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Mercury exposure and mechanism of response in large game using the Almadén mercury mining area (Spain) as a case study ☆

Juan J. Berzas Nevado [a], Rosa C. Rodríguez Martín-Doimeadios [b,*], Rafael Mateo [c], Nuria Rodríguez Fariñas [b], Jaime Rodríguez-Estival [c], María J. Patiño Ropero [b]

[a] Facultad de Ciencias Químicas, Universidad de Castilla-La Mancha, 13071 Ciudad Real, Spain
[b] Facultad de Ciencias Ambientales y Bioquímica, Universidad de Castilla-La Mancha, 45071 Toledo, Spain
[c] Instituto de Recursos Cinegéticos, IREC (CSIC, UCLM, JCCM), Ronda de Toledo s/n, 13071 Ciudad Real, Spain

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ABSTRACT

Mercury (Hg) accumulation, transfer, defense mechanism and adverse effects were studied in red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) from the Almadén mining district (Spain), the largest (285,000 t of Hg) and the oldest (more than 2000 years) Hg mine/refining operation site in the world. Red deer ($n=168$) and wild boar ($n=58$) liver, kidney, bones (metacarpus), testis and muscle were analyzed for total Hg and selenium (Se) within a range of distances to the Almadén mining district. The highest Hg concentrations were found in kidney (0.092 and 0.103 μg/g d.w. for red deer and wild boar, respectively) followed by the levels in liver (0.013 and 0.023 μg/g d.w. for red deer and wild boar, respectively). A significant correlation ($r=-0.609$, $p=0.007$) was found between Hg concentrations and distance to the Almadén Hg mining district. However, both red deer and wild boar closest to the mining area still showed mercury concentrations well below the concentration associated with clinical signs of Hg poisoning. Highest Se concentrations were found in kidney (2.60 and 6.08 μg/g in red deer and wild boar, respectively) and testis (2.20 μg/g in red deer). For selenium, differences between red deer and wild boar were statistically significant ($p<0.05$) in all tissues, concentrations being higher in wild boar than in red deer. In the diagnosis of Se deficiency, the vast majority of the examined red deer livers were deficient. A significant correlation was found between Hg and Se in kidney ($r=0.386$, $p>0.001$ for red deer and $r=0.567$, $p=0.005$ for wild boar). Liver GSSG concentrations were negatively correlated to total mercury and Hg:Se molar ratio in male deer, which could indicate a hormetic response to Hg exposure. Moreover, a positive association was found between the antioxidant element Se and antioxidant vitamins in red deer tissues.

1. Introduction

The environmental transport and fate of mercury (Hg) is a matter of global concern because it is a non-essential toxic element that occurs in different chemical and physical forms (Mason and Benoit, 2003; Watras and Huckabee, 1992). The most important forms are elemental Hg (Hg^0), inorganic Hg (Hg^{2+}) and methylmercury (MeHg), which differ with respect to kinetics and toxicology (WHO, 1990, 1991; Clarkson, 1997; ATSDR, 1999; Rodrigues et al., 2010). MeHg is known as a very important neurotoxicant that bioacumulates in the aquatic food chain, with fish or seafood being the main source for human exposure (Watras and Huckabee, 1992; Clarkson, 1997). Most studies have been devoted to the aquatic environments, while little attention has been paid to Hg in terrestrial ecosystems until now (Cristol et al., 2008; Gnamus et al., 2000; Wren, 1986; Lodenius, 1994; Lodenius, 1995; Boudou and Ribeyre, 1997). However, the majority of total Hg (approximately 60%) is estimated to be deposited on terrestrial environments close to contamination sources, leading to significant accumulation in local food webs (Fitzgerald and Mason, 1996). Therefore, there are several reasons why the study of Hg in terrestrial animals is of great interest. Firstly, major differences exist compared to the aquatic ecosystem; thus the mechanisms of Hg accumulation and transfer are probably very different. Additionally, part of human exposure to Hg pollution occurs through the consumption of terrestrial animal tissues. Finally, important information about the adverse impact of metal pollution, in terms of lethal and/or sublethal effects, and protection/detoxification mechanisms can be obtained from the study of

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* Corresponding author. Fax: +34 925 268 840.
E-mail address: RosaCarmen.Rodriguez@uclm.es (R.C. Rodríguez Martín-Doimeadios).

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Hg and Se status of these animals, and may be extrapolated to estimate the impact on humans.

In this context, the study of Hg accumulation and transfer in terrestrial animals in sites with past or active Hg mining constitutes a unique opportunity. Metal mining generated large volumes of crushed waste rock (spoil) and tailings with high concentrations of residual heavy metals. Heavy metal from mining and smelting commonly contaminates air, water and soil, and this contamination affects biota. A special case of interest for the study of Hg in terrestrial biota is the area of the recently closed Almadén Hg mine (Ciudad Real Province, Southern Spain). The Almadén mining district has produced one third of the total Hg production worldwide (285,000 t of Hg). Mining operation began more than 2000 years ago and no other region in the world has been influenced by Hg for such a long time. Therefore it is one of the largest Hg-contaminated sites and it continues to generate significant atmospheric emissions despite its closure in May 2002. Some recent studies have been conducted to evaluate the environmental impact and potential hazards related to Hg contamination in the Almadén ecosystem (Berzas Nevado et al., 2003; Gray et al., 2004; Higueras et al., 2006). Mercury contamination of soil, water, sediment and the atmosphere has been reported at Almadén (Berzas Nevado et al., 2003; Gray et al., 2004; Higueras et al., 2006; Hildebrand et al., 1980; Ferrara et al., 1998; Rodríguez Martín-Doimeadios et al., 2000; Berzas Nevado et al., 2009). On the other hand, the area is largely devoted to and occupied by hunting estates, where several hundred red deer and wild boar are hunted every year. Exposure of wildlife species to Hg can occur through food chains or through the direct ingestion or licking of soil to attain essential elements (Beyer and Fries, 2003; Beyer et al. 2007). The study of Hg distribution in different tissues from these terrestrial animals can provide important information regarding Hg exchanges and transformations since the long-term Hg exposition in Almadén probably yielded a steady cycling state. Moreover, the interactions between Hg and selenium (Se) should be examined, owing to the fact that Se has been described as an antagonist to Hg and the ratio of Hg to Se concentration has been suggested to be a more significant parameter to define the level of intoxication than the Hg concentration alone (Carvalho et al., 2008, 2010; Ralston et al., 2008; Ralston and Raymond, 2010; Yang et al., 2008).

