Chemosphere 76 (2009) 387–394

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Metals in liver and kidneys and the effects of chronic exposure to pyrite mine pollution in the shrew *Crocidura russula* inhabiting the protected wetland of Doñana

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ARTICLE INFO

Article history:
Received 11 December 2008
Received in revised form 5 March 2009
Accepted 16 March 2009
Available online 23 April 2009

Keywords:
Shrew
Heavy metals
Morphometric parameters
Genotoxicity
Protected areas

ABSTRACT

Historically impacted by anthropogenic activities, the nature reserve of Doñana (SW Spain) was affected by an unprecedented spillage of mud and acidic water from the Aznalcóllar pyrite mine in April 1998. Although several studies have addressed the influence of this spill on soils, water, and biota, there is little information on mammals, especially carnivorous species. We measured the concentrations of Fe, Mg, Pb, Hg, Cd, Zn, Cu, Mn, Mo, Co, and Cr in specimens of the greater white-toothed shrew, *Crocidura russula*, inhabiting the protected area affected by the mine spillage. We also examined other parameters to approach at the physiological effects of pollution. We found an increase in non-essential metals (Pb, Cd, and Hg), and morphometric, histological and genotoxic alterations. Age and gender were two significant factors explaining metal bioaccumulation: adults had higher Hg and Cd levels than juveniles, whereas males bioaccumulated more Pb and Co and less Mo than females. The micronucleus frequencies in blood erythrocytes were significantly higher in specimens from the polluted site than animals from the control site. Shrews from the impacted area also had hepatic alterations, namely increased liver-body ratio, focal necrosis, and signs of apoptosis in hepatocytes. Due to the relevance of small mammals in the diet of endangered species such as carnivorous birds and mammals, the findings of our study are of practical use for the management of the Doñana wildlife reserve and other protected Mediterranean wetlands.

1. Introduction

Doñana wildlife reserve is inhabited by several protected and endangered species, especially birds and mammals. On 25 April 1998, an incident at the pyrite mine "Los Frailes" in Aznalcóllar discharged about 5 Hm^3 of acidic waters and toxic mud carrying heavy metals such as Pb, Cd, Hg, Zn, Sb, and Tl into the Agrio river, thereby affecting this protected wetland. Since this event, several studies have provided abundant information on metal pollution in sediments and waters as well as on bioaccumulation and effects on plants, invertebrates, fish, reptiles, birds, rodents, carnivorous mammals, and human populations (e.g. Bonilla-Valverde et al., 2004; Fletcher et al., 2006; Gil et al., 2006; Madejón et al., 2007; Smits et al., 2007; Millán et al., 2008; Turner et al., 2008; Márquez-Ferrando et al., 2009). However, information on metal concentrations and their effects on insectivorous mammals is scarce, with only one study reporting a great increase in Tl in shrew tissues (Sánchez-Chardi, 2007).

Several heavy metals lead to clastogenic effects as a result of DNA breakage and can induce the generation of reactive oxygen species (ROS), which can lead to cell damage or death. Depending on the nature of the injury, the damage to the DNA may be repaired, or it may induce mutation or lead to apoptosis (Bragadin et al., 2003; Leonard et al., 2004), necrosis (Pereira et al., 2006; Jadhav et al., 2007) or other important cell alterations in somatic and germinative tissues (e.g. Damek-Poprawa and Sawicka-Kapusta, 2004; Nordberg et al., 2007). Effects are generally assessed or modelled in laboratory animals or culture cells in controlled conditions for a single compound or a few metals. However, biota are often exposed to a mixture of several chemical pollutants (Bellés et al., 2002). Field studies on ecotoxicological effects are useful to identify bioindicator species and assess the quality of the environment. Insectivorous mammals (shrews, hedgehogs, and moles) have been widely used as site-specific bioindicators of anthropogenic pollution, including heavy metals (e.g. Talmage and Walton, 1991; Ma and Talmage, 2001; D'Have et al., 2006). When non-degradable pollutants are released into the environment, they can be taken

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doi:10.1016/j.chemosphere.2009.03.036

up and transferred through food chains and may accumulate in predators such as the greater white-toothed shrew *Crocidura russula* (Alleva et al., 2006; Wijnhoven et al., 2007). In particular, this species has small home ranges, and shows high food requirements and a high metabolic rate (e.g. Talmage and Walton, 1991; Ma and Talmage, 2001; Stewart et al., 2005).

Here we examine the effect of a recent large increase in the environmental concentration of heavy metals to *C. russula* from the protected reserve of Doñana. We measure the bioaccumulation of Fe, Mg, Pb, Cd, Hg, Zn, Cu, Mn, Mo, Co, and Cr, and assessed several morphometric, genotoxic, and histopathological parameters as biomarkers of environmental metal pollution in shrews. Moreover, the relation between age and sex and the bioaccumulation patterns was evaluated as inherent sources of variability.

