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PIOMBO E RAME IN CINGHIALI CACCIATI E VALUTAZIONE RADIOGRAFICA DELLA FRAMMENTAZIONE FRA DIVERSE MUNIZIONI

Lead and copper in hunted wild boars and radiographic evaluation of fragmentation between ammunitions

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Index

Abstract

The purpose of the present study was to evaluate the content of lead in carcasses of wild boars shot with lead bullets, in comparison with that of copper caused by lead-free ammunitions. Radiographic images of hunted boars were obtained in order to assess the degree of bullet fragmentation in the carcasses. Samples of meat were collected from different body areas at increasing distance from bullet trajectory, to be analysed by ICP-MS for lead and copper levels. In wild boars shot with lead ammunitions, a massive dispersion of bullet fragments and very high lead levels were detected. By contrast, in wild boars killed with copper ammunitions no radiographic signs of bullet fragmentation were observed. Copper ammunitions seem therefore a safer alternative to standard lead-core ones, due to their minimal fragmentation and the relatively low toxicity of this metal.

1. Lead and heavy metals in the environment

1.1. Lead properties

Lead is a bright silvery heavy metal. Heavy metals are generally referred to as those metals which possess a specific density of more than 5 g/cm^3 or weight more than 40.04 (the atomic mass of Ca) (Jarup, 2003; Ming-Ho, 2005). They are introduced into the environment by natural and anthropogenic means and their distribution is governed by metals' properties and influences of environmental factors.

1.2. Sources of lead contamination

Common sources, artificial and natural, that take part in lead diffusion in the environment include: natural weathering of the earth's crust, mining, soil erosion, industrial discharge, urban runoff, sewage effluents, pest or disease control agents applied to plants, air pollution fallout and a number of others (Ming-Ho, 2005; Khlifi and Hamza-Chaffai, 2010). Nowadays, lead is still used and it is crucial in several industrial processes: production of batteries, ammunition, metal items like solder and pipes, and X-ray shielding devices. In the last years, it has be seen increasing concerns in public opinion on exposure and absorption of heavy metal by humans. This has led to a drastic reduction of its use in several products like gasoline, paints, and pipe solder (Martin and Griswold, 2009; Morais et al., 2012). Lead is a highly toxic metal with low geochemical mobility. Yet, its widespread use by man activities has determined an extensive environmental contamination, with consequent accumulation and health problems in many parts of the world. (Oehlenschläger, 2002, Jaishankar et al., 2014).

1.3. Industrial lead chain

Lead and lead compounds can be found as metallic lead, inorganic ions and salts in all parts of our environment, included air, soil, and water. The contamination chain of heavy metals, and of lead in particular, almost always follows the same order. First, industries use lead in their processes, producing waste. Then, those can be dispersed directly in atmosphere, soil, and water. In particular, the pollution of the air causes a large and indirect dispersion of heavy metals with the rains in water and soil. Once the latter have been contaminated, heavy metals and other toxic products can enter the food chain in lot of ways, causing health problems in animals and humans. For example, in areas with high concentration of lead in the soil, it can be absorbed and bioaccumulated by plants. If these plants are eaten by animals or humans, exposure to harmful levels of metals can happen, even through the soil that sticks to plants that is not easy to be completely removed during the preparation for human use. This is only one small part of all the possible paths that can carry lead to human consumption and result in the fact that diet, today, is the main route of exposure to lead for most of the population (Harrison, 2001; Castro-González and Méndez-Armenta, 2008; Martin and Griswold, 2009; Morais et al., 2012).

2. Lead effect on organisms

2.1. Lead poisoning

Lead poisoning was considered to be a classic disease and the signs, that were seen in children and adults, were mainly pertaining to the central nervous system and the gastrointestinal tract (Markowitz, 2000).It has no beneficial effects in living organisms, there is no known homeostasis mechanism for it and virtually no level of lead exposure can be considered harmless in consideration of its many sublethal, debilitating, and often irreversible effects (Needleman, 2004; Draghici et al., 2010; Vieira et al., 2011).

2.2. Lead acute toxicity

Lead causes an acute poisoning after accidental assumption of a large amount, or exposition to high environmental level. No controlled studies in humans have evaluated acute Pb poisoning, information have principally been obtained from case reports. Acute Pb toxicity is characterized by symptoms of abdominal pain/colic, vomiting, constipation, peripheral neuropathy, and cerebral edema and encephalopathy, which can lead to seizures, coma, and death. Children are more susceptible than adults to acute Pb poisoning.

2.3. Lead chronic toxicity

Today, acute toxicity from lead is rare and mainly restricted to developing countries. More often it is responsible of chronic toxicity following repeated exposure to small doses, and this latter form is the most insidious and concerning. Lead is mainly neurotoxic, but it can also damage kidneys, bones, cardiovascular and immune systems, and cause infertility (Goyer, 1989; Carmouche et al., 2005; ATSDR, 2007; Navas-Acien et al., 2007; Vigeh et al., 2011; Assi et al., 2016). It is associated with impaired motor function, attentional dysfunction, reduced somatic growth, decreased brain volume and spontaneous abortion (Borja-Aburto et al., 1999; Braun et al., 2006; Cecil et al., 2008; Hauser et al., 2008). Children present a more efficient absorption with diet that make them more susceptible to lead toxic action. Even low concentrations are associated with permanent cognitive damage in children, including those prenatally exposed (de Winter-Sorkina et al., 2003; Lanphear et al., 2005; Schnaas et al., 2006).

2.3.1. Lead chronic effects on bones

Adults who were exposed in the past, due to environmental contamination or their employment in industries that made large use of lead, maintain elevated blood levels. This can be explained by the integration of lead within the hydroxyapatite crystals during the bones' calcification process. The bones and the teeth of an adult contain more than 95% of the total body burden of lead.

During periods of high bone turn over like menopause, pregnancy and fractures, blood lead level increased (Wittmers et al., 1988; Silbergeld et al., 1988, 1993; Tellez-Rojo et al., 2004; Carmouche et al., 2005; Fischer, 2009). Even the aging process has been shown to increase the release of Pb from the bones (Barnes et al., 1999; Shih et al., 2007). Moreover, inorganic lead compounds are listed in the 2A group by the IARC (International Agency for Research on Cancer), being probably carcinogenic for humans (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006).

