



Effects of lead exposure on oxidative stress biomarkers and plasma biochemistry in waterbirds in the field [☆]

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ABSTRACT

Medina lagoon in Andalusia has one of the highest densities of spent lead (Pb) shot in Europe. Blood samples from waterbirds were collected in 2006–2008 to measure Pb concentration (PbB), δ -aminolevulinic acid dehydratase (ALAD), oxidative stress biomarkers and plasma biochemistry. PbB above background levels ($> 20 \mu\text{g}/\text{dl}$) was observed in 19% ($n=59$) of mallards (*Anas platyrhynchos*) and in all common pochards (*Aythya ferina*) ($n=4$), but common coots (*Fulica atra*) ($n=37$) and moorhens (*Gallinula chloropus*) ($n=12$) were all $< 20 \mu\text{g}/\text{dl}$. ALAD ratio in mallards and coots decreased with PbB levels $> 6 \mu\text{g}/\text{dl}$. In mallards, an inhibition of glutathione peroxidase (GPx) and an increased level of oxidized glutathione (oxGSH) in red blood cells (RBC) were associated with PbB levels $> 20 \mu\text{g}/\text{dl}$. In coots, PbB levels were negatively related to vitamin A and carotenoid levels in plasma, and total glutathione in RBCs; and positively related with higher superoxide dismutase and GPx activities and % oxGSH in RBCs. Overall, the results indicate that previously assumed background levels of PbB for birds need to be revised.

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1. Introduction

Lead (Pb) shot pellets present in hunted environments are often inadvertently ingested by waterbirds, which confuse them with grit required to grind food in their gizzards (Trost, 1981; Moore et al., 1998; Mateo and Guitart, 2000). Pb poisoning in birds owing to Pb shot ingestion has been described since the early 20th century (Mateo, 2009). Pb shot pellets spent in hunting activities have been accumulated in wetlands worldwide, with densities in the upper 20 cm of wetland sediments $> 100 \text{ shot}/\text{m}^2$ at many locations (Pain, 1992; Mateo, 2009). Pb shot ingestion by waterbirds and subsequent Pb poisoning has caused mortality rates in wintering waterfowl populations of 1.5% in North America and 9% in Europe (before regulations were implemented to reduce Pb shot use; Anderson et al., 2000; Mateo, 2009).

Once ingested, Pb shot can remain in the gizzard for more than 20 days (Pain, 1996). During this time, dissolved Pb may be absorbed through the intestinal wall into the bloodstream, and Pb may then be deposited and accumulated in soft tissues such as the liver or kidney and ultimately in bone (Pain, 1996). Pb acts at the molecular level and may result in a range of sublethal and acute effects that have been widely described in waterfowl (Pain, 1996; Beyer et al., 1998). In this way, threshold values of Pb concentration in waterfowl tissues for diagnostic criteria have been reported according to the impairment of biological functions and clinical signs of poisoning (Pain, 1996).

Unlike other tissues, blood may be used for non-destructive biomonitoring (Fossi, 1994). Pb in the bloodstream has a high affinity for the sulfhydryl (SH) group and can inhibit enzymes having this functional group, and also has antagonistic activity with some essential metals needed for antioxidant enzyme activities (Gurer and Ercal, 2000). The enzyme δ -aminolevulinic acid dehydratase (ALAD) is one of the first that is inhibited by Pb, via direct binding of Pb to the SH groups, which are essential for the catalytic activity of the enzyme (Gurer and Ercal, 2000; Kendall et al., 2001). ALAD is involved in the initial step in the mitochondrial biosynthetic pathway of heme to maintain hemoglobin content in erythrocytes (Bloom and Brandt, 2001). Furthermore, ALAD activity correlates negatively with blood Pb concentration (PbB) in Pb exposed waterfowl and it is used as a specific biomarker for Pb monitoring in birds (Scheuhammer,

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1987; Pain, 1989). Here, together with the Pb activity itself, the cellular accumulation of δ -aminolevulinic acid (ALA) due to the inhibition of ALAD activity induces the generation of reactive oxygen species (ROS), triggering an impaired oxidant/antioxidant balance and cellular oxidative stress (Gurer and Ercal, 2000; Ahamed and Siddiqui, 2007a; Halliwell and Gutteridge, 2007). Pb and induced ROS can oxidize membrane fatty acids initiating lipid peroxidation, modify proteins, oxidize non-protein SH and damage DNA (Bechara, 1993; Ahamed and Siddiqui, 2007a). Free radical scavenging and damage prevention and repair are provided by enzymatic and chemical scavengers and quenchers, which taken together compose the antioxidant system (Ahamed and Siddiqui, 2007a; Halliwell and Gutteridge, 2007). Thus, the measure of antioxidant enzymes activities, levels of antioxidant compounds and biochemistry values are used as biomarkers of Pb exposure in birds (Mateo et al., 2003a; Kaminski et al., 2009). Furthermore, recent studies have addressed the role played by antioxidants in wild birds with special attention to circulation of non-enzymatic low weight molecules, i.e. vitamins, carotenoids and uric acid (Costantini, 2008; Cohen and McGraw, 2009; Pérez-Rodríguez, 2009; Koivula and Eeva, 2010).

The aim of this study was to evaluate the exposure of Pb in waterbirds in the wetland with the highest Pb shot density in sediments reported from Southern Europe, the Medina lagoon in Spain (Mateo, 2009). Blood from live-trapped birds, was analyzed for PbB, ALAD activity, antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), endogenous antioxidants such as GSH, dietary antioxidants such as vitamins and carotenoids, and plasma biochemistry parameters.

2. Materials and methods

2.1. Study area

The study was carried out in the Medina lagoon (Province of Cádiz, 36°36'58"N, 06°02'47"W, Southern Spain), where Pb shot densities of 148 and 399 shot/m² have been reported in the first 10 and 30 cm of sediments, respectively (Mateo et al., 2007). Additional sampling was carried out in Salada lagoon, a brackish and shallow lagoon of 33 ha with a density of 59 Pb shot/m² in the first 10 cm of sediment, located in Puerto de Santa María (Cádiz, 36°38'40"N, 06°14'10"W) 16 km to the west of Medina lagoon (Mateo et al., 2007).

