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ALTERED PORPHYRIN EXCRETION AND HISTOPATHOLOGY OF GREYLAG GEESE (*ANSER ANSER*) EXPOSED TO SOIL CONTAMINATED WITH LEAD AND ARSENIC IN THE GUADALQUIVIR MARSHES, SOUTHWESTERN SPAIN

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Abstract—Greylag geese (*Anser anser*) in the Guadalquivir Marshes (southwestern Spain) can be exposed to sources of inorganic pollution such as heavy metals and arsenic from mining activities or Pb shot used for hunting. We have sampled 270 fecal excreta in different areas of the marshes in 2001 to 2002 to evaluate the exposure to Pb, Zn, Cu, Mn, and As and to determine its relationship with soil ingestion and with the excretion of porphyrins and biliverdin as biomarkers. These effects and the histopathology of liver, kidney, and pancreas were also studied in 50 geese shot in 2002 to 2004. None of the geese had ingested Pb shot in the gizzard. This contrasts with earlier samplings before the ban of Pb shot for waterfowl hunting in 2001 and the removal of Pb shot in points of the Doñana National Park (Spain) in 1999 to 2000. The highest exposure through direct soil ingestion to Pb and other studied elements was observed in samples from Entremuros, the area of the Doñana Natural Park affected by the Aznalcóllar mine spill in 1998. Birds from Entremuros also more frequently showed mononuclear infiltrates in liver and kidney than birds from the unaffected areas, although other more specific lesions of Pb or Zn poisoning were not observed. The excretion of coproporphyrins, especially of the isomer I, was positively related to the fecal As concentration, and the ratio of coproporphyrin III/I was positively related to fecal Pb concentration. Biliary protoporphyrin IX concentration was also slightly related to hepatic Pb concentration. This study reflects biological effects on terrestrial animals by the mining pollution in Doñana that can be monitored with the simple noninvasive sampling of feces.

Keywords—Heavy metals Lead shot Birds Heme metabolism Noninvasive biomarkers

INTRODUCTION

The Guadalquivir Marshes (southwestern Spain) are one of the most important wetlands in Europe, with an area of 230,000 ha. Part of the Guadalquivir Marshes are protected as Doñana National Park (50,720 ha), Doñana Natural Park (54,250 ha), and Brazo del Este Natural Site (1,336 ha). Parts are also protected as European Union Special Protection Area for Birds, Ramsar Site, Biosphere Reserve and World Heritage Site [1]. Despite these protection efforts, the risk of contamination of the Guadalquivir Marshes with heavy metals through sources like mining activity or hunting with Pb shot are still present. Doñana, the popular name given to these wetlands, depend on pluvial and superficial contributions arriving from the Rocina and Partido streams from the northwest, the Guadiamar River from the northeast, the Guadalquivir River from the east, and the Atlantic Ocean from the south [1,2]. The basin of the Guadiamar River is rich in zinc, lead, copper, and manganese deposits, and constant inputs to Doñana have long been recognized from mining activities upstream [3,4]. However, on April 25, 1998, part of the tailings dam of the Los Frailes pyrite mine in Aznalcóllar collapsed, releasing 4 million cubic meters of acidic water and 2 million cubic meters of sludge to the Agrio River, a tributary of the Guadiamar River. This sludge contained high concentrations of Fe (34–38%), S (35–40%), Zn (0.8%), Pb (0.8–1.2%), As (0.5–0.6%), and Cu (0.2%) [2,5]. The surface affected by the pyritic sludge was of 4,286 ha. The mine waste contaminated 2,656 ha of the Natural Park but only 98 ha of the National Park, which was protected by dykes constructed immediately after the spill in Entremuros [2]. Soil remediation after the mining accident included the removal of pyritic slurry by bulldozing and soil treatment by the addition of calcium carbonate and iron oxyhydroxides to prevent metal solubilization, although a residual pollution load of soils remained after these mitigating actions [6,7].

Soil contamination may represent a significant hazard for birds feeding on these areas through ingestion of soil or contaminated food [5]. Moreover, birds in the Guadalquivir Marshes are also exposed to Pb from shot pellets used as ammunition for waterfowl hunting. The density of Pb shot pellets in a site used for grit ingestion by greylag geese (*Anser anser*) called Cerro de los Ánsares (''Geese hill'') located in the National Park was approximately 4.7 shot/m^2 on the surface in 1997 because intensive hunting was carried out there until 1983 [8]. The prevalence of Pb shot ingestion in greylag geese in 1994 to 1996 was around 10%, and 21% of geese found

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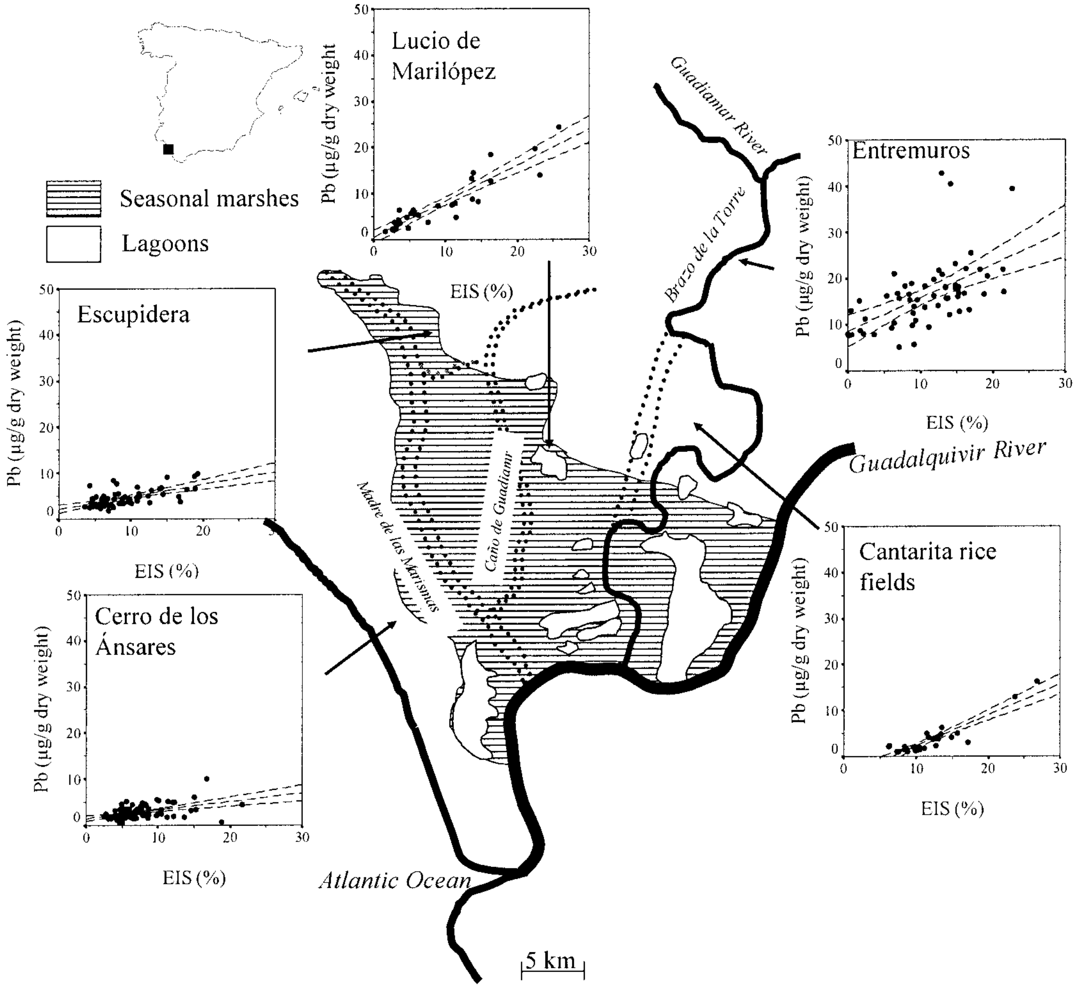

