Universidade de Trás-os-Montes e Alto Douro

Biomonitoring of arsenic, cadmium, lead and mercury with raptors from the Iberian Peninsula

PhD thesis in Veterinary Sciences - Clinic

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FCT
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“Ao meu filho”

"Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me a descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante dos meus olhos"

Isaac Newton

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RESUMO

A poluição ambiental é um problema de crescente relevância por constituir uma ameaça na estabilidade dos ecossistemas e consequentemente afetar a saúde humana. Entre os diferentes tipos de poluentes, os metais tóxicos têm merecido grande preocupação porque persistem muito tempo no meio ambiente, são ubíquos e apresentam uma pronunciada bioacumulação nos organismos e biomagnificação na cadeia alimentar. Por estas razões, a monitorização destes poluentes é de extrema importância. As aves de rapina por estarem no topo da cadeia alimentar e apresentarem uma vida longa, são adequadas para a monitorização de poluentes bioacumuláveis e biomagnificáveis como os metais tóxicos.

Na Península Ibérica, principalmente em Espanha, têm sido realizados alguns estudos de biomonitorização de metais com aves de rapina, em determinadas áreas geográficas. Contudo, em Portugal este tipo de estudos é escasso e em Espanha ainda faltam informações relativamente à exposição de algumas espécies e em algumas áreas geográficas.

O principal objetivo do presente trabalho foi avaliar o grau e tipo de exposição aos principais metais e metaloides tóxicos em três espécies de aves de rapina e assim determinar indiretamente a qualidade ambiental dos ecossistemas terrestres relativamente a estes poluentes. Para alcançar este propósito, utilizando a técnica de espetrometria de massa com plasma acoplado indutivamente (ICP-MS), determinaram-se as concentrações de arsénio (As), cádmio (Cd), mercúrio (Hg) e chumbo (Pb) em amostras de sangue, fígado e rim obtidas a partir da espécie Águia-d'asa-redonda (*Buteo buteo*) e em amostras de sangue do Milhafre-preto (*Milvus migrans*) admitidos em diferentes centros de reabilitação de animais selvagens (CRAS) de Portugal. Também foram analisadas amostras de sangue de Grifos (*Gyps fulvus*) capturados e admitidos em CRAS da Península Ibérica para determinar as concentrações de Cd, Hg e Pb.

Na espécie Águia-d'asa-redonda, o metal mais identificado foi o Hg e o menos identificado foi o As, nos três tipos de amostras recolhidas. De entre as diferentes amostras analisadas, as concentrações mais elevadas foram identificadas no rim para todos os elementos, o que sugere a existência de uma acumulação dos metais e do As neste órgão. As aves adultas apresentaram concentrações hepáticas e renais de Cd mais elevadas do que as juvenis. As concentrações sanguíneas de Pb e Hg parecem apresentar uma associação com a época de caça e a muda das penas, respetivamente.

Considerando a espécie Milhafre-preto, as concentrações sanguíneas de metais mais elevadas foram obtidas para o Pb e Hg nas aves que vivem em liberdade. O grupo de aves que vive em cativeiro apresentou as concentrações mais baixas para todos os elementos químicos estudados, tendo-se observado diferenças significativas ($p<0.01$) com as aves de vida livre apenas para o Hg e Pb.

Nos Grifos avaliados não foram detectadas concentrações sanguíneas de Cd e de Hg em mais de 95% dos animais, no entanto o Pb foi identificado em todos os animais. A intoxicação por Pb foi confirmada em três Grifos. A população de Grifos capturada na Catalunha (Espanha) apresentava as concentrações sanguíneas de Pb mais elevadas possivelmente devido ao contexto ecológico do habitat destas aves: área urbana e industrial, onde as lixeiras parecem fornecer uma fração significativa das suas necessidades alimentares. Os Grifos admitidos aos CRAS apresentaram concentrações de Pb sanguíneas significativamente mais baixas ($p<0.05$) que os animais capturados. Esta diferença pode ser explicada pelo facto da desnutrição ter sido a principal causa de admissão destes animais nos CRAS. Estes resultados indicam que os Grifos admitidos aos CRAS talvez não sejam representativos das populações locais quanto à exposição recente ao Pb.

Comparando as três espécies estudadas, a Águia-d’asa-redonda apresentou as concentrações sanguíneas de Hg mais elevadas, o Grifo as de Pb e o Milhafre-preto as de As. O Cd sanguíneo não foi detetado numa grande percentagem de animais (>90%) nas três espécies de rapinas estudadas. Esta diferença interespecífica observada para o As, Hg e Pb sanguíneo parece estar relacionada principalmente com diferenças nos hábitos alimentares.

Perante os nossos resultados pode-se afirmar que embora as aves de rapina estejam mais predispostas aos processos de biomagnificação que podem ocorrer na cadeia alimentar, as concentrações dos metais analisados e do As identificadas são compatíveis com uma exposição de baixo risco. Contudo, na Águia-d’asa-redonda os resultados obtidos sugerem uma exposição crónica a estes poluentes e o potencial risco do Hg deve ser considerado uma vez que esta espécie apresentou níveis relativamente elevados deste metal. O risco de toxicidade pelo Pb pode ser elevado principalmente no Grifo, e poderá ser um factor limitante para a conservação desta espécie.

Palavras-chave: Metais tóxicos, Águia-d’asa-redonda, Milhafre-preto, Grifo, Portugal, Espanha

ABSTRACT

Pollution is a problem of growing relevance because it is a serious threat to the stability of ecosystems and it affects the human health. Unlike many other pollutants, toxic metals have been contaminants of great concern because they are persistent, ubiquitous and they have a pronounced bioaccumulation in tissues and biomagnification in food chains. For these reasons, monitoring the toxic metals has become increasingly important. Raptors are considered especially suitable for monitoring bioaccumulable and biomagnificable pollutants mainly due to their position at the top of trophic chain and their susceptibility to bioaccumulate contaminants over time.

In the Iberian Peninsula, although several biomonitoring studies using several raptor species have been performed in Spain, still remains lack of information regarding the exposure to metals in some raptor species and about environmental contamination in some geographical areas. In Portugal, biomonitoring studies with raptors have been scarce.

The main goal of the present work was to evaluate in three raptors species the degree and type of exposure to the toxic metals and arsenic (As) and thus indirectly determine the environmental quality of terrestrial ecosystems related to these pollutants. For this purpose, the concentrations of As, cadmium (Cd), mercury (Hg) and lead (Pb) were determined by inductively coupled plasma mass spectrometry (ICP-MS) in blood, liver and kidney samples taken from Common buzzards (*Buteo buteo*) and in blood samples taken from captive and free-living Black kites (*Milvus migrans*) admitted to different Portuguese wildlife rehabilitation centres (WRCs). Blood samples taken from Griffon vultures (*Gyps fulvus*) caught in the wild or admitted to WRCs in the Iberian Peninsula were also analysed for Cd, Hg and Pb.

In the Common buzzard, Hg and As were the elements which appeared in the highest and lowest concentrations, respectively. The kidney was the analyzed sample which showed the highest concentrations of each element, what suggests an accumulation of the toxic metals. Adults' Common buzzards showed higher hepatic and renal Cd concentrations than juveniles, because this metal is accumulated throughout the life span of the birds. Blood Pb and Hg concentrations seem to show an association with the hunting and molting season, respectively.

Regarding the Black kite, the highest mean blood concentrations were found for Pb in free-living birds followed by Hg. The captive birds had the lowest blood concentrations for all analyzed toxic elements, but significant differences (p<0.01) with the free-living birds were

only observed for Hg and Pb. Therefore, an available source of Pb and Hg seems to be present in the habitat of the Black kite.

In the studied Griffon vultures, Cd and Hg were not detected in more than 95% of the birds, while Pb was detected in all birds. Pb poisoning was confirmed in three Griffon vultures. The population of vultures captured in Catalonia (Spain) has the highest mean blood Pb concentration possible due to the ecological context of the bird's habitat: an urban and industrial area, where rubbish dumps provide a significant fraction of their trophic needs. Birds admitted to WRCs had significantly lower Pb concentrations ($p<0.05$) than animals caught in the wild. This may be explained by the fact that malnutrition was the main cause of their admission to WRCs, once the ingestion has been described as the most significant pathway for Pb exposure in raptors. Therefore the Griffon vultures admitted to WRCs do not seem to be representative of the local free-living populations in terms of recent Pb exposure.

The comparison between the three species shows that the Common buzzard had the highest blood Hg concentrations, while the highest blood Pb concentrations were obtained for Griffon vulture. Blood As was higher in the free-living Black kites. A high percentage of birds (>90%) with not detected blood Cd concentrations were found in the three species. The interspecific difference observed for blood As, Hg and Pb concentrations seems to be mainly related with the different dietary habits.

Although raptors are potentially exposed to any biomagnification processes that may occur in a food web, the concentrations of metals and As detected in the samples collected from the three raptor species could be considered compatible with a low-risk exposure. However, chronic exposure to these metals was verified for Common buzzard and the potential risk of Hg should be considered due to the relatively high Hg concentrations found in this species. In certain cases, Pb can be related to toxic side effects and it could be a limiting factor for the Griffon vulture conservation.

Keywords: Toxic metals, Common buzzard, Black kite, Griffon vulture, Portugal, Spain.

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List of abbreviations and symbols

AAS	-	Atomic Absorption Spectrometry
As	-	Arsenic
ASV	-	Anodic Stripping Voltammetry
Ar	-	Argon
Ba	-	Barium
BSE	-	Bovine spongiform encephalopathy
Cd	-	Cadmium
CERAS	-	Centro de Estudos e Recuperação de Animais Selvagens
CERVAS	-	Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens
Cl	-	Chlorine
Co	-	Cobalt
CR	-	Critically endangered
Cu	-	Copper
CV	-	Cold Vapor
DD	-	Data deficient
d.w.	-	Dry weight
EU	-	European Union
Fe	-	Iron
GM	-	Geometric mean
GF	-	Graphite Furnace
Hg	-	Mercury
HNO_3	-	Nitric acid
H_2O_2	-	Hydrogen peroxide
I	-	Indeterminate
ICP-AES	-	Inductively Coupled Plasma-Atomic Emission Spectrometry
ICP-MS	-	Inductively Coupled Plasma-Mass Spectrometry
LC	-	Least concern
LOD	-	Limit of detection
LOQ	-	Limit of quantification
MeHg	-	Methylmercury
Mn	-	Manganese

Mo	-	Molybdenum
S.D.	-	Standard deviation
n.d.	-	Not detected
NT	-	Near threatened
Pb	-	Lead
PT	-	Portugal
RE	-	Regionally extinct
RIAS	-	Centro de Recuperação e Investigação de Animais Selvagens
SCT-UB	-	Scientific-Technical Services of the University of Barcelona
Se	-	Selenium
SP	-	Spain
UP	-	Unknown period
USA	-	United States of America
VU	-	Vulnerable
w.w.	-	Wet weight
WRC	-	Wildlife rehabilitation centre
Zn	-	Zinc

LIST OF PUBLICATIONS

Peer-reviewed publications included in this doctoral thesis:

Carneiro M, Colaço B, Faustino-Rocha A.I., Colaço A, Lavin S, Oliveira PA. Biomonitoring of metals and metalloids with raptors from Portugal and Spain: a review. *Environmental Reviews*. doi: 10.1139/er-2015-0051.

Carneiro M, Oliveira, PA, Brandão R, Nicolas Francisco O, Velarde R, Lavín S, Colaço B. (2015). Lead poisoning subsequent to lead-pellet ingestion in Griffon vultures (*Gyps fulvus*) from the Iberian Peninsula. *J Avian Med Surg.* Accepted for publication.

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Carneiro M, Colaço B, Brandão R, Ferreira C, Santos N, Soeiro V, Colaço A, Pires MJ, Oliveira PA, Lavin S. (2014). Biomonitoring of heavy metals (Cd, Hg, and Pb) and metalloid (As) with the Portuguese common buzzard (*Buteo buteo*). *Environ Monit Assess.* 186(11), 7011-7021.

Articles submitted for publication based on material of this doctoral thesis:

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Carneiro M, Colaço B, Brandão R, Ferreira C, Santos N, Soeiro V, Colaço A, Pires M, Oliveira P, Lavin S. (2012). Blood Concentrations of Heavy metals (Ba, Cd, Hg and Pb) and Metalloid (As) in Raptor Species (*Buteo buteo*, *Milvus migrans* and *Gyps fulvus*) from Portugal. In: *6th SETAC World Congress / SETAC Europe 22nd Annual Meeting*, Berlin, Germany, 20-24 May.

Carneiro M, Colaço B, Brandão R, Santos N, Soeiro V, Colaço A, Pires M, Oliveira P, Lavin S. (2011). Biomonitoring of heavy metals with common buzzard (*Buteo buteo*) from Portugal. In: II Simpósio de Selvagens e Exóticos, UTAD, Vila Real, Portugal, 18 e 19 Novembro.