2.4. Lead cellular effects

Lead can virtually affect every body's system. Its power toxic action is due to 2 main mechanisms: oxidative stress and replacement of cations in biological processes. Under lead influence there is a decrease of antioxidants and an increase in ROS production. This unbalance causes an oxidative stress for the cell, that results in lipid peroxidation and structural damage to membrane, proteins, nucleic acid and cells (Flora et al., 2008; Mathew et al., 2011; Wadhwa et al., 2012;). Furthermore, lead ions are able to replace other cations like Ca^{2+}, Na^{2+}, Fe^{2+} in cellulars' reactions. This impair the cell metabolism and cause alterations in protein folding, cellular signaling, enzyme regulation, neurotransmission and various other functions necessary to cell communication and functions (Flora et al., 2008).

3. Residues of lead in food

3.1. Lead from diet

Lead toxic potential and its long term effect on the population can result in an increment in public spending. The levels of lead in bones, hair, and teeth increase with age, suggesting a gradual accumulation of lead in the body. For these reasons, lead contamination of food is of major importance to humans. Therefore, the possibility of chronic lead intoxication through the diet need constant monitoring. In 2006 the European Commission has established maximum accepted levels in food; for instance, 0,1 mg/kg has been set as a limit for lead concentration in the meat of cattles, pigs, lambs and chickens, whereas a maximum accepted lead level for game meat has still not been established (European Commission 2006; European Food Safety Authority 2010).

3.2. Lead fragments in game meat

Meat from game animals shot using lead ammunition is a potential source of lead. Some studies have proved that regular consumption of game meat is associated with increased blood lead levels (Janssen, 1997; Iqbal et al., 2009). It was previously thought that the majority of lead bullets used to shot game animals remained as a single mass or large fragments in a carcass, and they were likely to be removed. However, recent studies have shown that in the meat of leadshot game animals, high levels of lead and a huge presence of lead fragments can be found (Hunt et al., 2006, 2009; Krone et al., 2009; Pain et al., 2010). Several factors are able to influence the level of exposure to lead that may occur eating game meat.

3.2.1. Lead bullets fragmentation

Lead softness and frangibility cause the dispersion of hundreds of bullet fragments inside the animal's carcass, and the entity of fragmentation may vary considerably depending on several factors, such as the calibre and type of the ammunition, the size of the animal, and the hardness of the tissues crossed by the bullet, especially if bones are hit. Small-size game, especially if hit by multiple shots, such as lead pellets in cartridges, may have a nearly generalised lead dispersion or, on the contrary, a single pellet could cut right through them, leaving virtually no trace of contamination. In large animals, lead bullets are generally subjected to a higher level of fragmentation, even though the dispersion may be more confined, as it tends to concentrate near the bullet's trajectory and to decrease radially within the animal body. However, fragments can be found even at 30 cm and more from obviously injured tissue.

3.2.2. Lead concentration and absorption

The high number of fragments, their small size and the distance from entry and exit wound of some of them make it very difficult, even for an experienced operator, to thoroughly remove the metal parts before the meat is sent to consumption. The smaller fragments are often invisible to human eye, even X-ray can fail in their detection. In another study, some carcass obtained after gunshot with few or no shot visible on X-rays showed, after analysis, high lead concentrations in the flesh (Pain et al., 2010). Moreover, smaller particles of metallic lead are more readily absorbed, especially those <50 micrometer diameter (Baltrop and Meek, 1979). Meat processing and cooking techniques could also play an important role in promoting lead poisoning. Lead is more soluble under acidic conditions. Vinegar, wine, lemon juice and other acid substances can be used for marinating the meat before cooking. If meat contains lead fragments, the action on the pH of these products, combined with long time cooking, can increase the conversion of lead from inorganic to organic form enhancing its absorption (Mateo et al., 2006).

3.3. Risk of lead toxicosis for hunters

Health risks related to heavy metal exposure are greater for some categories of consumer. This is true especially for hunter populations and their families which tend to have an average consumption of game meat bigger than the general population. The risk may be particularly high if animals are shot with lead bullets and the tissues are not carefully discarded (Burger, 2002; Vahteristo et al., 2003; Lazarus et al., 2008; Hunt et al., 2009; Tsuji et al., 2009; Morales et al., 2011).

3.3.1. Sustained consumption of game meat

Many studies found that lead concentrations in game meat were sufficiently high for sustained consumption to be potentially hazardous to human health, even though all visible shot were removed from the tissues before analysis. Furthermore, there is a correlation between reported levels of consumption of meat from animal killed using lead shots and blood lead levels in adult (Dewailly et al., 2001; Johansen et al., 2004; Bjerregaard et al., 2004; Pain et al., 2010). An analysis of North Dakota residents showed that recent (< 1 month) consumers of game meat had higher covariate-adjusted blood lead concentrations than those with a longer interval (< 6 month) since last consumption (Iqbal, 2008). Hunting has augmented in the last years (Geisser and Reyer, 2004; Bieber and Ruf, 2005).

3.3.2. Availability of wild boar meat

Wild ungulate populations are increasing throughout Europe which has resulted in a rise in distribution range and population density (Saez-Royuela and Telleria, 1986; Carnevali et al., 2009; Delibes et al., 2009; Meriggi et al., 2011). These factors contribute to increase the amount of wild boar meat available for human consumption (Ramanzin et al., 2010). Whilst some European countries have an established game meat market, in other, like in Italy, most of the game meat is consumed in domestic settings or is supplied in small amounts directly to final consumers and local retailers, with no traceability (Danieli et al., 2012).

The more game is consumed the higher the chance to consume contaminated meat (Gerofke et al., 2018).

