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Compliance with the ban of lead ammunition in a Mediterranean wetland, the Ebro delta

Núria Vallverdú Coll

Directores: Dr. Rafael Mateo y Dr. Manuel E. Ortiz-Santaliestra

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Trabajo presentado por

Núria Vallverdú Coll

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VºBº Directores

Dr. Rafael Mateo Dr. Manuel E. Ortiz-Santaliestra

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Abstract

The ingestion of lead (Pb) shot used for hunting continues being the main cause of Pb poisoning in waterfowl. In the Ebro delta (Spain), protected wetlands are surrounded by rice fields where waterbirds feed, and where Pb ammunition is still allowed. High Pb shot densities in sediments, and in turn high ingestion prevalence in waterfowl, have been detected. The use of Pb ammunition and the accumulation of Pb by birds after shot ingestion may pose a risk for human health due to consumption of contaminated meat. We assessed the degree of compliance with the ban on Pb ammunition in the Ebro delta wetlands, and studied the effect of the ban on the prevalence of Pb shot ingestion in waterbirds and on Pb levels in game meat. Waterfowl carcasses were collected and X-rayed from hunting bags (2007-2011) to determine the percentage of Pb and non-toxic embedded shot. Concentrations of Pb were analyzed in livers and muscles. In addition, gizzards were collected from hunting bags (2007-2012) and examined to determine the percentage of Pb shot ingestion. During the first study season minimum hunter compliance, estimated as the percentage of waterbirds having only embedded steel shot (the non-toxic alternative) was 48.75%, while 26.88% of birds had only embedded Pb shot (minimum hunter noncompliance). These values changed in the subsequent seasons to 68.95% and 1.13%, respectively. The little compliance detected during the first study season led local administration to notify hunters that a total prohibition of hunting in protected wetlands would enter into force if the prohibition was not observed. Pb ingestion prevalence in 2007-2008 (28.6%) was not different from the pre-ban value (30.2%), but decreased significantly to values below 17.9% in the following seasons. Birds continue ingesting Pb shot at a relative high proportion, although their prohibition slowly contributes to reduce prevalence of ingestion. Pb muscle concentrations decreased significantly after the ban, in spite of which most species present individuals with Pb liver and muscle concentrations over the maximum safety limits. Whereas muscle Pb levels were determined by the presence of both ingested and embedded shot (all $p<0.001$), liver levels largely depended on ingested shot ($p<0.001$). Thus, besides restrictions in Pb ammunition use, additional mechanisms to reduce Pb ingestion prevalence in waterfowl are necessary to reduce risks for human consumers.

Keywords

Lead shot, non-toxic ammunition, compliance, waterfowl, wetland, human health.

Resumen

La ingestión de perdigones de plomo (Pb) utilizados para la caza sigue siendo la principal causa de intoxicación en aves acuáticas. En el delta del Ebro (España), los humedales protegidos se encuentran rodeados por campos de arroz donde muchas aves acuáticas se alimentan, y donde todavía está permitido el uso de munición de Pb, habiéndose encontrado altas densidades de perdigones de Pb en los sedimentos, así como una elevada prevalencia de ingestión de perdigones de Pb en aves acuáticas. El uso de munición de Pb y la acumulación de Pb en los tejidos de las aves después de la ingestión de perdigones puede suponer un riesgo para la salud humana debido al consumo de carne contaminada. Se evaluó el grado de cumplimiento de la prohibición de la munición de Pb en los humedales protegidos del Delta del Ebro, y se estudió el efecto de la prohibición en la prevalencia de ingestión de perdigones de Pb en las aves acuáticas y en los niveles de Pb en la carne de caza. Se obtuvieron cadáveres de aves acuáticas de las bolsas de caza (2007-2011) y se tomaron radiografías para determinar el porcentaje de perdigones de Pb y de perdigones no tóxicos incrustados. Se analizaron las concentraciones de Pb en hígado y en músculo. Además, se obtuvieron mollejas de las bolsas de caza (2007-2012) y se examinaron para determinar el porcentaje de ingestión de perdigones de Pb. Durante la primera temporada del estudio, el cumplimiento mínimo, estimado como el porcentaje de aves acuáticas que tienen únicamente perdigones de acero incrustados (la alternativa no tóxica) fue del 48,75%, mientras que el 26,88% de las aves presentaron únicamente perdigones de Pb incrustados (incumplimiento mínimo). Estos valores fueron del 68,95% y 1,13%, respectivamente, en las temporadas posteriores. El bajo nivel de cumplimiento detectado durante la primera temporada de estudio llevó a las autoridades locales a amenazar a los cazadores con una prohibición total de la caza en los humedales protegidos si no respetaban la prohibición. La ingestión de perdigones de Pb en el período 2007-2008 (28,6%) no fue diferente al valor pre-prohibición (30,2%), pero disminuyó significativamente a valores por debajo del 17,9% en las siguientes temporadas. Las aves acuáticas siguen ingiriendo perdigones de Pb en una proporción relativamente alta, aunque su prohibición contribuye lentamente a una reducción de la prevalencia de ingestión. Las concentraciones de Pb en músculo disminuyeron significativamente después de la prohibición, a pesar de que la mayoría de las especies presentaron individuos con concentraciones de Pb por encima de los límites máximos de seguridad tanto en hígado como en músculo. Mientras que los niveles musculares de Pb estuvieron determinados por la presencia tanto de perdigón ingerido como de perdigón incrustado (ambos p<0,001), en el caso del hígado las concentraciones dependieron en gran medida de la presencia de perdigones ingeridos (p <0,001). En consecuencia, además de las restricciones en el uso de munición de Pb, son necesarios mecanismos adicionales para reducir la prevalencia de ingestión de Pb en aves acuáticas y así reducir los riesgos en la salud humana por parte de los consumidores.

Palabras clave

Perdigón de plomo, munición no tóxica, cumplimiento, aves acuáticas, humedales, salud humana.

1. Introduction

Ingestion of shot pellets or fishing weights is the main cause of lead (Pb) poisoning in avifauna (United States Fish and Wildlife Service 1986). Among wild birds, waterfowl present the highest prevalence of Pb shot ingestion (Pain 1996; Mateo 2009). In sediments of wetlands located in the lower reaches of rivers or in coastal areas there is an elevated density of accumulated Pb shot pellets, as well as a low availability of grit (grain particles or gastroliths) (Pain et al. 1992; Mateo et al. 1999). Shot pellets are confused with the grit that birds need for the proper functioning of the gizzard (Pain 1990; Mateo et al. 2000a). Ingested Pb fragments may be regurgitated or excreted, but they can also be retained in the gizzard until they are completely dissolved, which may take up to 20 days (Jordan & Bellrose 1951). Pb salts resulting from dissolution can be absorbed into the bloodstream, thus causing Pb toxicosis which, in birds, commonly manifests in distension of the proventriculus, green watery faeces, weight loss, anaemia and drooping posture (Mateo et al. 1998). Liver Pb levels >5 µg/g of dry weight (d.w.) are considered as diagnostic of abnormally Pb exposure (Guitart et al. 1994) and are often accompanied by the aforementioned clinical signs. Furthermore, Friend (1987) described levels >20 µg/g d.w. as indicative of acute Pb poisoning, associated with several physiologic effects and likely to cause death. In the United States, Grinnell (1894) was the first to report Pb poisoning in waterbirds on the western migration route, attributing it to the ingestion of shot. But it was not until 1991 when the use of Pb shot for waterfowl hunting was completely banned in the US, not only to conserve wild populations of waterfowl, but also to protect Bald Eagle (*Haliaeetus leucocephalus*) and Golden Eagle (*Aquila chrysaetos*) from secondary Pb poisoning (Anderson 1992).

