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Pollutant accumulation patterns in nestlings of an avian top predator: biochemical and metabolic effects

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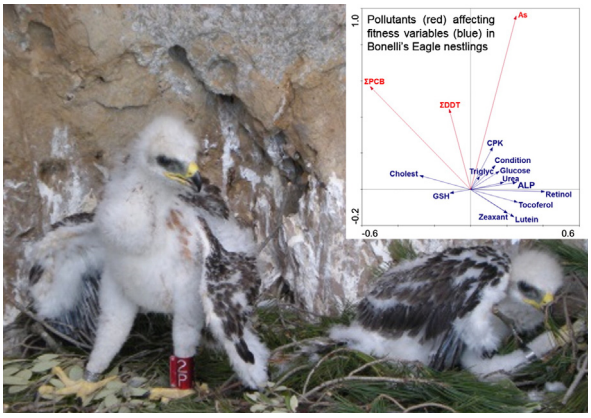
HIGHLIGHTS

- We studied organochlorine and metal accumulation in the endangered Bonelli's eagle.
- High PCBs and As levels in industrialized areas were related to oxidative damage.
- Increased plasma DDT level was associated with signs food stress.
- Hg uptake from non-preferred prey was inversely related to nestling productivity.
- Territory quality is essential to reduce pollutant exposure in sedentary raptors.

GRAPHICAL ABSTRACT



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ABSTRACT

The exposure to persistent pollutants such as organochlorine compounds (OCs) or metals has been associated with declines in top predator populations, which can accumulate high amounts of these pollutants from their prey. However, understanding how variation in OC and metal accumulation in wild species affects their biochemical and physiological responses is a big challenge, especially for endangered predators like the Bonelli's eagle (*Aquila fasciata*). This bird of prey is an interesting study model because the differences in diet composition among populations and territories can account for important pollutant uptake variations. We compared OC and metal accumulation in blood of Bonelli's eagle nestlings from three populations across Spain as a function of origin, age class (nestlings vs. adults), sex and number of siblings per nest, and related accumulation patterns to responses indicative of body condition, biochemistry and antioxidant status. Nestlings from Catalonia, the most industrialized area, showed the highest concentrations of PCBs and arsenic, and the lowest concentrations of zinc. The two former substances, together with DDTs, exerted an overall influence on nestling's physiology.

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PCBs and arsenic were associated with reduced retinol levels, pointing to oxidative damage in exposed individuals, which was also consistent with the low zinc levels in individuals from the polluted region. Increased plasma DDT levels were related to reduced body condition and lower levels of triglycerides. Mercury accumulation in Castile and Leon was higher in nestlings that were alone in the nest than in nestlings that shared it with a sibling; this suggests an increased mercury uptake from secondary prey in territories where preferred prey (i.e. rabbits) are scarce, which are also the territories where productivity is reduced. Overall, the results reveal a spatial variation in pollutant accumulation patterns and associated physiological effects, and suggest the major role that territory quality may have in such patterns.

1. Introduction

The accumulation of persistent contaminants in natural food webs constitutes one of the main toxic risks for top predators (Drouillard et al., 2001; Henny et al., 2003). Organochlorine compounds (OCs) are released as part of many industrial activities and have potential for long-distance transportation and further atmospheric deposition in apparently pristine areas (Elliott et al., 2012). Furthermore, because of their high persistence and slow environmental degradation, traces of the formerly used organochlorine insecticides (e.g. DDT, lindane) are still found in agricultural areas, several decades after their replacement by less persistent pesticides (Orton et al., 2013). Metals are also present in the environment at concentrations unnaturally high because of human activities, including mining or groundwater extraction that contribute to mobilize metals present in the Earth's crust, but also within industrial effluents, as part of some pesticide applications (e.g. copper-based fungicides) (Facchinelli et al., 2001) or, in the case of lead, as hunting ammunition (Mateo et al., 2014). Because OCs, as well as some metals like methylmercury, show potential for bioaccumulation and biomagnification in trophic webs, they pose a risk for top predators like raptors, which can ingest large amounts of contaminated prey and accumulate pollutant levels capable of affecting their health.

One of the responses most commonly associated with pollutant exposure is oxidative stress; the metabolism of harmful chemical substances, including OCs and metals, generates reactive oxygen species (ROS), which interact with biological systems causing an unbalance in the redox status that can damage membrane lipids, proteins and nucleic acids (Apel and Hirt, 2004). To cope with oxidative stress, the organism possesses an antioxidant system that consists of a series of enzymes (e.g. superoxide dismutase –SOD– and glutathione peroxidase –GPx–) and antioxidant molecules (e.g. glutathione –GSH–). Furthermore, some exogenous substances like vitamins or carotenoids, available only from the diet, might also play an important role in the antioxidant system. Carotenoids can capture free electrons from the oxygen radicals (Young and Lowe, 2001), although some studies question their antioxidant role in birds (Costantini and Møller, 2008). α-Tocopherol, the most common form of vitamin E, can neutralize the lipid radicals generated during lipid oxidation, thus preventing from the lipid peroxidation chain reaction to continue. Oxidized α-tocopherol can be transformed back and reused through a reduction in which another vitamin, retinol (i.e. the active, antioxidant form of vitamin A) is involved (Sies et al., 1992).

Much of the information about biochemical and metabolic responses of animals to pollutant exposure is retrieved from laboratory model species, which, in the case of birds, are mostly poultry and small or medium-sized wild species. Raptors have been identified as sentinels for biomonitoring pollutants and their potential toxic effects (see reviews in Henny and Elliott, 2007 and Gómez-Ramírez et al., 2014) because their high position in trophic webs makes them susceptible of pollutant accumulation. However, the difficulties of working with wild raptors have constrained the number of studies relating physiological responses with pollutant exposure in this group of birds. For instance, Sonne et al. (2012) found significant correlations between several organohalogen compounds and a number of biochemical parameters in raptors from Norway, suggesting the alteration of biochemical pathways because of pollutant exposure; however, they could not elucidate whether such alteration would manifest in health effects. Due to the conservation problems associated with pollutant exposure and accumulation in some endangered raptor species such as the Bonelli's eagle (*Aquila fasciata*) in Europe (BirdLife International, 2004), raptors in general, and endangered species in particular, should be studied as a priority.

The Bonelli's eagle is a medium-sized, long-lived, territorial raptor distributed from southeast Asia and the Middle East to the western Mediterranean (Ferguson-Lees and Christie, 2001). The European population of the species declined a 20–50% over the last three decades of the 20th century (Real and Mañosa, 1997). In Spain, where about three fourths of the European population inhabit, population declines are mostly attributed to habitat degradation because of changes in land-use, involving the development of urban areas and infrastructures as well as abandonment of traditional extensive farming, decrease of prey availability and, especially, increased mortality caused by persecution (e.g. poisoning and shooting by hunters and pigeon fanciers, Real et al., 2001) and electrocution in power lines (Ontiveros et al., 2004). These factors cause a demographic imbalance associated with the increased pre-adult and adult mortality (Hernández-Matías et al., 2013, 2015).

The Bonelli's eagle is a suitable model species for studying pollutant accumulation and its relationship with biochemical and metabolic biomarkers because of its marked intra- and inter-population dietary differences across its western European distribution range (Moleón et al., 2012a; Resano-Mayor et al., 2014a), which is usually related to different environmental characteristics among territories. Due to the capacity of Bonelli's eagle to exploit a wide variety of food resources, its diet depends on the availability of optimal and suboptimal prey species (Resano et al., 2011; Moleón et al., 2012a,b; Resano-Mayor et al., 2014a). In consequence, pollutant accumulation can be highly variable among different territories depending on the diet composition (Palma et al., 2005). We tested the hypothesis that pollutant accumulation will influence the antioxidant status and general biochemistry of individuals, and that such influence will vary among regions with expected differences in pollutant sources and diet composition. With this purpose we collected blood samples of nestlings and adults from three different populations across Spain to analyse pollutant accumulation (OCs and metals), as well as plasma biochemical parameters, vitamins, carotenoids, and oxidative stress biomarkers.