Fig. 1. Location of sampling areas in Doñana (southwestern Spain) with the relationship between the estimated ingested soil (EIS; % in the diet) and the lead (Pb) concentration in fecal excreta of wintering greylag geese.

dead in the Guadalquivir Marshes were diagnosed as Pb poisoned [9].

One of the topics discussed in the first studies on the accumulation of heavy metals in birds from Doñana after the Aznalcóllar accident was the difficulty of discerning between Pb exposure due the ingestion of contaminated soil or to Pb shot ingestion [10,11]. Some attempts to identify the origin of Pb have been developed by the analysis of the ratio of stable isotopes of Pb, suggesting that the mine waste was the main source of pollution in one colony of white storks (*Ciconia ciconia*) [12]. However, for species more susceptible to Pb shot ingestion like waterfowl, the wide variety of geographical origins of Pb used in shot pellets in Doñana makes this approach problematic. Two of the elements in higher concentrations in the contaminated Aznalcóllar sludge were Pb and Zn; thus, the risk of poisoning of waterfowl inhabiting the Guadalquivir Marshes may resemble the scenario of the Coeur d'Alene River basin in Idaho, USA, where analyses of fecal excreta were used to relate soil ingestion to the exposure to heavy metals in birds [13]. Moreover, this noninvasive sampling provides evidence of toxic effects of heavy metals and As in birds from the analysis of porphyrins and biliverdin in fecal excreta [14].

In this study, we have quantified soil ingestion and exposure to heavy metals and As in greylag geese in Doñana via the analysis of their fecal excreta. Concentrations in feces were related to concentrations in geese blood and livers. We also assessed the toxicity of contaminants from the Aznalcóllar spill or Pb shot by analyzing changes in the profile of porphyrins in bile and excreta and through the pathological study of the sampled geese.

METHODS

Sample collection

Fresh greylag goose excreta were collected in five locations of the Guadalquivir Marshes during the 2001–2002 winter season (Fig. 1). Locations were visited at three times (end of October–November, December, and January), and if geese flocks were present in the area, 30 samples were collected at each site at each visit. Cantarita rice fields, Entremuros channel, Lucio de Marilópez lagoon, and Escupidera grasslands are feeding sites for greylag geese, while Cerro de los Ánsares dune is a site of grit ingestion [8]. All samples were kept in plastic bags and frozen at $-20°C$ until analysis. In order to establish the relationship between the noninvasive sampling of excreta and tissue accumulation of heavy metals and As, 50 greylag geese were randomly shot with steel pellets under license by personnel of the Estación Biológica de Doñana in Entremuros (n = 7), Escupidera (n = 6), and the marshes

Table 1. Soil and plant characteristics of collection sites for geese excreta

	Soil		Predominant plants			
Zone	*n*	AIA (%)[a]	Species	*n*	AIA (%)	Digestibility (%)[b]
Cantarita	3	64.6 ± 2.1	*Oryza sativa*	3	0.44 ± 0.23	45
Cerro de los Ánsares	3	98.4 ± 0.8	*Scirpus* sp.	3	0.73 ± 0.11	55
Entremuros	3	68.6 ± 0.7	Several[c]	9	2.20 ± 0.45	37
Marilópez	3	69.6 ± 0.5	*Hordeum vulgare*	3	3.86 ± 0.43	40
Escupidera	3	70.7 ± 0.9	*Plantago* sp.	3	3.46 ± 0.23	37

[a] Acid-insoluble ash.
[b] Estimated using different studies [17,36].
[c] *Plantago* sp., *Hymenolobus procumbens,* gramineae.

around Cerro de los Ánsares ($n = 37$). Birds were necropsied, and liver, kidney, and pancreas were studied for histopathological changes associated with Pb or Zn poisoning [15,16]. Blood from heart, liver, muscle, bone, and content of large intestine were collected for elemental analysis and kept frozen at −20°C.

Study of soil ingestion

Soil ingestion was estimated from the analysis of acid-insoluble ash present in excreta following the method described by Beyer et al. [17]. Acid-insoluble ash in excreta, together with acid-insoluble ash analyzed in goose food and Doñana soils, and food digestibility data compiled from the literature (Table 1) were used to estimate soil ingestion with the equation given by Beyer et al. [17].

Chemical analyses

Excreta, blood, and tissues (0.2–0.3 g of dry sample) were analyzed for heavy metals by graphite furnace (Pb) or flame (Zn, Cu, Mn, Fe, Al) atomic absorption spectroscopy [18] and for As by hydride generation/atomic absorption spectroscopy [7]. Limits of detection in dry samples were approximately 0.036 μg/g for As and <0.01 μg/g for the other elements. Acceptable recoveries of the elements with the analytical methods (91–109%) were obtained with spiked blanks, spiked samples, and certified reference materials of soil (State Bureau of Technical Supervision, Beijing, China, GBW07406), bone meal (Standard Reference Material, SMR1486), and bovine liver (SRM1577b). All element concentrations were expressed on a dry-weight basis in feces and tissues and on a wet-weight basis in blood. Tissue concentrations were interpreted following the reviewed threshold levels of toxicity of Pb [9,19], Zn [16], and As [20].

