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Blood biomarkers and contaminant levels in feathers and eggs to assess environmental hazards in heron nestlings from impacted sites in Ebro basin (NE Spain)

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High levels of organochlorine and mercury levels in eggs and feathers were related with altered blood biomarkers of heron nesting chicks.

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ABSTRACT

Blood biomarkers and levels of major pollutants in eggs and feathers were used to determine pollution effects in nestlings of the Purple Heron *Ardea purpurea* and the Little Egret *Egretta garzetta,* sampled on three Ebro River (NE Spain) areas: a reference site, a site affected by the effluents of a chlor-alkali industry and the river Delta. The two impacted heron populations showed mutually different pollutant and response patterns, suggesting different sources of contamination. In the population nesting near the chlor-alkali plant, elevated levels of hexachlorobenzene (HCB) and polychlorobiphenyls (PCBs) in eggs, and mercury in feathers in *A. purpurea* chicks were related with reduced blood antioxidant defenses and increased levels of micronuclei. In Ebro Delta, high levels of plasmatic lactate dehydrogenase in *A. purpurea* chicks and high frequency of micronuclei in blood of both species were tentatively associated with intensive agricultural activities taking place in the area. These results provide the first evidence of a biological response in heron chicks to the release of pollutants at a chlor-alkali plant.

1. Introduction

Piscivorous birds such as herons and egrets (family Ardeidae) are suitable bio-indicators of environmental pollution in aquatic systems (Furness and Camphuysen, 1997; De Luca-Abbott et al., 2001; Connell et al., 2003; Champoux et al., 2006). They are top consumers in the trophic chain of aquatic ecosystems, and can potentially accumulate persistent organic pollutants and metals (Mañosa et al., 2001). The combined use of biomarkers and chemical analyses in the last decade has allowed assessing and identifying adverse health effects of endocrine disrupting persistent organic pollutants, pesticides and metals on several waterbirds (Fossi et al., 1994; Cordi et al., 1997; Muir et al., 1999; Vos et al., 2000; Strum et al., 2008). More recently the use of non-lethal biological techniques such as the analyses of biomarkers in blood, and contaminants in feathers and eggs have been applied when studying endangered species (Muir et al., 1999; Vos et al., 2000; Champoux et al., 2002, 2006; Franson et al., 2002; Murvoll et al., 2006; Quirós et al., 2008).

Antioxidant defenses represent the detoxification pathway of reactive oxygen species (ROS) in aerobic organisms (Livingstone, 2001). When ROS exceed antioxidant defenses, oxidative damage of lipids within cell membranes, of DNA molecules and proteins may occur leading to a pathological condition, generally termed oxidative stress (Livingstone, 2001). In this way, knowledge about the responses of organisms to oxidative stress may provide valuable information about their health status. Recently, some authors (Winston et al., 1998; Regoli, 2000), developed an analytical method to evaluate the total oxyradical scavenging capacity (TOSC) of any given tissue, defined as the capacity of the biological sample to neutralize ROS. For a given tissue lower TOSC values are likely to be related to lower capacity to detoxify ROS and hence a greater susceptibility to oxidative stress. The method was originally developed for rat liver (Winston et al., 1998), but in recent times it has been applied to the blood of birds in environmental surveillance programs (Corsolini et al., 2001). Plasmatic enzymes, such as the activity of lactate deshydrogenase and of B esterases such as butyrylcholinesterase and carboxylesterases may also provide

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valuable information on liver damage and the neurotoxic effects of pesticides in birds, respectively (Champoux et al., 2006; Fossi et al., 1994; Cordi et al., 1997; Strum et al., 2008). The presence of micronuclei in peripheral erythrocytes has also been used to evaluate the environmental impact by genotoxicants in different species, including birds (Kim et al., 2003; Stoncius and Lazutka, 2003; Quirós et al., 2008). Micronucleated cells originate when mitotic cells with chromatid breaks or dysfunctional mitotic apparatus undergo abnormal anaphase distribution of chromatin. The resulting micronuclei can be quantified either by microscopy or by flow cytometry analysis. The latter methodology represents a major advance in terms of sensitivity, reliability and throughput. Indeed in a recent study conducted in the Ebro river (NE Spain), the presence of micronuclei measured by flow cytometry analysis in peripheral erythrocytes in heron nestlings evidenced higher micronuclei counts in samples from sites heavily contaminated with metals, organochlorine residues and pesticides (Quirós et al., 2008).

