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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).