Avian Digestive Tract Simulation To Study the Effect of Grit Geochemistry and Food on Pb Shot Bioaccessibility

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Lead shot dissolution was investigated in a dynamic in vitro simulated avian gizzard-intestine system. The method allows simulated digestive fluid to pass (at intervals) from a gizzardlike environment to an intestine-based one, and then considers the dissolution of Pb shot (0–3 pellets) in the presence of differing grit geochemistries (siliceous and calcareous) and variable amounts of food (0–4 g of partially milled wheat seed). Dissolved Pb levels in simulated gizzards were consistently higher in the presence of siliceous, than with calcareous, grit. This was also seen in simulated intestines, except when less food was used (0–1 g), when Pb levels in solution were higher in calcareous systems. The Pb concentrations in gizzard and intestine solutions increased directly with the number of Pb shot used. In all treatments Pb levels in intestine liquids were lower than in gizzard liquids. Calcareous grit simulations maintained 25–34 times more Ca in solution than those that used siliceous grit. Dietary supplementation with calcareous grit may reduce Pb bioaccessibility of ingested Pb shot in birds by reducing gizzard acidity, by enhancing Pb precipitation (as Pb-carbonate), and by promoting higher dissolved Ca levels in the intestine, which may then compete with Pb for intestinal absorption.

Introduction

Birds commonly ingest and maintain grit in their muscular gizzards to help grind up food (1). However, ingestion can pose an ecotoxicological risk to birds if, for example, Pb shot from hunting is mistakenly consumed (2, 3), or if grit is naturally or anthropogenically enriched with heavy metals (4–6). Shot ingestion and associated Pb poisoning has been shown to be a significant cause of mortality for many avian species, in numerous scenarios globally (7, 8). Shot ingestion is currently estimated to cause mortality of almost one million waterfowl per wintering season in Europe alone, which represents 8.7% of the wintering populations of >11 million birds from 17 species (8). Waterfowl population trends in Europe are also inversely correlated with Pb shot ingestion prevalences for different species (8), suggesting that Pb poisoning is having effects on population levels at a global scale. In North America, before waterfowl hunting with Pb shot was prohibited, annual mortality from lead poisoning was estimated at ≈2–3% of the total waterfowl population (9, 10). Lead poisoning from ingestion of spent lead ammunition has also been implicated in the decline of several globally endangered species, such as white-headed duck (Oxyura leucocephala) (11) and Californian condor (Gymnogyps californianus) (12).

While conservationists continue to call for a worldwide ban on the use of Pb shot to protect birds, finding effective methods of mitigating the risks posed by shot already present in the environment is also a priority. Several studies (7, 13, 14) have suggested that where Pb shot pellets are abundant in certain habitats and grit availability is limited, dietary supplementation with clean grit may promote reduced shot ingestion in susceptible species. The amount of grit normally ingested by a bird is related to species (15) whereas grit type (or geochemistry) is limited by local environmental availability (1). In wild birds, two geochemically dissimilar grit types are commonly found in gizzards, i.e., quartz/quartzite based (siliceous grit) and limestone/calcite based (calcareous grit). While facilitating the physical breakdown of food, such grit also acts as an important source of major and trace elements (1). Likewise, the elements Ca or P and carbonaceous minerals affect both the solubility (16) and absorption of Pb within the digestive tract (17). One competitive metabolic interrelationship between Pb and Ca (for example) is already well-known (18, 19) whereby, with low dietary Ca, the parathyroid gland increases synthesis of intestinal Ca-binding proteins (to increase Ca absorption), but these also have a high affinity for Pb. Hence, birds exposed to Pb (via drinking water or Pb shot) and a low Ca diet develop more severe clinical signs of Pb poisoning, and deposit elevated amounts of Pb in soft tissues (20, 21). As well as competing with Ca, under certain Eh and pH conditions, Pb in solution will also readily form complexes or precipitates with (for example) carbonates and organic compounds (16).

