, glutathione peroxidase, superoxide dismutase, catalase, glutathione-S-transferase and thiobarbituric acid-reactive substances) in RBC, after homogenisation (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). We obtained levels of total glutathione (tGSH) 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 with the Ransel and Ransod kits (Randox Laboratories), respectively, using spectrophotometer (A25-Autoanalyzer, BioSystems), following descriptions of Reglero et al. (2009) with some modifications for RBC. To determine the GPx and SOD respectively, we diluted homogenised samples at 1:20 and 1:25 (v:v) with Ransel diluting agent and Ransod sample diluents (Randox Laboratories). The GPx and SOD results were expressed as Units per gram protein.

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 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 GSH. This reaction is catalysed 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 result was expressed as nanomoles produced per minute per milligram of protein.

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

### 2.5. Statistical analysis

We used the SPSS v.15.0 statistical package for all analyses. Reported metal concentrations and oxidative stress biomarker values represent the mean ± standard deviation, median and range. We tested the data for normality using a Kolmogorov–Smirnov test and when necessary, data were normalised using log-transformation. We used ANOVA to calculate the differences in metal concentrations and biomarker values between areas. Correlations between metals and 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 and with pooled data of all individuals. 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 analysed. We performed Generalised linear models (GLMs) with normal distribution and identity function to study combined effects of metals and their interactions on the biomarkers. The biomarker value was the response variable, metal concentrations were selected as covariates and studied areas were selected as factors (when the two populations were pooled together). 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 the 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

Table 1 shows metal concentrations in blood samples, and enzyme activities, glutathione levels and lipid peroxidation in red blood cells of Griffon vulture. Table 2 shows a review of metal concentrations in tissues of vultures from the Accipitridae family. Blood Pb concentrations in Griffon vultures (mean ± SD) were 15.3 ± 8.3 μg/dl wet weight in Alcoy ($n = 36$) and 41.4 ± 79.5 μg/dl in Cinctorres ($n = 30$) (Table 1). Mean Pb levels were significantly higher in vultures from Cinctorres ($p < 0.01$) (Table 1). Regarding Hg, the levels were 2.3 ± 2.2 μg/dl in Alcoy and 1.7 ± 1.4 μg/dl in Cinctorres (Table 1). Cd concentrations in blood were 0.018 ± 0.027 and 0.025 ± 0.043 μg/dl in Alcoy and Cinctorres, respectively. Finally, concentrations of the essential elements in the bloods of Griffon vultures were 332.2 ± 65.4 and 347.1 ± 80.9 μg/dl for Zn, and 20.4 ± 5.9 and 26.8 ± 23.1 μg/dl for Cu, in Alcoy and Cinctorres, respectively.

### 3.2. Oxidative stress biomarkers

Regarding the antioxidant biomarkers, Griffon vultures from Cinctorres displayed significantly higher CAT and GST activities ($p < 0.001$), and higher concentrations of tGSH and TBARS in red blood cells ($p < 0.001$) than vultures from Alcoy (Table 1). However, there were no significant differences in GPx and SOD activities between vulture populations (Table 1).

Table 3 shows the simple linear regression analysis that was conducted to check for relationships between single metal concentrations in bloods of Griffon vultures and biomarker response. In order to increase the number of samples, we also conducted linear regression with a pool of both vulture populations. Simple linear regression analysis showed that several metals were related to GPx, SOD, CAT, tGSH and TBARS. We also developed Generalised Linear Models (GLMs) (Table 4) to evaluate combined effects of metals and their interactions on the biomarkers. Biomarkers were related to one or to several metals, and some of them were affected by area when all individuals were pooled together. Pearson correlations were also conducted among oxidative stress biomarkers in Griffon vultures (Table 5), and we found that some biomarkers were correlated with each other (tGSH–GPx, GPx–SOD, GPx–CAT, CAT–SOD, SOD–GST, CAT–GST, CAT–TBARS, GST–TBARS, tGSH–TBARS) (Table 5).

Almost all the relationships found between metal concentrations and oxidative stress biomarker responses were found in vultures from Alcoy or when all samples were pooled together.

**Table 1**
Metal concentration in blood samples (μg/dl), enzyme activities, glutathione levels and lipid peroxidation in red blood cells of Griffon vulture.