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Pollution has increased in the last century, affecting ecosystems and human health. Unlike many other pollutants in the environment, metals and arsenic (As) have been identified among the most toxic elements to nearly all living beings. Metals have been considered contaminants of great concern because they are persistent, ubiquitous, non-biodegradable and some of them have a pronounced bioaccumulation in organisms and biomagnification in food chains [2, 26]. They can make their way in aquatic and terrestrial systems through different ways, such as atmospheric fallout, waste dumping and accidental leaks [17, 38, 39].

Despite some prohibitions and restrictions imposed by the European legislation regarding the release of metals to the environment that have been important for the continuous reduction of toxic metals emissions in Europe during the last 40 years, they persist in the environment and several anthropogenic activities (production of energy, industrial activities, incineration of wastes, hunting activities, mining and smelting) continue releasing relatively large amounts of these contaminants. For example, during the last decade numerous regulatory steps were enacted to reduce the deposition of lead (Pb) in the environment, namely restriction of the utilization of leaded fuels, reduction of the allowed Pb emissions, restrictions of Pb-based products and banning the use of Pb shot for the hunting of waterfowl. However, Pb shot continues to be used for hunting upland game and for other recreational shooting [28]. In the specific case of mercury (Hg), emissions in other regions may probably affect the concentrations of this pollutant in the air and precipitation of this pollutant in Europe [35-37, 39]. Furthermore, future emissions of toxic metals are forecasted on the basis of various scenarios such as economy growth, industrial development, resources use and population changes [35-37, 39].

Exposure to metals is potentially harmful especially for those metal-compounds which do not have any physiological role in the metabolism of cells, such as cadmium (Cd), Hg and Pb. Although the functionality of As in living beings is not perfectly understood, it is known that exposure to this metalloid is also potentially harmful. In addition to the acute toxicity of these toxic elements, prolonged exposure to relatively low doses can result in sub-lethal effects in living beings and therefore may cause population declines in a large number of species [5, 8, 19, 20, 48, 49].

In birds, highly toxic inorganic As may act as an endocrine disruptor, disrupt reproduction or trigger sublethal effects. It has also been shown that it negatively affects the central nervous system [11, 29, 30, 47]. Exposure to Cd can cause tissue damages (intestine, kidney and testis), changes in behavior, disruption of calcium metabolism, decrease in food intake and thinning of eggshells [6, 10, 19]. Methylmercury (MeHg), the most common organic form of Hg in living beings, has high toxicity due to its chemical stability and lipophilicity. It has been shown that it adversely affects birds, particularly through its neurotoxicity and negative effects on reproduction. Some of these effects are neurobehavioral underdevelopment, induction of eggshell thinning and malformations, inhibition of egg production and embryotoxic effects [13, 27, 31, 42, 44, 49]. In contrast, due to the high affinity of inorganic Hg for renal tissue, the major effects of this metal occur in the kidneys where it causes necrosis of cells from the proximal tubule [43, 49]. Pb poisoning is the most frequent type of metal poisoning in birds of prey and the chronic exposure can lead to population declines in more sensitive species. Pb is a non-specific poison that affects all body systems, causing alterations at vascular, haematological, nervous, renal, immune and reproductive systems, as well as behavioral changes [7, 8, 14, 16, 20, 32, 41, 43].

For the reasons mentioned above, monitoring the toxic metals and As has become increasingly important. Their release in the environment results in their distribution in water, air, soil and in the organisms living therein. To assess the pollution in a certain environment, it is impossible to analyze all these different compartments. In addition, it is very difficult to quantify the impact on organisms by measuring concentrations in the environment only. Therefore monitoring the concentrations in the environment and measure the bioavailability and resultant uptake by biota or people is often performed by means of biomonitoring [4, 18, 25]. Biomonitoring refers to a set of scientific techniques that assess environmental pollution based on sampling and analysis of tissues and fluids from individual organisms. These techniques take advantage of the knowledge that contaminants that have entered into the organisms leave marks reflecting the exposure. The marks may be the contaminant itself. It may also be a breakdown product of the chemical or some biological changes in the organisms that are the result of the action of the chemical on the individual. The results of these measurements provide information about the amounts of contaminants that have entered and remained in the organisms and the corresponding induced effects [50].

Compared to other vertebrate classes, birds have overwhelming been used to evaluate environmental contamination because they play an important role as bioindicators and biomonitors: they are abundant, widely distributed, relatively easy to observe and one of the best studied groups of organisms, they are in the focus of public interest and care and they are particularly sensitive to anthropogenic contamination. Amongst birds, the raptors (birds of prey, owls and scavengers) are considered especially suitable to monitor bioaccumulable and biomagnificable pollutants, mainly due to their position at the top of trophic chain and since they feed over a large geographical area, they reflect pollutants levels over a broad area and less samples may be necessary. Monitoring such substances in raptors could be useful not only to evaluate the health condition of the involved species, but also to assess the degree of contamination in the ecosystem where they live. Another advantage is that they reproduce the effect of biomagnification at trophic levels close to humans, an aspect that is not guaranteed by other bioindicators at low trophic levels, which may be useful in terms of public health. Furthermore, some raptors exhibit greater degrees of sensitivity to certain contaminants than other kinds of birds [3, 15, 18, 24, 33, 40, 46].

Several birds' tissues have been used to monitor avian exposure and to assess the risk of toxic metals. At present, blood, feathers and eggs are preferred to this kind of research because they can be obtained easily, repeatedly from the same individual and is not necessary to sacrifice the animals [1, 25]. Whereas blood is used to monitor recent exposure in many species of wild birds at any time in their life, eggs and feathers can be used to assess levels and bioaccumulation of environmental contaminants only at specific times in the avian lifecycle [45]. Although less frequently used, other interesting samples can be collected such as excrements, preen oil, and regurgitated pellets. Several birds' internal tissues (e.g. liver, kidneys, brain, muscle, lungs, intestine, fat and bones) have been also used to monitor avian exposure to the toxic metals [21, 25]. Despite the difficulties inherent to the collection of internal tissues, birds find dead in the field or that die after admission to wildlife rehabilitation centres (WRCs) can provide enough samples. The viscera reflect mainly the concentrations of metals obtained from the diet and which will accumulate in these organs over time. Therefore they are the best indicators of chronic exposure and they can contain higher concentrations of some metals than those generally found in the blood and other tissues [9, 12, 22, 23, 34]. Due to the different accumulation of the same metal in the different tissues, it is essential to measure its concentrations in different tissues of a species in order to evaluate at population level. Additionally, it is also important taking into account that metals tend to be held in one particular tissue at much higher level than others, for example, Cd concentrates in kidneys, Pb in bones and Hg in liver and kidneys [43].

During the last decades several studies using raptor species have been carried out worldwide in order to monitor environmental metals pollution. In the Iberian Peninsula, some studies have been performed in Spain, while in Portugal these studies have been scarce. Previously to this work, were only published three studies on raptors from Portugal, two of which share the same sampled individuals. Therefore there is little information regarding the abundance of the toxic metals in the Portuguese terrestrial ecosystems and their bioavailability in the animals at the top of food chain. Furthermore, taking into account that a large number of raptor species breeding in the Iberian Peninsula has conservation problems, it is necessary to determine the degree and type of exposure to these contaminants. Due to the metals toxicity and their persistence in the environment and continuous release into the atmosphere, it is of utmost importance continuing with biomonitoring studies to assess spatial and temporal trends in metal levels.

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Chapter 2

Aims

2. AIMS

Environmental pollution by persistent contaminants, such as toxic metals, is a worldwide problem, and represents a serious threat for the stability of ecosystems and living organisms. The need to determine the environmental quality of terrestrial ecosystems and the degree and type of exposure to the toxic metals and metalloids in raptors from the Iberia Peninsula led to this biomonitoring study.

Therefore, the following particular objectives were defined:

- Review the biomonitoring studies published from Iberian Peninsula using raptors taking into account the following specific issues: monitored raptor species, sampling periods, monitored areas, type of samples, analytical techniques used and the analyzed metals and metalloids.

- Determine the As, Cd, Pb and Hg concentrations in whole blood, liver and kidneys samples of the Common buzzard (*Buteo buteo*) from different areas of Portugal. Evaluate the influence of age, gender and origin of the Common buzzards on blood, hepatic and renal toxic-metal concentrations. Investigate also the influence of the season on blood-metal concentrations.

- Determine the concentrations of As, Cd, Hg and Pb in whole blood samples of Black kites (*Milvus migrans*) kept in captivity and from free-living Black kites nesting in Portugal, and to assess if there are differences between these two groups of birds.

- Investigate the blood metal (Cd, Pb and Hg) concentrations in Griffon vultures (*Gyps fulvus*) from different areas of Portugal and from Catalonia, Spain and to determine if metals in the blood of weak and/or injured Griffon vultures admitted to WRCs reflect profiles of contamination in the local, free-living and outwardly healthy Griffon vulture population. Investigate also differences between sampled areas.

CHAPTER 3

BIOMONITORING OF METALS AND METALLOIDS WITH RAPTORS FROM PORTUGAL AND SPAIN: A REVIEW

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3. Biomonitoring of metals and metalloids with raptors from Portugal and Spain: a review

3.1. Introduction

Metals are natural constituents of all ecosystems that are present at low concentrations in soils, plants and living organisms. Their distribution in the environment results from natural processes and various anthropogenic activities. However, human activities significantly increase their presence in the environment and these anthropogenic inputs exceed removals by natural biogeochemical cycles of metals in many ecosystems [49, 114].

Some metals, such as copper (Cu), cobalt (Co), iron (Fe), molybdenum (Mo), manganese (Mn), selenium (Se) and zinc (Zn) are considered important micronutrients once they are required in relatively small amounts for many physiological processes in living beings; their supply below physiological requirements can result in deficiency diseases and in larger amounts they may be harmful and even toxic to the organisms. Other metals such as Cd, Pb and Hg are not necessary for any physiological process, they have toxic effects on living organisms and are classified as contaminants or non-essential elements [41, 49, 73]. These are the most dangerous metals for both humans and animals, not only because of their high toxicity but also due to their persistence and pronounced biological accumulation and biomagnification in food chains [17, 18, 31, 59]. As is a metalloid or a semi-metal, although there is some uncertainties regarding to its necessity for physiological processes, it is well known that it has toxic effects in living beings [39, 84, 125, 133]. Unlike other toxic metals, As has a low rate of bioaccumulation and it is not biomagnified in the food chain [39, 84].

Environmental contamination by non-essential elements is a worldwide problem and represents a serious threat to the stability of ecosystems and living organisms [7]. The need to determine the degree of exposure and the effects of contaminants in both animals and humans have led to numerous biomonitoring studies. Direct monitoring of air, soil, water and sediments can be useful to determine the degree of contamination in a particular area; however, it does not provide a measure of bioavailability and resultant uptake by biota or people. It is only through direct biomonitoring (the analysis of contaminants in fluids and tissues of organisms) that the actual exposure of organisms can be properly determined and related to the levels in the physical environment [11, 67]. In general, the objectives of monitoring sentinel animals include data collection in order to: (1) estimate human health risks; (2) identify contamination of the food-chain; (3) determine the levels of environmental contamination; (4) identify adverse effects on animals (5) examine spatial or temporal trends in the contamination levels [67, 110].

Many of these studies are carried out with birds, that are particularly sensitive to the anthropogenic contamination [2-4, 8, 20, 33, 109]. Amongst birds, raptors (birds of prey, owls and scavengers) are considered especially suitable for monitoring bioaccumulable and biomagnificable pollutants, mainly due to their position at the top of trophic chain [51]. They may also provide information about the integration of contaminants that reaches other species in the top of trophic chain, such as humans [47, 51, 138]. Other key characteristics make raptors good sentinels for environmental contamination, namely relatively large home range, relative ease with which individuals (particularly nestlings) can be captured and non-destructive samples (blood, feather, preen gland oil) can be collected, and relative ease with which populations can be quantified and monitored [9, 67]. Moreover, raptors are known to have measurable responses to persistent, bioaccumulative and toxic chemicals, ranging from residue accumulation to population decline [13, 42, 54, 90, 99, 112].

The aim of this work is to provide synthesized information of the studies carried out in Portugal and Spain on the biomonitoring of metals and metalloids using raptors, through a systematic search of the published literature. Considering only the studies that performed a direct biomonitoring, the information is summarized taking in account the following specific issues: monitored raptor species, sampling periods, monitored areas, type of samples, the analytical techniques used in the determination of the metals, the analyzed metals and metalloids and the concentrations obtained.

3.2. Monitored raptors species

Raptor species, have been an important group of species used for monitoring purposes**,** not only because many of them are excellent sentinels of environmental contamination indicating specific environmental change, but also because they are valued natural resources in their own right with an important ecological role (providing important cultural and regulatory ecosystem), that may be adversely affected by toxicant exposure [9, 65].

