Lead in terrestrial game birds from Spain

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ABSTRACT

We analysed exposure to Pb and its relationship with lead-based ammunition in seven species of terrestrial game birds – Common Woodpigeon (*Columba palumbus*), Rock Dove (*Columba livia*), Stock Dove (*Columba oenas*), European Turtle-dove (*Streptopelia turtur*), Red-Legged Partridge (*Alectoris rufa*), Barbary Partridge (*Alectoris barbara*) and Common Quail (*Coturnix coturnix*) – from rural and urban areas in different parts of Spain (Valencia, Castilla-La Mancha, Castilla y León, Madrid, Islas Canarias and Navarra). A total of 530 liver samples were analysed and the presence of Pb pellets was studied in the crop, gizzard and intestine; the state and appearance of these organs was also analyzed. The number of specimens suspected to have ingested Pb shot was 28 (5.6%) and the geometric mean concentration of hepatic Pb was 0.054 μg g^{-1} (wet weight, ww). A low percentage of samples (4.8%) were above the abnormal exposure threshold (0.65 μg g^{-1} ww) and in these specimens renal Pb concentrations were determined. Common Woodpigeons and Rock Doves from Madrid were found to have high concentrations of Pb in their livers, and so both species can be considered to be good bioindicators of Pb contamination in rural (Common Woodpigeons) and urban (Rock Doves) environments. Partridges bred for hunting may be more prone to ingesting pellets from the environment, a fact that should be taken into account in management decisions.

KEY WORDS: ammunition, game birds, lead, liver, Spain, terrestrial habitats

1. INTRODUCTION

Lead (Pb) is regarded as a priority pollutant by the Agency for Toxic Substances and Disease Registry (ATSDR 2014) and the US Environmental Protection Agency (US EPA 2014). This heavy metal is common in contaminated ecosystems (Steinnes 2013) and has been extensively used by humans for centuries (Franson and Pain 2011) in a non-renewable way (Harrison 2012). In ecosystems, Pb is ubiquitous at all levels and is incorporated into the food chain through the atmosphere, soil, water, plants and animal tissues. However, the main source of Pb contamination is anthropogenic (Shotyk and Le Roux 2005) and its use as an anti-knocking agent in gasoline has contributed to its accumulation in the environment (US EPA 2003); nevertheless, currently, the major source of Pb in many areas of the world is secondary Pb produced by recycling processes (Ellis and Mirza 2010; Zhan et al. 2016), generally from discarded Pb acid batteries.

In recent decades, numerous studies have been conducted on the presence of Pb in the environment, many of which have looked at its effects on human and animal health (see review in Assi et al. 2016; and Tokar et al. 2013) and on wild birds (see review in Williams et al. 2017), as well as its presence in food (see review in EFSA 2010) and as a hazard to human health caused by Pb shot in game meat (Pain et al. 2010; Mateo et al. 2011). However, interest in wild birds' exposure to Pb due to the ingestion of pellets has increased in recent years and today is thought to be the most significant and widespread non-regulated source of Pb affecting wild animals (Pain et al. 2015), as well as one of the most frequent causes of clinical lead poisoning and death in birds (De Francisco et al. 2003; Fisher et al. 2006; Johnson et al. 2013; Pain et al. 2015).

Pb pellets are dispersed over the ground in hunting areas and may accumulate in certain places such as wetland sediments, where densities can be extremely high (up to 399 pellets/m^2, Mateo et al. 2007). Nevertheless, very few studies have ever been conducted on Pb pellet densities in terrestrial habitats and the few that have suggest densities range from 0.46

pellets/m^2 (Hungary, Ákoshegyi 1997) to 7.4 pellets /m^2 (Spain, Ferrandis et al. 2008). These pellets may be ingested by granivorous birds (aquatic and terrestrial) as they are mistaken for grit particles, since these birds need hard structures to grind down their food (Mateo et al. 2007; Pain et al. 2009). Birds may eliminate Pb pellets from their digestive tracts rapidly with little absorption, or they may be retained until they are eroded away, solubilised and absorbed, or may be processed in any intermediate stage (Pain et al. 2019). Rates of Pb digestive absorption and particle transit times depend on the species (Holladay et al. 2012). The digestive retention time has been reported to be between two weeks and one month, approximately, in Japanese Quail (*Coturnix japonica*), Northern Bobwhite Quail (*Colinus virginianus*) and Domestic Pigeon (Yamamoto et al. 1993; Kerr et al. 2010; Holladay et al. 2012). The Pb passes into the bloodstream and into soft tissues such as the liver and kidney, although a part may be excreted. Finally, it reaches the bones, where it is deposited and remains for many years (Patrick 2006; Pokras and Kneeland 2009).

In live animals (blood) and corpses (tissues), concentrations of Pb serve as an indicator of exposure to this heavy metal. According to Franson and Pain (2011), the basal hepatic concentration of Pb in birds (Anseriformes, Falconiformes and Columbiformes) is less than 2 mg kg^{-1} (ww), whereas clinical exposure is considered to have taken place at above 6 mg kg^{-1} (ww). Clinical signs of Pb poisoning in birds have been described by several authors (e.g. De Francisco et al. 2003; Patte and Pain 2003; Franson and Pain 2011), and include abnormal behaviour, anaemia, emaciation, green diarrhoea, lethargy, leg paralysis, wing droop, paralysis of the digestive tract, and convulsions. Sick birds become easy targets for predators (De Francisco et al. 2003), although some birds die rapidly without any apparent signs of having been poisoned (Pain et al. 2019). The poisoning affects a wide range of physiological and biochemical systems including the immune, haematopoietic, nervous, renal, reproductive and vascular systems (Pain et al. 2019), with severe consequences at individual and population level.