2. Materials and methods

2.1. Study area and data collection

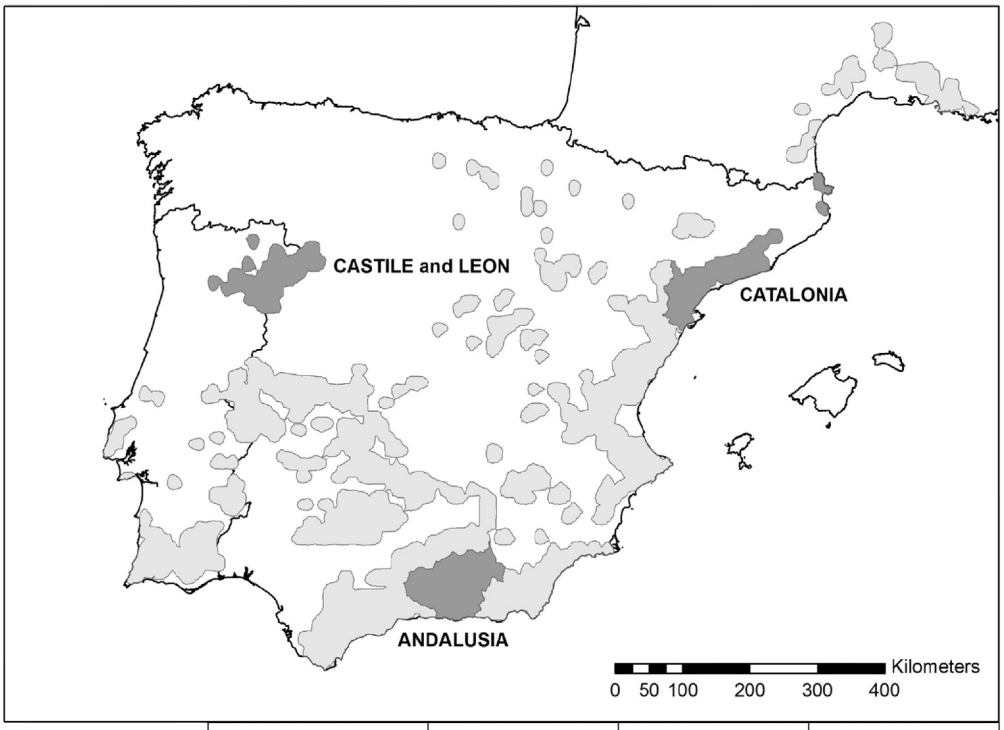
During 2010 and 2011 we monitored 57 breeding territories of Bonelli's eagle in three local populations across Spain: Catalonia, Castile and Leon, and Andalusia (Fig. 1). Catalonia, in spite of being one of the most industrialized and highly populated regions in Spain, supports a good population in terms of breeding density and fledging productivity, though adult survival shows relatively low values. In Castile and Leon, where the Bonelli's eagle population mainly occupies the protected area of the Natural Park of Arribes del Duero, the population shows a poor conservation status and all demographic parameters show very low values. In contrast, Andalusia supports the largest population of

Bonelli's eagle in Europe, with about 35% of the breeding pairs of the continent (del Moral, 2006), and the highest values of density, productivity and adult survival. This region is also where the largest percentage of preferred prey (i.e. European rabbits *Oryctolagus cuniculus* and Red-legged partridges *Alectoris rufa*; Moleón et al., 2012b) consumption has been observed (Resano-Mayor et al., 2014a).

Between January and March we checked the presence of territorial birds and breeding activity (i.e. incubation behaviour). In late March and April, occupied nests were checked to detect the presence, number (i.e. one or two), and age of nestlings, which was estimated by backdating from laying date (Gil-Sánchez, 2000). In total, 71 occupied nests were monitored and 117 nestlings were sampled. Once nestlings were 30–50 days old, experienced climbers accessed breeding nests to catch the chicks, which were safely transported up to the cliff for morphometric measurements and blood collection. Morphometric measurements included body mass (nearest 1 g) and tarsus length (nearest 0.01 mm), which were used to calculate individual body condition using the scaled mass index (M) reported by Peig and Green (2009). This index is based on the equation $M = M_i(L_a / L_i)^{b_{SMA}}$, where M_i and L_i are the individual mass and length, respectively, L_a is the average length considering all individuals, and b_{SMA} is the quotient between the slope of the regression of the natural log-transformed body mass on the natural-log transformed length of each individual and the Pearson's correlation coefficient of such regression. We also measured the length of the seventh primary to estimate nestling age with high accuracy (Mañosa et al., 1995), although this parameter was used only to determine the range of ages in our nestling samples and was not used in further statistical analyses. Blood (2 ml) was collected in heparinized vials from the brachial vein and kept refrigerated until processed in the laboratory within 12 h. Once in the lab, blood samples were centrifuged to separate plasma from the blood pellet. Both sets of samples were immediately frozen at −80 °C until analysis. Plasma was used to quantify OCs, vitamins, carotenoids and 14 different biochemical parameters. Blood pellet was used to quantify metal contents, to measure oxidative stress biomarkers and to sex each individual following the method by Fridolfsson and Ellegren (1999). In addition, seven territorial adults were captured in Catalonia (four males and three females), and morphometric measurements and blood samples were also collected from those individuals.

2.2. Organochlorine analysis

Solvents and reagents used were analytical grade or equivalent high purity grade and purchased from Merck (Darmstad, Germany) or Panreac (Montcada i Reixac, Spain). Mixtures of pesticides and PCBs (Pesticide-Mix 13 and PCB-Mix 20) and PCB 209 standard were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Pesticide-Mix 13 includes aldrin, cis-chlordane, trans-chlordane, o,p′-DDE, p,p′-DDE, o,p′-DDD, p,p′-DDD, o,p′-DDT, p,p′-DDT, dieldrin, α-endosulfan, β-endosulfan, endrin, HCB, α-HCH, β-HCH, γ-HCH, δ-HCH, ε-HCH, heptachlor, heptachlor-exo-epoxide, isodrin, methoxychlor, mirex and PCBs 28, 52, 101, 138, 153, and 180. PCB-Mix 20 includes PCBs 28, 31, 52,



Population	Density (pairs/ha)	Productivity (nestlings/ breeding pair)	Adult survival rate
Andalusia	0.85	1.230	0.926
Castile and Leon	Not available	0.605	0.883
Catalonia	0.64	1.130	0.889

Fig. 1. Distribution of Bonelli's eagle in the Iberian Peninsula (shadowed areas), and location and main demographic characteristics (retrieved from Hernández-Matías et al., 2013) of the three studied populations (dark–grey shadowed).

77, 101, 105, 118, 126, 128, 138, 153, 156, 169, 170 and 180. Sample preparation, based on the sulphuric acid clean-up of an n-hexane extraction procedure, was performed as described previously (Mateo et al., 2012).