2. Materials and methods

2.1. Study areas

During November and December 1999, 29 specimens of the greater white-toothed shrew, *C. russula* were trapped. Of these, 19 were caught in an area affected by the spill from the Aznalcóllar mine (“Entremuros, Parque Nacional”, 37°01′12″N, 6°16′38″W) and 10 were collected at a reference site not affected by this pollution (“La Vera, Reserva Biológica”, 36°59′25″N, 6°26′41″W). Traps were placed 5–8 m apart for about 400 m in a transect running along the intertidal zone. Both sites are situated in the protected reserve of Doñana (Fig. 1), an extensive ecological coastal wetland in SW Spain inhabited by several protected species. The total capture effort was 1950 traps per night. Shrews were transported to the laboratory and killed by cervical dislocation. The study was conducted in accordance with the European and Spanish legislation for laboratory animals (Guideline of the comission of the European Communities 86/609/EEC of 24/11/1986 and Real Ordinance, 1201/2005 of 10/10/2005). Sex was determined during dissection. Specimens were classified into two relative age classes (juveniles and adults) on the basis of the degree of tooth wear (Vesmanis and Vesmanis, 1979). In the polluted area, three females were pregnant and another two more lactating, whereas only three males showed signs of sexual activity. In the reference site, one male presented sexual activity and one female was lactating.

2.2. Morphometric parameters

The body weight (BW) to the nearest 0.01 g and head and body length (BL) to the nearest 0.01 mm of all specimens were measured during dissection. The residual index (RI) is calculated by the regression of BW and BL (Jakob et al., 1996). Positive values are an indication for specimens with relative higher body condition than expected for their weight and length. Animals with negative values have a relative lower body condition than predicted by the BW to BL ratio. Hepatic and renal weight to the nearest 0.001 g was measured on a wet weight basis (WW). The relative weights were calculated as a percent ratio of somatic tissue (100× tissue weight/body weight).

2.3. Chemical analyses

About 300–500 mg of the liver and right kidney of each specimen were dissected, dried to constant weight (48 h, 60 °C), weighed, and then digested in Teflon vessels with 2 mL of nitric acid and 1 mL of hydrogen peroxide (Instra, Baker Analized). Duplicate subsamples diluted (1:5), with rhodium as internal standard, were measured for Pb, Hg, Cd, Zn, Cu, Mn, Mo, Co, and Cr using a Perkin–Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) and for Fe and Mg by a Perkin–Elmer OPTIMA-3200RL Inductively Coupled Plasma Optical Spectrometer (ICP-OES). Moreover, standard reference material (Bovine Liver SRM-1577a) and 6 blanks were included in the analyses. Metal concentrations were expressed in $\mu g\ g^{-1}$ on a dry weight basis (DW). All chemical analyses were performed at the Elemental Analysis Facility of the Scientific-Technical Services at the University of Barcelona.

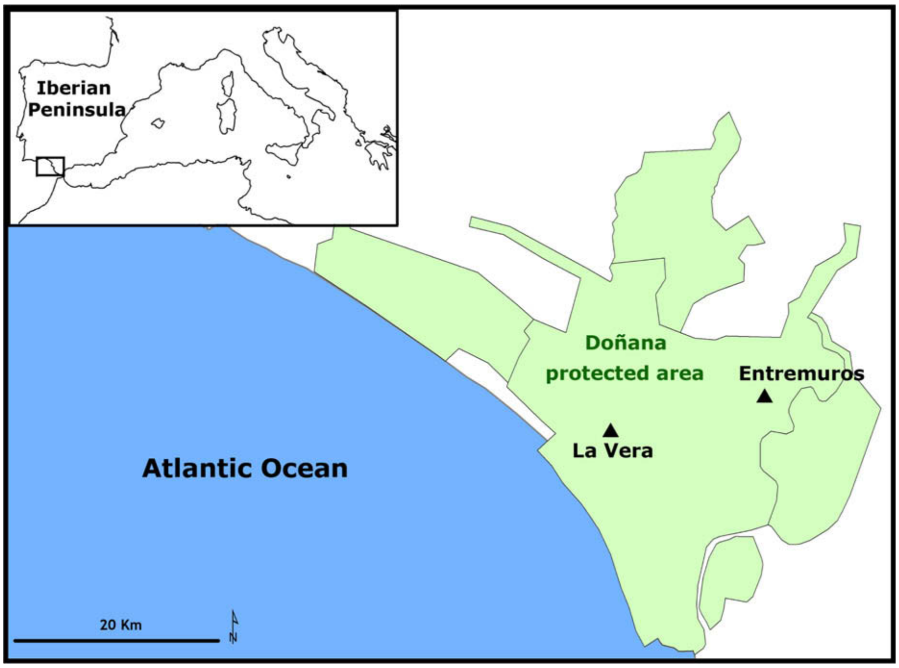

Fig. 1. Map showing the geographical location of the sites studied in Doñana protected area (▲): “Entremuros” (polluted site) and “La Vera” (reference site).