In the table 3.1 is summarized the main characteristics (trophic position and the most important prey, conservation status and migratory habits) of the raptors species breeding in Portugal and Spain, specifying which ones were used for monitoring purposes of metals and the total number of analyzed individuals. The selection of the raptor species for monitoring metals and metalloids in Portugal and Spain has been mainly influenced by the availability of samples, which in turn is conditioned by particular features of each species, such as abundance and conservation status, geographical distribution, type of habitat and migration habits. For example, the collection of samples from animals in their natural environment can sometimes be difficult to accomplish in raptors from wooded or mountainous areas, especially out of the breeding season and the cost and inherent difficulty in obtaining samples are disadvantages [55]. The collaboration with the WRCs can be useful for environmental biomonitoring, mainly when it is difficult to collect samples from animals in their natural environment, as it was in the sample collection of the Common buzzard, one of the most commonly used species in biomonitoring studies in both countries [26, 27, 57, 59, 60, 103, 118].

The type of diet has also been an important factor in the selection of the bird species, since it influences the bioaccumulation of metals [60, 116]. Mateo et al. [103] and García-Fernández et al. [60] found the highest Pb concentrations in tissues of bird species that usually feed on carrion, and the piscivorous species are capable of accumulating higher concentrations of Hg in their tissues [45, 131]. Most of the species frequently monitored in Portugal and Spain are mixed feeders, often taking a mixture of mammalian, avian, fish and, in some cases, invertebrate prey, which is considered by some authors a suitable characteristic in biomonitoring studies of different contaminants [8]. However, diet variation may have major confounding effects in studies of biomonitoring environmental contamination using birds of prey. For this reason, some authors recommended the use of bird species with narrow and inflexible diets in contamination studies, rather than generalist predators [83, 105, 116]. However, true dietary specialists are hard to find and constant diets across space and time is unwarranted.

The aim of the work is also a determining factor in the selection of the biomonitor bird species. For example, species having an unfavorable conservation status such as Bearded vulture, Egyptian vulture and Spanish imperial eagle were used in researches that mainly aimed to assess the adverse effects of the metals on their populations [38, 68, 76, 81, 115, 120].

Most of the raptor species breeding in Portugal and Spain (20 out of 26 species of diurnal raptors and 6 out of 8 nocturnal ones) have been used at some stage for monitoring metals. However, in many cases, the number of analyzed specimens was very low, being fewer than

20 (Cinereous vulture, Golden eagle, Short-toed eagle, Montagu`s harrier, Hobby, Peregrine falcon, Long-eared owl and Scops owl). Amongst the monitored raptors, Eagle owl (n=979), Black kite (n=428) and Common buzzard (n=408) are the species from which the greatest number of individuals has been analyzed. The Spanish imperial eagle is found only in the Iberian Peninsula and it is considered one of the most threatened birds of prey in Europe [120]. Several studies have analyzed the risk of metals for the conservation of this species in a relatively large number of individuals (n=257). In Portugal, only the raptor species Common buzzard, Griffon vulture, Eagle owl, Bonelli's eagle, Little owl, Tawny owl and Barn owl were used for monitoring purposes, wherein were sampled less than 20 individuals in the last three referred species [25, 26, 47, 93, 116]. Eagle owl and Common buzzard gather relevant features, such as favorable conservation status, wide geographical distribution, non-migratory habits, and high territoriality and diversified diet, which make them suitable biomonitors. Although Black kite is a migratory bird, the other life history traits make it also a suitable species for contaminants monitoring. They often breed in habitats highly affected by agricultural, industrial and urban pollution. Therefore, they were important indicators of environmental contamination in an environmental disaster (Aznalcóllar mining accident) and in polluted areas [3, 5, 6, 12, 14]. Furthermore, some studies have shown that Black kites are susceptible to metal toxicity and contamination has been suspected as an important factor that contributes to the decline of their populations. In general, the less sampled species for metals monitoring purposes are those with an unfavorable conservation status and/or with migratory habits.

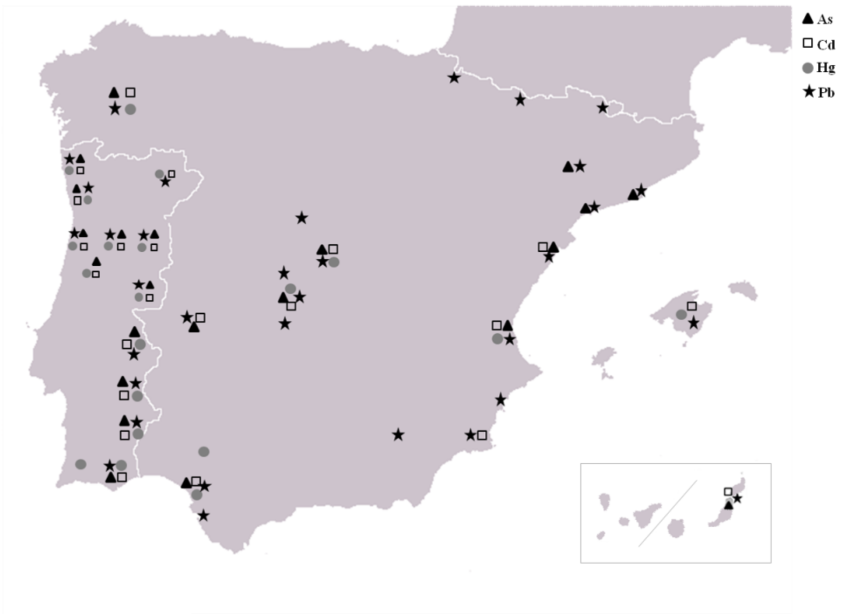
In future studies, other raptor species such as Eurasian sparrowhawk, Common kestrel, Little owl, Tawny owl and Barn owl can be used more frequently for biomonitoring purposes of metals in Portugal and Spain, once they gather the essential characteristics to be suitable biomonitors, namely their relative high abundance that can easily provide a large number of samples for environmental biomonitoring. Future efforts should also be focused on the study of those species that have demonstrated their usefulness in matters of conservation, such as Black-winged kite, Bonelli's eagle, Cinereous vulture, Egyptian vulture, Golden eagle, Red kite and Osprey.

3.3. Sampling periods and monitored areas

In the table 3.2 are listed the sampling periods and the bird species studied by each monitored area of Portugal and Spain. There is a striking difference between Portugal and Spain regarding to the studies performed on the biomonitoring of metals using raptors. To our knowledge, to date only six studies were performed in Portugal. Although these studies have enclosed several areas of the country, there is still a lack of information especially in the central and southern coast of the country. In Spain, despite several studies were carried out, there are still many areas not monitored (Fig. 3.1).

Amongst the most sampled bird' species, the Common buzzard stands out as having been monitored over practically the entire Iberian Peninsula, except in the Islands, while the Eagle owl and the Black kite were mostly sampled in Murcia, Southeastern Spain and in Doñana National Park, Southwestern Spain, respectively. Concerning to the analyzed metals, unlike Hg that was mostly analyzed in Portugal and Southwest Spain, Pb was monitored over a larger number of geographic areas (Fig. 3.1).

Figure 3.1 Areas of Portugal and Spain (approximate indication) in which the most toxic metals and metalloids were monitored with raptors, specifying the analyzed elements in each area.



The monitored area is often closely related to the purpose of each study and the location of specific sources of metals such as environmental disasters, industry, agriculture, hunting activities or urbanization. For example, in the Southwest of Spain, some studies were carried out after an environmental disaster (Aznalcóllar toxic spill) in order to evaluate its effects on bird populations [5, 6, 12]. The selection of the area to monitor is also related with the distribution of the species intended to be used as monitors and its consequent conservation status. For example, research with threatened species like the Spanish imperial eagle has been made in Central Spain and Doñana National Park, Southwestern Spain. The Bearded vulture in the Iberian Peninsula is only distributed by the Pyrenees, and thus researches with this species are limited to this geographical area.

Furthermore, the geographic location of the research groups also influences the monitored areas. The research groups who have taken on the monitoring of raptors as a major branch of their research are localized in Spain, namely in Doñana (Southwest), Madrid (Central), Barcelona (Northeast) and Murcia (Southeast). For this reason, Central, Northeast and South of Spain are the areas in which a greatest number of studies have been performed. The research groups in Central and Southwest Spain have focused with a high degree in two species with an unfavorable conservation status (Black kite and Spanish Imperial eagle) and the studies carried out in the Southeast and Northeast Spain have covered a greater variety of species with a more favorable conservation status. In Portugal, because there is not any research group mainly directed to monitor environmental contaminants with raptors, have been performed only few isolated studies.

The research carried out in Portugal and Spain has been sporadic in both time and space. The earliest monitoring studies started in 1980s in Central and Southwest of Spain mainly with the Spanish Imperial eagle species [68, 76, 78]. In Portugal the first study was published in 2005 with the Bonelli’s eagle [116]. It was during the period between 1997 and 2005 that the majority of the investigations were performed and therefore a greater number of species and areas were evaluated. In recent years there has been a decrease of investigations directed to the biomonitoring of toxic metals with raptors in the Iberian Peninsula and there is not a significant number of long term monitoring studies which could be potentially important to assess time trends in contaminants concentrations in birds.

3.4. Type of samples

The 41 biomonitoring studies with raptors carried out in Portugal and Spain have measured metals in a variety of different biological samples. In the table 3.3 are listed the metals and metalloids measured in each raptor species from Portugal and Spain *per* sample type and the total number (n) of the different analyzed samples for each species.

Choosing the type of samples to be used depends directly on the endpoint and, in a great degree, on the kinetics of the metals to be analyzed. Furthermore, the number of available samples is conditioned by several issues such as ethics legalities, census, and human and financial resources [55]. For example, the conservation status of a species is a conditioning factor in selecting the sample type, wherein the samples that could be collected from live birds in a non-destructive way are preferred.

Blood has been the preferred sample in most biomonitoring studies performed in Portugal and Spain, in order to measure the concentrations of all studied elements. Blood samples provide the most direct and non-lethal assessment of dietary exposure, reflecting recent exposure primarily and recently metabolized contaminants to a lesser extent [3, 45, 126]. Moreover, blood levels strongly correlate with concentrations in other internal tissues and blood samples can be easily and repeatedly obtained from the same individual if required [19, 59, 87, 88].

Non-invasive samples such as eggs and feathers have also been collected in some species. In the first periods of biomonitoring with raptors in Spain, eggs were frequently collected to evaluate a variety of different metals in twelve raptor species [68, 69, 76-78, 111]. Maybe because eggs are not indicative of exposure in all individuals of the population, such as males and juvenile birds, and it is difficult to obtain a sufficient number of samples, they have not been often collected in more recent studies. However, they are a homogenous sample that can be directly related with reproductive effects, which is particularly important in the case of birds with an unfavorable conservation status [67, 72, 76, 111].

In recent years the usefulness of feathers as a biomarker of metals exposure has been investigated and in Portugal and Spain they have been frequently used in recent studies to evaluate mainly Hg and Pb exposure. The use of feathers has several advantages over other tissues for the measurement of metals contamination in birds. Feathers are easy to collect non-invasively, from both dead and living birds or can be easily found in nests, which is particularly appropriate for rare and declining species; and once they do not require refrigeration, feathers can be easily stored for decades being useful for long-term studies [1, 91, 108]. Moreover, the specific incorporation of metals in feathers renders profiles inert and stable and may lead to higher concentrations than in internal tissues, particularly the Hg, so those concentrations in feathers can be more easily measured [20, 21, 33, 53], and some studies indicate that it is possible to correlate metal levels in feathers with concentrations in blood and internal tissues [32, 96, 97]. However, there are some factors that influence the deposition of metals into feathers such as variation between feathers, variation between different parts of a feather, molting patterns and changes of diet during molting, making difficult the interpretation of the metal levels [34, 35, 85]. Excepting for Hg, besides the endogenous deposition, the external contamination (atmospheric deposition or during preening) may have an important impact on the metals concentrations found in feathers, which can also difficult the interpretation of the results [32, 34, 86, 91].

Different non-invasive methods, such as the collection of regurgitated pellets, have also been realized and used to evaluate Pb exposure [98, 100, 101]. Like the blood samples, they can give information on the exposure frequency, seasonality and the food items associated with metal exposure, however these methods can sometimes be difficult to accomplish in raptors from wooded or mountainous areas, especially out of the breeding season [100].

Despite the difficulties inherent to the collection of internal tissues, they have also been collected for monitoring metals exposure and assessing its risk on bird populations from Portugal and Spain. Most metals are stored in internal tissues such as liver, kidneys and bones that have a tendency to accumulate them over time, consequently these tissues contain higher concentrations of some metals than those that are generally found in the blood and other tissues of the birds [40, 57, 59, 109]. The concentrations of metals in internal tissues represent the levels of metals in the diet, which in turn reflect the degree of contamination of the ecosystem [27, 106]. The collaboration with the WRCs can provide a sufficiently large number of such samples. However, the interpretation of results requires a great care, since the cause of death or illness may interfere with the kinetics of certain compounds. In Spain, most studies were preferentially performed using liver and bone followed by the kidney. Liver is the first target of most elements and consequently it can be a good indicator of recent exposure [8, 15, 123], for this reason it was used to measure the concentrations of all studied metals in many bird species. Bone was the preferred sample for evaluating the Pb exposure because it is a long-term repository for this metal, containing approximately 90% of the total body burden in mammals and birds and reflecting life time exposure [54, 57, 103, 123]. Kidney has been mainly analyzed to measure Cd concentrations once this organ accumulates about one half of the Cd total body burden and its Cd content is extremely stable. Therefore kidney is considered an adequate biomarker of chronic exposure to Cd [59, 123]. Other internal tissues, such as brain are not frequently used for monitoring metals due to its low rate of accumulation [57, 59].