Chromatographic analyses were carried out on an Agilent Technologies 6890 N series equipped with a 30 m fused silica capillary column of 0.32 mm ID and 0.25 μm of film thickness (HP-5 from J&W Scientific, USA), coupled to an electron capture detector (ECD), in conditions optimized for the analytes. The oven initial temperature was 145 °C, then raised to 275 °C at a rate of 2.5 °C/min. Injector and detector temperatures were 290 °C and 310 °C, respectively. Carrier gas (He) was set at an average velocity of 52 cm/s. Make-up gas (N_2) was adjusted at a flow of 30 ml/min. Organochlorine pesticides and PCB congeners were identified by their retention time. Quantification of organochlorine pesticides was done with calibration curves prepared with Pesticide-Mix 13. Individual PCBs were quantified with calibration curves of the congeners present in PCB-Mix 20. Concentrations of individual PCB congeners as well as the sum of PCBs (ΣPCB) were given for those present in PCB-Mix 20. The recovery of the method was calculated with plasma samples spiked with Pesticide-Mix 13 at four different concentrations ($n = 3$ per concentration level). Except for cyclodienes such as endrin and dieldrin that are completely lost in the clean-up step, the recovery of all analysed compounds ranged from 79.1% to 107.5%. Corrections based on recovery data were not taken into account for quantification. Detection limits of pesticides and PCB congeners, calculated as 3SD of peak integrations obtained from blanks, were all <0.01 ng/ml. As an approach to estimate potential toxicity of accumulated PCBs, we converted tissue concentrations of coplanar PCBs present in PCB-Mix 20 into toxic equivalents (TEQ). These coplanar PCBs were 105, 118, 126, 156 and 169. Toxic equivalency factors (TEF) were retrieved from Van den Berg et al. (2006) and multiplied by the concentration of each corresponding congener to obtain TEQ.

2.3. Metal analysis

Blood pellets were freeze-dried (Christ Alpha 1e2, Braun Biotech) and analysed for lead (Pb), cadmium (Cd), arsenic (As), copper (Cu), zinc (Zn) and mercury (Hg). All concentrations are given relative to dry weight (d.w.). Pb, Cd and As were analysed using graphite-furnace atomic absorption spectroscopy (GF-AAS; AAnalyst800 with autosampler AS800, Perkin-Elmer). For Cu and Zn we used flame-AAS, and Hg was analysed using a mercury/hydride system (MHS-15, Perkin-Elmer) coupled to the AAS. Dried samples were digested in a quartz digest tube with 1 ml of HNO_3 (69%) and heated overnight at 70 °C. Then, we added 1 ml of H_2O_2 (30% v/v) and heated the tube at 110 °C for 6 h. Digest solutions were diluted to a final volume of 15 ml with Milli-Q grade water. We prepared calibration standards from commercial solutions containing 1 g/l of each element (Panreac) and Milli-Q water. A certified reference material (Lobster Hepatopancreas; TORT-2, National Research Council, Canada) with certified levels (μg/g; mean ± 95% CI) of Pb (0.35 ± 0.13), Cd (26.7 ± 0.6), As (21.6 ± 1.8), Cu (106 ± 10), Zn (180 ± 6), Hg (0.27 ± 0.06) was analysed ($n = 6$) to ensure the quality of the methodology. Mean ± SD recovery values were 83.0 ± 15.0% for Pb, 99.1 ± 9.5% for Cd, 113.9 ± 12.4% for As, 104.7 ± 16.5% for Cu, 137.6 ± 24.5% for Zn, and 105.3 ± 16.5% for Hg. The detection limits were 0.030 μg/g Pb, 0.006 μg/g Cd, 0.072 μg/g As, 0.005 mg/g Cu, 0.006 mg/g Zn, and 0.052 μg/g Hg.

2.4. Oxidative stress biomarkers and biochemistry

We measured oxidative stress biomarkers in blood pellets, previously homogenized in phosphate buffer, following the spectrophotometrical methods described in Reglero et al. (2009). We quantified lipid peroxidation levels, estimated as thiobarbituric acid reactive substances (TBARS), as well as total (GSH) and oxidized (GSSG) glutathione concentrations. We used Ransel and Ransod kits (Randox Laboratories, Crumlin, UK) to measure the activities of glutathione peroxidase (GPX, EC 1.11.1.9) and superoxide dismutase (SOD, EC 1.15.1.1), respectively. Enzyme activities were calculated relative to mg of protein.

We determined the levels of free retinol, α-tocopherol and carotenoids (zeaxanthin and lutein) in plasma using high performance liquid chromatography (HPLC, Agilent Technologies 1100 Series) coupled to a photodiode detector (DAD) and a fluorescence detector (FLD) following the extraction and chromatographic methods described in Rodríguez-Estival et al. (2010).

We measured the following biochemical parameters in plasma samples with an automatic spectrophotometer analyser A25 using the reaction kits available for each enzyme or analyte (BioSystems, Barcelona, Spain): alkaline phosphatase (ALP), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), total protein, glucose, cholesterol, triglycerides, calcium, magnesium, phosphorus, creatinine, urea and uric acid.

2.5. Data analysis

We checked the effects of local population, age (nestlings vs. adults from Catalonia), sex, and number of siblings per nest on log-transformed pollutant levels with general linear models (GLM). For statistical analysis, we assigned samples below detection limit a value equal to half the detection limit. To assess the effects of contaminant levels on the physiological variables and nestling body condition (used as response variables), we firstly performed a detrended correspondence analysis (DCA) that revealed a gradient length < 3 for axis 1, so we decided to use a model with linear response curve. Therefore, we conducted a redundancy analysis (RDA) (ter Braak, 1994) run on a correlation matrix. The most significant variables were determined using a forward selection according to a Monte Carlo permutation test (999 permutations). In order to test the effects of each pollutant on the response variables, we ran bivariate correlations of every fitness (i.e. oxidative stress, biochemistry and body condition) variable with the contaminants selected by the RDA as significant to explain health changes. For the rest of the pollutants, we applied a Bonferroni correction in order to minimize the risk of type I error after multiple comparison testing. In order to check the potential influence of the population of origin on the relationship between contaminants and fitness variables, we ran for each significant correlation a generalized linear model (GzLM) adjusted to a linear response, with the fitness variable as dependent, the population as fixed factor, and the pollutant concentration as covariate. We tested for the effect of interaction between population and pollutant on the dependent variable, and when significant, we explored the correlation between pollutant and fitness variable in each population separately. RDA was conducted with CANOCO 4.5 (Microcomputer Power, Ithaca, NY, USA), and the rest of data analyses with SPSS Statistics 21 (IBM, Armonk, NY, USA).

3. Results

3.1. Contaminant levels by local population, age, sex and number of siblings

Less than 3% of the analysed samples had levels of Cd, Cu, PCB 77, o,p′-DDD, o,p′DDE and organochlorine insecticides other than DDTs above detection limits, so we did not consider these chemicals in further analyses. Nestlings from Catalonia had the highest plasma levels of ΣPCB and of most individual congeners in the PCB-Mix 20 (all post-hoc $p < 0.001$), whereas nestlings from Andalusia showed the lowest OC levels (Table 1). TEQ were also higher in nestlings from Catalonia than in those from the other two populations (post-hoc $p \leq 0.036$). Within Catalonia, adults showed higher levels of most PCB congeners ($p \leq 0.049$ for the pool of significantly different congeners, Table 1), as well as of TEQ ($p = 0.035$), than nestlings, although no differences were detected in ΣPCB ($p = 0.087$). The only case in which Catalonian

nestlings showed higher plasma concentrations than adults was that of PCB congeners 28 and 31 ($p = 0.017$). We did not find significant differences among populations or between age groups (nestlings vs. adults) within Catalonia either in total DDTs (ΣDDT, the sum of all isoforms of DDT and its metabolites) or in any particular parent compound or metabolite isoform. Neither sex nor number of siblings had any effect on OC levels in plasma of Bonelli's eagles.

Concentrations of As in nestlings' blood pellet were significantly higher in individuals from Catalonia (post-hoc $p \leq 0.013$, Table 2) than in the other two populations, while Zn levels were lower in Catalonia (post-hoc $p < 0.001$). Furthermore, Zn levels were higher in nestlings than in adults from Catalonia ($p < 0.001$). We only found a sex-dependent effect in accumulation of Zn in adults from Catalonia (Mean ± SD: males: 22.11 ± 0.52 μg/g d.w.; females: 24.99 ± 0.46 μg/g d.w. $p = 0.001$), although the sample size was too low (4 males and 3 females). No sex effects were found in metal accumulation in nestlings. Number of siblings related to a difference in Hg values in blood pellet of nestlings from Castile and Leon ($p = 0.014$), with higher values in nestlings that were alone in their nests (mean ± SD: 0.189 ± 0.15 μg/g d.w.) than in nestlings sharing the nest with a sibling, which showed Hg concentrations below the detection limit (0.052 ng/g d.w.).