Medina lagoon is a wetland of 120 ha, with a catchment area of 1900 ha, situated in a sedimentary region defined by the Guadalquivir River depression and by the foot of the Betic Mountain Range (Fig. 1). This is the second largest "semi permanent" natural lagoon in Andalusia, with maximum water depth of 3.5 m, drying out completely in extreme droughts (most recently in 1999–2000). The sediments are composed mainly of marl and clays (Páez, 1991). Medina lagoon is important for breeding and wintering waterbirds (Amat, 1984; Martí and del Moral, 2003), with monthly censuses often exceeding 10,000 birds, mainly common coots (*Fulica atra*). Medina lagoon is especially important for the threatened white-headed duck (*Oxyura leucocephala*), red-knobbed coot (*Fulica cristata*) and marbled duck (*Marmaronetta angustirostris*).

Bird abundance in Medina lagoon led to intense hunting activity in the past. For example, in the early days of the hunting season in 1961, 5000 birds were shot per day by 80 hunters (Bernis, 1964). This intense hunting activity in Medina lagoon lasted until 1982, when the wetland was declared a hunting refuge and was acquired by the regional Government (Granados, 1991). It has since been declared an Integral Reserve in 1987, a Natural Reserve and a wetland of

international importance under the Ramsar convention in 1989, and a Special Protection Area and Site of European Community Importance in 2000. Salada is included in the same Ramsar site (Bernués, 1998).

2.2. Animal sampling

Bait trapping at Medina lagoon was done during the wintering period November 2006–February 2007, and post-breeding period August–October 2007 and August–October 2008, using four funnel traps. In addition, during the wintering period 2006/2007, one trap was installed in Salada lagoon. Traps were set on the shore or in shallow waters used by waterbirds. The shore and traps were baited with abundant grain. Every day, traps were checked, ensuring no bird was in a trap for > 24 h. Birds were removed from the traps and held in boxes or bags for less than 2 h, prior to banding and sample collection. The species captured at Medina lagoon were mallard (*Anas platyrhynchos*) ($n=59$), common coot ($n=37$), moorhen (*Gallinula chloropus*) ($n=12$) and common pochard (*Aythya ferina*) ($n=4$). Seven additional moorhens were captured at Salada lagoon.

Birds were weighed, and tarsus, beak and wing length were measured. Sex and age (juveniles or adults) were determined using plumage and beak criteria (Baker, 1993). After marking with metal rings, blood samples were taken with syringes from the brachial, femoral, or jugular vein before release. Blood was collected in heparinised tubes, and kept cool in a portable fridge until processed in the laboratory the same day. Once in the laboratory, hematocrit was recorded using a capillary tube reader after centrifugation at 4000g during 5 min. After this, an aliquot of whole blood was immediately separated and the remaining blood was centrifuged at 10,000g during 5 min at 4 °C in order to obtain the plasma and red blood cell (RBC) fractions. Then RBC samples were washed three times with cold saline solution followed by centrifugation at 10,000g during 5 min at 4 °C. Finally, two vials with plasma and RBC were stored at –80 °C and another vial with whole blood was frozen in liquid nitrogen until analysis. The time elapsed between taking blood samples in the field and storing them properly in the laboratory was around 6 h.

2.3. Blood Pb and ALAD analysis

Pb in blood samples diluted 1:10 with 0.1% triton was analyzed using graphite furnace atomic absorption spectroscopy (GF-AAS; Analyst800 with autosampler AS800, Perkin-Elmer) following Mateo et al. (1999). Calibration standards were prepared from commercial solutions containing 1 g/l of Pb (Panreac) and ultra-pure grade water. A certified reference material (blood BCR-196) for Pb was analyzed to ensure the quality of the methodology within a recovery of $96 \pm 7\%$ ($n=24$). The detection limit was < 0.6 $\mu\text{g}/\text{dl}$ of Pb in blood. Blood Pb concentration (PbB) between 20 and 50 $\mu\text{g}/\text{dl}$ has been associated with subclinical poisoning in waterfowl, between 50 and 100 $\mu\text{g}/\text{dl}$ with clinical poisoning, and > 100 $\mu\text{g}/\text{dl}$ with severe clinical poisoning (Pain, 1996).

ALAD activity and the ratio between the non-activated and the *in vitro* activated enzyme were determined, using a spectrophotometer (Ultrospec 2100 pro UV/Visible, Amersham Biosciences), according to Pain (1989) with some minor modifications (more detail is given in Supplementary Material). This method is based on the incubation of the enzyme ALAD with an excess of δ -aminolevulinic acid (ALA), and with and without the presence of Zn (an enzyme reactivator). The ALA is converted to porphobilinogen (PBG), which is mixed with a modified Ehrlich reagent and the color developed is measured spectrophotometrically against an Ehrlich's reagent blank at 555 nm. The amount of PBG formed is a measure of the activity of ALAD. ALAD is an allosteric enzyme with SH groups which could be activated in presence of Zn, a co-factor of this enzyme, which acts additionally by displacing Pb from the enzyme. The ratio between the non-activated and the *in vitro* activated enzyme was calculated because this has previously been shown to be more efficient than ALAD activity for screening of Pb intoxication in humans (Farant and Wigfield, 1982) and birds in the field (Pain, 1989).

2.4. Plasma biochemistry

Plasma biochemistry and the total antioxidant status (TAS) were measured using an A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain). The plasma constituents analyzed were albumin, glucose, cholesterol, triglycerides, calcium, magnesium, phosphorus, creatinine, urea, uric acid and total protein. The plasma enzyme activities analyzed were alkaline phosphatase (ALP; EC 3.1.3.1), alanine aminotransferase (ALT; EC 2.6.1.2), aspartate aminotransferase (AST; EC 2.6.1.1), γ -glutamyl transferase (γ -GT; EC 2.3.2.2), lactate dehydrogenase (LDH; EC 1.1.1.27) and creatine phosphokinase (CK; EC 2.7.3.2) with commercial kits from BioSystems S.A. TAS in plasma was measured with a kit of Randox Laboratories (Crumlin, Northern Ireland, UK; ref. NX2332).