The Ebro catchment is the largest river basin in Spain, covers an area of 85,362 km^2 and receives the potential influence of 3 million people, including some heavily industrialized areas. One of these areas is the Flix site, where an organochlorine industry operates since the beginning of the 20th century. This long operational period, along with the construction of a dam next to the factory around 1960, resulted in the accumulation of high amounts of heavy polluted sediments in the adjacent riverbed (Fernández et al., 1999). The surroundings of the Flix dam have become a valuable wetland, with profusion of nesting birds and small mammals. Pollutants originated at Flix site are carried downstream by the Ebro river to its Delta 90 km away, an UNESCO wildlife and bird reserve (Pastor et al., 2004). Ebro Delta land is also affected by an intensive agricultural activity, with significant impact on wildlife (Mañosa et al., 2001). Therefore both Flix and the Ebro Delta could be considered impacted sites. Aiguabarreig, a marsh site and bird sanctuary located upstream of Flix represents an appropriate reference site due to its high ecological value (Quirós et al., 2008).

The aim of this study is to test the usefulness of using a set of non-destructive markers to monitor environmental hazards of organochlorine and metallic persistent pollutants in nestlings of two engaged piscivorous bird species (*Ardea purpurea* and *Egretta garzetta*) along the Ebro river (NE Spain). By combining and comparing the response of blood biomarkers with levels of selected pollutants in eggs and feathers it was intended to identify potential contaminants casing detrimental effects on the studied bird species. Nestlings instead of adults were used to minimizing the effect of bioaccumulation because the time of exposure is known and similar for all samples. Furthermore, since all chicks are provisioned with prey caught by parents in the surroundings of the colony, the results should reflect only the impact of pollutants ingested at that site. Provided that herons and egrets and persistent organic and metallic pollutants are distributed world wide, the methods and results obtained in this study may apply or be used to assess the effects of pollution in other areas of concern.

2. Materials and methods

2.1. Sampling and tissue preparation

A total of 37 eggs and feathers and blood samples of 59 chicks from two species were sampled and analyzed from three locations in the eastern portion of the Ebro basin, Aiguabarreig, Flix and Ebro Delta (Fig. 1, Table 1). Egrets do not nest in Flix, neither in surrounded areas, thus the study was restricted to Aiguabarreig and Ebro Delta site. Sampling was conducted in spring 2006 at two time periods. First, one egg per nest was collected at the end of the laying period. Later, chicks were sampled 1 week before the estimated peak fledgling time. During sampling, unusual adverse climatic and river flow conditions limited the number of eggs and chicks sampled in the same locations and species. To avoid pseudoreplication, only one egg and one

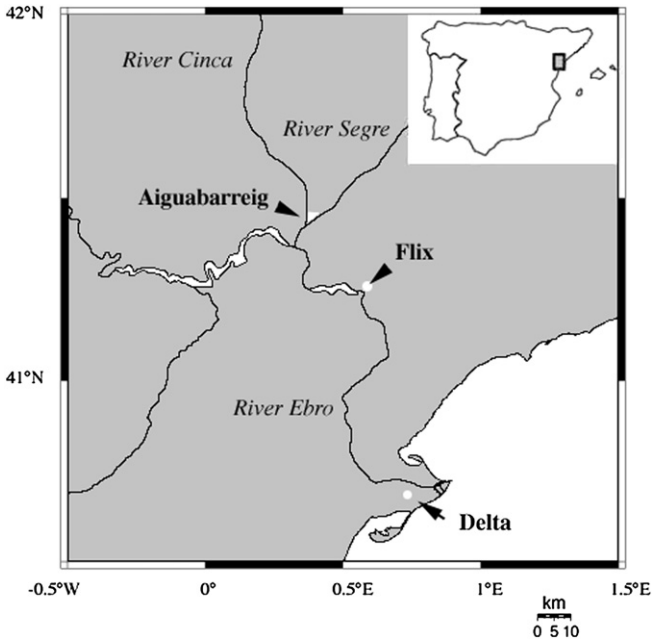

Fig. 1. Map of sampling sites in NE Spain. The inset shows the approximate position of the sampling area in the Iberian Peninsula.

chick per nest were sampled. Chicks were measured with a digital caliper (0.1 mm) for tarsus length and weighed with a Pesola spring balance to the nearest gram. In all chicks two feathers from the back were obtained for metal analyses. In 45 chicks 1 ml of blood was extracted using a heparinized syringe by punction of the tarsal vein and transferred to heparinized vials and kept refrigerated at 4 °C. In the lab blood samples were split into two 0.5 ml aliquots. One was preserved cold and immediately used for micronuclei determination by flow cytometry as described in Quirós et al. (2008). The remaining was centrifuged at 1500 rpm for 2 min and the plasma gently removed, frozen with liquid N_2 and stored at −80 °C until biomarker determination. Entire eggs (37) were labelled and kept refrigerated in individual glass containers packed with cotton to avoid breakage until arriving at laboratory. Eggs were kept frozen (−20 °C) until organochlorine residue analysis.

