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Identifying Sources of Pb Exposure in Waterbirds and Effects on Porphyrin Metabolism Using Noninvasive Fecal Sampling

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Supporting Information

ABSTRACT: Waterbird feces (mainly mallard *Anas platyrhynchos* and coot *Fulica atra*) were collected from four wetlands in Southern Spain in the field or during capture (n = 558 and n = 59, respectively) to study lead (Pb) shot ingestion. Lead and aluminum (Al) concentrations along with Pb isotope signatures were used to identify sources of Pb exposure. The profile and concentrations of porphyrins and biliverdin in feces were used as biomarkers of toxicological effects. Feces with Pb concentrations $\geq$34 μg/g d.w. showed higher Pb/Al ratios, together with lower $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ ratios, and higher $^{208}Pb/^{206}Pb$ ratios, than feces with <34 μg/g d.w. Isotope signatures and Pb/Al ratios together indicated that Pb shot ingestion was the likely cause of the high Pb levels in some samples, whereas sediment ingestion was linked to lower/background levels. Coproporphyrin I and protoporphyrin IX were also higher in feces with Pb $\geq$34 μg/g d.w., indicating measurable disruption in heme synthesis. Noninvasive fecal sampling permits study of the degree and source of Pb exposure and physiological effects, with low-effort and minimal disturbance to waterbirds.

INTRODUCTION

Lead (Pb) shot ingestion and resultant Pb poisoning has caused high mortality rates in wintering waterfowl populations in North America (of 1.5%) and Europe (of 9%) before regulations were implemented to reduce Pb shot use.[1,2] Heavily hunted wetlands worldwide can have 100−500 Pb shot/m^2 in sediments.[2] Pb shot densities can be even higher in wetland sediments at clay pigeon shooting ranges where densities >2000 shot/m^2 [2] have been reported. Many wetlands are low-energy sedimentary environments containing fine-grained silt/clay assemblages. In such areas, there is often a shortage of large sand or gravel >1 mm in diameter.[3,4] These larger particles, grit, are used by waterbirds to break up and grind food in their gizzard.[5] Thus, in hunted environments where Pb shot pellets are more abundant than natural grit, Pb shot ingestion presents a potentially lethal risk for waterbirds.[6,7]

Ingested Pb shot are eroded within the avian gizzard until they are either expelled in feces or completely dissolved.[8] During this process, dissolved Pb may be absorbed through the intestine wall into the bloodstream, or, will remain unabsorbed and be excreted in feces.[8] Once in the bloodstream, a primary toxic effect of Pb is to cause anemia, by indirectly impairing heme synthesis and shortening erythrocyte life span.[9] Pb inhibits several key enzymes that are involved in heme synthesis, such as δ-aminolevulinic acid deshidratase (ALAD), coproporphyrinogen oxidase, and ferrochelatase.[9] This enzyme inhibition can then cause changes in the measurable porphyrin profile in blood and feces, which can be used as a powerful biomarker of toxicological effects.[10,11]

Pollutant-induced alterations in the profile of excreted porphyrins and biliverdin have been noted previously in birds and have been used successfully in noninvasive studies (using feces) to monitor the effects of toxic elements such as Pb and As.[11,12] Fecal sampling has been used to study the exposure of birds to Pb through the ingestion of food,[13] soil,[11,14] and Pb shot.[15] Further, correlations between Pb and aluminum (Al) in feces have been used to discriminate between Pb sources, that is, between soil or Pb shot ingestion in waterfowl.[14,15] Recently, Pb concentrations in feces from mallards (*Anas platyrynchos*) in otherwise unpolluted wetlands were shown to correlate significantly with the number of Pb shot ingested, and the weight of Pb shot remaining in the gizzard and fecal Pb concentrations $\geq$34 μg/g d.w. were specifically indicative of Pb shot ingestion.[15] In addition, Pb isotope ratios in blood, bone, kidney, liver, muscle, feathers, or feces have been used to distinguish between different Pb exposure sources in birds.[16−21]

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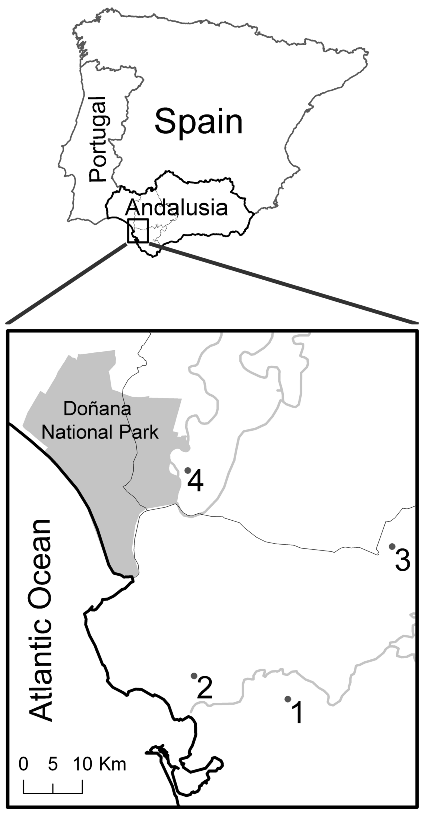

Figure 1. Map of the study area showing study sites located within the Guadalquivir valley (Andalusia, Spain). 1, Medina lagoon; 2, Puerto Santa María lagoons; 3, Espera lagoons; 4, Veta la Palma (Doñana Natural Park).