Levels of vitamin A (free retinol in alcoholic form) and their esterified forms with fatty acids, vitamin E (α -tocopherol) and carotenoids (zeaxanthin and lutein) were determined in plasma by high pressure liquid chromatography (HPLC,

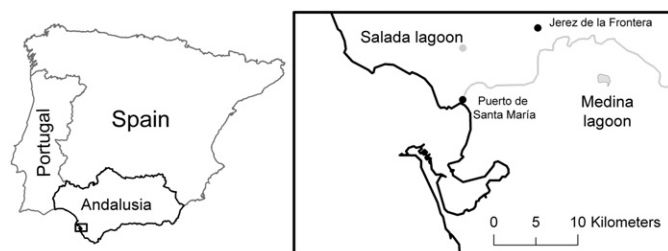


Fig. 1. Geographic location of Medina lagoon (Jerez de la Frontera) and Salada lagoon (Puerto de Sta. M^a) in the Iberian Peninsula.

Agilent Technologies 1100 Series) coupled to fluorescence and diode array detectors (Rodríguez-Estival et al., 2010).

2.5. Red blood cell analysis

Several oxidative stress parameters 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-Alvarez et al. (2008) with a spectrophotometer (Ultrospec 2100 pro, UV/vis, Amersham Biosciences). Total sulfhydryl (TSH) and nonprotein sulfhydryl (NPSH) concentrations were measured spectrophotometrically according to Sedlak and Lindsay (1968), and protein sulfhydryl (PSH) concentration calculated as the difference between them. Levels of total glutathione (tGSH) and GSH in oxidized form (oxGSH, 2 oxGSH = GSSG) were obtained as described by Reglero et al. (2009) with an automated spectrophotometer A25-Autoanalyzer (BioSystems). The oxGSH was expressed as a molar concentration and as a percentage of tGSH. The activities of glutathione peroxidase (GPx; EC 1.11.1.9) and superoxide dismutase (SOD; EC 1.15.1.1) were determined spectrophotometrically (A25-Autoanalyzer, BioSystems) using the Ransel and Ransod kits, respectively (Randox Laboratories), following descriptions of Reglero et al. (2009) with minor 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. Enzyme activities were expressed relative to grams of protein in the homogenates calculated spectrophotometrically using the method of Bradford (1976).

2.6. Statistical analysis

PbB was log-transformed so as to fit a normal distribution. Generalized Linear Models (GLMs) for each of the studied biomarkers were used with PbB and a body condition index (Peig and Green, 2009) as covariables, and year of sampling, sex and age as factors (Fairbrother et al., 1990). Due to low sample size, the effects of factors without their interactions were considered in the models. These analyses were only performed for species with $n > 10$ and at Medina lagoon. Samples obtained from recaptured birds were not included in the analysis. In those species in which some birds had PbB higher than background levels, the PbB level was included in the model as a factor instead of a covariable in order to test differences in the studied biomarkers among the different degrees of Pb exposure. PbB was classified considering background ($< 20 \mu\text{g/dl}$), subclinical (20–50 $\mu\text{g/dl}$) and clinical poisoning ($> 50 \mu\text{g/dl}$) as defined by Pain (1996). As few birds showed clinical PbB levels, this factor was also reclassified in two levels as $< 20 \mu\text{g/dl}$ (background exposure) and $\geq 20 \mu\text{g/dl}$ (elevated exposure). Significant differences in biomarkers among different PbB levels were tested with Tukey tests. A backward stepwise procedure was used to select the final models, excluding the predictor variables when they had non-significant effects. Biochemical measurements were only performed with birds captured during the post-breeding period (August–October) to reduce seasonal effects (Fairbrother et al., 1990), and because the number of birds sampled during the winter was very small (five mallards). The relationship between the different parameters analyzed was studied using Pearson correlations.

Because of the limited amount of blood collected from some birds, sample sizes were not the same for all the parameters analyzed. All tests were performed using SPSS 17.0, with a level of statistical significance of $p \leq 0.05$.

3. Results

3.1. Blood Pb concentration

Blood Pb concentrations of moorhens and coots captured in Medina lagoon were at background levels (Table 1). Conversely, all four pochards had PbB above background levels and two showed levels associated with severe clinical poisoning. For mallards in the post-breeding seasons 2007 and 2008, respectively, 19% ($n=37$) and 24% ($n=17$) had PbB above background levels, whereas 3% and 12% had PbB associated with severe clinical poisoning. All mallards ($n=5$) captured during the winter of 2006/2007 had PbB within background levels, so the overall prevalence of $> 20 \mu\text{g/dl}$ was 19% ($n=59$). PbB levels in mallards captured during post-breeding season were higher in males than in females ($F_{1,50}=10.984$, $p=0.002$; geometric means of PbB 19.0 $\mu\text{g/dl}$ ($n=14$) and 9.3 $\mu\text{g/dl}$ ($n=40$), respectively). The body condition index was not affected by PbB in any of the species. Moorhens captured in Salada lagoon showed PbB associated with subclinical poisoning in 43% of cases (Table 1).

Twenty-one waterbirds (11 mallards, one pochard, one moorhen and eight coots) were recaptured and blood samples were taken and analyzed, but no conversions from background to exposure level or vice versa were observed between these captures separated in time by 4–42 days (mean \pm SD: 16.3 ± 12.3 days). One mallard with 38 $\mu\text{g/dl}$ showed 22 $\mu\text{g/dl}$ 42 days later, and another mallard with 92 $\mu\text{g/dl}$ showed 47 $\mu\text{g/dl}$ 19 days later. One pochard with 36 $\mu\text{g/dl}$ increased to 226 $\mu\text{g/dl}$ six days later. The rest of these recaptured birds had PbB $< 20 \mu\text{g/dl}$ on both occasions.