4. Environmental dispersion of lead bullets

In addition to direct contamination, lead ammunition may also cause indirect harm by means of environmental dispersion. The concentration of metals in wild animals may vary considerably from location to location (Kålås et al., 1995; Petersson-Grawe et al., 1997; Wlostowski et al., 2006; Bilandzic et al., 2009, 2010). Ingestion and inhalation are the two most common entry routes of lead into animals (Demayo et al., 1982; Eisler, 1988; Pain, 1995; Mateo, 1998; Guitart et al., 1999). Many scavenging, predatory birds and mammals swallow lead gunshot or bullets along with their food, including viscera discarded by hunters, unretrieved quarry, and prey animals with ingested gunshot in the digestive tract or which have been shot but have survived, carrying lead pellets in their flesh.

4.1. Lead intoxication in birds

More than 50 bird species have been documented to have ingested lead or to have suffered lead poisoning from ammunition sources, including ten Globally Threatened or Near Threatened species (Mateo, 2009; Pain et al., 2009). In a past study, 5 bald eagles (*Haliaeetus eucocephaluksi*) have been feeded with shotgun pellets that killed 4 of them, and severe clinical signs prompted euthanization of the fifth (Hoffman et al., 1981; Pattee et al., 1981). The likelihood of a bird becoming poisoned is related to the retention time of lead items, frequency, and history of exposure to lead, and factors such as nutritional status and environmental stress (Pattee and Pain, 2003).

4.1.1. Sintomes of lead toxicosis in birds

The effects of toxicosis in birds commonly include distension of the proventriculus, green watery faeces, weight loss, anaemia and drooping posture (Redig et al., 1980; Reiser and Temple, 1981; Franson et al., 1983; Custer et al., 1984; Sanderson and Bellrose, 1986; Mateo, 1998). Sub-lethal toxic effects are exerted on the nervous system, kidneys, and circulatory system. Even vitamin metabolism and immune system can be affected (Baksi and Kenny, 1978; Redig et al., 1991; Grasman and Scanlon, 1995). In some birds, it has been seen a correlation between lead and thin eggshells and a decreased egg production (Grandjean, 1976; Edens and Garlich, 1983). As a result of physiological and behavioural changes, birds may become increasingly susceptible to predation, starvation, and infection by disease, improving the probability of death from other causes (Scheuhammer and Norris, 1996). Lead exposure may also reduce the likelihood of birds to return to an area to breed (Mateo et al., 1999).

4.2. Removal of lead shots from the environment

Various approaches to alleviate the problems caused to birds by accumulation of shots in the environment have been trialed, however, the only demonstrably effective solution is a ban on hunting or a move to non-toxic shot (Mudge, 1992; Scheuhammer and Norris, 1995). In areas where lead shots were banned for hunting, but only in some species, the blood lead concentration of raptors became lower but the prevalence of poisoning was the same. These may be attributed, in part, to offal piles and carcass from hunter-killed animal. Many hunters tend to leave them in the forest after hunting trips, and afterward, Wild animals can eat the abandoned entrails (Kramer and Redig 1997; Murrell et al., 2000). Indeed, in geographical areas where lead ammunitions have been banned, positive environmental effects have been reported. The contaminations of the environment and of the food chain may represent an additional risk for human health (Guitart et al., 2002; Fisher et al., 2006). It should be taken into account to replace lead ammunition with copper ammunition.

5. Copper and its distribution

Copper is a reddish metal that occurs naturally in rock, soil, water, sediment, and, at low levels, air. It is extensively mined and processed and it is primarily used as metal or alloy in the manufacture of wires, sheets, pipes, and other metal products. Copper compounds, like copper sulfate, are most commonly used in agriculture to treat plant diseases, or for water treatment and as preservatives for wood, leather, and fabrics.

5.1. Copper effects on organisms

Copper occurs naturally in all plants and animals and is an essential element for all known living forms at low levels of intake. Exposure to high levels of copper will result in the same types of effects in children and adult: nausea, vomiting, stomach cramps, diarrhea, liver and kidney damage and even death (ATSDR, 2004). Copper tolerable upper intake levels are 1 to 5 mg per day, depending on age. It is stored in the liver and is excreted via the bile (EFSA, 2006).

5.2. Copper residues in meat

The maximum residue level (MRL) for copper in food of animal origin such as pigs, cattle, sheep, goats, horses, poultry and other farm animals is 5 mg/kg (fresh weight) according to regulation (EC) No 149/2008 and the amending regulation (EC) No 396/2005. It is far less toxic than lead and it is even less frangible. Studies have shown that, while lead ammunitions generate hundreds of fragments, copper bullets retain almost 95% of their mass. Each carcass obtained with one copper shot has less than 10 fragments. Moreover, the level of copper in game meat is comparable to those regularly detected in the meat of farm animals (pork, beef, sheep) or in products made from them (Hunt et al., 2006; Schitling et al., 2017).

6. Copper bullets

6.1. Copper bullets in public opinion

Despite the scientific evidence, lead substitutes in ammunition are deemed inadequate, especially by hunters community. In Norway, the total ban on lead shot used for hunting outside the wetlands has been rescinded in 2015 with the vote of 79 out of 95 member of parliament. Associations for hunters and manufacturers of sporting ammunition described this decision as a victory, a great success for hunters community (Arnemo et al., 2016).

6.2. Hunter community and copper bullets

Bans or restricts on lead ammunition are often viewed as an anti-hunting practice. Nevertheless, in Denmark the prohibition of ownership and use of lead shot has not had a long-term detrimental effect on both participation in hunting and numbers of animals taken with non-toxic shot substitutes (Kanstrup, 2015). Most of the argumentation used by the hunters community against copper is not based on scientific literature. Their concern about the transition to new types of ammunition are mainly anecdotal and oral but they are able to influence public attitudes and the course of government policy (Thomas et al., 2016). The World Forum on Shooting Activities (WFSA) stated that: “metallic lead in ammunition has no significant impact on human health and the environment as compared to other forms of lead. Lead fragments in game meat, if ingested, cannot be directly absorbed by the human body because they are in metallic form" (AFEMS, 2015). There is an obvious rejection of all modern studies about lead intoxication. Old experiments on projectile toxicity focused mostly on shotgun pellets (Hoffman et al., 1981), but this may underestimate the effects of rifle bullet fragment. These latter are irregularly shaped and their absorption by the body can be greater than those of shotgun spherical pellets of comparable mass, which have less surface (Hunt et al., 2006). Moreover, humans, can absorb about 5 to 15% of ingested inorganic lead, without taking into consideration all the factors that can enhance this process (see below) (Ming-Ho, 2005).