Porphyrins and biliverdin were determined in excreta and bile of geese by high-pressure liquid chromatography with ultraviolet detection [14]. As porphyrins and biliverdin have a common biliary origin in bird excreta, porphyrin concentrations in excreta were expressed relative to biliverdin in order to compensate for variation in the amount of feces excreted due to variation in the diet.

Statistical analyses

Element concentrations were log-transformed to fit a normal distribution. Undetected values were assumed to be half the detection limit of each element. The variation in soil ingestion during the wintering season was studied with excreta collected in Cerro de los Ánsares in November, December, and January and Entremuros and Escupidera in December and January (2001–2002). Concentrations of heavy metals and As in excreta or animal tissues were compared between locations with analyses of variances and Tukey tests. Tissue concentrations of Pb and As were not lognormally distributed, and means were compared using generalized linear models with normal error and logarithmic link function. The relationship between heavy metals and As in excreta and tissues was studied with Pearson correlation coefficients in order to assess the predictive value of the noninvasive sampling of feces. The relationships among element concentrations in excreta were also studied using correlation coefficients. The linear regression between estimated ingested soil (predictor variable) and element concentrations (dependent variable) in excreta, with the location as a covariant, was used to explore the differences between locations in soil contamination (the interaction between soil ingestion and location) or in food contamination (the main effect of location).

The use of excreta to reflect changes in the metabolism of porphyrins and biliverdin in geese was validated with the correlations of these compounds in excreta (content of the large intestine) and bile of shot geese. Moreover, the effects of the studied contamination on the metabolism of porphyrins and biliverdin focused on two elements, Pb and As, both with well-described effects on the heme synthesis/degradation pathway [21]. Porphyrin concentrations relative to biliverdin were log-transformed to fit a normal distribution, and the effects of Pb and As concentrations and of sampling location were studied with generalized linear models. The frequency of lesions found in the geese from Entremuros and the areas not affected by the mine spill were compared with chi-square or Fisher exact probability tests.

RESULTS

Validation of the noninvasive sampling

The concentration of Pb in the intestinal content of geese was significantly correlated with the concentrations in liver ($n = 49$, $r = 0.407$, $p = 0.004$) and blood ($n = 39$, $r = 0.433$, $p = 0.006$) of these birds, which support the usefulness of this noninvasive technique for wildlife monitoring surveys of Pb poisoning. Moreover, porphyrin composition in feces reflects composition in bile, as shown by the significant correlation in the ratio coproporphyrin III/coproporphyrin I between bile and intestinal content of shot geese (Fig. 2). The concentrations of the different porphyrins were significantly correlated with biliverdin concentrations in feces, especially coproporphyrin III ($n = 150$, $r = 0.559$, $p < 0.001$) and protoporphyrin IX ($n = 150$, $r = 0.459$, $p < 0.001$). This can be explained by their common biliary origin, and consequently the porphyrin concentrations relative to biliverdin may be used to test the effects of heavy metals and As in geese feces or intestinal content.

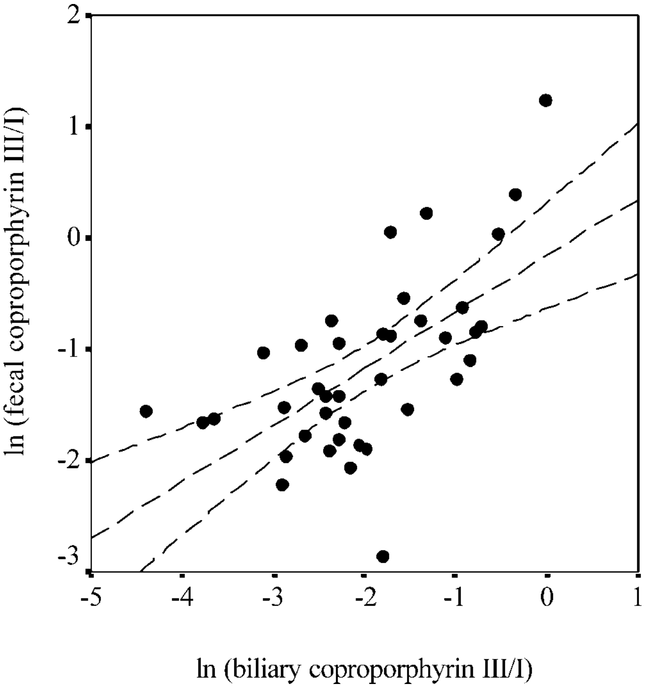

Fig. 2. The relationship between biliary and fecal (intestinal) coproporphyrin III/I ratio supports the use of the noninvasive sampling of feces ($r = 0.616$, $p < 0.001$).

Biliary concentrations of both coproporphyrins and protoporphyrin IX were also significantly correlated with biliverdin (r range, 0.398–0.693). Biliverdin concentrations were higher in feces with visually detected green stained urates (geometric means of 628 vs 80 nmol/g dry wt, $t_{148} = 6.9$, $p < 0.001$); thus, this simple observation may be useful to assess changes in biliverdin excretion. We may consider goose feces a good indicator of local contamination in this species because the plant material found in feces (rice in Cantarita, barley *Hordeum vulgare* and *Scirpus* in Marilópez, *Plantago* in Entremuros and Escupidera, and *Scirpus* in Cerro) corresponded to plants located around each sampling point (Table 1).

Sources of contamination and differences among areas

Lead and As were the only elements that were significantly at higher concentrations in tissues of geese from Entremuros than in the other areas (Table 2). Lead concentrations in the feces collected in Entremuros were also higher than in the other areas (Tables 2 and 3). Lead shot is probably not the source of such contamination because none of the birds had ingested Pb shot in the gizzard. Moreover, the feces collected in Entremuros also contained the highest levels of the other heavy metals and As (Table 3), and the estimation of soil ingestion was significantly correlated with the concentrations of most of the elements in the feces collected in the different areas (Table 4). Concentrations of the different elements were also significantly correlated among them (r range, 0.467–0.957). Soil ingestion estimated from feces collected in Cerro de los Ánsares decreased progressively between October and January ($p = 0.003$), and this decrease was more markedly observed in feces collected in Entremuros and Escupidera ($p < 0.001$; Fig. 3).