Here, we focus on the study of total Hg and Se concentration in red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) from the Ciudad Real Province (Southern Spain), where samples were collected within a range of distances to the Almadén mining district. The selected tissues were liver, kidney, bones (metacarpus), testis and muscle. The objectives of the present study were as follows: (1) to examine the environmental impact of Hg pollution in wildlife and species of interest for hunting; (2) to assess the route of Hg and its transfer into terrestrial organisms tissues and (3) to identify possible toxicity effects and defense mechanisms (Se interaction).

2. Material and methods

2.1. Study area

The area under investigation is in the northern part of the Eastern Sierra Morena in the Province of Ciudad Real (Spain). In this area there are various Hg mines with the most important one being the mine in the town of Almadén itself (Fig. 1). In this area there are also several old lead mines and prospects, the majority of which targeted lead–zinc veins (Palero-Fernández and Martin-Izard, 2005). The region lies at altitudes between 500 and 1300 m. The climate of the region is defined as Mediterranean with marked seasons and with an average annual rainfall of 500 mm. During the sampling year 2005, Spain had the lowest annual rainfall since 1974, with only 279 mm in this area (Spanish Meteorological Agency, http://www.aemet.es). Some of the sites further from the Almadén district were located in the Montes de Ciudad Real and Toledo region, which has similar geomorphological, climatic and biogeographic characteristics as those of the Almadén area of study, but was not subjected to mining and not known to be rich in mineralized zones with high metal contents. Sampling points represent a gradient of Hg contamination depending on the distance to Almadén rather than control vs. polluted sites. The density of red deer in managed hunting states in the study area (18.3–32.4 deer/100 ha) is within the range found in the

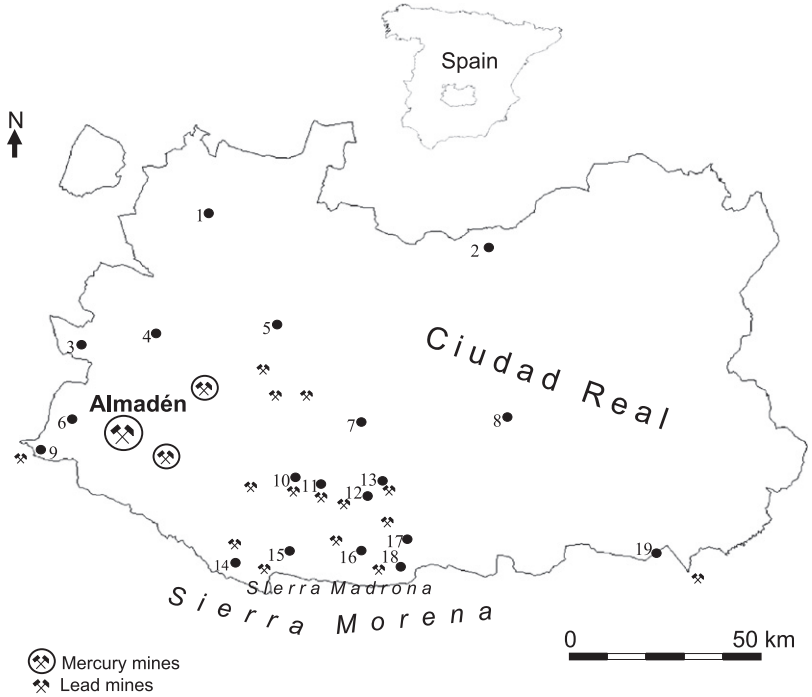

Fig. 1. Study area in the Province of Ciudad Real (Southern Spain). The most important Hg mines in the area are shown. Also the presences of Pb mines close to the sampling sites are shown. The sampling hunting estates were: El Rostro (1), Peñas Amarillas (2), Lagunillas (3), Torneros (4), Hornias Bajas (5), Los Manchones (6), Aeropuerto (7), Pozo del Borrico (8), Los Baldíos (9), El Girote (10), Zamorillas (11), El Burcio (12), La Gallega (13), La Garganta (14), Navalmartina (15), Umbría de Montoro (16), Tembladera (17), El Hoyo (18) and Navalcaballo (19).

Montes de Ciudad Real and Toledo area (11.3–90.1 deer/100 ha; Vicente et al., 2007).

2.2. Sampling

In all, 168 red deer and 58 wild boar were obtained from regular shooting allocations during the hunting seasons 2004–2005 (74 red deer) and 2005–2006 (94 red deer and 58 wild boar) between October and March. In the hunting season of 2004–2005, only male red deer samples were collected. Sampling took place after "monterías" (large driven hunts), which usually last 3 h, at the end of which the animals are butchered on site. Liver, kidney, muscle, testis and bone (metacarpus) were taken during this process and immediately cooled (at 4 °C) in a portable cooler. Testes were not sampled from wild boar, and muscle of both red deer and wild boar was collected only in the 2005–2006 season. Within 2 h, samples were transferred to a −80 °C freezer and stored until analysis. Sex was recorded at the time of sampling. Red deer were classified as 130 male and 38 female. Sex was reported only for 30 wild boar samples: 15 were male and 15 were female.