Here, we analyze Pb concentrations in feces from waterbirds from several wetlands contaminated with spent Pb shot (from historical hunting activities) and establish the toxicological relevance of any Pb shot ingestion observed. We use the relationship between Pb and Al concentrations in feces, and the Pb isotope ratios ($^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{208}Pb/^{207}Pb$), to discriminate between Pb exposure sources in these wild birds. Finally, we use the porphyrin and biliverdin profiles and concentrations in feces as biomarkers of measurable adverse or toxicological effects related to Pb shot ingestion.

Material and Methods

Study Area. The study was carried out in southern Spain at three endorheic lagoon complexes (Medina, Puerto de Santa María, and Espera) and a seminatural marshland (Veta la Palma, VLP). These wetlands contain a wide range of Pb shot densities in their sediments and are situated in a sedimentary area defined by the Guadalquivir River depression at the foot of the Betic Mountain Range (Figure 1). Medina lagoon has some of the highest Pb shot densities in sediments ever recorded, with 148 shot/m^2 and 399 shot/m^2 in the first 10 and 30 cm of sediment, respectively.[4] The other wetlands studied were Puerto de Santa María (Puerto), which has 12−59 shot/m^2, Espera with 27 shot/m^2, and VLP where no Pb shot has been detected in the upper 10 cm sediment layer.[4] Further details of the study area are given in the Supporting Information.

Sample Collection. Each site was visited and sampled twice per year (once during the breeding and once during the wintering season) during 2006, 2007, and 2008. Flocks of birds resting on the lagoon shorelines were located with binoculars and a telescope. Then, fresh feces were collected, normally from sites where only one species was present (mallard or coot *Fulica atra*). Some samples were recorded as purple gallinule (*Porphyrio porphyrio*), unidentified ducks (mallard or gadwall *Anas strepera*), or unidentified waterbird (coot or duck). Additionally, feces expelled from bait trapped birds (mallard, coot, moorhen *Gallinula chloropus*, and common pochard *Aythya ferina*) during capture and handling at Medina lagoon[22] were collected. All feces were carefully picked up by hand and any adhered soil was removed. Samples were placed in zip-lock plastic bags and frozen at −20 °C until analysis.

Sediment samples were collected from points along transects at the Puerto and Espera lagoons in September 2006 (when lagoons were completely dry). In Medina lagoon, sediment samples were collected in 2007 from points around the lagoon shoreline, and from the primary stream, which drains water into the lagoon. We used previously published Pb concentrations and Pb isotopic ratios for sediments from the area of VLP (Guadalquivir marshes).[23,24]

Sample Analysis. Feces (n = 617) and sediment (n = 93) samples (~0.25 g dry weight, d.w.) were acid digested after freeze-dried to determine Pb and Al concentrations using graphite furnace and nitrous oxide-acetylene flame atomic absorption spectroscopy system, respectively (AAnalyst800, PerkinElmer), following methods described previously with minor modifications (ref 15, also Supporting Information for more detail about methodology and quality control).

Lead isotopes (^{206}Pb, ^{207}Pb, ^{208}Pb) were analyzed in a selection of collected feces and sediment digestions (n = 95) at the Department of Analytical Chemistry and Food Technology in Toledo (Faculty of Environmental Sciences, UCLM). The analysis was conducted using an inductively coupled plasma mass spectrometry system (ICP-MS, Thermo X series). A certified NIST Pb isotope standard was used (SRM 981), which has an isotopic composition (mean ±95%) for ^{206}Pb of 24.1442 ± 0.0057%, for ^{207}Pb of 22.0833 ± 0.0027%, and for ^{208}Pb of 52.3470 ± 0.0086%. All isotope ratios determined for SRM 981 during analysis were within 1% of the certified value (before the nominal rolling correction was applied). Procedure details are given in the Supporting Information.

Porphyrins and biliverdin were determined in a selection of collected feces (n = 74) by liquid chromatography coupled to mass spectrometry (LC/MS) following a method described previously with some modifications (ref 25, Supporting Information and Table S1 of the Supporting Information)

Statistical Analysis. Where necessary, data were log-transformed prior to statistical analyses to achieve the required parametric assumptions. Differences in Pb, Al, Pb/Al sediment concentrations, and Pb isotope ratios among localities were analyzed using one-way ANOVAs. Where significant effects were observed, posthoc Tukey tests were used. Differences in mean Pb concentrations in feces for mallard and coot were tested using GLMs, with locality and season (breeding or wintering) as factors. Because high concentrations of Pb in sediment are not expected (e.g., mining activities or other sources of Pb), feces were classified according to Pb concentration in two levels, assuming that birds that had ingested Pb shot were indicated by fecal Pb concentrations ≥34 μg/g d.w.[15] This classification was then used to compare Pb/Al ratios and Pb isotope ratios using one-way ANOVAs. Differences in porphyrin and biliverdin concentrations were tested with GLMs, using fecal Pb level (<34 vs ≥34 μg/g d.w.), species, locality, and season as factors. Differences in fecal Pb levels among localities were tested with X^2.