In Europe, Pb poisoning was first described as a disease in wild birds in 1842 in Germany (von Fuchs 1842). The first countries that adopted the ban on Pb shot for hunting in wetlands (i.e. Denmark, Norway, the Netherlands, Finland and Sweden) did it so in the late 1980s. In 1995, some European and African countries agreed to implement bans on the use of Pb for shooting over wetlands by the year 2000 following the African-Eurasian Waterbird Agreement (AEWA). The use of Pb shot for hunting has been regulated so far in 14 European countries, but there is no common

European Union regulation on Pb ammunition. In Spain, the use of Pb shot for hunting in wetlands was initially banned in the Balearic Islands in 1995 and in Castilla-La Mancha in 1999. In the rest of Spain the prohibition was limited to Ramsar areas and other protected wetlands in 2001 (Royal Decree 581/2001 of 1st June), and then extended to all the Natura 2000 wetlands in 2007.

Pb shot in sediments can remain unaltered for decades, and therefore high densities can still be found in wetland sediments despite of the ban of using Pb ammunition in these areas. Data until 2002 indicate Pb shot densities >100 shot/m^2 in the upper 20 cm of wetland sediments from Denmark, France and Spain, and between 10 and 50 shot/m^2 in most wetlands from the UK (Mateo 2009). However, there is little recent information to analyse the influence of the ban on densities of Pb shot in sediments at the mid or long term. In the case of Tablas de Daimiel (Central Spain), around 100 shot/m^2 were found in 1993, three decades after the ban of hunting in 1963 (Mateo et al. 1998). Wetlands in the Mediterranean area show the highest Pb shot densities in sediments, which is due to the fact that large concentrations of waterbirds and hunters occur in few remaining wetlands during migration or wintering. This is the case of the Ebro delta (Catalonia, North-eastern Spain), where waterfowl hunting has been carried out for more than one century and Pb shot densities in the upper 20 cm of sediment in 1991-1996 were 97-266 shot/m^2 in the lagoons and 6-83 shot/m^2 in the surrounding rice fields (Guitart el al. 1994; Mateo et al. 1997, 1999).

Mediterranean wetlands also present the highest prevalence of Pb shot ingestion by waterfowl. Thus, in El Hondo (Valencia, Spain), a wetland considered a refuge within Europe for endangered waterfowl species like the white-headed duck (*Oxyura leucocephala*), prevalence of Pb shot ingestion was up to 32% in *Oxyura* spp. (Mateo et al. 2001). Recent data confirm that levels of Pb in tissue of white-headed duck are still abnormally high (Taggart et al. 2009). In the Ebro delta prevalence of Pb shot ingestion during the pre-ban period (1991-1996) was 74.2% in Northern pintail (*Anas acuta*), 69.2% in common pochard (*Aythya ferina*) and 30.2% in mallard (*Anas platyrhynchos*) (Mateo et al. 2000b).

Consumption of birds poisoned with Pb as a consequence of shot ingestion can suppose a source of indirect poisoning. Furthermore, shot embedded may also pose a significant risk to human health because regular consumption of meat from game animals hunted with Pb ammunition is associated with increased blood Pb levels (Iqbal et al. 2009). Recent studies have shown the risk for humans of consuming game birds shot with Pb ammunition (Mateo et al. 2011; Green & Pain 2012). Moreover, bioaccessibility of Pb from ammunition in game meat is affected by cooking treatment (Mateo et al. 2011). Mateo et al. (2007) demonstrated the effect of the acidic conditions generated by the vinegar on transfer of Pb from the embedded shot to the meat during cooking. Recently, the Spanish Agency of Food Safety and Nutrition (AESAN) recommended children under six years old and pregnant women not to eat meat of animals killed with Pb ammunition, because its negative effects on developing central nervous system.

Mediterranean wetlands present high concentration of waterbirds and waterfowl hunters during the last months of the hunting season (January-March). This is the case of the Ebro delta, where the available literature reports, as mentioned above, high Pb shot densities on sediments and large prevalence of Pb shot ingestion, as well as high Pb tissue concentration in waterfowl (Mateo et al. 1997). Even though the Pb ammunition prohibition in 2001 was an important step for the conservation of this wetland and for the general public health, it is crucial to assess if hunters are actually complying with Pb ammunition ban and the use of non-toxic shot. In this regard, comparisons between existing data about Pb prevalence in waterfowl with new data obtained after the prohibition could be very helpful. With this purpose, we have monitored during the 2007-2012 period the presence of embedded shot in hunted birds, the prevalence of Pb shot and non-toxic shot ingestion, and Pb concentration in liver and meat of waterfowl. Considering this information, the aims of this study are (i) to assess the degree of compliance with the ban on Pb ammunition in the Ebro delta wetlands, (ii) to study the effect of the ban on the prevalence of Pb shot ingestion in waterbirds and (iii) to evaluate the impact of this measure on Pb levels in game meat under the perspective of food safety.

2. Material and methods

2.1 Study area

The Ebro delta is an alluvial plain situated in the NE of Spain with a surface of 320 km^2 (Fig. 1). More than 360 bird species have been cited in the area, which includes breeding colonies of some of the most important seabirds in the Mediterranean. It was catalogued as an Important Bird Area (IBA) and included in the List of Wetlands of International Importance (Ramsar Convention) on 26 March 1993.

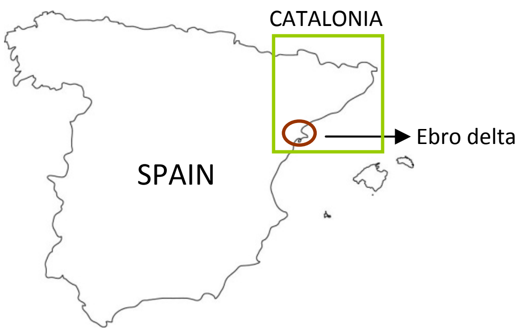

Figure 1: Geographic location of the study area in the Iberian Peninsula (left), and location of sampling sites at the lagoons of Illa de Buda (1), Encanyissada (2), Garxal (3) and the rice fields of Bombita-Canal Vell (4), Dacsa (5), Migjorn (6) and Muntells (7) (right).

Crops occupy 77% of the surface (66% of rice fields and 11% of orchards). The rest of the area, occupied by natural wetlands (6% lagoons and 17% marshes) (Martínez-Vilalta 1995), is included within the legally protected natural space. Hunting with Pb ammunition is banned in the protected wetlands, being still allowed in adjacent rice fields which are not part of the protected area (Fig. 1).