2.2. Contaminant analyses

2.2.1. Metal analysis

For metals analysis, feathers were washed with a 0.1 molar NaOH solution and dried out at 50 °C. Levels of Cu, Hg and Pb were determined in acid digested samples by Perkin Elmer model Elan 6000 inductively coupled plasma mass spectrometer (ICP-MS) following Barata et al. (2005). Samples ($n = 3$) of similar weight of a certified reference material of Human Hair (CRM 397, Commission of the European Community) for Hg and Pb and lobster hepatopancreas (Tort 2, National Research Council, Canada) for Cu were digested during each analytical run. Mean ± SD recoveries ($n = 6$) were 98.5 ± 2.1, 99.1 ± 4.0 and 100 ± 4.8 for total Hg, Cu and Pb, respectively, therefore no corrections were done. Detection and quantification (LOD/LOQ) limits were calculated from blank measurements ($n = 10$) being these values (in ng/g): 0.01/0.02, 0.07/0.19 and 0.19/0.29 for Hg, Pb and Cu, respectively.

2.2.2. Organochlorine compound analysis

Organochlorine compounds were extracted and analyzed following minor modifications of the procedures of Catalan et al. (2004). The egg content (1.5 g) of individual eggs was separated from the shell, weighed, ground down with activated sodium sulphate to obtain a fine powder and then introduced into previously cleaned cellulose cartridge. This mixture was Soxhlet-extracted during 24 h with *n*-hexane-dichloromethane (4:1 v/v) adding 1,2,4,5-tetrabromobenzene (TBB) and PCB 200 as recovery standards. Extracts were concentrated under vacuum and cleaned-up with sulphuric acid until a colourless transparent *n*-hexane layer was obtained. Extract solutions were then transferred to vials and evaporated just to dryness under a gentle stream of nitrogen (10–20 °C). The cleaned extract was re-dissolved in 50 μl of isooctane for instrumental analysis.

Hexachlorobenzene (HCB), α-, β- and γ-hexachlorocyclohexanes (HCHs), the congeners of PCB nos. 28, 52, 101, 118, 153 and 180, *o,p′*-DDE, *p,p′*-DDE, *o,p′*-DDD, *p,p′*-DDD, *o,p′*-DDT and *p,p′*-DDT were analyzed in a Hewlett-Packard gas chromatograph Model HP-5890 equipped with an electron-capture detector and an HP-7673-A autosampler. Separation was achieved with a 60 m × 0.25 mm I.D. DB-5 capillary column (J&W Scientific, Folsom, CA) coated with 5% phenyl methyl

Table 1
Sample size (n) and descriptive statistics for chick morphometric measurements and levels of heavy metals and organochlorine residues by species and sampling points. Within each species, different letters indicate significant ($P < 0.05$) differences following Student's t-test or ANOVA and post hoc Tukey's tests for *A. purpurea* or *A. garzetta data*, respectively. Abbreviations are explained in the text. Differences in sample size are due to missing values.

Sampling	n	Weight[a]	Tarsus[b]	Hg[c]	Pb[c]	Cu[c]	n	HCB[d]	DDTs[d]	HCHs[d]	PCBs[d]
A. purpurea chicks and feathers							*A. purpurea* eggs				
Aiguabarreig	7	659.2 ± 89.9a	83.9 ± 7.1a	1.6 ± 0.2a	1.4 ± 0.2a	8.7 ± 0.4a	10	4.1 ± 1.2a	223.7 ± 55.2a	8.2 ± 2.1b	262.2 ± 45.3a
Flix	10	646.5 ± 65.8a	88.4 ± 5.8a	8.16 ± 1.1b	1.7 ± 0.1ab	8.1 ± 0.3a	5	18.2 ± 10.4b	684.4 ± 144.3b	1.3 ± 0.4a	878.8 ± 86.5b
Delta	4	792.5 ± 144.1a	113.2 ± 13.7a	2.4 ± 0.2a	2.1 ± 0.2b	11.3 ± 0.6b	14	5.5 ± 2.1a	477.3 ± 98.8b	3.1 ± 0.8a	205.3 ± 38.3a
E. garzetta chicks and feathers							*E. garzetta* eggs				
Aiguabarreig	16	318 ± 18.2A	67.3 ± 2.7A	1.6 ± 0.3A	0.7 ± 0.2A	10.7 ± 1.1A	4	3.4 ± 1.2A	556.5 ± 111.2A	1 ± 0.2A	426.1 ± 145.3A
Delta	22	328.6 ± 10A	69.1 ± 1.8A	1.7 ± 0.1A	0.9 ± 0.1A	12.3 ± 1A	4	11.8 ± 2.2B	550.2 ± 119.7A	6 ± 4.2A	532.4 ± 200.3A

[a] Grams, average ± standard error.
[b] Millimeters, average ± standard error.
[c] Metal levels in feathers, μg/g dry weight, average ± standard error.
[d] Organochlorine residues in egg, ng/g wet weight, average ± standard error.

polysiloxane (film thickness 0.25 mm). The oven temperature was programmed from 90 °C (holding time 2 min) to 130 °C at 15 °C/min and finally to 290 °C at 4 °C/min, keeping the final temperature for 10 min. Injector and detector temperatures were 280 and 320 °C, respectively. Injection was performed in the splitless mode, keeping the split valve closed for 35 s. Helium was the carrier gas (1.5 ml/min) and nitrogen was used as the make-up gas for the detector (60 ml/min). For quantification a solution of PCB 142 was added to the vial prior to injection in order to correct for instrumental variability. Calibration curves were performed for each compound. Data were corrected for surrogate recoveries. The mean recoveries for TBB and PCB 200 were 63 ± 12 and 115 ± 15%, respectively. The quantification of blanks ($n = 10$) within each batch of analysed samples was used in order to calculate the limits of detection (LOD) of the compounds, these ranged between 0.02 ng/g for HCB, 0.04–0.09 ng/g for PCBs, 0.06–0.33 ng/g for HCHs and 0.09–0.84 ng/g for DDTs.