6.3. Comparison between lead and copper ammunition

The principals concerns from hunters about the transition to metallic bullets are related to their cost, their capacity to rapidly kill and their accuracy.

6.3.1. Copper bullets price

In a study from Thomas (2013) has been done a comparison between nine commonly used calibers of assembled rifle ammunition available in the USA. It has been found that prices for the two types of ammunition were generally comparable, and when the non-lead bullets cost more, the increase was not enough to deny purchase and use. Even in Europe, non-lead rifle ammunitions are largely available in all normal calibers at prices comparable to equivalent lead products. Only small calibers (< 6 mm) are less available for now, but this problem can be crossed with extensive adoption of metallic bullets from European states (Gremse and Rieger, 2012; Kanstrup, 2015).

6.3.2. Copper bullets accuracy

The lower density of copper bullets, compared with lead ones, has to be compensated with a greater volume to achieve an equal bullet mass. It may be counteracted by reducing bullet weight which results in a need for higher velocity to achieve the same striking power, ballistic and accuracy of lead bullets. To generate a higher velocity must be changed the twist rate of the rifle. The twist rate is designed to stabilize the range of bullets and their respective velocities used in a particular caliber, and, in most existing rifles, it is designed for lead-core bullets. The twist rate in a given rifle cannot be modified, but the barrel can be replaced to achieve the same performance of lead ammunition (Thomas et al., 2016). Niels Kanstrup personally tested a lead bullet designed rifle for accuracy at 100m with non-lead bullets. The accuracy wasn't acceptable, but after a barrel change it became so. The price for the change was around 600 Euros. This problem concern only old rifle already purchased by hunters, after a transition to new bullets rifle would be adopted by industries.

6.3.3. Copper bullets fragmentation

Non-lead bullets may be either fragmenting or non-fragmenting types. Some hunters view the bullet core fragmentation as a positive adjunct to a swift kill and view negatively the performance of bullets that pass through the entire animal intact (Caudell et al., 2012; Thomas, 2013). An exit wound with the consequent blood trail may allow easier pursuit of a wounded animal (Gremse and Rieger, 2012). Lead-free non-fragmenting bullets are designed not to disintegrate during passage through animal tissues. Bullet manufacturers now produce non-lead bullets whose anterior region is engineered to fragment into four to six large pieces upon entry. Each piece assumes its own trajectory in the animal and continues to wound, while the intact posterior remnant of the bullet continues along its initial trajectory. These bullets behave in the same way as unbonded lead-core bullets. Their performance has been evaluated and have shown same killing efficiency of traditional lead-core bullets (Knott et al., 2009; Trinogga et al., 2013).

6.4. Residues of copper bullets

Many studies have highlighted that there is no risk of toxicity by ingestion of metallic copper fragments for birds, mammals and human (Thomas et al. 2007; Thomas and McGill 2008; Franson et al. 2013). The levels of copper residues remaining in game meat killed with non-lead bullets have been measured by Irschik et al. (2013) and Schuhmann-Irschik et al. (2015). The only concern about them is that metal fragment if not removed could abrade the mucosa and gingiva of animals and humans that ingest them. Only pure deforming non-fragmenting bullets are suited to avoid even this risk for human consumption. (Nadjafzadeh et al., 2015), while for hunting of game not marketable for human consumption all type of non-lead bullets can be used.

7. Aim of the study

The aim of this study was to assess the level of the dispersion and the concentration of lead in the carcasses of wild boars (*Sus scrofa*) killed with lead ammunitions, in comparison with lead-free ones, by means of radiographic examination and ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) analysis, with the final goal to promote the development of correct procedures for consumer protection.

8. Materials and methods

8.1. Boar collection

In accordance with the administration of the Carrega Woods Regional Park (Parma, Italy), the carcasses of eight wild boars of both sexes killed by rangers within the containment plan established by Emilia-Romagna region were collected for our study. Six boars were killed with a single shot by using lead ammunitions (Remington Bronze Point, .270 Winchester calibre, 130 grains), whereas two boars were killed with lead-free copper ammunitions (Federal Vital Shock, .270 Winchester calibre, 130 grains). Sex, weight, and identification tags are reported in Table 1. Wild boar collection was conducted between 3 September 2017 and 18 February 2018, between 6.00 and 7.30 a.m. The animals were immediately tagged, following national regulations, band carried to our Department for radiographic assessment and sample collection.

Identification tag	Sex	Weight (kg)
34073	Male	19.5
34074	Female	78.5
34076	Male	52.0
34077	Male	58.0
34078	Female	22.1
34080	Male	61.3
01679	Female	22.5
01680	Female	19.6

Table 1. Wild boar description

8.2. Radiographic examination

The boar carcasses were subjected to radiographic examination in order to assess the localisation and dispersion of lead bullet fragments throughout the body. At least two radiographic projections were taken (lateral and sagittal plane) in order to examine a wide area of the carcass.

8.3. Necropsies and tissue sampling

Necropsies were performed by two pathologists, and, after removing the skin, samples of muscle from three different areas were taken (about 6 g each) starting from the entry wound. The first set of samples (A) was taken in the area closer to the wound channel, within 15 cm radially along the visible bullet trajectory. The second (B) and third sets of samples (C) were excised from areas 15–25 cm, and >30 cm from the bullet trajectory, respectively. According to the size of the animal, a total of 20–25 muscle samples from each wild boar were collected; when a macroscopic bullet fragment was found, it was removed, in order to simulate, as closely as possible, the carcass finishing made by hunters. In addition, samples of whole blood (5 mL), bone, and liver were taken; blood was collected from thoracic or abdominal cavity, and bone samples were taken from area A. In wild boars shot with lead-free copper ammunitions, only samples from area A and C were collected. All samples were immediately put in numbered vials and frozen at −20°C until analysis. For comparison, lead and copper concentrations were measured also in wild boars killed with non-lead and lead ammunitions, respectively.