| **Metal concentrations in Griffon vulture blood samples (μg/dl)** | | | | | | |
|---|---|---|---|---|---|---|
| **Metal** | **Alcoy, Alicante** | | | **Cinctorres, Castellón** | | |
| | *n* | **Mean ± SD** | Median (range) | *n* | **Mean ± SD** | Median (range) |
| Cd | 36 | **0.018 ± 0.027** | 0.006 (0.006–0.1610) | 30 | **0.025 ± 0.043** | 0.006 (0.006–0.217) |
| Pb | 36 | **15.32 ± 8.28**** | 12.37 (7.03–45.61) | 30 | **41.44 ± 79.50** | 16.26 (9.31–362.13) |
| Cu | 36 | **20.39 ± 5.92** | 19.31 (13.77–44.41) | 30 | **26.85 ± 23.11** | 19.58 (9.89–134.70) |
| Zn | 36 | **332.16 ± 65.40** | 327.48 (248.55–629.82) | 30 | **347.12 ± 80.89** | 351.22 (146.57–497.95) |
| Hg | 36 | **2.27 ± 2.24** | 1.53 (0.55–10.03) | 30 | **1.72 ± 1.35** | 1.17 (0.62–6.75) |
| **Enzyme activities, glutathione levels and lipid peroxidation in red blood cells of Griffon vultures** | | | | | | |
| **Biomarker** | **Alcoy, Alicante** | | | **Cinctorres, Castellón** | | |
| | *n* | **Mean ± SD** | Median (range) | *n* | **Mean ± SD** | Median (range) |
| GPx[a] | 36 | **415.56 ± 91.45** | 408.22 (246.57–626.86) | 29 | **483.09 ± 239.80** | 425.88 (217.60–1247.78) |
| SOD[b] | 36 | **929.92 ± 202.78** | 911.26 (588.39–1445.87) | 30 | **827.72 ± 314.55** | 782.99 (411.48–2058.73) |
| CAT[c] | 36 | **0.55 ± 0.15*** | 0.55 (0.18–0.84) | 30 | **1.18 ± 0.39** | 1.12 (0.60–2.13) |
| GST[d] | 36 | **7.26 ± 1.59*** | 7.07 (4.67–10.82) | 29 | **9.79 ± 2.24** | 9.37 (6.73–16.41) |
| tGSH[e] | 36 | **4.56 ± 0.99*** | 4.49 (1.86–7.05) | 30 | **5.42 ± 0.93** | 5.24 (4.11–7.16) |
| TBARS[f] | 36 | **0.0338 ± 0.0074*** | 0.032 (0.024–0.052) | 30 | **0.0499 ± 0.0038** | 0.0499 (0.0438–0.0565) |

* *Note*: Significant differences between areas: $p < 0.001$.
** *Note*: Significant differences between areas: $p < 0.01$.
[a] 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).

**Table 2**
Literature on metal concentrations in blood (μg/dl) and other tissues (μg/g dry weight) of vultures (Accipitridae family).

| **Species[a]** | **Mean metal concentrations in blood (μg/dl) and tissues (μg/g dry weight)[b]** | **Observations** | **Sampling area** | **Year** | **Reference** |
|---|---|---|---|---|---|
| G fu | B($n$=36): Pb=15.32, Cd=0.018, Cu=20.39, Zn=332.16, Hg=2.27<br>B($n$=30): Pb=41.44, Cd=0.025, Cu=26.85, Zn=347.12, Hg=1.72 | Alcoy<br>Cinctorres | Spain | 2011 | Present study |
| G fu | B($n$=23): Pb=43.07 | Outside hunting season | Spain | 2003 | García-Fernández et al. (2005a) |
| G fu | B($n$=26): Pb=123 | During hunting season | Spain | 2006 | García-Fernández et al. (2008) |
| G fu | B($n$=6): Pb=37.9, Cd=0.11 | | Spain | 1993 | García-Fernández et al. (1995) |
| G fu | B($n$=9): Pb=10.4, As= < 1, Cd= < 1, Hg= < 2, Se=46.2<br>B($n$=7): Pb=14.2, As= < 1, Cd= < 1, Hg=1.2, Se=36.5<br>B($n$=9): Pb=8.4, As= < 1, Cd= < 1, Hg=1.3, Se=42.4 | Juvenile<br>Subadult<br>Adult | Israel | 2007 | Shlosberg et al. (2012) |
| G fu | BN($n$=4): Pb=5.54*, As=18** | | Spain | 1998–2001 | Mateo et al. (2003b) |
| G fu | BN($n$=20): Pb=9.30<br>F($n$=20): Pb=0.208(rachis), 1.919(barbs), Al= < LOD[c](rachis), Al=1978 (barbs) | | Spain | – | Cardiel et al. (2011) |
| G ba | B($n$=40): Pb=2.33*, BN($n$=12): Pb=1.46*, L($n$=10): Pb=0.57*<br>B($n$=15): Pb=4.56*, BN($n$=3): Pb=2.71*, L($n$=3): Pb=0.83*<br>B($n$=20): Pb=4.20*, BN($n$=11): Pb=2.87*, L($n$=5): Pb=1.15*<br>B($n$=26): Pb=5.45*, BN($n$=17): Pb=3.16*, L($n$=12): Pb=1.36* | Fledgling<br>Juvenile<br>Subadult<br>Adult | Spain and France | 2008 | Hernández and Margalida (2009) |
| N pe | B($n$=32): Pb=0.56–21.73 range, BN($n$=11): Pb=6.17*<br>B($n$=137): Pb=0.51–178 range, BN($n$=28): Pb=7.42*<br>B: Pb (During hunting season, $n$=47)=9.33*<br>B: Pb (Outside hunting season, $n$=90)=2.88* | Iberian Peninsula<br>Canary Islands<br>Canary Islands<br>Canary Islands | Spain | 1999–2005 | Gangoso et al. (2009) |
| N pe | B ($n$=26): Pb=14.6, Cd=0.113, Cu ($n$=11)=0.047, Zn=361.5 | | Spain | 1998–2001 | Donázar et al. (2002) |
| A mo | BN($n$=3): Pb=11.13<br>F($n$=3): Pb= < LOD[c](rachis), Pb=0.265(barbs), Al= < LOD[c](rachis), Al= < LOD[c](barbs) | | Spain | – | Cardiel et al. (2011) |
| G fu | F($n$=3): Hg=0.93 (Secondary feather), Hg=1.16 (Tail feather) | | Iran | 2005 | Zolfaghari et al. (2007) |

[a] G fu=Gyps fulvus, Griffon vulture; A mo=Aegypius monachus, Cinereous vulture; G ba=Gypaetus barbatus, Pyrenean bearded vulture; N pe=Neophron percnopterus, Egyptian Vulture.
[b] B=Blood, BN=Bone, L=Liver, F=Feathers.
[c] LOD=Limit of detection.
* Geometric mean.
** Median.

