PRESENCE OF PELLETS AND HEPATIC LEAD LEVELS IN GAME BIRDS HUNTED WITH LEAD-FREE AMMUNITION

Report prepared by

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1 INTRODUCTION

1.1 ENGAGEMENT AND AIM

This report was commissioned by the Spanish Sectoral Federation of Weapons and Ammunition (FSA) and it aims to further broaden the report submitted in 2019 entitled "Incidence and Repercussion of Lead Pellets Introduced into the Terrestrial Environment on Terrestrial Game Birds" (hereinafter referred to as the "2019 Report") by adding an additional variable to include hunting with firearms (shotguns) using ammunition other than lead (steel) to obtain samples. This engagement therefore intends to provide a response to the third conclusion and fifth recommendation contained in the 2019 Report, which are quoted below:

- Third conclusion: "There are doubts about the possible existence of samples with Pb fragments embedded in their tissues, especially given the high concentrations found in some specimens coinciding with an absence of any clinical signs. New studies on other tissues may clear up this doubt."
- Fifth recommendation: "Analysis of samples from game birds killed with lead-free ammunition."

In order to carry out the work, the FSA entered into agreements with:

- University of Murcia (UMU): Diego Romero García (Tenured Professor at the University of Murcia).
- Juan Manuel Theureau de la Peña and Antonio de José Prada (Forest Engineers).

The following people have also collaborated:

- Juan Bautista Torregrosa Soler (Tenured Professor at the Polytechnic University of Valencia) and Andrés Ferrer Gisbert (Tenured Professor at the Polytechnic University of Valencia).

The engagement's other specifications state that:

- An assessment should be carried out of the game birds that threw up data with higher Pb concentrations by sampling group in the 2019 Report.
- The study should be conducted by attempting to obtain samples from the same territories and/or conditions chosen by game bird species as the ones used for the 2019 Report.

This report offers the final results obtained in order to be able to assess all the information obtained to date.

1.2 RESEARCH PHASES

- Review of the bibliography. Done by Antonio de José Prada.
- Fieldwork consisting of obtaining game specimens. Done by Antonio de José Prada and Juan Manuel Theureau de la Peña.

- Primary data collection and sample taking, consisting of measuring several physical parameters of the game specimens and processing them to obtain samples from each specimen. Done at the University of Murcia by Diego Romero García.
- Obtaining secondary values (chemical analysis of biological samples and physical analysis of crops, gizzards and intestines) done at the University of Murcia by Diego Romero García.
- Analyses and conclusions. Done by Antonio de José Prada, Diego Romero García, Juan Manuel Theureau de la Peña, Juan Bautista Torregrosa Soler and Andrés Ferrer Gisbert.

1.3 SIMILAR PRECEDING STUDIES

Though the scientific studies conducted to date on terrestrial environment birds and the possible effect of lead pellets on their populations are rare, the studies which obtain samples from specimens shot with ammunition other than lead are even rarer, seeing as the immense majority of samples are obtained from specimens hunted down with lead-based ammunition.

In the review conducted for the 2019 Report, only one of the 13 papers found on terrestrial environment birds had obtained its samples from specimens hunted down with ammunition other than lead. Of the aforementioned 13 papers, it turned out that in eight of them the specimens had been obtained using lead-based ammunition (61.5%), in four of them the specimens had been found dead (30.8%), in two of them the specimens were not specified (15.4%) and only in one of them (7.7%) had the specimens been obtained from birds hunted down with steel and lead-based ammunition.

2 TIME FRAMES OF THE RESEARCH WORK

The work began in July 2019 by choosing the multidisciplinary team to determine the methodology and the aims to be pursued.

The species were chosen in the initial phase, along with the sampling areas based on them. The action protocol established for collecting samples in the field and the analyses to be conducted on the specimens obtained over the course of the study was the same as the one used in the preceding study and was as set out in the 2019 Report.

The first game specimens were obtained in August 2019 (2019/20 hunting season). The batches and number of samples selected for this study were completed in January 2020 (the same 2019/20 hunting season).

The processing and the analyses of specimens overlapped during this time and were completed in March 2020.

3 FIELDWORK

Taking into account the findings obtained by batches for the different species in the 2019 Report, an effort was made to obtain a similar number of game specimens per batch as in the preceding study (2019 Report) in order to ensure the study would be as equally representative.

The quantifiable variables observed were as follows:

- Species
- Method of capture
- Territory
  - General characteristics
  - Environmental contamination
- Hunting intensity

3.1 DEFINITION OF THE DIFFERENT VARIABLES

3.1.1 *SPECIES Variable*

Species chosen

Game bird species having a terrestrial environment habitat which require grit in their digestive process, preferably granivores, were chosen from the range of possible species susceptible of being sampled. More specifically, an effort was made to replicate the batches in which the presence of lead in the liver was higher in the 2019 Report's results, but this time hunting them with steel ammunition.

Table 1. *Species chosen for this study*

	Sedentary	Summer migratory	Other (**)
Gallinaceans	*Alectoris rufa*	*Coturnix coturnix (*)*	-
Pigeons	-	-	*Columba palumbus*

*Not strictly migratory. ** *Columba palumbus* has territories in which it is permanently present, present in the summer, and wintering populations with marked migration routes.

The replicated batches amounted to three, one for each of the species appearing in Table 1. Said batches coincided with the ones in the 2019 Report that were found to have a higher number of specimens with lead concentrations in the liver above the limit set as environmental contamination (> 0.65 µg/g). See Table 2.

It should be noted the study's possibilities were limited to three batches, as it was not possible to replicate the other batches considered in the 2019 Report, even though it would have been of interest to do so.

The 2019 Report's replicated batches and results were the following.

Table 2. *Percentage of specimens with a lead concentration higher than 0.65* µg */g in 2019 Report*

	Origin of the batch	n	% of specimens (> 0.65 µg/g)
Coturnix coturnix	**Zamora**	**31**	**6.5**
Alectoris rufa	Alicante	36	5.6
Alectoris rufa	Navarre	26	0.0
Alectoris rufa	Zamora	30	0.0
Alectoris rufa	Valencia	30	0.0
Alectoris rufa	**Ciudad Real**	**97**	**9.3**
Columba palumbus (H.C.)	Zamora	30	3.3
Columba palumbus (H.C.)	Alicante	30	0.0
Columba palumbus (H.C.)	Madrid	30	6.7
Columba palumbus (S.)	**Zamora**	**12**	**8.3**

H.C. – Half-Closed Season S. – General Season

Table 3. *Pb concentrations (µg/g, wet weight) in the liver in 2019 Report for replicated batches*

	n	Geometric mean±SE	% of specimens (> 0.65 µg/g)
Common quail	31	0.121±0.213	6.5
Red-legged partridge	97	0.065±0.166	9.2
Common woodpigeon	12	0.018±0.230	8.3

Number of species chosen per batch

A stratified sampling was conducted due to the size of the population. To achieve this, the management unit (game reserve) was deemed as a stratum or segment, and the target species were captured on a random basis within each unit (stratum).

It was considered that specimens from the same place would show a similar contamination profile in order to the determine the size of the sample. The availability of samples is usually a limiting factor in environmental contamination monitoring studies. Numerous studies are therefore conducted based on a small number of samples, especially when they deal with well-preserved tissues or organs. It is assumed in this study that 30 samples of the same species per strata are enough to provide information on lead levels in tissues. The distribution of the specimens obtained is shown in Table 4 below:

Table 4. *Samples foreseen and obtained in the different sampling locations*

Area	Species	Type	Samples foreseen	Samples obtained	Batch
Castilla y León	*Coturnix coturnix*	Wild	30	32	Complete
	Columba palumbus	Migratory	30	31	Complete
Castilla-La Mancha	*Alectoris rufa*	Acclimated	30	31	Complete
			90	**94**	

All the batches exceeded the 30 specimens foreseen. This was because we did not wish to subjectively discard specimens exceeding the 30-specimen limit when the last specimens of a batch were delivered at the same time by the hunters.

Total number of species

The total number of game specimens on which a study was finally performed amounted to 94, which were distributed by species as follows:

- *Alectoris rufa* 31 specimens
- *Coturnix coturnix* 32 specimens
- *Columba palumbus* 31 specimens

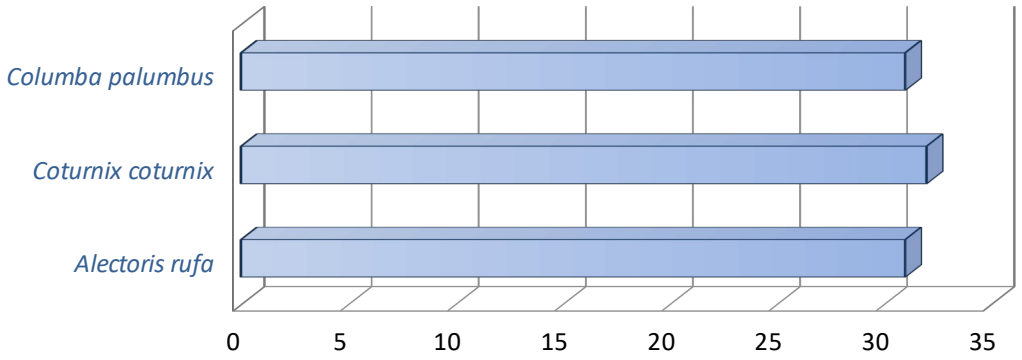


Figure 1. *Distribution of the number of game specimens by species*

Identification code designation

The code used to identify each of the game specimens captured is made up of several sub-codes:

AA-BB-CC-00

AA: species and type (PA: acclimated partridge, CS: wild quail, PT: common woodpigeon)
BB: province of capture (ZA: Zamora, CR: Ciudad Real)
CC: month captured (in Roman numerals)
00: sort number of the sample (in Arabic numerals)

3.1.2 *METHOD OF CAPTURE variable*

The 94 specimens sampled were all obtained through the use of firearms. The ammunition used in all cases was steel shot of different calibres: number 6 and 7 pellets with a 30-gram charge for common quails and common woodpigeons, and number 6 pellets with a 32-gram charge for red-legged partridges.

3.1.3 *TERRITORY variable*

General characteristics

A brief summary is set out below of the main characteristics (approximate values) of each of the areas in two regional authorities where the specimens were captured:

Castilla y León:

The game specimens were obtained from the province of Zamora.