The dynamics of rice cultivation greatly determines the feeding behaviour of waterbirds in the Ebro delta and their risk of Pb shot ingestion. Rice cycle starts in November after the harvest, when the water supply is closed and some rice fields are being dried as the hunting season (October-March) progresses, while those with game importance are kept flooded. In winter, rice grains are a major food source for the thousands of waterfowl wintering in the area (Llorente 1984). In consequence, large concentrations of waterbirds and waterfowl hunters occur in the few remaining flooded fields and lagoons, which motivate the accumulation of Pb shot at high densities in these areas.

2.2 Sampling and analysis of embedded shot

A total of 523 waterfowl carcasses were collected from hunting bags between 2007-08 and 2010-11 hunting seasons. Collected species included Northern pintail, Northern shoveler (*Anas clypeata*), common teal (*Anas crecca*), common pochard, tufted duck (*Aythya fuligula*), Eurasian wigeon (*Anas _enelope*), mallard, gadwall (*Anas strepera*), common coot (*Fulica atra*), common moorhen (*Gallinula chloropus*), common snipe (*Gallinago gallinago*), red-crested pochard (*Netta rufina*) and Northern lapwing (*Vanellus vanellus*). We recorded body mass of all animals.

Carcasses were X-rayed to carry out an initial detection of shot pellets, which were then removed during necropsies. In addition, the gizzard was also collected to determine the prevalence of shot ingestion. The liver and pectoral muscles were also obtained to analyse their Pb levels. Once separated, pectoral muscles were X-rayed again to detect embedded pellets. In this regard, steel shot were easily distinguished in the X-ray because they are larger and rounder than Pb shot. Furthermore, Pb pellets are not attracted to a magnet, which was also tested during necropsies. Livers and muscles were stored at -20 °C until analysis.

The material of embedded pellets was used as an indicator of the degree of compliance of the ban of Pb use. In this regard, some animals showed both steel and Pb embedded shot, which seems a consequence of the repeated encounters with hunters (Guillemain et al. 2007). In these cases, as the use of steel ammunition is relatively recent, motivated by the ban of Pb, we considered that animals would have been killed by steel shot. Then, embedded Pb shot came from old shot that could have happened before the ban or in areas where the use of Pb is not prohibited.

Apart from the gizzards obtained from these carcasses, 418 additional gizzards (from the same species mentioned above), were collected from hunting bags during the 2011-2012 hunting season.

2.3 Gizzard examination

A total of 941 gizzards were examined externally to observe the presence of shot entry holes in order to establish whether any of the shot found inside the gizzard could have been shot-in (Mateo et al. 1997). Each gizzard was cut with scissors and its content washed with water and dried. Vegetation was removed by flotation in trichloromethane, which was found to be useful in the separation of organic from inorganic material. The precipitates were dried and placed in plastic Petri dishes. If no holes or signs of shot entry were found in the gizzard, all pellets present in the precipitates were assumed to be ingested. Furthermore, pellets that had entered the gizzard ballistically were uneroded, deformed by impacts caused when fired, and larger and shinier than ingested shot (Martínez-Haro et al. 2010). The presence of pellets in the gizzard contents was determined using a binocular magnifying lens (x10).

2.4 Analysis of Pb in liver and muscle

Given that not all samples could be stored from all collected carcasses, 465 livers and 327 muscles were available for analysis. The whole organs were weighed. Pieces of liver and muscle were homogenized and lyophilized. Pb analyses were performed following the methodology described by Mateo et al. (2006) and Rodríguez-Estival et al. (2011) with slight modifications. Lyophilized samples (0.3-0.5 g) were digested with 3 ml of HNO_3 (69% Analytical grade, Panreac, Spain) in Pyrex tubes in a heating block (Micro, for 40 tubes, Selecta) with an electronic controller (RAT-2, Selecta). Tubes were heated for 5 h at 50 °C, then 5 h at 100 °C, and then left at room temperature overnight. The next day, 2.5 ml of H_2O_2 (30% v/v Suprapur, Merck, Germany) were added, the tubes where heated 45 min at 90 °C, and then 2 h at 120 °C. Digests were diluted to 50 ml whit H_2O (Milli-Q grade).

The analysis of Pb in the digested samples was carried out using a graphite furnace-atomic absorption spectroscopy system (AAnalyst 800, Perkin Elmer) equipped with an autosampler (AS 800, Perkin Elmer). Limit of detection (LOD back-calculated to in tissue concentrations) was 0.015 µg/g of d.w. Blanks were processed in each batch (N=3) of digestions. Pb standards were used to determine Pb concentrations in samples by linear regression. Certified bovine liver samples (BSR 185R, Community Bureau of Reference) containing known Pb levels were also analyzed in each batch of digestions to assure the quality of the methodology, and the percentage recovery (mean ± RSD) was 93.2 ± 2.33 for liver and 93.0 ± 0.34 for muscle. All concentrations are given in d.w.

2.5 Statistical analyses

The compliance of the use of non-toxic shot was assessed by means of the percentage of waterbirds killed with non-toxic shot. The presence of embedded Pb shot may be as a consequence of the legal use of Pb shot in the non-protected areas (mostly rice fields), but also to the illegal use of Pb shot in the protected areas. As all the X-rayed waterbirds had been killed in the protected areas, the presence of only embedded Pb shot was considered as failure to comply with the ban. The presence of only steel shot was considered as a minimum compliance of the ban, although birds with steel and Pb were probably killed with steel shot and carried Pb shot from previous shootings at the non-protected areas. Therefore, the data used to evaluate the trend in the non-compliance of the Pb ban has been the percentage of birds with only embedded Pb shot (as a minimum value of non-compliance). These percentages of non-compliance were compared among seasons (2007-08, 2008-09, 2009-10, 2010-11) for the whole sample with χ^2 tests. As some differences may exist between species, we performed the same analyses only with mallards, which is the species with the highest sample size and more appropriate as a bioindicator. Moreover, the number of embedded pellets was compared between animals shot only with steel and animals shot only with Pb with a Mann-Whitney test. This comparison was performed with a non-parametric test because the number of embedded shot did not fit a normal distribution (even after a logarithmic transformation). As the number of shot that impact a bird may depend on the size of the animal, we studied the linear correlation between the number of embedded shot and the body mass of each individual. This correlation was also performed interspecifically with the mean values of each species with a Spearman correlation test.

Differences in the prevalence of Pb shot ingestion were compared between pre and post-ban periods for all the studied species with χ^2 tests. Moreover, differences between the prevalence of Pb shot ingestion in mallard among the hunting seasons within the post-ban period were also studied with χ^2 tests. Although the indicator of the ban success would be the reduction of Pb shot ingestion, the prevalence of steel shot and any type of shot ingestion were also compared among seasons. The prevalence of Pb, steel and overall shot ingestion were also compared between mallards hunted in lagoons and in rice fields with a Fisher test.

As a diagnosis of abnormally high Pb exposures, we compared the percentage of animals showing liver Pb concentrations above 5 μg/g (d.w.) between (i) pre- and post-ban periods with a Fisher test, and (ii) among species with a χ^2 test. We used an analysis of the variance (ANOVA) to determine statistical differences between species in log-transformed Pb liver and muscle concentrations. The influence of the presence of ingested or embedded Pb pellets on Pb concentrations in liver and muscle were analysed with general linear models GLMs using the Pb concentration as dependent variable, and the presence of embedded Pb shot, the presence of ingested Pb shot and the species as factors. The relationships between Pb concentrations in liver and muscle for each species were also explored with linear correlations.