However, in Griffon vultures from Cinctorres only two significant relationships were found (Tables 3 and 4).

### 3.2.1. Glutathione peroxidase (GPx)

In vultures from Alcoy, GPx activity was directly correlated with blood Cd and Cu concentrations (Table 3). Concentrations of Cd ≥ 0.05 μg/dl in blood produced an induction of 33.3% in GPx activity. Besides, in vultures from Cinctorres, GPx activity was inversely related with blood Pb concentrations (Table 3, Fig. 2). When both populations were pooled together, GPx was inversely correlated with Pb concentrations (Table 3), and the best-fitting GLM for GPx activity only included an effect of Pb (Table 4). Concentrations of Pb ≥ 15 μg/dl in blood produced an inhibition of 12.5% in GPx activity, and Pb levels ≥ 25 μg/dl produced an inhibition of 16.7% in GPx activity in Griffon vultures. When only individuals from Cinctorres were selected, the inhibition of GPx was 18.3% and 25.2% with Pb concentrations ≥ 15 and 25 μg/dl, respectively (Fig. 2).

### 3.2.2. Superoxide dismutase (SOD)

As single metals, both the levels of Hg and Cu showed significant positive relationship with SOD activity in Griffon vultures from Alcoy (Table 3), a population with higher, though not significant, Hg concentrations (Table 1). When GLMs were performed both Hg and Cu showed up in the top-ranked model (Table 4), and SOD activity seemed to depend mostly on Hg

**Table 3**
Linear regression analysis of biomarker response on single metal concentrations in Griffon vulture.

| Biomarker response (Y)[a] | Metal (X) | Intercept (*a*) | Regression coefficient (*b*) | *F* | *p* | *r* | *n* |
|---|---|---|---|---|---|---|---|
| Alcoy, Alicante | | | | | | | |
| GPx | LogCd | 588.66 | 88.56 | 5.39 | 0.026 | 0.370 | 36 |
| GPx | Cu | 298.82 | 5.727 | 5.42 | 0.026 | 0.371 | 36 |
| SOD | Cu | 652.68 | 13.6 | 6.36 | 0.016 | 0.397 | 36 |
| SOD | LogHg | 874.13 | 256.538 | 7.02 | 0.012 | 0.414 | 36 |
| CAT | LogCd | 0.8 | 0.129 | 4.34 | 0.045 | 0.336 | 36 |
| CAT | Cu | 0.343 | 0.01 | 6.58 | 0.015 | 0.403 | 36 |
| CAT | LogPb | 0.818 | −0.238 | 3.97 | 0.055 | −0.323 | 36 |
| TBARS | Zn | 0.013 | 6.14E-05 | 14.08 | 0.001 | 0.541 | 36 |
| Cinctorres, Castellón | | | | | | | |
| LogGPx | LogPb | 2.908 | −0.2 | 5.27 | 0.03 | −0.404 | 29 |
| CAT | Zn | 0.531 | 0.002 | 4.62 | 0.04 | 0.376 | 30 |
| All individuals | | | | | | | |
| LogGPx | LogPb | 2.798 | −0.143 | 6.49 | 0.013 | −0.306 | 65 |
| SOD | LogHg | 832.008 | 279.818 | 7.34 | 0.009 | 0.321 | 66 |
| TBARS | Zn | 0.027 | 4.28E-05 | 6.71 | 0.012 | 0.308 | 66 |
| TBARS | LogPb | 0.027 | 0.011 | 9.19 | 0.004 | 0.354 | 66 |
| tGSH | LogCu | 2.993 | 1.481 | 3.92 | 0.052 | 0.240 | 66 |

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, tGSH=Total Glutathione, TBARS=Thiobarbituric acid-reactive substances.

**Table 5**
Pearson correlations for oxidative stress biomarkers from Griffon vultures.

| Parameters[a] | Alcoy, Alicante | | | Cinctorres, Castellón | | | All individuals | | |
|---|---|---|---|---|---|---|---|---|---|
| | *n* | *r* | *p* | *n* | *r* | *p* | *n* | *r* | *p* |
| *Pearson correlation for oxidative stress biomarkers* | | | | | | | | | |
| GPx–SOD | 36 | 0.650 | 0.001 | 29 | 0.370 | 0.047 | 65 | 0.390 | 0.001 |
| GPx–CAT | 36 | 0.600 | 0.001 | 29 | 0.470 | 0.011 | 65 | 0.360 | 0.004 |
| GPx–tGSH | | | | 29 | 0.510 | 0.004 | 65 | 0.390 | 0.001 |
| SOD–CAT | 36 | 0.410 | 0.013 | 30 | 0.330 | 0.078 | | | |
| SOD–GST | | | | 29 | 0.510 | 0.005 | | | |
| SOD–tGSH | | | | 30 | 0.350 | 0.060 | | | |
| SOD–TBARS | | | | 30 | −0.350 | 0.058 | | | |
| CAT–GST | | | | 29 | 0.360 | 0.052 | 65 | 0.570 | 0.001 |
| CAT–tGSH | | | | 30 | 0.350 | 0.056 | 66 | 0.450 | 0.001 |
| CAT–TBARS | | | | | | | 66 | 0.570 | 0.001 |
| GST–TBARS | | | | | | | 65 | 0.420 | 0.001 |
| tGSH–TBARS | | | | | | | 66 | 0.290 | 0.020 |