2.3. Elemental analysis

Tissues were freeze-dried (Christ Alpha 1–2, Braun Biotech) and dry samples (0.5 g) digested with 3 mL of HNO_3 (65% Suprapur), 1 mL of H_2O_2 (30% v/v Suprapur) and 4 mL of H_2O (ultrapure water, 18.2 MΩ cm) with a Milestone Ethos plus microwave oven (Monroe, CT, USA; 10 min at 180 °C after a 15 min ramping time). Digested samples were diluted to a final volume of 50 mL with ultrapure water and the extracts were kept refrigerated until analysis. Blanks were processed in each batch of digestions. The total Hg and Se analyses were carried out with an inductively coupled plasma mass spectrometer (ICP-MS) equipped with a collision cell (CCT; Thermo Electron Model XSeries II). The instrument was operated in standard (non-CCT) mode for acquiring data for ^{202}Hg, while CCT mode (using H_2/He as the collision gas) was used for measuring ^{78}Se. Solutions used for calibration were prepared from commercial certified stock standards with 1 g/L of each element. The limits of detection (LODs, in ng/g dry weight, back calculated to 0.5 g in tissue) were for Hg: 0.49 ng/g and for Se: 0.53 ng/g. Reference bovine liver (NCS ZC 71001) was analyzed and adequate recovery was obtained in all cases (92.7 ± 2.4% for Hg and 94.7 ± 0.6% for Se, $n=3$). All concentrations are given in dry weight (d.w.).

2.4. Analysis of oxidative stress biomarkers

Oxidative stress biomarkers were analyzed in some red deer and wild boar tissues, with the values published in Reglero et al. (2009a, 2009b) and Rodriguez-Estival et al. (2011). Here we use the data on oxidative stress biomarkers obtained in these studies to explore potential relationships with Hg pollution. The selected biomarkers measured in red deer liver ($n=66$) and testis ($n=38$) were TBARS (thiobarbituric acid-reactive substances), GSH (glutathione), GSSG (oxidized GSH), SOD (superoxide dismutase) and GPX (GSH peroxidase). A full method description can be found in Reglero et al. (2009a). Moreover, levels of vitamins A and E in liver ($n=66$) and testis ($n=38$) of red deer and liver of wild boar ($n=22$) were analyzed as described by Rodriguez-Estival et al. (2011).

2.5. Statistical analysis

Data below the detection limit were assigned values of half of the respective LOD for each element. Tissue concentrations of Hg and Se and Hg:Se molar ratio were log-transformed to approach a normal distribution. Differences in Hg and Se levels between hunting seasons were tested in males of red deer with Student *t*-tests. The effects of gender, age (juvenile or adult) or species were studied by means of General Linear Models (GLM). As age and gender of some animals were not determined, differences in tissue concentrations of the studied elements between species were performed with Student *t*-tests in order to test the species effect with a larger sample size. The relationship between mean Hg levels in tissues of wild boar and red deer from each hunting estate and the distance to Almadén was tested by means of linear correlations. A relationship between tissue concentrations of each element and the relationship between both elements within each tissue were also established using linear correlation analyses, as was the relationship between liver Hg, Se or Hg:Se concentration and oxidative stress biomarkers in the liver and testis of red deer. As the study area had also an important Pb pollution, the observed effects of Hg, Se or Hg:Se on oxidative stress biomarkers were confirmed by means of GLM including Pb levels as a covariant. Statistical significance was set at $p<0.05$. Statistical analysis was performed with IBM SPSS Statistics version 19.

3. Results

The highest Hg concentrations were found in kidney (0.092 and 0.103 μg/g d.w. for red deer and wild boar, respectively) followed by the levels in liver (0.013 and 0.023 μg/g d.w. for red deer and wild boar, respectively; Table 1). The lowest total Hg concentrations were found in bone and muscle. Differences between red deer and wild boar Hg concentrations were significant for muscle, with higher levels in the wild boar than in the red deer (*t*-test, $p<0.001$). Wild boar also had higher levels of Se in liver, muscle and kidney than red deer (*t*-tests, $p<0.001$).

Red deer sampled in the 2005–2006 season showed higher levels of Hg (*t*-test, $p=0.006$, Fig. 2) and Se (*t*-test, $p<0.001$) in kidney and lower levels of Se in liver (*t*-test, $p<0.001$) than in the 2004–2005 hunting season. As the sample collected in the first hunting season was only of male red deer, further GLM analyses including species, gender and age (juvenile or adult) as factors were performed only with the samples collected in the 2005–2006 hunting season. In these analyses, the effect was significant for gender on Hg in muscle (GLM, $p=0.017$), with the males showing higher levels than females in both species (Fig. 3). The differences seen between species were confirmed for Hg in muscle, Se in liver and Se in kidney using the models applied (GLM, $p<0.001$).

The Hg concentration in the animal tissues was correlated with the spatial distribution of the respective animals within the study area. Here we found higher Hg concentrations for animals living closer to the Almadén Hg district (Fig. 4), showing that the mean Hg level in kidney of ungulates of each hunting estate is negatively correlated with the distance to the Almadén Hg mine ($r=-0.609$, $p=0.007$).