The samples from the province of Zamora were obtained from two management units (game reserves). One of them is situated within the municipal boundaries of Palacios del Pan, more precisely in the private game reserve registered as ZA-10303. The other is located within the municipal boundaries of Zamora, more precisely the private game reserve registered as ZA-10719. In general terms, they could be considered as a single stratum, since the distance between one area and the other is just 20 kilometres. However, they will be differentiated for descriptive purposes due to the proximity of the private game reserve registered as ZA-10719 to the city of Zamora:

Table 5. *General characteristics of the ZA-10303 and ZA-10719 Stratums*

	ZA-10303	ZA-10719
Surface area (ha)	1280	1354
Average rainfall (mm)	474.3	360.8
Average temperature (°C)	11.7	11.8
Warm period (months)	0	0
Cold or frosty period (months)	6	8
Dry or arid period (months)	3.5	4
Altitude (m)	720	640
Protected spaces	NO	NO
Land uses	80% agricultural – 20% forestry	97% agricultural – 3% forestry
Main agricultural crop	Dryland cereals	Irrigated cereals

Castilla-La Mancha:

The game specimens were obtained from the province of Ciudad Real.

The samples from the province of Ciudad Real were obtained from two management units (game reserves). Both of them are located within the same local authority, Torre de Juan Abad. Due to the proximity of the two management units (CR-10420 and CR-10517), they will be considered as a single stratum for the purpose of the territory's characteristics.

Table 6. *General characteristics of the CR-10420-CR-10517 Stratum*

	CR-10420 - CR-10517
Surface area (ha)	1544
Average rainfall (mm)	525.6
Average temperature (°C)	13.6
Warm period (months)	2
Cold or frosty period (months)	6
Dry or arid period (months)	4
Altitude (m)	820
Protected spaces	NO
Land uses	65% agricultural – 35% forestry
Main agricultural crops	Dryland cereals, grapevines and olive trees

Table 7. *Distribution of game specimens captured*

Regional Authority	Province	Local Authority	Management unit	No. of specimens	Species
Castilla y León	Zamora	Palacios del Pan	ZA-10303	32	*Coturnix coturnix*
				11	*Columba palumbus*
		Zamora	ZA-10719	20	*Columba palumbus*
Castilla-La Mancha	Ciudad Real	Torre de Juan Abad	CR-10420	24	*Alectoris rufa*
			CR-10517	7	*Alectoris rufa*

The distribution of the total number of birds captured by provinces and local authorities is shown in the following graphs (Figures 2 and 3):

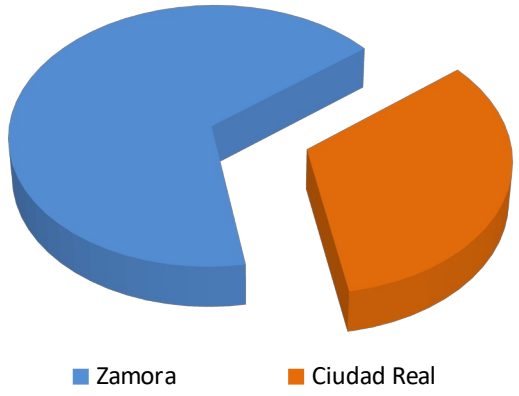


Figure 2. *Distribution of the number of game specimens by provinces*

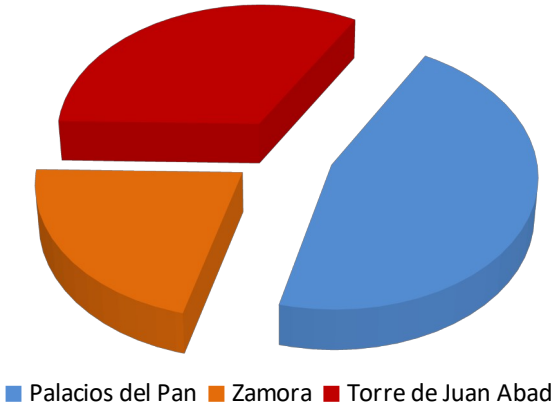


Figure 3. *Distribution of the number of game specimens by local authorities*

Environmental contamination

We considered as a variable the presence of environmental lead (soil) — where cumulative environmental contamination may affect the chemical or biological values of

the living being populations to be found in said areas — based on the Geochemical Atlas of Spain of the Geological and Mining Institute of Spain, which is dependent on the Government of Spain's Ministry of Science, Innovation and Universities (http://www.igme.es/actividadesIGME/lineas/CartoGeo/geoquimica/geoquimicaEsp.htm).

More specifically, in the case which concerns us here, the following values for lead (Pb) were found in the soil samples of the different management units sampled, which are associated to the local authority (Table 8).

Table 8. *Parts per million of Pb in soil samples*

Regional Authority	Province	Local Authority	Management unit	Pb (ppm)
Castilla y León	Zamora	Palacios del Pan	ZA-10303	20.7 - 32.0
		Zamora	ZA-10719	17.0
Castilla-La Mancha	Ciudad Real	Torre de Juan Abad	CR-10420	69.3 - 69.7
			CR-10517	

The following graph (Figure 4) shows from least to most the concentrations found in soil samples by local authorities according to the Geochemical Map of Spain:

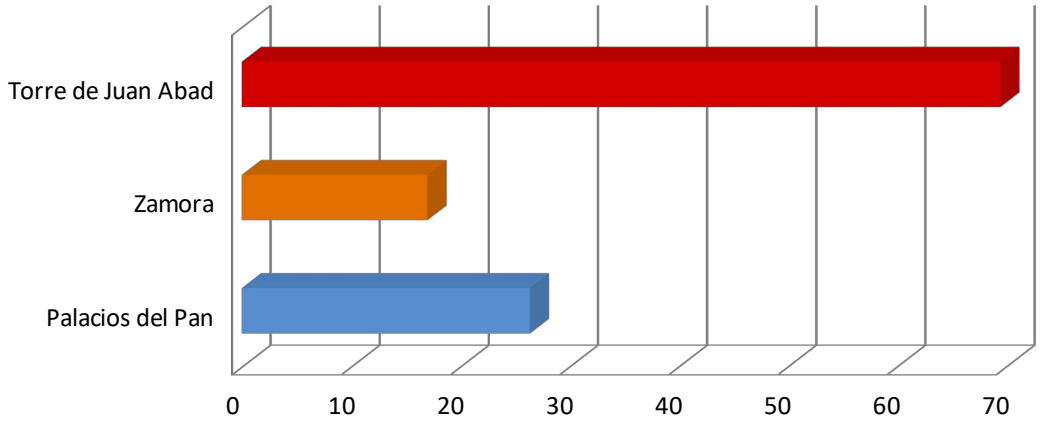


Figure 4. *Distribution of lead concentrations (ppm) in soil by local authorities*

3.1.4 *HUNTING INTENSITY variable*

The number of gunshots per hectare and thus the weight of lead pellets which end up in the terrestrial environment can be estimated on the basis of the captures declared by the owners or users of the different management units where hunting activities are carried out (game reserves). The following have thus been estimated per management unit taking the most unfavourable scenario into consideration, pellet cartridges weighing 34 g:

Table 9. *Intensity of gunshots per hectare and season in management units*

Management unit	Gunshots/ha and season	g of Pb/ha and season
ZA-10303	2.01	68.34
ZA-10719	3.32	112.88
CR-10420	24.61	836.74
CR-10517	24.61	836.74

The graph below (Figure 5) shows from least to most the estimated amounts of lead in grams per hectare and hunting season which are supposedly introduced into the environment:

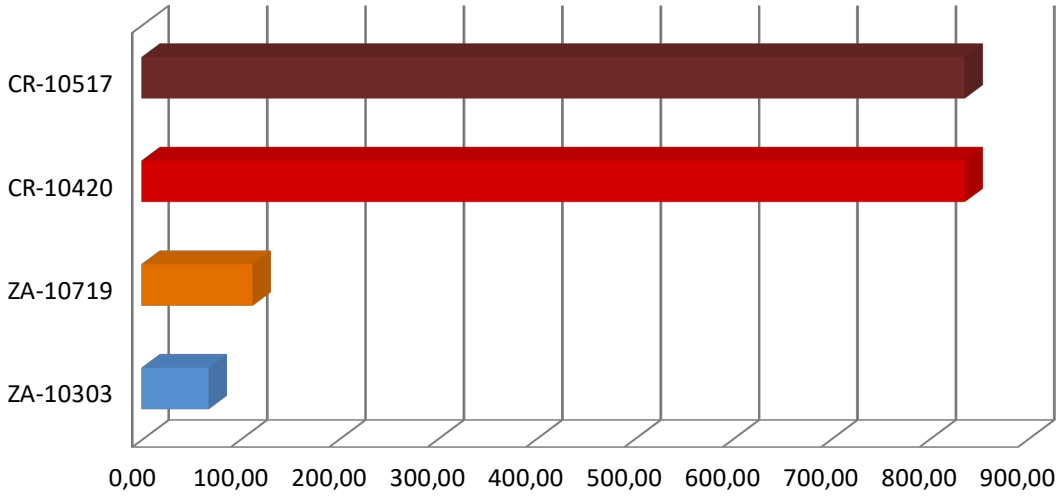


Figure 5. *Weight of lead from cartridges in gr/ha and season*

3.2 SUMMARY OF THE VARIABLES

Table 10 contains a summary of the different variables determined for this study:

Table 10. *Summary of the variables considered associated to codes and number of samples*

Species	Type	Capture	Management unit	Contamination	Hunting intensity	Code	N
Alectoris rufa	Acclimated	Hunted	CR-10420	69.3 - 69.7	High	PA1-CR	24
	Acclimated	Hunted	CR-10517	69.3 - 69.7	High	PA2-CR	7
Coturnix coturnix	Wild	Hunted	ZA-10303	20.7 - 32.0	Low	CS-ZA	32
Columba palumbus	Wild	Hunted	ZA-10303	20.7 - 32.0	Low	PT1-ZA	11
	Wild	Hunted	ZA-10719	17.0	Low	PT2-ZA	20

3.3 ACTION PROTOCOL TO OBTAIN SPECIMENS

3.3.1 *Obtaining specimens in the field*

The specimen batches were obtained from captures, taking advantage of the hunting season at the game reserves.

To do so, the team responsible for getting the specimens first obtained (depending on the programme) the relevant permits or commitments from the different game reserves or groups of hunters.

All collaborating hunters were given the relevant instructions beforehand. The percentage of samples obtained by the technicians in charge of the sampling amounted to 69.1% (65 game specimens), while 30.9% (29 game specimens) were obtained by collaborating hunters.

3.3.2 *Shipment of specimens*

In all cases, one of the two people responsible for the fieldwork went to the place of capture to take samples directly or collect recently hunted specimens.

The specimens were then shipped in portable refrigerators on the same day and all the specimens were frozen in bags and labelled according to the place of capture and date they were hunted. As mentioned above, the place and date of capture were recorded on the bag label.