The percentage of waterbirds with liver and muscle Pb concentrations above the maximum residue levels (MRL) in offal (0.5 μg/g wet weight = 1.5 μg/g d.w.) and meat (0.1 μg/g wet weight = 0.3 μg/g d.w.) for human consumption in the European Union (European Commission, 2006) were calculated for each species. These percentages were compared among hunting seasons for all the sampled animals and for mallards only with ANOVAs. Similarly, one way-ANOVAs followed by Tukey post-hoc tests were used to determine differences in Pb muscle concentrations among hunting seasons.

Statistical procedures were carried out with the software SPSS Statistics 19 (IBM).

3. Results

3.1 Embedded shot pellets

Waterfowl hunted in the Ebro delta between 2007 and 2011 had only embedded steel shot in 64.3% of birds, embedded steel and Pb shot together in 10.9% of birds, only embedded Pb shot in 9.2% of birds and no presence of embedded shot in 15.6% of birds. The number of embedded shot depended on the body mass of the birds (n=518, Rs=0.422, *p*<0.001; see Fig. 2 for the interspecific relationship), and thus higher numbers of shot were retained in large species like mallard or red-crested pochard than in small species like common teal and Northern shoveler. The number of embedded pellets in mallards was similar in animals shot only with Pb (n=24, median=4, range=1-24) and in those shot with steel (n=164, median=5, range=1-30) (Mann-Whitney test, Z=1.186, *p*=0.236).

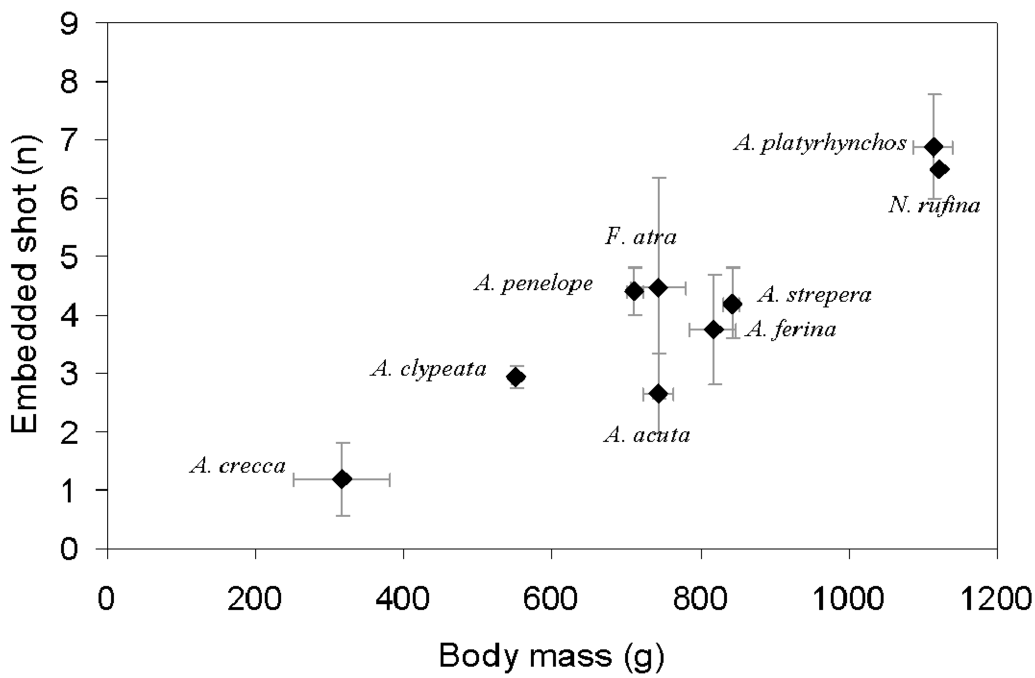

Figure 2. Correlation between body mass of waterfowl and the number of embedded pellets by species.

The compliance of the ban of Pb shot increased during the study period. In 2007-08, the percentage of waterbirds shot only with Pb pellets was 26.97%, and it declined significantly in the three following seasons to ≤2% (χ^2_3=86.8, *p*<0.001; Fig. 3). In the specific case of mallards, these values were 35.7% in 2007-08 and ≤3.1%

afterwards (χ^2_3=55.8, *p*<0.001; Fig. 3). For the entire post-ban study period, 64.1% of mallards presented only embedded steel shot.

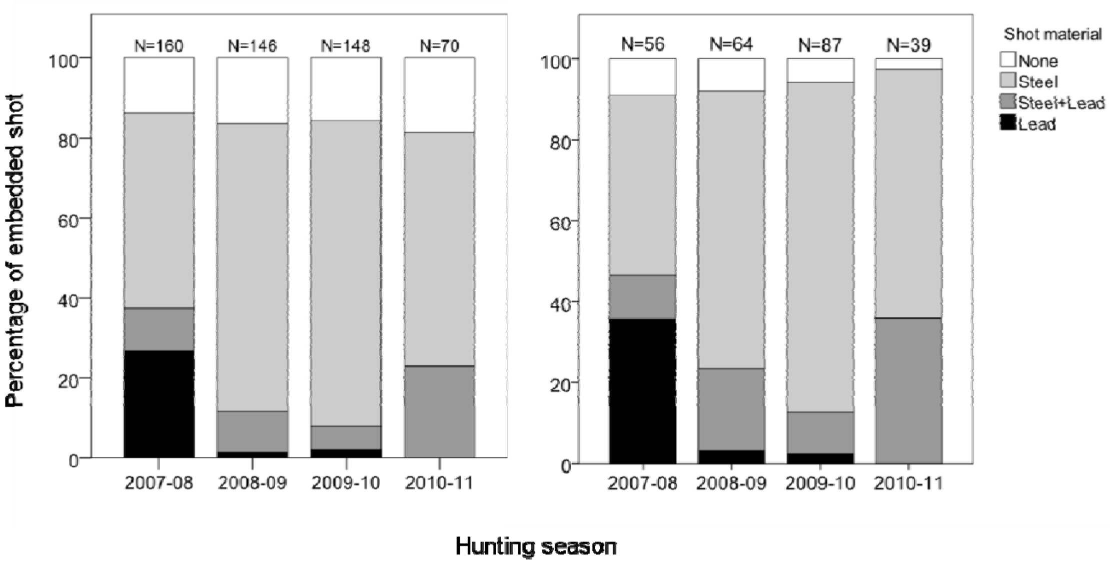

Figure 3. Percentages of hunted waterbirds (left) and mallards (right) with embedded shot (lead, steel or both) during four hunting season in the Ebro delta, Spain.

3.2 Ingested shot pellets

The prevalence of Pb shot ingestion of the different species ranged from 0 to 76% (Table 1), being significantly lower in the post-ban period (2007-12) than in the pre-ban one (1991-96) for Northern shoveler, common teal, common pochard and mallard (χ^2 tests, all *p*≤0.047). The Northern pintail showed the highest prevalence of Pb shot ingestion, with a value not different from the pre-ban prevalence (χ^2_1=0.033, *p*=0.856). In the post-ban period, the ingestion of steel shot has started to be found in most of the species with values up to 30% in common pochard (Table 1).