Differences in soil ingestion were observed between the feces collection areas, but this did not explain the high concentrations of several elements found in Entremuros. When studying the linear regressions of soil ingestion and Pb concentrations in geese feces, we observe relatively high slopes in Lucio de Marilópez, Entremuros, and Cantarita rice fields and lower slopes in Escupidera grasslands and Cerro de los Ánsares dune (Table 4). Similar patterns are recorded for the other elements. This may reflect a similarity in soil concentrations of Pb and other elements in the northern areas of the marshes that had historically received the Guadiamar effluents (Fig. 1). However, differences in the concentrations in feces of all the elements exist between Entremuros and Marilópez/Cantarita, and these are not related to the amount of ingested soil or the element concentration in soil (Table 3). Thus, these differences in element concentrations in feces must be due to differences in the concentrations of elements in plants ingested by geese between Entremuros and Marilópez/Cantarita, which are reflected by the constants of the regressions between soil ingestion and feces concentrations (Table 4). Entremuros again has the highest constant value for several elements, including Pb (Table 4 and Fig. 1).

Effects on geese health: Pathology and porphyrins

Liver Pb concentrations suggested subclinical poisoning in three birds from Entremuros (>5 μg/g dry wt), but none of them reached levels of clinical poisoning (>18 μg/g dry wt).

Table 2. Geometric mean (range) of the concentrations of four elements in blood (μg/g wet wt) and other samples (μg/g dry wt) of greylag geese shot in Doñana (southwestern Spain)[a]

Sample	Zone	*n*	Pb	Zn	Cu	As
Intestinal content	Entremuros	6	7.6A (1.9–18)	193A (82–447)	23 (7–44)	1.3 (0.40–3.0)
	Escupidera	6	1.5B (0.43–12)	69B (49–121)	14 (8.0–30)	0.36 (0.05–3.0)
	Cerro de los Ánsares	37	0.71C (0.18–3.4)	43B (19–145)	19 (6.5–95)	0.65 (0.07–4.1)
Blood	Entremuros	4	0.80A (0.12–3.3)	4.3 (2.7–6.4)	0.47 (0.10–2.8)	ND
	Escupidera	6	0.08AB (0.05–0.12)	4.7 (2.9–6.1)	0.78 (0.40–1.2)	ND
	Cerro de los Ánsares	30	0.06B (0.04–0.49)	4.8 (3.5–6.1)	0.69 (0.21–3.3)	ND
Liver	Entremuros	7	1.6A (0.32–13)	172A (112–298)	129 (41–417)	0.05A (ND–0.20)
	Escupidera	6	0.36AB (0.22–0.59)	196A (146–245)	84 (16–232)	0.05AB (ND–0.08)
	Cerro de los Ánsares	37	0.27B (0.18–3.9)	102B (64–209)	90 (35–398)	NDB
Muscle	Entremuros	6	0.21 (0.12–0.45)	52B (36–130)	5.4 (4.7–8.2)	ND
	Escupidera	6	0.20 (0.18–0.29)	111A (71–173)	7.1 (5.4–9.5)	ND
	Cerro de los Ánsares	37	0.22 (0.18–0.45)	80B (51–131)	6.5 (4.1–13)	ND (ND–0.06)
Bone	Entremuros	7	11 (4.0–28)	266 (108–261)	3.3B (2.4–6.0)	0.04 (0.04–0.05)
	Escupidera	6	7.0 (0.93–45)	195 (147–265)	5.7A (5.0–7.0)	ND (ND–0.05)
	Cerro de los Ánsares	37	4.8 (0.45–36)	209 (166–261)	5.5[a] (3.6–7.6)	ND (ND–0.09)

[a] Means with different capital letters differed significantly among locations (analysis of variance with post hoc Tukey test for log-normally distributed data—Zn, Cu—and generalized linear model with normal error and logarithmic link function for the Pb and As). ND = not detected.

Table 3. Geometric mean (range) of acid-insoluble ashes (AIA; %) with the corresponding estimated ingested soil (EIS; %) and concentrations of heavy metals and arsenic (μg/g dry wt) in goose excreta collected in Doñana (southwestern Spain)[a]

Zone	*n*	AIA	EIS	Pb	Zn	Cu	Mn	Fe	Al	As
Cantarita	30	12AB (7.6–26)	11B (6.1–27)	2.6A (1.0–16)	20A (8.0–334)	7.1A (2.4–52)	93A (43–219)	1,203A (237–7,391)	931A (231–4,517)	0.5A (0.2–2.6)
Cerro de los Ánsares	90	11A (5.7–29)	6.6A (2.5–22)	2.5A (0.6–10)	28A (5.2–156)	12B (4.2–111)	106A (45–231)	1,999B (441–7,328)	1,171A (517–4,820)	1.2B (0.4–5.9)
Entremuros	60	13AB (2.9–36)	8.0B (0.1–38)	15D (5.1–43)	308C (61–840)	27D (10–55)	208B (32–601)	4,269C (515–15,840)	2,336B (302–9,125)	2.8C (0.3–14.5)
Marilópez	30	15B (8.1–53)	7.6B (1.6–62)	6.5C (1.8–31)	53B (21–94)	13BC (5.6–24)	169B (78–547)	3,061BC (337–21,284)	211B (244–13,747)	0.9B (0.1–4.7)
Escupidera	60	12AB (7–22)	8.2AB (3.5–19)	4.3B (1.8–9.9)	27A (8.6–81)	16C (5.8–55)	108A (60–228)	2,832BC (827–11,860)	2,074B (652–12,071)	0.8B (0.3–3.5)

[a] Means with different capital letters differed significantly among locations (analysis of variance and post hoc Tukey test).

Five birds showed Pb concentrations in blood above the commonly accepted background level (0.2 μg/g wet wt), and three of those birds that were shot in Entremuros had levels suggesting clinical poisoning (>0.5 μg/g wet wt). These birds were apparently healthy when shot, and no lesions attributable to Pb poisoning were observed. However, geese sampled in Entremuros showed higher prevalences of mononuclear infiltrates in kidney and liver (Table 5). Zinc concentrations in liver and blood were below the levels usually detected in clinically Zn-poisoned geese (>1,000 μg/g dry wt in liver and >6 μg/g wet wt in blood), and no lesions compatible with Zn poisoning were observed. Arsenic and Cu levels can be considered within background values.