Once a batch was completed, it was shipped by road from Valencia or Zamora to the University of Murcia by the people responsible for the fieldwork. The samples were removed from the freezers and shipped from Valencia (a trip of 2 hours and 15 minutes) in portable refrigerators. From Zamora (a trip of 6 hours), they were transported in WAECO Coolfun refrigerator freezers, which generate cold through an electrical connection to the vehicle.

4 PRIMARY DATA AND SAMPLE TAKING

4.1 MATERIALS USED

The materials used for this action are set out below:

- Gloves
- Self-closure bags
- Indelible marker pen
- Scissors
- Tweezers
- Scalpel
- Conical microtubes (*Eppendorf* 1.5 ml)
- Sample containers (50 and 100 ml)
- Scales (accuracy of 1 gr)
- Refrigerator
- Freezer

4.2 PROTOCOL FOLLOWED

The specimens were checked on a workbench equipped with a nearby sink according to the following protocol:

- **Weighing the specimen**: Each specimen was weighed at origin prior to being frozen.
- **General visual examination** of the specimen to note down any observations that might turn out to be relevant.

- **Examination of primary remiges' moulting stage**. It was recorded if the moulting was complete or which feather of the remiges was in the process of moulting. To determine which primary remiges were moulting or which was the last one to be shed, the number of primary remiges was counted (if one had been recently shed) and the wing's internal feathers covering the shaft were pulled out. After counting the feathers, the moulting remiges were recorded. In case of any doubt, the left wing's remiges were pulled out to examine the shafts' insertion into the wing. If it was observed that one was more tender, that feather was recorded as the last one to undergo moulting. If any feather had recently been shed without any signs of regrowth, said feather was recorded as the moulting remiges.
- **Examination of feather wear in farm-bred red-legged partridges acclimated** to the site. Partridges acclimated to the wild before the hunting season generally show very characteristic damage to the feathers. It can even be clearly seen which remiges have moulted in the wild and which have moulted at the farm. The marks on primary and secondary remiges of farm partridges were recorded as abundant, a few, scarce or none. If the marks were clearly visible, the first remiges which had moulted in the wild were recorded.
- **The specimen's age**. After the aforementioned examinations were done, we then proceeded to assign the specimen's age, differentiating between young (0+) and adult (>1+), without prejudice to any observations related to age due to double spurs or any other sign which allows an age above 2+ to be attributed.

4.3 SUMMARY OF PRIMARY DATA RESULTS

The data set out below were recorded for each specimen and subsequently compiled on a spreadsheet:

- The specimen's code
- Species
- Origin (management unit: game reserve registration number)
- Date of capture
- Nature (in this section a differentiation is made about whether the bird's origin is from wild or acclimated populations)
- Environment (in this section a first impression is recorded about the environment's population density, differentiating between rural and peri-urban environments)
- Age (differentiating between young or adult)
- Sex
- The specimen's weight
- Any other observations not indicated in the sections above

The summary by stratum is given in Table 11.

Table 11. *Hunted specimens' primary data*

Species	Management unit	O	N	En	A	S	W
Alectoris rufa	CR-10420	A	24	R	22/1	13/11	422.9
Alectoris rufa	CR-10517	A	7	R	6/1	5/2	461.8
Coturnix coturnix	ZA-10303	Wd	32	R	17/15	15/17	95.0
Columba palumbus	ZA-10303	Wd	11	R	1/10	6/5	453.3
Columba palumbus	ZA-10719	Wd	20	P	3/17	10/10	453.1

O: origin (Wd-wild; A-acclimated); N: number of specimens sampled; En: environment (R-rural; P-peri-urban); A: age (young/adult); S: sex (male/female); W: weight (in grams)

5 SECONDARY VALUES: ANALYSES, CROPS, GIZZARDS AND INTESTINES

5.1 PROTOCOL FOLLOWED

The samples (complete specimens) were received at the laboratory of the University of Murcia's Toxicology area in a frozen state (-20°C). Once they were identified and recorded, said specimens were kept in the same frozen state until their subsequent processing.

To begin with, X-rays were taken of all the specimens (game specimens), which were kept frozen throughout the process. To do so, said specimens were placed on X-ray plates at the facilities of the University of Murcia's Veterinary Clinical Hospital (Photographs 1, 2 and 3). Once the X-rays had been taken, the specimens were conserved once more at -20°C. The number of pellets in each specimen was recorded and any cases in which the pellets could be in the abdomen, thorax or an anatomical area compatible with crop were identified.

Each specimen was photographed before the samples were taken. The liver was weighed and samples were taken of the liver (n=2), kidney (n=1) and pectoral muscle (n=1) from each specimen. In order to do so, the animal was defrosted and the samples were immediately taken and placed into 1.5 ml microtubes. The samples were then frozen immediately to prevent the cold chain from breaking. Each of the specimens was then studied and photographs were taken during this process (Photographs 4, 5 and 6). A systematic search for pellets was conducted and each specimen's pellets were kept separately. One of the wings and the head were separated for subsequent studies (where appropriate). In the case of pellets located (according to the X-ray image) in areas that could potentially correspond to the crop, gizzard or intestine (see example in Photographs 7 and 8), a thorough examination of said organs was made, including opening them up and recording them in photographs (Photographs 9, 10, 11, 12, 13, 14, 15 and 16). This part of the study of the digestive apparatus organs was done in the way described in the 2019 Report. The chemical analysis of the liver was also done in the way described in the 2019 Report.

5.2 RESULTS

The data of each specimen are shown below (Tables 12, 13 and 14).

Table 12. *Common quail. Number of pellets found in each specimen and their anatomical location. Hepatic Pb concentration (µg/g, wet weight).*

Specimen	Weigh t	Sex	Age	Hepatic Pb	No. of steel pellets	Location of pellets (X-ray)	Presence of Pb pellets in digestive apparatus
CS-ZA-VIII-1	96.1	Male	Adult	0.182	0	-	Negative
CS-ZA-VIII-2	89.2	Female	Adult	0.327	0	-	Negative
CS-ZA-VIII-3	82.0	Male	Young	0.229	3	Thorax and abdomen	Negative
CS-ZA-VIII-4	81.7	Male	Young	0.159	1	Thorax	Negative
CS-ZA-VIII-5	101.0	Male	Young	0.387	1	Thorax	Negative
CS-ZA-VIII-6	100.6	Female	Young	0.312	1	Neck	Negative
CS-ZA-VIII-7	98.4	Female	Adult	0.832	0	-	Negative
CS-ZA-VIII-8	97.2	Male	Adult	0.121	3	Thorax	Negative
CS-ZA-VIII-9	93.9	Female	Young	0.185	3	Entry to thorax and abdomen	Negative
CS-ZA-VIII-10	81.8	Male	Young	0.160	1	Back limb	Negative
CS-ZA-VIII-11	115.7	Female	Adult	0.343	0	-	Negative
CS-ZA-VIII-12	88.8	Female	Young	0.103	1	Thorax	Negative
CS-ZA-VIII-13	97.6	Female	Young	0.891	1	Abdomen	Negative
CS-ZA-VIII-14	105.6	Female	Young	0.267	2	Head and thorax	Negative
CS-ZA-VIII-15	98.4	Female	Young	0.065	2	Head and thorax	Negative
CS-ZA-VIII-16	96.6	Female	Adult	0.164	0	-	Negative
CS-ZA-VIII-17	101.0	Female	Adult	0.229	2	Entry to thorax	Negative
CS-ZA-VIII-18	75.4	Female	Adult	0.105	0	-	Negative
CS-ZA-VIII-19	93.9	Male	Adult	0.142	0	-	Negative
CS-ZA-VIII-20	97.9	Male	Adult	0.207	1	Neck	Negative
CS-ZA-VIII-21	96.7	Male	Young	0.244	0	-	Negative
CS-ZA-VIII-22	103.8	Male	Young	0.291	2	Head	Negative
CS-ZA-VIII-23	102.4	Male	Young	0.136	0	-	Negative
CS-ZA-VIII-24	81.3	Male	Adult	0.098	0	-	Negative
CS-ZA-VIII-25	94.3	Male	Adult	0.199	1	Abdomen	Negative
CS-ZA-VIII-26	79.5	Male	Adult	0.168	2	Entry to thorax	Negative
CS-ZA-VIII-27	93.0	Male	Young	0.809	0	-	Negative
CS-ZA-VIII-28	85.4	Female	Young	1.052	0	-	Negative
CS-ZA-VIII-29	97.1	Female	Adult	0.137	0	-	Negative
CS-ZA-VIII-30	91.2	Female	Young	0.315	1	Peri-cloacal area	Negative
CS-ZA-VIII-31	112.1	Female	Young	0.247	0	-	Negative

CS-ZA-VIII-32	102.2	Female	Adult	0.288	1	Head	Negative

Table 13. *Red-legged partridge. Number of pellets found in each specimen and their anatomical location. Hepatic Pb concentration (µg/g, wet weight).*

Specimen	Weight	Sex	Age	Hepatic Pb	No. of steel pellets	Location of pellets (X-ray)	Presence of Pb pellets in digestive apparatus
PA-CR-XI-1	419.8	Female	Young	0.086	2	Abdomen and peri-cloacal area	Negative
PA-CR-XI-2	354.3	Female	Indet.	0.097	1	Thorax	Negative
PA-CR-XI-3	436.0	Female	Young	0.012	4	Abdomen	Negative
PA-CR-XI-4	487.2	Male	Young	0.025	3	Thorax (or wing), peri-cloacal area and back limb	Negative
PA-CR-XI-5	450.6	Male	Young	0.134	4	Thorax, abdomen, and peri-cloacal area	Negative
PA-CR-XI-6	434.2	Male	Young	0.229	2	Wing and abdomen	Negative
PA-CR-XI-7	398.2	Female	Young	0.451	6	Abdomen, neck and peri-cloacal area	Negative
PA-CR-XI-8	405.1	Female	Young	0.132	3	Thorax and back limb	Negative
PA-CR-XI-9	488.8	Male	Young	0.052	7	Head, thorax and abdomen	Negative
PA-CR-XI-10	482.0	Male	Young	0.327	0	-	Negative
PA-CR-XI-11	399.8	Female	Young	0.096	2	Thorax and abdomen	Negative
PA-CR-XI-12	374.4	Female	Young	0.139	9	Thorax and abdomen	Negative
PA-CR-XI-13	415.2	Male	Young	0.084	15	Abdomen, thorax and head	Negative
PA-CR-XI-14	514.1	Male	Young	0.080	8	Wing, back limb, thorax, abdomen	Negative
PA-CR-XI-15	366.7	Female	Young	0.097	0	-	Negative
PA-CR-XI-16	535.1	Male	Young	0.012	1	Thorax	Negative
PA-CR-XI-17	439.5	Male	Young	0.021	0	-	Negative
PA-CR-XI-18	364.8	Male	Young	0.163	1	Neck	Negative
PA-CR-XI-19	422.9	Female	Adult	0.064	2	Entry to thorax and abdomen	Negative
PA-CR-XI-20	503.0	Male	Young	0.077	5	Thorax, abdomen, neck and back limb	Negative
PA-CR-XI-21	267.1	Female	Young	0.138	1	Thorax	Negative
PA-CR-XI-22	371.8	Female	Young	0.088	13	Thorax and abdomen	Negative
PA-CR-XI-23	428.0	Male	Young	0.092	4	Thorax and abdomen	Negative
PA-CR-XI-24	471.5	Male	Young	0.185	2	Wing and neck	Negative
PA-CR-XI-25	419.2	Female	Young	0.050	0	-	Negative
PA-CR-XI-26	493.9	Male	Young	0.128	3	Neck and abdomen	Negative
PA-CR-XI-27	431.4	Male	Young	7.189	10 (*)	Abdomen, back limb, peri-cloacal area	Positive
PA-CR-XI-28	512.4	Male	Young	0.068	5	Abdomen and wing	Negative
PA-CR-XI-29	462.1	Female	Adult	0.058	4	Abdomen	Negative
PA-CR-XI-30	453.9	Male	Young	0.079	14	Thorax, abdomen, back limb, peri-cloacal area	Negative
PA-CR-XI-31	459.6	Male	Young	0.618	4	Thorax and abdomen	Negative