*Note*: $n$=number of samples, $r$=Pearson's correlation coefficient, $p$=significance.

[a] GPx=Glutathione peroxidase, SOD=Superoxide dismutase, CAT=Catalase, GST=Glutathione-S-Transferase, tGSH=Total Glutathione, TBARS=Thiobarbituric acid-reactive substances.

**Table 4**
Generalised linear models (GLMs) evaluating combined effects of metals and their interactions on the oxidative stress biomarkers response.

| Biomarker response[a] | Model[b] | AIC[c] | ΔAIC[d] | Akaike weight[e] | $X^2$ | *p* | *n* |
|---|---|---|---|---|---|---|---|
| Alcoy, Alicante | | | | | | | |
| GPx | Cu (0.017) | 426.96 | 0.00 | 0.36 | 5.32 | 0.021 | 36 |
| SOD | Cu (0.011)+LogHg (0.008) | 478.92 | 4.30 | 0.05 | 12.70 | 0.002 | 36 |
| CAT | Cu (0.008) | −37.25 | 0.49 | 0.20 | 6.37 | 0.012 | 36 |
| tGSH | LogCd (<0.001)+LogPb (0.004)+Cu (0.004)+LogCd × Cu (0.003) | 96.12 | 0.00 | 0.60 | 16.32 | 0.003 | 36 |
| TBARS | LogPb (0.002)+Zn (<0.001)+LogHg (0.037) | −265.11 | 0.00 | 0.50 | 23.22 | <0.001 | 36 |
| Cinctorres, Castellón | | | | | | | |
| LogGPx | LogPb (0.017) | −13.35 | 0.00 | 0.47 | 5.17 | 0.023 | 29 |
| CAT | Zn (0.026) | 30.45 | 3.41 | 0.06 | 4.58 | 0.032 | 30 |
| All individuals | | | | | | | |
| LogGPx | LogPb (0.010) | −66.06 | 2.65 | 0.10 | 6.37 | 0.012 | 65 |
| SOD | LogHg (0.006) | 920.41 | 0.00 | 0.36 | 7.16 | 0.007 | 66 |
| CAT | Area (<0.001)+LogPb (0.048) | 26.03 | 0.11 | 0.27 | 55.96 | <0.001 | 66 |
| GST | Area (<0.001) | 272.36 | 0.00 | 0.40 | 24.05 | <0.001 | 66 |
| tGSH | Area (<0.001) | 186.31 | 0.36 | 0.31 | 12.39 | <0.001 | 66 |
| TBARS | Area (<0.001)+LogPb (0.039)+LogHg (0.046)+Zn (<0.001) | −494.39 | 0.45 | 0.32 | 86.02 | <0.001 | 66 |

The model with the greatest AIC weight and lowest AIC value indicates the closest to unknown reality. $n$=number of samples.

[a] GPx=Glutathione peroxidase, SOD=Superoxide dismutase, CAT=Catalase, GST=Glutathione-S-Transferase, tGSH=Total Glutathione, 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] Akaike weight is the likelihood that a given model is the best among all candidate models.

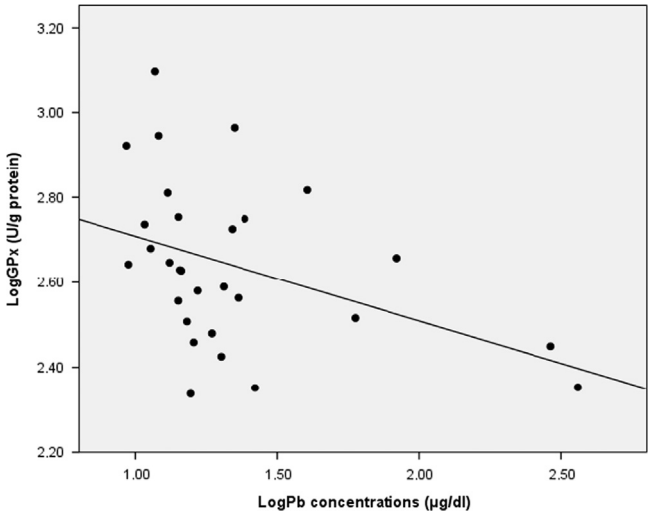


**Fig. 2.** Effect of blood lead concentrations on GPx activity in Griffon vultures from Cinctorres ($r=-0.404$, $p=0.03$, $n=29$).