3.2. ALAD activity

The values of ALAD ratios in whole blood were specifically related to PbB in mallards and coots ($F_{1,52}=187.849$, $p < 0.001$; $F_{1,33}=13.079$, $p=0.001$; Table 2). Negative relationships between ALAD ratio and PbB were found in both species (mallard: $r = -0.885$, $p < 0.001$, $n=54$) and (coot: $r = -0.533$, $p=0.001$, $n=35$), despite PbB being at background concentrations in coots.

Table 1
Summary statistics of Pb in whole blood ($\mu\text{g/dl}$) from birds captured in Medina and Salada lagoons, and percentage of animals within the different Pb exposure levels.

Species	N	Blood Pb concentration ($\mu\text{g/dl}$)		% of birds		
		G. Mean (95% CI)	Min–Max	Background ($< 20 \mu\text{g/dl}$)	Subclinical (20–50 $\mu\text{g/dl}$)	Clinical ($> 50 \mu\text{g/dl}$)
Common pochard	4	92.6 (8.0–1067.0)	20.7–634.0	0	50	50
Coot	37	2.0 (1.4–2.8)	nd–13.3	100	0	0
Mallard	54	11.2 (8.6–14.5)	0.7–240.7	80	15	6
Moorhen	6	0.6 (0.2–2.1)	nd–3.3	100	0	0
Mallard ^a	5	7.4 (3.3–16.3)	3.2–15.9	100	0	0
Moorhen ^a	6	2.7 (1.7–4.3)	2.1–6.0	100	0	0
Moorhen ^{a,b}	7	10.6 (4.1–27.1)	2.1–26.7	57	43	0

nd=below detection limit.

^a Birds captured during wintering period.

^b Birds captured in Salada lagoon.

Table 2

Mean values (\pm SE) of hematocrit (Hct) percentage, ratio of δ -aminolevulinic acid dehydratase (ALAD) activity, and ALAD activity (nmol ALA/ml red blood cell) in whole blood from captured birds in Medina and Salada lagoons.

Species	Parameter	Background (< 20 μ g/dl)		Subclinical (20–50 μ g/dl)		Clinical (> 50 μ g/dl)	
		N	Mean \pm SE	N	Mean \pm SE	N	Mean \pm SE
Common pochard	Hct %			2	41.20 \pm 2.01	2	32.29 \pm 2.80
	ALAD			2	10.46 \pm 3.75	2	6.23 \pm 5.72
	ALAD ratio			2	0.26 \pm 0.14	2	0.06 \pm 0.04
Coot	Hct %	37	34.85 \pm 1.04				
	ALAD	35	49.71 \pm 3.18				
	ALAD ratio	35	1.79 \pm 0.13				
Mallard ^a	Hct %	47	41.19 \pm 0.86	8	39.46 \pm 1.57	3	44.99 \pm 2.65
	ALAD	44	71.26* \pm 4.00	8	24.09* \pm 6.65	3	1.33* \pm 0.76
	ALAD ratio	43	0.78* \pm 0.02	8	0.31* \pm 0.06	3	0.02* \pm 0.01
Moorhen ^{a,b}	Hct %	5	35.16 \pm 5.20	2	39.64 \pm 0.84		
	ALAD	5	73.44 \pm 16.47				
	ALAD ratio	8	2.11* \pm 0.24	2	0.73* \pm 0.15		

^a Including birds captured during wintering and post-breeding seasons.

^b Birds captured in Medina and Salada lagoons.

* Significant difference $p \leq 0.05$.

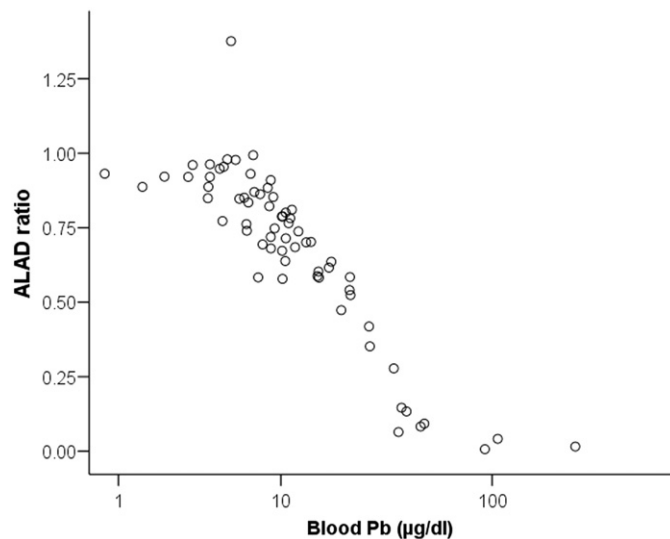


Fig. 2. Relationship between blood Pb level and ALAD ratio (ALAD activity/ALAD reactivated activity) in mallards captured in Medina lagoon.

In mallards, this relationship was also confirmed when excluding those animals with severe clinical poisoning, which showed a strong ALAD inhibition ($r = -0.825$, $p < 0.001$, $n = 51$; Fig. 2).

In fact, ALAD ratio was significantly lower in mallards with clinical than with subclinical PbB levels, and significantly lower in those with subclinical levels than with background levels ($F_{2,51} = 79.230$, $p < 0.001$; post-hoc Tukey). Hematocrit values in coots were positively correlated with PbB ($r = 0.397$, $p = 0.015$, $n = 37$). Mean values of ALAD ratio and ALAD activity of pochards with PbB levels of clinical poisoning were 0.26 and 10.46 nmol ALA/ml of blood, respectively (Table 2). These values decreased to 0.06 and 6.23 nmol ALA/ml in birds with severe clinical poisoning levels. Finally, in moorhens, lower ALAD ratio was also found in birds with PbB ≥ 20 μ g/dl than those with background level (considering all captured animals, $F_{1,8} = 7.396$, $p = 0.026$; Table 2).