Table 1
Mean ± SEM values for morphometric parameters (BW, BL, RI, weights and relative weight ratios) of *C. russula* by site.

	Reference site ($n = 10$)	Polluted site ($n = 19$)
BW (g)	6.57 ± 0.18	6.14 ± 0.20
BL (mm)	66.80 ± 0.47	64.88 ± 0.73
RI	−0.559 ± 0.246	0.265 ± 0.226
Liver (g)	0.263 ± 0.016	0.324 ± 0.019*
% Liver	4.02 ± 0.28	5.47 ± 0.27**
Kidneys (g)	0.102 ± 0.005	0.115 ± 0.009
% Kidneys	1.58 ± 0.09	1.87 ± 0.15

* $p \leqslant 0.05$.
** $p \leqslant 0.01$.

2.4. Micronucleus test (MNT)

Blood samples are obtained by cardiac punction with a heparinized syringe. For each specimen, duplicate smears were made on pre-cleaned microscope slides, fixed with heat and stained with conventional May Grünwald-Giemsa staining. Micronuclei (MN) frequencies were scored by two observers on 2,000 blood erythrocytes for each specimen through an oil immersion objective (100×) on a Leica Leitz DMRB microscope. The frequencies were expressed as arithmetic mean and standard error of the mean ($M \pm$ SEM).

2.5. Histopathology

A fraction of liver and the left kidney of six adult shrews from the polluted site and eight from the reference site were fixed by 10% neutral-buffered formaldehyde and prepared for histological studies following conventional procedures, as described in Sánchez-Chardi et al. (2008). For the qualitative analysis, the stained sections were analysed under a Leica Leitz DMRB light microscope using a Sony Cyber-Shot, 7.2 mega pixels, to capture the images. Four images from each slide were captured under a 40× objective. These images were then processed by Image Tools software for quantitative analysis. The endpoints used were the presence of necrosis areas or apoptosis, inflammatory response, fibrosis, neoplasia, pre-neoplasic focuses and cytoplasmic vacuolization. For the quantitative analyses, we measured the perimeter, elongation, roundness, and compactness of a total of 1588 cells.

2.6. Statistical analyses

After log-transformation and test for normal distribution (Shapiro–Wilk test) and for homogeneity of variance (Levene, F-test), a three-way multivariate analysis of variance (MANOVA) was performed to obtain an overall estimation of the effects of on the parameters evaluated of site, age, and sex, together with their interactions. In order to increase sample size, the data were pooled and the effects of age and sex on metal bioaccumulation were analysed for each site. The differences in metal concentrations and morphometric parameters were analysed for site, age and gender classes with Student's tests (t). The same comparisons of MNT and histopathology were performed with Mann–Whitney tests (U). In order to increase sample size, sexual activity was not considered as factor since significant differences had not been found in previous analyses. Significant differences were assumed at $p < 0.05$. For all statistical analyses, SPSS 14.0 (2005) was used.

3. Results

Among the morphometric parameters, liver weight and relative liver weight were significantly higher in specimens from the polluted area than reference specimens ($t = 2.544$, $p = 0.017$; $t = 3.233$, $p = 0.003$, respectively). Moreover, the former tend to have greater relative renal weights and lower body weight than reference shrews (Table 1).

In liver, significant differences in metal levels between sites ($F = 33.421$, $p < 0.001$), ages ($F = 48.191$, $p < 0.001$), and sexes ($F = 52.930$, $p < 0.001$) were found. Hepatic mean concentrations of Hg and Cd were significantly higher in shrews from the polluted area, while more Cr was found in high concentrations in specimens from the reference site (Table 2). Adults from the polluted site had more Hg ($t = 3.240$; $p = 0.005$) and Cd ($t = 3.252$; $p = 0.005$) than the juveniles (Fig. 2A). Moreover, levels of Pb, Mo and Fe tend to increase and Cu and Cr decrease in adults. Among gender differences, males had significantly high mean values of Pb and Co at the polluted site ($t = 2.520$; $p = 0.023$, $t = 2.358$; $p = 0.031$, respectively), whereas females bioaccumulated more Mo ($t = -3.119$; $p = 0.017$) at the reference site (Table 3).

In renal tissue, the importance of the factors regarding metal differences were more site related ($F = 3.624$, $p = 0.024$), than sex related ($F = 1.315$, $p = 0.329$) and to a lesser extent age related ($F = 1.133$, $p = 0.415$). In a similar pattern to that of hepatic tissue, the shrews from the polluted site had elevated Mg, Hg, Cd, Cu, and Co concentrations (Table 2). Moreover, adults from the polluted site had higher Hg ($t = 3.068$; $p = 0.018$) and Cd ($t = 3.886$; $p = 0.001$) concentrations than juveniles (Fig. 2B). No significant sex-dependent variation was detected in kidneys; however, males and females showed a similar pattern of metal bioaccumulation as that detected in livers (Table 3).