3.5. Analytical techniques used in the determination of metals and metalloids

The analytical techniques commonly used for metals detection in biological samples of raptors from Portugal and Spain have been the atomic spectroscopy methods such as Atomic Absorption Spectrometry (AAS), the voltammetric stripping technique Anodic Stripping Voltammetry (ASV), Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Table 3.4).

The element(s) to be analyzed, the sensitivity of the technique to the respective element(s) and the associated costs were the main factors which influenced the selection of the detection method. Except for Hg determination, Graphite Furnace (GF) - AAS and ASV have been the most frequently performed techniques to determine most of the metals due to their relation cost and sensitivity of the technique. For determination of Hg, Cold Vapor (CV) - AAS method has always been the preferred technique, the most recent investigations used a single-purpose atomic absorption spectrometer (direct Hg analyser) by applying additional steps in the CV-AAS procedure.

In the first researches, the Flame AAS was the analytical technique used to determine Pb, Cd, Cu and Zn concentrations in eggs, while in the recent researches this same technique has been less used and restricted to the measurement of Zn and Cu concentrations, mainly due to its low sensitivity. GF-AAS analysis times are longer and relatively more expensive than those for Flame, but the enhanced sensitivity of GF-AAS, and its ability to analyze very small samples, significantly expands the capabilities of atomic absorption. The technique Hydride generation-AAS is one of the best available techniques for trace analysis of As. ICP-MS is also very sensitive, having already been applied to various biological matrices, but it is important take into account the possible existence of spectral interferences due to the presence of Ar-Cl [50]. The major drawback of AAS techniques (flame, graphite furnace, hydride generation and cold vapor) is the impossibility to perform simultaneous multi-elemental analysis. ASV has been also widely used for monitoring trace or ultra-trace metals because it is very sensitive, offers speciation capabilities and it has low purchase and running costs [16]. Although ASV can be used to measure a number of elements, it has been mainly used to determine Pb concentrations. However, when is necessary to measure very low blood Pb concentrations, techniques with highest sensitivity and accuracy should be employed [136]**.** This method modified by García-Fernández et al. [60] has demonstrated to be very useful mainly in the measurement of blood Cd concentrations, having detection limits equivalent or lower than those of the ICP-MS [42, 59, 60]. The major drawbacks are the difficulties associated with handling, storage and disposal of its Hg based electrodes, which becomes its application undesirable. In fact, environmental regulations and health considerations restrict its continuous application due to the high toxicity of Hg [127].

ICP-MS technique is a very powerful tool for the determination of metals at ultra-trace levels in different matrices, allowing simultaneous multi-elemental analysis with high sensitivity. ICP-MS combines the simultaneous multi-element capabilities of ICP-OES techniques with exceptional detection limits equivalent or lower than those of GF-AAS [30, 130]. However, its major drawbacks, high purchase and running costs and highly skilled laboratory operator required are the main reasons for its less frequent use in the biomonitoring studies with raptors in the Iberian Peninsula. Although the purchase cost of an ICP-MS device is high, it has a high productivity and becomes comparatively economical when many samples and/or elements need to be determined.

A number of methodological issues complicate the literature concerning the levels and effects of metals on organisms, populations, and ecosystems and influence our ability to compare tissue levels. These include mainly changes in technology over the years, use of wet weight (w.w.) vs. dry weight (d.w.), statistical calculation (for example, use of arithmetic mean and geometric mean) and the non standardization of the quantification limits calculation. Changes in technology influence mainly the detection levels, which in turn affects the number of samples with detectable levels.

3.6. Metals and metalloids studied and concentrations obtained

According to the results found in the literature, are listed in the table 3.5a and 3.5b the ranges of the arithmetic means of the metals and metalloid concentrations found in blood and tissues of the studied raptor species from Portugal and Spain.

Both essential and non-essential metals have been analyzed, but the essential metals have received the least interest. Cu and Zn were the essential metals most frequently analyzed; Cu has been analyzed mainly in eggs and Zn in liver and also in eggs of different raptor species. Se was only evaluated in blood of three raptors species from the Southwest of Spain. In general the concentrations of Zn, Cu and Se in the bird species studied were in the range of physiological levels in several health bird species, including different raptor species [3, 5, 6, 14, 42, 44, 118].

Regarding the non-essential elements, Pb and Cd have been the most studied, while Hg and As have received the least interest.

Pb is one of the most toxic metals and has historically been shown to affect wildlife [10, 48, 70]. In the Iberian Peninsula, contamination deriving from hunting activities has increased in relative importance in recent decades and consequently it has received special concern particularly when the birds have scavenger feeding habits and an unfavorable conservation status. Numerous authors have presented the Pb risk to raptors in Spain, including predators and scavengers [38, 56, 81, 98, 118]. It has been a limiting factor for the conservation of some endangered species, as the Pyrenean bearded vulture [81], Egyptian vulture [38, 54] and Spanish imperial eagle [115, 120]. Pb shot in regurgitated pellets of raptors has been documented in Red kite [98, 101], Marsh harrier [100, 101], Spanish imperial eagle [98, 101] and Peregrine falcon [101]. Pb poisoning has been described in the Golden eagle [29], Spanish Imperial eagle [79], Eagle owl, Red kite [103] and in four species of vultures living in Spain: Griffon vulture [102], Cinereous vulture [80], Bearded vulture [81] and Egyptian vulture [121]. Elevated concentrations of Pb have been detected in blood of Griffon vultures both in Portugal and Spain [25, 42, 56].

Cd, as well as Pb, is a persistent and ubiquitous metal that can cause several deleterious effects in living beings [18]. In general, in the studies carried out in Portugal and Spain, it has been the element with the lower concentrations in all types of analyzed samples (Table 3.5a, b) and the blood Cd concentrations are often below the detection limits of commonly used techniques [25, 26, 60]. The highest blood Cd concentrations were obtained for the Black kite feeding in the area affected by the toxic spill (Doñana National Park, Southwest Spain) [12] and for the Northern goshawk from a non-polluted Mediterranean forest (Murcia, Southeastern Spain), the researchers considered that concentrations were generally low [96]. Regarding the concentrations in internal tissues, the highest concentrations were observed in the liver of Tawny owl and Long-eared owl from Galicia (Northwest Spain) [118]. Scheuhammer [123] suggested that Cd levels above 3 µg/g d.w. in liver and above 8 µg/g d.w. in kidney might indicate increased environmental exposure. According to these thresholds, it is possible that there is an increased environmental exposure to Cd mainly in Northwest Spain**.** Renal and hepatic Cd concentrations described in raptors from Portugal and

Spain are lower than the suggested threshold tissue concentrations, above which Cd acute intoxication might be expected (40 µg/g w.w. in liver and 100 µg/g w.w. in kidney) [52] (Table 3.5a). The Cd concentrations in eggs are not believe to have caused adverse effects on reproductive success or shell thickness [68, 69, 76-78].

Comparatively with the Pb and Cd, few studies have evaluated the exposure to Hg in raptors from Portugal and Spain. However, Hg has been a contaminant of concern because it accumulates in the tissues of wildlife species and can adversely affect the reproduction, especially in higher trophic level species [124, 129, 137]. Although Hg biomagnification in predatory birds is generally greater in aquatic than in terrestrial food webs, some studies have found large accumulations of the Hg in birds that are at the top of terrestrial trophic chains [47, 93, 116]. Furthermore, the relatively high blood Hg concentrations observed in the Peregrine falcon (Southwest of Spain) and in Common buzzard (Portugal) [3, 26] support the hypothesis of the transport of aquatic Hg into the adjacent terrestrial food web, proposed by Cristol et al. [31]. Although all mean blood-Hg levels detected in the studies performed in the Portugal and Spain were lower than the threshold reported as of high risk for avian piscivores (i.e. Common loon, Gavia immer) of 300 µg/dl [46], the sensitivity of the species to toxic elements vary considerably and toxicity threshold levels have not yet been established for raptors. According to the Hg threshold level of 3 µg/dl associated with alterations in antioxidant enzymes’ activity [42, 44], many birds could end up being susceptible to suffer sub-lethal effects related with oxidative stress. Regarding Hg levels in feathers and eggs, Palma et al. [116] found a strong relationship between them, with feather levels of 4.1 µg/g w.w. corresponding to eggs containing the benchmark of 1.0 µg/g w.w, which is the lowest concentration associated with deformities of particularly sensitive embryos, though it is unlikely to affect more than a small percentage of eggs [74]. Therefore, it is unlikely that Hg contamination can negatively affect the reproduction of the studied bird populations. The first researches (1980s) carried out with eggs in the Southwest and Central Spain, reached this same conclusion [68, 69, 76, 78]. However, sub-lethal effects are likely to occur at concentrations far lower than those required to produce more pronounced pathological effects [123, 131].

As was the least studied toxic element in raptors from Portugal and Spain, perhaps because despite being a toxic metalloid, it has a low rate of bioaccumulation and it is not biomagnified in the food chain [39, 84]**.** The As concentrations obtained in the studied species could be considered as indicative of low and background amounts, with no ecotoxicological concern [5, 6, 14, 26, 118].

3.7. Conclusion

In conclusion, the analysis of toxic metals in different tissues of raptors has been an important tool for assessing the environmental contamination by these elements in Portugal and Spain. Several studies using a range of sentinel raptor species have been carried out since 1980s in order to identify adverse effects on the animals and their populations, to identify the contamination of the food-chain, to determine the levels of environmental contamination and to estimate human health risks. However, there is a striking difference between the number of studies performed in Portugal and Spain. Central, Northeast and South of Spain are the areas in which a greatest number of studies have been performed.

Most of the raptor species breeding in the Portugal and Spain have at some stage been analyzed for exposure to metals. Eagle owl, Black kite and Common buzzard were the species from which the greatest number of individuals has been analyzed. Due to conservation problems, the Spanish Imperial eagle was also subject of several biomonitoring studies which analyzed metals exposure in a relatively large number of individuals. The favorable conservation status, wide geographical distribution, non-migratory habits and diversified diet of the Eagle owl and Common buzzard make them suitable for biomonitoring studies. Despite the migratory habits of Black kite, it has also been considered a suitable biomonitor species due to its sensitivity to environmental pollution and to breed in areas highly affected by pollution. While the Common buzzard has been monitored over practically the entire of Iberian Peninsula, the Eagle owl and Black kite were mostly sampled in Murcia, Southeastern Spain and Doñana National Park, Southwestern Spain, respectively. The study of other raptor species that have demonstrated their usefulness in matters of conservation and environmental risk assessment is recommended.

The studies performed have measured the metals concentrations in a variety of different biological samples (blood, liver, kidneys, brain, bones, feathers and eggs) employing different analytical techniques. Blood was the type of sample collected in a great number, followed by the feathers, liver and bone. Due to the several advantages of the feathers over other tissues in the monitoring of metals, these have been frequently collected in recent studies to evaluate mainly Hg and Pb exposure. Blood and liver were used to measure the concentrations of all studied metals in many bird species, while bone was mainly collected to evaluate the Pb chronic exposure. Regarding the analytical techniques, AAS has been the most frequently performed technique to determine most of metals and metalloids, mainly due to its relatively low cost and good sensitivity. The ICP-MS, by allowing simultaneous multi-elemental analysis with higher sensitivity, has been applied in some recent studies. However, the high cost has limited its application. Although the purchase cost of an ICP-MS device is high, it has a high productivity and becomes comparatively economical when many samples and/or elements need to be determined.

Both essential and non-essential metals have been analyzed, but the essential metals and the metalloid As have received the least interest. In general, the concentrations of metals detected in raptor samples from Portugal and Spain are generally low and of an insufficient level to produce toxic side effects. Only Pb, in certain cases, can be related to toxic side effects and it has been a limiting factor for the conservation of some endangered species, such as the Pyrenean bearded vulture, Egyptian vulture and Spanish imperial eagle. Although in Spain, Griffon vulture has a positive population trend, in Portugal Pb exposure could be a risk for the conservation of this endangered species. Pb is the metal that has received great concern among researchers and although it was monitored in practically all species and in the various types of samples, more efforts are necessary to assess its risk in the conservation of other endangered species and in the human health. Regarding Hg, although the results obtained by the studies performed were not compatible with Hg levels that could compromise the reproductive success of birds and cause other toxic effects, the transport of aquatic Hg into the adjacent terrestrial food web and the lack of information concerning its exposure in many raptor species support the need for further biomonitoring studies of this metal. Furthermore, species vary considerably in their sensitivity to toxic elements and threshold Hg levels have yet to be established for raptors.