3.2. Influence of contaminant levels on nestling physiology and body condition

The results of body condition, biochemical parameters and oxidative stress biomarkers per population and age class (nestlings vs. adults) are shown in Table 3. Three contaminants were selected in the RDA as significantly influencing the full set of fitness variables measured in nestlings: ΣPCB, ΣDDT and As (Fig. 2). Thus, we considered only three canonical axes in the RDA, which explained a low cumulative percentage variance of species occurrence data (6.4%) (Table 4). However, there was a strong relationship between the contaminant levels and responses of nestlings, with a responses–predictors correlation of 0.68 in the first axis. Carotenoids and free vitamin forms were the response variables most negatively correlated with ΣPCB ($R \leq -0.321$, $p \leq 0.001$) and As ($R \leq -0.209$, $p \leq 0.025$) accumulation. However, when we controlled the effect by population, only retinol levels were negatively associated with ΣPCB ($\chi^2 = 6.967$, 2 d.f., $p = 0.031$) and As ($\chi^2 = 9.869$, 2 d.f., $p = 0.007$). Increasing concentrations of ΣPCB and As were associated with reduced retinol plasma levels in nestlings from Catalonia (Fig. 3). The three pollutants were selected by the model as significantly related to increased plasma concentrations of CPK ($R \geq 0.222$, $p \leq 0.018$), although only in the case of As this relationship was not related to differences between populations ($\chi^2 = 6.278$, 2 d.f., $p = 0.043$); the positive correlation between CPK and As was observed in nestlings from Andalusia ($R = 0.407$, $p = 0.025$). Levels of As also correlated with increased levels of glucose in plasma ($R = 0.230$, $p = 0.013$), but in this case the relationship was not independent of the regional variability ($\chi^2 = 1.490$, 2 d.f., $p = 0.475$). ΣPCB plasma concentrations correlated positively with cholesterol and GSH ($R \geq 0.221$, $p \leq 0.041$), and negatively with urea and ALP ($R \leq = -0.196$, $p \leq 0.037$). The only case in which we found that the relationship between ΣPCB and plasma biochemistry was not consequence of differences among populations was that of ALP ($\chi^2 = 6.551$, 2 d.f., $p = 0.038$); we observed a negative correlation between ΣPCB and ALP plasma levels in nestlings from Castile and Leon (Fig. 4). Plasma levels of ΣDDT increased with decreased body condition and plasma concentration of triglycerides ($R \leq -0.229$, $p \leq 0.022$). The relationship with body condition remained after controlling inter-population variability ($\chi^2 = 8.054$, 2 d.f., $p = 0.018$); significant negative relationships of ΣDDT were observed with body condition of nestlings from Castile and Leon and Catalonia (Fig. 5).

Concerning pollutants not selected by the RDA as significant for the full set of variables, after controlling for inter-population effects by means of the GzLM and further application of Bonferroni corrections, we only found significant relationships of Zn levels with retinol ($\chi^2 =$

Table 1
Geometric means and (maximum) levels of organochlorine compounds (ng/ml) in plasma samples of Bonelli's eagle nestlings and adults from Spain: Andalusia, Castile and Leon and Catalonia. Superscript letters define significant differences among nestlings from different populations. Asterisks (*) indicate significant differences between adults and nestlings from Catalonia. Limit of detection: 0.01 ng/ml. PCB congeners 28 and 31 are shown together because they co-eluted.

	Andalusia (nestlings)	Castile and Leon (nestlings)	Catalonia (nestlings)	Catalonia (adults)
N	31	11	71	7
PCB 28 + 31	0.02^a	0.29^b	1.79^c	0.06*
	(5.38)	(6.19)	(64.11)	(3.88)
PCB 52	0.01	0.01	0.02	0.02
	(0.63)	(0.49)	(7.29)	(1.03)
PCB 101	<0.01	<0.01	0.01	0.01
	(<0.01)	(<0.01)	(1.69)	(0.18)
PCB 105	$<0.01^a$	$<0.01^a$	0.02^b	0.13
	(<0.01)	(<0.01)	(4.72)	(1.65)
PCB 118	0.05^a	0.33^a	1.32^b	3.08
	(3.29)	(1.50)	(16.71)	(10.23)
PCB 126	0.01^a	0.01^{ab}	0.03^b	0.35*
	(1.35)	(0.91)	(4.01)	(3.93)
PCB 128	0.01^a	$<0.01^a$	0.06^b	0.24
	(0.84)	(<0.01)	(7.46)	(2.94)
PCB 138	0.01^a	0.26^b	1.33^c	7.85*
	(3.85)	(1.86)	(88.82)	(35.71)
PCB 153	0.02^a	1.03^b	3.81^c	17.65*
	(8.69)	(6.13)	(152.00)	(66.67)
PCB 156	0.02^a	0.06^{ab}	0.23^b	1.77*
	(0.92)	(1.39)	(8.87)	(6.12)
PCB 169	0.01	<0.01	<0.01	<0.01
	(0.49)	(<0.01)	(<0.01)	(<0.01)
PCB 170	0.01^a	0.24^b	1.25^c	4.90
	(1.20)	(3.83)	(36.87)	(16.88)
PCB180	0.01^a	0.36^b	0.49^b	14.41*
	(4.64)	(5.21)	(105.38)	(43.55)
ΣPCB	0.58^a	6.59^b	17.67^c	54.06
	(21.45)	(17.51)	(425.49)	(187.84)
p,p′-DDE	0.03	0.06	0.10	0.02
	(8.65)	(7.79)	(174.73)	(68.97)
p,p′-DDD	<0.01	<0.01	0.01	<0.01
	(<0.01)	(<0.01)	(6.98)	(<0.01)
o,p′-DDT	<0.01	<0.01	0.01	<0.01
	(<0.01)	(<0.01)	(2.37)	(<0.01)
p,p′-DDT	<0.01	<0.01	0.01	<0.01
	(<0.01)	(<0.01)	(2.91)	(<0.01)
ΣDDT	0.03	0.06	0.14	0.02
	(8.65)	(7.79)	(177.11)	(68.97)
TEQ ($\times 10^3$)	0.01^a	0.05^a	0.84^b	21.78*
	(0.13)	(0.91)	(0.40)	(0.39)

Table 2
Geometric means and (maximum) levels of metals (μg/g dry weight) in blood pellet samples of Bonelli's eagle nestlings and adults from Spain: Andalusia, Castile and Leon and Catalonia. Superscript letters define significant differences among nestlings from different populations. Asterisks (*) indicate significant differences between adults and nestlings from Catalonia.

	Andalusia (nestlings)	Castile and Leon (nestlings)	Catalonia (nestlings)	Catalonia (adults)
N	30	11	75	7
Arsenic (As)	0.051^a	0.048^a	0.101^b	0.103
	(2.326)	(0.222)	(1.117)	(0.348)
Mercury (Hg)	0.043	0.050	0.047	0.043
	(0.376)	(0.346)	(0.716)	(0.093)
Lead (Pb)	0.137	0.093	0.060	0.098
	(0.452)	(0.695)	(0.900)	(0.527)
Zinc (Zn)	39.48^b	37.83^b	31.67^a	23.30*
	(55.75)	(43.52)	(39.82)	(25.38)

Table 3
Mean ± SE body condition of Bonelli's eagle nestlings, and mean ± SE values of plasma biochemistry, vitamins and carotenoids, as well as oxidative stress biomarkers in blood pellet measured in nestlings and adults from Spain: Catalonia, Castile and Leon and Andalusia. Data are compared to those that Balbontín and Ferrer (2002) measured in Bonelli's eagle nestlings from Andalusia. Asterisks (*) indicate significant differences between adults and nestlings from Catalonia. Superscript letters define significant differences among nestlings from different populations. Bold values in the data from Balbontín and Ferrer (2002) indicate parameters for which the 95% confidence interval does not overlap with measurements retrieved from Andalusia in the present study.