2.3. Biochemical determinations

TOSC was determined using gas chromatography (Winston et al., 1998; Corsolini et al., 2001). Two radicals were measured: peroxyl radicals (ROO• TOSC) were generated by the thermal homolysis of 20 mM 2–2′-azo-bis-(2 methyl-propionamidine)-dihydrochloride (ABAP) in 100 mM potassium phosphate buffer, pH 7.4. Hydroxyl radicals (OH• TOSC) were generated from the Fenton reaction of iron-EDTA (1.8 mM Fe 3+, 3.6 mM EDTA) plus ascorbate (180 mM) in 100 mM potassium phosphate buffer. Reactions were conducted at 35 °C in gastight sealed vials (Mininert valves, Supelco) in a final volume of 1 ml, and 200-μl aliquots of the headspace were analyzed at 10- to 12-min intervals for a total duration of 96 min; ethylene production was measured with a 6000 Vega Series 2 (ICU 600 Carlo ERBA Instruments) gas chromatograph equipped with a Supelco (Bellefonte, PA) packed Alumina F1 80/100 and a flame ionization detector. The oven, injection and detector temperatures were, respectively, 80, 80 and 120 °C; helium was the carrier gas (1 ml/min flow rate), and a split ratio 20:1 was used. TOSC values were quantified from the equation $TOSC = 100 - \int SA/\int CA \times 100$; where $\int SA$ and $\int CA$ are the integrated areas calculated under the kinetic curve produced during the reaction course for, respectively, sample (SA) and control (CA) reactions. Samples were diluted to obtain experimental TOSC ranging from 20 to 40, which generally corresponded to 2 μl of plasma in the assay. For all the samples, a specific TOSC (referred to 1 μl of plasma) was calculated.

Plasmatic enzymatic activities were measured at 42 °C in a Multi-Detection Microplate Reader, BioTek ® (Vermont, USA) following the methods described by Diamantino et al. (2001). For lactate deshydrogenase activity (LDH) determination, the reaction mixture contained 100 mM phosphate buffer (pH 7.5), 0.1 mM NaOH, 0.2 mM NADH and 1 mM pyruvate. LDH activity was evaluated by using the extinction factor coefficient of 6.3 mM/cm and expressed in international units ($U = \mu mol/$ min) per 1 l of plasma. BChE and CbE activities were determined using the Ellman method in the presence of 1 mM butyrylthiocholine for BChE or 0.6 mM phenylthioacetate for CbE and 0.3 mM 5,5′ dithiobis-2-dinitrobenzoic acid (DTNB), and the increase of absorbance measured at 405 nm. Activities were expressed as μmol/min per ml of plasma. Plasmatic proteins were also measured by the Bradford method using γ-globulin as standard.

2.4. Data analysis

Quantile plots and Shapiro–Wilks tests showed that data was normally distributed. Therefore differences in biometrical, contaminant residues and biochemical responses across sites and between species were compared by ANOVA procedures using Student's t-tests or one way ANOVA tests followed by post hoc Tukey's like multiple comparison tests for *A. purpurea* data (Zar, 1996). Relationships between the studied parameters were assessed using parametric Pearson correlation coefficients.

3. Results

3.1. Biometric measurements and contaminant residues

As expected from the sampling scheme, no significant differences in size among chicks of *A. purpurea* or *E. garzetta* were found across sites (Table 1).

Eggs and feathers of both species nesting at Aiguabarreig and Ebro Delta sites showed similar levels of contaminants except for Hg and Cu, which were significantly different ($P < 0.05$, based on t-tests). Analysis of the presence of different pollutants in eggs and feathers of *A. purpurea* nestlings showed significant variations in pollution loads among the three studied populations (Table 1). The Flix population showed maximal levels of Hg in feathers, and HCB, DDT and total PCBs in eggs, whereas the Aiguabarreig population showed the lowest levels for all analysed contaminants. The Ebro Delta population showed significant high levels of Pb and Cu in feathers and DDTs in eggs; in the case of the two heavy metals, the levels surpassed the values obtained in the Flix population, suggesting that the source for these pollutants should be downstream Flix. In contrast, *E. garzetta* chicks showed much less variation in contaminant loads among populations, as only HCB showed significantly high levels in eggs collected in the Ebro Delta population compared to the Aiguabarreig population (Table 1).