As significant differences in Pb shot ingestion were found among species, a more accurate analysis of the trend of prevalence in the post-ban period was focused on mallard because of its larger sample size (n=380). The prevalence of Pb shot ingestion in the 2007-08 hunting season (28.6%) was not different from the pre-ban value (30.2%) (χ^2_1=0.045, *p*=0.832). A significant decrease in the prevalence of Pb ingestion was found in the 2008-09 season (5.1%) (χ^2_1=8.227, *p*=0.004), but it increased again in the three following years (13.8-17.9%) to values nevertheless not significantly different than in 2008-09 (χ^2_1=2.047, *p*=0.152; χ^2_1=3.140, *p*=0.076;

χ^2_1=2.230, *p*=0.135, respectively for 2009-10, 2010-11 and 2011-12; Fig. 4). Prevalence of steel shot ingestion was 12.5% in 2007-08, 12.8% in 2008-09,12.6% in 2009-10, 5.1% in 2010-11 and 6.9% in 2011-12, and no significant differences among hunting seasons were observed (χ^2_4=4.196, *p*=0.380). The percentage of total intake of pellets (regardless of the material) was 35.7% in 2007-08, 15.4% in 2008-09, 19.5% in 2009-10, 17.9% in 2010-11 and 18.9% in 2011-12. Significant differences among hunting seasons were neither observed in this case (χ^2_4=8.799, *p*=0.066).

Table 1. Comparing the prevalence of Pb and steel shot ingestion and the proportion of animals with Pb levels in liver >5 µg/g between the pre-ban period (1991-1996) and the post-ban period (2007-2012).

Species	1991-1996[a]				2007-2012[b]					
	Shot ingestion		Liver Pb		Shot ingestion				Liver Pb	
	n	Pb (%)	n	>5 ppm (%)	n	Pb (%)	Steel (%)	Either (%)	n	>5 ppm (%)
A. acuta	97	74.2	24	75.0	25	76	20	84	15	100.0
A. clypeata	36	27.8	36	22.2	102	7.8*	2.9	7.9	37	5.4*
A. crecca	35	22.9	31	19.3	170	10.6*	0.6	11.1	77	10.4
A. ferina	26	69.2	26	53.9	20	35*	30	45	6	33.3
A. penelope	25	4	20	10.0	16	12.5	0	12.5	11	9.1
A. platyrhynchos	86	30.2	43	27.9	380	15.5*	10	21	216	20.4
A. strepera	25	8	24	8.3	40	0	2.5	2.5	9	0.0
F. atra	28	3.6	28	3.6	93	2.2	23.7	25.8	91	0.0
G. gallinago	-	-	2	0.0	18	5.6	0	5.6	-	-
N. rufina	21	19	21	9.5	16	12.5	25	31.3	2	0.0

[a] Guitart et al. (1994), Mateo et al. (1997, 2000b). [b] Present study. * Significantly different between 1991-1996 and 2007-2012 periods (χ^2 tests).

Regarding the habitat where the mallards were hunted, the prevalence of steel shot ingestion was higher in the lagoons (11.6%) than in the rice fields (3.8%) (Fisher test, two-tailed, *p*=0.019). On the contrary, the prevalence of Pb shot ingestion in the lagoons (n=276, 16.7%) was not significantly different than in the rice fields (n=104, 12.5%) (Fisher test, two-tailed *p*=0.345). The overall ingestion of either type of shot was also not different between areas (23.2% and 15.5%, respectively; Fisher test, two-tailed *p*=0.120).

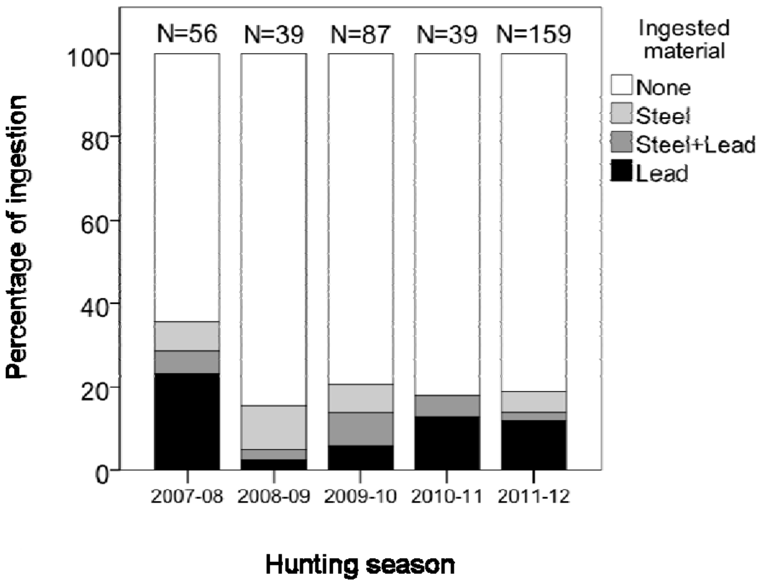

Figure 4. Percentages of hunted mallards with ingested shot (lead, steel or both) during five hunting season in the Ebro delta, Spain.

3.3 Liver and muscle Pb concentrations

Percentage of birds with >5 ppm of Pb in liver differed significantly among species (χ^2_8=110.5, *p*<0.001). This value declined significantly after the ban of Pb shot only in the Northern shoveler (Fisher test, two-tailed *p*=0.046; Table 1). Pb concentrations were significantly different among waterbird species in liver ($F_{8,455}$=21.49, *p*<0.001) and muscle ($F_{8,317}$=5.79, *p*<0.001; Table 2). Liver Pb levels were largely determined by the presence of ingested Pb shot in the gizzard ($F_{1,441}$=186.93, *p*<0.001; Fig. 5), but not by the presence of embedded Pb shot ($F_{1,441}$=0.019, *p*=0.890). Muscle Pb level was largely determined by the presence of ingested shot ($F_{1,303}$=75.258, *p*<0.001; Fig. 5), but there was also a significant effect of the presence of embedded Pb shot ($F_{1,303}$=63.317, *p*<0.001).

Liver Pb concentration was correlated with muscle Pb concentration (r=0.561; *p*<0.001). This correlation was stronger in Northern pintail (r=0.852, *p*<0.001), mallard (r=0.645, *p*<0.001), and Northern shoveler (r=0.614, *p*=0.002).

The percentage of birds with >1.5 µg/g d.w. of Pb in liver ranged from 0 to 100% (Table 3). No significantly differences among hunting seasons were observed in the entire pool of hunted animals ($F_{2,72}$=0.076, *p*=0.927) or in mallard ($F_{2,43}$=1.627, *p*=0.208).

Table 2. Concentrations of Pb (µg/g d. w.) in liver and muscle by species.