(*) 4 lead pellets

Table 14. *Common woodpigeon. Number of pellets found in each specimen and their anatomical location. Hepatic Pb concentration (µg/g, wet weight).*

Specimen	Weight	Sex	Age	Hepatic Pb	No. of steel pellets	Location of pellets (X-ray)	Presence of Pb pellets in digestive apparatus
PT-ZA-XI-1	424.3	Female	Adult	0.358	21	Thorax and abdomen	Negative
PT-ZA-XII-2	454.4	Male	Adult	0.037	0	-	Negative
PT-ZA-XII-3	430.0	Male	Young	0.216	2	Neck and thorax	Negative
PT-ZA-XII-4	435.7	Female	Adult	0.241	2	Thorax	Negative
PT-ZA-I-5	443.3	Female	Adult	0.347	0	-	Negative
PT-ZA-I-6	507.8	Male	Adult	0.260	6	Wing, abdomen and neck	Negative
PT-ZA-I-7	463.2	Male	Adult	0.259	0	-	Negative
PT-ZA-I-8	470.8	Male	Adult	0.304	0	-	Negative
PT-ZA-I-9	482.9	Male	Adult	0.348	1	Wing	Negative
PT-ZA-I-10	431.3	Female	Adult	0.478	0	-	Negative
PT-ZA-I-11	442.4	Female	Adult	0.311	1	Thorax	Negative
PT-ZA-I-12	443.6	Male	Adult	0.320	0	-	Negative
PT-ZA-I-13	465.6	Male	Adult	0.319	5	Head, neck and abdomen	Negative
PT-ZA-I-14	467.3	Female	Adult	0.280	3	Thorax and abdomen	Negative
PT-ZA-I-15	480.6	Male	Adult	1.230	2	Thorax	Negative
PT-ZA-I-16	376.6	Female	Young	0.267	7	Head, thorax and abdomen	Negative
PT-ZA-I-17	457.7	Female	Adult	0.289	4	Neck and thorax	Negative
PT-ZA-I-18	422.6	Male	Young	0.310	0	-	Negative
PT-ZA-I-19	372.8	Female	Young	0.234	6	Neck, thorax and abdomen	Negative
PT-ZA-I-20	500.2	Male	Adult	0.232	8	Neck, thorax and abdomen	Negative
PT-ZA-I-21	477.1	Indet.	Adult	0.308	5	Thorax	Negative
PT-ZA-I-22	483.7	Female	Adult	0.260	8	Thorax, back limb and abdomen	Negative
PT-ZA-I-23	472.8	Male	Adult	0.314	2	Head and thorax	Negative
PT-ZA-I-24	519.0	Female	Adult	0.230	1	Thorax	Negative
PT-ZA-I-25	477.8	Female	Adult	0.319	11	Thorax, back limb and abdomen	Negative
PT-ZA-I-26	425.2	Male	Adult	0.316	1	Neck	Negative
PT-ZA-I-27	461.0	Male	Adult	0.340	5	Thorax, abdomen, and peri-cloacal area	Negative
PT-ZA-I-28	426.0	Female	Adult	0.369	3	Wing and abdomen	Negative
PT-ZA-I-29	418.5	Female	Adult	0.569	8	Head, wing, thorax, abdomen and gizzard	Negative
PT-ZA-I-30	501.8	Male	Adult	0.638	4	Head, thorax and abdomen	Negative
PT-ZA-I-31	412.6	Female	Adult	0.288	2	Thorax	Negative

The descriptive statistics for the three species are shown in Tables 15 and 16.

Table 15. *Descriptive statistics of Pb concentrations (µg/g, wet weight) in the liver of quails, red-legged partridges and common woodpigeons*

	n	Geometric mean±SE	Minimum	Maximum	% of specimens (> 0.65 µg/g)
Coturnix coturnix	32	0.231±0.044	0.065	1.052	12.5
Alectoris rufa	31	0.103±0.229	0.012	7.189	3.2
Columba palumbus	31	0.304±0.035	0.037	1.230	3.2

Table 16. *Percentages of specimens with a presence of pellets in the digestive tract*

		Steel			Lead		
	n	Crop	Gizzard	Intestine	Crop	Gizzard	Intestine
Coturnix coturnix	32	0%	0%	0%	0%	0%	0%
Alectoris rufa	31	3.2% (1)	9.7% (3)	3.2% (1)	0%	3.2% (1)	0%
Columba palumbus	31	0%	6.5% (2)	3.2% (1)	0%	0%	0%

6 ANALYSIS OF RESULTS

6.1 ANALYSIS OF RESULTS FOR COMMON QUAILS

In the case of quails, the specimens that might have pellets in the crops according to the X-ray images were the following: CS-ZA-VIII-(8-9-14-17-26). No pellets were found in the crop of these specimens. Furthermore, the pellets that were in this area were made of steel.

In the case of quails having a high Pb concentration (>0.65 µg/G), the crop contents are shown below (the crop of specimen CS-ZA-VIII-28 was empty):

Specimen CS-ZA-VIII-7	Specimen CS-ZA-VIII-13	Specimen CS-ZA-VIII-27

Figure 6. *Details of crop contents of common quails CS-ZA-VIII (7, 13 and 27)*

The data and images taken of the common quail specimens whose hepatic Pb concentration was above 0.65 µg/g (wet weight) are shown below (Figures 7, 8, 9 and 10), taking the most restrictive value for Pb environmental exposure according to the scientific literature (Guitart et al., 1994; Ferrandis et al., 2008; Franson and Pain, 2011; Berny et al., 2015; Bingham et al., 2015), and considered in the 2019 study (Romero et al., 2019).

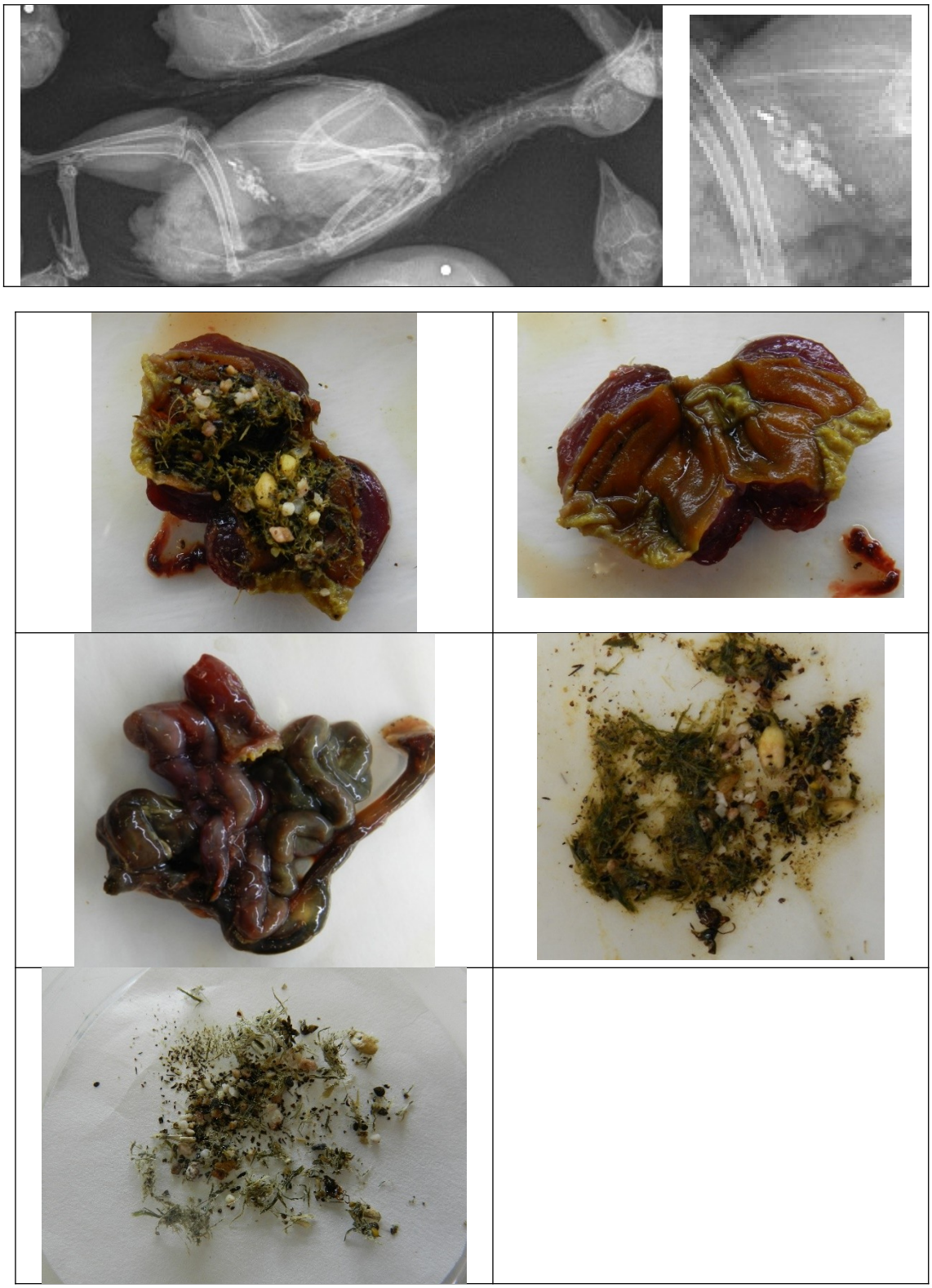


Figure 7. *SPECIMEN CS-ZA-VIII-7. Stratum ZA-10303. Date of capture 15/08/2019. Female. Adult. No. of pellets (according to X-ray): 0; Pb concentration (µg/g): 0.832 (liver); 14.471 (kidney); 0.038 (muscle tissue).*