3.2. Biochemical responses

From the six biochemical parameters analyzed in the plasma of chicks (Table 2), only levels ROO• TOSC and the activities of BChE and CbE differed significantly between egrets and herons chicks at Aiguabarreig and Ebro Delta sites ($P < 0.05$ based on t-tests). Biochemical parameters in the plasma of *A. purpurea* chicks showed a similar profile to the one observed with contaminant levels (Table 2). The Aiguabarreig population showed a lower impact than the other two populations, whereas the Flix population showed a significantly low scavenging capacity (ROO• TOSC and Total TOSC). In addition, the Delta population showed a significant increase on LDH activity, indicating again a specific pollution source downstream Flix. None of the studied biochemical parameters showed significant differences between the Aiguabarreig and Delta populations of *E. garzetta* (Table 2). Table 2 also includes the data on MN abundance in chicks blood (Quirós et al., 2008), which agrees with the general picture of a low impact in the Aiguabarreig site compared to the other two sites. In this case, the difference between sites was observed with both species.

Table 2
Sample size (n) and descriptive statistics for protein levels, total scavenging capacity (TOSC) for peroxyl and hydroxyl radicals (ROO•, OH•), their sum (TOT), buthirylcholinesterase (BhCE), carboxylesterase (CbE) and lacatate deshydrogenase (LDH) activities and number of micronucleus (MN). Within each species different letters indicate significant ($P < 0.05$) differences following ANOVA and post hoc Tukey's tests for *A. purpurea* and Student's t-tests for *E. garzetta* data. Differences in sample size are due to missing values.

	n	Protein[a]	ROO• TOSC[b]	OH• TOSC[b]	TOT TOSC[b]	BChE[c]	CbE[c]	LDH[d]	MN[e]
A. purpurea									
Aiguabarreig	5	25.0 ± 0.7 a	17.42 ± 1.22 a	2.70 ± 0.27 a	20.10 ± 1.31 a	0.21 ± 0.02 a	2.03 ± 0.23 a	290 ± 61 a	12.1 ± 2.6 a
Flix	10	27.9 ± 2.2 a	11.11 ± 0.65 b	2.22 ± 0.14 a	13.68 ± 0.75 b	0.22 ± 0.02 a	2.90 ± 0.20 a	216 ± 20 a	68 ± 12.1 b
Delta	4	26.7 ± 5.0 a	14.10 ± 5.95 a b	2.78 ± 0.22 a	16.87 ± 3.15 a b	0.22 ± 0.01 a	2.32 ± 0.52 a	444 ± 50 b	68.6 ± 10.8 b
E. garzetta									
Aiguabarreig	11	28.4 ± 2.4 A	10.90 ± 0.57 A	4.68 ± 2.28 A	15.59 ± 2.29 A	0.28 ± 0.02 A	0.27 ± 0.02 A	252 ± 14 A	18.9 ± 5.8 A
Delta	15	28.3 ± 2.1 A	10.07 ± 0.97 A	2.67 ± 0.13 A	12.73 ± 0.94 A	0.27 ± 0.02 A	0.31 ± 0.03 A	244 ± 29 A	30.3 ± 5.1 B

[a] mg/ml plasma, average ± standard error.
[b] TOSC units/μl plasma, average ± standard error.
[c] μmol/min per ml plasma, average ± standard error.
[d] μmol/min per l plasma, average ± standard error.
[e] Counts per 50,000 particles counted (data from Quirós et al., 2008).

3.3. Relationships

Bivariate comparison of trace metals in feathers, biometric, plasmatic biochemical parameters and the frequency of micronuclei (MN) are depicted in Table 3. For brevity, only the sum of TOSC radicals (TOT TOSC) was considered. Most biochemical parameters and trace metals were independent of chick size, except for Cu and LDH in *A. purpurea* and Cu and Pb levels in *E. garzetta*. In all these cases, larger animals showed higher levels than smaller ones (Table 3). Several biochemical parameters in plasma also showed significant ($P < 0.05$) correlation with trace metals in feathers, but only for *A. purpurea*. In this species, levels of Hg were inversely related with TOT TOSC values and both Hg and Cu levels were positively related with MN indexes and LDH activities, respectively (Table 3, Fig. 2). A further partial correlation analysis accounting for tarsus length still denoted a significant ($P < 0.01$; 0.64, $n = 15$) relationship between LDH and Cu. These results indicate that the above mentioned relationships were unaffected by size. Conversely, no significant correlations were observed between metal levels and any of the studied biomarkers for *E. garzetta* samples (Table 3, Fig. 2). Organochlorine levels were measured in whole eggs and hence could not be compared with measured responses in chicks.