	Liver						Muscle					
Species	n	G Mean	95% C.I.		Min.	Max.	n	G Mean	95% C.I.		Min.	Max.
A. acuta	15	41.57	25.55	67.64	6.95	161.58	15	1.40	0.77	2.55	0.14	5.64
A. clypeata	37	0.31	0.16	0.60	<LOD	175.20	25	0.08	0.04	0.14	<LOD	2.91
A. crecca	77	0.19	0.12	0.31	<LOD	52.89	43	0.11	0.06	0.23	<LOD	20.75
A. ferina	6	1.44	0.17	12.06	0.08	23.69	6	0.31	0.07	1.46	<LOD	1.62
A. fuligula	1	0.63					1	0.06				
A. penelope	11	0.30	0.10	0.89	<LOD	11.95	12	0.11	0.06	0.22	<LOD	1.08
A. platyrhynchos	216	1.10	0.86	1.40	<LOD	180.17	135	0.10	0.08	0.13	<LOD	36.25
A. strepera	9	0.37	0.22	0.60	0.18	1.49	10	0.09	0.04	0.19	<LOD	1.40
F. atra	91	0.40	0.34	0.46	0.09	2.25	78	0.07	0.05	0.10	<LOD	446.41
N. rufina	2	0.37			0.26	0.52	2	0.05			0.04	0.06

LOD=Limit of detection.

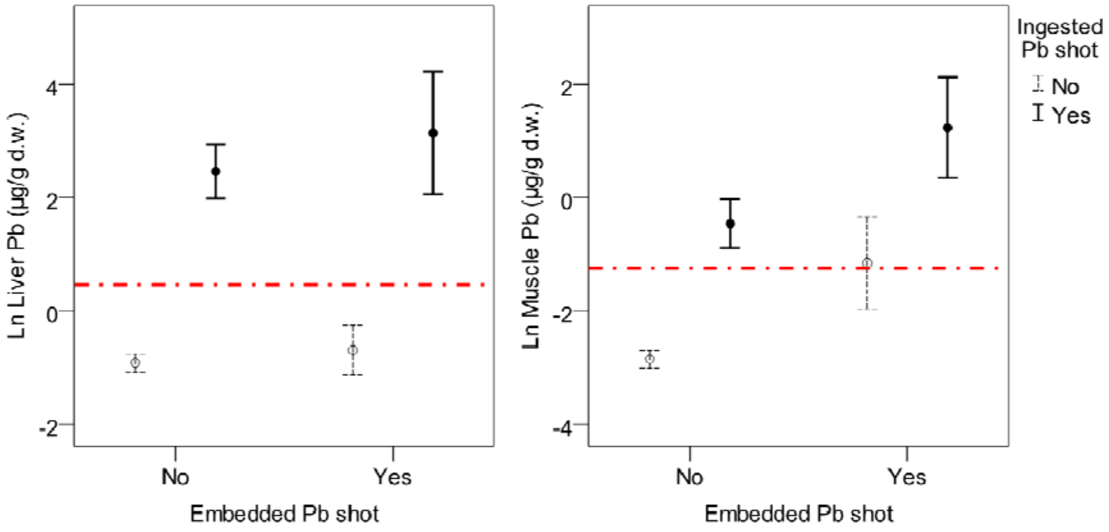

Figure 5. Liver (left) and muscle (right) Pb concentrations in waterbirds according to the presence of ingested and embedded shot. The highest Pb levels were found in birds with ingested Pb shot, independently of the additional ingestion of steel shot. Bars represent means ±95% confidence interval. The red line indicates Ln of maximum residue levels in offal (0.4 µg/g d.w.) and meat (-1.2 µg/g) for human consumption, according to European Legislation.

Table 3. Proportion of animals with Pb levels in liver >1.5 µg/g d.w. and Pb levels in muscle >0.4 µg/g d.w.

	Liver		Muscle	
Species	n	>1.5 µg/g d.w. (%)	n	>0.3 µg/g d.w. (%)
A. acuta	15	100.0	15	86.7
A. clypeata	37	16.2	25	12.0
A. crecca	77	16.9	43	30.2
A. ferina	6	50.0	7	71.4
A. fuligula	1	0	1	0
A. penelope	11	9.1	12	16.7
A. platyrhynchos	216	42.1	135	20.0
A. strepera	9	0	10	10.0
F. atra	91	1.1	78	7.7
N. rufina	2	0	2	0

The percentage of animals with >0.3 µg/g d.w. of Pb in muscle varied from 0 % to 86.7% (Table 3). No significantly differences among hunting seasons were observed in the entire pool of hunted animals ($F_{2,72}$=1.669, p=0.196) or in mallard ($F_{2,43}$=2.229, p=0.120). Pb muscle concentrations decreased significantly from the ban in the entire pool of hunted animals ($F_{2,324}$=27.964, p<0.001), as well as in mallard ($F_{2,132}$=12.437, p<0.001) (Fig. 6).

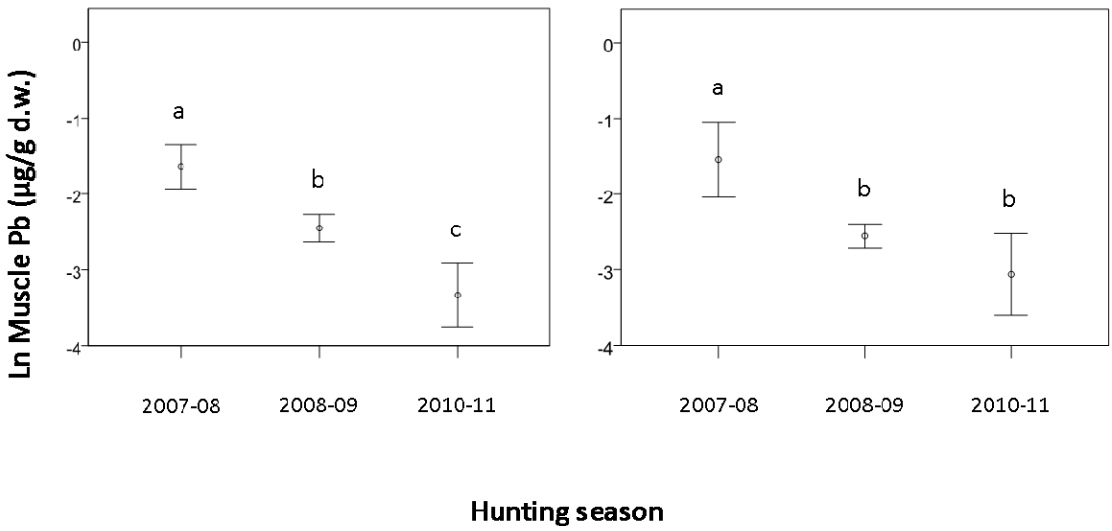

Figure 6. Pb muscle concentration in the entire set of hunted animals (left) and in mallard (right) during the three hunting seasons sampled after the ban of Pb shot use. Bars represent means ±95% confidence interval. Lower case letters (a, b, c) represent significant differences in Pb muscle concentrations between hunting seasons (Tukey test, p<0.05).