The prevalence of Pb pellet ingestion and concentrations Pb in tissues in water birds has been the subject of numerous studies in Europe (see review in Descalzo and Mateo 2018). Indeed, the use of lead-based pellets in wetlands to hunt water birds is today restricted in several European countries. The European Chemicals Agency (ECHA) recommends the adoption of measures designed to regulate the used of lead-based ammunition in terrestrial environments (ECHA 2018). Nonetheless, only a few studies on terrestrial granivorous birds have ever been conducted in Europe, some of which are out-of-date: Denmark (Clausen and Wolstrup 1979), Spain (Soler-Rodríguez et al. 2004; Ferrandis et al. 2008), the United Kingdom (Calvert 1876; Holland 1882; Keymer 1958; Anger 1971; Keymer and Stebbings 1987; Butler et al. 2005; Potts 2005) and Hungary (Ákoshegyi 1997). The presence of birds with pellets or high Pb concentrations in their tissues constitutes a danger for any hunters who consume this kind of game (Johansen et al. 2006; Tranel and Kimmel 2009; Johnson et al. 2013). Pb concentrations can also affect predators such as birds of prey, which can be poisoned via this type of exposure (Mateo et al. 2014) leading to clinical signs such as coordination problems, no appetite, lethargy and general weakness (Carpenter et al. 2003; Patee et al. 2006).

Data describing Pb exposure in game birds in Spain is limited. The aim of this study was to evaluate this exposure in several species of game birds from different areas of Spain using analyses of liver Pb concentrations and the presence of shot in digestive tracts. This information will be useful for monitoring the presence and the availability of this heavy metal in hunting areas and as an aid when taking decisions regarding the use of Pb shot in terrestrial ecosystems.

2. MATERIALS AND METHODS

2.1. Sample collection

A total of 530 birds belonging to seven game-bird species were studied: Common Woodpigeon (n=107, *Columba palumbus*), Rock Dove (n=99, *Columba livia*), Stock Dove (n=30, *Columba oenas*), European Turtle-dove (n=31, *Streptopelia turtur*), Red-legged Partridge (n=219, *Alectoris rufa*), Barbary Partridge (n=13, *Alectoris barbara*) and Common Quail (n=31, *Coturnix coturnix*). The samples were collected in 2016, 2017 and 2018 from six Spanish regions (Fig. 1) and were obtained in two ways: by hunting or by capture as part of bird control programs at airports and seaports. Data regarding sampling areas, bird characteristics and causes of death are given in Table 1. The ammunition used in firearms and compressed air guns was Pb, while captures were made using falcons and net traps. A control group with no contact with Pb pellets was set up consisting of birds (*A. rufa*) bred on a farm in Navarra. For classification of the hunting intensity – and in the absence of any official or scientific classification – we took into account the captures declared by the owners or users of the management units where hunting activities are carried out (game reserves), which we transformed into gunshots per hectare and season: very high (>30), high (10–30), moderate (5–10), low (1–5), very low (<1), and zero-intensity hunting. Rural, urban and periurban areas were described in terms of land uses: agricultural and forestry areas were catalogued as 'rural' and unproductive areas as 'urban'. The 'periurban' variable was only applied to a single area with agriculture and forestry in the vicinity of the built-up area around Madrid (20 km). The specimens hunted in Ciudad Real came from three game reserves that have been repopulated with birds from Navarra. The migratory character of species was determined according to species (*S. turtur* and *C. coturnix*) and date and place of hunting (*C. palumbus*).

Once birds had been hunted or euthanized, they were weighed, and their age determined by studying their feathers (primary remiges) or by other means including the presence of double spurs. Specimens were subsequently dissected to determine their sex, extract their digestive tract and take liver samples, which were transferred to microtubes and stored at -20°C until analysis. Kidney samples were also taken for analysis from specimens with high hepatic Pb concentrations; in these cases we took 0.65 µg g^{-1} to be the more restrictive environmental exposure threshold of Pb, according the scientific literature (Guitart et al. 1994; Ferrandis et al. 2008; Franson and Pain 2011; Berny et al. 2015; Bingham et al. 2015).

The extraction of crops was carried out with great care and they were examined for holes and pellets (Supplementary material, Fig. S1). X-rays were taken of the rest of the digestive tract to check for Pb pellets (from the junction of the proventriculus and the gizzard up to the cloaca). The gizzard and intestines were inspected for shot entry orifices and then opened up to locate any pellets detected by the X-ray and to evaluate their state (Supplementary material, Figs. S2–S5). In specimens with pellets in their digestive tracts but no entry holes, shot was considered as ingested. Finally, the colour and appearance of the gizzard mucous membrane from specimens with high hepatic Pb concentrations was studied.

2.2. Metal analysis

Tissue samples were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES, ICAP 6500 Duo, Thermo Scientific, with One Fast System) to determine Pb content. Liver and kidney samples were treated with trace mineral grade nitric acid (69% Suprapure, Merck) and 33% H2O2 (Suprapure, Merck) in special Teflon reaction tubes, which were heated in a microwave digestion system (UltraClave-Microwave Milestone®) for 20 min at 220°C and finally diluted to 10 ml with double deionised water (MilliQ). The detection limit was 0.001 µg g^{-1}. Two replicates were analyzed for every sample; the concentration values used were the mean of the two readings. To check for possible contamination, one blank sample for every eleven samples was also analysed in the ICP-OES. Multi-element calibration standards (SCP Science, in 4% nitric acid) were prepared with specific concentrations of Pb, taking as a reference UNE-EN ISO 11885 for the determination of elements by ICP atomic emission spectroscopy. Furthermore, intermediate patterns of all elements were prepared. The calibration device was established per batch, with a minimum of three points for every single lot. Each run started out with the calibration standards, continued with samples and intermediate patterns, and finished with the series with intermediate patterns (10% variation coefficient). The wavelength was 220.353 nm. The recovery rate for reference materials (Standard Reference Material L577b, Bovine Liver) was 98.47%. Pb concentrations were expressed in micrograms per gram in wet weight (µg g^{-1} ww).