Uroporphyrin, coproporphyrins I and III, protoporphyrin IX, and biliverdin were detected in bile of geese, and all but uroporphyrin were detected in intestinal content or feces (Table 6). Arsenic had a significant effect on the increase of both coproporphyrins in feces collected in the field and especially on the increase of the isomer I ($F_{1,144} = 17.7$, $p < 0.001$; Fig. 4a). This result was also observed in the intestinal content of shot geese ($F_{1,45} = 5.3$, $p < 0.026$; Fig. 4b) but not in bile. On the other hand, Pb concentration in the intestinal content of shot geese was positively related to the ratio copro III/I, although this effect is evident only at high Pb concentrations (Fig. 5a). This effect was also observed in bile ($p = 0.001$) but not in feces collected in the field. Concentration of protoporphyrin IX in bile of shot birds was positively related to liver Pb concentration ($p = 0.019$; Fig. 5b), but this effect was not observed in the intestinal content or the feces. Concentrations of biliverdin in bile or feces were not affected by liver Pb concentration.

DISCUSSION

The low importance of Pb shot ingestion in the exposure of greylag geese observed during the study contrasts with earlier studies in Doñana. The prevalence of Pb shot ingestion in 1994–1996 was 10% ($n = 20$) [9] and 7.9% in 1998–1999 ($n = 38$, R. Baos et al., Estación Biológica de Doñana, Seville, Spain, unpublished data), but no ingested shot were found in 85 birds sampled between 1999 and 2004 ([22]; present study). Although the sample size is small, these temporal trends suggest a decrease in Pb shot ingestion. This may result from a partial cleanup of Cerro de los Ánsares conducted by Sociedad Española de Ornitologia-Birdlife (Spanish Association of Ornithology) volunteers in the summers of 1999 and 2000 [23], when about 100 kg of Pb shot were manually removed from this important site of grit ingestion for geese. Cerro de los Ánsares in 1997 contained 26.1 kg of Pb shot/ha in the upper 20 cm of the sand dune [8]; thus, the amount of Pb removed may have been significant. Current Pb shot densities in Cerro de los Ánsares should be studied. Geese themselves may also have contributed to the decontamination of the dune to a lower extent. Lead shot ingestion could be estimated at 5.5 kg/year from an extreme assumption of 10% of prevalence in 60,000 geese wintering for five months in Doñana. Moreover, Pb shot was banned for waterfowl hunting in protected wetlands in Spain by 2001, although no information exists about the compliance by hunters.

Nowadays, the ingestion of contaminated soil with heavy metals and As possibly represents a higher concern for the wintering geese in Doñana than Pb shot. However, Pb is the element more clearly present at higher concentrations in birds from the spill-contaminated area (Entremuros) than from other

Table 4. Regression of the concentrations of heavy metals and arsenic in excreta of greylag goose with the estimated ingested soil showing constant (a), slope (b), and the coefficient of determination (r^2)

Dependent variable		Cantarita n = 30	Cerro n = 90	Entremuros n = 59	Marilópez n = 29	Escupidera n = 60
Pb	r^2	0.824	0.206	0.309	0.839	0.377
	a	−4.4 ± 0.73D	1.5 ± 0.3BC	8.6 ± 1.8A	0.62 ± 0.75C	2.2 ± 0.5B
	b	0.66 ± 0.06A	0.18 ± 0.04C	0.72 ± 0.14AB	0.78 ± 0.07A	0.27 ± 0.05BC
Zn	r^2	0.481	0.210	0.235	NS	0.082
	a	−72 ± 22C	10 ± 5.4B	189 ± 46A		21 ± 4.1B
	b	8.8 ± 1.7B	3.2 ± 0.66C	16 ± 3.8A		0.94 ± 0.42D
Cu	r^2	NS	NS	0.255	0.368	0.132
	A			19 ± 2.6A	9.8 ± 1.2B	12 ± 2.2B
	B			0.94 ± 0.21A	0.42 ± 0.11A	0.67 ± 0.22A
Mn	r^2	0.681	0.111	0.482	0.756	0.382
	a	15 ± 12C	84 ± 10A	83 ± 25ABC	89 ± 12A	57 ± 11B
	B	7.2 ± 0.9BC	4.2 ± 1.3C	15 ± 2.0A	9.4 ± 1.0AB	6.4 ± 1.1C
Fe	r^2	0.877	0.191	0.617	0.918	0.553
	a	−2,468 ± 319C	1,221 ± 266A	861 ± 554AB	−485 ± 342B	−336 ± 494B
	b	360 ± 25B	150 ± 33C	432 ± 45AB	522 ± 30A	422 ± 50A
Al	r^2	0.859	0.150	0.503	0.862	0.551
	a	−1,367 ± 216BC	713 ± 181A	661 ± 342A	−323 ± 320B	−545 ± 404B
	b	226 ± 17B	88 ± 22C	212 ± 28B	363 ± 28A	344 ± 41A
As	r^2	0.840	0.118	0.455	0.830	0.440
	a	−0.73 ± 0.12C	0.79 ± 0.20A	0.49 ± 0.55ABC	0.15 ± 0.10AB	0.05 ± 0.15B
	b	0.12 ± 0.01B	0.08 ± 0.02BC	0.31 ± 0.05A	0.10 ± 0.01C	0.10 ± 0.02BC

[a] Element concentration (μg/g dry wt) = a + b × estimated ingested soil (%). Parameters with different capital letters differed significantly among locations. NS = not significant.

areas of Doñana. Thus, our discussion focuses on this element. Concentrations of Pb after the Aznalcóllar spill were about 10,000 μg/g in the mine tailing sludge accumulated at the lower stretch of the Guadiamar River, and affected soils at depth between 0 and 10 cm contained 2,000 μg/g before cleaning operations [6,24]. Soil remediation activities reduced, on average, soil contamination to prespill metal concentrations in surface sediments [25]. In the case of Pb, Guadiamar soils just above the Entremuros area contained between 58 and 416 μg/g of Pb [6,25], not very different from the prespill alluvium concentrations of the basin of 15 to 540 μg/g [25]. Guadiamar River waters used to flood the northern part of Doñana through the Caño (channel) del Guadiamar, before their waters were canalized in the Entremuros (between walls) area in the mid-20th century. We can infer from the study of geese feces that soil Pb concentrations are currently similar in Cantarita rice fields, Entremuros, and Lucio de Marilópez. This scenario resembles that previous to the spill, with 30 to 80 μg/g in soils of Marilópez and Cantarita and 30 to 100 μg/g in soils of Entremuros, possibly because of the proximity of these areas to the Guadiamar River, which has received heavy metal and As contamination by mining activity upstream since long before the accident [3,4,25].