	Catalonia (adults)	Catalonia (nestlings)	Castile and Leon (nestlings)	Andalusia (nestlings)	Andalusia (nestlings) Balbontín and Ferrer (2002)	
					Males	Females
N	7	75	11	31	5–14	8–14
Body condition[1]		1613.2 ± 32.3	1632.2 ± 37.4	1724.0 ± 40.9		
Alkaline phosphatase (U/l)	18.9 ± 2.0*	847.2 ± 18.5	851.5 ± 72.4	935.0 ± 62.3	**2148 ± 696**	**2280 ± 327**
Aspartate aminotransferase (U/l)	301.7 ± 29.8*	217.6 ± 3.0	225.5 ± 11.0	210.4 ± 7.67	**171.6 ± 18.2**	203.2 ± 31.5
Lactate dehydrogenase (U/l)	1356.4 ± 56.7*	2268.8 ± 80.1	2187.0 ± 136.7	2453.0 ± 103.7	**1647 ± 160**	**1828 ± 355**
Creatine phosphokinase (U/l)	1086.3 ± 159.2*	4259.9 ± 84.0	3730.9 ± 269.9	3910.3 ± 388.6	3859 ± 883	3853 ± 918
Total protein (g/l)	34.63 ± 1.39*	32.03 ± 0.35^{ab}	30.88 ± 1.05^{a}	33.34 ± 0.66^{b}	**29.8 ± 1.5**	**30.1 ± 2.8**
Glucose (mg/dl)	444.6 ± 16.4*	319.6 ± 3.0	303.6 ± 4.7	305.6 ± 6.9	283.3 ± 37.6	**252.5 ± 25.0**
Cholesterol (mg/dl)	186.4 ± 10.9	181.1 ± 2.7	166.8 ± 8.2	179.9 ± 6.8	168.2 ± 23.2	175.2 ± 28.2
Triglycerides (mg/dl)	51.00 ± 11.24	99.05 ± 10.53	91.64 ± 13.65	113.50 ± 13.45	71.3 ± 35.1	81.7 ± 36.6
Calcium (mg/dl)	8.93 ± 0.53*	10.33 ± 0.13^{ab}	9.56 ± 0.58^{a}	10.90 ± 0.34^{b}	**8.60 ± 1.50**	**13.20 ± 1.38**
Magnesium (mg/dl)	1.78 ± 0.24*	1.37 ± 0.04	1.18 ± 0.10	1.39 ± 0.09	**1.87 ± 0.10**	**1.75 ± 0.17**
Phosphorus (mg/dl)	2.21 ± 0.36*	6.57 ± 0.12	6.46 ± 0.28	6.77 ± 0.21	**4.80 ± 0.71**	**4.62 ± 0.46**
Creatinine (mg/dl)	0.274 ± 0.016	0.346 ± 0.011	0.351 ± 0.013	0.336 ± 0.012	**0.280 ± 0.050**	**0.289 ± 0.040**
Urea (mg/dl)	9.86 ± 2.21*	16.47 ± 0.51^{a}	17.81 ± 1.92^{ab}	21.00 ± 1.78^{b}	**13.62 ± 3.36**	**15.12 ± 5.88**
Uric acid (mg/dl)	5.09 ± 0.93*	12.42 ± 0.56	12.39 ± 1.98	12.64 ± 1.04	11.49 ± 4.70	13.89 ± 5.17
Retinol (μM)	8.11 ± 0.60*	5.04 ± 0.09^{a}	4.67 ± 0.39^{a}	6.91 ± 0.40^{b}		
α-Tocopherol (μM)	15.55 ± 1.93*	19.60 ± 0.55^{ab}	17.71 ± 1.67^{a}	22.21 ± 0.89^{b}		
Lutein (μM)	2.19 ± 0.38*	2.88 ± 0.10^{a}	3.56 ± 0.24^{ab}	4.10 ± 0.29^{b}		
Zeaxanthin (μM)	1.16 ± 0.12	1.28 ± 0.05	1.15 ± 0.11	1.57 ± 0.11		
TBARS (μmol/g)	0.058 ± 0.003	0.060 ± 0.002	0.050 ± 0.002	0.058 ± 0.003		
GSH (μmol/g)	3.11 ± 0.13*	4.93 ± 0.10	4.57 ± 0.37	4.51 ± 0.17		
GSSG (μmol/g)	0.389 ± 0.183*	0.887 ± 0.064^{b}	0.504 ± 0.145^{a}	0.658 ± 0.128^{ab}		
GSSG/GSH (%)	6.67 ± 3.21	8.95 ± 0.61^{b}	5.47 ± 1.60^{a}	7.10 ± 1.29^{ab}		
GPX (IU/mg prot.)	0.366 ± 0.018*	0.279 ± 0.010	0.285 ± 0.025	0.300 ± 0.019		
SOD (IU/mg prot.)	1.179 ± 0.118	1.196 ± 0.034^{a}	1.224 ± 0.099^{a}	1.473 ± 0.080^{b}		

[1] See methods for details on body condition calculation.

9.580, 2 d.f., p = 0.008) and LDH (χ^2 = 9.288, 2 d.f., p = 0.010). Nestlings from Catalonia showed increased levels of both biochemical parameters as Zn concentrations increased, and the same happened with Castile and Leon nestlings when comparing Zn and LDH levels, although in the latter case the correlation was entirely driven by the values measured in a single nestling and the significance disappeared when it was removed from the analyses (Fig. 6).

4. Discussion

4.1. Pollutant accumulation in Bonelli's eagles

PCBs, including 10 out of the 13 detected congeners and ΣPCB, As and Zn were the only contaminants for which we found significant variation among study populations. PCB congeners and ΣPCB, as well as As, showed higher levels in nestlings from Catalonia than in those from the other two regions. Industrial activity has been associated with contamination by PCBs in raptors. For instance, Gómara et al. (2004) measured on average 22 ng/ml of ΣPCB in plasma of Egyptian vultures (*Neophron percnopterus*) sampled from an industrial area in northern Spain, whereas mean concentrations observed in rural areas from the centre and south of the country ranged from 5.8 to 8.5 ng/ml. Working in the British Columbia (Canada), Elliott et al. (2000) found that osprey (*Pandion haliaetus*) eggs from the Columbia River basin had a ΣPCB concentration four times higher than eggs from the Fraser River valley, which the authors attributed to the development of hydroelectric generation and related industries in the former area. These results are consistent with the regional variation found in our study between highly industrialized (Catalonia) and less industrialized (Andalusia and Castile and Leon) areas. Plasma OC concentrations in nestling raptors are related to levels measured in their prey (Bignert et al., 1998), and thus variation in prey types may account for differences in OC accumulation by nestlings (Mañosa et al., 2003). In our study area, some territories from Catalonia are highly altered by direct human disturbance, so the abundance of the most preferred prey (i.e. rabbits or partridges) is scarce, and Bonelli's eagles can consume higher amounts of alternative prey like gulls (Resano et al., 2011; Resano-Mayor et al., 2014b), which could have higher potential of PCB accumulation because of its feeding in aquatic environments. In this regard, Elliott et al. (2009) observed in the bald eagle (*Haliaeetus leucocephalus*) that trophic chains were larger for individuals feeding on pelagic marine systems than in those feeding on terrestrial systems, and that OC concentrations generally increased with trophic level and marine input. Moreover, the decline of rabbit populations due to viral diseases (i.e. myxomatosis and haemorrhagic disease) that have forced European Bonelli's eagles to prey more frequently on birds (Moleón et al., 2009) could have contributed to reach the elevated OC exposure levels frequently observed in bird-eating raptors (Mañosa et al., 2003; van Drooge et al., 2008). This could be a previously unreported example of the indirect negative effects that emerging infectious diseases affecting prey species may exert on their predators.