2.3. Data analysis

The data given for the metal concentrations are geometric mean, standard error, and minimum and maximum concentrations. Data below the detection limit were expressed as half of this (0.0005) in order to be able to perform the statistical analysis. To check for data normality, the Shapiro-Wilk and Kolmogorov-Smirnov tests were used (according to the number of samples). A log transformation of the data was carried out on the concentration variable, and parametric mean comparison tests were conducted (ANOVA and Student's t-test; Tukey and Games Howell *post-hoc* test; Levene's test for equality of variances). We compared Pb concentrations in liver by age group and gender, by location for species (*A. rufa*, *C. palumbus* and *C. livia*) and by species for location (Zamora, Madrid and Alicante), and by hunting intensity. A Chi-square test was performed to evaluate possible associations between Pb shot ingestion and liver Pb concentrations above 0.65 µg g^{-1} (ww). The significance level for all tests was set at 0.05. All statistical analyses were performed with SPSS v.19.0 for Windows.

3. RESULTS

Specimens' weights and frequency by sex and age are given in the Supplementary material (Table S1). In all, 53.0% of birds were male and 44.5% females (the sex of the remaining 2.5% could not be determined); 49.2% were adults and 45.3% juveniles (the age of the remaining 5.5% could not be determined).

Table 2 shows the concentrations of Pb detected in livers, and specimens with Pb shot due possibly to ingestion. The overall population's mean hepatic Pb concentration was 0.055±0.102 $\mu g\ g^{-1}$ (0.054±0.107 $\mu g\ g^{-1}$ if we exclude the 26 farm specimens from Navarra). The percentage of samples with Pb concentrations above the detection limit for the entire study population was 94.3%; the number of specimens with liver Pb concentrations above 0.65 $\mu g\ g^{-1}$ was 24. There were no statistically significant differences between groups in terms of either gender or age.

Hepatic Pb concentration in birds with shot in gizzard due to possible ingestion (n=14) was 0.133±0.815 $\mu g\ g^{-1}$ (nd-10.379 $\mu g\ g^{-1}$) while in the remaining birds, the geometric mean was 0.053±0.102 $\mu g\ g^{-1}$ (nd-35.567 $\mu g\ g^{-1}$). In birds with Pb shot in crop (n=6), the hepatic Pb concentration was 0.192±5.190 $\mu g\ g^{-1}$ (0.005-31.253 $\mu g\ g^{-1}$), while it was 0.054±0.084 $\mu g\ g^{-1}$ (nd-35.567 $\mu g\ g^{-1}$) in birds without Pb shot in this organ. Finally, regarding birds with Pb shot in intestine (n=10), the hepatic Pb concentration was 0.134±0.774 $\mu g\ g^{-1}$ (0.005-7.885 $\mu g\ g^{-1}$), and 0.054±0.103 $\mu g\ g^{-1}$ (nd-35.567 $\mu g\ g^{-1}$) in birds without Pb shot. Two specimens presented pellets located in two tissues: intestine and gizzard, and in intestine and crop. Differences between groups with and without Pb shot were no significant.

Hepatic Pb concentrations in *A. rufa*, *C. palumbus* and *C. livia* by location are shown in Table 3. In the case of Red-legged Partridges, the mean hepatic concentration of Pb from all locations was below 0.100 $\mu g\ g^{-1}$. In this species, the highest mean concentration was detected in birds from Alicante, followed by birds from Ciudad Real and Navarra (farm partridges); there were no statistical differences between these birds, but there were differences from birds from Valencia and Zamora. The analysis of the results for Common Woodpigeons reveals a higher hepatic mean concentration of Pb ($p<0.05$) in pigeons from Madrid than in birds from Zamora and Alicante; there were no differences ($p>0.05$) between specimens from Zamora and Alicante. In the case of Rock Doves, the highest mean concentrations were found in doves from Madrid, followed by Valencia; there were no statistical differences between these birds, but there were significant differences with the Rock Doves from Zamora and Alicante.

Pb concentrations in liver by location are shown in Table 4. In Zamora, there were differences ($p<0.05$) between *C. coturnix* and *C. palumbus* and the remaining species, but no differences ($p>0.05$) between pigeon species from Madrid. In Alicante there were only statistically significant differences between the Rock Dove and the Red-legged Partridge, and between the Rock Dove and the Common Woodpigeon.

In terms of environments, there were statistically significant differences in hepatic Pb concentrations between specimens from areas with different hunting intensity (Table 5). By species, there were no significant differences between specimens from areas of low and very high hunting intensity in the case of Common Woodpigeons (0.047 vs 0.055 µg g^{-1}); birds from areas with high hunting intensity (Madrid, 0.180 µg g^{-1}) had greater Pb concentration ($p<0.05$) than specimens from low and very high hunting intensity. In Rock Doves, there were no significant differences between specimens from areas of low and very high hunting intensity (0.022 vs 0.020 µg g^{-1}); there were significant differences between specimens from areas with high-intensity hunting (0.187 µg g^{-1}) and specimens from areas with very high (0.020 µg g^{-1}) and low hunting intensity (0.022 µg g^{-1}). In the case of Red-legged Partridges, there were statistically significant differences between the specimens hunted in low hunting intensity areas (0.024 µg g^{-1}) as opposed to high and very high hunting intensity areas (0.065 and 0.070 µg g^{-1}, respectively). There were no statistical differences in liver Pb concentration between both pigeon species from Madrid, but there were differences ($p<0.05$) between each pigeon species from Madrid and partridges from Ciudad Real.

The number of pellets found in birds' crops was 22 from a total of 20 specimens; we found shot entry orifices on the crop's surface in 16 specimens. The number of pellets found in the rest of the digestive tract were 78 inside the gizzard (55 specimens) and 18 inside the intestine

(13 specimens). We found 21 pellets (19 specimens) embedded in the gizzard muscle layers and serous membrane. Of the specimens with pellets in the gizzard and/or intestinal lumen, no apparent shot entry orifices were found in 24. The total number of specimens containing pellets inside one or more of these three organs but without an apparent shot entry-orifice totalled 28 (5.3% of the total population, 5.6% if we exclude the farm specimens, Table 2).