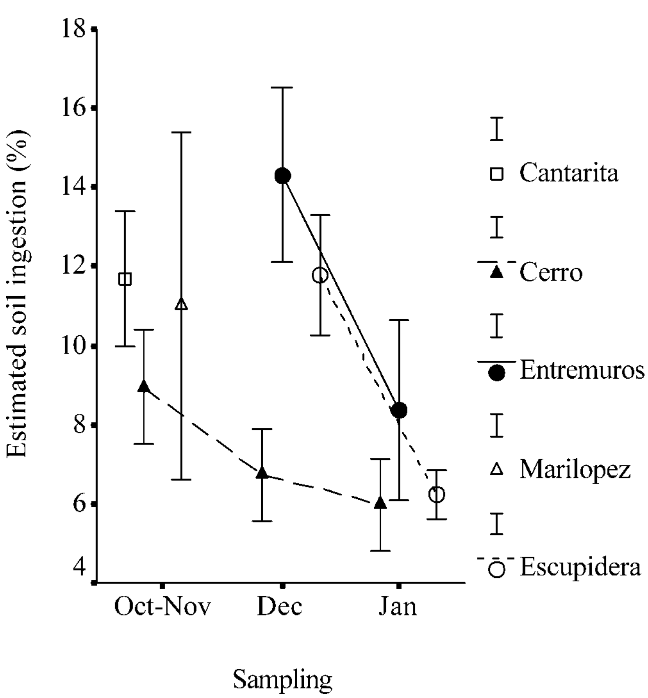

Fig. 3. Trends of the estimated ingested soil (mean % in the diet with 95% confidence interval) in greylag geese during the wintering season in Doñana (southwestern Spain).

Solubility and the consequent bioavailability of heavy metals to plants in the spill affected soils may have been increased by soil acidification due to the oxidation of metallic sulfides [24,26,27]. Plants growing in these affected soils accumulated higher concentrations of several heavy metals and As [26,28–31]. In the case of Pb, concentrations of 0.5 to 7.5 μg/g were found in different parts of sunflower (*Helianthus annuus*) and sorghum (*Sorghum bicolor*) plants grown in affected soils with 58 to 97 μg/g of Pb just after the spill [32]. Similar concentrations (0.3–5.2 μg/g) were obtained two years after the spill in sunflower grown in soils with 113 μg/g of Pb [31]. However, other plant species such as *Amaranthus blitoides* grown on soils with 203 μg/g of Pb accumulated 86 μg/g in their shoots, and *Plantago* sp., one of the plants present in the diet of geese, accumulated 25 μg/g at soils with 382 μg/g [29]. Wild grasses such as *Cynodon dactylon* and *Sorghum halepense* grown in sludge-contaminated soils accumulated 47 to 148 μg/g in aboveground tissues, but levels in remediated soils diminished to 5 to 16 μg/g [30]. From the regression of soil ingestion with fecal Pb concentrations in samples from Entremuros, we may estimate that plant tissues and soil present in fecal excreta contain 8.6 and 72 μg/g, respectively (Table 4 and Fig. 1).

Table 5. Histopathological changes observed in geese captured in three locations in Doñana (southwestern Spain)

Histopathologic findings	Cerro $n = 37$	Entremuros $n = 7$	Escupidera $n = 6$
	n with lesions (%)		
Kidney			
Discrete vacuolization of tubular epithelium	1 (2.7)	1 (14.3)	0 (0)
Interstitial mononuclear infiltrate	0 (0)	2 (28.6)[a]	0 (0)
Presence of *Eimeria* sp. in tubular epithelium	0 (0)	1 (14.3)	0 (0)
Pancreas			
Focal interstitial mononuclear lymphocitary infiltrate	3 (8.1)	1 (14.3)	0 (0)
Liver			
Focal necrosis	1 (2.7)	0 (0)	1 (16.7)
Focal/diffuse congestion	2 (5.4)	0 (0)	0 (0)
Periportal mononuclear infiltrate	0 (0)	4 (57.1)[a]	0 (0)
Periportal polinuclear infiltrate	0 (0)	1 (14.3)	0 (0)

[a] Higher presence of the lesion in Entremuros than in the other two unpolluted locations (chi-square test or Fisher exact probability tests).

These concentrations do not differ too much from the values reviewed previously for plant and soil Pb concentrations. However, we must take into account that fecal analysis shows the unabsorbed Pb ingested, and consequently soil and plant concentrations of Pb or the other elements must be higher than the concentrations in feces.

The availability of water may be important for geese to wash food items before ingesting them. Soil ingestion was significantly affected by time of sampling possibly because, when the marshes in winter are completely flooded, geese can wash their food items more efficiently before ingesting them. *Cynodon dactylon* and *S. halepense* from remediated soils in Doñana reached toxic levels of Pb for animals in the unwashed plants but not in their tissues [30]. Amat et al. [33] have observed that geese spend more time handling rhizomes of *Scirpus* after these were extracted when walking in shallow waters than when swimming.

Liver concentrations of Cu, Zn, and Pb were within the ranges detected in greylag geese before the spill in Doñana, although the ingestion of two Pb shot was then responsible for the highest Pb concentration detected (8.1 μg/g dry wt) [9]. Other Anatidae species in Doñana showed similar levels of Zn and Cu in blood and liver a few months after the spill, but As concentrations were markedly higher than in geese studied here [10,11]. Lead was the only element present at concentrations in our geese above the threshold levels associated with toxicosis, and these levels were reached only in Entremuros. Blood Pb levels detected there were 0.1, 0.9, 1.2, and 3.3 μg/g wet weight, and Pain [19] suggested that concentrations >1 μg/g wet weight are indicative of severe clinical poisoning. Histopathological examination also revealed the presence of discrete mononuclear infiltrates in liver and kidney in a higher percentage of birds from Entremuros, although these changes cannot be considered life threatening. Renal intranuclear inclusion bodies were not observed in any of the geese, which may have been expected because this is a finding observed in mallards with >1 μg/g and not even detected in Canada geese with 2.5 μg/g wet weight of Pb in blood [13].