In red deer, Hg concentrations (all log-transformed) in liver were positively correlated with Hg concentrations in kidney ($n=144$, $r=0.280$, $p=0.001$) and negatively with Hg concentrations in testis ($n=111$, $r=-0.371$, $p<0.001$). Selenium levels in liver and kidney were negatively correlated ($n=144$, $r=-0.258$, $p=0.002$), while kidney levels of Se and Hg were positively correlated ($n=144$, $r=0.349$, $p<0.001$, Fig. 5). In wild boar, there was a positive correlation between Hg in liver and kidney ($n=19$, $r=0.537$, $p=0.018$) or muscle ($n=31$, $r=0.431$, $p=0.016$), and between kidney and muscle ($n=17$, $r=0.494$, $p=0.044$). Also in wild boar, Hg and Se concentrations were positively correlated in kidney ($n=19$, $r=0.656$, $p=0.002$; Fig. 5) and muscle ($n=31$, $r=0.501$, $p=0.004$). The correlation between renal Hg and Se concentrations was more significant (and the slope of the regression was greater) in wild boar than in red deer (Fig. 5).

The Hg:Se molar ratios have been reported to be characteristic of the association between Hg and Se. Results are shown in Table 2. Higher molar ratios (Hg over Se) were found in red deer than in wild boar (*t*-test, $p=0.002$ in liver and $p=0.018$ in kidney) and in liver than in kidney (*t*-test, $p<0.001$ for red deer and wild boar).

The observed Hg levels have produced few changes on oxidative stress biomarkers. Liver levels of Hg in red deer were inversely correlated with liver GSSG levels ($n=66$, $r=-0.345$, $p=0.005$). Liver Hg:Se molar ratio in red deer was also inversely correlated with liver GSSG level ($n=66$, $r=-0.445$, $p<0.001$). However, this effect was gender related and significant only in males (GLM, interaction gender × Hg:Se, $p=0.003$; Fig. 6). Moreover, an association was found between the antioxidant element Se and the antioxidant vitamins in red deer tissues, because Se levels were positively correlated with α-tocopherol in liver ($n=66$, $r=0.442$, $p<0.001$) and retinol in testis ($n=35$, $r=0.349$, $p=0.040$). Similarly, Se levels were positively correlated in liver with retinyl docosapentaenoate ($n=66$, $r=0.361$, $p=0.003$) and retinyl palmitoate ($r=0.329$, $p=0.007$) levels.

Table 1
Total mercury and selenium concentrations (μg/g dry weight) in different tissues of wild ungulates (red deer and wild boar) from Southern Spain.

	Red deer (*Cervus elaphus*)					Wild boar (*Sus scrofa*)				
	n	Mean	SE	Min	Max	*n*	Mean	SE	Min	Max
Selenium										
Bone	7	0.014	0.005	0.003	0.018	10	0.023*	0.008	0.016	0.040
Liver	161	0.311	0.132	0.120	0.914	51	0.835*	0.354	0.280	1.730
Testis	116	2.204	0.476	0.003	3.130	0	–	–	–	–
Muscle	22	0.121	0.075	0.0005	0.331	33	0.334*	0.133	0.142	0.671
Kidney	146	2.600	0.829	0.142	5.630	23	6.078*	1.402	2.700	9.020
Mercury										
Bone	7	ND	ND	ND	ND	10	ND	ND	ND	ND
Liver	161	0.013	0.011	0.0002	0.061	51	0.023	0.027	0.0006	0.125
Testis	116	0.004	0.013	0.0009	0.093	0	–	–	–	–
Muscle	22	0.001	0.002	0.0005	0.010	33	0.017*	0.018	0.008	0.103
Kidney	146	0.092	0.106	0.0005	0.991	23	0.103	0.083	0.016	0.316

ND: less than the limit of detection.
*Significantly different between red deer and wild boar at $p<0.05$ (*t*-test with log-transformed data). The species with higher concentration is marked with an asterisk.

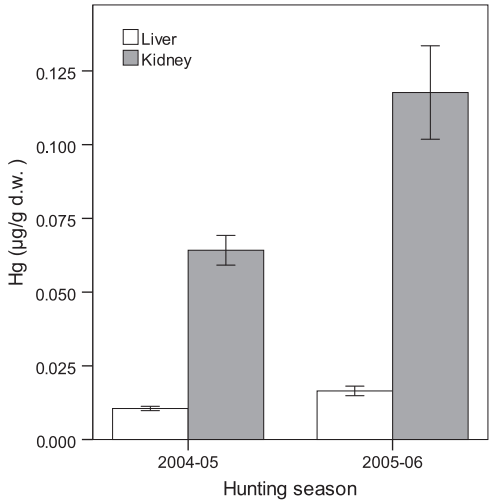

Fig. 2. Differences by hunting season in liver and kidney Hg concentrations (mean ± SE) of red deer in 2004–2005 and 2005–2006.

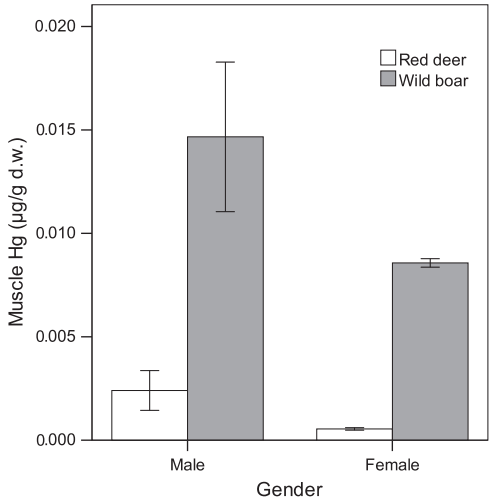

Fig. 3. Differences by sex in the Hg concentrations (mean ± SE) in muscle of red deer and wild boar.