3.3. Oxidative stress biomarkers and plasma biochemistry

Mallards with PbB level ≥ 20 μ g/dl had higher levels of oxGSH ($F_{1,47} = 4.329$, $p = 0.043$), lower GPx activity ($F_{1,51} = 8.139$, $p = 0.006$) and higher ALP activity ($F_{1,47} = 9.113$, $p = 0.004$) than those with

Table 3

Mean values (\pm SE) of oxidative stress biomarkers in plasma and red blood cells from captured mallards in Medina lagoon during the post-breeding seasons (2007–2008) according to different Pb exposure levels.

Parameter	Units	Mallard			
		< 20 μ g/dl		≥ 20 μ g/dl	
		N	Mean \pm SE	N	Mean \pm SE
<i>Plasma</i>					
TAS	mmol/L	43	1.83 \pm 0.13	11	1.82 \pm 0.19
Lutein	μ g/ml	40	6.70 \pm 0.55	11	6.75 \pm 0.69
Zeaxanthin	μ g/ml	39	1.09 \pm 0.11	11	0.89 \pm 0.14
Retinol	μ g/ml	40	4.93 \pm 0.36	11	4.29 \pm 0.87
Tocopherol	μ g/ml	40	7.35* \pm 0.34	11	9.33* \pm 1.47
<i>Red blood cell</i>					
TBARS	μ mol/g	43	0.0436* \pm 0.0012	11	0.0397* \pm 0.0021
TSH	μ mol/g	43	51.13 \pm 0.45	11	51.10 \pm 0.93
NPSH	μ mol/g	43	6.15 \pm 0.53	11	7.06 \pm 1.09
PSH	μ mol/g	43	44.98 \pm 0.55	11	44.04 \pm 0.73
tGSH	μ mol/g	43	4.56 \pm 0.09	11	4.69 \pm 0.17
oxGSH	μ mol/g	43	0.49* \pm 0.07	11	0.76* \pm 0.20
oxGSH	%	43	11.06 \pm 1.58	11	15.32 \pm 3.87
SOD	U/g _{protein}	43	1109.51 \pm 45.59	11	1096.72 \pm 109.96
GPx	U/g _{protein}	43	307.82* \pm 12.20	11	275.85* \pm 37.85

* Significant difference $p \leq 0.05$.

background levels (Tables 3 and 4). These exposed mallards also had a marginally higher level of vitamin E in plasma ($F_{1,48} = 3.990$, $p = 0.051$). Lipid peroxidation (TBARS) in RBC decreased significantly in those birds with PbB > 100 μ g/dl ($F_{2,49} = 3.185$, $p = 0.050$).

In coots (Tables 5 and 6), PbB within background levels were inversely associated with plasma values of several antioxidants such as uric acid ($F_{1,32} = 4.186$, $p = 0.049$), carotenoids (lutein: $F_{1,28} = 5.985$, $p = 0.021$; zeaxanthin: $F_{1,29} = 7.915$, $p = 0.009$) and vitamin A ($F_{1,25} = 6.189$, $p = 0.020$). The antioxidant status measured in RBC showed a similar effect because GSH concentration was negatively related to PbB ($F_{1,34} = 5.841$, $p = 0.021$), and positively related to % oxGSH ($F_{1,34} = 4.698$, $p = 0.037$). The activity of SOD increased with PbB ($F_{1,33} = 25.838$, $p < 0.001$) and the activity of GPx showed a similar but not significant trend ($F_{1,34} = 4.071$, $p = 0.052$). Negative correlations were found between SOD and ALAD activities ($r = -0.525$, $p = 0.001$, $n = 34$). PbB levels were also inversely associated with plasma activities of ALP ($F_{1,30} = 15.637$, $p < 0.001$) and ALT ($F_{1,34} = 7.875$, $p = 0.008$). On the contrary,

creatinine ($F_{1,31}=7.983$, $p=0.008$) and Ca ($F_{1,31}=8.473$, $p=0.007$) levels in plasma increased with higher PbB levels.

Both mallards and coots showed positive correlation between TAS and uric acid ($r=0.697$, $p<0.001$, $n=58$; $r=0.918$, $p<0.001$, $n=34$). The mean values of oxidative stress and plasma biochemistry parameters for pochards, coots and moorhens are presented in Tables 5 and 6. All individuals of these species were within one category of Pb exposure (background or clinical), and therefore comparisons between exposure groups were not possible.

4. Discussion

4.1. Blood Pb blood concentration and ALAD activity

More than 25 years after the ban on hunting waterbirds at Medina lagoon are exposed to Pb and affected by Pb shot ingestion,

Table 4

Mean values (\pm SE) of biochemistry parameters in plasma from mallards captured in Medina lagoon during the post-breeding seasons (2007–2008) according to different Pb exposure levels.

Parameter	Units	Mallard			
		< 20 μ g/dl		\geq 20 μ g/dl	
		N	Mean \pm SE	N	Mean \pm SE
ALP	U/L	39	175.54* \pm 12.45	10	260.09* \pm 26.65
ALT	U/L	43	60.95 \pm 5.07	11	67.80 \pm 14.30
AST	U/L	43	112.49 \pm 12.53	11	123.78 \pm 29.07
CK	U/L	31	977.34 \pm 119.39	9	561.34 \pm 166.86
g-GT	U/L	43	5.05 \pm 0.44	11	6.86 \pm 1.43
LDH	U/L	36	1548.34 \pm 103.67	8	1573.49 \pm 341.47
Albumin	g/L	43	19.20 \pm 0.99	11	18.61 \pm 1.73
Bilirubin total	mg/dl	42	7.25 \pm 0.37	11	6.68 \pm 0.58
Calcium	mg/dl	42	11.86 \pm 0.28	11	10.97 \pm 0.52
Cholesterol	mg/dl	43	192.55 \pm 8.84	11	197.79 \pm 17.69
Creatinine	mg/dl	43	0.61 \pm 0.03	11	0.67 \pm 0.06
Glucose	mg/dl	43	326.64 \pm 13.90	11	327.19 \pm 30.31
Magnesium	mg/dl	43	1.76 \pm 0.10	11	1.82 \pm 0.23
Phosphorus	mg/dl	42	3.07 \pm 0.33	11	2.33 \pm 0.26
Total protein	g/L	43	40.82 \pm 1.11	11	40.93 \pm 1.50
Triglycerides	mg/dl	42	292.23 \pm 25.72	11	265.43 \pm 33.89
Urea	mg/dl	43	3.93 \pm 0.50	11	4.67 \pm 0.94
Uric acid	mg/dl	42	14.12 \pm 1.15	11	15.10 \pm 2.36

* Significant differences $p \leq 0.05$.