Table 1. Geometric Mean with 95% Confidence Interval for Pb, Al, and Pb/Al Concentrations (μg/g d.w.) in Sediments from the Medina Lagoon, and the Puerto de Santa María, and Espera Lagoon Complexes; Also, the Mean (±SD) for Pb Isotopes for Sediment Samples, and Pb Shot Densities Present in Each Lagoon

	N	Medina	*N*	Puerto Sta M[a]	*N*	Espera
Pb shot/m^2		148.3		58.9		26.7
Pb μg/g	14	25.86^A	49	31.17^A	30	12.56^B
		(17.65−37.89)		(24.95−38.94)		(9.80−16.10)
Al μg/g	14	20 824^{AB}	49	14 132^A	30	13 242^B
		(16 753−25 886)		(8579−23 279)		(10 575−16 581)
Pb/Al	14	0.0012^{AB}	49	0.0022^A	30	0.0009^B
		(0.0008−0.0019)		(0.0015−0.0031)		(0.0007−0.0012)
^{206}Pb/^{207}Pb	7	1.1830^A ± 0.0086	13	1.1803^A ± 0.0038	13	1.1768^A ± 0.0058
^{208}Pb/^{206}Pb	7	2.0843^A ± 0.0110	13	2.0875^A ± 0.0055	13	2.0907^A ± 0.0073
^{208}Pb/^{207}Pb	7	2.4693^A ± 0.0123	13	2.4649^A ± 0.0028	13	2.4630^A ± 0.0050

[a] Shot densities (in the upper 10 cm of sediment) taken from Mateo et al.[4] Sediments were not taken at the fourth wetland, Veta La Palma. Means sharing a superscript letter were not significantly different among localities (Tukey test, $p > 0.05$).

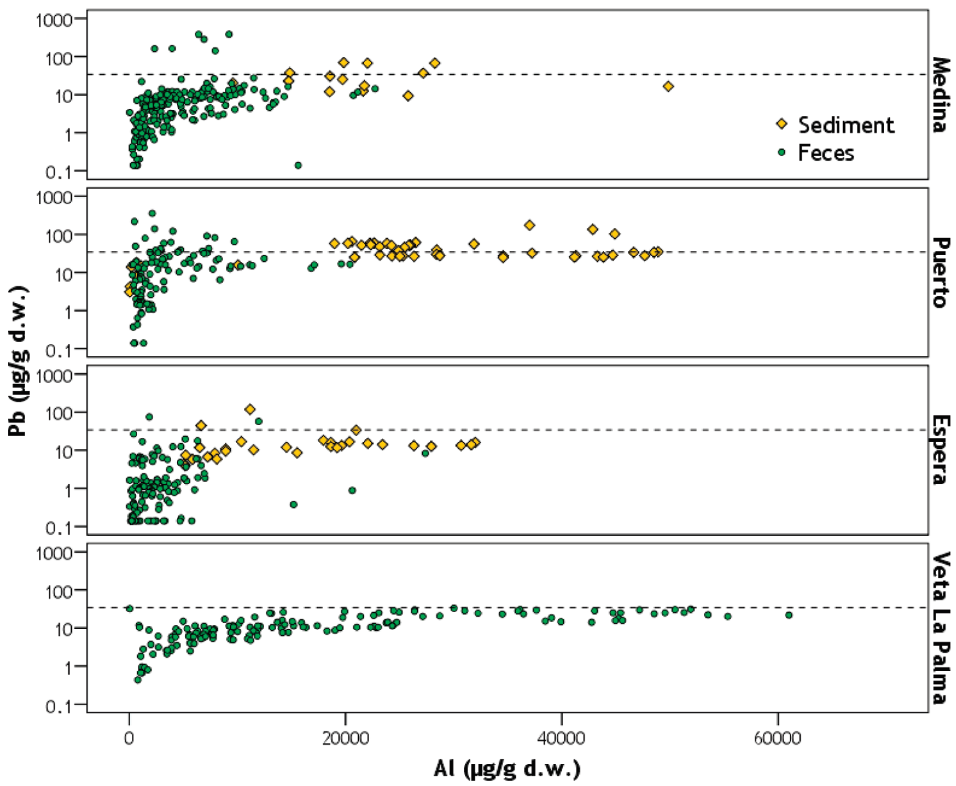

Figure 2. Relationship between Pb and Al concentration (μg/g d.w.) in sediment (yellow) and feces (green) when including all samples taken at each wetland. The dotted line shows the 34 μg/g d.w. Pb level. Those feces with Pb concentrations above the control line are highly likely to have ingested Pb shot in their gizzard.[15].

The relationship between Pb and Al concentrations in feces and in sediments was tested with Pearson correlations as was the correlation between Pb concentration in feces and blood taken from live captured birds (blood Pb data are taken from Martinez-Haro et al.[22]). All tests were performed using SPSS 17.0, with a level of statistical significance of $p \leq 0.05$.

RESULTS

Sediments. Mean Pb concentrations in sediments were higher in Medina and Puerto than in Espera ($F_{2,90} = 14.879$, $p < 0.001$; Table 1). Higher Al concentration and Pb/Al ratio were observed in Puerto than in Espera ($F_{2,90} = 6.445$, $p = 0.002$ and $F_{2,90} = 6.620$, $p = 0.002$, respectively; Table 1). Correlations between Pb and Al concentrations in sediments were significant in Puerto ($r = 0.780$, $p < 0.001$, $n = 49$) and Espera ($r = 0.375$, $p = 0.041$, $n = 30$), but not in Medina ($r = -0.026$, $p = 0.931$, $n = 14$) (Figure 2). No differences in the mean Pb isotope ratio in sediments were detected among localities (Table 1).