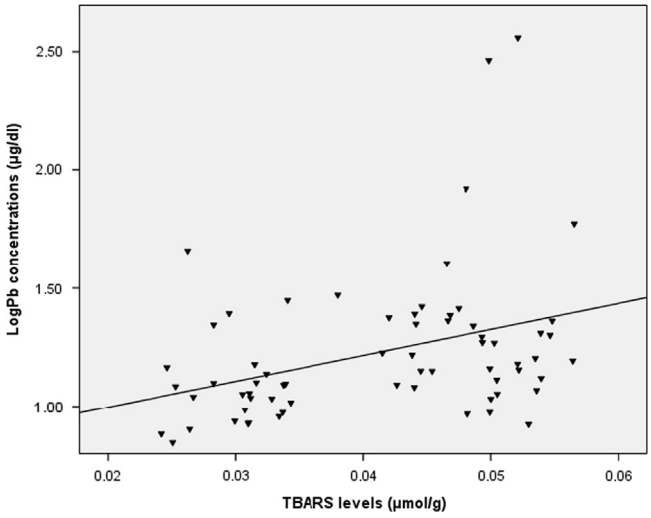


**Fig. 3.** Effect of blood lead concentrations on TBARS levels in Griffon vultures ($r=0.354$, $p=0.004$, $n=66$).

concentrations when all individuals were pooled together (Tables 3 and 4). Hg concentrations in blood ≥ 3 µg/dl produced an induction of 10% in SOD activity.

### 3.2.3. Catalase (CAT)

CAT activity was positively correlated with Cd and Cu concentrations in blood of vultures from Alcoy (Table 3). In this regard, Cd concentrations ≥ 0.05 µg/dl in blood induced CAT activity of 44%. In addition, we noticed an almost significant negative relationship between Pb and CAT activity in vultures from Alcoy (Table 3). When all individuals were pooled together, the top-ranked model for CAT activity included area and Pb concentrations effect (Table 4). Concentrations of Pb ≥ 15 µg/dl in blood produced an inhibition of 11.3% in CAT activity, while Pb levels ≥ 20 µg/dl resulted in a CAT inhibition of 15.8%.

### 3.2.4. Lipid peroxidation

The positive relationship of TBARS concentrations with the levels of Pb and Zn was significant when all individuals were pooled together (Table 3, Fig. 3). The best-fitting model for TBARS concentrations was constructed with Pb, Zn and Hg concentrations as covariates in vultures from Alcoy, and the same variables including area when all individuals where pooled together (Table 4). Pb concentrations ≥ 15 µg/dl produced a TBARS induction of 10.7%, while Pb levels ≥ 30 µg/dl produced an induction of 13.4% in red blood cells of Griffon vultures.

## 4. Discussion

In general, the plasma biochemistry parameters were similar to those described by several authors (Dobado-Berrios et al., 1998; Polo et al., 1992; Villegas et al., 2002) as baseline data in Egyptian vultures (*Neophron percnopterus*), Griffon vultures and Black vultures (*Aegypius monachus*), and are indicative of normal health.

### 4.1. Metal concentrations in blood

Mean blood Pb concentrations in Griffon vultures from Alcoy were similar to those in Griffon vultures from Israel and Egyptian vultures from Canary Islands (Donázar et al., 2002; Shlosberg et al., 2012), but higher than those in Pyrenean bearded vultures (*Gypaetus barbatus*) from the Iberian Peninsula and France (Gangoso et al., 2009; Hernández and Margalida, 2009) (Table 2). Regarding mean blood Pb concentrations in vultures from Cinctorres, they were significantly higher than those found in Alcoy in this study and by other authors in vultures from different areas (Gangoso et al., 2009; Hernández and Margalida, 2009; Shlosberg et al., 2012), but similar to those in Griffon vultures from Cazorla Natural Park (Southern Spain) outside hunting season and from Murcia, Southeast Spain (García-Fernández et al., 1995, 2005a) (Table 2). However, Pb concentrations in both populations were lower than those in Griffon vultures from Cazorla Natural Park during hunting season (García-Fernández et al., 2008) (Table 2).

Since the sampling in this study was done in September–November at the beginning of the hunting season in the Community of Valencia (Order 1, 2011), and the concentrations detected were much lower than those in Griffon vultures sampled during the hunting season (García-Fernández et al., 2008) (Table 2), Pb levels in the most Griffon vultures from Alcoy and Cinctorres (median levels of 12.37 and 16.26 µg/dl, respectively) may be normal or background levels, in vultures feeding on carcasses of pork origin all year round. Although we did not analyse the metal content in the food, the pork carcasses are from agricultural sources and therefore the Pb levels are expected to be low in the carcasses in accordance with the legislation (0.1 mg/kg wet weight for meat and 0.5 mg/kg wet weight for offal of pig; EU, 2006). However, Pb concentrations were high in three individuals from Cinctorres (83, 290 and 362 µg/dl). The blood levels of Pb found in these three vultures probably indicate recent exposure to large amounts of this metal (García-Fernández et al., 1995), and can be related to the ingestion of the metallic form of Pb (García-Fernández et al., 2008; Hoffman et al., 1981). Several authors have demonstrated that Pb ammunition can produce hundreds of small fragments that contaminate animal carcasses and discarded viscera that serve as food for scavengers (Hunt et al., 2006; Knopper et al., 2006). In Spain, the use of Pb shots are only banned in wetlands included on Ramsar's List due to the risk entailed for waterfowl (Royal Decree 581, 2001). However, Pb ammunition is still used in big- and small-game hunting. In the Community of Valencia, big-game hunting is allowed and regulated for Iberian wild goat (*Capra pyrenaica*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), wild boar (*Sus scrofa*), mouflon (*Ovis aries*) and barbary sheep (*Ammotragus lervia*) (Order 1, 2011). There are also many species allowed for small-game hunting such as partridge (*Alectoris rufa*), European