4. Discussion

4.1 Compliance of the Pb shot ban

During the period between 2007 and 2012, there has been a significant increase in the compliance of the ban of Pb shot use in the Ebro delta, a Spanish wetland under a long-term monitoring program of lead poisoning. The non-compliance values have declined from 27% in 2007-08 to less than ≤2% in the three following seasons. The ban of Pb shot has been translated into a significant reduction in the prevalence of Pb shot ingestion in four waterfowl species and a significant decrease in Pb levels in game meat. This decrease can be attributed to the reduction of the prevalences of Pb shot ingestion and to the elimination of the risk of contamination by Pb ammunition around the injury when steel shot were used.

The contribution of non-toxic shot regulations to waterfowl conservation has been evaluated almost exclusively in the US, where the use of Pb shot for waterfowl hunting was completely banned in 1991. Values of minimum hunter compliance are generally higher than what we have detected in the Ebro delta, e.g., 92.2% in New Mexico, 63.6% in New Jersey, 84.6% in mallard from Illinois and 80% in South Dakota (Weingarten 1986; Widjeskog 1987; Illinois Department of Conservation 1988; Simpson 1989), although the method used for determining the compliance percentage in those cases was different from ours, and consisted in counting Pb and steel shot shell wads found in the field. With a methodology similar to ours (i.e. analysing steel and Pb embedded shot in hunted waterfowl), Havera et al. (1994) studied the waterfowl hunter compliance with non-toxic ammunition shot regulations in mallard and geese (*Branta canadensis*) from zones where Pb ammunition is banned since 1986 in Illinois. They found, after five years, minimum hunter compliance of 98.9% (mallard) and 96.5% (geese), and minimum hunter noncompliance of 1.1% (mallard) and 1.8% (geese). These rates are similar than what was observed at the end of the present study. In the US and Canada, compliance with legislation appears to be high, which has been attributed to the support of waterfowl hunters and conservation police officers to the non-toxic shot programme (Anderson et al. 2000; Stevenson et al. 2005). Furthermore, once was implemented the total ban on the use of Pb shot for hunting waterfowl, industry responded by developing high quality non-toxic shot shells. On the other hand, the European Union allowed member states to determine their policy on the use of Pb shot, simply encouraging but not requiring them to phase out the use of Pb shot by 2000 (Thomas & Guitart 2010).

The minimum hunter non-compliance found for mallards from the Ebro delta was much lower than the 68% (2001-2002) and 70% (2008-2010) values reported in England for mallards after the ban of Pb ammunition (Cromie et al. 2002, 2010). We must take into account that, whereas we considered that only animals with Pb and not steel embedded pellets had been recently killed with Pb ammunition, Cromie et al. (2002, 2010) distinguished between old and new Pb pellets at necropsy, and thus the source data to calculate non-compliance ratios were not obtained using the same method. Nevertheless, if we considered all the individuals with Pb embedded (both with only Pb and with Pb + steel) the noncompliance in the Ebro delta would be 20.1%, still clearly lower than values from England. Contrary to the Ebro delta, the ban of Pb shot in England is for all the wetland sites, so the percentage of birds without embedded Pb shot should be higher in England than in the Ebro delta.

During the first season of monitoring we measured relatively high percentages of embedded Pb shot in waterbirds (27%). This apparent inefficacy of the ban of Pb use led to the local authorities to notify that, if this elevated presence of Pb shot in hunted birds remained during the next season, all hunting practices would be banned in protected wetlands. As a consequence, a marked decrease in the percentage of embedded Pb shot (≤2%) was observed in the 2008-2009 and subsequent hunting seasons. During these seasons steel ammunition, which was not used before the ban, gradually replaced Pb and this have been followed by an increase of the presence of steel shot ingestion in several waterbird species (up to 30% in *Aythya ferina*).

One of the arguments used by hunters against replacing Pb ammunition by non-toxic alternatives is that Pb shot tend to remain embedded in the bodies, while the steel ones pass through, leaving badly injured birds without killing them. In contrast, we found that the number of embedded pellets in mallards was similar in individuals shot only with Pb and in those shot with steel. The efficiency of steel shot has been investigated in several scientific studies, showing that efficiency is more related to hunters experience and shooting distances rather than to the performance of the cartridge, and in turn that the performance of the cartridge is more critical than the shot material itself (Kanstrup & Andersen 2010). In addition, we have observed that large species are generally more likely to carry embedded shot than small ones. Previous studies have demonstrated a relationship between body size and pellet incidence, which could be explained because large birds have a greater surface area for pellets to hit than small ones (Elder 1955; Evans et al. 1973).

The establishment of Pb hunting restriction and reinforcement by authorities has been linked to a reduction in illegal Pb shooting, with an increasing legislative compliance since the 2007-2008 hunting season. Although we were in the 2007-08 hunting season far from achieving the compliance levels obtained in the US, there has been a positive trend regarding the replacement of Pb by non-toxic ammunition. The prevalence of Pb has dropped over the hunting seasons, and the use of steel has grown from zero to 64.3% of hunted birds, plus an additional 10.9% of birds carrying both steel and Pb.

4.2 Effects on waterfowl health

We have observed clear inter-specific differences in prevalence of Pb shot ingestion, which may be explained from variability among species in grit consumption as was already observed in the studies of the nineties (Mateo et al. 2000b). The quantity of grit in the gizzard depends on the phenotype of species, and grit size is a good predictor of Pb shot ingestion (Figuerola et al. 2005). In this regard, mallards are very useful for monitoring Pb poisoning because of their high population abundances and nearly worldwide distribution (Guitart et al. 1994), as well as because the prevalence of Pb shot ingestion in this species is moderate to high among waterfowl (Mateo 2009). The prevalence of Pb shot ingestion in the 2007-08 hunting season (28.6%) was not different from the pre-ban value (30.2%; Mateo et al. 2000b). A significant decrease in the prevalence of Pb shot ingestion was found in the following seasons (15.5%). Prevalence of steel shot ingestion was 10%. Among mallards, the total percentage of pellet ingestion was 21%.

As could be expected and according to the high compliance observed, prevalence of Pb shot ingestion in the US is lower than in Europe. White and Stendell (1977) sampled several natural areas nationwide during the 1974-75 hunting season (before the nationwide Pb ban but after the implementation of regulation in several states) and found 7.8% and 3.6% of Pb and non-toxic shot ingestion, respectively. Anderson et al. (2000) analysed birds from the Mississippi flyway during the 1996-1997 hunting season, 5-6 years after nationwide ban of Pb shot in 1991, and reported a 2.8% and 6.1% of Pb and non-toxic shot ingestion, respectively. Our results show, compared to these surveys in the US, a higher prevalence of Pb shot ingestion in the Ebro delta despite the ban, as well as a higher prevalence of steel ingestion, which could be explained because the total percentage of shot ingestion is higher too.