The specimens from the polluted site showed a significant increase in micronuclei frequencies in peripheral erythrocytes compared with those from the reference site (1.222 ± 0.169 *vs.* 0.250 ± 0.119‰; $U = 1.500$, $p < 0.001$).

The histopathological findings did not reveal the lesions that are commonly described in animals exposed to toxic metals or other

Table 2
Mean ± SEM values for several metals (Fe, Mg, Pb, Hg, Cd, Zn, Cu, Mn, Mo, Co, and Cr) in tissues of *C. russula* compared by site (in $\mu g\ g^{-1}$ DW).

	Liver				Kidneys			
	Reference site ($n = 10$)	Polluted site ($n = 19$)	t	p	Reference site ($n = 9$)	Polluted site ($n = 19$)	t	p
Fe	732.16 ± 230.29	952.37 ± 170.16	–	–	351.36 ± 15.08	362.49 ± 12.99	–	–
Mg	390.17 ± 20.92	394.14 ± 46.08	–	–	239.52 ± 21.19	313.83 ± 9.83	3.635	0.001
Pb	2.16 ± 0.39	2.79 ± 0.37	–	–	2.09 ± 0.19	2.43 ± 0.19	–	–
Hg	0.53 ± 0.12	1.28 ± 0.13	4.167	<0.001	1.08 ± 0.54	3.04 ± 0.24	3.976	<0.001
Cd	1.57 ± 0.21	5.56 ± 1.04	4.008	0.001	4.66 ± 1.01	16.74 ± 2.76	2.670	0.014
Zn	52.18 ± 3.52	49.34 ± 5.92	–	–	160.10 ± 11.34	177.12 ± 6.44	–	–
Cu	51.97 ± 10.30	56.16 ± 7.87	–	–	37.63 ± 4.98	48.95 ± 1.78	2.720	0.012
Mn	36.78 ± 1.83	38.27 ± 7.31	–	–	17.81 ± 2.23	20.02 ± 0.89	–	–
Mo	4.27 ± 1.22	6.81 ± 0.91	–	–	3.32 ± 0.58	3.75 ± 0.24	–	–
Co	0.64 ± 0.11	0.54 ± 0.10	–	–	0.62 ± 0.12	1.09 ± 0.10	2.589	0.016
Cr	3.00 ± 0.48	1.74 ± 0.60	2.955	0.006	2.07 ± 0.27	1.91 ± 0.19	–	–

pollutants; lesions such as fibrosis, vacuolization of the cytoplasm, inflammatory response, or neoplasic focus. However, our data showed some hepatic alterations in specimens from the polluted area, namely the occurrence of necrotic areas in four out of six individuals (Fig. 3) and high incidence of apoptotic figures (Fig. 3). When alterations were observed in hepatocyte nuclei, 1036 cells from specimens from the polluted site were compared by image analysis with 552 cells from specimens from the reference site. There was no significant difference in these parameters. However, shrews exposed to metals showed a tendency to increase the cellular perimeter when compared with reference shrews (7.531 ± 0.356 *vs.* 6.914 ± 0.222 μm), whereas the elongation, roundness, and compactness remained invariant between sites ($M \pm \mathrm{SEM}$: 1.190 ± 0.020 *vs.* 1.193 ± 0.021 μm; 0.724 ± 0.021 *vs.* 0.755 ± 0.010 μm; 0.912 ± 0.009 *vs.* 0.917 ± 0.007 μm, respectively). These parameters were significantly correlated with Pb, Mn and Cr contents in liver. The perimeter was correlated with Pb and Cr ($r = -0.665$, $p = 0.018$; $r = -0.597$, $p = 0.041$, respectively), the roundness with Pb ($r = -0.597$, $p = 0.041$), Mn ($r = -0.597$, $p = 0.041$), and Cr ($r = -0.597$, $p = 0.041$), and compactness with Mn ($r = -0.597$, $p = 0.041$). Moreover, no alterations related to toxic metals were detected in renal tissue. Given the small number of animals examined, micronucleus and histopathological data were not analysed to assess the effect of age or gender or correlations with metal bioaccumulation.

4. Discussion

4.1. Bioaccumulation of metals

Since the Aznalcóllar mine spillage, several studies have reported an increase in heavy metal concentrations in soil and water (Tovar-Sanchez et al., 2006) which lead to toxic effects for wildlife of Doñana (e.g. Blasco et al., 1999; Bonilla-Valverde et al., 2004; Sánchez-Chardi, 2007). After this ecological disaster, most of the sludge from the floodplains was removed to minimize pollution effects. However, even after this remediation measure, later studies reported the continued presence of metals in the environment (e.g. Fletcher et al., 2006; Tovar-Sanchez et al., 2006; ; Smits et al., 2007; Millán et al., 2008; Márquez-Ferrando et al., 2009).