4. Discussion

Except for Cu and Hg levels in feathers, which were greater in *E. garzetta* and *A. purpurea* chicks, respectively, contaminant levels were similar in the studied two species regardless of their market size differences. Although *A. purpurea* eats larger (and presumable more contaminated) fish than the small mosquito fish and cray fish typically eaten by *E. garzetta* (Gonzalez-Martin et al., 1992), differences in prey contaminant load could only explain the observed greater levels of Hg in the bigger species. The highest Cu levels found in feathers of *E. garzetta* could be either related to a higher content of this metal in the diet (e.g., Cu in the carapace of crayfish) or due to interspecific differences (Kim and Koo, 2008).

Organochlorine compound levels in eggs from Flix and the Ebro Delta nesting sites of the studied heron species (HCB 3.4–18.2 ng/g ww, DDTs 223.7–684.4 ng/g ww, PCBs 262.2–878.8 ng/g ww), were moderate in relation to ranges reported in previous studies from North America (Mississippi, CA), Europe (Dabube Delta, N Italy) and Asian (Hong Kong) contaminated sites in great blue, grey and black-crowded night herons, little and great egrets (3–20 ng/g ww of HCB, 200–6000 ng/g ww PCB, 100–12,000 ng/g ww DDTs; Elliott et al., 1989; Custer et al., 1997; Fasola et al., 1998; Aurigi et al., 2000; Connell et al., 2003; Henny et al., 2008). The concentrations of Pb, Cu and total Hg at the contaminated studied sites (0.9–2.1, 8.1–12 and 1.7–8.1 μg/g ww, respectively) were within the range reported elsewhere in feathers of heron nestlings (0.1–9, 3–9 and 0.4–2 μg/g ww, respectively; Fasola et al., 1998; Custer et al., 2007, 2008), the exception being the quite high levels of Hg (>30 μg/g ww) reported by Henny et al. (2002) in heron and egret nestling feathers from Carson River (Nevada).

Concentrations of *p,p′*-DDE and PCBs above 4000 and 12,000 ng/g ww in eggs could be regarded as concentrations affecting reproductive success in herons (Fasola et al., 1998). In the present study, over 95% of measured total DDT levels were *p,p′*-DDE and averaged levels of DDTs and PCBs in eggs were roughly one order of magnitude lower than those reported to affect the reproduction of herons.

A study on mercury effects on birds from the Carson River (NV) found severe adverse health effects of Hg in hero and egret chicks having 30–35 μg/g ww of Hg in their feathers (Henny et al., 2002). Studying the distribution of heavy metals in two heron species from Korea, Kim and Koo (2008) concluded that levels of Cu and Pb in feathers ranking from 9–30 to 0.2–0.5 μg/g ww, respectively, were within the background levels of wild herons and far below those having adverse effects.

The above mentioned Hg levels are about four fold the average level of the Flix population, the most contaminated one in our study. Similarly, levels of Cu and Pb found in our study are within the background range for herons. Taking this into consideration it is unlikely that the measured contaminants could cause acute effects on the studied populations. However, sublethal effects are likely to occur at much lower concentrations than those at which pathological effects become pronounced.

The studied biochemical response in plasma and of micronuclei frequency in blood of chicks varied across species and nesting sites. TOT TOSC ranks in both species (12.16–20.48 U/μl plasma) were of similar magnitude to those reported by Corsolini et al. (2001) in Adélie and Emperor penguins from Wood Bay, North Victoria (10–25 U/μl plasma). Activity levels of BChE varied little in both species (0.21–0.28 μmol/min per ml), but plasmatic CbE activities in *E. garzetta* (0.27–0.31 μmol/min per ml) were ten fold lower than those of *A. purpurea* (2–2.9 μmol/min per ml). Reported values for plasmatic B esterase activities were highly variable across bird species ranging from 0.7 to 5 μmol/min per ml for BChE and from 0.2 to 11 μmol/min per ml for CbE (Fossi et al., 1996). According to Fossi et al. (1996), B esterase activities tend to change depending on specimen size or species diet. In our study the observed differences in CbE activities between chicks of *E. garzetta* and *A. purpurea* could be explained by differences in weight (a factor of 2) since both are piscivorous. Measured plasmatic LDH activities in the studied chick

Table 3
Bivariate Pearson correlations of log transformed studied parameters by species. Abbreviations are explained in the text.