Several in vitro methods have been described to investigate the dissolution of Pb shot in simulated avian digestive systems (22), to look at either the effect of grit on the breakdown of plant material (23) or the bioaccessibility of metals from contaminated soil (24, 25), and these can provide practical viable alternatives to in vivo methods. However, while current techniques provide valid information, many perhaps lack the degree of complexity that really exists in an avian digestive tract. Here, we develop and use a dynamic in vitro simulation and consider interactions among several factors that have not been considered together previously. The proposed novel system allows for variations in grit composition (and/or volume), the amount of food ingested (and/or type), and the number of Pb shot (or potentially, other toxicants). We apply the technique to investigate the dissolution of varying numbers of Pb shot in the presence of variable amounts of food and grit types of differing geochemistry. Further, while the system monitors Pb dissolution over time within a simulated gizzard environment, it also permits the transfer of portions of gizzard solution (at intervals) into differing simulated intestine conditions (where Pb may precipitate out). Since absorption of Pb in solution predominantly occurs in the intestine, and not the gizzard (26), we suggest that the physical break down of fluid from one set of conditions (the gizzard) to another (the intestine) is an important element in an effective simulation of this type.
**Experimental Section**

**Justification for Experimental Design.** The avian digestive tract is complex, dynamic, and markedly different from that of mammals. Many birds have powerful muscular gizzards where food is predigested, dissolved, degraded, and physically compressed/ground down [often with the aid of grit (23)]. The gizzard contains enzyme-rich digestive fluid with a basal pH of ≈2.6, containing gastroliths, pepsin, HCl, and sodium chloride (27). Secretion of this fluid is promoted by the presence of food (27), and it occurs continuously during digestion. In chickens, the rate of secretion has been calculated to be ≈15.4 mL h⁻¹ (27). As food is digested, digestive liquid containing the finest particulates is allowed to pass into the intestine, where a different intestinal fluid is secreted. These secretions are of a higher pH (5.2—7.2) and also contain bicarbonate ions, bile salts, and pancreatin (25, 27). Undigested food material is ultimately removed as an intestinal pellet (in feces). The duration of the whole digestion process (depending on the ingesta type/size) is between 6—7 h in mallards (*Anas platyrhynchos*; 29).

To simulate avian gizzard digestive juice (GizzDJ) we prepared a solution of 1 N NaCl (Prolabo) containing 10 g L⁻¹ of pepsin (Merck), adjusted to pH 2.0 with concentrated HCl (Panreac; after (22) and (24)). To simulate intestinal digestive juice (IntDJ) we prepared a concentrated solution containing bile extract (3.5%) and porcine pancreatin (0.35%; Sigma) which was diluted upon experimental addition to effective concentrations of 0.35% and 0.035% (after (25)), respectively (see below). We used two grit types, siliceous (Gritsil) with 99% SiO and 0.6% CaO and calcareous (Gritcal) from a floor tile factory (*Sanluquen˜ a de Pavimentos*, S.A.). Before undertaking experiments, grit was thoroughly washed with Milli-Q water. The grit size used was 1—2 mm as this is most commonly found in gizzards of waterfowl species that are more frequently affected by Pb poisoning (15, 30). For all experiments we used 2 g of grit, a typical amount previously reported in hunted mallards (30). For food, we used briefly milled wheat seed with a particle size ranging from fine flour to whole seed. We created one batch of this material which was subdivided into portions and then used for all experiments. Pb shot pellets used were size no. 6, with an average weight of 109 mg (±8 mg). All digestive solutions used in experiments were heated to 42 °C before use [average mallard body temperature (31)], and all chemicals used were analytical grade or better. High purity nitric acid (69% Analytical grade, Panreac) and hydrogen peroxide (30% v/v Suprapur, Merk) were used for the total digestions mentioned.

**Simulated Gizzard-Intestine.** Gizzard simulations were carried out in triplicate in 50 mL polycarbonate centrifuge tubes. Initially, 12 mL of GizzDJ was added to the tube, then 2 g of grit (Gritsil or Gritcal), Lead dissolution was investigated using 1 Pb shot and a variable amount of food material (0, 1, 2, or 4 g). We undertook a control experiment free of Pb with 2 g of grit and 4 g of food, and another experiment using 4 g of food but 3 Pb shot. A fourth replicate of all treatments was used solely to monitor pH change during each experiment; this replicate was undertaken first.