The research carried out in Portugal and Spain has been sporadic in both time and space. Financial support has been the main limiting factor to implement biomonitoring studies with raptors at a large spatial and temporal scale. However, given the toxicity of the metals and As, it is advisable to establish at a national scale, both in Portugal and Spain, biomonitoring programmes using raptors in order to conduct long-term studies that would link the survival of birds to their contaminant burdens. A network needs to be developed between the two countries and between toxicologists, veterinarians and ornithologists studying raptor populations to encompass analysis of samples collected in the WRCs and by ornithologists.

Table 3.1 Main characteristics (trophic position, diet, conservation status and migratory habits) of the raptors species breeding in Portugal and Spain, specifying which ones were used for monitoring purposes of metals and the total number of analyzed individuals.

Common name	Scientific name	Trophic position	Most important prey	Conservation status[22, 94] PT	Conservation status[22, 94] SP	Migratory habits	Total number of analyzed individuals	References
Accipitridae Family								
Northern goshawk	*Accipiter gentilis*	Bird and mammal predator[28, 119]	Domestic pigeon[28, 119]	VU	LC	Resident	68	[27, 35, 75, 78, 96, 97, 103, 118]
Eurasian sparrowhawk	*Accipiter nisus*	Bird predator[28]	Passeriformes[28]	LC	LC/ VU	Resident	22	[59, 60, 75, 118]
Cinereous vulture	*Aegypius monachus*	Carrion consummer[28, 107]	Livestock carcasses[28, 107]	CR	VU	Resident	18	[24, 75, 78]
Spanish Imperial eagle	*Aquila adalberti (heliaca)*	Bird and mammal predator[28]	European rabbit[28]	CR	EN	Resident	257	[68, 69, 76, 78, 103, 115, 120]
Golden eagle	*Aquila chrysaetos*	Bird and mammal predator/Carrion consumer[28, 122]	European rabbit[28, 122]	EN	NT	Resident	8	[29, 59, 77, 103]
Common buzzard	*Buteo buteo*	Bird and mammal predator[28, 95, 128]	Small mammals[28, 95, 128]	LC	LC	Resident	408	[26, 27, 57, 59, 60, 75, 77, 96, 97, 103, 118]
Short-toed eagle	*Circaetus gallicus*	Predator[28]	Reptiles (snakes and lizards)[28]	NT	LC	Reproductive/ Migratory	3	[59]
Marsh harrier	*Circus aeruginosus*	Bird and mammal predator [23, 28]	Small mammals and birds[23, 28]	VU	LC	Resident/ Visitor	79	[3, 78, 100]
Hen harrier	*Circus cyaneus*	Bird and mammal predator[28]	Small mammals and birds[28]	CR/ VU	LC	Resident/ Visitor	-	-
Montagu´s harrier	*Circus pygargus*	Predator[28]	Insects, small mammals, birds[28]	EN	VU	Reproductive Migratory	10	[59, 75]
Black-winged kite	*Elanus caeruleus*	Predator[28]	Small mammals, birds, reptiles, insects[28]	NT	NT	Resident	-	-
Bearded vulture	*Gypaetus barbatus*	Carrion consummer[81]	Bones of wild and domestic ungulates[81]	RE	EN	Resident	130	[81]
Griffon vulture	*Gyps fulvus*	Carrion consummer[28, 37]	Wild and domestic ungulates[28, 37]	NT	LC	Resident	245	[24, 25, 42, 56, 60, 103]

Table 3.1 (Continued) Main characteristics (trophic position, diet, conservation status and migratory habits) of the raptors species breeding in Portugal and Spain, specifying which ones were used for monitoring purposes of metals and the total number of analyzed individuals.

Common name	Scientific name	Trophic position	Most important prey	Conservation status[22, 94] PT	Conservation status[22, 94] SP	Migratory habits	Total number of analyzed individuals	References
Accipitridae Family								
Bonelli´s eagle	*Hieraaetus fasciatus*	Bird and mammal predator[28, 104]	Rabbits, pigeons, partridges[28, 104]	EN	EN	Resident	31	[47, 59, 60, 103, 116]
Booted eagle	*Hieraaetus pennatus*	Bird and mammal predator[28, 61]	Songbirds[28, 61]	NT	LC	Reproductive Migratory	144	[59, 60, 69, 77, 96, 97, 103]
Black kite	*Milvus migrans*	Predator/Carrion consummer[28, 135]	European rabbits, birds, fish, carrion[28, 135]	LC	LC	Reproductive Migratory	428	[3, 5, 6, 12, 14, 24, 59, 69, 77, 78, 103]
Red kite	*Milvus milvus*	Predator/Carrion consummer[28, 62]	Small mammals, birds, fish, carrion[28, 62]	CR	EN	Resident/ Visitor	37	[24, 69, 75, 103]
Egyptian vulture	*Neophron percnopterus*	Carrion consumer [38, 82]	Carcasses of mammals and birds, garbage dumps [38, 82]	EN	EN	Reproductive Migratory	234	[38, 54]
Honey buzzard	*Pernis apivorus*	Invertebrate predator[28]	Larvae of hymenopterans[28]	VU	LC	Reproductive Migratory	-	-
Pandionidae family								
Osprey	*Pandion haliaetus*	Fish predator[28]	Fish[28]	CR	EN	Resident/ Visitor	-	-
Falconidae family								
Eleonora´s falcon	*Falco eleonarae*	Predator[94]	Insects[94]	RE	NT	Reproductive migratory	-	-
Lesser kestrel	*Falco naumanni*	Predator[28, 94, 117]	Insects[28, 94, 117]	VU	VU	Reproductive Migratory	41	[111]
Hobby	*Falco subbuteo*	Bird and invertebrate predator[28, 94]	Small birds and insects[28, 94]	VU	NT	Reproductive Migratory	7	[69]
Barbary falcon	*Falco pelegrinoides*	Bird predator[94]	Domestic pigeon[94]	I	EN	Resident	-	-
Peregrine falcon	*Falco peregrinus*	Bird predator[92]	Domestic pigeon [92]	VU	LC	Resident	18	[3, 77, 103]
Common kestrel	*Falco tinnunculus*	Predator[28, 64]	Insects, rodents, birds[28, 64]	LC	LC	Resident	94	[57-60, 75, 93, 118]

Table 3.1 (Continued) Main characteristics (trophic position, diet, conservation status and migratory habits) of the raptors species breeding in Portugal and Spain, specifying which ones were used for monitoring purposes of metals and the total number of analyzed individuals.

Common name	**Scientific name**	**Trophic position**	**Most important prey**	**Conservation status**[22, 94]		**Migratory habits**	**Total number of individuals analyzed**	**References**
				PT	**SP**			
Strigidae family								
Tengmalm´s owl	*Aegolius funereus*	Mammal predator[89]	Voles[89]	-	NT	Resident	-	-
Short-eared owl	*Asio flammeus*	Bird and mammal predator[28, 94]	Small mammals, birds, insects[28, 94]	EN	NT	Visitor/ Reproductive	-	-
Long-eared owl	*Asio otus*	Mammal predator[28, 36]	Small mammals[28, 36]	DD	LC	Resident	5	[59, 118]
Little owl	*Athene noctua*	Predator[28, 36, 132]	Insects, small mammals [28, 36, 132]	LC	LC	Resident	52	[57, 59, 60, 93, 118]
Eagle owl	*Bubo bubo*	Bird and mammal predator[28, 66]	European rabbit[28, 66]	NT	LC	Resident	979	[43, 44, 57, 59, 60, 66, 93, 103, 113]
Scops owl	*Otus scops*	Predator[28]	Insects[28]	DD	LC	Reproductive Migratory	1	[59]
Tawny owl	*Strix aluco*	Predator[28, 134]	Invertebrates, rodents (rats)[28, 134]	LC	LC	Resident	74	[27, 35, 93, 118]
Tytonidae family								
Barn owl	*Tyto alba*	Predator[28, 63]	Rodents (rats)[28, 63]	LC	LC	Resident	33	[59, 93, 118]

PT (Portugal), SP (Spain), I (Indeterminate), DD (Data deficient), LC (Least concern), NT (Near threatened), VU (Vulnerable), EN (Endangered), CR (Critically endangered), RE (Regionally extinct)

Table 3.2 Sampling periods and bird species studied by each monitored area of Portugal and Spain

Areas	*Sampling Periods*	*Species (n)*
Northwest, Spain	1997-2005 [27] 1999-2005[75] UP [35, 118]	Common buzzard (n=126), Tawny owl (n=71), Northern goshawk (n=32), Barn owl (n=16), Eurasian sparrowhawk (n=16), Common kestrel (n=6), Long-eared owl (n=4), Little owl (n=3)
Northeast, Spain	1990-1991[29] 1992-1995[100] 1998-2001[103] 2002[54] 2010-2012[25] 2008[81]	Bearded vulture (n=130), Common buzzard (n=53)*, Griffon vulture (n=52)*, Marsh harrier (n=47), Eagle owl (n=21)*, Northern goshawk (n=9)*, Black kite (n=8)*, Red kite (n=6)*, Booted eagle (n=5)*, Egyptian vulture (n=10), Peregrine falcon (n=4)*, Bonelli’s eagle (n=3)*, Golden eagle (n=3), Spanish Imperial eagle (n=1)*
Weast, Spain	1999-2005[75]	Montagu´s harrier (n=8), Cinereous vulture (n=6), Griffon vulture (n=5), Red kite (n=5), Common buzzard (n=3)
Central, Spain	1979-1984[68] 1982-1985[78] 1985-1986[77] 1986-1987[76] 1997-2008[120] 1998-2001[103] 2002-2003[113] 2003-2004[54] 2001[14]	Spanish Imperial eagle (n=127)*, Black kite (n=91)*, Common buzzard (n=56)*, Eagle owl (n=53)*, Egyptian vulture (n=19), Marsh harrier (n=17), Northern goshawk (n=11)*, Booted eagle (n=9), Peregrine falcon (n=8)*, Red kite (n=6)*, Golden eagle (n=4)*, Bonelli’s eagle (n=3)*, Griffon vulture (n=2)*
East, Spain	2011[42]	Griffon vulture (n=66)
South, Spain	1980-1999[115] 1988-1991[111]	Spanish Imperial eagle (n=75), Lesser kestrel (n=41)
Southwest, Spain	1980-1983[69] 1982-1985[78] 1985-1986[77] 1986-1987[76] 1999[12] 1999-2000[3] 1999,2001-2003[5] 1999-2002[6] 2003[54] 2003-2007[93]	Black kite (n=318), Spanish Imperial eagle (n=54), Eagle owl (n=30)*, Marsh harrier (n=15), Red kite (n=10), Booted eagle (n=7), Hobby (n=7), Little owl (n=7)*, Barn owl (n=6)*, Peregrine falcon (n=6), Egyptian vulture (n=3), Common buzzard (n=2), Tawny owl (n=1)*
Southeast, Spain	1993[59, 60] 1994[57] 1995-1997, 2001[58] 1999-2000[96, 97] 2003[56] 2003-2007[66] 2006-2012[43] 2011-2012[44]	Eagle owl (n=844), Booted eagle (n=123), Common kestrel (n=87), Common buzzard (n=43), Little owl (n=34), Griffon vulture (n=29), Northern goshawk (n=16), Eurasian sparrowhawk (n=6), Barn owl (n=4), Bonelli´s eagle (n=4), Short-toed eagle (n=3), Black kite (n=2), Montagu´s harrier (n=2), Golden eagle (n=1), Long-eared owl (n=1), Scops owl (n=1)
Balearic Islands, Spain	1982-1985[78]	Cinereous vulture (n=9)
Canary Islands, Spain	1998-2001[38] 1999-2005[54]	Egyptian vulture (n=191)
Portugal	1992-2001[47, 116] 2003-2007[93] 2007-2012[25, 26]	Common buzzard (n=125), Griffon vulture (n=71), Eagle owl (n=31)*, Bonelli’s eagle (n=21), Little owl (n=8)*, Barn owl (n=7)*, Tawny owl (n=2)*, Common kestrel (n=1)

UP (unknown period), *The author does not provide the number of species collected in each area and thus an estimate (mean) was performed.
The author and their collaborators, Cardiel et al. [24] does not specify the sampling area and period for the species Griffon vulture (n=20), Cinereous vulture (n=3), Black kite (n=9) and Red kite (n=10).

Table 3.3 The metals and metalloids measured in each raptor species from Portugal and Spain *per* sample type and the total number (n) of the different samples analyzed for each species.