Feces. Mean Pb concentrations in mallard feces collected in the field varied by locality ($F_{3,337} = 29.776$, $p < 0.001$; Table 2) and season ($F_{1,337} = 79.376$, $p < 0.001$), with higher Pb concentrations

Table 2. Geometric Mean with 95% Confidence Interval for Waterbird Fecal Pb Concentrations (μg/g d.w.), and Percentage of Feces that Had Pb Concentrations ≥34 μg/g d.w. (Potentially Indicative of Pb Shot Exposure, ref 15) for Four Wetlands (Data Exclude Samples from Captured and Unidentified Birds)

species		Medina	Puerto Sta M[a]	Espera	Veta la Palma
mallard	N	75	109	71	90
	G. Mean	3.78^{B}	9.59^{A}	2.21^{C}	8.99^{A}
	95% CI	2.75−5.19	7.02−13.10	1.63−3.01	7.19−11.24
	≥34 μg/g (%)	3^{b}	19^{a}	3^{b}	0^{c}
coot	N	35		52	56
	G. Mean	5.36^{B}		0.43^{C}	10.10^{A}
	95% CI	4.03−7.12		0.30−0.62	8.85−11.53
	>34 μg/g (%)	0		0	0
purple gallinule	N	5		3	
	G. Mean	1.24		0.43	
	95% CI	0.42−3.64		0.02−9.48	
	≥34 μg/g (%)	0		0	

[a] Means sharing a superscript capital letter were not significantly different among localities (Tukey test, $p > 0.05$). Percentages sharing a superscript lowercaseletter were not significantly different among localities (X^2 test, $p > 0.05$).

Table 3. Geometric Mean with 95% Confidence Interval for Pb/Al Ratios of Sediment, and Fecal Samples Analyzed According to Fecal Pb Level Detected (μg/g d.w.); Also, from Samples Selected for a More Complete Analysis, Mean (±SD) for Pb Isotope Signatures, and Marginal Geometric Mean with 95% Confidence Intervals (From Generalized Linear Models) for Porphyrins and Biliverdin Concentrations (nmol/g d.w.)[a]

			feces			
	N	sediment	N	Pb <34 μg/g	N	Pb ≥34 μg/g
Pb/Al	93	0.0015^{B}	583	0.0011^{B}	27	0.0231^{A}
		(0.0012−0.0020)		(0.0010−0.0020)		(0.0148−0.0358)
$^{206}Pb/^{207}Pb$	33	$1.1795^{A} \pm 0.0062$	45	$1.1761^{A} \pm 0.0054$	17	$1.1690^{B} \pm 0.0061$
$^{208}Pb/^{206}Pb$	33	$2.0881^{C} \pm 0.0077$	45	$2.0924^{B} \pm 0.0062$	17	$2.1005^{A} \pm 0.0053$
$^{208}Pb/^{207}Pb$	33	$2.4651^{A} \pm 0.0068$	45	$2.4623^{A} \pm 0.0068$	17	$2.4554^{B} \pm 0.0086$
coproporphyrin I			55	0.4835^{b}	13	1.1876^{a}
				(0.2843−0.8221)		(0.6423−2.1959)
coproporphyrin III			58	1.8967^{a}	15	3.1574^{a}
				(1.1414−3.1516)		(1.7649−5.6488)
mesoporphyrin IX			33	0.2654^{a}	8	0.1471^{a}
				(0.0971−0.7252)		(0.0496−0.4368)
protoporphyrin IX			59	0.9446^{b}	15	2.4704^{a}
				(0.5503−1.6214)		(1.3301−4.5883)
biliverdin			59	24.1310^{a}	14	45.1757^{a}
				(10.7599−54.1182)		(17.7761−114.8084)

[a] Means sharing a superscript capital letter were not significantly different by fecal Pb level (Tukey test, $p > 0.05$). Means sharing a superscript lowercase letter were not significantly different by fecal Pb level ($p > 0.05$).

in feces collected during the winter than in the breeding season. For coot, Pb in feces also differed by locality ($F_{2,139} = 128.426$, $p < 0.001$; Table 2). Only mallard feces contained Pb ≥34 μg/g d.w. indicative of possible Pb shot ingestion), and the percentage of feces above this threshold was higher in Puerto (19%) than in other wetlands (Table 2).

Fecal Pb and Al concentrations were correlated in all study localities when excluding those samples with Pb ≥34 μg/g d.w. (at $r > 0.336$, $p > 0.001$ in all cases). In Espera, Medina, and Puerto, there were 2, 6, and 19 outliers (with Pb ≥34 μg/g d.w.) respectively when including all samples. Each outlier had high Pb and relatively low Al and thus fell outside the overall Pb−Al relationship observed for feces at each area (Figure 2). Furthermore, fecal samples with Pb ≥34 μg/g d.w. showed significantly higher Pb/Al ratios than those with <34 μg/g d.w. and sediment samples ($F_{2,700} = 86.259$, $p < 0.001$; Table 3). This same trend was detected in each locality (Medina: $F_{2,242} = 43.514$, $p < 0.001$; Puerto: $F_{2,154} = 25.090$, $p < 0.001$; Espera: $F_{2,153} = 5.162$, $p = 0.007$). Significant differences in Al concentrations in feces were not detected between groups of samples with Pb concentrations above or below 34 μg/g d.w.