8.4. Lead and copper level analysis

High purity deionised water was obtained by Evoqua Water Technologies (Barbsbüttel, Germany); nitric acid was purchased by J.T. Baker (Center Valley, PA, USA); hydrochloric acid was from Sigma Aldrich (St. Louis, MO, USA). Each sample was homogenised and two subsamples (weigthing 3 g each) were obtained, put in screw cap polypropylene sample tubes (50 mL Digi-Tubes, SCP Science, Montreal,Canada), and 10 mL of nitric acid were added; the samples were then placed in a Digi-Prep system (SCP Science,Montreal, CA) at 75°C overnight. After cooling, the samples were diluted to a final volume of 200 mL with an acid solution and analysed by means of ICP/MS (7700 Agilent Technologies, Santa Clara, CA, USA) with an ASX-500 CETAC Autosampler (Cetac Technologies, Omaha, NE, USA). The analytical method has been previously validated according to international regulations (Reg. 333/2007/EU and Reg. 882/2004/EU Annex 3). The method validation was performed evaluating for Pb linearity (solvent and matrix), limit of quantification, specificity, precision (under repeatability conditions), trueness and intra-laboratory reproducibility. Linearity was performed analysing standard concentrations from 0.1 to 100 μg/L (six concentrations five replicates/concentration) and blank matrices (muscle) fortified with defined Pb concentrations from 0.003 to 2mg/kg (ten concentrations-six replicates/concentrations). Limit of quantification was evaluated at least 10 times over the signal of blank sample (0.005 mg/kg for each metal). Specificity was performed by analysing negative samples (n = 20) and by verifying the absence of inferences. Precision was calculated by fortifying samples at three different levels (six replicates per level) in the validation range and evaluating repeatability as coefficient of variation (CV < 10%). Trueness was evaluated by the recovery percentage calculated on the same samples (>80% for Pb). Reproducibility (CV < 15%) was evaluated on the same samples at the same concentrations but repeating the analysis on three different times during a month. Uncertainty, from 20% to 22%, was calculated with "inhouse data" using the bottom-up approach (Ellison and Williams 2011). The analysis was continuously monitored with BCR (Community Bureau of Reference, Brussels, Belgium) sample CR-185R in every batch and evaluating that the parameters were within the predicted values. This is a bovine liver with 0.172 mg/kg Pb and a defined uncertainty of 0.009 mg/kg. During time more than 100 replicates were performed with a mean value of 0.173 mg/kg and a SD of 0,005 mg/kg. The laboratory participates regularly in proficiency tests organised by national and international committees, such as the Italian Istituto Superiore di Sanità (the Italian Health Institute in Rome, e.g. PT 2017.1: "Heavy metals in mussels," with an assigned value of 70 µg/kg for lead, z-score 0.8), the EURL (European Union Reference Laboratory) and FAPAS (the Food Analysis

Performance Assessment Scheme, York, UK). Recent FAPAS results were a z-score of 1.9 for metallic contaminants in canned crab meat with an assigned value of 52.6 µg/kg, and a z-score of 0.2 for metallic contaminants in offal with an assigned value of 481 µg/kg, both for lead (FAPAS 2016, 2018).

9. Results

9.1. Radiographic examination

The radiographic images of all boars killed by using lead ammunitions showed a widespread dissemination of bullet shreds of variable size, ranging from few tenths of a mm up to about 20 mm. The lead fragments were present not only close to the bullet trajectory but often surprisingly far from the entry or exit wound (Figures 1 and 2). By contrast, radiographs of wild boars killed with copper ammunitions showed a complete absence of visible bullet fragments (Figures. 3 and 4).

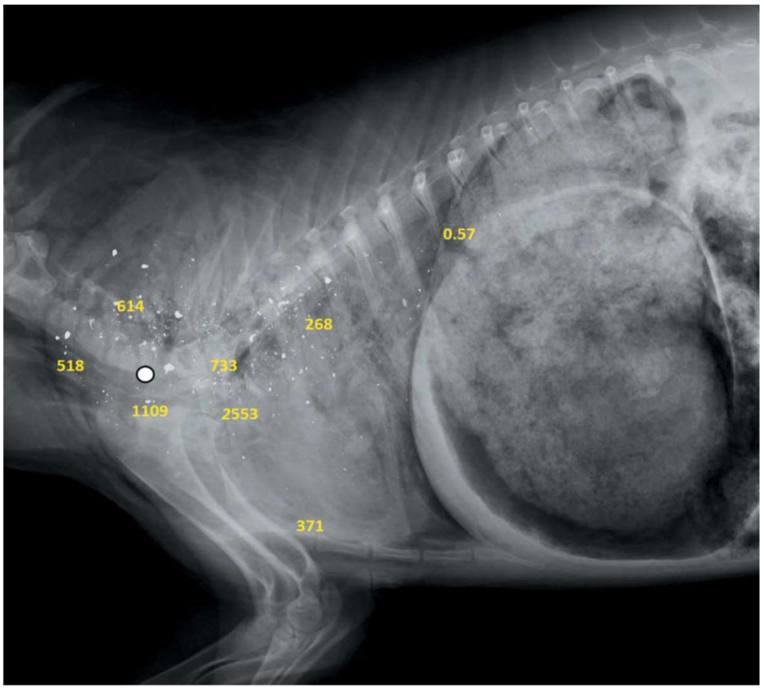

Figure 1. Latero-lateral radiographic projection of wild boar n. 34073, killed with lead ammunition. Several bullet fragments of variable size are clearly visible. Some lead levels are reported on the image in their respective sampling point. The position of the entry wound (left shoulder) is shown by a white dot.