There was a weak association (V of Cramer= 0.190; $p<0.05$) between the possible ingestion of Pb shot and liver Pb concentrations above 0.65 μg g^{-1}. By hunting intensity area, we only found this statistical association in birds from high hunting intensity areas (V of Cramer= 0.269); by location, this statistical association only existed for Ciudad Real (V of Cramer= 0.359).

4. DISCUSSION

4.1. Pb in livers of the studied bird groups

In terrestrial habitats, most of the studies conducted to date have analyzed the presence of Pb ammunition in bird corpses and its role as the cause of death, and its impact on birds of prey that feed on them (Fisher et al. 2006; Pain et al. 2009; Berny et al. 2015; Carneiro et al. 2016; Williams et al. 2017). On the other hand, few studies have ever evaluated tissue concentrations of Pb in apparently healthy game bird populations (DeMent et al. 1987; Soler-Rodríguez et al. 2004; Ferrandis et al. 2008; Kreager et al. 2008). In our study, the hepatic mean value found for the entire population (n=530) was 0.055 μg g^{-1} (0.054 μg g^{-1} if we exclude the 26 farm specimens); only the groups from Madrid and two groups from Zamora (the Rock Dove from Santa Cruz de Tenerife with a value of 0.161 μg g^{-1} was not considered, since there was only a single specimen) exceeded the value of 0.100 μg g^{-1} (Table 2). For livers of *A. rufa* from Spain, Ferrandis et al. (2008) reported means of 2.17 and 0.58 μg g^{-1} (dry weight) of Pb, higher values than those found in our study (according Franson and Pain 2011, 1 μg g^{-1} wet weight is equal to approximately 3.1 μg g^{-1} of the liver's dry weight). The data reported by

Soler-Rodríguez et al. (2004) for the same species gives a geometric mean of 0.073 µg g^{-1} ww, a value slightly higher than that obtained in our study. If we consider the above-mentioned criteria regarding the more limited environmental exposure threshold of Pb (0.65 µg g^{-1} ww), 95.5% of the studied population (95.2% if the farm partridges are excluded) had a hepatic Pb concentrations below that value. According Franson and Pain (2011), basal Pb concentrations in the liver are less than 2 µg g^{-1} (ww); in our study 96.8% of specimens (96.6% if the farm partridges are excluded) had Pb liver concentrations below that level. Thus, most of the populations did not have abnormal Pb concentrations.

When the total population of birds was analyzed, we found that there were no statistically significant differences between age groups and sexes, which coincides with the findings reported by Cui et al. (2013) in homing pigeons.

4.2. Analysis by species and locations

In Red-legged Partridges we found no differences between specimens originating from locations with very different characteristics (Table 3): an area dedicated to breeding birds in captivity (Navarra) and rural areas of high or very high hunting intensity (Ciudad Real and Alicante). However, Pb concentrations in partridges from these locations were higher ($p<0.05$) than those found in Zamora. According the Geochemical Atlas of Spain (2019), Pb concentrations in soil from the sampling areas in Zamora are 20.7–32.0 µg g^{-1}, as opposed to 69.3–69.7 µg g^{-1} from Ciudad Real and 39.1 µg g^{-1} from Alicante. Also, the hunting intensity in Zamora was the lowest, which could explain these results. However, Pb concentrations in soil from Navarra were very low (12.9–17.9 µg g^{-1}), which not explain the liver Pb concentrations that we found for this area. On the other hand, the mean Pb concentrations were very low in the five locations (<0.070 µg g^{-1}) and, as we have indicated above, lower than those reported in partridges from other areas of Spain (Soler-Rodríguez et al. 2004; Ferrandis et al. 2008).

The statistical differences observed between Alicante and Zamora for partridges were not replicated in either species of pigeons (Table 3). Several authors have reported that Rock Doves are good indicators of environmental contamination (Antonio García et al. 1988; Nam and Lee 2006; Cui et al. 2013; Cai and Calisi 2016), and they have been used in several countries to determine exposure levels to heavy metals in urban environments. However, and according our results (with no statistical differences between pigeons from Zamora and Alicante), these species do not seem to be the best indicators of exposure levels to this heavy metal in rural environments. Liver Pb concentrations in both Common Woodpigeon and Rock Doves from Madrid were higher ($p<0.05$) than those found in rural environments (Zamora and Alicante), with no differences between them (Table 4). As well, in Valencia Pb liver concentrations in *C. livia* were higher ($p<0.05$) than those we found in *A. rufa* (Table 4). According to Schilderman et al. (1997), hepatic concentrations of Pb in pigeons in control zones in the Netherlands were 0.13–0.16 μg g^{-1} (ww). Other authors give higher concentrations for pigeons in control zones, reaching 2.01 μg g^{-1} dry weight (Hutton and Goodman 1980, equivalent to 0.65 μg g^{-1} in wet weight, applying the conversion factor of 3.1 suggested by Franson and Pain 2011), and 1.57 μg g^{-1} ww (Nam and Lee 2006). Although the Pb concentrations in the livers of Common Woodpigeons and Rock Doves in our study were low, we can at least affirm that there is a relationship between a periurban environment and higher concentrations of Pb in pigeon species. This finding is supported by the fact that no differences were observed ($p>0.05$) in liver Pb concentrations in Rock Doves from Madrid and Valencia (seaport) (Table 3), the latter an unproductive area with high Pb concentrations in the soil (104 μg g^{-1}, Geochemical Atlas of Spain 2019).

Common Quails had the highest Pb liver concentrations (above 0.100 μg g^{-1}, Table 4) in Zamora. Although this is a migratory species, Common Quails live in hunting areas from spring onwards and several authors have reported that this species can be either migrants, partial migrants or sedentary (Fontoura et al. 2000; Mur 2009). According to Nadal et al. (2019), several parts of the Iberian Peninsula are home to sedentary Common Quail populations. As well, 61.2% of the hunted specimens in our study were juveniles (see Supplementary material, Table S1) born in the study area, so this species could be a bioindicator for monitoring in this area.