rabbit (*Oryctolagus cuniculus*), red fox (*Vulpes vulpes*) and several pigeon species (*Columba sp.*) (Order 1, 2011). Although the diet of the Griffon vultures in this study is mainly based on pork carcasses provided at feeding stations, they can also feed from dead wild species found in the field (Donázar, 1993). Therefore, it is probable that they ingest game meat with bullet fragments in carcasses or with Pb shots embedded in their flesh. This may be the major cause of the blood Pb concentrations in the three individuals from Cinctorres with high blood Pb concentrations.

The Pb levels of 27 vultures from Alcoy (75% of total population studied) and 18 vultures from Cinctorres (60% of total population studied) measured below the 20 μg/dl mentioned by Franson (1996) as the minimum blood Pb level necessary in Falconiformes for considering physiological effects. Besides, the Pb concentrations of 9 individuals from Alcoy (25% of total population) and 8 from Cinctorres (27% of total population) measured between the 20 and 50 μg/dl considered as the threshold value for physiological effects (Franson, 1996). Finally, the Pb levels of 4 individuals from Cinctorres (13% of total population) measured above 50 μg/dl, with 2 vultures (290.48 and 362.13 μg/dl) presenting concentrations higher than those considered by Franson (1996) as threshold value in individuals with probable clinical symptoms (100 μg/dl). In spite of the threshold of 20 μg/dl set by Franson (1996), more recent studies have found that Pb concentrations below 15 μg/dl in blood can cause sublethal effects such as inhibition of δ-aminolevulinic acid dehydratase (ALAD) activity in raptors and waterbirds in the field (Gómez-Ramírez et al., 2011; Martínez-Haro et al., 2011; Martínez-López et al., 2004). Therefore, judging from the concentrations found in this study, some individuals could be susceptible to suffer sublethal effects due to Pb exposure.

Regarding Hg, few papers have studied the concentrations of this metal in blood of terrestrial birds of prey (Espín, 2013; Shlosberg et al., 2012) mainly due to the methylation and bioaccumulation of methylmercury in the aquatic systems. Only one report has documented Hg concentrations in blood of a vulture species (Shlosberg et al., 2012), with concentrations slightly lower than those found in this study (Tables 1 and 2). Hg concentrations in blood of Griffon vultures in this study were similar to those in nestlings Eagle owls (*Bubo bubo*) from Southeastern Spain (Espín, 2013), and seem to be too low to cause any adverse effects on vultures. In fact, levels of Hg in blood considered as no-observed adverse effect level in adult Common loon (*Gavia immer*) are two orders of magnitude larger (1 μg/ml) (Evers et al., 2004).

With regards to Cd concentrations in blood of Griffon vultures in this study, levels were lower than those found in Egyptian vultures by Donázar et al. (2002) and in Griffon vultures by García-Fernández et al. (1995) (Tables 1 and 2). Levels that may produce sublethal effects are unknown for Cd, but concentrations were below those found in raptors from unpolluted zones (0.1 μg/dl) (García-Fernández et al., 1995).

Finally, in this study, Zn concentrations in blood of Griffon vultures were similar to those found in Egyptian vultures by Donázar et al. (2002), although Cu levels were higher than those published by these authors. There are no commonly accepted toxicity thresholds for sublethal effects for Cu and Zn in the blood of birds, but concentrations found in this study are in the range of Cu and Zn levels for healthy birds (García-Fernández et al., 2005b) and seem to be too low to produce any adverse effects on vultures. Therefore, Zn and Cu concentrations found in this study could be considered as physiological in this species.

### 4.2. Oxidative stress biomarkers

#### 4.2.1. Oxidative stress biomarkers in Griffon vultures from two areas of Spain

The general trend observed in the present study is an increase in enzymatic and non-enzymatic antioxidant mechanisms in Griffon vultures from Cinctorres (Table 1). Antioxidant response of vultures from Cinctorres to ROS, while still operating, may not be sufficient to maintain oxidative damage at the same level of vultures from Alcoy, since TBARS concentrations are higher in vultures from Cinctorres (Table 1). The enhanced activities of CAT and GST, and concentrations of tGSH in vultures from Cinctorres may be interpreted as protective response against the higher TBARS levels, since these mechanisms may contribute together to the scavenging of ROS, and alleviate oxidative damage. In this sense, it seems that a mild exposure to oxidative attacks could results in a more permanent up-regulation of the antioxidant defence (Rattan, 2008). Besides, as explained above, Pb concentrations were significantly higher in blood of Griffon vultures from Cinctorres than in vultures from Alcoy. This may be related with the highest TBARS levels found in vultures from Cinctorres, since it is known that Pb may induce generation of ROS, which is associated with lipid peroxidation in erythrocytic membranes (Gurer and Ercal, 2000).