Fecal samples with Pb <34 μg/g d.w. had similar $^{206/207}Pb$ and $^{208/207}Pb$ ratios to sediment samples ($F_{2,92} = 18.421$, $p < 0.001$; Table 3; Figure 3). Fecal samples with Pb <34 μg/g d.w. and sediments also had higher ratios ($^{206/207}Pb$ and $^{208/207}Pb$) than those with Pb ≥34 μg/g d.w. ($F_{2,92} = 10.288$, $p < 0.001$; Table 3;

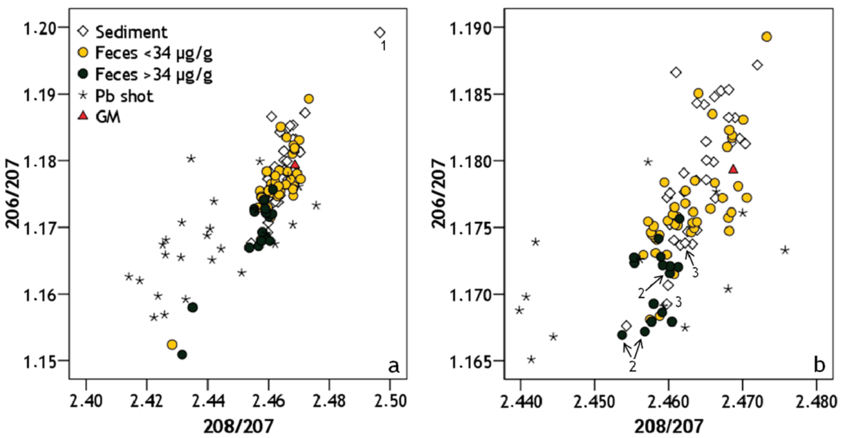

Figure 3. Relationship between the isotope ratios $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ in sediment and feces is shown in (a) for all data and without the peripheral points in (b). Birds with ≥34 μg/g d.w. of fecal Pb are highly likely to have ingested Pb shot in their gizzard.[15] One = sediment from a Medina stream, 2 = feces of captured pochard, 3 = samples of sediments with Pb >100 μg/g d.w. Pb isotope ratios for Pb shots and sediment from Guadalquivir marshes (GM) taken from literature.[17,23]

Figure 3). Likewise, sediment and fecal samples with Pb <34 μg/g d.w. had lower $^{208/206}Pb$ ratio values than fecal samples with ≥34 μg/g d.w. ($F_{2,92} = 19.740$, $p < 0.001$; Table 3). Furthermore, feces of two pochards captured in Medina with high blood Pb concentrations (226−634 μg/dl) had both Pb ≥34 μg/g d.w. (Figure 3).

In terms of biomarkers, concentrations of coproporphyrin I and protoporphyrin IX in feces were significantly higher in fecal samples from birds with ≥34 μg/g d.w. Pb (when including all samples analyzed; $F_{1,58} = 6.937$, $p = 0.011$ and $F_{1,64} = 7.706$, $p = 0.007$, respectively; Table 3). Lastly, higher mean concentrations of fecal coproporphyrin I and protoporphyrin IX were observed for mallards with ≥34 μg/g d.w. Pb in feces ($F_{1,36} = 7.911$, $p = 0.008$ and $F_{1,40} = 10.464$, $p = 0.002$, respectively; Table S3 of the Supporting Information); and mean coproporphyrin III and biliverdin concentrations were also higher in these birds, although differences were not statistically significant.

Lead in feces from birds captured in Medina was closely correlated with blood Pb ($r = 0.967$, $p < 0.001$, $n = 43$ [fecal Pb = $a + b \cdot$ blood Pb; $a = 1.907 \pm 2.209$, $b = 0.490 \pm 0.020$ (mean ± SE)]; blood Pb data derived from Martinez-Haro et al.[22]). Birds with fecal Pb ≥ 34 μg/g d.w. ($n = 3$) had 226−634 μg/dl Pb in blood. All other birds (with fecal Pb <34 μg/g d.w., $n = 40$) had blood Pb concentrations of less than 40 μg/dl.

■ DISCUSSION

Although overall sediment Pb concentrations can be affected by in situ Pb shot dissolution in soils,[26,27] the background geochemical composition of the sediment, and porewater conditions within it, are also important. The mean Pb concentration in sediments from Medina, Puerto lagoons, and the Guadalquivir Marshes were all similar,[24] despite the fact that higher Pb shot densities occur in Medina. In Espera, where Pb shot densities are lower, Pb concentrations in sediments were also lower. In Medina, no significant correlation was observed between Pb and Al in sediments, indicating high heterogeneity in this locality. This may be related to complex sedimentary dynamics in the lagoon, or, to the sporadic sediment contamination caused by the spent Pb shot pellets deposited there. Lead shot in sediment will slowly weather, disintegrate, dissolve, and settle into deeper sedimentary layers. These processes will occur at variable rates depending on sedimentary dynamics, and the environmental conditions in the sediment (such as prevailing pH, redox potential, particle size distribution, biotic activity, etc; refs 26−28). In the three lagoon complexes where sediments were sampled, certain samples showed high Pb values (up to a maximum of 173 μg/g d.w.). In terms of their Pb isotope ratios, such samples showed lower $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$, and higher $^{208}Pb/^{206}Pb$. Ratios were also consistent with values reported previously for European Pb shot (Figure 3, Table S2 of the Supporting Information). Moreover, in Medina, sediment present in the stream feeding this lagoon showed a higher $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ ratio and a lower $^{208}Pb/^{206}Pb$ ratio than that for European Pb shot (Figure 3, Table S2 of the Supporting Information). Hence, it seems likely that the degree of Pb heterogeneity observed in these sediments is due to the release of Pb from spent shot pellets.