The 50 mL tubes containing each simulation [12 mL of GizzDJ, 2 g grit (2 types), 0—4 g food, and 0—3 Pb shot pellets] were incubated for a total of almost 3 h at 42 °C, and constantly shaken on their sides at high speed on a flat bed revolving shaker (at 350 rpm). Shaking was used to partially simulate the physical action of the muscular gizzard, i.e., permitting aggressive contact between grit, food, and Pb shot. To simulate the flow of gizzard solution (GizzDJ) containing suspended material into the intestine, and the associated excretion of fresh GizzDJ that would normally occur in an avian gizzard, after 6 min of shaking, tubes were opened, 3 mL of existing GizzDJ was removed, and 3 mL of fresh GizzDJ was added. Tubes were then closed and placed back on the heated shaker for another 6 min. In each experiment GizzDJ was added 10 times and the total run time (including the steps required to add or remove liquid, purge headspace, etc.) was approximately 180 min. Before starting each simulation, and after each addition of fresh GizzDJ, tube headspace was purged with N₂ to maintain a low oxygen environment within the tubes.

Each 3 mL of GizzDJ removed from each treatment were immediately divided into two 1.5 mL aliquots. The first was immediately centrifuged at 9500 g for 10 min, and 1 mL of supernatant was then digested in 0.5 mL 35% HNO₃ and 0.5 mL of 30% H₂O₂ for 1 h, at 90 °C, in 15 mL polypropylene centrifuge tubes in a water bath. This was diluted to 10 mL with Milli-Q water, and stored at 4 °C until analysis. The second 1.5 mL aliquot was immediately taken forward for further incubation under simulated intestine conditions.

The second 1.5 mL aliquot (in a 1.5 mL microtube) of GizzDJ was adjusted to pH 6.2 using a saturated solution of NaHCO₃ (Merck; after (25)). The amount required to reach this pH (20 µL for Gritsil, 0—10 µL for Gritcal) was predetermined from the control replicate used to monitor pH change during each experiment. After vortex mixing, 150 µL was discarded, and then 150 µL of IntDJ (10 times concentrate) was added. The tube headspace was then purged under a stream of N₂, and solutions were incubated for 3 h at 42 °C in an end over end shaker at slow speed (to simulate slow intestinal passage). Finally, intestinal solutions were centrifuged at 9500 g for 10 min, and 1 mL of supernatant was acid digested in a 15 mL polycarbonate centrifuge tube (as above). Remaining solid material (effectively the feces) was oven-dried at 40 °C for 48 h, and then digested in quartz tubes. A 1 mL portion of concentrated HNO₃ was added to the sample, tubes were left at room temperature overnight, then heated for 1 h at 90 °C. A further 1 mL of 30% H₂O₂ was added, and the tubes were heated for another 2 h at 90 °C. Finally, digests were diluted to 20 mL with Milli-Q water and stored at 4 °C until analysis. In order to attain enough solid material, all three replicate intestinal pellets were combined for total digestion.

**Quality Control/Assurance and Analytical Procedure.** Certified reference material (bush, branches and leaves, NCS DC 73349) was digested using the procedures noted above (for the intestinal pellet) to provide quality control data (Table S1, Supporting Information). Mean Pb and Ca recoveries were 101% (n = 6) and 102% (n = 6), respectively.

The triplicate gizzard and intestine liquid digests, and the combined digest for the intestine pellet, were analyzed using graphite furnace atomic absorption spectroscopy [GF-AAS; AAnalyst800 with autosampler AS800 (Perkin-Elmer)] for Pb, using 50 µg of NH₄H₂PO₄ and 3 µg of Mg(NO₃)₂ as matrix modifiers for each atomization. One of the triplicate gizzard and intestine liquid digests was also analyzed using flame atomic emission spectroscopy [AES; AAnalyst800 with AS90 plus autosampler (Perkin-Elmer)] for Ca.