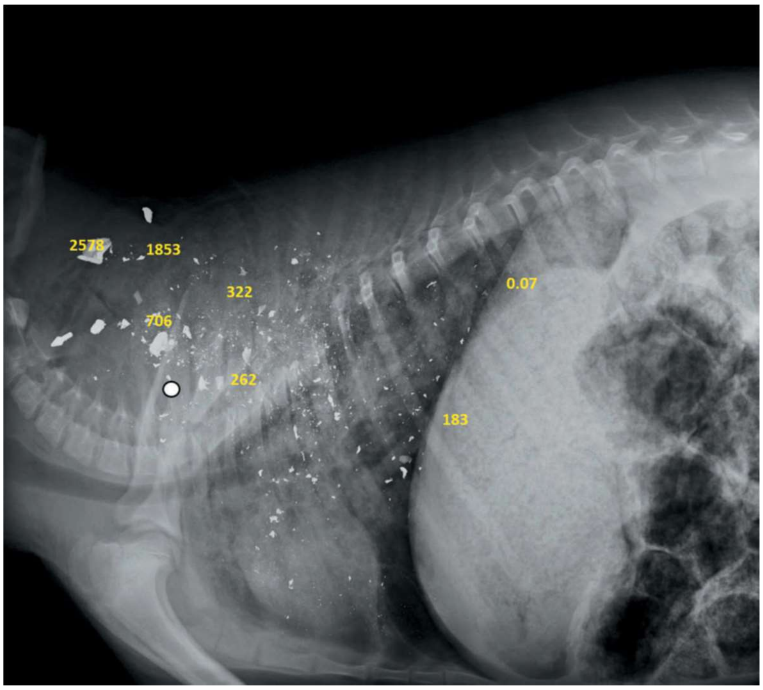

Figure 2. Latero-lateral radiographic projection of wild boar n. 34078, killed with lead ammunition. Several big bullet fragments are visible among a scattered number of shreds of variable size. Some lead levels are reported on the image in their respective sampling point. The position of the entry wound (right cervical region) is shown by a white dot.

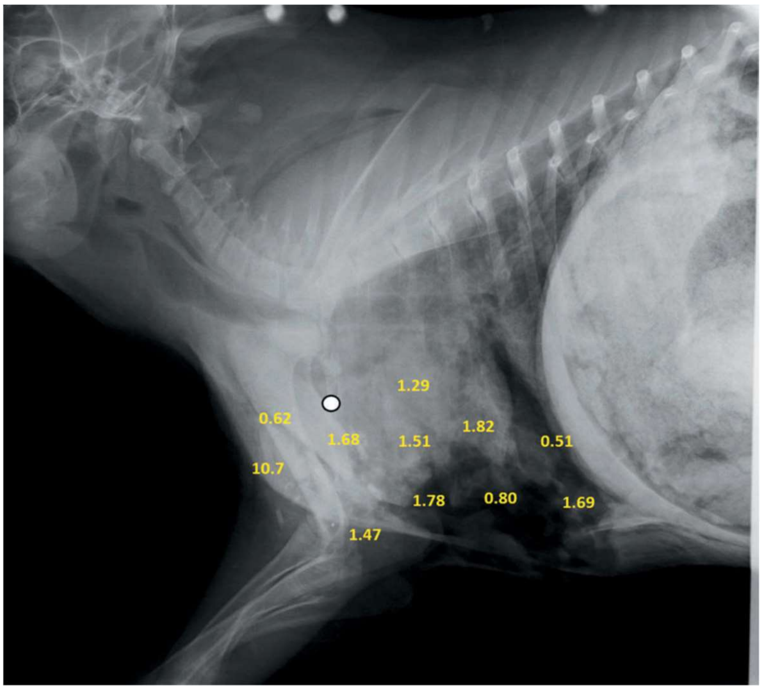

Figure 3. Latero-lateral radiographic projection of wild boar n. 01679, killed with copper ammunition. No bullet fragments are visible in the radiograph. Some of the copper concentrations are reported on the image in their respective sampling point. The position of the entry wound (right shoulder) is shown by a white dot.

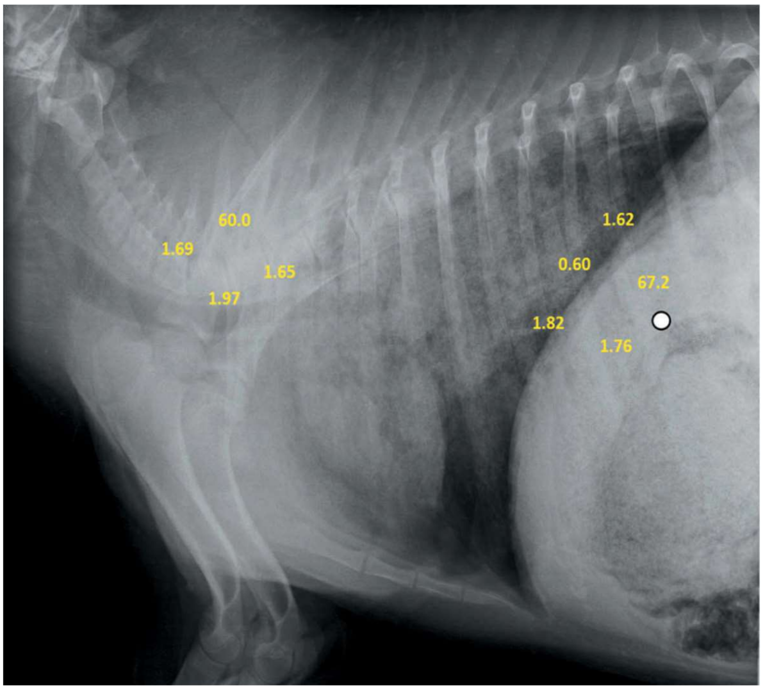

Figure 4. Latero-lateral radiographic projection of wild boar n. 01680, killed with copper ammunition. Despite the extensive damage caused by the bullet to the right humerus, no bullet fragments are visible in the radiograph. Some of the copper concentrations are reported on the image in their respective sampling point. The position of the entry wound (left abdominal region) is shown by a white dot.