In Alicante (where hunting was most intense), four species (Common Woodpigeon, Rock Dove, European Turtle-dove and Red-legged Partridge) were hunted but there were only statistically significant differences between the Rock Dove and the Red-legged Partridge, and between the Rock Dove and the Common Woodpigeon. Differences between these two species of pigeon were found in both Zamora and Alicante. As mentioned above, the Rock Dove is an ideal bioindicator species in urban environments (Antonio García et al. 1988; Nam and Lee 2006; Cui et al. 2013; Cai and Calisi 2016) and we suggest that the Common Woodpigeon could be a better bioindicator species in rural environments than the Rock Dove.

4.3. Differences associated with hunting intensity and shot ingestion

Pigeons from high hunting intensity areas (Madrid) were hunted in places with Pb concentrations in soil of 60.6 μg g^{-1}, similar concentrations to those reported from Ciudad Real (69.3–69.7 μg g^{-1}) (Geochemical Atlas of Spain 2019), which suggests that differences between both locations (Table 5) were derived from the other sources. Pigeons samples were taken in an area 20 km from Madrid, one of the largest metropolitan areas in Europe. Heavy metals such as Pb are released in different particle sizes in the urban atmosphere remaining in urban soils for a long time and act as sources of further pollution (Argyropoulos et al. 2012; Peña-Fernández et al. 2015), which could have affected these results.

For Red-legged Partridges, we have found differences between areas with low (0.024 μg g^{-1}) and high and very high hunting intensity (0.065 and 0.070 μg g^{-1}, with no differences between them). Additionally, we also found a statistical association between liver Pb concentrations above 0.65 μg g^{-1} and possible shot Pb ingestion, even though this association was derived from partridges from Ciudad Real (high hunting intensity). Wild birds ingest Pb pellets resembling weed seeds and grain or grit particles to grind down food (Conti 1993; Mirarchi and Baskett 1994; Kendall et al. 1996; Mateo et al. 2007; Pain et al. 2009). According to several authors, hunters leave high quantities of Pb shot in relatively small areas (Castrale 1989; Best et al. 1992; Kendall et al. 1996; Schulz et al. 2002), which accumulate during the hunting season. Thus, specimens from areas with different levels of hunting intensity should show differences in liver Pb concentrations. However, in the seven specimens (four pigeons and three partridges) and eight (seven pigeons and one partridge) with possible Pb shot ingested from areas with very high and low hunting intensity, there were no statistical associations with high liver Pb concentrations and so it would seem that the species was important in determining how much shot was ingested.

4.4. Specimens with Pb shot in their digestive tracts and hepatic Pb concentrations above the threshold for environmental exposure

We found hepatic Pb concentrations over 0.65 μg g^{-1} (the lowest value found in the bibliography as a threshold for environmental exposure) in 24 specimens (4.5% of the total population under study, 4.8% without taking the farm partridge population into account) (Supplementary material, Table S2). Of these specimens, only eight had Pb pellets in their crops, gizzards or intestines, and only six birds had no apparent shot entry orifices (1.2% of the population, without taking farm specimens into account). If we assume that an intake of pellets as grit only occurred in these cases, our findings are similar to or lower than those reported by other authors (Table 6). Moreover, if we analyse these results by species, we find that it includes 0.9% of Common Woodpigeons, 0% of Common Quails, Rock Doves, Stock Doves, European Turtle-doves and Barbary Partridges, and 2.6% of Red-legged Partridges (without taking into account farm partridges). Thus, in our study, the percentage for the latter species is lower than the data reported previously from Spain (Table 6).

However, the rest of the specimens may have expelled the pellets. In order to analyse the results with this possibility in mind, we took the following into account: (1) renal Pb concentrations and (2) the colour and appearance of the gizzard mucous membrane (Supplementary material, Table S2). Furthermore, we also considered ideas put forward by other authors: (1) renal concentrations of Pb tend to be higher than hepatic concentrations (DeMent et al. 1987; Franson and Pain 2011); (2) the concentrations of Pb in the soft tissues of birds that have ingested metal Pb tend to be very biased or have atypical values (Dieter 1979; Beyer et al. 1998); (3) there are sometimes erroneous values in measurements when ammunition fragments are left embedded in the tissue (Kreager et al. 2008); (4) gizzards tend to be dark in colour when exposed to Pb (De Francisco et al. 2003; Franson and Pain 2011); and (5) concentrations of Pb in the liver of birds over the aforementioned threshold of 0.65 $\mu g\ g^{-1}$ have been found in large cities (Hutton and Goodman 1980; Schilderman et al. 1997; Nam and Lee 2006).

Based on all the cited scientific references, there seems to be some evidence of high Pb concentrations resulting from a possible ingestion of Pb pellets in fifteen birds (Supplementary material, Table S2, specimens 3, 4, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 24, the final one at the limit of the threshold), which corresponds to 3.0% of all the specimens studied (without taking farm specimens into account). There were also another four specimens that should be treated with caution, since the Pb concentration in one of their organs was very low but excessively high in the other (specimens 1, 2, 20 and 23), although the colour of their gizzards showed no evidence of Pb shot ingestion. If these specimens' tissue concentrations are correct and there were no embedded ammunition fragments, we should have found clinical signs of poisoning, which was not the case. However, several authors have reported a greater resilience to Pb poisoning in some bird species, including partridges and pigeons (Barthalmus et al. 1977; Bannon et al. 2011; Franson and Pain 2011). If we were to include these specimens, the percentage would rise to 3.8% (19 specimens), a percentage still below those reported from Spain (Soler-Rodríguez et al. 2004; Ferrandis et al. 2008), Hungary (Ákoshegyi 1997), Canada (Kreager et al. 2008) and USA (DeMent et al. 1987; Walter and Reese 2003; Larsen et al. 2007; Bingham et al. 2015).