**Statistical Analyses.** Lead levels in solution in the gizzard (Pb-Gizzsoln), the intestine (Pb-INTsoln) liquid, and the intestine solid (Pb-INTsolid) were log-transformed to attain a normal distribution and analyzed using generalized linear models (GLMs), to examine which factor variations caused significantly different concentration levels. The factors examined were grit type, number of Pb shot, amount of food (g), and time point. Posthoc Tukey tests were then used to examine differences found in both grit systems, due to the amount of food used, or time point. Differences between treatments in pH and Ca levels in the different compartments were tested using Mann—Whitney and Kruskal—Wallis tests.
since we observed a lack of homogeneity of variances when using GLMs. Correlations between Pb-GIZZsoln and Pb-INTsoln levels as well as between Pb-INTsoln and Pb-INTsolid levels were tested with Pearson correlations for each treatment. Differences between Pb-GIZZsoln and Pb-INTsoln were tested with a paired student t-test. The level of statistical significance used was $p < 0.05$, and all tests were performed using SPSS 15.0.

Results
Irrespective of treatment, (i.e., 1 or 3 shot, and 0, 1, 2, or 4 g of food), Pb-GIZZsoln levels were consistently higher in simulations with Grit\textsuperscript{sil} than Grit\textsuperscript{cal} (GLM $p < 0.001$; Figure 1, Table 1). For Pb-INTsoln levels, this was also the case with 2 or 4 g of food and 1 or 3 Pb shot, but higher Pb-INTsoln concentrations were recorded in Grit\textsuperscript{cal} simulations without food and, at the temporal end of the simulation, with 1 g of food and 1 Pb shot (Figure 1; Figure S1, Supporting Information). For Pb-INTsolid in simulations with Grit\textsuperscript{sil}, Pb levels were always higher than in those with Grit\textsuperscript{cal} (GLM $p < 0.001$, Figure 1).

For experiments using 4 g of food, where grit type, number of shot (1 or 3), and time were factors, all factors were significant for Pb-GIZZsoln, Pb-INTsoln, and Pb-INTsolid levels. As might be expected, Pb-GIZZsoln concentrations increased with the number of Pb shot used (GLM $p < 0.001$, Figure 1), as did Pb-INTsoln and Pb-INTsolid levels (GLM both $p < 0.001$). In solution, levels were 3.7 (GIZZ\textsuperscript{sil}), 3.3 (GIZZ\textsuperscript{cal}), 2.3 (INT\textsuperscript{sil}), and 2.1 (INT\textsuperscript{cal}) times higher in systems with 3 shot rather than 1. In INT\textsuperscript{solid}, levels were 4.6 and 4.7 times higher in Grit\textsuperscript{sil} and Grit\textsuperscript{cal} systems, respectively (Table 1). Without Pb shot, Pb concentrations in solution were below our limits of detection for all treatments.

In treatments with 1 Pb shot, the amount of food used in each simulation affected Pb-GIZZsoln and Pb-INTsoln levels (GLM both $p < 0.001$, Table 1). In gizzards with Grit\textsuperscript{sil}, the Pb-GIZZsoln concentrations increased as food content decreased (Tukey all at $p < 0.001$). With Grit\textsuperscript{cal}, Pb-GIZZsoln levels were also lowest with 4 g of food, but were highest (higher than with no food and 2 g) with 1 g of food ($p < 0.001$). In terms of Pb-INTsoln levels, with Grit\textsuperscript{sil}, concentrations were similar with 4 g and no food, and higher with 1 g and then 2 g of food (Tukey $p < 0.001$). Using Grit\textsuperscript{sil}, increased amounts of food (1–4 g) caused lower Pb-INTsoln levels (Tukey $p < 0.001$), and again, at the temporal end of the 1 g food simulation, levels were higher than when using no food (Figure 1). With time, Pb-GIZZsoln concentrations increased, especially in simulations where food was included (GLM $p < 0.001$). Pb-INTsoln levels varied with time in all treatments (GLM $p < 0.001$), with maximum concentrations during the middle and at the end of simulations with Grit\textsuperscript{sil} and Grit\textsuperscript{cal} respectively (Figure 1).