Liver Hg:Se molar ratio in red deer was inversely correlated with retinyl docosapentaenoate ($n=66$, $r=-0.275$, $p=0.025$) and retinyl palmitoate ($r=-0.292$, $p=0.018$) levels. In the liver of wild boar, significant associations were found between Se and α-tocopherol ($n=22$, $r=0.594$, $p=0.004$) or retinyl linoleate ($r=-0.452$, $p=0.035$). No associations were found between Hg, Se or Hg:Se and oxidative stress biomarkers in testis of red deer.

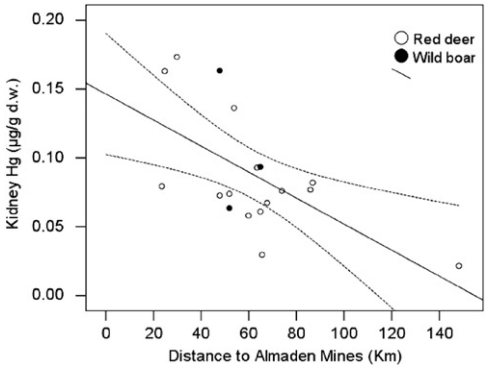

Fig. 4. Relationship between kidney Hg concentration and distance to Hg mines ($r=-0.609$, $p=0.007$) in red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*).

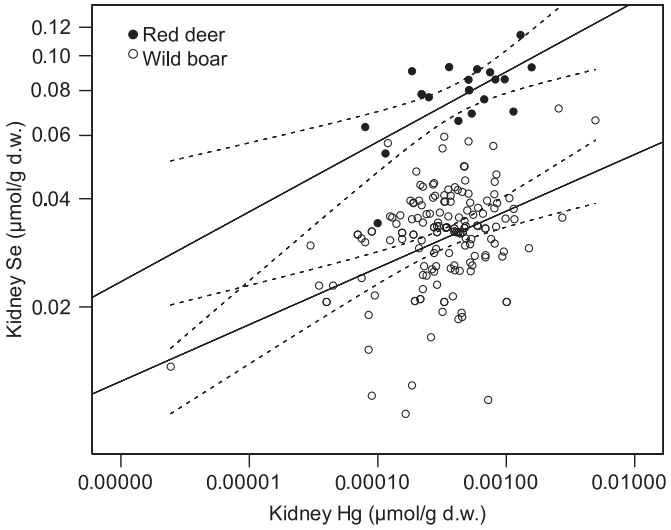

Fig. 5. Molar relationship between Hg and Se in kidney ($n=144$, $r=0.349$, $p<0.001$ for red deer and $n=19$, $r=0.656$, $p=0.002$ for wild boar).

Table 2
Tissue mercury:selenium molar ratios[a].

	Red deer		Wild boar	
	Liver ($n=161$)	Kidney ($n=144$)	Liver ($n=51$)	Kidney ($n=19$)
Mean	0.019	0.014	0.011	0.006
SE	0.017	0.013	0.011	0.004
Minimum	0.0001	0.0002	0.0002	0.0012
Maximum	0.091	0.076	0.052	0.017

[a] Molar ratio individually calculated using tissue mercury and selenium molar concentrations.

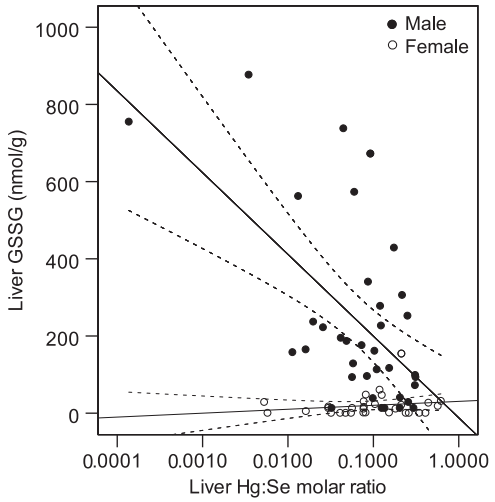

Fig. 6. Relationship between Hg:Se molar ratio and GSSG in liver of red deer. The correlation was significant in males ($n=36$, $r=-0.588$, $p<0.001$) but not females.

4. Discussion

The Almadén mining district has been exploited for over 2000 years producing one third of the total Hg worldwide production, and therefore wildlife species in the surrounding areas have been exposed to Hg. The Hg concentrations found in red deer and wild boar tissues are related to the distance to the main Hg mine. This elevated Hg exposure may have induced a Se defense mechanism, even in a situation of Se deficiency. However, biomarkers of oxidative stress were not affected by Hg exposure and only liver GSSG levels seem to be related.

4.1. Interpretation of Hg concentrations in red deer and wild boar tissues

Despite Hg exposure, red deer and wild boar showed liver and kidney concentrations of Hg well below the concentration associated with clinical signs of Hg poisoning (30 μg/g w.w., Thompson, 1996). In terms of implications for human consumption, there is no EU legislation for meat. All legislative efforts are concentrated on fish and seafood, in which Hg is bioaccumulated in the form of MeHg, which is well known for its neurotoxicity, and is more toxic than inorganic Hg. Other food sources have so far been excluded from the legislative framework, because Hg is predominantly in the inorganic form. However, inorganic Hg is also toxic, and the risk posed to the consumers may be underestimated. High exposure to inorganic Hg may result in damage to the gastrointestinal tract, the nervous system and the kidneys (WHO, 2003). The Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives has established regulatory guidelines regarding Hg intake (JECFA, 2000) provisionally recommending a tolerable weekly intake (PTW1) of 300 μg total Hg (THg) per person, of which no more than 100 μg should be present as MeHg. These amounts are equivalent to 5 μg/kg body weight (b.w.) of THg and 1.6 μg/kg b.w. of MeHg (WHO, 2003). Therefore, additional regulations considering other food sources and Hg forms become very important. With respect to our case study, the risk for human consumption of meat is minimal since low contents were found here in this tissue. However, significant Hg accumulation is found in other tissues, especially in kidney with values up to 0.991 μg/g (d.w.).