The concentrations of some PCB congeners in plasma also differed between nestlings and adults from Catalonia. Adults generally showed higher plasma PCB levels than nestlings, which is in the line of findings of most studies (Kenntner et al., 2003; Goutner et al., 2011) and can be attributed to a progressive accumulation of PCBs over time because uptake rate is higher than dilution rate. Surprisingly, we found the opposite trend for the specific case of congeners PCB 28 + 31. We are not aware of any previous paper reporting such finding. The reason for this result could have to do with a recent ingestion of contaminated items by nestlings. Blood PCB levels in raptors are known to vary because of recent feeding, especially for those congeners that are quickly metabolized (Olsson et al., 2000). Whereas PCB 28 seems to be persistent (Borlakoglu and Walker, 1989), PCB 31 has meta-para vicinal

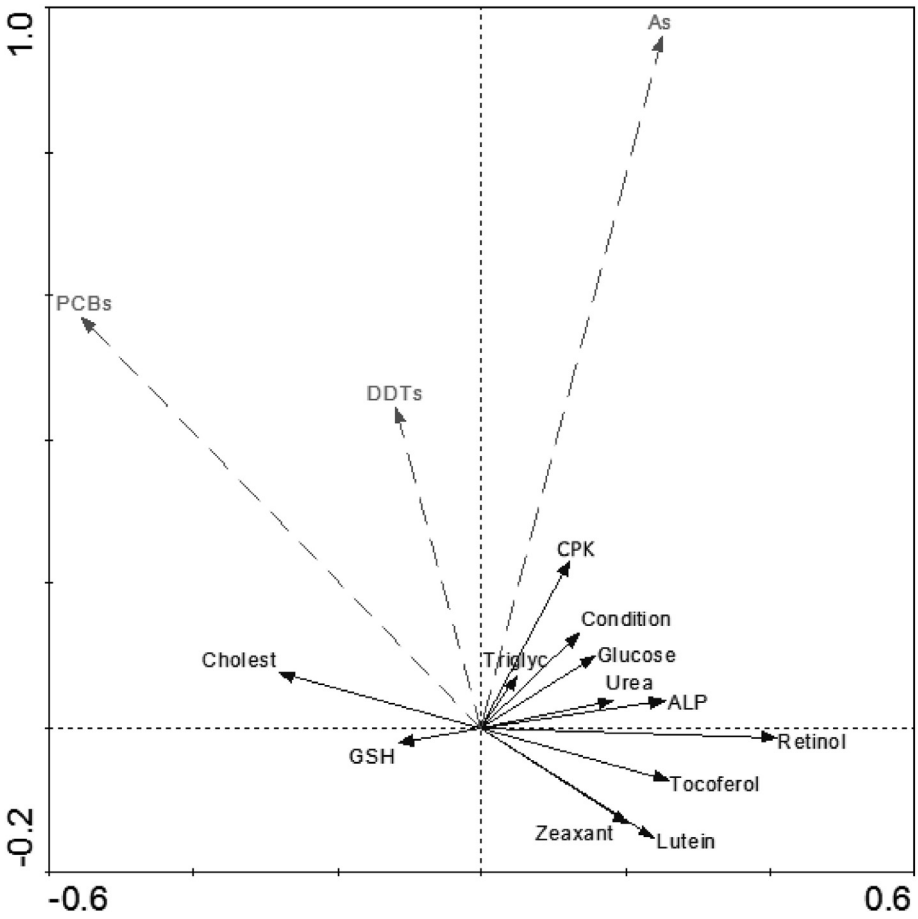


Fig. 2. Graphic representation of the results of the RDA and Monte Carlo permutation test to analyse the effect of pollutants (dashed arrows) on fitness variables (continuous arrows). Among the latter, only those significantly correlated with the selected pollutants are shown.

hydrogens, which characterizes the most readily metabolized congeners (Drouillard et al., 2001).

Proximity to industrialized areas could also explain the inter-population trends observed in As accumulation. There is limited information on threshold values of As in nestling blood that may cause detrimental effects; Burger and Gochfeld (1997) proposed that normal As blood levels in raptors should be below 20 ng/ml. Although we did not measure metal levels referred to whole blood volumes, an estimation using the average water content in the pellet (65.1%), the average haematocrit value of the analysed samples (30.04%; range 23.16–37.26), and an approximate density of 1.1 g/ml of the blood pellet, would result in average levels below 12 ng/ml in all populations. However, one nestling from Castile and Leon (9% of the samples), three from Andalusia (10%), 12 from Catalonia (16%), and four of the seven adults from Catalonia presented estimated As levels above 20 ng/ml, suggesting that despite As is more abundant in industrialized zones, it can still pose at risk individuals from all other areas.

Zn blood levels were lower in Catalonian individuals than in those from the other two populations, but, as pointed by Rattner et al. (2008), modest fluctuations between regions in Zn levels are common in raptors. We also found higher Zn levels in nestlings than in adults from Catalonia. Because Zn is involved in keratinization of feathers and in tissue proliferation (Honda et al., 1986), high levels of this essential element are required during the growing phase.

Hg concentrations were higher in Castile and Leon nestlings that were alone than in those sharing the nest with a sibling. Gill and Elliott (2003) observed that nest productivity in bald eagles was positively related to prey availability, while pollutant accumulation in

Table 4
Eigenvalues and percentage of variance explained by the redundancy analysis (RDA), with Pearson's correlations between the three canonical axes and the predictor variables (i.e. contaminants) selected as significant, and results of the Monte Carlo permutations tests to check the significance of canonical axes.

		Axis 1	Axis 2	Axis 3
Eigenvalues		0.037	0.016	0.011
Predictors–responses correlation		0.677	0.446	0.508
Cumulative percentage data of response variables		3.7	5.3	6.4
Cumulative percentage data of responses–predictors correlation		41.6	59.9	72.2
Selected predictor variables (contaminants)	ΣPCB	−0.361	0.257	0.268
	ΣDDT	−0.077	0.201	0.391
	As	0.162	0.434	−0.056

Monte Carlo test	F	P
Significance of first canonical axis	4.137	0.002
Significance of all canonical axes	1.514	0.006

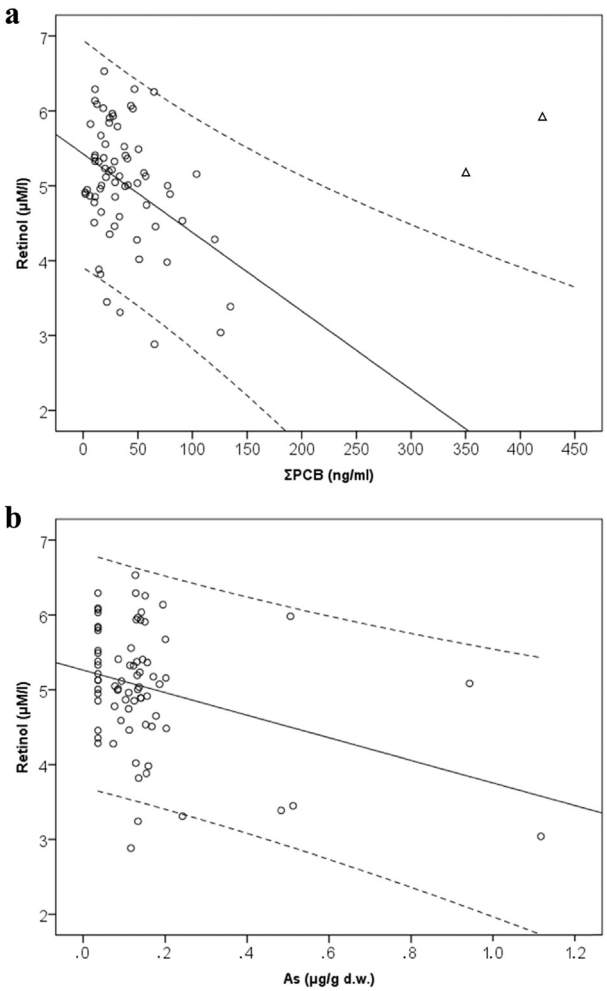


Fig. 3. Linear fit (±95% confidence intervals) of the relationship between retinol plasma levels and (a) concentrations of ΣPCB in plasma or (b) concentrations of arsenic (As) in blood pellets, in nestlings from Catalonia. The correlations were significant for ΣPCB ($R = -0.321$, $p = 0.001$), after removing the outlier values (open triangles), and for As ($R = -0.467$, $p < 0.001$).