In all experiments, for both grit types, Pb-INT\textsuperscript{solid} concentrations were lower than Pb-GIZZ\textsuperscript{solid} levels (paired Student $t$ test $p < 0.001$; Figure 1, Table 1). Pb-INT\textsuperscript{solid} and Pb-GIZZ\textsuperscript{solid} levels were also correlated in all Grit\textsuperscript{sil} simulations (all at $r > 0.79$, $p < 0.001$), but only with 1 Pb shot + 4 g food when using Grit\textsuperscript{cal} (Table S2, Supporting Information). Pb-INT\textsuperscript{solid} and Pb-INT\textsuperscript{solid} levels were also correlated in Grit\textsuperscript{cal} simulations with no food and 1 g of food ($r > 0.738$, $p < 0.05$), and also with 4 g of food and 3 Pb shot ($r = 0.672$, $p = 0.05$). However, with Grit\textsuperscript{sil}, levels were negatively correlated when using 1 g of food ($r = -0.883$, $p = 0.001$).

GIZZ\textsuperscript{solid} pH was consistently lower in simulations with Grit\textsuperscript{cal} than with Grit\textsuperscript{sil} (Mann–Whitney $p < 0.001$). With Grit\textsuperscript{cal}, in the gizzard phase of experiments, the increased presence of food caused higher Pb-GIZZsoln levels, with the Pb-GIZZsoln levels being highest with 4 g of food. Pb-INTsoln levels were also highest in simulations with Grit\textsuperscript{cal} and 4 g of food, but were lowest (lower than with no food and 2 g) with 1 g of food (Tukey $p < 0.001$). Pb-INTsolid levels were also highest with 1 g of food and 4 g of food, but were lowest (lower than with 1 g and 2 g) with 4 g of food (Tukey $p < 0.001$). Pb-INTsolid levels were also highest with 4 g of food, but were lowest (lower than with no food and 2 g) with 1 g of food (Tukey $p < 0.001$). Pb-INTsolid levels were also highest with 4 g of food, but were lowest (lower than with no food and 2 g) with 1 g of food (Tukey $p < 0.001$). Pb-INTsolid levels were also highest with 4 g of food, but were lowest (lower than with no food and 2 g) with 1 g of food (Tukey $p < 0.001$). Pb-INTsolid levels were also highest with 4 g of food, but were lowest (lower than with no food and 2 g) with 1 g of food (Tukey $p < 0.001$). Pb-INTsolid levels were also highest with 4 g of food, but were lowest (lower than with no food and 2 g) with 1 g of food (Tukey $p < 0.001$).
of food tended to cause less acidic conditions, with the most acidic conditions (pH 1.9–2.0) observed without food (Kruskal–Wallis p < 0.001, Table 1). In contrast, with Grit\textsuperscript{cal}, food caused a reduction in pH (to around 5.2–5.5) in comparison to systems without food, where the pH was 6.1–6.4.

Ca-GIZZ\textsuperscript{soln}, Ca-INT\textsuperscript{soln}, and Ca-INT\textsuperscript{solid} levels were all higher in simulations using Grit\textsuperscript{cal} than with Grit\textsuperscript{cal} (Mann–Whitney all at p < 0.001; Table 1, Figure S2, Supporting Information). Ca-GIZZ\textsuperscript{soln} and Ca-INT\textsuperscript{soln} levels decreased with increasing food in Grit\textsuperscript{cal} simulations (Kruskal–Wallis both p < 0.001), but increased in Grit\textsuperscript{cal} systems (Kruskal–Wallis both p < 0.001).