Species	TOT TOSC	LDH	MN	BChE	CbE	Lt	W	Hg	Pb	Cu
A. Purpurea										
Proteins	0.08 (18)	−0.19 (18)	−0.08 (18)	0.31 (18)	0.27 (18)	0.13 (18)	0.20 (17)	0.10 (18)	−0.0 (18)	−0.21 (18)
TOT TOSC		0.24 (18)	−0.41 (18)*	−0.27 (18)	−0.44 (18)*	0.32 (18)	0.35 (17)	−0.57 (18)**	0.04 (18)	0.39 (19)
LDH			0.05 (18)	0.29 (18)	−0.22 (18)	0.47 (18)*	0.46 (17)*	−0.43 (18)*	0.35 (18)	0.78 (18)***
MN				0.01 (18)	0.14 (18)	0.16 (19)	−0.22 (17)	0.64 (19)***	0.37 (19)	0.05 (19)
BChE					0.31 (18)	0.27 (18)	0.35 (17)	0.11 (18)	−0.02 (18)	0.12 (18)
CbE						−0.39 (18)	−0.37 (17)	0.39 (18)	−0.24 (18)	−0.28 (18)
Lt							0.90 (17)***	0.05 (21)	0.26 (17)	0.62 (21)***
W								−0.24 (17)	−0.14 (17)	0.38 (17)
Hg									0.16 (21)	−0.28 (21)
Pb										0.35 (21)
E. garzetta										
Proteins	−0.14 (14)	−0.28 (14)	−0.43 (12)	0.39 (14)	0.28 (14)	−0.07 (13)	0.30 (14)	0.27 (14)	0.14 (14)	−0.31 (14)
TOT TOSC		−0.28 (13)	−0.22 (14)	0.01 (14)	−0.27 (14)	−0.25 (15)	−0.35 (16)	0.38 (16)	−0.37 (16)	−0.22 (16)
LDH			0.35 (13)	0.06 (14)	−0.08 (14)	0.11 (14)	−0.12 (15)	0.08 (15)	0.38 (15)	0.01 (15)
MN				0.11 (12)	0.06 (12)	−0.13 (27)	−0.23 (27)	−0.08 (27)	−0.22 (27)	−0.12 (27)
BChE					0.34 (14)	0.14 (13)	0.22 (14)	−0.03 (14)	0.27 (14)	−0.23 (14)
CbE						−0.24 (13)	−0.17 (14)	0.10 (14)	0.30 (14)	−0.18 (14)
Lt							0.88 (38)***	−0.19 (37)	0.37 (38)**	0.52 (38)***
W								0.08 (38)	0.29 (38)	0.36 (38)**
Hg									−0.04 (38)	0.44 (38)***
Pb										0.56 (38)***

Sample size is depicted between brackets. Differences in sample size are due to missing values *$0.05 < P < 0.1$; **$0.01 < P < 0.05$; ***$P < 0.01$.

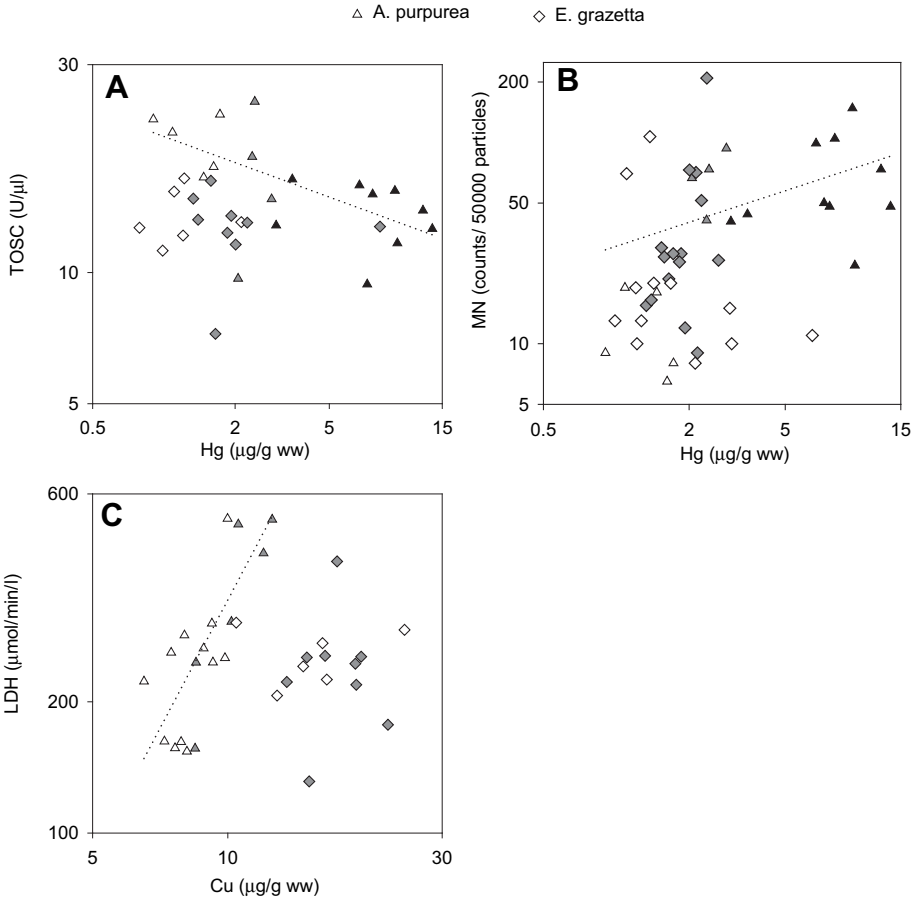

Fig. 2. Bi-plots of mercury levels in feathers versus total scavenging capacity – TOT TOSC (A), micronucleated erythrocites MN (B) and of levels of Cu versus lactate deshydrogenase – LDH (C) in *A. purpurea* and *E. garzetta*. Each symbol corresponds to a single chick. Dotted lines indicate significant linear relationships, which only occurred in *A. purpurea* ($P < 0.05$). White, black and grey symbols correspond to values from Aiguabarreig, Flix and Delta sites, respectively. Data are depicted in log scale.

species (216–444 U/l) were comparable to those reported in nestlings and fledglings of great egret and great blue herons (200–1700 U/l; Hoffman et al., 2005; Champoux et al., 2006).