9.2. Lead and copper levels

In all boars killed with lead bullets, the muscle samples collected from area A, closer to the wound channel, showed a very high lead concentration of 511 ± 130 mg/kg (Figure 5(a), Table 2). Mean lead concentrations in muscle samples from area B and C were much lower: 1.65 ± 0.87 mg/kg and 0.10 ± 0.04 mg/kg, respectively (Figure 5(b,c) and Table 2). Mean lead concentration in all meat samples (A + B + C) was 319 ± 83.8 mg/kg. In bone, blood, and liver samples lead levels of 557 ± 241, 73.6 ± 72.2, and 3.57 ± 2.74 mg/kg, respectively, were measured. Mean copper level in meat samples of wild boars killed with lead bullets was 1.48 ± 0.06 mg/kg (Table 2). The concentrations of copper detected in muscle samples of wild boars killed with lead-free ammunitions were considerably lower. Indeed, in muscle samples taken in the area near the wound channel (area A), mean copper concentration was 6.65 ± 3.07 mg/kg, while in muscle distant from bullet trajectory (area C) a mean level of 1.48 ± 0.26 mg/kg was measured (Figure 6). In these wild boars mean lead concentration was 0.04 ± 0.01 mg/kg (Table 2).

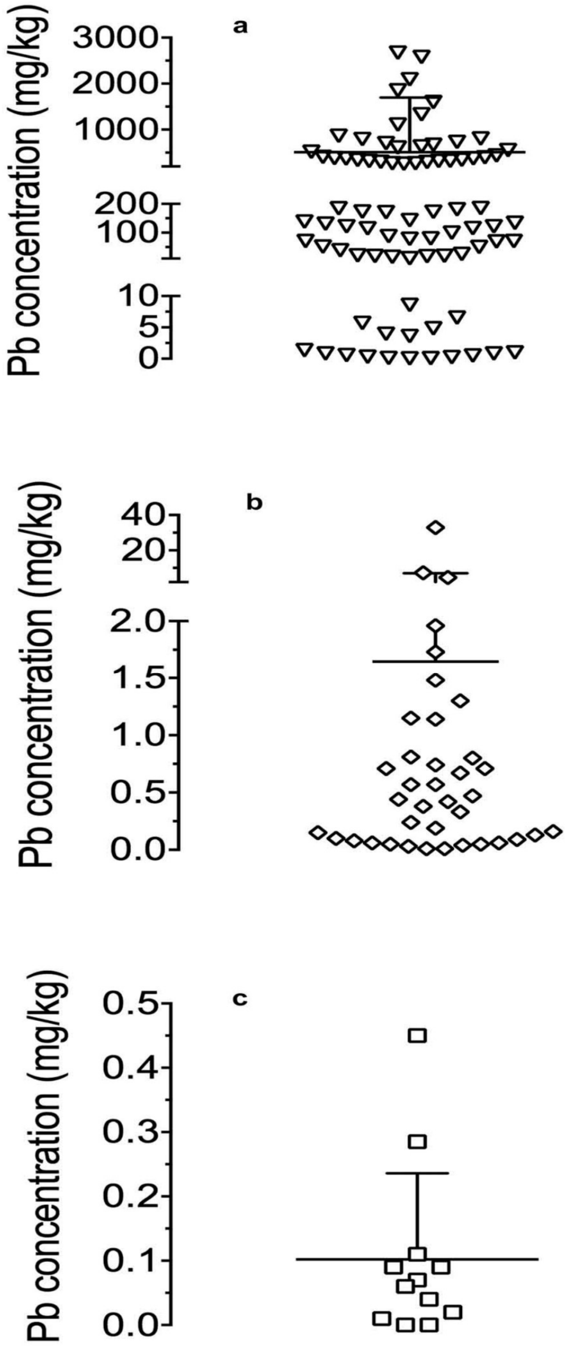

Figure 5. Symbols represent the lead concentrations (mg/kg) measured in each meat sample from area A (within 15 cm from bullet channel), area B (15–25 cm from bullet channel), and area C (>30 cm from bullet channel). The two highest lead levels from area A (8704 and 5188) are not shown for a better graph clarity. Mean and SEM are also shown in the graph

Bullet	Sample area	Pb level (mean ± SD)	Max Pb level	Mean Cu level (mean ± SD)	Max Cu level
Lead	A	511 ± 130	8704	1.56 ± 0.08	4.49
Lead	B	1.65 ± 0.87	32.93	1.27 ± 0.06	2.18
Lead	C	0.10 ± 0.04	0.45	1.49 ± 0.13	2.67
Lead	A+B+C	319 ± 83.8	8704	1.48 ± 0.06	4.49
Lead-free	A	0.04 ± 0.01	0.21	6.65 ± 3.07	67.2
Lead-free	C	0.02 ± 0.01	0.03	1.48 ± 0.26	1.88
Lead-free	A+C	0.04 ± 0.01	0.21	6.01 ± 2.70	67.2

Table 2. Lead and copper content (mg/kg) in meat samples of wild boars killed with lead or lead-free ammunitions.

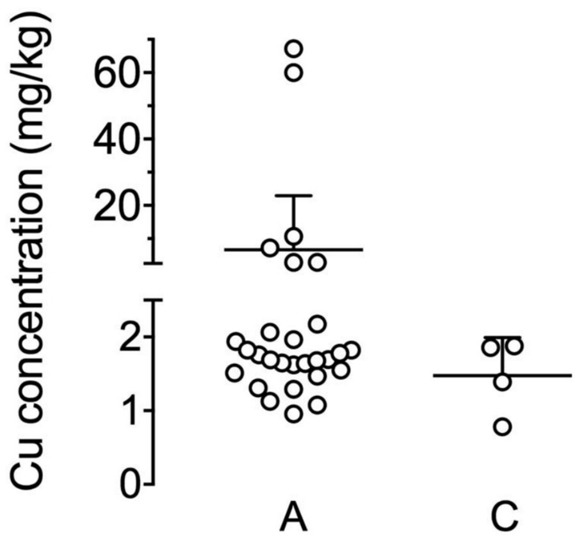

Figure 6. Symbols represent copper concentrations (mg/kg) measured in each meat sample from area A (within 15 cm from bullet channel), and area C (>30 cm from bullet channel). Mean and SEM are also shown in the graph.