Eighteen months after this environmental disaster, the availability of potentially toxic metals such as Cd and Hg in the impacted site was still higher than in the reference site and they were accumulated in the tissues of shrews. In general, these non-essential elements are available to biota over a long time and are usually strongly accumulated in soft tissues of mammals recently exposed to contaminated air, food or water. Cd is a highly bioavailable metal and is mobile in less acidic soils such as those found in the Doñana area. It is retained with Fe oxides (Kraus and Wiegand, 2006) and is one of the main pollutants of mine residues. Mercury was also present in considerable amounts in the residues (e.g. Bonilla-Valverde et al., 2004). The low increase in Pb concentrations detected in shrews from the polluted area could be explained by low bioavailability several months after the spillage, probably because this metal is immobile in less acidic soils (Kraus and Wiegand, 2006). Zn, Cu, Co, Mn, and Mo were abundant in waters and slurry spilled from the mine and remained in high concentrations in the water and soil of the polluted area several years after the incident (Tovar-Sanchez et al., 2006).

Essential metals had similar concentrations in tissues from shrews from the polluted and reference sites, as previously reported in the Algerian mouse *Mus spretus* (Bonilla-Valverde et al., 2004). This is a result of metabolic regulation that prevents disruption of metabolic turnover and high bioaccumulation in mamma-

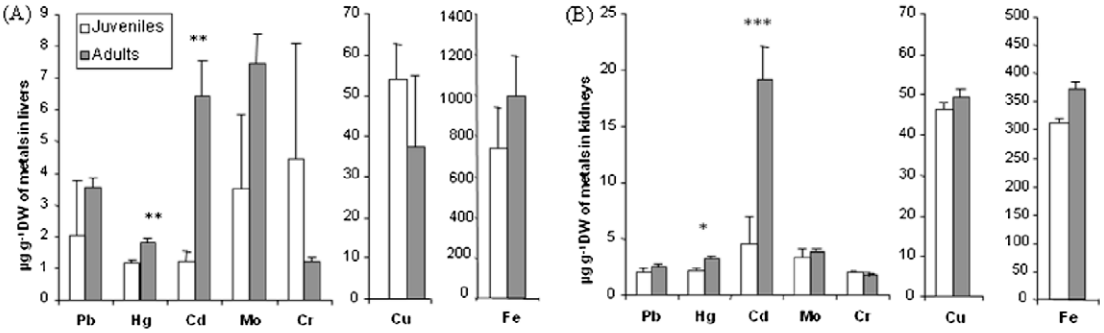

Fig. 2. Mean ± SEM values for Pb, Hg, Cd, Mo, Cr, Cu, and Fe in *C. russula* collected at the polluted site, by age in liver (A) and kidneys (B) (in $\mu g\ g^{-1}$ DW), ($^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.01$; $^{***}p \leqslant 0.001$).

Table 3
Mean ± SEM values for metals (Fe, Pb, Hg, Zn, Mo, and Co) in *C. russula* by tissue, site and sex (in $\mu g\ g^{-1}$ DW).

		Reference site		Polluted site	
		Males ($n = 6$)	Females ($n = 3$)	Males ($n = 9$)	Females ($n = 10$)
Liver	Fe	803.27 ± 223.87	589.92 ± 248.45	1086.3 ± 361.3	845.2 ± 119.8
	Pb	2.88 ± 0.53	2.52 ± 0.56	3.20 ± 0.69	1.56 ± 0.18*
	Hg	0.83 ± 0.07	0.38 ± 0.14	1.49 ± 0.15	1.11 ± 0.20
	Zn	24.54 ± 2.41	63.94 ± 22.96	67.93 ± 22.37	77.85 ± 15.71
	Mo	2.41 ± 0.22	8.00 ± 2.69*	6.07 ± 1.30	7.74 ± 1.25
	Co	0.63 ± 0.08	0.67 ± 0.25	0.63 ± 0.19	0.29 ± 0.05*
Kidneys	Fe	162.30 ± 19.67	136.77 ± 19.67	161.41 ± 14.92	163.36 ± 20.86
	Pb	2.40 ± 0.33	2.14 ± 0.70	2.36 ± 0.21	2.52 ± 0.27
	Hg	2.57 ± 0.56	1.00 ± 0.51	3.09 ± 0.29	3.03 ± 0.37
	Zn	157.31 ± 7.89	163.81 ± 0.51	175.78 ± 11.81	178.19 ± 7.34
	Mo	3.44 ± 0.62	3.27 ± 1.26	3.37 ± 0.38	4.05 ± 0.27
	Co	0.64 ± 0.09	0.59 ± 0.27	0.86 ± 0.09	1.26 ± 0.14

* $p \leqslant 0.05$.