The differences found between red deer and wild boar can be related to different feeding habits. The diet of red deer in Spain is based on grass between autumn and spring and on leaves of bushes and trees during the summer (Reglero et al., 2008). Additionally, the plant species more commonly consumed by red deer in Spain are not known as active accumulators of Hg from soil or air (Carranza, 2004; Gnamus and Horvat, 1999; Gnamus et al., 2000). Wild boar may have higher concentrations compared to red deer, because they commonly feed by rooting in the soil, and as such ingest significantly more soil, and eat more roots, bulbs or tuber, which tend to contain higher metal levels than above ground plant parts (Reglero et al., 2009a; Rodríguez-Estival et al., 2011). Moreover, wild boar also includes animals in its diet, so higher Hg biomagnification than in the herbivorous red deer may be expected.

The differences found between hunting seasons (Fig. 2) could be explained by the fact that 2005–2006 was an unfavorable season because of the intense drought that affected the region (Reglero et al., 2009b). Drought probably reduced the quantity and quality of food and this could affect the mechanism of Hg detoxification (Chapman and Chan, 2000; Furst, 2002). Moreover, the absence of rain can increase the amount of dust adhered to the plant surface, leading to an increase in the exposure to metals from polluted soils in herbivorous animals (Mendez and Maier, 2008). Drought condition effects have been reported by other authors (Henny et al., 2007; Hill et al., 2008; Hoffman et al., 2009). In birds (snowy egrets) drought conditions were found to exacerbate Hg-related effects for overall productivity as well as physiological effects. A variable threshold of tolerance to Hg associated with habitat quality (food type and abundance) was proposed (Henny et al., 2007; Hill et al., 2008; Hoffman et al., 2009).

There is limited and contradictory information about age and gender effects on metal concentrations in wildlife organs. In the study of Iberian lynx (*Lynx pardinus*) and other wild carnivores from Southern Spain, Millán et al. (2008) found that females had higher Se and Hg concentrations in liver than males. However, in a study of arctic foxes (*Alopex lagopus*) and wolverines (*Gulo gulo*) in the Canadian Arctic, Hoekstra et al. (2003) did not find any significant influence of age, body length and gender on the Hg concentrations in liver and kidney.

As previously stated, the Hg levels detected in wild boar and red deer are well below those associated with clinical mercury poisoning, but sublethal effects cannot be discarded. Oxidative stress (disturbance of the prooxidant–antioxidant balance) is recognized as one cause of contaminant toxicity in wildlife and may ultimately result in cellular dysfunction and damage. Reduced glutathione (GSH) and associated antioxidant enzymes are major combatants of oxidative stress that influence redox status. Several studies with mallards and other species of aquatic birds have confirmed the utility of such measurements for Hg exposure in both the laboratory and the field (Hoffman and Heinz, 1998). Changes in glutathione status are thought to increase the likelihood of injury to liver and other tissues. The significant negative correlation occurring between hepatic THg and GSSG concentration in red deer in this study may indicate a hormetic mechanism by reducing oxidized GSH in response to early signs of oxidative stress induced by Hg. Similar compensatory effects have been reported by other authors in fish eating birds exposed to

mercury in the mining area of Carson River, Nevada (Hoffmann et al., 2009, 2011; Henny et al., 2002).

There is not much information on Hg in herbivores for comparison in the literature. Additional problems are different result units (dry or wet weight), mean calculations (arithmetic, geometric or median values), differing age and sex distributions or different species and localities. For consistency in the comparison, all data on Hg concentrations have been transformed to wet weight (w.w.) using wet/dry weight ratio of 2.76 (about 36% dry matter) and 3.66 (about 27% dry matter) for liver and kidney, respectively (Frøslie et al., 2001). The transformed data finally used for comparison are summarized in Table 3. Liver and kidney Hg levels in red deer and wild boar from different parts of Europe were reviewed by Frøslie et al. (2001). Thus, Hg hepatic concentrations below 0.1 μg/g w.w. and renal concentrations an order of magnitude higher are found, especially in regions contaminated by human activities. Liver tends to reflect short-term exposure and it acts as a metabolizing and excretion organ, while kidney is a target organ of inorganic Hg accumulation (Frøslie et al., 2001). Our results are consistent with the main finding for both tissues and species distribution; thus higher concentrations were found in kidney than in liver and in wild boar than in red deer. The mean value (w.w) ranges were 0.0003–0.34 and 0.01–8.6 μg/g w.w. in red deer liver and kidney, respectively, and 0.008–5.7 and 0.03–34.0 μg/g w.w. in liver and kidney from wild boar. Our mean values were within this range: 0.005–0.169 and 0.02–3.7 μg/g w.w. in red deer liver and kidney, respectively, 0.017–0.347 μg/g and 0.06–1.17 μg/g w.w. in wild boar liver and kidney. More recently, Lazarus et al. (2005) have studied Hg concentrations in tissues of red deer ($n=57$) from Eastern Croatia. The median mass fraction of Hg in the kidney was 0.362 μg/g w.w. (0.092–0.883 μg/g w.w.). Bilandzic et al. (2010) found 0.04–0.152 μg/g Hg concentration in wild boar kidney ($n=169$) also in Eastern Croatia. The most similar case for comparison to our area of study is the Idrija mine in Slovenia (Gnamus et al., 2000). This is the second largest Hg mine in the world; it is in the Mediterranean area and it was recently closed. In the immediate vicinity of the Hg mine in Idrija, Slovenia, hepatic Hg concentrations in roe deer (*Capreolus capreolus*) were many times higher (0.295–2.270 μg/g w.w., mean value 0.845 μg/g w.w., $n=9$) than in the surrounding areas (0.074–0.664 μg/g w.w, mean value 0.237 μg/g w.w., $n=4$). In kidney, Hg concentrations in the close vicinity to the mine were 2.980–56.2 μg/g w.w. (mean value 18.7 μg/g w.w., $n=9$), also higher than in the surroundings (1.210–4.640 μg/g w.w., mean value 2.840 μg/g w.w., $n=4$). The maximum concentrations described in this area are several times higher than in the present study because our samples are not from the mine smelter complex. Samples were collected from a large distance that corresponds to the one noted as surrounding area in the Idrija study. Moreover, comparison with the Gnamus et al. (2000) study should be carefully taken because the herbivorous species are not the same (red deer and wild boar in Almadén vs. roe deer in Idrija) and also because in this last study there is a very limited number of samples (less than 10).