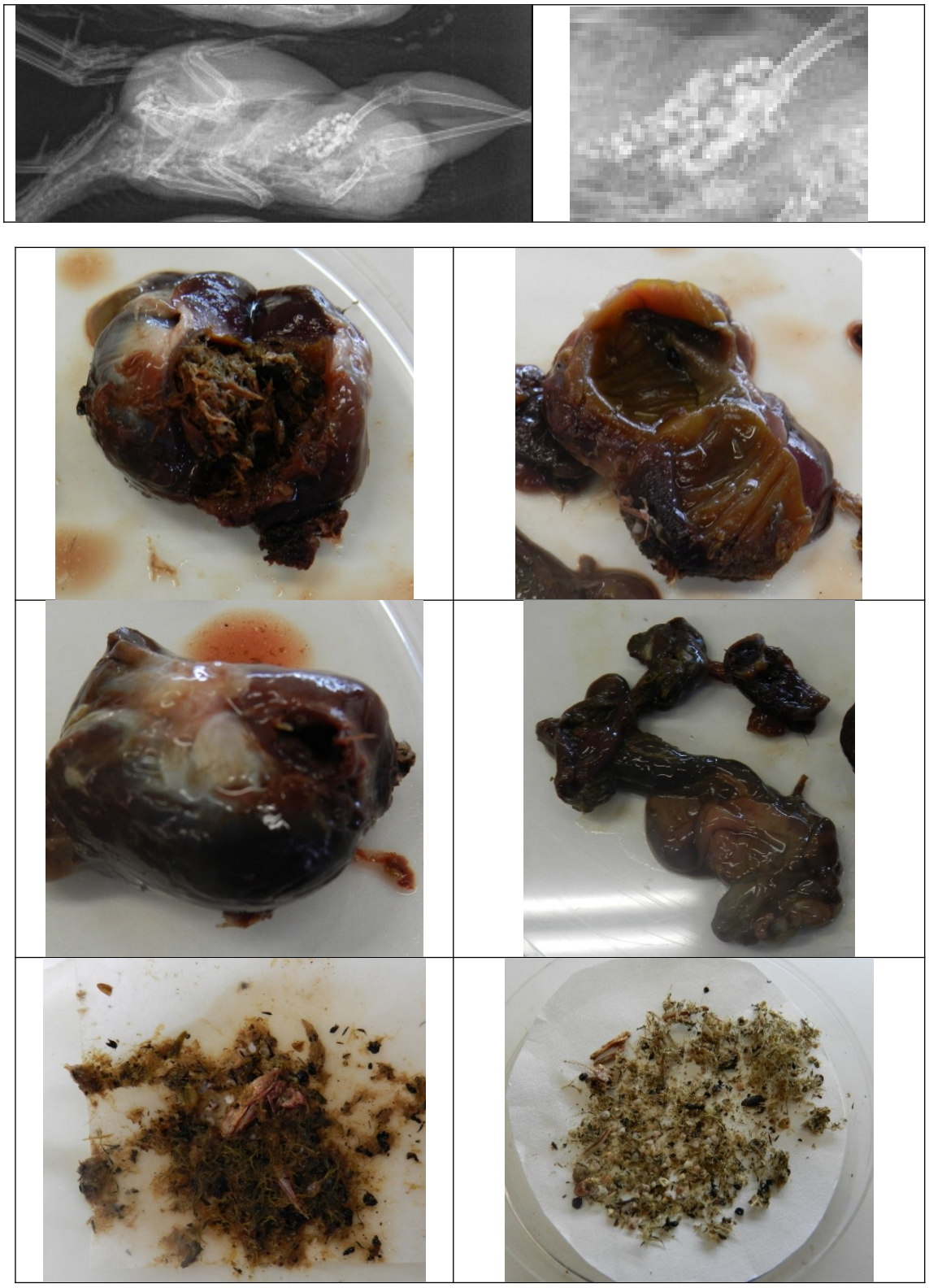


Figure 8. *SPECIMEN CS-ZA-VIII-13. Stratum ZA-10303. Date of capture 15/08/2019. Female. Young. No. of pellets (according to X-ray): 1 (anatomical area: gizzard); Pb concentration (µg/g): 0.891 (liver); 0.036 (muscle tissue); kidney not determined (shattered by gunshot); orifice in gizzard; steel pellet.*

Figure 9. *SPECIMEN CS-ZA-VIII-27. Stratum ZA-10303. Date of capture 22/08/2019. Male. Young. No. of pellets (according to X-ray): 0; Pb concentration (µg/g): 0.809 (liver); 8.650 (kidney); 0.036 (muscle tissue).*

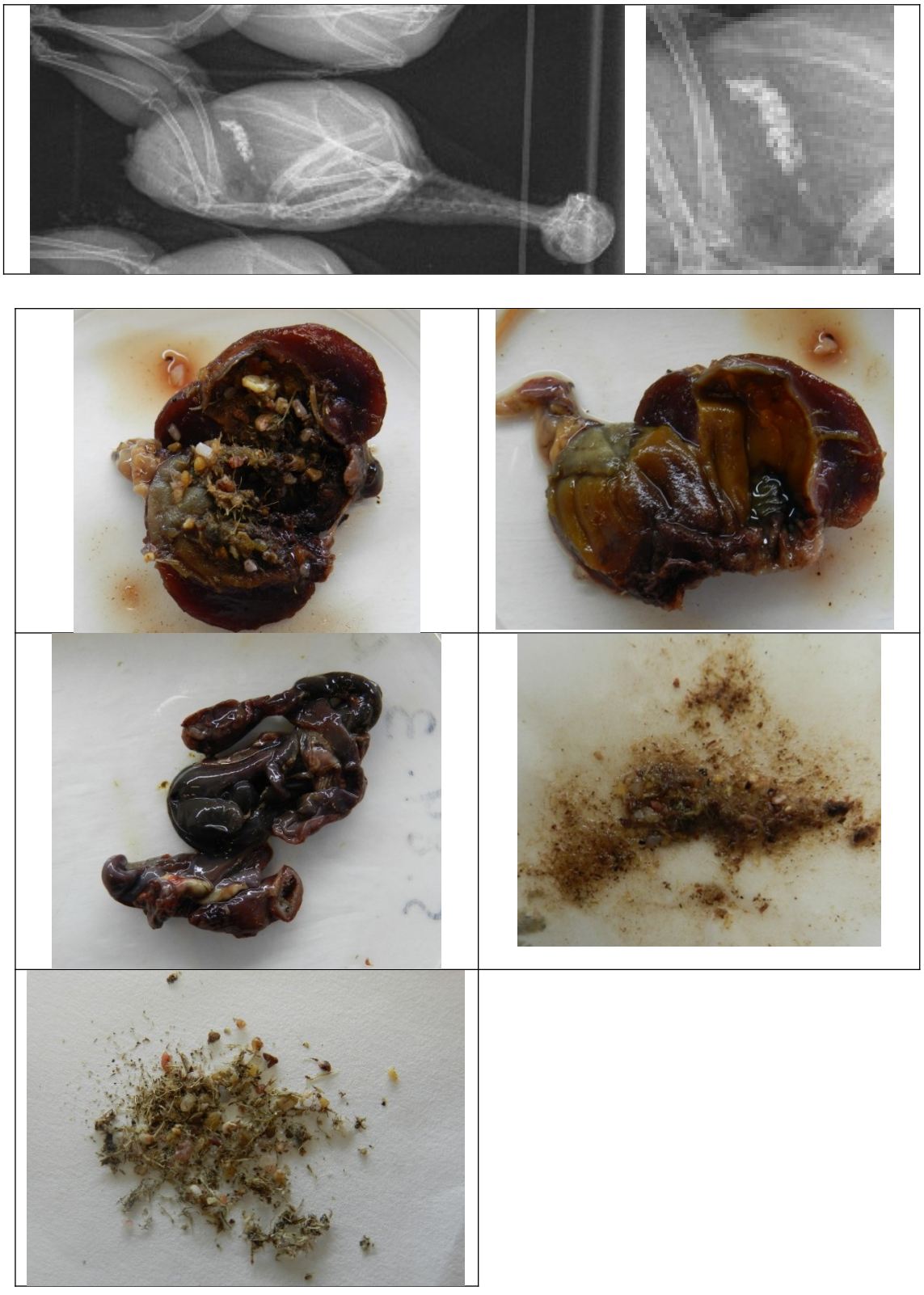


Figure 10. *SPECIMEN CS-ZA-VIII-28. Stratum ZA-10303. Date of capture 22/08/2019. Female. Young. No. of pellets (according to X-ray): 0; Pb concentration (µg/g): 1.052 (liver); 2.887 (kidney); 0.038 (muscle tissue).*


CS-ZA-VIII-26 (0.168 µg/g). Adult male, 2 steel pellets (gunshot)


CS-ZA-VIII-17 (0.229 µg/g). Adult female, 2 steel pellets (gunshot)


CS-ZA-VIII-24 (0.098 µg/g). Adult male, 0 pellets


CS-ZA-VIII-1 (0.182 µg/g). Adult male, 0 pellets

CS-ZA-VIII-5 (0.387 µg/g). Young male, 1 steel pellets (gunshot)

Figure 11. *Gizzards and contents of other specimens belonging to the same batch, their Pb concentration and the number of pellets found in each specimen.*

As can be seen in Figure 11, the colour hues of these specimens' gizzard mucosa are similar to those of the specimens shown above (hepatic lead concentration above 0.65 µg/g, wet weight). The absence of lead shot in these specimens, along with a colour hue of the gizzard mucosa, which is similar to that of the specimens having a low hepatic Pb concentration, do not allow us to affirm that there was a possible ingestion of lead pellets that would explain the hepatic concentration found.

6.2 ANALYSIS OF RESULTS FOR RED-LEGGED PARTRIDGES

As far as the anatomical area compatible with the crop is concerned, pellets were only found in specimen 19 of specimens (PA-CR-XI-) 2-8-13-14-18-19-21-26-30-31, which were steel pellets with ammunition entry and exit orifices.