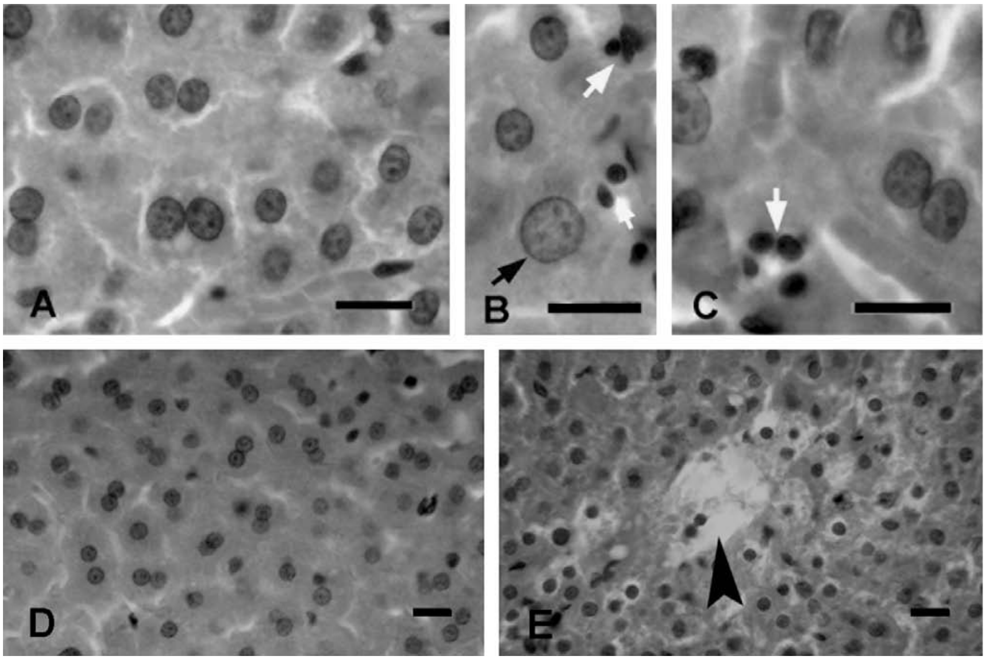

Fig. 3. Hepatic sections showing normal and altered tissue in *C. russula*. (A, D) Healthy livers from shrews collected at the reference site; (B, C, E) livers from shrews collected at the polluted site: observe the enlarged nuclei (black arrow) (B), the apoptotic figure (white arrow) (B, C), and the foci of cell necrosis (black arrow) (E). Haematoxilin and eosin stains. (Scale bars: A, D, E = 50 μm; B, C = 20 μm).

lian tissues despite substantially elevated dietary intake (e.g. Talmage and Walton, 1991; Goyer, 1997; Ma and Talmage, 2001). In contrast, the higher mean values of Cr, another essential element, in specimens from the reference site, could be partially explained by the effect of fertilizers, which load the waters and soils of the reference area with metals (Tovar-Sanchez et al., 2006). The Doñana area has been exposed to agricultural practices and mining extraction for centuries, which contributed metals to the environment before the spillage of 1998 (Arambarri et al., 1996; Fletcher et al., 2006).

The adult shrews from Doñana had higher Cd accumulation than juveniles, which is consistent with previous reports in soft tissues of *C. russula* (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007b) and other small mammals species exposed to mine pollution (Bonilla-Valverde et al., 2004; Smith and Rongstad, 1982). This age-dependent bioaccumulation is associated with detoxification mechanisms, namely the formation of cadmium-metallothionein in liver, which is transported by blood and then stored mainly in the renal cortex (e.g. Bonilla-Valverde et al., 2004; Sánchez-Chardi et al., 2007b). A similar mechanism could explain the age-dependent accumulation of Hg and Pb, as also reported for other small mammals species (e.g. Pankakoski et al., 1993, 1994; Stansley and Roscoe, 1996; Damek-Poprawa and Sawicka-Kapusta, 2004). The tendency of essential elements that are physiologically well regulated in mammals to increase with age (Fig. 2) could be related to the metabolic requirements of adult specimens as well as to interferences with non-essential elements (Pankakoski et al., 1994; Goyer, 1997; Bellés et al., 2002; López Alonso et al., 2004). The decrease in Cr detected is concordant with a lower intestinal absorption of this essential metal in adults (Outridge and Scheuhammer, 1993; Sánchez-Chardi et al., 2007a).

Gender differences in the bioaccumulation pattern of non-essential metals have been reported in several species of mammals including human (Smith and Rongstad, 1982; Komarnicki, 2000; Clark et al., 1992; Vahter et al., 2007). Low Hg and Pb levels in females could be related mainly to the reduction of metal burden mobilized and transferred during gestation to the foetus and/or during lactation to the young (see references in Sánchez-Chardi et al., 2007b). No sex-dependent variation was reported for Mo in *Talpa europaea* (Pankakoski et al., 1993). In contrast, the females of *C. russula* collected in a pyrite mine site and in a landfill site had higher Mo concentrations than males (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007a). More Fe was detected in males of different species including human (Khan et al., 1995; Clark et al., 1992). Zn also tends to increase in soft tissues in the females of some mammals such as the mole (Komarnicki, 2000). More specifically, steroid hormone receptors are Zn-finger proteins and Zn is also part of several enzymes, including some related to antioxidant systems such as cytosolic superoxide dismutase (Lopes et al., 2002). Generally speaking, the gender differences of these essential elements (Co, Fe, Zn, and Mo) may be associated with differences in the metabolic profile of metals involved in the activity of sexual hormones, the intake or uptake of metals, nutritional requirements or interactions between elements (Goyer, 1997; Chmielnicka and Sowa, 2000; Bellés et al., 2002; Lopes et al., 2002; López Alonso et al., 2004; Vahter et al., 2007).