	Blood **(n=1962)**	***Liver*** **(n=591)**	***Kidney*** **(n=318)**	***Brain*** **(n=193)**	***Bone*** **(n=592)**	***Feathers*** **(n=815)**	***Eggs*** **(n=268)**
Northern goshawk	**Cd**[96], **Pb**[97] (n=6)	**As**[75, 118], **Cd**[27, 75, 118], **Hg**[27], **Pb**[27, 75, 118], **Zn**[75, 118] (n=22)	**Cd**[27], **Hg**[27], **Pb**[27] (n=16)		**As**[103], **Pb**[103] (n=18)	**Cd**[27, 96], **Hg**[27], **Pb**[27, 35, 97] (n=36)	**Cd**[78], **Cu**[78], **Hg**[78], **Pb**[78], **Zn**[78] (n=2)
Eurasian sparrowhawk	**Cd**[59] (n=1)	**As**[75, 118], **Cd**[59, 60, 75, 118], **Pb**[60, 75, 118], **Zn**[75, 118] (n=25)	**Cd**[59, 60], **Pb**[60] (n=9)	**Cd**[59, 60] , **Pb**[60] (n=9)	**Cd**[59, 60], **Pb**[60] (n=9)		
Cinereous vulture		**As**[75], **Cd**[75], **Pb**[75], **Zn**[75] (n=6)			**Al**[24]**, Pb**[24] (n=3)	**Al**[24], **Pb**[24] (n=3)	**Cd**[78], **Cu**[78], **Hg**[78], **Pb**[78], **Zn**[78] (n=9)
Spanish Imperial eagle		**Pb**[120] (n=15)			**As**[103], **Pb**[103, 115, 120] (n=90)	**Pb**[115, 120] (n=195)	**Cd**[68, 69, 76, 78], **Cu**[68, 69, 76, 78], **Hg**[68, 69, 78], **Pb**[68, 69, 76, 78] , **Zn**[68, 69, 76, 78] (n=95)
Golden eagle	**Cd**[59] (n=1)	**Cd**[59] , **Pb**[29] (n=4)	**Cd**[59], **Pb**[29] (n=4)	**Cd**[59] (n=1)	**As**[103] , **Cd**[59] , **Pb**[103] (n=4)		**Cd**[77] , **Cu**[77], **Hg**[77], **Pb**[77] , **Zn**[77] (n=3)
Common buzzard	**As**[26] **, Cd**[26, 59, 60, 96]**, Hg**[26]**, Pb**[26, 57, 60, 97] (n=113)	**As**[26, 75, 118]**, Cd**[26, 27, 59, 60, 75, 118]**, Hg**[26, 27]**, Pb**[26, 27, 57, 60, 75, 118]**, Zn**[75, 118] (n=191)	**As**[26]**, Cd**[26, 27, 59, 60]**, Hg**[26, 27], **Pb**[27, 57, 60] (n=91)	**Cd**[59, 60], **Pb**[57, 60] (n=17)	**As**[103]**, Cd**[59, 60], **Pb**[57, 60, 103] (n=120)	**Cd**[27, 96] , **Hg**[27], **Pb**[27, 97] (n=56)	**Cd**[77], **Cu**[77] , **Hg**[77], **Pb**[77], **Zn**[77] (n=4)
Short-toed eagle	**Cd**[59] (n=2)	**Cd**[59] (n=3)	**Cd**[59] (n=3)	**Cd**[59] (n=3)	**Cd**[59] (n=2)		
Marsh harrier	**Hg**[3], **Pb**[100], **Se**[3] (n=52)						**Cd**[78] , **Cu**[78], **Hg**[78], **Pb**[78], **Zn**[78] (n=17)
Montagu´s harrier	**Cd**[59] (n=1)	**As**[75], **Cd**[59, 75], **Pb**[75], **Zn**[75] (n=9)	**Cd**[59] (n=1)	**Cd**[59] (n=1)	**Cd**[59] (n=1)		

Table 3.3 (Continued) The metals and metalloids measured in each raptor species from Portugal and Spain *per* sample type and the total number (n) of the different samples analyzed for each species.

	Blood (n=1962)	*Liver* (n=591)	*Kidney* (n=318)	*Brain* (n=193)	*Bone* (n=592)	*Feathers* (n=815)	*Eggs* (n=268)
Bearded vulture	**Pb**[81] (n=101)	**Pb**[81] (n=30)			**Pb**[81] (n=43)		
Griffon vulture	**Cd**[25, 42, 60], **Cu**[42], **Hg**[25, 42], **Pb**[25, 42, 56, 60], **Zn**[42] (n=216)	**As**[75], **Cd**[75], **Pb**[75], **Zn**[75] (n=5)			**Al**[24]**, As**[103], **Pb**[24, 103] (n=24)	**Al**[24], **Pb**[24] (n=20)	
Bonelli´s eagle	**Cd**[59, 60], **Pb**[60] (n=4)				**As**[103], Pb[103] (n=6)	**Hg**[47, 116] (n=21)	
Booted eagle	**Cd**[59, 60, 96], **Pb**[60, 97] (n=58)	**Cd**[59, 60], **Pb**[60] (n=8)	**Cd**[59, 60], **Pb**[60] (n=7)	**Cd**[59, 60], **Pb**[60] (n=7)	**As**[103], **Cd**[59], **Pb**[103] (n=17)	**Cd**[96], **Pb**[97] (n=60)	**Cd**[69, 77] , **Cu**[69, 77], **Hg**[69, 77], **Pb**[69, 77], **Zn**[69, 77] (n=7)
Black kite	**As**[5, 6, 12, 14], **Cd**[5, 6, 12, 14, 59], **Co**[12], **Cu**[5, 6, 12, 14], **Hg**[3], **Pb**[5, 6, 12, 14], **Se**[3], **Sb**[12], **Zn**[5, 6, 12, 14], **Tl**[12] (n=334)	**Cd**[59] (n=2)	**Cd**[59] (n=2)	**Cd**[59] (n=2)	**Al**[24]**, As**[103], **Cd**[59], **Pb**[24, 103] (n=31)	**Al**[24], **Pb**[24] (n=9)	**As**[14], **Cd**[14, 69, 77, 78], **Cu**[14, 69, 77, 78], **Hg**[69, 77, 78], **Pb**[14, 69, 77, 78], **Zn**[14, 69, 77, 78] (n=70)
Red Kite		**As**[75], **Cd**[75], **Pb**[75], **Zn**[75] (n=5)			**Al**[24]**, As**[103], **Pb**[24, 103] (n=22)	**Al**[24], **Pb**[24] (n=10)	**Cd**[69], **Cu**[69], **Hg**[69] **Pb**[69], **Zn**[69] (n=10)
Egyptian vulture	**As**[38], **Cd**[38], **Cu**[38] **Pb**[38, 54], **Zn**[38] (n=195)				**Pb**[54] (n=39)		
Lesser kestrel							**Cd**[111], **Cu**[111], **Hg**[111], **Pb**[111], **Zn**[111] (n=41)
Hobby							**Cd**[69], **Cu**[69], **Hg**[69] **Pb**[69], **Zn**[69] (n=7)

Table 3.3 (Continued) The metals and metalloids measured in each raptor species from Portugal and Spain *per* sample type and the total number (n) of the different samples analyzed for each species.

	***Blood* (n=1962)**	***Liver* (n=591)**	***Kidney* (n=318)**	***Brain* (n=193)**	***Bone* (n=592)**	***Feathers* (n=815)**	***Eggs* (n=268)**
Peregrine falcon	**Hg**[3], **Se**[3] (n=6)				**As**[103], **Pb**[103] (n=9)		**Cd**[77], **Cu**[77], **Hg**[77], **Pb**[77], **Zn**[77] (n=3)
Common kestrel	**Cd**[59, 60], **Pb**[57, 60] (n=26)	**As**[75, 118], Cd[59, 60, 75, 118], **Pb**[57, 58, 60, 75, 118], **Zn**[75, 118] (n=93)	**Cd**[59, 60], **Pb**[57, 58, 60] (n=85)	**Cd**[59, 60], **Pb**[57, 58, 60] (n=87)	**Cd**[59, 60], **Pb**[57, 58, 60] (n=72)	**Hg**[93] (n=1)	
Little owl	**Cd**[59, 60], **Pb**[57, 60] (n=11)	**As**[118], **Cd**[59, 60, 118], **Pb**[57, 60, 118], **Zn**[118] (n=64)	**Cd**[59, 60], **Pb**[57, 60] (n=34)	**Cd**[59, 60], **Pb**[57, 60] (n=34)	**Cd**[59, 60], **Pb**[57, 60] (n=19)	**Hg**[93] (n=15)	
Long-eared owl		**As**[118], **Cd**[59, 118], **Pb**[118], **Zn**[118] (n=6)	**Cd**[59] (n=1)	**Cd**[59] (n=1)	**Cd**[59] (n=1)		
Eagle Owl	**Cd**[44, 59, 60], **Cu**[44] **Hg**[43, 44], **Pb**[44, 57, 60, 66], **Zn** [44] (n=834)	**Cd**[59, 60], **Pb**[57, 60] (n=26)	**Cd**[59, 60], **Pb**[57, 60] (n=26)	**Cd**[59, 60], **Pb**[57, 60] (n=26)	**As**[103], **Cd**[59, 60], **Pb**[57, 60, 103] (n=58)	**Hg**[43, 93, 113] (n=322)	
Scops Owl		**Cd**[59] (n=1)	**Cd**[59] (n=1)	**Cd**[59] (n=1)	**Cd**[59] (n=1)		
Tawny Owl		**As**[118], **Cd**[27, 118], **Hg**[27], **Pb**[27, 118], **Zn**[118] (n=51)	**Cd**[27], **Hg**[27], **Pb**[27] (n=34)			**Cd**[27], **Hg**[27, 93], **Pb**[27, 35] (n=54)	
Barn owl	**Cd**[59] (n=1)	**As**[71, 118], **Cd**[59, 71, 118], **Pb**[71, 118], **Se**[71], **Zn**[118] (n=25)	**Cd**[59] (n=4)	**Cd**[59] (n=4)	**Cd**[59] (n=3)	**Hg**[93] (n=13)	

Table 3.4 Analytical techniques used in the determination of metals in biological samples of the raptors from Portugal and Spain.

Analytical technique	*Elements*
Atomic Absorption Spectrometry (AAS)	
• Flame	**Al**[24], **Zn** [5, 6, 12, 14, 38, 69, 76-78], **Cu** [14, 38, 69, 76-78], **Pb** [69, 76-78, 81], **Cd** [69, 76-78]
• Graphite furnace	**As** [5, 6, 12, 14, 38], **Cd** [5, 6, 12, 14, 27, 38], **Co** [12], **Cu** [5, 6, 12, 14], **Pb** [5, 6, 12, 14, 24, 27, 35, 38, 54, 81, 100, 103, 115, 120], **Sb** [12], **Tl** [12]
• Hydride generation	**As** [103]
• Cold vapor	**Hg** [3, 27, 42-44, 47, 69, 78, 93, 113, 116]
Inductively Coupled Plasma (ICP)	
• Atomic/Optical Emission Spectrometry (AES)/(OES)	**Pb** [54, 100]
• Mass Spectrometry (MS)	**As** [26, 118], **Cd** [25, 26, 118], **Hg** [25, 26], **Pb** [25, 26, 118], **Se** [3], **Zn** [118]
Anodic Stripping Voltammetry (ASV)	**Cd** [42, 44, 59, 60, 96], **Cu** [42, 44], **Pb** [42, 44, 56-58, 60, 66, 97], **Zn** [42, 44]

Table 3.5a Range of arithmetic mean concentrations of the studied metals and metalloids in blood, liver and kidney samples of the studied raptors species from Portugal and Spain.

	Blood (µg/dl)	***Liver (µg/g d.w.)***	***Kidney (µg/g d.w.)***
As	**0.8** (Black kite)[12] **–26.24** (Black kite)[6]	**0.10** (Common buzzard) [26] – **6.88** (Tawny Owl)[118]	**0.18** (Common buzzard)[26]
Cd	**n.d. (**Griffon vulture)[25] – **0.68** (Black kite)[12]	**0.05** (Booted eagle)[60] –**5.52 (**Tawny Owl)[118]	**0.13** (Booted eagle)[60] – **1.81** (Common kestrel)[60]
Hg	**n.d.** (Griffon vulture)[25] – **20. 94** (Common buzzard)[26]	**1.39** (Common buzzard)[26]	**2.09** (Common buzzard)[26]
Pb	**2.78** (Black kite)[6] **– 43.07** (Griffon vulture)[56]	**0.39** (Common buzzard)[60] – **6.64 (**Common kestrel)[118]	**0.63** (Common buzzard)[60] – **1.65** (Eurasian sparrowhawk, Eagle owl)[57, 60]
Cu	**0.05 (**Egyptian vulture)[38] **– 36.86** (Black kite)[6]	Not measured	Not measured
Zn	**330 (**Black kite)[12] – **528 (**Black kite)[6]	**134.24 (**Griffon vulture)[75] – **360.3** (Tawny Owl)[118]	Not measured
Se	GM*[3]	Not measured	Not measured
Al	Not measured	Not measured	Not measured

d.w. (dry weight), **w.w**. (wet weight), **n.d.** (not detected), **GM (**Geometric mean), * result of the arithmetic mean not available

Table 3.5b Range of mean concentrations of the studied metals and metalloids in brain, bone, feathers and eggs samples of the studied raptors from Portugal and Spain.