A pellet was found in the gizzard of specimen PA-CR-XI-13, but it was a steel pellet. Specimen PA-CR-XI-19 had a pellet in the crop, but an entry orifice was observed, and the pellet was made of steel. Specimen PA-CR-XI-22 had a steel pellet in the gizzard and an entry orifice was observed in it. Specimen PA-CR-XI-26 had a pellet in the gizzard, but it was a steel pellet. Specimen PA-CR-XI-27 had 10 pellets, four of which were in the gizzard and made of lead (there was no entry orifice in the gizzard) and one was in the intestine (steel). It was a specimen which had moulted up to the fourth remiges in a farm, showing unequivocal signs of having had its origin in a farm. There was evidence in this latter specimen of it having ingested pellets, which were in the gizzard.

The data and images taken of the red-legged partridge specimen whose hepatic Pb concentration was above 0.65 µg/g (wet weight) are shown below (Figure 12), taking the most restrictive value for Pb environmental exposure according to the scientific literature (Guitart et al., 1994; Ferrandis et al., 2008; Franson and Pain, 2011; Berny et al., 2015; Bingham et al., 2015), and considered in the 2019 study (Romero et al., 2019).

Figure 12. *SPECIMEN PA-CR-XI-27. Stratum CR-10517. Date of capture 30/11/2019. Male. Young. No. of pellets found: four in the gizzard, one in the intestine (steel), five in the rest of the body (steel); Pb concentration (µg/g): 7.189 (liver); 33.212 (kidney); 0.147 (muscle tissue).*

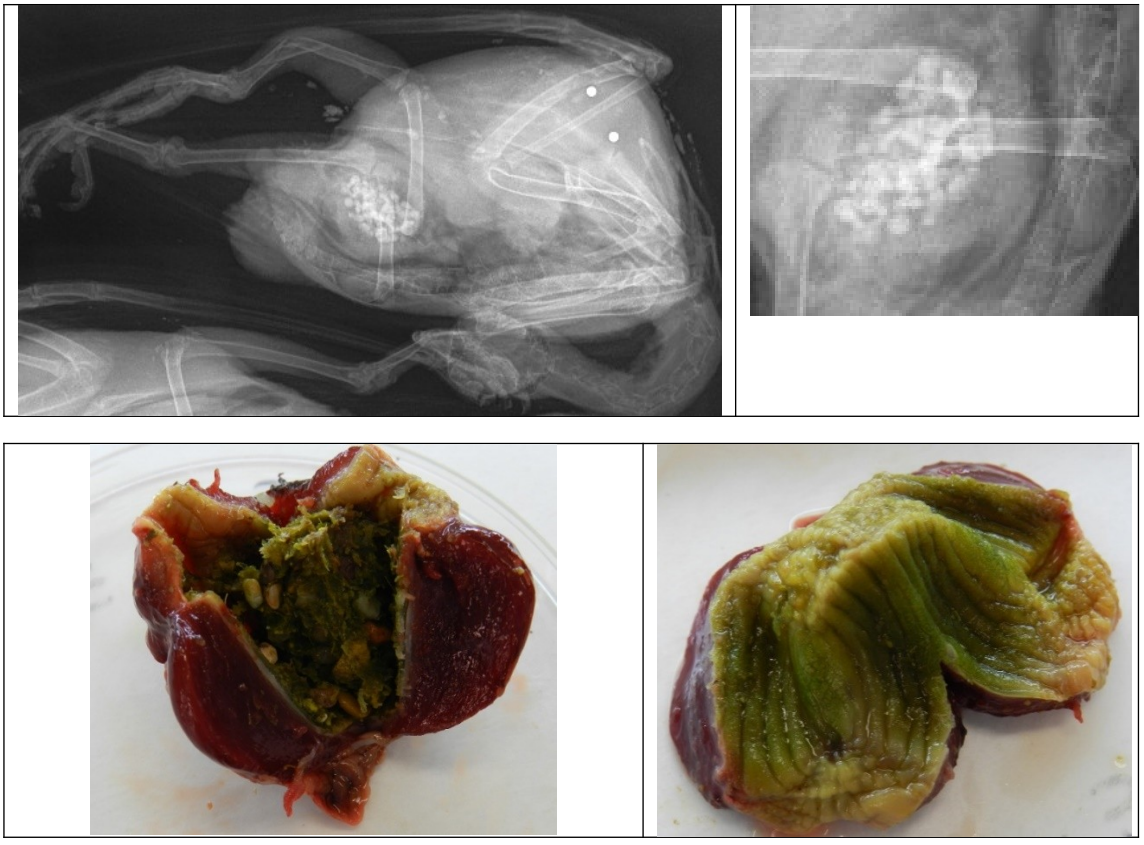
There is clear the evidence that this specimen had ingested lead pellets from the environment, thereby justifying the high tissue concentrations found, the highest of which was the renal concentration detected.

6.3 ANALYSIS OF RESULTS FOR COMMON WOODPIGEONS

The common woodpigeon specimens having a possible presence of pellets in the crop according to the X-ray images were the following: (PT-ZA-I-) 6-13-20-22-26 and 30. None of the pellets (steel) were eventually found inside the crop.

A steel pellet was found in the gizzard of specimen PT-ZA-I-14. Specimen PT-ZA-I-16 had a steel pellet the gizzard. There was a steel pellet in the gizzard of specimen PT-ZA-I-19.

The data and images taken of the common woodpigeon specimen whose hepatic Pb concentration was above 0.65 µg/g (wet weight) are shown below (Figure 13), taking the most restrictive value for Pb environmental exposure according to the scientific literature (Guitart et al., 1994; Ferrandis et al., 2008; Franson and Pain, 2011; Berny et al., 2015; Bingham et al., 2015), and considered in the 2019 study (Romero et al., 2019).



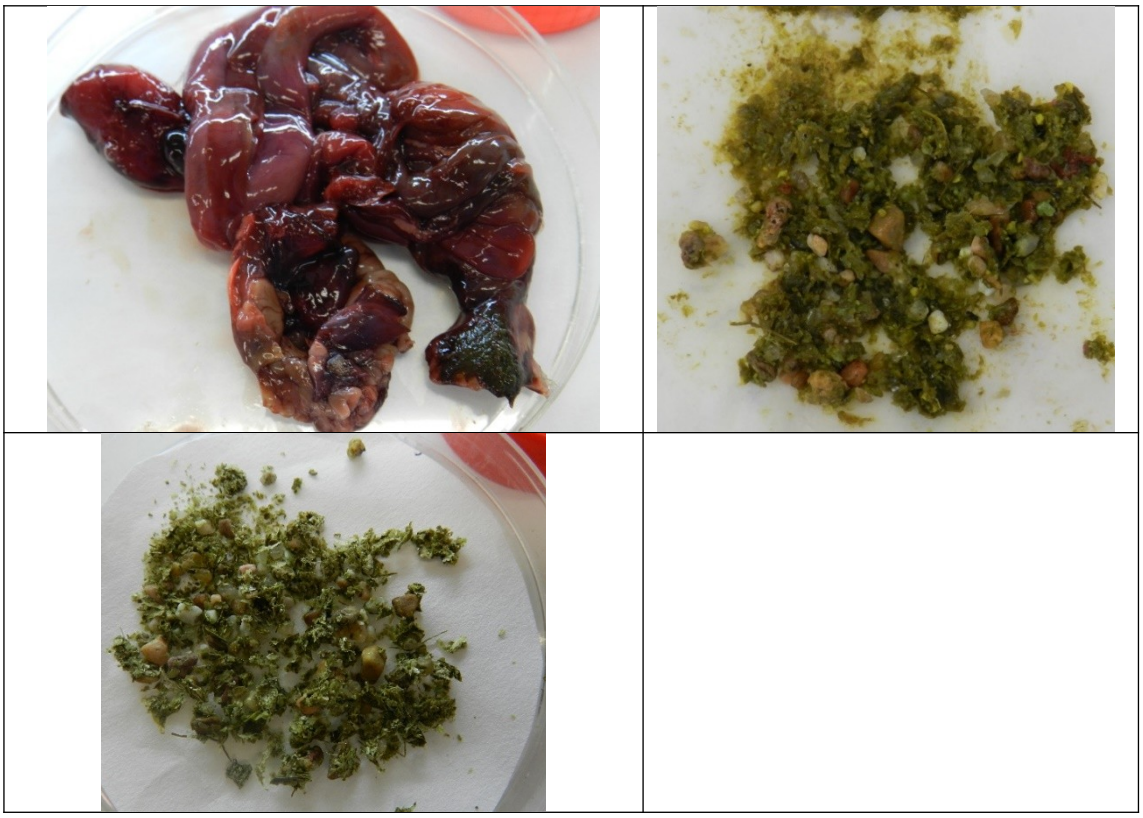
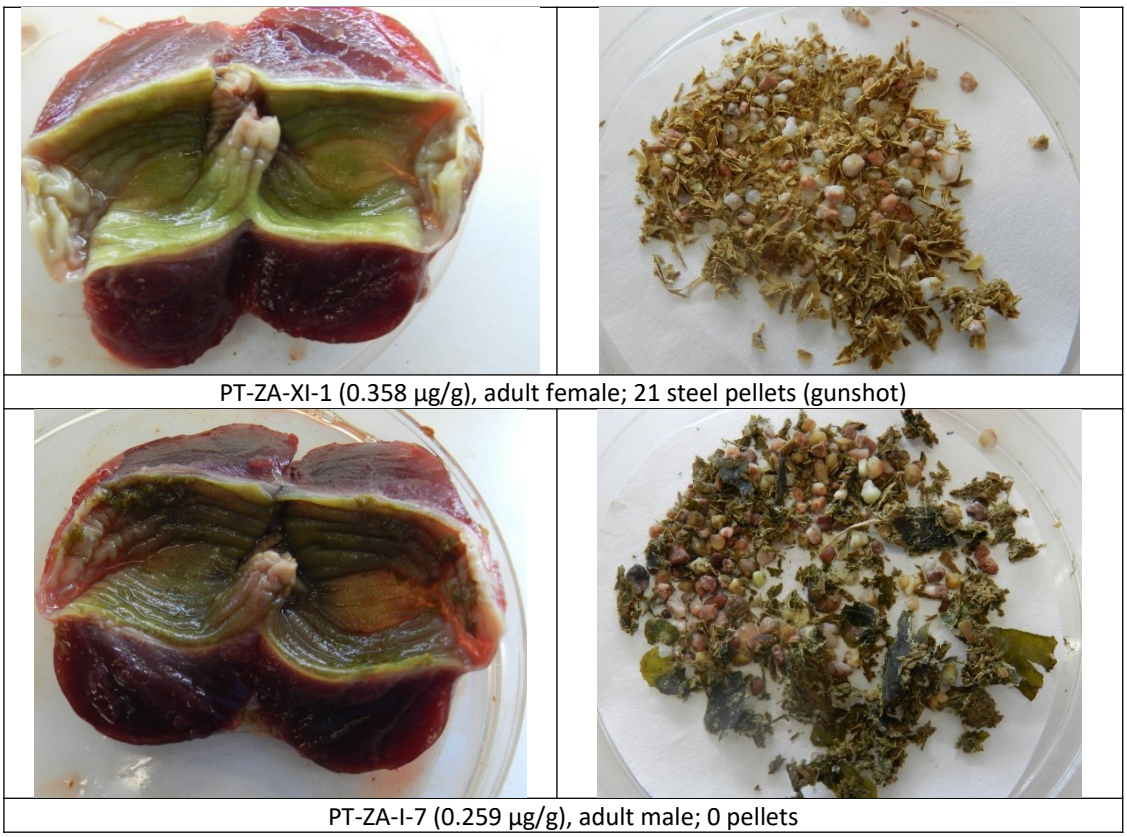
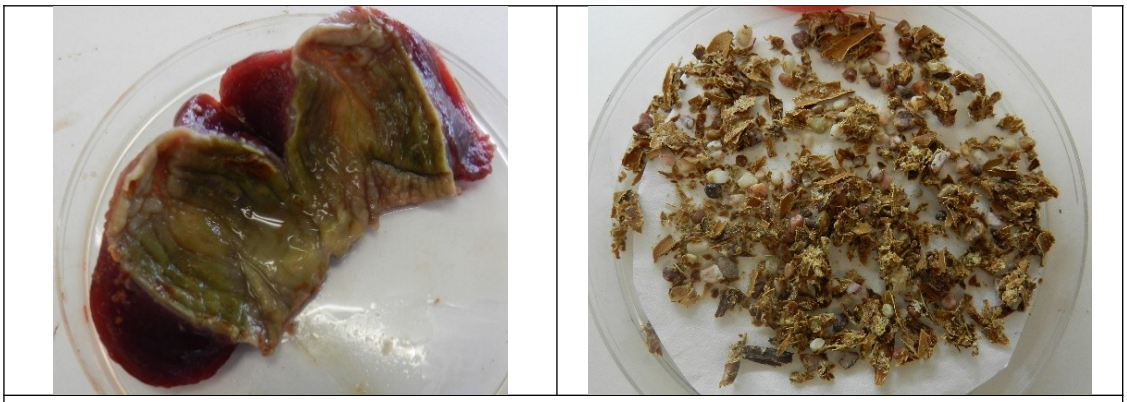