4.2. Genotoxicity

The micronucleus numbers found in shrews from the central part of the Doñana reserve ("La Vera") could be considered references for genotoxicity rates and are in concordance with data previously reported (Tanzanella et al., 2001; Festa et al., 2003; Mateos et al., 2008). The MN frequencies were higher in shrews exposed to metals from the pyrite mine, thereby indicating the clastogenic effects of this kind of pollution, which is bioavailability to biota over a long period of time. Similar results were reported by Festa et al. (2003) and Tanzanella et al. (2001) in a survey of the Algerian mouse, *M. spretus*, collected at the same sites during the same period. These authors speculate on the contribution of the chronic pollution that occurred before the mine accident to genotoxicity. In fact, the Guadiamar River was severely affected by anthropogenic activities before this accident (some mines have been operating since Roman times), thereby causing increased levels of toxic substances in the area (e.g. Arambarri et al., 1996; Fletcher et al., 2006). However, the significant effect of the spillage in 1998 on the bioavailability of toxic metals and on the genotoxic damage to the Doñana reserve is evident, as shown by the increase in metal concentrations in soil, water, plants and animals (e.g. Blasco et al., 1999; Bonilla-Valverde et al., 2004; Fletcher et al., 2006; Gil et al., 2006; Kraus and Wiegand, 2006; Tovar-Sanchez et al., 2006; Madejón et al., 2007; Smits et al., 2007).

4.3. Morphometric parameters

In shrews from the polluted site, the increase in liver weight and the tendency to show increased renal weight and decreased body weight may be indicative of considerable physiological and histological alterations (Shore and Douben, 1994; Sánchez-Chardi et al., 2007b, 2008). The presence we detected of both histological alterations in the livers and genotoxic effects in the blood of shrews from the polluted area are consistent with these morphological findings and could be explained by exposure to toxic levels of non-essential metals. Similar results of morphometric parameters were previously obtained for small mammals exposed to metals (Ma and Talmage, 2001; Pereira et al., 2006; Sánchez-Chardi et al., 2007a,b). In mammals, the main exposure route to pollutants is through diet. The liver is the most important detoxifying tissue. This organ plays a crucial role in food conversion, biotransformation of xenobiotics, and vitellogenesis for reproduction purposes. Consequently, the impairments of hepatic function have a number of negative consequences on growth, health, life expectancy and reproductive success of individuals and may therefore adversely affect whole populations.

4.4. Histopathology

Histopathological endpoints could also contribute to the sensitivity of organs to heavy metal pollution and could provide information on the mechanism of action of pollutants and on target organs (Wester et al., 2002). Cellular biomarkers act as early warning signals of stress by organisms exposed to contamination. They may provide information on the level of the developing stress, ranging from initial biological effects to the impact on cell physiology. There are wide descriptions that exposure to heavy metals generates ROS via Fenton-type or Haber-Weiss-type reactions. Heavy metals such as Tl, Cd, Co, Fe, Pb, and Cr also react directly with cell molecules within the cytosol and cause a wide range of cellular responses such as apoptosis and finally necrosis (e.g. Bragadin et al., 2003; Leonard et al., 2004; Chia et al., 2005; Oliveira Ribeiro et al., 2005), depending on the level of oxidative stress generated. The occurrence of cell cycle arrest (necrosis and apoptotic figures) in liver of *C. russula* chronically exposed to high concentrations of Tl, Pb, Cd, and Hg corroborates the above effects reported in the laboratory. In addition, the nuclear alterations described here are also additional evidence that the uptake and high bioaccumulation of toxic metals in livers of *C. russula* are related to these findings. Theses alterations may represent chromatin disorganization and have serious consequences on gene expression by altering the transmission of information from the nucleus to the cytoplasm and outside the cell. In fact, the presence of distinct nuclear forms reported here provides evidence that the toxic mechanism of metals also disturbs the DNA and protein array within the nucleus.

A few studies have described renal alterations (see references in Sánchez-Chardi et al., 2008) in Soricine species of shrews. However, the response of *C. russula*, a Crocidurine species, to metal pollution is more similar to that shown by rodents (Damek-Poprawa and Sawicka-Kapusta, 2004; Pereira et al., 2006). Given their particular metabolic characteristics (e.g. Stewart et al., 2005) and differences in phylogenic origin and ecological strategy, further studies including biochemical, physiological or morphological parameters, are required to explain the differences observed between groups of shrews in response to exposure to heavy metals.