In the remaining five specimens Pb pellet ingestion does not seem to have occurred given the normal appearance of their gizzards' internal surfaces, and given the existence of concentrations situated between the two threshold values set for hepatic Pb (0.65–2.0 μg g^{-1}, specimens 19, 21 and 22), their renal Pb concentrations within the limits considered (specimen 19), their high hepatic concentrations but low renal concentrations (specimens 5 and 8), and origin from urban areas (specimens 19 and 22). The dark-green staining of the gizzard membrane could be associated with the ingestion of lead shot many years ago (Locke and Bagley 1967; McConnell 1968) and is considered an important diagnostic sign (De Francisco et al. 2003).

Finally, we observed that there was some evidence that at least eight of these 24 specimens had been bred and released into the wild for hunting (cage marks on feathers). Partridges are commonly released in intensive hunting estates where lead shot densities in soil could be high, which means that the study of Pb shot densities could be relevant to this result.

Conclusion

We can affirm that the wild bird populations analysed had low hepatic Pb concentrations (median of 0.054 μg g^{-1}) and that the Pb concentrations in a large percentage (95.2%) of the specimens were below the environmental exposure threshold. There was some evidence of the ingestion of Pb pellets in a small percentage of birds (3.0–3.8%), and in some specimens, exposure to Pb seems to be due to urban sources. Birds bred for hunting seem to be more prone to ingesting pellets from the environment, probably because of the high hunting intensity in their release areas, a fact that should be taken into consideration when taking management decisions. There appears to be a relationship between the hepatic concentrations of Pb in pigeons and their life in urban environments. In low-intensity hunting environments, Common Quails and Common Woodpigeons should be chosen for Pb contamination monitoring studies. The Rock Dove has been suggested by several authors as a good indicator of contamination in urban environments, while the Common Woodpigeon could be a good indicator in rural environments.

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Figure 1. Location of sampling areas from Spain.

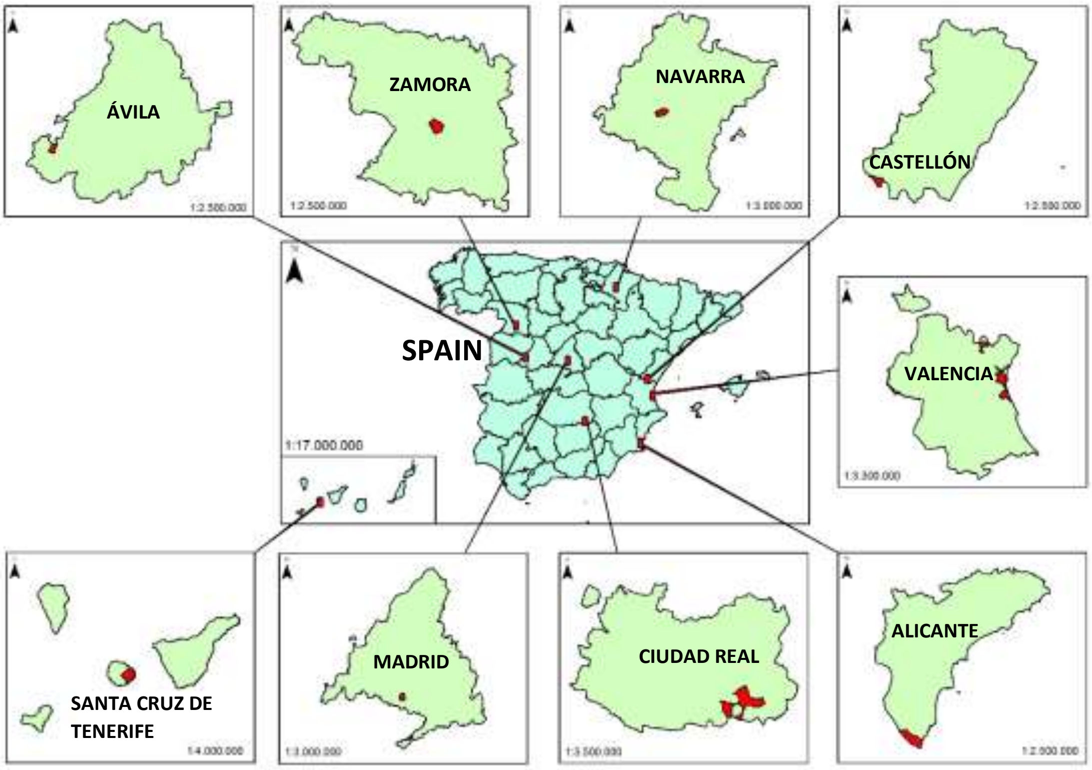

Table 1. Characteristics of the studied groups of birds, sampling areas and causes of death.

Region	Species	Area	n	Nature	Behaviour	Environment	Sampling method	Cause of death	Hunting intensity	Other
Comunidad Valenciana (n=191)	*Alectoris rufa*	Valencia	30	Wild	Sedentary	Urban	Urban control program	Bird of prey (24) Compressed-air guns (6)	Zero	Airport
	Alectoris rufa	Alicante	36	Wild	Sedentary	Rural	Hunting	Firearms	Very high	Claim hunting
	Columba livia	Valencia	30	Wild	Sedentary	Urban	Urban control program	Lethal gas*	Zero	Seaport
	Columba livia	Alicante	30	Wild	Sedentary	Rural	Hunting	Firearms	Very high	Half-closed season
	Columba palumbus	Alicante	30	Wild	Sedentary	Rural	Hunting	Firearms	Very high	Half-closed season
	Columba palumbus	Castellón	2	Wild	Sedentary	Rural	Hunting	Firearms	Low	Half-closed season
	Columba palumbus	Castellón	2	Wild	Migration	Rural	Hunting	Firearms	Low	General hunting season
	Streptopelia turtur	Alicante	31	Wild	Summer migration	Rural	Hunting	Firearms	Very high	Half-closed season
Madrid (n=40)	*Columba livia*	Madrid	10	Wild	Sedentary	Periurban	Hunting	Firearms	High	Half-closed season
	Columba palumbus	Madrid	30	Wild	Sedentary	Periurban	Hunting	Firearms	High	Half-closed season
Castilla-La Mancha (n=97)	*Alectoris rufa*	Ciudad Real (HR 1)	32	Repopulation	Sedentary	Rural	Hunting	Firearms	High	Start of season
	Alectoris rufa	Ciudad Real (HR 2)	24	Repopulation	Sedentary	Rural	Hunting	Firearms	High	Middle of season
	Alectoris rufa	Ciudad Real	17	Repopulation	Sedentary	Rural	Hunting	Firearms	High	End of