Twenty five years after hunting was banned in the Medina, Puerto, and Espera lagoons, waterbirds continue to be exposed to spent Pb shot pellets deposited in sediments. In mallard, 3% of feces from the Medina and Espera lagoons, and 19% of those from Puerto, had Pb concentrations ≥34 μg/g d.w., a threshold indicative of Pb shot ingestion.[15] Furthermore, clear statistical differences in Pb/Al ratios and Pb isotope ratios were found in feces with more or less than 34 μg/g d.w. Feces with ≥34 μg/g d.w. had higher Pb/Al ratios (indicating an additional Pb source was being ingested, apart from soil when this is not polluted; Figure 2), and also lower $^{206/207}Pb$ and $^{208/207}Pb$, and higher $^{208/206}Pb$, which was consistent with a Pb shot signature. Mean $^{206}Pb/^{207}Pb$ ratios for European Pb shot were described previously[20] and are 1.1620 ± 0.0087 (mean ± SD) when excluding UK sources (for which isotope ratios are generally lower). Lead shot ingested by white-headed duck (*Oxyura leucocephala*) in Spanish wetlands had a $^{206}Pb/^{207}Pb$ ratio of 1.1699 ± 0.0117, a $^{208}Pb/^{206}Pb$ ratio of 2.0885 ± 0.0156, and a $^{208}Pb/^{207}Pb$ signature of 2.4433 ± 0.0193.[17] A lower $^{208}Pb/^{207}Pb$ ratio of 2.4120 ± 0.0292 was reported for Pb shot embedded in hunted red grouse (*Lagopus lagopus scotica*) from the UK.[18] Furthermore, in VLP, where Pb shot pellets are not present in sediment,[4] the Pb isotope profile in waterbird feces was similar to that reported for unpolluted soils from the Guadalquivir Marshes (ref 23, and Table S2 of the Supporting Information). Such data tend to support our suggestion that Pb shot is the most likely source in feces with outlying Pb concentration (≥34 μg/g d.w).

Since the use of Pb isotopes to discriminate between sources of Pb intake in birds was first described,[16] this technique has been used successfully to study Pb shot exposure in several avian species. Once Pb shot is ingested and, while the shot remains in the gizzard (or digestive tract as a whole), a fraction of the Pb that dissolves per unit time is absorbed into the avian bloodstream through the intestine. This then passes to and accumulates in soft tissues such as the liver and kidney (in the short term) but is subsequently deposited in bones (in the longer term, ref 10) or excreted in bile, urine, or feathers. Liver, kidney, and bone samples are often obtained from hunter-shot birds or from birds found dead in the field. However, such destructive or opportunistic sampling methods are not always ideal, feasible, or desirable, that is, when studying endangered or threatened species, which will be encountered only rarely in a fresh state in the field and cannot be hunted. Hence, Pb concentrations and Pb isotope signatures derived from blood and feathers have also been used to monitor exposure in rare species such as Californian condor (*Gymnogyps californianus*).[19] However, blood sampling is invasive, involves capture, animal manipulation and also requires significant time and money efforts, whereas feathers are often not ideal sample media due to surface contamination.[29] Analyzing Pb concentrations and Pb isotopes in feces is another useful noninvasive option to study Pb exposure without the need for capture. Feces sampling also requires far less effort, and large, statistically powerful sample sizes can be obtained comparatively easily.

Aluminum concentrations in waterfowl feces have been used previously to indicate the degree of soil ingestion,[14] and Pb−Al relationships can help discriminate between birds ingesting soil or soil plus Pb shot.[15] Soil or sediment ingestion in waterbirds can be an important heavy metal exposure route, especially in mining or other polluted areas.[30,31] Soil ingestion depends on feeding behavior, habitat, and diet,[32] but soil can typically represent <12% of the material (as dry mass) ingested by herbivorous waterfowl.[11,14,32] A similar value of soil ingestion (10.5%) can be estimated from our Al concentrations in waterfowl feces and sediments and assuming a 50% digestibility of dietary plant material.[11] Because the maximum Pb concentration observed in the sediments of wetlands studied here was between 69 and 173 μg/g d.w. (concentration that would then be diluted ∼5−10 fold in feces), 34 μg/g d.w. would seem an appropriate threshold value that could be used to discriminate between soil and Pb shot ingestion. Furthermore, feces with Pb concentrations ≥34 μg/g d.w. plotted well outside the distribution noted between Pb and Al concentrations in the other feces (Figure 2).

In terms of fecal porphyrins, coproporphyrin I and protoporphyrin IX concentrations were significantly higher in those birds with feces with Pb concentrations ≥34 μg/g d.w. (Table 3). Fecal coproporphyrin I and biliary protoporphyrin IX have previously been linked with exposure to heavy metals and metalloids, in particular to fecal As and liver Pb concentrations (respectively) in geese.[11] In addition, increased biliary excretion of protoporphyrin IX has been previously described in Pb poisoned mallards, which suggests that increased protoporphyrin IX in feces would be expected in birds being poisoned by Pb.[25] Increased fecal protoporphyrin IX has also been described in pigeons (*Columba livia*) exposed to urban air pollution.[33] Elevated Pb exposure in birds leads to hemolytic anemia, which manifests itself by causing gall bladder enlargement, viscous bile, and biliverdinuria (green-stained feces).[34] Biliverdin is a bile pigment that is produced during the biodegradation of heme, which increases in bile of Pb poisoned birds;[25] here, although no statistically significant effect on this pigment was observed, mean concentrations were twice as high in mallards with ≥34 μg/g d.w. Pb when compared to those with less than this level (Table S3 of the Supporting Information).