Figure 13. *SPECIMEN PT-ZA-I-15. Stratum ZA-10719. Date of capture 16/01/2020. Male. Adult. No. of pellets (according to X-ray): two in thorax; Pb concentration (µg/g): 1.230 (liver); 0.455 (kidney); 0.018 (muscle tissue).*

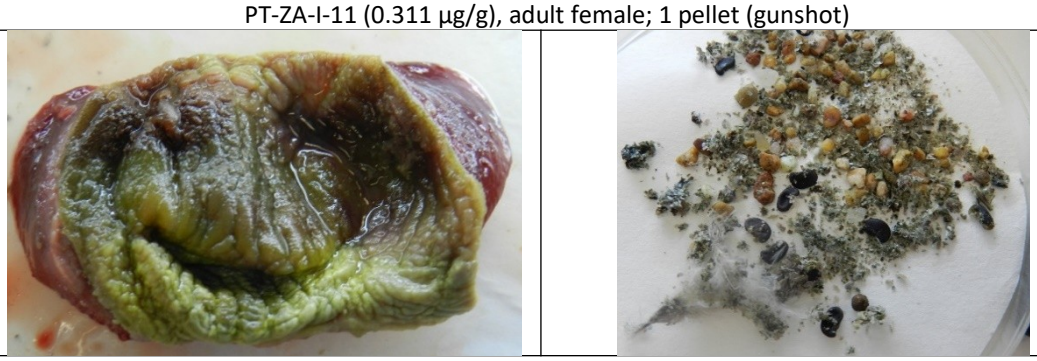


PT-ZA-XI-1 (0.358 µg/g), adult female; 21 steel pellets (gunshot)

PT-ZA-I-7 (0.259 µg/g), adult male; 0 pellets

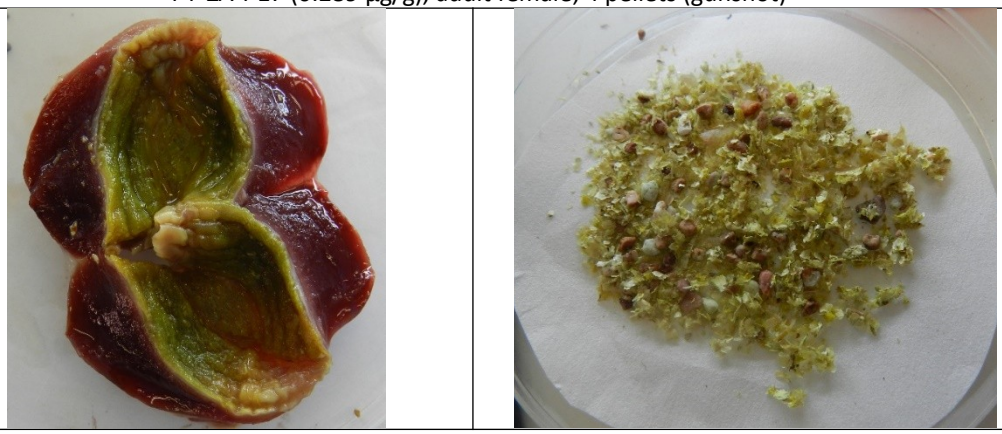


PT-ZA-I-11 (0.311 µg/g), adult female; 1 pellet (gunshot)



PT-ZA-I-16 (0.267 µg/g), young female, 7 pellets (gunshot); 1 of them in the gizzard (steel)

PT-ZA-I-17 (0.289 µg/g), adult female; 4 pellets (gunshot)



PT-ZA-I18 (0.310 µg/g), young male, 0 pellets

Figure 14. *Gizzard samples of other specimens belonging to the same batch, their Pb concentration and the number of pellets found in each specimen.*

Neither can the hepatic lead concentration found in specimen PT-ZA-I-15 be associated to consumption of lead pellets from the environment based on the colouring of the gizzard's inner surface.

The concentration detected in this study's two groups (rural environment, n=11 and peri-urban environment, n=20) were compared, but there were no statistically significant differences between them in so far as hepatic Pb concentrations are concerned: 0.252±0.033 (0.037-0.478 µg/g, wet weight, rural environment) and 0.337±0.051 (0.230-1.230 µg/g, wet weight, peri-urban environment).

6.4 COMPARISON OF RESULTS WITH 2019 REPORT

Both studies showed a very low average Pb concentration which is far below the value of 0.65 µg/g (the wet weight threshold concentration for environmental exposure described as the most restrictive in the bibliography). Nevertheless, an increase in the hepatic Pb concentration ($p<0.05$) was observed in this study among the three species under study. The concentrations found in the 2019 Report (geometric mean and standard error) can be consulted in Romero et al. (2019).

Table 17. *Comparison of descriptive statistics for Pb concentrations (µg/g, wet weight)*

		Origin of the batch	n	% of specimens with hepatic Pb > 0.65 µg/g	Pb concentration (geometric mean ± standard error
Coturnix coturnix	2019 study	Zamora	31	6.5	0.121±0.213
Coturnix coturnix	2020 study	Zamora	32	12.5	0.231±0.044
Alectoris rufa	2019 study	Ciudad Real	97	9.3	0.065±0.166
Alectoris rufa	2020 study	Ciudad Real	31	3.2	0.103±0.229
Columba palumbus	2019 study	Zamora	12	8.3	0.018±0.230
Columba palumbus	2020 study	Zamora	31	3.2	0.304±0.035

In the case of quails, the rise in the percentage of specimens with a value above 0.65 µg/g should be highlighted, which is considered by several authors as an indicator of environmental contamination (as has already been mentioned). In this case, three of the four specimens with a Pb concentration above this value were young specimens (the two quail specimens in the report of 2019 with hepatic Pb concentrations higher than the threshold value were also young specimens). The insectivorous habit of this species at a young age is well known and insects are an important path for the transfer of metals along food chains (Butt et al., 2018). Hence, this habit might perhaps explain these results.

7 CONCLUSIONS

As this is a results report containing conclusions based on them, a discussion of the results is not undertaken (as was also the case in the 2019 Report), which is left for the study's publication (should it come about).

The conclusions which can be drawn from this report are as follows:

First.- The Pb concentrations in the liver of the three species remain very low, as was observed in the samples analysed in the preceding study (2019 Report). The batch having the highest value in this study (0.302 µg/g for common woodpigeons) is still far below the value of 0.65 µg/g (wet weight threshold concentration for environmental exposure described as the most restrictive in the bibliography).

Second.- Evidence of the consumption Pb pellets from the environment was only found in a specimen of red-legged partridge with sufficient proof to affirm that it was an acclimated specimen and therefore came from a farm. Hence, the percentage of specimens in this study showing evidence of having consumed pellets from the natural environment amounted to 1.06%, a value which is lower than the one described in the 2019 Report, as indicated in Romero et al. (2019) (3.0-3.8%).

Third.- Apart from the partridge mentioned in the second conclusion, four quail specimens and one common woodpigeon specimen were found to have Pb concentrations in the liver above 0.65 µg/g (wet weight) (concentrations of between 0.809 and 1.230 µg/g, wet weight). They can therefore be considered as examples of environmental contamination, though without any evidence of having ingested Pb pellets from the environment. Tracking these species and locations is therefore recommended to determine other sources of Pb in the environment.

Fourth.- The renal Pb concentration was very high in the red-legged partridge specimen (33.212 mg/kg, wet weight). Its relationship with exposure to the pellets found in its gizzard is therefore clear. The renal Pb concentration was not high (0.455 µg/g, wet weight) in the case of the common woodpigeon with a Pb concentration in the liver above the threshold value. In the case of the quails that exceeded said threshold value in the liver, kidney concentrations were between the values indicated above (2.887-14.471 µg/g, wet weight).