Changes in porphyrin metabolism may appear at subclinical levels of exposure of As and Pb. The increase in coproporphyrin I excretion was significantly associated with As exposure even when including Pb in the statistical analysis, and

Table 6. Porphyrins and biliverdin concentrations (geometric means with range) in excreta (nmol/g dry wt) and bile (nmol/g wet wt) of greylag geese from Doñana (southwestern Spain)[a]

	Sample	Cantarita	Cerro	Entremuros	Marilópez	Escupidera
		Zone				
Uroporphyrin	Bile	—	NDC (ND–0.46)	NDB	—	0.36A (0.16–0.57)
Coproporphyrin I	Bile	—	1.5 (0.39–6.1)	1.8 (1.2–4.2)	—	1.5 (1.0–2.1)
	Intestine	—	0.85 (0.33–3.7)	0.46 (0.25–0.81)	—	0.45 (0.21–0.71)
	Excreta	0.54 (0.05–6.4)	0.41 (0.12–16)	1.0 (0.07–7.0)	0.38 (0.06–1.3)	0.33 (0.05–2.2)
Coproporphyrin III	Bile	—	0.14B (ND–1.6)	0.86A (0.28–2.0)	—	0.71AB (0.54–1.2)
	Intestine	—	0.19 (0.02–0.89)	0.70 (0.19–1.5)	—	0.31 (0.07–1.0)
	Excreta	1.2 (0.40–9.0)	0.94 (0.13–9.2)	0.97 (0.09–2.6)	0.71 (0.08–4.1)	0.46 (0.07–4.9)
Protoporphyrin IX	Bile	—	1.6 (0.55–7.5)	1.3 (0.71–2.3)	—	1.3 (0.59–2.3)
	Intestine	—	1.9 (0.35–19)	1.5 (0.67–4.2)	—	0.90 (0.27–2.5)
	Excreta	1.6 (0.20–4.5)	0.99 (0.10–5.4)	0.66 (0.07–2.9)	0.51 (0.05–2.9)	0.09 (0.05–0.15)
Biliverdin	Bile	—	119 (18–569)	202 (29–479)	—	278 (127–581)
	Intestine	—	172 (26–1,096)	51 (0.65–363)	—	360 (127–709)
	Excreta	189 (12–5,934)	356 (16–4,506)	85 (7.7–1,857)	52 (4.7–654)	47 (3.9–1,060)
Sample size	Bile	—	32	4	—	4
	Intestine	—	37	6	—	6
	Excreta	30	30	30	30	30

[a] Intestinal content of dead birds can be considered equivalent to excreta. Biliary concentrations with different capital letters differed significantly among locations (analysis of variance and post hoc Tukey test). Differences in fecal or intestinal concentrations of porphyrins were calculated for values normalized by biliverdin and are commented in Figures 4 and 5.

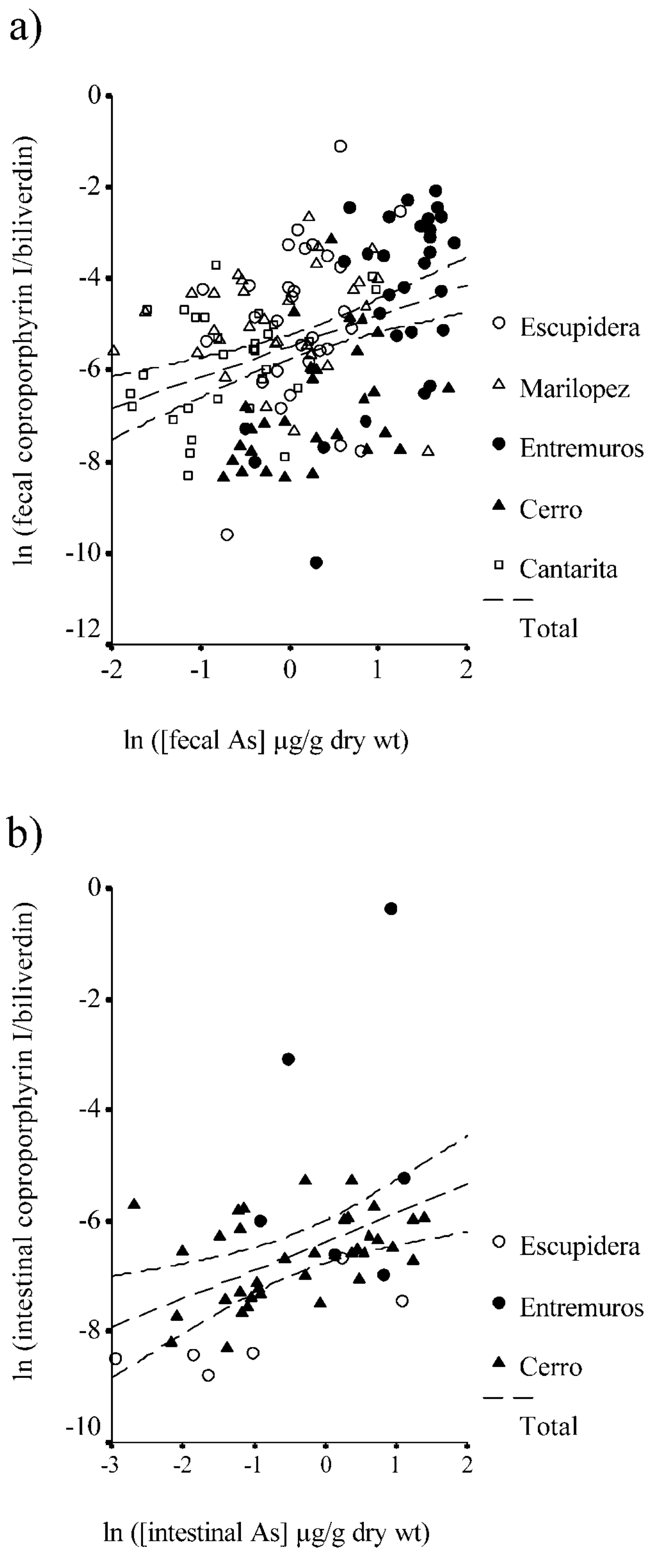

Fig. 4. Relationship between arsenic (As) concentration and coproporphyrin I standardized by biliverdin in feces (**a**) and intestinal content (**b**) of greylag geese wintering in Doñana (southwestern Spain). Significant effects of As and zone were observed in feces (As: $F_{1,144} = 17.7$, $p < 0.001$; zone: $F_{4,144} = 10.4$, $p < 0.001$) and intestinal content (As: $F_{1,45} = 5.3$, $p = 0.026$; zone: $F_{1,45} = 12.0$, $p < 0.001$). Pairwise comparisons of the estimated marginal means were significant between Cerro (southwestern Spain) and the other areas in feces and among all studied areas in intestinal content.