Finally, the results obtained from the analysis of fecal excreta were consistent with those reported from blood analysis for birds captured in Medina during the breeding season,[22] common pochard, and mallard being the species with higher exposure to Pb, with blood Pb concentration ≥20 μg/dl (the level associated with subclinical poisoning in waterfowl, ref 10). In contrast, none of the coots analyzed had blood Pb concentrations above this threshold,[22] and none of their fecal samples had Pb concentrations ≥34 μg/g d.w. Furthermore, as was previously reported,[11] fecal Pb has also been correlated with blood Pb in captured birds. Nevertheless, the noninvasive sampling and analysis of feces has several key advantages, that is, it is simple, cheap, and quite large numbers of samples can be gained quickly from a range of species. Critically, it permits the detection of adverse effects as picked up by biomarkers such as porphyrins, as described here.

■ ASSOCIATED CONTENT

S Supporting Information. Includes further information about the study area, sample analysis, and three tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information

Identifying sources of Pb exposure in waterbirds and effects on porphyrin metabolism using non-invasive fecal sampling

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Study area

Medina is the second largest natural lagoon in Andalusia (120 ha, with a catchment area of 1900 ha) and has a maximum water depth of 3.5 m. It does however dry out completely during periods of drought (e.g., in 1999-2000). There are three seperate lagoons at Puerto: Salada (34 ha), Chica (11 ha) and Juncosa (7 ha). These are located in a sedimentary area composed of silt rich marl (*1*). The Espera wetland is also composed of three lagoons: Salada (25 ha), Dulce (12 ha) and Hondilla de Zorrilla (5 ha). Sediments here are rich in gypsum, with occasional limestone and dolomite outcrops (*1*). The Puerto and Espera lagoon complexes both have a maximum water depth of 2.5 m, and frequently dry out in summers. The fourth wetland studied, Veta La Palma (VLP), is a private estate with a complex of extensive brackish fish ponds (3000 ha). The estate is built upon former natural marshes that run alongside the Guadalquivir river in the Doñana Natural Park (*2, 3*).

The three first wetland areas support a variety of waterbirds (*4*) and are especially important for red-knobbed coot (*Fulica cristata*), and the Globally Endangered white-headed duck (*Oxyura leucocephala*, *5*). The latter is also especially susceptible to Pb shot ingestion (*6, 7*). Waterfowl hunting was common in all of these wetland areas before they were protected as Integral Reserves in 1987. Veta la Palma is within the Doñana Natural Park, is extremely important for wintering waterbirds (*3, 8*), and is also one of the most important wetlands in Europe for the Globally Vulnerable marbled duck (*Marmaronetta angustirostris*; *9*). All these areas are classified as wetlands of International Importance under the Ramsar Convention.

Pb and Al analysis

Samples were acid digested using a microwave digestion system (Ethos E, Milestone, Italy), with 3 ml of HNO_3 (69% Analytical grade, Panreac, Spain), 1 ml of H_2O_2 (30% v/v Suprapur, Merk, Germany) and 4 ml of H_2O (Milli-Q grade). Samples were then analyzed for Pb and Al by graphite furnace (Pb) or flame (Al) atomic absorption spectroscopy. Blanks, a certified soil reference material (GBW07406) and a certified bush, branches and leaves reference material (NCS DC 73349), were processed in each batch of digestions to provide quality control data. Limits of detection (LODs) in dry samples were 0.28 µg/g for Pb and 88.80 µg/g for Al. Mean percentage Pb recoveries (± %RSE) were 106.9% (± 3.4%, n = 8) for soil and 99.5% (± 3.5%, n = 9) for bush, branches and leaves. For Al, the mean % recovery (± %RSE) for the bush, branches and leaves CRM was 100.4% (± 1.69%, n = 6).

Lead isotopes analysis

The analysis was conducted using an inductively coupled plasma mass spectrometry system (ICP-MS; Thermo X series). The instrument was calibrated for Pb concentration using a diluted certified NIST Pb isotope standard (SRM 981) solution (containing 1000 mg/l, diluted to the 5-100 µg/l range). Full calibration was performed at the beginning of every set of measurements. Factors affecting the precision and accuracy of the isotope ratio measurements were carefully evaluated. Data acquisition parameters were: a dwell time of 10 ms for ^{206}Pb and ^{207}Pb and 5 ms for ^{208}Pb, three points per peak, and 800 sweeps. The % relative standard deviation for all isotope ratio measurements was <0.1%. To correct for mass bias, the lead isotopic standard SRM 981 was remeasured after every 5 samples. Isotope ratio measurements were then corrected for mass bias by applying a rolling correction identical to that required to achieve the certified isotope reference value.

Porphyrin and biliverdin analysis

Porphyrins and biliverdin were determined by liquid chromatography coupled to mass spectrometry (LC/MS) following a method described previously with some modifications

(*10*). The analytical system is formed by Agilent 1100 series and Agilent 6110 Quadrupole LC/MS with a multimode (MM) source. Porphyrins and biliverdin were detected and quantified using positive ion monitoring in SIM mode (Table S1) with the following MM-ESI source settings. Nebulizer pressure was set at 60 psi, drying gas flow was 5 l/min, drying gas temperature was 350 ºC, vaporizer temperature was 100 ºC, capillary voltage was 2000 V, charging voltage was 1000 V, and fragmentation voltage was 210, 240, and 300 V for different compound. Chromatograms were also monitored with UV-Vis detector in order to confirm the identity of some peaks by their absorption spectra (*10*).