Besides possible breaches of the prohibition, there are several reasons for high prevalence of Pb shot ingestion in waterbirds. First, the high density of accumulated Pb shot in sediments of wetlands where waterbirds live and are hunted. Due to the inert nature of Pb the rate of Pb shot decomposition has been estimated to be up to 300 years (Jorgensen & Willems 1987). Thus, Pb gunshot may remain accessible to feeding waterbirds long after deposition (Mudge 1984; Rooney et al. 2007). Second, the low availability of grit (grain particles or gastroliths) in sediments of many wetlands increases the risk of Pb shot ingestion (Pain et al. 1992; Mateo et al. 1999). Shot are confused with grit which birds need for the proper functioning of the gizzard (Pain 1990; Mateo et al. 2000b). Furthermore, in the Ebro delta Pb is still legally deposited in areas accessible to waterfowl, with high risk of exposure and ingestion, particularly on rice fields where many waterbirds go to feed. Rice fields are commonly located in areas adjacent to protected wetlands, acting as temporary wetlands when they are flooded. Mateo et al. (2000b) found that the proportion of ingested grit with size >2-3 mm was positively correlated to rice ingestion, as well as to the prevalence of Pb shot ingestion, and these two variables were positively correlated between them. Thus, species like the mallard feeding on rice (76.3% of occurrence of rice in mallard gizzards) have larger grit in the gizzard and higher Pb shot ingestion prevalence (30.2%) than herbivorous species like the Eurasian wigeon (36% of occurrence of rice and 4% of Pb shot prevalence in gizzards) (Mateo et al. 2000b). Prevalence of Pb shot ingestion in birds was no different between lagoons, where Pb shot use is banned, and rice fields, where it is allowed. This could be due to the fact that, regardless of where they were killed, birds move frequently around the territory. On the contrary, steel shot ingestion was higher in lagoons than in rice fields, which shows the change to the use of non-toxic ammunition in protected wetlands. In essence, this long-term shot ingestion study indicates that birds continue to ingest shot pellets at a relative high proportion, but Pb shot seems to be slowly being replaced by non-toxic pellets.

As a toxic heavy metal, Pb from shot ingestion can affect virtually every physiological system in animals (Franson & Pain, 2011). Ragarding this, Pb residues in tissues have been generally accepted as a good criterion for evaluating Pb exposure in waterfowl, and we have observed a good concordance between prevalence obtained by gizzard examination and liver Pb concentration. The International Association of Fish and Wildlife Agencies (IAFWA) proposed the finding of liver concentrations of 2.0 µg/g wet weight of Pb (approximately 5 µg/g d.w. of Pb) in ≥5% of samples as a decision criterion for recommending conversions to non-toxic shot for waterfowl hunting (United States Fish and Wildlife Service 1986). We have found concentrations over 5 µg/g of d.w. (associated to measurable physiological effects) in ≥5% of samples in Northern pintail (100%), common pochard (33.3%), mallard (20.4%), common teal (10.4%), Eurasian wigeon (9.1%) and Northern shovelers (5.4%), representing more than half of the studied species. Pb liver concentrations in the Ebro delta are in order of those described in other Mediterranean wetlands such as the Evros delta and the Camargue (Pain & Handrinos 1990; Pain et al. 1992, respectively). On the other hand, all mallards analyzed in the Goskü delta in Turkey (Ayas & Kolankaya 1996) had <5 µg/g d.w. in liver.

4.3 Effects on human health

Pb gunshot can suffer a fragmentation on impact with game birds, contaminating their muscle and increasing exposure to Pb of human consumers of game (Frank 1986; Scheuhammer et al. 1998; Pain et al. 2010). Recent studies (Mateo et al. 2011; Green & Pain 2012) have shown the risk for humans of consuming game birds shot with Pb ammunition.

The European Union does not establish any safety limit of Pb concentrations in game meat or other tissues (European Commission, 2006) and, although consumption of game meat by humans is generally low, it can locally reach relevant levels especially among hunters and their families. Given this lack of safety limits for game birds, we may assume the criteria established for poultry offal (1.5 µg/g d.w.) and meat (0.3 µg/g d.w.).

We found that seven out of the 10 studied species presented individuals with >1.5 µg/g d.w. of Pb in liver, and that eight of the studied species presented individuals with >0.3 µg/g d.w. of Pb in muscle. In both cases Northern pintail presented the highest percentages of individuals over these safety limits (100% and 86.7%, respectively) and common coot the lowest ones (1.1% and 7.7%, respectively). This means that a portion of the population is consuming meat and tissues with Pb concentrations above safety limit. Green and Pain (2012) used food consumption in the United Kingdom and Pb concentration data to estimate risks of game bird consumption. They found that consumption of <1 meal per week may be associated with a reduction in the Intelligence Quotient in children, and 1.2–6.5 meals per week may be associated with increased systolic blood pressure, occurrence of chronic kidney disease, and increased rates of spontaneous abortion. Moreover, cooking treatment can affect Pb bioaccessibility in meat of animals with embedded shot, sometimes magnifying the risk of consumption of game birds hunted with Pb ammunition (Mateo et al. 2011).

Pb muscle concentrations decreased significantly since the ban and Pb level was determined by the presence of (a) ingested and (b) embedded Pb shot. Thus, the decrease of Pb concentrations in muscle results not only from increasing compliance with the prohibition of Pb but also from decreasing prevalence of Pb shot ingestion. In this regard, analysis of meals prepared using meat from game birds showed the Pb concentration in meals was significantly correlated with the number of shot visible in X-rays (Pain et al. 2010). Regarding liver, most studies coincide with ours in pointing that Pb concentrations are usually linked to ingestion of Pb shot (e.g. Mateo et al. 1998, 2001; Taggart et al. 2009), which in the Ebro delta is also decreasing as a consequence of the ban, although at a slower pace than embedded shot.

Although the results of this study show a positive trend in the enforcement of the ban of Pb ammunition in protected wetlands, this does not seem enough to resolve the great environmental problem that Pb is still today. Pb gunshot deposited in protected wetlands remains in the environment, and the problem continues increasing in areas where hunting with Pb ammunition is still allowed. Our results reflect the effort that is being made by hunters in certain zones. The involvement of hunters is necessary to ameliorate the impacts of Pb on wildlife in general, and on game species in particular, and thereby educational programs for hunting community should be carried out to promote a collaborative environment (Friend et al. 2009). These programs should also highlight the potential risk that hunting with Pb poses to human health, especially to foetuses and children (Stroud & Hunt 2009). We propose two remediation measures: i) at the short-term, an extension of the ban on Pb to those rice fields that act as temporary wetlands, and ii) at the long-term, a complete restriction in the use of Pb ammunition in favour of other available non-toxic alternatives.

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6. Conclusions

1. Compliance with the ban on Pb has increased during the 2007-2011 period, especially as a consequence of the surveillance.
2. Although Pb shot ingestion has decreased after the ban, moderate prevalence of Pb shot ingestion by waterfowl is still found, thus affecting the health of birds.
3. The higher prevalence of steel shot ingestion in protected lagoons than in rice fields reflects the compliance with the ban at these protected sites.
4. In spite of the decrease related to reduced percentages of embedded and ingested Pb after the ban, Pb concentrations in bird muscle and liver, especially in those species showing higher ingestion prevalence, exceed in occasions the maximum safety limits for human consumption.
5. Northern pintail and common pochard, species feeding largely rice, present higher prevalence of lead shot ingestion, higher lead concentration in liver and muscle, and higher percentage of individuals with concentrations above maximum safety limits.
6. Our results show the need of the extension of the ban to the rice fields, because these sites are used by birds for feeding and the continued use of Pb shot there reduces the outcome of the implemented regulation.

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