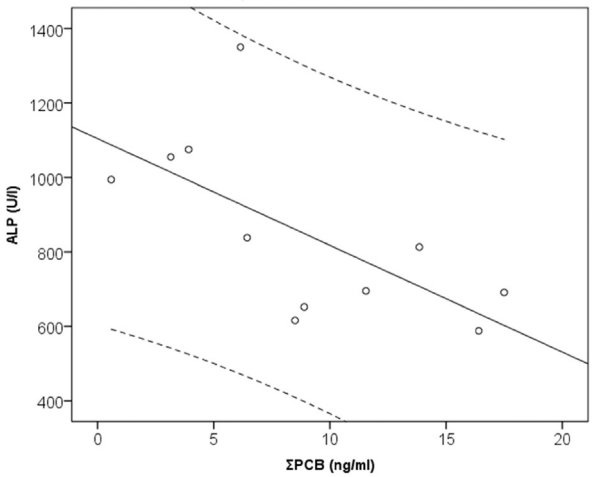


Fig. 4. Linear fit (±95% confidence intervals) of the relationship between plasma levels of alkaline phosphatase (ALP) and ΣPCB in nestlings from Castile and Leon. The correlation was significant ($R = -0.657$, $p = 0.028$).

nestlings had generally no effect on productivity. Thus, the quality of the territory could explain this trend; in Bonelli's eagle, productivity is positively correlated to high or moderate consumption of rabbits (Resano-Mayor et al., 2015), but negatively correlated with diet diversity (Moleón et al., 2012b; Resano-Mayor et al., 2014a). This pattern arises because when preferred prey like rabbits are scarce, eagles consume higher amounts of alternative prey (e.g. small to medium birds), which occupy higher position in trophic chains and can therefore accumulate more Hg than rabbits. Palma et al. (2005) found that Hg levels in nestling Bonelli's eagles from southwest Portugal were positively correlated with the dietary proportion of insectivorous and omnivorous birds (e.g. egrets, corvids, and thrushes) and negatively correlated with the ingestion of herbivore prey (e.g. rabbits). Furthermore, this study showed that variations among territories in prey Hg burden determined the differences of concentrations measured in nestlings.

For the rest of contaminants measured we did not find any effect of population, age, sex or number of siblings. In fact, the concentrations of all those contaminants were generally low compared to other studies (e.g. Garcia Fernandez et al., 1995; van Wyk et al., 2001), even below detection limits for Cd and Cu. Garcia Fernandez et al. (1995) reported a mean Pb concentration of 18 µg/dl in whole blood samples of Bonelli's eagles, well above the maximum concentration detected in our samples (10.4 µg/dl if we estimate the concentration relative to whole blood as we did above for As). These low Pb values in Bonelli's eagles are consistent with the low frequency of consumption of shot animals (dead or injured) or Pb-poisoned waterfowl (Mateo et al., 2014). However, some studies have reported abnormal effects of Pb on raptors through the inhibition of the δ-aminolevulinic acid dehydratase (δ-ALAD) at concentrations as low as 4–5 µg/dl (Gómez-Ramírez et al., 2011; Espín et al., 2015). With regards to ΣDDT, in spite of its high persistence, several studies have already shown decreasing trends in its bioaccumulation in biota from those areas, like Spain, where it was banned in the 1970s (Mateo et al., 2012). The concentrations of p,p′-DDE in the samples were generally more than 100 times higher than the concentrations of p,p′-DDT, which reflects the expected profile in areas where DDT has not been used for a long time (van Wyk et al., 2001).

4.2. Influence of contaminant levels on nestling physiology and body condition

We are aware of a single study having analysed biochemical parameters in Bonelli's eagle; Balbontín and Ferrer (2002) sampled 28 nestlings from Cádiz, within Andalusia region. In order to make a statistical comparison between levels measured in that study and

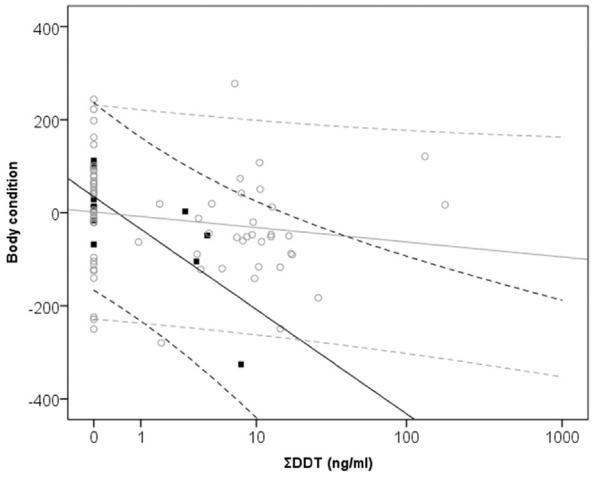


Fig. 5. Linear fit (±95% confidence intervals) of the relationship between body condition calculated according to Peig and Green (2009) and plasma levels of ΣDDT in nestlings from Castile and Leon (black squares, black lines) and Catalonia (open circles, grey lines). The correlations were significant for Castile and Leon ($R = -0.705$, $p = 0.015$) and Catalonia ($R = -0.261$, $p = 0.028$).

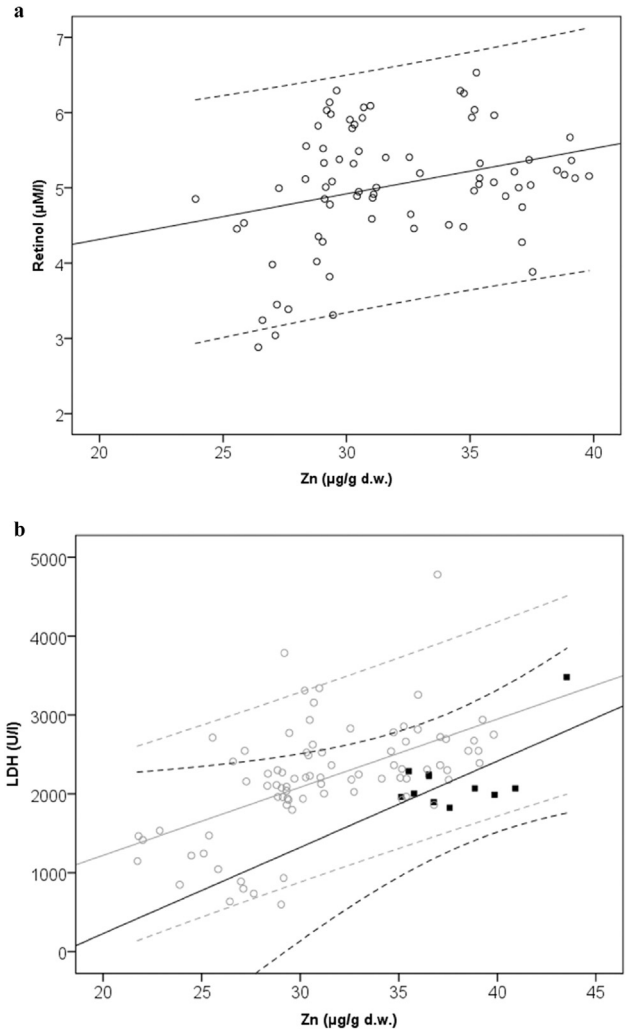


Fig. 6. Linear fit (±95% confidence intervals) of the relationship between zinc (Zn) concentrations in blood pellets and (a) plasma levels of retinol in nestlings from Catalonia, or (b) plasma levels of lactate dehydrogenase (LDH) in nestlings from Castile and Leon (black squares, black lines) and from Catalonia (open circles, grey lines). The correlations were significant for retinol ($R = 0.292$, $p = 0.011$) and for LDH in Catalonia ($R = 0.410$, $p < 0.001$) and in Castile and Leon ($R = 0.631$, $p = 0.037$), although in the latter case significance was lost after removing the nestling with highest plasma LDH and Zn concentrations ($R = -0.183$, $p = 0.612$).

those measured by us, we calculated the 95% confidence intervals of mean values from both studies and look for the absence of overlaps (Table 3). All the significant differences that we found between both studies could be explained due to age and/or nutritional status.