GSH is a major antioxidant in aerobic organisms with an important role in the protection of cells, since it binds to free radicals and many metals (Klaassen et al., 1985), and an up-regulation of GSH concentrations may be interpreted as a protective response against metals and/or raised amount of ROS. Several studies have shown enhanced total GSH levels in Pb-fed birds and rats (Hoffman et al., 2000a; Hsu, 1981; Mateo and Hoffman, 2001; McGowan and Donaldson, 1986). It is also known that the enzyme γ-glutamylcysteine synthetase, involved in GSH synthesis, can be induced by heavy metals and oxidative stress (Griffith, 1999). Besides, the increased activity of antioxidant enzymes such as CAT, GST and GPx, and lipid peroxidation has also been previously described in different bird species from polluted sites compared with reference sites (Berglund et al., 2007; Kamiński et al., 2009). GST catalyses the conjugation of GSH with cytotoxic aldehydes produced during lipid peroxidation (Halliwell and Gutteridge, 1999) and GSH conjugation with pollutants, and some GST isozymes have non-Se-dependent GPx activity (Prohaska and Ganther, 1977). Hence, an induction of GST activity could be an indication of a detoxification process (Jemec et al., 2007). In addition, the higher GST activity in vultures from Cinctorres could imply increased GSH concentrations due to higher GSH requirements for conjugation reactions of detoxification (Josephy, 1997). Regarding CAT and GPx, these enzymes could be enhanced to cope with an increment in $H_2O_2$ levels (Gurer et al., 1998; Shaikh et al., 1999). We found a low CAT activity, which could be due to GPx being the main enzyme used by Griffon vulture for catalyse $H_2O_2$ as suggested by Hernández-García (2010) and Koivula et al. (2011) in other bird species. In fact, the normal rate of $H_2O_2$ production is mainly balanced by GPx that uses $H_2O_2$ to oxidise GSH, but CAT becomes more important at enhanced $H_2O_2$ formation because of its ability to directly catalyse the transformation of $H_2O_2$ to $H_2O$ and $O_2$ (Halliwell and Gutteridge, 1999).

Antioxidant defence responds differently depending on pollution levels and species (Berglund et al., 2007; Ji et al., 2006; Martínez-Haro et al., 2011; Mateo and Hoffman, 2001). Therefore, it is necessary to use several biomarkers for oxidative stress as supported by several authors (Berglund et al., 2007; Halliwell and Gutteridge, 1999). In this study, it seems that the increased lipoperoxidation in vultures from Cinctorres modifies antioxidant status causing an up-regulation of the antioxidant defence system (CAT and GST activity, and tGSH concentrations) as a possible protective response.

#### 4.2.2. Effect of metal concentrations in oxidative stress biomarkers

*4.2.2.1. Glutathione peroxidase (GPx).* GPx enzyme uses $H_2O_2$ as substrate, and Cd exposure has been shown to increase $H_2O_2$ levels in rat pituitary membrane (Pillai et al., 2002), although its ROS

generation is indirect (Price and Joshi, 1983). This could explain the direct correlation that exists between GPx activity and Cd concentrations in vultures from Alcoy (Table 3). In addition, GPx activity was inversely related with blood Pb concentrations (Table 3). GPx enzyme requires selenium (Se) as a cofactor (ExPASy, 2012), but Schrauzer (1987) indicated antagonistic effects between Pb and Se, resulting in reduced Se uptake that may affect GPx activity. In fact, Se has a protective effect against Pb attributed to the formation of inactive Se–Pb complex (Gurer and Ercal, 2000). Experimental studies with Pb-treated birds have found an inhibition of this enzyme (Mateo et al., 2003a; Somashekaraiah et al., 1992).

*4.2.2.2. Superoxide dismutase (SOD).* Results clearly show that Hg has an effect on this enzyme (Tables 3 and 4) in spite of the low Hg levels found in this study (Table 1). It is known that Hg stimulates the activity of Cu–ZnSOD (Gurer and Ercal, 2000). Changes in SOD enzyme are dependent on exposure time and level of Hg (Ji et al., 2006), such that low-dose Hg exposure would result in increased levels of SOD as protective response of the redox-defence system (Elia et al., 2003). Moreover, the effect of Cu was expected in SOD activity (Tables 3 and 4) since Cu is a cofactor of this enzyme (ExPASy, 2012).

*4.2.2.3. Catalase (CAT).* CAT catalyses $H_2O_2$ to $H_2O$ and oxygen (Koivula and Eeva, 2010). As explained before, Cd exposure has been shown to increase $H_2O_2$ levels in rat pituitary membrane (Pillai et al., 2002), which may explain the positive correlation found between CAT activity and Cd concentration (Table 3).

Regarding the effect of Pb levels in CAT activity (Tables 3 and 4), CAT enzyme has heme as the prosthetic group (ExPASy, 2012), and Pb is known to reduce the absorption of iron, present in this group, in the gastrointestinal tract and to inhibit the heme biosynthesis (Gurer and Ercal, 2000). In fact, several authors have noticed CAT activity inhibition in Pb-exposed animals (Sandhir and Gill, 1995; Sandhir et al., 1994).