### Discussion

**Grit Geochemistry and Associated pH Modification.** Results show that Pb shot dissolution and concentrations within the digestive tract (gizzard and intestine) are affected significantly by coingested grit geochemistry. With Grit\textsuperscript{cal} in the gizzard, Pb-GIZZ\textsuperscript{soln} concentrations were consistently higher than when Grit\textsuperscript{cal} was present. This difference is probably due to geochemical interactions and/or the linked effect of grit geochemistry on prevailing gizzard liquid pH, since systems with Grit\textsuperscript{cal} maintained a more acidic environment than did those with Grit\textsuperscript{cal}. In both cases pH values were within the active range for duck pepsin (28). In previous in vitro digestion models, prevailing pH has also been described as one of the most important limiting factors for Pb solubility (25, 32, 33). Gastric fluid is the most acidic digestive liquid in an avian digestive tract, and tends to solubilize the greatest fraction of various metals (25, 32, 34). Ultimately, our results are intrinsically linked to Pb solubility under differing Eh/pH conditions (35). At ambient temperature/pressure, in a Pb\textsuperscript{2+} + H\textsubscript{2}O + CO\textsubscript{2} system (with 1 × 10\textsuperscript{-6} mol L\textsuperscript{-1} Pb\textsuperscript{2+}), under the Eh conditions likely in a digestive tract (mildly reducing), Pb\textsuperscript{2+} tends to remain in solution up to pH 5.9. Above this, and up to pH 7.7, PbCO\textsubscript{3} will tend to precipitate. Pb-INT\textsuperscript{soln} levels were also lower than Pb-GIZZ\textsuperscript{soln} levels in all treatments, and this indicates that Pb taken into solution in the gizzard subsequently precipitated out (with, for example, carbonates) within the intestine phase. In Grit\textsuperscript{cal} systems, 66–92% of Pb in solution at the gizzard stage did not remain so during the intestine phase, while in Grit\textsuperscript{soln} systems 30–63% reductions were recorded. Hence, while higher levels of Pb in solution were maintained in the gizzards with Grit\textsuperscript{cal} than with Grit\textsuperscript{cal}, the decreases within the intestine were also proportionally greater. Mean levels in solution within the intestine were not actually very dissimilar for the two grit types; Pb-INT\textsuperscript{solid} levels were 0.6–2.5 times higher in Grit\textsuperscript{cal} systems, while Pb-GIZZ\textsuperscript{solid} levels were 1.9–5.4 times higher in Grit\textsuperscript{cal} systems (Table 1). Lower Pb concentrations in intestine phases, than in gizzard phases, have also been reported in previous in vitro digestion experiments.

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**TABLE 1. Mean (±SE) Concentrations in Solution of Pb (in µg L\textsuperscript{-1}) and Ca (mg L\textsuperscript{-1}) in Gizzard and Intestine Liquids and in the Intestine pellet (mg kg\textsuperscript{-1}) with Ranges in Parentheses and pH Range Recorded During Each Experiment**

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<th>1 g food</th>
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<td>Pb</td>
<td>10\textsuperscript{a}</td>
<td>6733 ± 273\textsuperscript{A1}</td>
<td>5660 ± 74\textsuperscript{B1}</td>
<td>4179 ± 257\textsuperscript{C1}</td>
<td>2099 ± 170\textsuperscript{D1}</td>
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<tr>
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<td>1631 ± 93\textsuperscript{A2}</td>
<td>3017 ± 203\textsuperscript{B1}</td>
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</table>

\textsuperscript{a}Mean of results for 30 digestions; 3 replicates were analyzed for each of 10 time points. Superscript capital letters illustrate whether means within the same row differ significantly or not (Tukey test p < 0.05), i.e., whether amount of food influences Pb in solution when 1 Pb shot was present.
In Gritsil systems, we noted consistent correlations between Pb-INTsoln and Pb-GIZZsoln levels, which might suggest that Pb dissolution here is controlled predominantly within the gizzard phase, with simple proportional precipitation occurring in the intestine. This may indicate that, in Gritsil systems, grit solubility, the subsequent generation of free carbonate, and associated pH increases were exerting key (somewhat linear) controls over Pb levels in solution (38, 39). In the Gritsil systems, we noted fewer correlations between Pb-INTsoln and Pb-GIZZsoln levels, indicating that Pb dissolution and subsequent precipitation/adsorption in this system is more complex (40). In Gritsil systems, high Pb-GIZZsoln levels were recorded, and a high proportion of this precipitated or became absorbed from solution in the intestine. However, simple proportional reductions were often not occurring. Hence, for Gritsil systems at least, it may be difficult to extrapolate in vitro data based solely on gizzard simulations, to toxicologically relevant intestine levels. It seems important in this case to couple and include the intestine phase since precipitation/adsorption reactions occur primarily within the intestine, as does Pb absorption into the bloodstream (26). Simple gizzard based models that do not consider the passage of fluid to very differing intestine conditions (or include grit, if appropriate) may ultimately seriously overestimate potential toxicological impacts. While considering worst case scenarios may be useful, more realistic simulations should also be designed.