Table 3
Mercury concentrations in liver (L) and kidneys (K) of red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*). Values are means/range of means (upper values in parentheses).

Species	Location (Sampling year)	Organ (n)	Hg Wet weight (μg/g)	Reference
Red deer (*Cervus elaphus*)	Germany (1980–82)	L(*136*)	0.013 (0.34)	Kleiminger (1983)
		K(*135*)	0.11 (8.6)	
	Germany	L(*219*)	0–0.08	Holm (1984)
		K(*220*)	0.016–2.2	
	Germany (1991–92)	L(*29*)	<0.01 (<0.01)	Lusky et al. (1992)
		K(*29*)	0.02 (0.07)	
	Slovakia	L(*52*)	0.004 (0.01)	Findo et al. (1993)
		K(*52*)	0.02 (0.06)	
	Germany (1991–92)	L(*9*)	<0.013–0.078[a]	Lusky et al. (1992)
		K(*9*)	<0.013–0.072[a]	
	Poland (1987–91)	L(*70*)	0.003–0.009 (0.035)	Falandysz (1994)
		K(*62*)	0.010–0.035 (0.054)	
	Germany (1993–94)	L(*105*)	0.01 (0.049)	Launer et al. (1996a)
		K(*102*)	0.047 (0.17)	
	Croatia (2002–03)	K(*57*)	0.362 (0.883)[b]	Lazarus et al. (2005)
	Almadén (Spain) (2005–06)	L(*161*)	0.036 (0.169)[c]	*This study*
		K(*146*)	0.33 (3.67)[c]	
Wild boar (*Sus scrofa*)	Austria (1984–87)	L(*195*)	0.011 (0.06)	Tataruch et al. (1979)
		K(*214*)	0.037 (0.29)	
	Germany (1980–82)	L(*142*)	0.26 (5.7)	Kleiminger (1983)
		K(*140*)	3.8 (100)	
	Germany	L(*175*)	0.03–0.45	Holm (1984)
		K(*182*)	0.31–17	
	Germany (1991–92)	L(*18*)	0.02 (0.07)	Lusky et al. (1992)
		K(*18*)	0.02 (0.09)	
	Germany (1975–80)	K(*459*)	0.10–32	Teuwsen (1982)
	Poland (1985–91)	L(*114/122*)	0.008–0.04 (0.04)	Falandysz (1994)
		K(*87*)	0.03–0.06 (0.3)	
	Germany (1991–92)	L(*294*)	0.03[d] (4.2)	Launer et al. (1996b)
		K(*413*)	0.13[d] (34)	
	Croatia (2008–09)	K(*169*)	0.077 (0.984)	Bilandzic et al. (2010)
	Almadén (Spain) (2005–2006)	L(*51*)	0.064 (0.347)[c]	*This study*
		K(*23*)	0.381 (1.17)[c]	

[a] Range.
[b] Kidney cortex alone.
[c] Calculated from dry weight (if we assume d.w. of liver is 36% and of kidney 27%).
[d] Median.

4.2. Selenium distribution and deficiency evaluation in red deer and wild boar

Selenium is both an essential and a highly toxic element, although Se toxicity does not play any significant role in wild ungulates (Frøslie et al., 2001). Most research on Se concentration has focused on farm animals because Se deficiency is a significant problem. There is little information available on Se concentration in organs of game animals (Frøslie et al., 2001). Selenium deficiency in free living animals is associated with the quantity and accessibility of this element in the soil and plants. In our study (Table 1), the highest concentrations corresponded to kidney (2.60 and 6.08 μg/g d.w. in red deer and wild boar, respectively) and testis (2.20 μg/g d.w. in red deer). The high Se levels in testis may be related to the important antioxidant system necessary to maintain healthy male reproductive function (Burk, 2002; Boitani and Puglisi, 2008). None of the species (red deer or wild boar) or tissues (liver, kidney or testis) were affected by age or gender.