		(HR 3)								season
	Alectoris rufa	Ciudad Real (HR 1)	24	Repopulation	Sedentary	Rural	Hunting	Firearms	High	End of season
Castilla-León (n=162)	*Alectoris rufa*	Zamora	30	Wild	Sedentary	Rural	Hunting	Firearms	Low	General hunting season
	Columba livia	Zamora	28	Wild	Sedentary	Rural	Hunting	Firearms	Low	General hunting season
	Columba palumbus	Zamora	30	Wild	Sedentary	Rural	Hunting	Firearms	Low	Half-closed season
	Columba palumbus	Zamora	12	Wild	Migration	Rural	Hunting	Firearms	Low	General hunting season
	Columba palumbus	Ávila	1	Wild	Migration	Rural	Hunting	Firearms	Low	General hunting season
	Columba oenas	Zamora	30	Wild	Migration	Rural	Hunting	Firearms	Low	General hunting season
	Coturnix coturnix	Zamora	31	Wild	Summer migration	Rural	Hunting	Firearms	Low	Half-closed season
Islas Canarias (n=14)	*Alectoris barbara*	Santa Cruz de Tenerife	13	Wild	Sedentary	Rural	Hunting	Firearms	Very low	General hunting season
	Columba livia	Santa Cruz de Tenerife	1	Wild	Sedentary	Rural	Hunting	Firearms	Very low	General hunting season
Navarra (n=26)	*Alectoris rufa*	Navarra	26	Livestock	Sedentary	Rural	Veterinary practice	Lethal injection**	Zero	Farm

* authorisation code BIO/FC/15661/ 21 03 2016; ** clinical veterinary practices, non-experimental; HR = hunting reserve

Table 2. Concentrations of Pb (µg g^{-1}, wet weight) in game-bird livers and specimens with Pb shot, possibly due to ingestion.

Region	Species	Area	n	n above DL	Geometric mean±SE	Min	Max	n (%) above 0.65 µg g^{-1} (*)	Specimens with Pb shot due to possible ingestion	
									Total specimens [%]	Pb shot in C/G/I and (hepatic Pb concentration)
Comunidad Valenciana	*Alectoris rufa*	Valencia	30	28	0.035±0.018	nd	0.434	0 (0)	0 [0]	0/0/0
	Alectoris rufa	Alicante	36	36	0.070±0.392	0.007	14.187	2 (5.6)	3 [8.3]	1 (0.083) / 1 (0.104) / 1 (0.701)
	Columba livia	Valencia	30	29	0.089±0.043	nd	1.171	2 (6.7)	0 [0]	0/0/0
	Columba livia	Alicante	30	24	0.020±0.008	nd	0.142	0 (0)	1 [3.3]	0 / 1 (0.036) / 0
	Columba palumbus	Alicante	30	27	0.055±0.018	nd	0.395	0 (0)	3 [10.0]	0 / 1 (nd) / 2 (0.119 and 0.029)
	Columba palumbus	Castellón	2	2	0.013±0.040	0.002	0.081	0 (0)	0 [0]	0/0/0
	Columba palumbus	Castellón	2	1	0.002±0.003	nd	0.006	0 (0)	0 [0]	0/0/0
	Streptopelia turtur	Alicante	31	30	0.042±0.029	nd	0.846	1 (3.2)	0 [0]	0/0/0
Madrid	*Columba livia*	Madrid	10	10	0.187±0.103	0.022	1.101	1 (10)	0 [0]	0/0/0
	Columba palumbus	Madrid	30	30	0.180±1.195	0.047	35.567	2 (6.7)	2 [6.7]	1 (0.166) / 0 / 1 (0.061)
Castilla-La Mancha	*Alectoris rufa*	Ciudad Real[1]	32	31	0.078±0.183	nd	5.946	1 (3.1)	0 [0]	0/0/0
	Alectoris rufa	Ciudad Real[2]	24	24	0.059±0.443	0.011	10.379	2 (8.3)	2 [8.3]	0 / 2 (10.379 and 0.017) / 0
	Alectoris rufa	Ciudad Real[3]	17	17	0.054±0.281	0.008	4.329	2 (11.8)	1 [5.9]	0 / 1 (4.329) / 0
	Alectoris rufa	Ciudad Real[1]	24	21	0.063±0.400	nd	7.885	4 (16.7)	7 [29.2]	3 (0.192, 0.130 and 0.005[¥]) / 3 (4.986, 0.132 and 0.064) / 2 (7.885 and 0.005[¥])
Castilla-León	*Alectoris rufa*	Zamora	30	28	0.024±0.018	nd	0.439	0 (0)	1 [3.3]	0 / 1 (0.023) / 0

	Columba livia	Zamora	28	24	0.022±0.010	nd	0.226	0 (0)	1 [3.6]	0 / 1 (0.135§) / 1 (0.135§)
	Columba palumbus	Zamora	30	30	0.111±1.038	0.034	31.253	1 (3.3)	5 [16.7]	1 (31.253) / 1 (0.082) / 3 (0.299, 0.176 and 0.049)
	Columba palumbus	Zamora	12	9	0.018±0.230	nd	2.788	1 (8.3)	0 [0]	0/0/0
	Columba palumbus	Ávila	1	0	nd	-	-	0 (0)	0 [0]	0/0/0
	Columba oenas	Zamora	30	28	0.033±0.006	nd	0.151	0 (0)	1 [3.3]	0 / 1 (0.151) / 0
	Coturnix coturnix	Zamora	31	31	0.121±0.213	0.028	6.270	2 (6.5)	0 [0]	0/0/0
Canarias	*Alectoris barbara*	S. C. Tenerife	13	13	0.091±0.879	0.002	8.766	3 (23.1)	1 [7.7]	0 / 1 (0.255) / 0
	Columba livia	S. C. Tenerife	1	1	0.165±	0.165	0.165	0 (0)	0 [0]	0/0/0
Navarra	*Alectoris rufa*	Navarra	26	26	0.060±0.011	0.006	0.213	0 (0)	0 [0]	0/0/0