Table 5

Mean values (\pm SE) of oxidative stress biomarkers in plasma and red blood cells from common pochards, coots and moorhens captured in Medina lagoon during the post-breeding seasons (2007–2008) according to Pb exposure level.

Parameter	Units	Coot (< 20 μ g/dl)		Moorhen (< 20 μ g/dl)		Common pochard (> 20 μ g/dl)	
		N	Mean \pm SE	N	Mean \pm SE	N	Mean \pm SE
<i>Plasma</i>							
TAS	mmol/L	37	2.11 \pm 0.14	3	1.42 \pm 0.15	4	1.01 \pm 0.12
Lutein	μ g/ml	30	1.71 \pm 0.1			4	2.22 \pm 0.73
Zeaxanthin	μ g/ml	31	0.33 \pm 0.03			4	0.43 \pm 0.16
Retinol	μ g/ml	27	4.28 \pm 0.22			4	3.52 \pm 1.11
Tocopherol	μ g/ml	31	10.3 \pm 0.53			4	7.03 \pm 0.79
<i>Red blood cell</i>							
TBARS	μ mol/g	37	0.0363 \pm 0.0012	3	0.0368 \pm 0.0003	3	0.0394 \pm 0.0040
TSH	μ mol/g	37	53.18 \pm 0.64	3	49.69 \pm 2.33	3	49.23 \pm 4.28
NPSH	μ mol/g	37	6.74 \pm 0.53	3	8.62 \pm 2.02	3	4.5 \pm 0.62
PSH	μ mol/g	37	46.44 \pm 0.59	3	41.06 \pm 1.29	3	44.73 \pm 4.89
tGSH	μ mol/g	37	5.27 \pm 0.11	3	4.97 \pm 0.1	3	5.47 \pm 0.68
oxGSH	μ mol/g	37	0.95 \pm 0.06	3	0.49 \pm 0.25	3	0.06 \pm 0.03
oxGSH	%	37	17.94 \pm 1.19	3	10.05 \pm 5.21	3	0.93 \pm 0.42
SOD	U/g protein	36	1255.57 \pm 56.68	3	1150.96 \pm 50.43	3	1087.84 \pm 190.94
GPx	U/g protein	37	723.86 \pm 41.12	3	579.93 \pm 271.00	3	263.75 \pm 18.26

as observed by elevated PbB levels and altered values of specific Pb biomarkers such as ALAD. In addition, antioxidant mechanisms and plasma biochemistry also appear to be affected by the level of Pb exposure. Pb pollution in Medina lagoon is likely to come mainly from the high density of Pb shot in sediments, thus PbB over background levels may be considered indicative of Pb shot ingestion (Daury et al., 1993). In a separate study, we have confirmed using Pb isotopes that Pb shot ingestion causes high Pb levels in waterbird feces from the Medina lagoon (authors, in prep.). The highest levels of exposure to Pb shot have been observed in mallards and pochards. All pochards and also 19% and 24% of mallards, captured during the 2007 and 2008 breeding seasons, respectively, had PbB above background levels. Two pochards and 3% and 12% of these mallards had PbB associated with severe clinical poisoning. Furthermore, PbB in one pochard increased from 36 to 226 μ g/dl in six days between two captures, suggesting that this animal ingested Pb shot within this period or just before the first capture. This animal increased the number of pochards with PbB associated with severe clinical poisoning to three out of four. Levels of Pb shot exposure for these two duck species in Medina lagoon are similar to those reported from hunted birds in other studies in Mediterranean wetlands (Pain, 1991; Mateo et al., 1997, 2007; Mateo, 2009). The results for pochards suggest that the white-headed duck is also exposed to Pb poisoning at Medina lagoon, which regularly holds several hundred individuals of this globally threatened species, and up to 25% of the Spanish population. These two species are both diving ducks with similar diets and grit size selection (Mateo et al., 2000, 2001). Furthermore, high prevalence of Pb shot ingestion has been reported for white-headed ducks in other parts of Spain, suggesting that this as a major threat for this species (Svanberg et al., 2006; Taggart et al., 2009).

Coots and moorhens appear to be less exposed to Pb shot ingestion in Medina lagoon, since PbB in all individuals was at background levels. Reported Pb shot ingestion in coots from wetlands with high Pb shot densities varies from 3.6% in the Ebro Delta (Mateo et al., 2000) to 19% in the Camargue (Rhone Delta, France; Pain, 1990). The relatively low prevalence of Pb shot in Rallidae is related to their feeding behavior, characterized by grazing at or below the water surface or along the shore. They do not share the ability of ducks to sift through bottom material with their complex beak (Gurd, 2007). Thus, Rallidae are only exposed to Pb shot that can be picked up from the surface of the sediment. Furthermore, the highest proportion of grit size reported in gizzards of hunted coots is < 1 mm, whereas in ducks it is

Table 6

Mean values (\pm SE) of biochemistry parameters in plasma from captured common pochards, coots and moorhens in Medina lagoon during the post-breeding seasons (2007–2008) according to Pb exposure level.