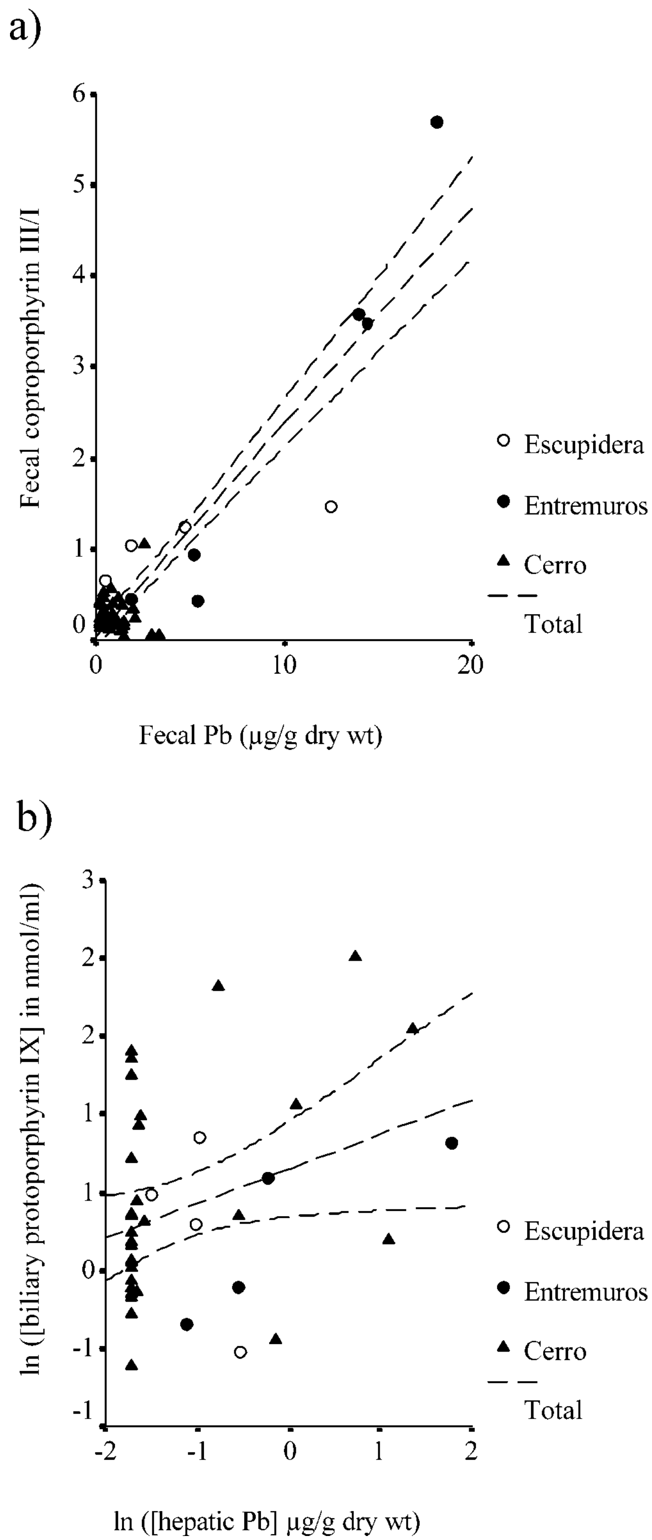

Fig. 5. Relationship between lead (Pb) concentrations and changes in patterns (**a**) or concentrations of porphyrins (**b**) in greylag geese wintering in Doñana (Spain). Fecal lead concentration and coproporphyrin III/I ratio were significantly related at high Pb concentrations (Pb: $F_{1,45} = 95.0$, $p < 0.001$; zone: not significant). Biliary protoporphyrin IX concentration was significantly related to liver Pb concentration (Pb: $F_{1,36} = 6.9$, $p = 0.012$; zone: not significant).

this finding was observed in feces collected in the field as well as in the intestinal content of shot geese. However, As concentrations in geese tissues were always close to the detection limit and significantly higher only in livers of birds from Entremuros than from Cerro de los Ánsares. Changes in coproporphyrin urinary excretion have been described in humans exposed to As, manifested as an increase in coproporphyrin and an altered ratio of coproporphyrin III/I [34–36]. The changes observed in the geese in relation to Pb exposure, such as the increase of the coproporphyrin III/I ratio in feces or the increase of protoporphyrin IX excretion in bile, have also been observed in bile of Pb-poisoned mallards [14]. Lead also causes an increase in urinary coproporphyrins in mammals [21,37] and more of the isomer III than the isomer I [38]. Other biological effects in animals reported after the Aznalcóllar spill associated with Pb, Cd, and As exposure include the evidence of increased oxidative stress and ethoxyresorufin-*O*-deethylase activity in Algerian mouse (*Mus spretus*) [39]. Damage on DNA has also been observed in lymphocytes of white storks and black kites (*Milvus migrans*), and this genotoxic effect has increased 2- to 10-fold between 1999 and 2002 [40].

Greylag geese wintering in Doñana may be subjected to a certain risk of Pb poisoning by the ingestion of contaminated soil in the area affected by the Aznacóllar spill. Although this area is relatively small compared with the overall surface of the marshes in the National Park, the number of geese feeding in Entremuros can be high in some periods of the wintering season or during years of drought because this is one of the few zones in Doñana with permanent waters. Given current plans to restore inputs of water from the Guadiamar River to the marshes in the heart of Doñana National Park, inputs of contaminated sediment should be evaluated to prevent a further increase in heavy metals and As concentrations in the soils of the marshes.

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