Reported nesting site effects on ROO•/TOT TOSC and micronuclei frequency provided the first evidence of detrimental effects of pollutants released by a chlor-alkali plant in chicks from heron species. In particular, nestling populations of *A. purpurea* chicks near the chlor-alkali plant showed the highest frequency and levels of micronuclei, Hg, HCB, DDTs and PCB and a reduced buffering capacity detoxify ROS (lowest values for ROO• or TOT TOSC). Moreover TOT TOSC was inversely related with mercury levels in feathers and micronuclei frequency. The observed lack of biological responses in *E. garzetta* chicks could be related to the absence of nesting sites at the most contaminated location (Flix). Exposure to various organic and inorganic contaminants are known to reduce TOT TOSC in bivalves and fish species and increase oxidative stress (Regoli, 2000; Regoli et al., 2005). More specifically, lab exposures showed that Cd, Cu, PCBs and PAHs caused oxidative stress, diminishing TOSC in bivalves (Lehmann et al., 2007; Company et al., 2008). Although there are no reported studies on effects of mercury in TOT TOSC, there is substantial information indicating that mercury decreases antioxidant defenses causing oxidative stress in adults and nestlings of egrets and herons (Hoffman et al., 2005; Henny et al., 2002). Therefore the observed site differences in ROO• or TOT TOSC in *A. purpurea* chicks agrees with previous studies and could be related to exposure to contaminants such as Hg, PCBs or DDTs.

Plasmatic B esterases like those in liver are known to act as alternative target sites for anticholinergic compounds, thus protecting brain and muscle cholinesterases from inhibitory substances (Chambers et al., 1994). Activity levels of B esterases were similar across sites in both species, thus indicating low or no exposure to anticholinergic compounds. This finding is environmentally relevant since the use of large quantities of organophosphate and carbamate insecticides in Ebro's Delta in the past decades had dramatic effects in wildlife (Mañosa et al., 2001).

Like the other parameters, only in *A. purpurea* were there significant differences across sites in LDH activity with chicks nesting at the Ebro Delta showing the greatest activities. LDH activities were also positively related with levels of Cu in feathers, and although marginally ($0.05 < P < 0.1$), inversely related with Hg. Exposure to mercury chloride and to the PCB mixture Aroclor 1254 has been observed to increase plasmatic LDH of the quail *Coturnix coturnix* (Dieter, 1974). In contrast, methylmercury inhibited plasmatic LDH activities of great egret nestlings (Hoffman et al., 2005). In the present study, total mercury levels measured in feathers were likely to be mainly methylmercury given that this is the preferred bioaccumulative form through the aquatic food web and for piscivorous birds that are at the top of the food chain (Henny et al., 2002). Under this point of view, the observed inverse relationship between LDH activity and Hg in chicks agrees with previous studies. In contrast, the observed higher LDH activities in Ebro Delta chicks and its direct correlation with Cu levels in feathers are difficult to explain. Observed differences in Cu levels and probably of LDH activities across Ebro Delta with the other studied nesting sites may be related to differences in diet or habitat related as Ebro Delta colonies were located near the sea (Champoux et al., 2006). Alternatively, the widespread use of herbicides such as propanil and endosulfan in the Ebro Delta (Mañosa et al., 2001), with known oxidative stress and LDH effects in mammals (Omurtag et al., 2008; Rankin et al., 2008) may have affected *A. purpurea* plasmatic parameters. Indeed, increased oxidative stress levels in chicks nesting at Ebro Delta may also explain the quite high frequency of micronuclei reported by Quirós et al. (2008) in both species.

In summary our results support previous findings (Quirós et al., 2008) and provide the first evidence of an association of biological responses with pollutants released by a chlor-alkali plant in chicks from heron species. In particular, in this study two impacted sites with altered biological responses on chick blood parameters have been identified: *A. purpurea* chicks nesting in a chlor-alkali impacted area (Flix) showed high frequency of micronuclei and high levels of Hg, HCB and PCB and a reduced buffering capacity to detoxificate reactive oxygen species. In Ebro Delta, biological response changes included high levels of plasmatic LDH in *A. purpurea* chicks and high frequency of micronuclei in blood of both species. We tentatively relate these alterations to the intensive agricultural activities taking place at the Ebro Delta.

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