	Brain (µg/g w.w.)	*Bone (µg/g d.w.)*	*Feathers (µg/g d.w.)*	*Eggs (µg/g w.w.)*
As	Not measured	GM*[103]	Not measured	**0.01 (**Black kite)[14] **– 0.02 (**Black kite)[14]
Cd	**0.003** (Booted eagle)[60] – **0.02 (**Common kestrel)[60]	**0.01** (Common buzzard)[60] – **0.125** (Eurasian sparrowhawk)[60]	**0.02** (Northern goshawk, Booted eagle)[96] – **0.03** (Common buzzard) [96]	**0.006** (Black kite)[14] – **0.18** (Black kite)[69]
Hg	Not measured	Not measured	**0.09** (Eagle owl)[113] - **2.43** (Bonelli´s eagle)[47, 116]	**0.19** (Booted eagle) – **0.45 (**Black kite)[69]
Pb	**0.02(**Common buzzard)[57, 60] – **0.14** (Common buzzard)[60]	**1.36** (Common kestrel)[58] – **46.35** (Eagle owl)[60]	**0.033** (Spanish Imperial eagle)[120] – **1.01**(Common buzzard)[97]	**0.05(**Black kite)[14] – **0.82** (Spanish Imperial eagle)[68]
Cu	Not measured	Not measured	Not measured	**0.65 (**Black kite)[14] – **2.07 (**Hobby)[69]
Zn	Not measured	Not measured	Not measured	**10.2** (Spanish Imperial eagle) – **14.79** (Black kite)[69]
Se	Not measured	Not measured	Not measured	Not measured
Al	Not measured	GM*[24]	GM*[24]	Not measured

d.w. (dry weight), **w.w**. (wet weight), **n.d.** (not detected), **GM (**Geometric mean), * result of the arithmetic mean not available

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CHAPTER 4

BIOMONITORING OF HEAVY METALS (Cd, Hg AND Pb) AND METALLOID (As) WITH THE PORTUGUESE COMMON BUZZARD (*Buteo buteo*)

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4. BIOMONITORING OF HEAVY METALS (Cd, Hg AND Pb) AND METALLOID (AS) WITH THE PORTUGUESE COMMON BUZZARD (*Buteo buteo*)

4.1. Introduction

Trace elements are present in the environment through the geological cycle and various anthropogenic activities, with the latter the most relevant [35, 40]. These elements can easily enter the food chain and at high doses they can be acutely lethal, while at lower doses they may have a wide range of health effects – such as mutagenicity, carcinogenicity, teratogenicity, immunosuppression, poor body condition and impaired reproduction in humans and animals [20, 47]. All of which make them a serious threat to the stability of ecosystems and living organisms [4, 36, 40].

Levels of trace elements and their effects on organisms are influenced by numerous factors related to habitat, physiology and life history [44]. Exposure to non-essential elements generally is below levels thought to be acutely toxic [29, 49, 53]. Although acute toxicity is unlikely to occur, chronic exposure to non-essential elements may interact with other environmental stressors namely parasitic infections or other pathogens and this could compromise bird's survival and reproduction [51]. It should be appreciated that potentially sub-lethal effects caused by chronic exposure to environmental contaminants are largely unknown in wild birds [48].

Biomonitoring of trace elements in the environment has enabled the identification of many sources of pollutants. Typically, the bioavailability of environmental pollutants is assessed by measuring chemical residues in tissues or fluids taken from animals living in specific habitats [36]. The direct measurement of contaminants in blood and internal tissues is the best indicator of the degree and type of exposure to them [24], presenting blood the advantage of being easily accessible, sampling can be relatively harmless and it is in contact with all tissues where chemicals are deposited and stored [18]. Some species have biological habits that increase the likelihood of exposure to contaminants and can produce relevant information that would be missed if only the area's water or soil were analyzed [4]. However, assessing an ecosystem's health adequately by means of biomonitoring requires the selection of species that are representative. Territorial birds of prey, ones that are non-migratory and have long life spans, are likely to reflect chemical contamination within their extended home ranges [45]. These localised, upper-trophic level species are also believed to be especially vulnerable to metals and play a very important role as environmental-contamination indicators [49, 54].

The Common buzzard, a diurnal bird of prey belonging to the order Accipitriformes and to the family Accipitridae, was the sentinel species selected for this study for several reasons: it is abundant within Portuguese territory; the Portuguese population of these birds is essentially resident, though in autumn and winter a comparatively small number of Common buzzards from northern Europe do reach the Iberian Peninsula. What is more, these birds are very territorial, are present in different habitats (forests, agricultural zones, mountain regions and sub-urban areas), they feed on a wide range of prey and are very opportunistic hunters [10].

The present study was carried out in order to evaluate the degree and type of exposure to trace elements that the Portuguese Common buzzard may be exposed and to monitor environmental pollution. For this purpose, we determine the As, Cd, Pb and Hg concentrations in whole blood, liver and kidney samples taken from Common buzzards from different areas of Portugal. Also, differences between their areas of origin, and the influence of age and gender on toxic-metal concentrations were studied. The influence of the season the samples were taken in, on blood-metal concentrations was also investigated.

4.2. Materials and methods

4.2.1. Sample collection

All samples collected from Common buzzards came from five Portuguese WRCs (*Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens* – CERVAS; *Centro de Recuperação de Animais Selvagens do Parque Nacional da Peneda do Gerês*; *Centro de Recuperação de Animais Selvagens do Parque Biológico de Gaia*, *Centro de Recuperação e Investigação de Animais Selvagens da Ria Formosa* – RIAS and *Centro de Estudos e Recuperação de Animais Selvagens de Castelo Branco* - CERAS) between November 2007 and January 2012.

Collected animals were either found dead or brought to the centres alive but injured or debilitated due to several potential reasons. These reasons were as follows: collision with a vehicle (n=18), collision with power lines (n=17), injury by Pb shot (n=22), fall from nest (n=14), malnutrition (n=3), and injury of unknown origin (n=51). The following data was registered for each bird: date of arrival at centre, area of origin, reason for being brought in, gender (male, female or unknown) and age (juvenile, adult or unknown). The birds' origin was divided into two different Portuguese regions: littoral (urban and industrial areas) and interior (rural and natural areas).

From a total of 125 Common buzzards, blood (n=93), liver (n=56) and kidney (n=36) samples were collected. The number of samples collected across different years, seasons, regions and for different gender and age classes is listed in table 4.1.

Table 4.1 Data relating to Common buzzards (*Buteo buteo*) of this study.

		Blood **(93n)**	***Liver*** **(56n)**	***Kidney*** **(36n)**
Year	2007	5	0	0
	2008	8	6	0
	2009	24	19	5
	2010	33	24	24
	2011	22	7	7
	2012	1	0	0
Season	Spring	15	11	6
	Summer	27	15	7
	Autumn	34	17	15
	Winter	17	13	8
Origin	Littoral	47	28	24
	Up-country	46	28	12
Age	Adult	30	19	16
	Juvenile	44	24	9
	Unknown	19	13	11
Sex	Female	21	15	8
	Male	35	26	19
	Unknown	27	15	9

n: number of samples

Blood samples were collected via the brachial vein at the moment of arrival to the rehabilitation centre and immediately transferred into collection tubes without the use of an anticoagulant. Liver and kidney samples were collected from animals that were found dead, died of natural causes or were sacrificed when their state of health indicated a potential recovery was unlikely. Liver and kidney samples were placed individually in plastic bags. In this study, only samples from animals that died in the rehabilitation centre within the first month after admission were included. All samples were stored at – 20 ºC until analysis.

4.2.2. Analytical procedure

Sample analysis was performed in the laboratories of the Scientific-Technical Services of the University of Barcelona (SCT-UB), Spain. Liver and kidney sub-samples (250-350 mg w.w.) were digested in Teflon reactors with 3 ml of 65% concentrated nitric acid (HNO_3) and 2 ml of 30% hydrogen peroxide (H_2O_2) at 90 ºC in an oven and left overnight. According to the volume of blood contained in the tubes, different amounts of HNO_3 and H_2O_2 were used until the digestion of blood samples was complete. After digestion, each liver and kidney sample was brought up to a volume of 40 ml with tetra-distilled purified water and according to the blood volume to be analyzed, blood samples were brought up to a volume of 20, 30 or 40ml with tetra-distilled purified water. All samples were transferred to the measuring vessel and then analyzed for As, Cd, Hg and Pb by ICP-MS (Perkin Elmer Model Elan 6000, Perkin Elmer, Waltham, USA). All material used in the digestion process was thoroughly acid-rinsed. A second set of identical liver and kidney samples (1 – 2 g) was oven-dried at 60 ºC until reaching a constant weight in order to calculate the percentage of humidity in each sample, which enabled the transformation of wet weight results into dry weight values [46].

An analytical quality-control programme was applied throughout the study, according to López-Alonso et al. [36]. Blank absorbance values were monitored throughout the survey and were subtracted from the readings before the results were calculated. The limits of detection (LOD) in the acid digest (set at three times the standard deviation (S.D.) of the reagent blanks) were in all cases < 0.5 µg/l and the limits of quantification (LOQ), expressed as a concentration in the blood and tissue, were calculated on the basis of the mean sample weight and volume analyzed. Analytical recoveries were determined from the certified standard reference materials (Whole-blood Seronorm, Trace Element, Whole Blood 2 - ref. 201605 and Whole Blood 3 - ref. 102405 from SERO AS, Norway and Bovine Liver – 1577b from National Institute of Standards and Technology, Gaithersburg, USA) analyzed together with the samples. The range of recovery rates (in view of the concentrations in the reference material) ranged between 93.67% for hepatic Pb to 138.43% for blood As.

4.2.3. Data analysis

Statistical analyses were performed with IBM SPSS Statistics for Windows, V.19.0. Each sample below the LOQ was assigned a value of one-half the LOQ and included in the data set for statistical treatment, a technique which minimizes nominal type I error rates [11]. A statistical significance level of $p<0.05$ was used for null hypothesis rejection.

Normal-distribution assumption was checked using the Kolmogorov-Smirnov test. When normal-distribution assumption was violated, the data sets were log-transformed before analysis and checked with the Kolmogorov-Smirnov test. However, most of the variables did not follow normal distribution even after transformation, so a non-parametric approach to the data analysis was necessary. The Mann-Whitney U test was used to test the statistical significance for area of origin, age and gender in the blood and tissues concentrations. Birds with unknown age and gender were not included in the statistical analysis when testing the significance for these variables. Comparisons across the different seasons in terms of blood concentrations and differences between metal concentrations in the blood, liver and kidney samples were tested using the Kruskal Wallis test followed by the Dunn's post-hoc test. A non-parametric Spearman's test was applied to test the correlation between blood and tissues and between tissue concentrations for each analyzed metal.

4.3. Results

Heavy metals and metalloid concentrations found in the blood, liver and kidney of Common buzzards are listed in the table 4.2.

Concerning As concentrations, blood As was not detected in 30.1% of total samples, in the liver and kidney samples it was not detected in 37.5% and 19.4%, respectively (Table 4.2). Mean As concentrations were significantly statistically different between animals' blood, liver and kidney ($p<0.01$): the lowest mean concentration was found in blood (0.014±0.014 µg/g w.w) and the highest in the kidney (0.041±0.0259 µg/g w.w.) (Table 4.2 and Fig. 4.1). As concentrations in kidney samples varied significantly with age and gender: adults showed higher ($p<0.05$) renal-As concentrations than juveniles and females showed renal-As concentrations that were nearly twice as high as average concentrations in males ($p<0.05$). No significant influence from any factor was detected in blood- and hepatic-As concentrations (Table 4.3).

When considering Cd results, this was not detected in 94.6% of total blood samples and for that reason the influence of age, gender, origin and season on concentrations of this metal in blood were not studied. In contrast, Cd was detected in 96.4% of liver samples and in all the kidney samples (Table 4.2). Mean Cd concentrations were significantly (nearly four times) higher ($p<0.05$) in the kidney (0.373±0.381 µg/g w.w.) than in the liver (0.089±0.097 µg/g w.w.) (Fig. 4.1). Cd concentrations in tissues varied significantly with age: adults showed higher hepatic- (twice as high, $p<0.01$) and renal- (three times as high, $p<0.05$) Cd concentrations than juveniles. No significant influence of origin and gender in hepatic and renal levels were detected for Cd accumulation (Table 4.3).

In this study, Pb was detected in most of the samples: 97.8% (blood), 87.5% (liver) and 94.4% (kidney) (Table 4.2). Mean Pb concentrations were very similar between blood (0.142±0.628 µg/g w.w.) and liver (0.152±0.194 µg/g w.w.). In the kidney, the highest mean Pb concentration (0.245±0.364 µg/g w.w.) was found, but this difference was not statistically significant (Fig. 4.1). Blood Pb was significantly affected by age ($p<0.01$) - adults had higher concentrations than juveniles, and by season ($p<0.01$) - blood samples collected in autumn had higher Pb concentrations than those collected in spring and summer. Hepatic and renal Pb concentrations were not significantly affected by age, gender and origin (Table 4.3).