Fifth.- In the case of muscle tissue, the concentration was very low in the case of the four quails that exceeded the threshold value of Pb in the liver (0.036-0.038 µg/g, wet weight) and also in the common woodpigeon (0.018 µg/g, wet weight). It was, however, higher in the case of the red-legged partridge that had ingested Pb pellets from the environment (0.147 µg/g, wet weight).

Sixth.- The percentages of specimens with a Pb concentration in the liver above the value deemed as environmental contamination (0.65 µg/g) fell in the case of partridges and common woodpigeons with regard to the same species and localisation (2019 Report), but rose in the case of quails. Taking into account the insectivorous habits of this latter species (especially at a young age) and the lack of any evidence of exposure to Pb resulting from the ingestion of Pb ammunition by the specimens which exceeded said threshold, it would be advisable to investigate whether such habits could condition the results observed in these cases. It is recommended that the specimens should always be obtained by using steel pellets.

Further studies should be conducted to establish (1) the origin of the Pb found in specimens having a high hepatic Pb concentration and with no evidence of having ingested Pb pellets from the environment, including studies on insects and other tissues (like the kidney); and (2) Pb concentration in the muscle tissue of game specimens to verify whether or not there is an ingestion risk due to the consumption of these pellets, for which the samples already taken of both this study and the preceding one are useful.

8 DATE AND SIGNATURES

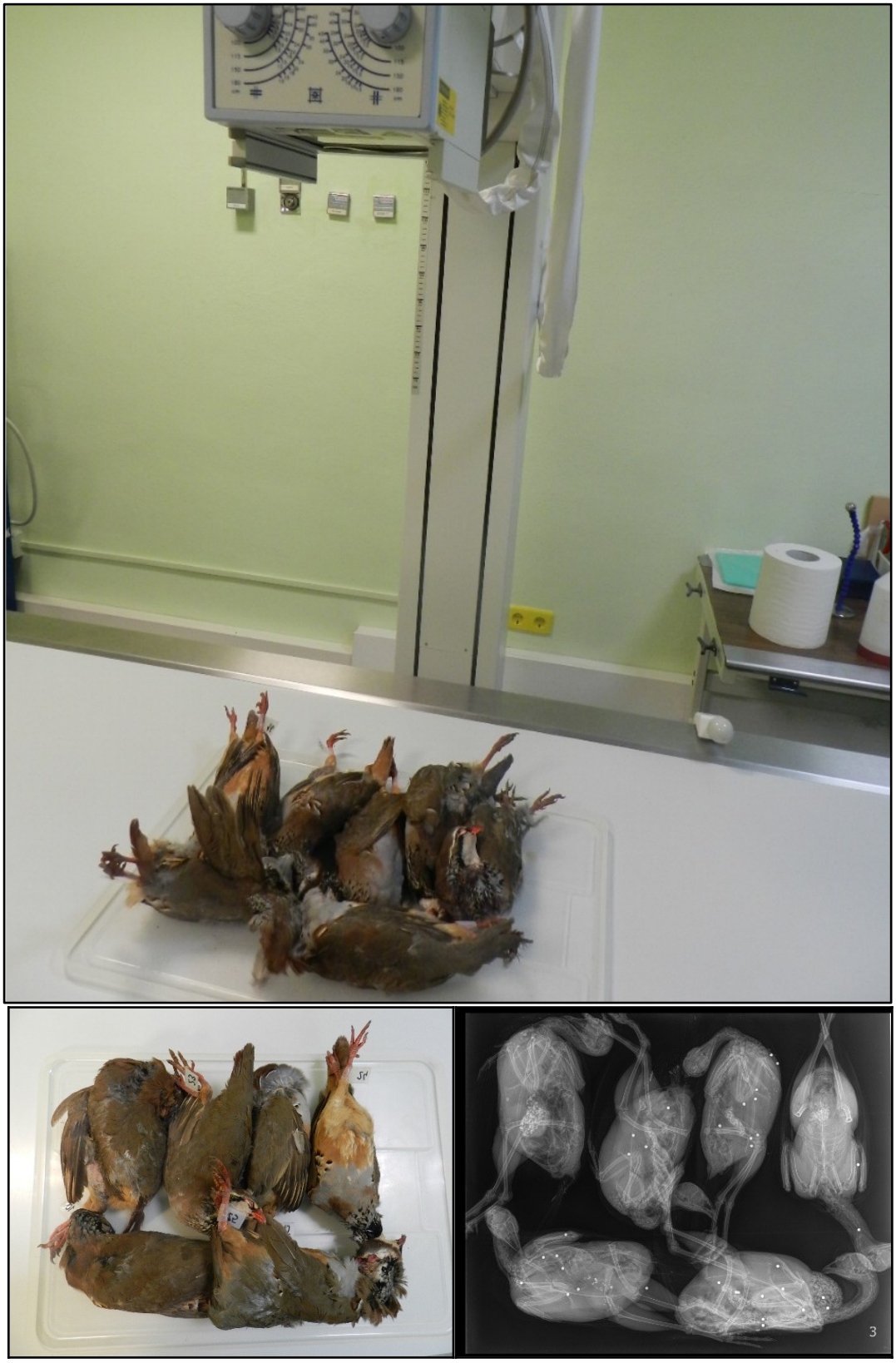
This report was prepared at the request of the Spanish Sectoral Federation of Weapons and Ammunition (FSA).

Date: 01 September 2020.

Authors: Antonio de José Prada, Diego Romero García, Juan Manuel Theureau de la Peña, Juan Bautista Torregrosa Soler and Andrés Ferrer Gisbert.

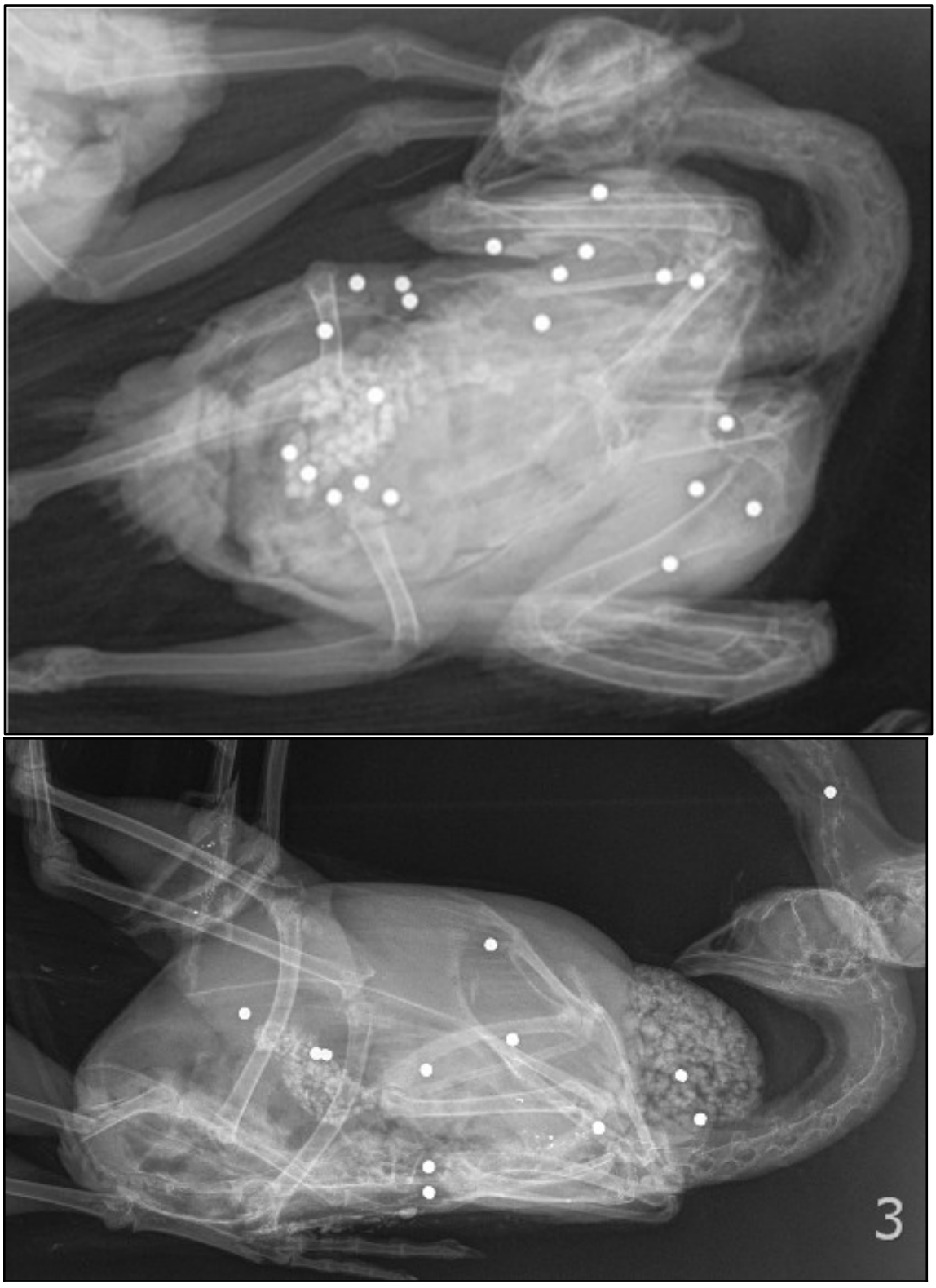
9 PHOTOGRAPHS

***Photographs 1, 2 and 3**: Details of X-rays taken of partridges*

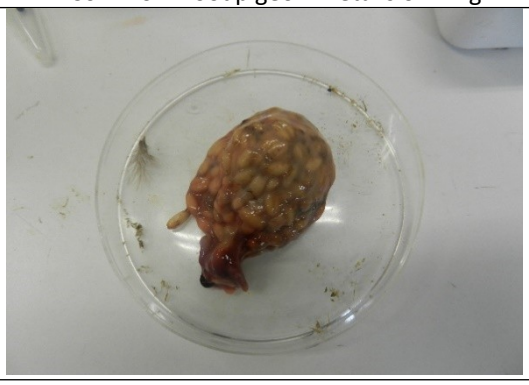


***Photographs 4, 5 and 6**: Details of some specimens sampled*

***Photographs 7 and 8**: Details of pellets in locations compatible with digestive apparatus organs*



***Photographs 9, 10, 11, 12, 13, 14, 15 and 16**: Detail of the physical study of specimens*

	
Common woodpigeon	Common woodpigeon. Details of wing
	
Partridge. Ammunition impacts in thorax	Partridge. Crop
	
Quail. Detail of gonads	Partridge. Impact orifice in crop
	
Quail. Subcutaneous pellet in abdomen	Quail. Pellet at entry to thorax

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