Parameter	Units	Coot (< 20 μ g/dl)		Moorhen (< 20 μ g/dl)		Common pochard (> 20 μ g/dl)	
		N	Mean \pm SE	N	Mean \pm SE	N	Mean \pm SE
ALP	U/L	32	650.78 \pm 66.23	2	763.82 \pm 124.02	4	279.20 \pm 69.97
ALT	U/L	36	40.40 \pm 3.74	2	65.78 \pm 23.30	4	33.80 \pm 15.09
AST	U/L	37	131.54 \pm 22.72	3	251.87 \pm 58.54	4	329.57 \pm 89.60
CK	U/L	15	1014.33 \pm 139.87	1	643.40	3	1491.35 \pm 520.18
g-GT	U/L	36	4.28 \pm 0.57	3	2.59 \pm 1.15	4	8.43 \pm 2.68
LDH	U/L	26	652.34 \pm 59.84	3	1261.96 \pm 744.69	4	2109.87 \pm 260.68
Albumin	g/L	36	16.41 \pm 0.82	3	10.80 \pm 3.94	4	12.15 \pm 2.19
Bilirubin total	mg/dl	36	4.36 \pm 0.19	3	4.53 \pm 0.35	4	5.83 \pm 1.11
Calcium	mg/dl	34	11.03 \pm 0.38	3	12.25 \pm 2.39	4	10.26 \pm 0.66
Cholesterol	mg/dl	37	164.12 \pm 11.74	3	147.59 \pm 41.76	4	183.15 \pm 14.85
Creatinine	mg/dl	34	0.68 \pm 0.02	3	0.55 \pm 0.24	4	0.87 \pm 0.09
Glucose	mg/dl	37	270.72 \pm 16.18	3	367.53 \pm 31.25	4	370.44 \pm 43.47
Magnesium	mg/dl	37	2.02 \pm 0.15	3	1.03 \pm 0.60	4	0.87 \pm 0.14
Phosphorus	mg/dl	34	3.35 \pm 0.36	3	2.50 \pm 1.33	4	2.16 \pm 0.36
Total protein	g/L	37	42.94 \pm 1.76	3	39.99 \pm 6.31	4	33.02 \pm 4.90
Triglycerides	mg/dl	35	106.03 \pm 11.04	3	47.33 \pm 23.91	4	298.99 \pm 62.26
Urea	mg/dl	32	4.08 \pm 0.49	3	5.32 \pm 2.99	4	5.42 \pm 1.91
Uric acid	mg/dl	34	12.90 \pm 1.20	3	9.84 \pm 1.44	4	9.24 \pm 1.12

> 1 mm (Pain, 1990; Mateo et al., 2000). Pb shot ingestion in hunted birds is positively related to grit size in gizzards, and also negatively related to the abundance of grit in the environment (Mateo et al., 2000; Figuerola et al., 2005). Medina lagoon has low abundance of grit of > 1 mm, but a high Pb shot density in sediment (Mateo et al., 2007), increasing the risk of Pb shot ingestion for those species selecting grit of > 1 mm.

The elevated Pb exposure observed in the moorhens from Salada lagoon (43%) can be explained by the high accessibility of Pb shot in the sediments due to the low water level at the time of capture. As water levels drop, more species are capable of reaching depths at which shot is deposited (Bolduc and Afton, 2008). Differences in PbB related to sex were found in mallards, with higher levels in males than females. This contrasts with the majority of studies, in which PbB or Pb shot ingestion were not related to gender (Pain, 1989, 1990; Mateo et al., 1997, 2000). However, Havera et al. (1992) also found higher PbB levels in male lesser scaup (*Aythya affinis*), but this difference was not observed for Pb shot ingestion. Higher PbB levels were also observed in males of several species of birds from Doñana National Park after an accidental mine spill (Benito et al., 1999). In our study, it is possible that male mallards had spent longer at Medina than females, as they may move to the lagoon to moult while females are still rearing their broods at other wetlands.

In addition to the values of PbB in captured waterbirds, Pb exposure was confirmed by the effect on the specific Pb biomarker ALAD. As reported previously (Pain, 1989; Henny et al., 2000; Beyer et al., 2004), ALAD ratio is a good predictor of PbB in waterbirds and a sensitive biomarker for screening Pb exposure and poisoning in the field. Physiological and heme-pathway disturbances due to Pb poisoning may be observed with PbB > 40 μ g/dl (Pain, 1989). In this study, ALAD ratio in mallards decreased linearly with PbB levels between 6 and 40 μ g/dl (Fig. 2). At higher concentrations of PbB, ALAD activity is very inhibited and ratio values remain very low. For pochards, in addition to the low ALAD ratios, ALAD activities were similar to those reported by Mautino and Bell (1986), in an experimental study with dosed ring-necked ducks (*Aythya collaris*).

4.2. Oxidative stress

Several effects on oxidative stress biomarkers were observed as a consequence of Pb exposure, and some differences in these

effects were found among species. In mallards, the oxidative stress generated by Pb exposure was indicated in RBC by an increase of GSSG, which is the oxidized form of GSH, the main intracellular antioxidant. This species also showed a lower activity of GPx in RBC, which is responsible for the transformation of H₂O₂ generated in tissues to a superoxide anion (O₂⁻) using GSH as a reducing agent (Meister and Anderson, 1983; Gurer and Ercal, 2000; Circu and Aw, 2010). Despite these effects, mallards did not show reductions in the levels of other dietary or endogenous antioxidants. On the contrary, there was a slight trend to increase vitamin E level in plasma in mallards with elevated PbB levels. This effect may be owing to a response of the organism to cope with the oxidative stress generated by Pb poisoning. Vitamin E can be mobilized from the liver to maintain plasma levels for delivery to other tissues (Bartlett et al., 1974; Mustacich et al., 2007). Under oxidative stress, the erythrocytic membranes are susceptible to lipid peroxidation, which has been associated with the hemolytic action of heavy metal ions (Gurer and Ercal, 2000). Despite the PbB found in mallards, no effect on hematocrit values was detected, and lipid peroxidation in RBCs did not increase in the Pb exposed mallards. Furthermore, a decrease of TBARS was detected in those animals with elevated PbB, which may be a result of the increase of vitamin E, described above, as a protective mechanism. Inhibition of GPx activity has been described in mallards experimentally dosed with Pb, and those birds supplemented with vitamin E, as an antioxidant, showed a slight decrease of TBARS (Mateo et al., 2003a).