All chromatographic conditions and quantification were controlled using ChemStation software. A Waters (Milford MA, USA) Spherisorb ODS 2 (5 µm particle size, 4.6 mm × 100 mm) chromatographic column was used. ACS grade methanol, GR grade sulphuric acid, glacial acetic acid, ammonium acetate and HPLC grade acetonitrile were purchased from BDH Prolabo (Leuven, Belgium). Porphyrin standards (CMK 1-A, B655-9, C654-3 and P562-9) were purchased from Frontier Scientific (Carnforth, UK). Sample extraction was undertaken using 0.02 – 0.03 g of liofilized feces. This was placed in an eppendorf, and 0.25 ml of HCl 3N, 0.3 ml of acetonitrile and 0.3 ml of water was added. Tubes were vortex mixed for 2 minutes and then centrifuged for 10 minutes at 14,000 RCF. Finally, 0.2 ml of supernatant was transferred to an amber glass vial for HPLC analysis. The recovery for the extraction procedure was calculated by comparing standard solutions with samples spiked (n = 6) with porphyrins and biliverdin. Mean % recoveries (± %RSD) for coproporphyrin I, III, mesoporphyrin IX, protoporphyrin IX and biliverdin were 107 ± 8, 73 ± 8, 51 ± 8, 18 ± 2 and 23 ± 6% respectively.

Table S1. Parent ions used for the quantification of the porphyrins and biliverdin in fecal samples and fragmentation conditions used for each compound.

Compound	Ion m/z	Fragmentation voltage (V)
Coproporphyrin I	655.2	210
Coproporphyrin III	655.2	210
Biliverdin	583.2	240
Mesoporphyrin	566.7	210
Protoporphyrin IX	563.2	300

Table S2. Lead isotope ratios (mean ± SD) reported for European Pb shot and Guadalquivir marshes sediment in previous works, and those for specific samples of the present work.

	N	$^{206}Pb/^{207}Pb$	$^{208}Pb/^{206}Pb$	$^{208}Pb/^{207}Pb$	References
Shot	8	1.1620 ± 0.0087			*11*
Shot	27	1.1699 ± 0.0117	2.0885 ± 0.0156	2.4433 ± 0.0193	*6*
Shot	3			2.4120 ± 0.0292	*12*
Guadalquivir marsh	3	1.1793	2.0932	2.4687	*13*
Feces pochards	3	1.1686 ± 0.0026	2.1024 ± 0.0025	2.4569 ± 0.0032	This study
Sediment > 100 µg/g	2	1.1715 ± 0.0032	2.0710 ± 0.0043	2.4610 ± 0.0018	This study
Stream sediment (Medina)	1	1.1992	2.0710	2.4966	This study
Feces VLP	15	1.1766 ± 0.0018	2.0957 ± 0.0031	2.4659 ± 0.0030	This study

Table S3. Geometric mean with 95% confidence intervals for porphyrins and biliverdin concentrations in feces (nmol/g d.w.) of mallard, common pochard, coot, and unidentified waterbirds according to the fecal Pb level detected (µg/g d.w.).

		Mallard		Common pochard	Coot	Unidentified duck[1]	Unidentified species[2]	
Fecal Pb		**<34 µg/g**	**≥34 µg/g**	**≥34 µg/g**	**<34 µg/g**	**<34 µg/g**	**<34 µg/g**	**≥34 µg/g**
	N	32	10	2	14	2	7	1
Coproporphyrin I	G. Mean	0.55^{B}	0.98^{A}	2.93	0.8	1.08	0.77	1.91
	95%CI	0.43 - 0.71	0.50 - 1.89		0.39 - 1.62	0.64 - 1.83	0.53 - 1.13	
	N	34	12	2	14	3	7	1
Coproporphyrin III	G. Mean	1.07^{A}	1.56^{A}	16.71	1.25	1.69	2.47	1.88
	95%CI	0.81 - 1.41	0.88 - 2.79		0.71 - 2.21	0.22 - 12.87	2.10 - 2.91	
	N	20	7	1	8	2		
Mesoporphyrin IX	G. Mean	0.384^{A}	0.224^{A}	0.595	0.328	0.241		
	95%CI	0.247 - 0.597	0.049 - 1.028		0.133 - 0.810			
	N	34	12	2	15	3	7	1
Protoporphyrin IX	G. Mean	0.45^{B}	0.79^{A}	27.29	1.69	0.61	1.07	3.08
	95%CI	0.35 - 0.57	0.40 - 1.58		0.94 - 3.04	0.14 - 2.73	0.57 - 2.01	
	N	34	11	2	15	3	7	1
Biliverdin	G. Mean	16.59^{A}	32.85^{A}	322.57	13.07	36.6	30.88	123.15
	95%CI	11.47 - 23.99	17.59 - 61.36		4.61 - 37.03	4.69 - 285.80	12.81 - 74.44	

[1]Most likely to be Mallard *Anas platyrhynchos* or Gadwall *A. strepera*.
[2]Most likely to be ducks or coot.
Means sharing a superscript letter were not significantly different by fecal Pb level ($p>0.05$).

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