10. Discussion

The present study showed that in all wild boars killed by means of lead ammunitions, a high degree of dispersion of bullet fragments is occurring. Despite the removal from the carcasses of all visible metal shreds, the ICP-MS analysis revealed very high levels of lead in the meat samples. In area A, close to the bullet trajectory, the mean lead concentration was more than five thousand times the maximum admitted level in livestock and chicken meat for human adults (0.1 mg/kg). This concentration is in accordance with the high number of bullet fragments observed in radiographic images, which are more numerous and of bigger size close to the wound channel. In samples of meat collected from area B (between 15 and 25 cm from bullet trajectory), however, mean lead concentration, even though considerably lower, was still about 16 times the maximum admitted level. A lower lead concentration, equal to maximum accepted level, was detected in muscle samples collected in area C, the most distant from the wound channel. In this study no tissue was discarded around the wound channel, thus it is possible that some samples of meat with high lead levels would have been removed in the finishing process by hunters in domestic conditions or by people processing meat for food preparations. However, given the wide area of dispersion of metal shreds, and the huge lead content, even an accurate excision of the meat crossed by bullet trajectory might unlikely prevent the potential intake of lead concentrations over the maximum limits fixed for other species. Moreover, the hardness of different organs hit by the bullet is able to greatly influence the fragmentation of lead core; indeed, radiographic images show that big lead shreds can be observed at a considerable distance from entry or exit wound, and a high lead concentration was found also in samples taken from area B, which is likely including marketable meat. The lead concentration detected in this study is considerably higher than those previously reported in the meat of wild boars killed with lead bullets (Amici et al. 2012; Danieli et al. 2012; Ertl et al. 2016; Gerofke et al. 2018). However, the differences in the calibre and weight of lead ammunitions used by hunters (which can range from 40 up to 300 grains, each corresponding to 0.0648 g lead), together with the unpredictability of bullet trajectory and fragment dispersion could be reasons of such discrepancies. Moreover, in those studies, either the meat that was visibly damaged by the bullet was discarded, or sampling was made very far from the wound channel, precluding the possible analysis of samples with very high lead concentration. Another factor to be taken into consideration is the possible contamination of the meat by means of the knife used by the hunters to prepare the meat for the consumer. In the study by Gerofke et al. (2018) the instruments were cleaned before the excision of each sample, while in our study they were not, in order to simulate as closely as possible what happens in usual hunting practice. The high lead levels detected in our study might be partially due to a contamination carried by the knife after collecting samples from the area near the bullet channel. Additionally, blood accumulated in thoracic or abdominal cavity, in which a very high lead concentration was detected, could also contribute to contamination, by coming in contact with meat also in portions of the carcass without bullet fragments. The lead level of muscle collected farthest from the wound channel is compatible with those measured in previous published studies (Danieli et al. 2012; Gerofke et al. 2018). Samples from area C were collected outside the area with visible presence of bullet parts, according to radiographs, and this could explain the low concentration of lead, compared to area A and B. Although the lead level detected in these muscle samples is equal to the accepted maximum concentration according to European regulations, the ingestion of meat from these parts of the carcass might not be devoid of risk for the consumer, because of the unpredictable trajectory of bullet shreds. Moreover, the risk of bioaccumulation in human body should not be underestimated, and repeated ingestion of low level of lead could damage health over time. Indeed, previous evidence exists that a regular consumption of game meat is linked to an increased risk of ingestion of relevant doses of lead (Johansen et al. 2004, 2006; Iqbal et al. 2009). In contrast with data obtained with lead-core ammunitions, radiographic images of carcasses of wild boars killed with copper ammunitions showed no visible metal fragments, and in both animals a clear exit wound was present, showing that the bullet was able to pierce through the bodies without a significant release of shreds. In accordance with radiographic analysis showing negative results for the presence of metal fragments, copper levels in the vast majority of muscle samples were low, even though mean concentration (6.01 ± 2.70 mg/kg) was higher than those found in a previous study (Schlichting et al., 2017), and above the suggested safe level for game meat (4 mg/kg) (EFSA, 2014). However, in one wild boar (identification tag n.01679), two of the meat samples taken on the border of entry and exit wounds had a high copper concentration (above 60 mg/kg), possibly due to the presence of tiny copper fragments, invisible by radiographic examination, and these values are the reason behind the relatively high mean level. In addition, other studies detected higher (Amici et al., 2012) or similar (Roślewska et al., 2016) copper levels in wild boars killed with standard ammunitions. It has to be also taken into consideration that bullets of lead-core ammunitions have usually an external jacket containing copper, and thus the fragmentation of this type of bullets might also cause a dissemination of copper shreds in the animal's body. Lead content in muscle samples of wild boars killed with lead-free ammunitions was 0.04 ± 0.01 mg/kg, and this value is likely attributable to the absorption of lead from the environment. Indeed, since this lead concentration was less than half compared to that of meat from area C in boars killed with lead ammunitions, the presence of bullet-derived lead also in parts of the carcass very distant from wound channel cannot be excluded, possibly due to contamination occurring during carcass finishing.

11. Conclusions

The results of this study suggest that commonly employed lead ammunitions, because of the high level of fragmentation, are able to disperse a high content of this toxic metal in the body of wild boars, raising serious concerns for the consumer's health. Even though the highest levels of lead were found in samples close to wound channel, a careful carcass finishing does not seem to grant sufficient protection against a dangerous lead absorption, since a toxic concentration of lead was detected also in the meat taken 15–25 cm from bullet trajectory. Moreover, a careless finishing of the carcass might greatly increase the risk of lead poisoning. By contrast, lead-free copper ammunitions, being subjected to a minimal fragmentation of the bullet, are able to grant very low dispersion of copper in the wild boar's body, as demonstrated by radiographic images and by copper analysis. Moreover, copper is a metal with physiologic properties, naturally present in the body, and therefore endowed with a much lower toxicity with respect to lead, which is considered a noxious element at any concentration. Copper ammunitions should therefore be considered a safer alternative to standard lead-based ones for the hunting of wild boars and other game, against the risks of heavy metal poisoning.

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