Pb exposure in coots was lower than in mallards, but several biomarkers of oxidative stress showed linear relationships with PbB. Dietary antioxidants, such as carotenoids (lutein and zeaxanthin) and vitamin A in plasma, showed negative relationships with PbB. In addition, TAS showed a strong correlation with uric acid in coots, as well as in mallards, which may reflect the role of uric acid as an antioxidant in birds (Cohen and McGraw, 2009). Plasma uric acid decreased in mallards exposed to Pb contaminated sediment (Hoffman et al., 2000). The effect of Pb exposure on plasma vitamin A has not been described previously; however, other environmental pollutants cause severe disturbances in vitamin A metabolism (Zile, 1992; Rolland, 2000; Rodríguez-Estival et al., 2011).

These negative effects on antioxidant levels in coots could be compensated by induced activities of SOD and GPx in RBC, which are positively related to PbB. Moreover, the increased activity of

GPx may explain the decrease of tGSH levels and increase of % oxGSH in RBCs. GSH is the most important cellular thiol, acting as a substrate for several transferases, peroxidases, and other enzymes that prevent or mitigate the deleterious effects of ROS (Meister and Anderson, 1983; Circu and Aw, 2010). Depletion of GSH by Pb toxicity has been widely reported (Gurer and Ercal, 2000; Ercal et al., 2001). Furthermore, a rise of % oxGSH is considered an indicator of oxidative stress (Costa et al., 1997; Gurer and Ercal, 2000; Circu and Aw, 2010; Koivula and Eeva, 2010). SOD and GPx are two of the main antioxidant enzymes in the cell (Halliwell and Gutteridge, 2007). In the erythrocyte, O_2^- is catalytically quenched by SOD, with the production of H_2O_2 which is in turn scavenged by catalase and GPx. SOD keeps the concentration of O_2^- at low levels and therefore plays an important role in defence against oxidative stress (Fridovich, 1997). The increased activity of the antioxidant enzymes has also been previously described in erythrocytes from workers exposed occupationally to Pb, suggesting that the enhancing of SOD and GPx activities may be interpreted as a protective response against the raised amount of ROS generated by ALA accumulation (Bechara, 1993; Costa et al., 1997). This may explain the negative correlation between ALAD and SOD activity found in coots in this study. Furthermore, mild exposure to oxidative attacks results in a more permanent upregulation of defence levels (Rattan, 2008).

The association between low level Pb exposure and oxidative stress in wild animals has not been explored systematically. However, Pb is a major environmental toxin that causes hematological, gastrointestinal and neurological dysfunction. In human epidemiological studies, low levels of Pb exposure can induce oxidative stress through induced generation of free radicals, and this has a graded association with several diseases such as hypertension, peripheral artery disease, kidney disease, neurodegenerative disease and cognitive impairment (Ahamed and Siddiqui, 2007b). Stressful conditions, such as Pb exposure, may be an important ecological and evolutionary force modulating adaptive responses of natural populations (Romero, 2004; Korte et al., 2005; Monaghan et al., 2009). Here, in order to obtain insight into Pb exposure, a wide range of components of the antioxidant system have been analyzed, both enzymes and molecules. The decrease of the non-enzymatic circulating antioxidants may indicate the increased generation of ROS under Pb exposure, thus the antioxidant depletion together with the increase of antioxidant enzymes activities may reflect the activation of defense mechanisms (Halliwell and Gutteridge, 2007; Koivula and Eeva, 2010). Thus, our findings suggest a protective response against the deleterious effects of ROS in coots. This is the first study in which oxidative stress related to low level Pb exposure is shown in wild birds.

4.3. Plasma biochemistry

In coots, increased PbB concentrations were accompanied by lower activity of plasma ALP. This effect was also observed in previous studies with mallard ducklings experimentally exposed to Pb with sediment contaminated with Pb acetate (828 $\mu\text{g/g}$ Pb dry weight; Hoffman et al., 2000), adult mute swans exposed to Pb contaminated sediment (700 $\mu\text{g/g}$ Pb dry weight; Day et al., 2003) and mallard ducks with Pb contaminated food (1840 $\mu\text{g/g}$ Pb dry weight; Mateo et al., 2003b). Alkaline phosphatase is an indicator of osteoblastic activity (Campbell, 1986), and Pb exposure has been associated with altered bone mineralization in birds (Gangoso et al., 2009). In contrast, mallards with PbB > 20 $\mu\text{g/dl}$ in the current study showed a higher ALP activity than birds with background levels.

The level of creatinine in plasma was also associated with PbB in coots, which may be an indicator of renal damage produced by Pb (Mateo et al., 2003a). This effect is consistent with recent

human epidemiological studies, which suggest that kidney function may be altered even at the lowest levels of PbB < 5 $\mu\text{g/dl}$ (Ahamed and Siddiqui, 2007b). The decrease of ALT activity in plasma observed in coots has been reported previously in mute swans exposed to low Pb contaminated sediment (Day et al., 2003). However, increases of ALT have been reported in mallards exposed to high Pb levels, which can be related to hepatic alterations (Mateo et al., 2003a).

5. Conclusion

This study provides information about oxidative stress in wild birds exposed to different levels of Pb contamination, and takes an important step towards elucidating the biochemical mechanisms underlying these processes. Knowledge about the potential role of oxidative stress, associated with Pb exposure, may be used to design treatments or environmental practices to enhance antioxidants in order to mitigate Pb-induced toxicity, as previously proposed for humans (Gurer and Ercal, 2000). The adverse effect of Pb on ALAD activity on mallards has been observed at PbB levels as low as 6 $\mu\text{g/dl}$, and coots showed effects on oxidative stress biomarkers at PbB levels < 20 $\mu\text{g/dl}$. Therefore, the no-effect level may be considered lower than the background value of 20 $\mu\text{g/dl}$ frequently assumed for birds, probably at levels ≤ 10 $\mu\text{g/dl}$ as found in humans (Gilbert and Weiss, 2006).

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Appendix A. Supplementary Material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2011.02.012.

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