*4.2.2.4. Lipid peroxidation.* In this study, Pb, Zn and Hg concentrations were the covariates in the best-fitting model for TBARS (Table 4). Several authors have noted an increase in lipid peroxidation after Pb exposure in birds (Hoffman et al., 2000a, 2000b; Mateo and Hoffman, 2001; Mateo et al., 2003a; Somashekaraiah et al., 1992) and after Hg exposure in birds and rats (Hoffman et al., 2005; Huang et al., 1996). In addition, we found that Pb levels $\geq 30$ μg/dl produced a TBARS induction of 13.4% in red blood cells of Griffon vultures. However, in Eagle owl, Pb concentrations $\geq 10$ μg/dl produced a TBARS induction of 28% (Espín, 2013), suggesting that Griffon vulture is more resistant to oxidative damage caused by Pb than other species. Thus, García-Fernández et al. (2008) found high Pb concentrations in Griffon vultures (750–1100 μg/dl) that did not showed observable effects, suggesting that this species may be more tolerant to Pb exposure.

Pb-induced lipid peroxidation has been associated with several mechanisms (Mateo and Hoffman, 2001). In this sense, Pb can produce ROS that attack membranes by its interaction with haemoglobin and by ALAD inactivation and the consequent accumulation of pro-oxidant aminolevulinic acid in erythrocytes. Moreover, Pb inhibits GSH because the binding of this molecule with Pb or aldehydic products of lipid peroxidation, but can also inhibit antioxidant enzymes involved in the protection of cells such as GPx, SOD or CAT. The effects on GSH and antioxidant enzymes reduce the protection of membranes to ROS attack and lipid peroxidation. Pb may also alter the membrane integrity and fatty acid composition increasing susceptibility of membranes to oxidative attack (Gurer and Ercal, 2000). As discussed above, in this study Pb concentrations showed inverse relationships with GPx and CAT activity, two important scavengers of $H_2O_2$. These correlations together with the positive effect of Pb concentrations on TBARS levels suggest that the inhibition of antioxidant enzymes can play a role in the increase of lipid peroxidation.

Regarding the positive correlation between Zn and TBARS (Table 3), also found in other bird species (Berglund et al., 2007), may be related to a protective effect by an increased amount of this essential metal. Several authors have proposed that one function of Zn is the maintenance of membrane structure and function (Bettger and O'Dell, 1981). In fact, dietary Zn deficiency was shown to increase the susceptibility to lipid peroxidation in rats (Sullivan et al., 1980).

*4.2.3. Correlations among oxidative stress biomarkers*

SOD, GPx and CAT are enzymes that collaborate together in the decomposition of $H_2O_2$ and $O_2^-$ to less detrimental forms. In this sense, SOD catalyses the transformation of $O_2^-$ into $H_2O_2$ and $O_2$, and then GPx and CAT catalyse the decomposition of $H_2O_2$ (Halliwell and Gutteridge, 1999). This important collaboration could explain the positive correlations found between these three enzymes (Table 5).

In addition, GPx oxidises GSH to GSSG, which supports the positive correlation between tGSH and GPx (Table 5). We also found positive correlations between SOD and CAT with GST (Table 5), which may be interpreted as a collaboration of GST with these enzymes since GST also removes $H_2O_2$ from cells through the GSH oxidation (Koivula and Eeva, 2010).

The toxic lipid peroxides accumulated in the system are generally metabolised by cytosolic defence enzymes to prevent any damage to the biological membranes (Somashekaraiah et al., 1992). In this regard, the significant positive correlations found between CAT, GST and tGSH with TBARS concentrations (Table 5) also support the protective effect of the antioxidant system.

## 5. Conclusions

In this study, since sampling was done at the beginning of the hunting season, Pb levels found in most Griffon vultures studied from Alcoy and Cinctorres could be considered normal or background levels in vultures feeding all year round on carcasses of pork origin. However, the high blood Pb concentrations found in three Griffon vultures from Cinctorres could be due to ingestion of game meat with bullet fragments in carcasses or with Pb shots embedded in their flesh.

The general trend observed in oxidative stress status is an increase in antioxidant mechanisms (CAT and GST activity, and tGSH concentrations) in Griffon vultures from Cinctorres, which may be interpreted as a protective response against the higher TBARS levels. Several metal-related effects were observed in antioxidant enzymes of Griffon vultures. Inverse relationships were found to exist between Pb and GPx or CAT activity. Besides, there were also direct correlations between Cd and GPx or CAT, and Hg and SOD. Pb was shown to bear significant effect on lipid peroxidation in Griffon vultures. The positive correlations found between some oxidative stress biomarkers prove that antioxidant defence operates as a balanced and coordinated system.

This study provides threshold concentrations at which metals can affect antioxidant systems derived from 66 samples of Griffon vulture. Blood Cd concentrations greater than 0.05 μg/dl produced an induction of 33% in GPx activity and of 44% in CAT activity in red blood cells of vultures from Alcoy. Hg concentrations in blood higher than 3 μg/dl produced an induction of 10% in SOD activity. Concentrations of Pb above 15 μg/dl in blood produced an inhibition of 12.5% in GPx activity and 11.3% in CAT activity, and a TBARS induction of 10.7% in red blood cells of Griffon vultures.

Our results suggest that antioxidant enzymes, particularly GPx, CAT and SOD, as well as lipid peroxidation as biomarker of oxidative damage, may function as useful biomarkers of metal induced effects on antioxidant system in Griffon vultures.

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