Effects Caused by Differing Amounts of Food. As noted previously (27, 33, 34, 41), pH and Pb dissolution were not only affected by grit geochemistry, but also by the presence of food in the gizzard in all treatments. In Gritsil systems, increased amounts of food caused pH-GIZZsoln values to rise, and Pb-GIZZsoln levels to fall. Again, more alkaline conditions and increased levels of food derived carbonate, phosphate, and organic ligands are likely to have promoted these reductions (34, 41). Elsewhere, such decreases have been linked to Pb precipitation with organic ligands such as phytic acid salts (38). In Gritsil systems, food had the opposite effect, whereby its presence within the system caused decreased pH, i.e., 5.2–5.5 in systems with food, but 6.1–6.4 in those without. Thus, with 1 g of food, mean Pb-GIZZsoln levels were actually higher than if no food had been added. This may be due to several factors. First (and most importantly), with food, the pH was more acidic and below 5.9 (the level below which Pb²⁺ tends to remain in solution), yet without food, it was above this level and dissolution rates are likely to have been lower, while precipitation with grit derived carbonate was more likely to occur. Second, food itself may have enhanced physical abrasion and predissolution oxidation of the Pb shot surface, and/or acted to bind hydroxide (OH⁻) from solution (resulting in a lowered pH). Both processes would act to allow higher Pb-GIZZsoln levels in systems with 1 g of food, versus those without food. Ultimately, simulations with increasing amounts of food (from 1–4 g) did however have progressively lower Pb levels in solution (as observed in the Gritsil system). In addition to the mechanisms already noted above, in this case higher Ca levels may also have enhanced the sorption of Pb to organic ligands such as phytic acid (42).

Including food in simulations such as this is complicated. However, we believe that, in doing so, more realistic results are generated. Many previous studies regarding in vitro gastrointestinal simulations use “fasted” (without food) conditions, since there is bound to be a degree of variation in determined Pb bioaccessibility, depending on the type and amount of food added (25, 32). However, diets high in calcium and protein, and low in fiber, can reduce Pb accumulation in tissues and result in toxicity (7, 20, 28, 43). While fasted system models may be simpler, and provide valid data, in reality birds such as waterfowl can survive for long periods with ingested Pb shot in their gizzards (7, and it is critical that interactions with food (and ingested grit) are considered, if realistic toxicological simulations are to be developed.

Ingestion/Grit Supplementation. Ingestion of grit (and/or phase or matrix) may increase voidance of shot from the gizzard, but it might also increase Pb shot erosion, and therefore exposure/mortality (7). Further, if grit supplements are given using, for example, a “corn grain + grit” mix, ducks tend to consume twice as much grit than those given “commercial duck pellets + grit”, and again, this may result (in the field) in a greater probability of Pb shot ingestion (2, 3). The only field scale experimental work we are aware of which has studied grit supplementation to reduce the probability of Pb shot ingestion in waterfowl was completed in the Camargue.
This and many other previous studies (physical/mechanical action is difficult to simulate in the literature cited, XRF methods and results, and supporting information available). M.M.-H. was supported by a project funded by the Consejería de Medio Ambiente, Junta de Andalucía, under a CSIC contract. This study was also funded by MICINN (under CGL2007-62797). We thank Eva García for the XRF analytical data shown.

Supporting Information Available
Raw data and graphical representations for Pb, Ca, and pH associated statistical results, XRF methods and results, and quality control/assurance data. This material is available free of charge via the Internet at http://pubs.acs.org.

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