ΣPCB, ΣDDT, and As were the contaminants associated with major impacts on the pool of biochemical and metabolic variables measured on Bonelli's eagle nestlings. Circulating levels of dietary antioxidants (i.e. vitamins and carotenoids) were the most reliable indicators of the effects caused by the contaminants selected by the RDA model as significant. Both PCB and As were related to decreased concentrations of these molecules. However, most of the relationships between contaminants and biochemical parameters were related to differences among the three study populations. In Bonelli's eagle, marked dietary differences exist among territories due to prey availability (Moleón et al., 2012a). Thus, we can expect both biochemical parameters and pollutant accumulation being highly influenced by diet, which in turn depends on the site of origin. In the case of circulating vitamins and carotenoids this is particularly relevant, as these substances are obtained from the diet. Only in the case of the retinol the influence of PCBs and As was still observed when considering only the individuals from Catalonia, the most contaminated region.

Exposure to PCBs or As is known to cause oxidative stress in birds. PCBs are well-known aryl hydrocarbon receptor agonists that induce cytochrome P450 activity, which is linked to the production of ROS (Schlezinger et al., 2000), whereas As can induce oxidative stress also through the generation of ROS (Koivula and Eeva, 2010). The overall negative associations of PCBs and As with vitamin and carotenoid levels could reflect the necessity of organisms to incorporate these exogenous antioxidants in redox processes to balance the increased oxidative stress caused by the exposure to pollutants. A common effect after exposure to pollutants generating oxidative stress is the depletion of GSH, the major intracellular antioxidant molecule that helps neutralizing free radicals (Apel and Hirt, 2004). However, in our study, PCB exposure was related to increased total GSH, which would be a compensatory response to the expected GSH conjugation with xenobiotic metabolites (DeLeve and Kaplowitz, 1991). A similar pattern of compensation of GSH levels has been observed in a recent study after exposure of red-legged partridges (*A. rufa*) to Pb, another chemical known to cause oxidative stress (Vallverdú-Coll et al., 2015). Besides exogenous antioxidants, As levels in nestlings were also positively associated with CPK concentrations, which are known to increase after pollutant-induced oxidative stress (Venkataraman et al., 2009).

Concentrations of plasma retinol were positively related to blood Zn levels in Catalonia. The regulation that Zn exerts on retinol has been widely studied (Christian and West, 1998). Two mechanisms are postulated to explain the dependence of retinol on Zn; on the one hand, Zn regulates the synthesis of retinol-binding protein in the liver, which reduces plasma retinol levels (Smith et al., 1974). On the other hand, Zn is essential for the activity of the retinol dehydrogenase enzyme, which plays a critical role in the retinol metabolic pathway (Huber and Gershoff, 1975). Zn blood levels were also associated with decreased activity of LDH. This enzyme is composed by four sub-units, each one containing a cysteine, and the interaction of Zn with thiol groups in the cysteine is known to cause enzyme inhibition (Holbrook and Gutfreund, 1973).

Increased PCB levels were associated with reduced concentrations of ALP in nestlings from Castile and Leon, an association shown also by Sonne et al. (2012) in white-tailed sea eagles (*Haliaeetus albicilla*). ALP activity is an indicator of osteoblastic activity and rises with age during the pre-adult stage, as we have previously detected in Bonelli's eagle nestlings within the range of ages of the individuals sampled in the present study (up to 52 days old, authors' unpublished data). Hoffman et al. (1996) dosed nestling American kestrels (*Falco sparverius*) with different levels of PCB 126 and observed that ALP in plasma decreased with increased PCB 126 concentrations. These authors attributed the observed result to decreased osteoblastic activity because of the decreased bone growth associated with increased toxicant exposure. Whereas, in view of this result, it would have been interesting to relate PCB accumulation to nestling growth, the fact of working with an endangered species whose nests are difficult to reach did not make possible to perform repeated measurements to estimate growth rates. Because the Castile and Leon population, where this negative association between ALP and ΣPCB was found, inhabits a protected area without industrial activity, little direct PCB uptake from the environment is expected, and therefore ΣPCB burden in nestlings would come mainly from maternal transfer. Thus, maternally-derived ΣPCB in nestlings would be maximal at hatching, being progressively diluted as the nestling grows and ALP levels increase.

ΣDDT levels associated with an impoverished body condition, which points to prolonged food stress in birds with higher ΣDDT levels. Two hypotheses can explain this association; on the one hand, animals suffering food stress will be forced to mobilize endogenous fats, which constitute the main storage reserve of ΣDDT (Perkins and Barclay, 1997). On the other hand, similar to what we suggested above for the case of Hg, the exploitation of alternative, less nutritive prey, which would likely lead to food stress, could also associate with increased

uptake of ΣDDT. Although influenced by inter-population differences, the observed association of decreased triglyceride levels with increased ΣDDT would support the influence of food stress as a determinant factor for the increase of plasma ΣDDT concentrations. According to the model of fasting described by Handrich et al. (1993), a phase I involving carbohydrate metabolism will be followed by a phase II during which lipids are mobilized and constitute the main source of energy. Animals in this phase II of fasting have normally undergone a rapid loss of body mass, occurring during phase I, and so will show an impoverished body condition. Thus, animals suffering food stress would be forced to mobilize endogenous fats in form of free fatty acids, which would also result in mobilization of DDT and other OC accumulated in the adipose tissue. If nutritional stress is prolonged in time, organisms can begin metabolizing muscle proteins, which will result in muscle damage and the consequent increase in CPK levels (Maceda-Veiga et al., 2015), as we have also observed with increased ΣDDT levels.

5. Conclusions

The results of the present study confirm the hypothesis that pollutant accumulation in nestlings of Bonelli's eagle vary among populations, likely because of dietary differences that result in different patterns of pollutant uptake. In highly industrialized regions, like Catalonia, nestlings accumulate higher levels of certain contaminants (i.e. PCBs, As) than in less-industrialized areas, and such increased pollutant exposure is associated with oxidative stress which manifests in varying circulating levels of antioxidant-related substances like retinol or Zn. At the local scale, availability of preferred herbivore prey (i.e. rabbits) in certain territories may also account for reduced chances of accumulating contaminants like Hg, which is expected to reach higher concentrations in those territories where eagles consume higher amounts of alternative prey located up in the food chain (e.g. Passeriformes, Accipitriformes, Ardeiformes), and especially those associated with marine food webs (e.g. Charadriiformes). Our understanding of all these patterns of variation in pollutant accumulation would benefit from further research combining residue analysis in prey from different territories and regions with stable isotope analysis to determine dietary patterns. The exposure to these persistent pollutants may have long-term effect on top predators, as shown by the mobilization of DDTs accumulated in fat tissues in animals with signs of starvation. Together with the elimination of pollutant sources, the availability of high quality territories is essential for preserving health of sedentary, long-lived predators like Bonelli's eagles, if we aim to reduce the chances of accumulation of pollutants still present in the environment.

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