4.5. Toxicity effects

Several studies with laboratory specimens have reported toxic effects of a single metal under controlled conditions. However, data on wild populations of species of higher trophic levels are scarce. The effects of heavy metals considered alone as well as cumulative toxicity rates or interactions such as synergism could explain the increase in genotoxicity and histological alterations observed in shrews from the Doñana reserve. No complete guideline on the toxic levels of the heavy metals addressed in this study is currently available for shrews. However, considered alone, the levels found for essential metals and Pb do not appear to be of toxicological hazard in the specimens collected in Doñana. In contrast, Cd and Hg levels in renal and hepatic tissues of shrews from the polluted area merit concern. The mean values of Hg higher than 1.1 $\mu g\ g^{-1}$ should be regarded as presumptive evidence of an environmental mercury problem in wild mammals (Eisler, 1987). Wild specimens with high loads of non-essential metals may suffer toxic effects such as impairment of reproduction or a decrease in life expectancy. When assessing the status of wild animals, the toxic effects of environmental pollution could be biased because of the limited number of animals studied as well as by the intra-specific variation and selection in natural populations, which may reduce the metal load or eliminate impacted animals that are easier preys to predators. These features may remove extreme values that can be measured in laboratory studies. In fact, laboratory rodents showed alterations such as necrosis, apoptosis, vacuolization and fibrosis when chronically exposed to realistic concentrations of metals (e.g. Jadhav et al., 2007).

4.6. Environmental quality assessment in protected sites

Inhabited by several endangered vertebrate species, including carnivorous birds and mammals, the area of Doñana is one of largest protected coastal sites and one of the last great wildernesses in Europe. These protected species feed on small mammals and one of the most abundant in Doñana is the greater white-toothed shrew (Cagnin et al., 1998). Could *C. russula* serve as a bioindicator to assess environmental quality? Given that this species has a high metabolic rate, food requirements and tolerance to toxins, it reacts to pollution by bioaccumulating higher concentrations of heavy metals than sympatric rodents (Alleva et al., 2006; Sánchez-Chardi and Nadal, 2007; Wijnhoven et al., 2007), as occurs with other insectivores (Talmage and Walton, 1991; Ma and Talmage, 2001). These metabolic characteristics as well as the ecological interest of abundant species of non-migratory and easily available carnivores make shrews as suitable biomonitoring species to detect very finely an increase of xenobiotics (Talmage and Walton, 1991; Shore and Douben, 1994; Komarnicki, 2000; Ma and Talmage, 2001). In contrast, the shrews appear to be more tolerant to pollutants than rodents as shown by less alteration of physiological parameters such as biochemical biomarkers (e.g. Ma and Talmage, 2001; Sánchez-Chardi et al., 2008). Comparative studies of sympatric species are required for correct assessment of environmental quality and risk in order to determine inter-specific sensitivity to pollutants and detect the most sensitive species. Thus, it may be possible to assess the critical loads, namely highest levels of pollutants without toxic effects, for wild populations and ecosystems. Moreover, a database for heavy metal concentrations in autochthonous biota with diverse trophic strategies is required to allow studies on the bioavailability, transfer and behaviour of chemicals through a variety of protected ecosystems. The study of temporal variation of metals throughout the ecosystems could also be important for these protected areas. In fact, our samples were collected a few months after the spillage from the pyrite mine. Later studies in Doñana showed differences in bioavailability of metals in soils, waters and plants. However, to our knowledge, terrestrial mammals at high trophic position have not been biomonitored. We consider that *C. russula* is a suitable species to assess environmental quality especially for bioaccumulable pollutants and recommend that it be included as part of management programmes for protected sites.

5. Conclusions

Here we have identified Hg and Cd as toxic elements that are highly bioaccumulated in terrestrial mammals in Doñana. We have also reported age and sex as two relevant factors to explain variability in bioaccumulation patterns in wild populations recently exposed to extremely high amounts of metals. Moreover, we have found morphometric, genotoxic, and histological effects of metal pollution in shrews from the area affected by the mine spillage. These effects and the considerable concentrations of non-essential metals in tissues of shrews from this protected area also indicate the need for frequent sampling to evaluate the food chain transfer of these long-term persistent pollutants. The high trophic position of shrews as well as their abundance makes them a suitable species for biomonitoring programmes especially in areas of ecologic value such as Doñana.

Acknowledgements

We thank M.A. Bravo, R. Laffitte, S. Moreno, F. Ibáñez, and P. Bayón and the rest of the staff at the Estación Biológica de Doñana for their field and laboratory assistance. We also thank M. Franch (Dpt. Animal Biology, Universitat de Barcelona) for laboratory assistance and R. Rycroft (Servei d'Assessorament Lingüístic, Universitat of Barcelona) for revising the English.

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