(*) threshold of abnormal exposure; C=crop, G=gizzard, I=intestine; nd=no detected; [1]hunting reserve 1; [2]hunting reserve 2; [3]hunting reserve 3; ¥ same specimen (one specimen had one Pb pellet in crop and another one in its intestine); § same specimen (the same specimen had one Pb pellet in its gizzard and another one in its intestine)

Table 3. Hepatic Pb concentrations (μg g^{-1} wet weight) in each species by location. For each species, the same small letter indicates statistically significant differences between locations.

Species	Area	n	Geometric mean±SE
Alectoris rufa	Alicante	36	0.070±0.392[a,b]
	Ciudad Real	97	0.065±0.166[c]
	Navarra	26	0.060±0.011[d]
	Valencia	30	0.035±0.018[a]
	Zamora	30	0.024±0.018[b,c,d]
Columba palumbus	Madrid	30	0.180±1.195[e,f]
	Zamora	42	0.065±0.743[e]
	Alicante	30	0.055±0.018[f]
	Castellón*	4	0.005±0.020
	Avila*	1	nd
Columba livia	Madrid	10	0.187±0.103[g,h]
	La Gomera*	1	0.165
	Valencia	30	0.089±0.043[i,j]
	Zamora	28	0.022±0.010[g,i]
	Alicante	30	0.020±0.008[h,j]

*excluded from the statistical analyses; nd=not detected

Table 4. Hepatic Pb concentrations (µg g^{-1} wet weight) in each location by species. For each location, the same small letter indicates statistically significant differences between species.

Area	Species	n	Geometric mean±SE
Zamora	*Coturnix coturnix*	31	0.121±0.213[a,b,c]
	Columba palumbus	42	0.065±0.743[d]
	Columba oenas	30	0.033±0.006[a]
	Alectoris rufa	30	0.024±0.018[b]
	Columba livia	28	0.022±0.010[c,d]
Madrid	*Columba livia*	10	0.187±0.103
	Columba palumbus	30	0.180±1.195
Alicante	*Alectoris rufa*	36	0.070±0.392[e]
	Columba palumbus	30	0.055±0.018[f]
	Streptopelia turtur	31	0.042±0.029
	Columba livia	30	0.020±0.008[e,f]
Valencia	*Columba livia*	30	0.089±0.043[g]
	Alectoris rufa	30	0.035±0.018[g]

Table 5. Hepatic Pb concentrations (µg g^{-1}, wet weight) in birds from areas with different hunting intensities, and possible Pb shot ingestion. The same small letter indicates statistically significant differences between intensity hunting areas. The same capital letter indicates statistically significant differences between the intensity hunting areas for the same species.

Hunting intensity	n	Geometric mean±SE	Possible Pb shot ingestion	Species	Geometric mean±SE	Possible Pb shot ingestion
Very high	127	0.044 ± 0.111^{a}	7	*Columba palumbus*, n=30	0.055 ± 0.018^{A}	3
				Columba livia, n=30	0.020 ± 0.008^{C}	1
				Streptopelia turtur, n=31	0.042 ± 0.029	0
				Alectoris rufa, n=36	0.070 ± 0.392^{F}	3
High	137	$0.088\pm0.286^{a,b}$	12	*Columba palumbus*, n=30	$0.180\pm1.195^{A,B}$	2
				Columba livia, n=10	$0.187\pm0.103^{C,D}$	0
				Alectoris rufa, n=97	0.065 ± 0.166^{E}	10
Low	166	0.041 ± 0.192^{b}	8	*Columba palumbus*, n=47	0.047 ± 0.665^{B}	5
				Columba oenas, n=30	0.033 ± 0.006	1
				Columba livia, n=28	0.022 ± 0.010^{D}	1
				Alectoris rufa, n=30	$0.024\pm0.018^{E,F}$	1
				Coturnix coturnix, n=31	0.121 ± 0.213	0
Very low	14	0.095 ± 0.819	1	*Alectoris barbara*, n=13	0.091 ± 0.879	1
				Columba livia, n=1	0.165	0
Zero*	86	0.057 ± 0.017	-	*Columba livia*, n=30	0.089 ± 0.043	-
				Alectoris rufa, n=56	0.045 ± 0.011	-

*excluded from the statistical analyses

Table 6. Pb pellet ingestion in game birds from terrestrial environments.

Species	n	Country	Birds with Pb pellets in gizzards (%)	Reference
Alectoris rufa	7	Spain	14.3	Soler-Rodríguez et al., 2004
	637	United Kingdom	0.16*	Butler et al., 2005
	144	United Kingdom	1.4**	Butler et al., 2005
	76	Spain	7.8	Ferrandis et al., 2008
Perdix perdix	62	Denmark	1.6	Clausen and Wolstrup, 1979
	77	United Kingdom	0	Watson, 2004
	1318	United Kingdom	1.4	Potts, 2005
Alectoris chukar	123	USA	5.7	Walter and Reese, 2003
	75	USA	10.7	Larsen et al., 2007
	76	Canada	8	Kreager et al., 2008
	461	USA	9.3	Bingham et al., 2015
Columba palumbus	142	Denmark	0.7	Clausen and Wolstrup, 1979
Columba livia	13	USA	23.1	DeMent et al., 1987
Phasianus colchicus	947	Hungary	4.75	Ákoshegyi, 1997
	437	United Kingdom	3	Butler, 2005
	47	Canada	34	Kreager et al., 2008

*historical; **current (2005)