In the diagnosis of Se deficiency in the liver of red deer, the vast majority of the examined red deer livers were below the threshold level of 0.6 μg/g d.w., by which this element is deficient according to the biochemical criteria used by Pilarczyk et al. (2009). Selenium concentration above 0.88 μg/g d.w. (optimum value) was observed only in 1% of animals. According to McDowel et al. (1995), Se concentration in the kidney of red deer below 3.0 μg/g (d.w.) is proof of Se deficiency. In our study, Se deficiency in the kidney of red deer was found in 88.7% of animals in season 2004–2005 and in 57.1% in season 2005–2006. Selenium concentration in the liver has been proposed to be a better indicator of Se deficiency than in kidney (Pilarczyk et al., 2009). For Se concentration in wild boar, there is limited literature data and a lack of reference values. A normal level was proposed for liver in pigs by Puls (1994) but they should be carefully used because pigs and wild boar have different feedings habits. Using the standards designed for the liver in pigs by Puls (1994), 23.5% of individuals were found to have optimal levels (0.4 μg/g w.w. or approximately 1.1 μg/g d.w., if we assume dry weight of liver is 32%), and only 3.9% were found to have Se deficiency (<0.3 μg/g d.w.; Frøslie et al., 2001). The low Se concentrations in liver and kidneys of red deer from Ciudad Real, Spain, may show that they live in areas deficient in this element. In the study developed by Oh et al. (1976), higher concentrations of Se were found in the kidney than in the liver when animals were fed with poor Se diet and the contrary situation was found when they were given feed rich in Se. Our results are consistent with a deficiency state for red deer and wild boar since higher values are found in kidney in both species.

The positive correlation association between Se levels and antioxidant levels (α-tocopherol and retinyl esters) may be explained by the role of this trace element as a cofactor in antioxidant enzymes such as Se-dependent GPX that can help to maintain the levels of other dietary antioxidants (Hsu and Guo, 2002; Sarma and Mugesh, 2008). Moreover, the effect of Hg contamination at the detected exposure levels was not significant on the oxidative biomarkers of these wild ungulates compared with Pb exposure; however sampling was especially designed to evaluate the impact of the Pb mining area (Reglero et al., 2009a, 2009b; Castellanos et al., 2010; Rodríguez-Estival et al., 2011).

4.3. Hg–Se interaction

The accumulation of certain essential elements can be affected by overexposure to other elements (essential or otherwise). A well known example relates to the accumulation of Se in tissues that is triggered by Hg exposure (Rooney, 2007).

The correlation between renal Hg and Se molar concentrations was more significant (and the slope regression was greater) in wild boar than in red deer (Fig. 5). This suggests that wild boar has a higher accumulation of Se and it is also more affected by Hg exposure than in red deer. The accumulation of Se in kidney can be related to the overexposure to Hg in this tissue (Rooney, 2007). This significant correlation further supports the hypothesis of functional association between these elements found in other biota (Hoekstra et al., 2003). It is also important to note the lack of correlation between Hg and Se in liver in contrast with that observed in a variety of marine mammals (Prestud et al., 1994; Woshner et al., 2001; Hoekstra et al., 2003), where Se levels were positively correlated with Hg levels. This difference between terrestrial and marine mammals could be related to the higher levels of exposure to Hg in the marine mammals. Moreover, hepatic Se concentrations in marine mammals were approximately 3-fold higher than levels in kidney. In contrast, the highest mean Se concentration in terrestrial species occurred in kidney. Thus, Hg–Se association can be found in the target organ (liver or kidney) depending on whether they are marine or terrestrial animals. This could be related to the Hg species they are exposed to. In the terrestrial ecosystem the predominant species is inorganic Hg, which targets on kidney tissue, while marine mammals are mainly exposed to organic Hg bioaccumulated in the aquatic food web and can be more widely distributed in the different body compartments, including liver.

Selenium is known to be very active in counteracting mercury toxicity (Cuvin-Aralar and Furness, 1991; Yang et al., 2008). With respect to Se "protective effect", it was initially presumed that Se binds to Hg, thereby preventing its harmful effect. However, recent investigations reversed this explanation since more has become understood about Se physiology and the mechanism of Hg toxicity (Ralston et al., 2008; Ralston and Raymond, 2010; Carvalho et al., 2008). It has been shown that Hg does not cause oxidative damage directly but secondarily through the inhibition of selenoenzymes such as GPX. The high affinity between Hg and Se results in Hg binding to Se, thus compromising Se biological functions and availability. Vital selenoenzyme activities are inhibited and formation of insoluble Hg selenide (HgSe) depletes availability of Se for subsequent cycles of selenoprotein synthesis. Selenoenzymes are required to prevent and reverse oxidative damage through the body, particularly in the brain and neuroendocrine tissues. Thus, mercury toxicity occurs through irreversible inhibition of selenoenzyme in vital-but-vulnerable tissues. Therefore the "protective effect" found for supplemental Se is due to the additional Se available to replace the Se lost due to Hg sequestration. This additional Se maintains normal selenoenzyme synthesis.

Therefore, mercury toxicity is not uniformly associated with mercury exposures, but it is instead associated with Hg:Se molar ratios (Ralston et al., 2008; Ralston and Raymond, 2010). High Hg:Se molar ratios were associated with toxicity and lower Hg:Se molar ratios were associated with diminished or no observed symptoms (Ralston and Raymond, 2010). In Table 3 are shown the Hg:Se molar ratios obtained in liver and kidney from both red deer and wild boar. In all cases, the ratio was <1 (i.e. maximum value 0.091 Hg/Se molar ratio in red deer liver). Little information about Hg:Se molar ratio is available for comparison. Ralston et al. (2008) found similar ratio to our values in rats fed with low-MeHg diets and no toxicity effects were evident. A better understanding of the interactions between essential and non-essential elements and impacts will need more comprehensive research of Hg and Se speciation.

5. Conclusions

Only minor information on Hg in herbivorous is available and the study of Hg exchanges and transformations in the terrestrial

biota of the Almadén mining district is a unique opportunity. In the present study, higher Hg concentrations are found in kidney than in liver and in wild boar than in red deer. However, the concentrations found are well below the ones associated with clinical signs of poisoning, and no sub-lethal effects can be associated with this element. A strong association between Hg and Se has been found even in a situation of Se deficiency. Additional information about Hg and Se speciation is necessary for a better understanding of interactions and impacts in this polluted terrestrial ecosystem.

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