Turning to Hg results, this metal was also detected in most of the samples: 88.2% (blood), 94.6% (liver) and 97.2% (kidney) (Table 4.2). Blood Hg concentrations were significantly lower than hepatic and renal Hg ($p<0.001$). Hg accumulation was mainly in the kidney, although there is no significant difference between hepatic and renal concentrations (Fig. 4.1). Blood Hg concentrations were significantly higher ($p<0.01$) in adults than in juveniles. The season the samples were taken in also had significant effects on blood Hg concentrations ($p<0.001$): higher levels were found in autumn and winter than in spring and summer, but significant differences were only verified between autumn and spring and autumn and summer. Hepatic and renal Hg was not significantly affected by any of the variation factors considered in this study (Table 4.3).

The existence of a statistical relationship between metal concentrations in blood and in the different tissues was studied by means of simple correlation coefficients (Table 4.4). The relationship between Cd contents in blood and tissues was not studied, since it was barely detected in blood. The highest correlation was observed between hepatic and renal Hg concentrations (r=0.946, $p<0.001$).

Table 4.2 Arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) concentrations in the blood, liver and kidney of Common buzzards from Portugal analyzed in the present study.

	Blood (n=93)		**Liver (n=56)**		**Kidney (n=36)**	
	µg/dl	**µg/g w.w.**	**µg/g d.w.**	**µg/g w.w.**	**µg/g d.w.**	**µg/g w.w**
As						
Mean ± S.D.	1.489 ± 1.457	0.014 ± 0.014	0.104 ± 0.136	0.029 ± 0.039	0.180 ± 0.133	0.041 ± 0.026
Median	1.391	0.013	0.074	0.022	0.149	0.036
Minimum	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Maximum	8.508	0.082	0.978	0.281	0.588	0.112
n < LOQ / %	28/ 30.1%		21/ 37.5%		7/ 19.4%	
Cd						
Mean ± S.D.	0.201 ± 0.567	0.002 ± 0.005	0.322 ± 0.361	0.089 ±0.097	1.553 ± 1.706	0.373 ± 0.381
Median	0.102	0.001	0.184	0.050	0.865	0.216
Minimum	n.d.	n.d.	n.d.	n.d.	0.033	0.009
Maximum	4.447	0.043	1.801	0.450	8.344	1.697
n < LOQ / %	88/ 94.6%		2/ 3.6%		0	
Pb						
Mean ± S.D.	14.711±65.156	0.142 ± 0.628	0.541 ± 0.687	0.152 ± 0.194	0.945 ± 1.356	0.245 ± 0.364
Median	5.864	0.056	0.284	0.079	0.443	0.102
Minimum	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Maximum	631.473	6.089	3.468	0.949	5.331	1.386
n < LOQ / %	2/ 2.2%		7/ 12.5%		2/ 5.6%	
Hg						
Mean ± S.D.	20.940±26.728	0.202 ± 0.258	1.387 ± 1.242	0.389 ± 0.346	2.086 ± 1.689	0.503 ± 0.310
Median	12.603	0.121	1.168	0.319	1.850	0.448
Minimum	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Maximum	164.895	1.590	5.314	1.479	5.945	1.482
n < LOQ / %	11/ 11.8%		3/ 5.4%		1/ 2.8%	

S.D.: standard deviation, n: number of samples, LOQ: limit of quantification, n.d.: not detected

Figure. 4.1 Bar chart showing the toxic metal concentrations (expressed as arithmetic means, µg/g w.w.) in the blood, liver and kidney in the 125 Common buzzards considered in this study.* p<0.05, ** p<0.01, *** p< 0.001

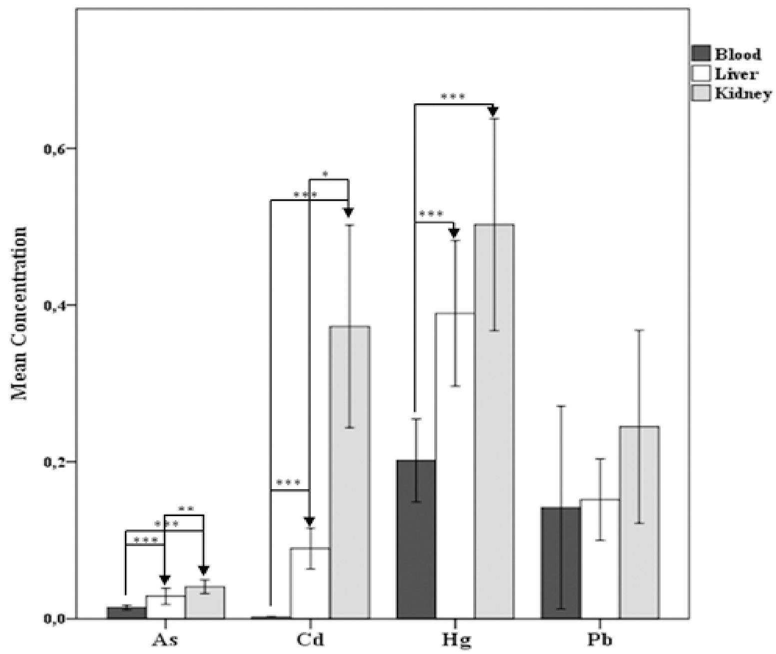

Table 4.3 Heavy metals in adults and juveniles birds, females and male birds, from littoral and up-country birds and in blood samples collected in the different seasons expressed as mean ± standard deviation (S.D.) µg/dl in blood and µg/g d.w. in tissues.

Sample			As Mean ± S.D.	Cd Mean ± S.D.	Pb Mean ± S.D.	Hg Mean ± S.D.
Blood (µg/dl)	**Age**	**Adult**	1.578 ± 1.178	-	9.865 ± 8.302[a]	20.853 ± 13.77[b]
		Juvenile	1.451 ± 1.647	-	5.704 ± 7.534[a]	14.856 ± 20.964[b]
	Gender	**Female**	1.698 ± 1.188	-	5.645 ± 8	14.688 ± 14.085
		Male	1.662 ± 1.734	-	6.291 ± 5.692	22.642 ± 26.565
	Origin	**Littoral**	1.389 ± 1.088	-	20.852 ± 91.3	27.605 ± 34.075
		Up-country	1.593 ± 1.769	-	8.436 ± 8.86	14.131 ± 13.409
	Season	**Spring**	2.267 ± 2.22	-	5.413 ± 6.493[c]	11.871 ± 15.363[d]
		Summer	1.297 ± 1.249	-	6.310 ± 9.278[c]	8.191 ± 9.571[d]
		Autumn	1.438 ± 1.271	-	9.608 ± 7.353[c]	30.268 ± 28.51[d]
		Winter	1.205 ± 1.265	-	9.899 ± 8.56	29.429 ± 38.931
Liver (µg/g d.w.)	**Age**	**Adult**	0.104 ± 0.055	0.460 ± 0.454[e]	0.443 ± 0.433	1.481 ± 1.33
		Juvenile	0.118 ± 0.192	0.209 ± 0.278[e]	0.441 ± 0.618	1.130 ± 1.132
	Gender	**Female**	0.096 ± 0.048	0.199 ± 0.196	0.323 ± 0.348	1.364 ± 1.341
		Male	0.083 ± 0.058	0.342 ± 0.405	0.508 ± 0.555	1.415 ± 1.385
	Origin	**Littoral**	0.077 ± 0.044	0.273 ± 0.359	0.484 ± 0.519	1.618 ± 1.489
		Up-country	0.132 ± 0.184	0.371 ± 0.364	0.596 ± 0.829	1.155 ± 0.903
Kidney (µg/g d.w.)	**Age**	**Adult**	0.217 ± 0.139[f]	2.165 ± 2.162[g]	0.828 ± 1.326	1.895 ± 1.725
		Juvenile	0.139 ± 0.068[f]	0.698 ± 0.975[g]	0.822 ± 1.558	1.816 ± 1.331
	Gender	**Female**	0.245 ± 0.106[h]	2.056 ± 1.793	0.706 ± 0.762	2.407 ± 1.705
		Male	0.166 ± 0.131[h]	1.496 ± 1.99	0.892 ± 1.533	1.745 ± 1.72
	Origin	**Littoral**	0.150 ± 0.092	1.620 ± 1.972	0.874 ± 1.207	2.455 ± 1.871
		Up-country	0.241 ± 0.179	1.421 ± 1.048	1.087 ± 1.665	1.348 ± 0.928

a (Z = -2.940, p<0.003), **b** (Z = -3.164, p <0.002), **c** (H = 11.639, p<0.008), **d** (H = 24.190, p<0.001), **e** (Z = -2.641, p<0.008), **f** (Z = -2.040, p <0.043), **g** (Z = -1.981, p <0.048), **h** (Z = -2.447, p <0.013).

Table 4.4 Correlations coefficients (r) between blood and tissues and between tissues for each element in Common buzzard.

	Blood*Liver (n=24)	Blood*Kidney (n=15)	Liver*Kidney (n=36)
As	r = 0.6**	r = 0.5*	r = 0.9***
Cd	-	-	r = 0.8***
Pb	r = 0.9***	r = 0.9***	r = 0.8***
Hg	r = 0.9***	r = 0.8***	r = 0.9***

***** p<0.05, ** p<0.01, *** p<0.001

4.4. Discussion

Previous data on the concentrations of non-essential elements in the blood and tissues of Common buzzard are scarce. In fact, this study reports the first data on the concentrations of trace elements (As, Cd, Hg and Pb) in blood and internal tissues of raptors residing in Portugal.

The Common buzzards, being birds of prey, are at the top of the food chain and consequently are potentially exposed to any biomagnification processes that may occur in a food web [27]. In the Common buzzard, concentrations of toxic elements reported in the literature were usually determined in the liver and kidney [4, 9, 40, 45]. Concentrations of heavy metals in the liver and kidney can be considered suggestive of chronic exposure to metals while concentrations in blood reflect recent exposure, and thus can be used as a real-time monitor for all stages of the birds' life-cycles. Better understanding of the relationship between heavy-metal concentrations in tissues and blood would enable researchers to assess whether high concentrations in blood warrant concern regarding their toxic effects, without having to use lethal sampling techniques [7, 9, 40, 52].

There have been few studies on the transfer of As through terrestrial food chains to predatory birds and on the presence of such metalloid in the raptor tissues, but it is known that vertebrate top predators experiencing higher As burdens [17, 34, 38]. In fact, published data on As levels in Common buzzard is sparse [40, 45] and this study reports the first data on blood As concentrations in this species, so it is difficult to compare our results with other studies. However, other species of birds sampled in natural areas [7] and near potential sources of metals [2, 3, 5] showed similar and higher blood As concentrations, respectively. With respect to As concentrations in the liver and kidney, our results are much lower than those observed for the Common buzzard in Galicia, Spain [45] and in Sicily, Italy [40] where the exposure to As was considered of no toxicological concern. However, we obtained similar or slightly lower concentrations than those obtained by Erry et al. [17] in Common kestrel from Britain, which have similar feeding habits to Common buzzard. Erry et al. [17] also measured hepatic and renal As concentrations in Barn owl, that despite having similar feeding habits with Common kestrel and Common buzzard, presented lower As concentrations than those obtained in these two species. Taking into account this previous information, the study of Common buzzards in Portugal showed a low level of exposure to this metalloid. However, the significantly higher As levels found in kidney samples of adult birds suggests that As is being bioconcentrated over time This mean that Common buzzards faces chronic exposure to this metalloid [16]. Despite the As burdens detected in Common buzzards were not likely to have been associated with adverse health effects (this element could not be considered as a threat for the survival of the studied birds), it could be involved in sub-lethal effects [37, 38].

Cd has been described as one of the most dangerous trace elements from environmental and toxicological standpoints, both for humans and animals, not only for its high toxicity but also for its persistence [4, 25, 35]. Cd distribution and bioaccumulation patterns observed in our study are consistent with previous studies in the Common buzzard [4, 9, 25, 33, 40]. The kidney was the organ with the highest concentration. In contrast, Cd in the blood was not detected in 94.6% of blood samples, which reflect its low concentrations in blood and/or may be associated with the LOD of our analytical method. In the blood samples where Cd was detected, the mean concentration was higher than that obtained by García-Fernández et al. [25] in samples of whole blood from wild birds. Comparison of the Cd concentrations in tissues obtained in this study with those obtained in other recent studies with the same species reveals that our results differ depending on the geographical area: similar with the results obtained in Italy by Alleva et al. [1], Battaglia et al. [4] and by Naccari et al. [40]; lower than those observed in Galicia, Spain by Pérez-López et al. [45] and Castro et al. [9] and higher than the results obtained by García-Fernández et al. [26] in Murcia, Spain. According to Scheuhammer [47], the hepatic and renal-Cd concentrations obtained in our study are indicative of a prolonged exposure to low and background amounts of this metal.

Pb is a highly toxic heavy metal that acts as a non-specific poison, affecting all body systems [30]. Blood-Pb concentrations obtained in this study were slightly higher than those obtained by García-Fernández et al. [24, 26] in Common buzzards from south-eastern Spain. According to Franson [21], 90.3% of the Common buzzards analyzed in our study had blood-Pb concentrations compatible with an absence of abnormal Pb exposure, 8.6% had Pb levels indicative of subclinical exposure and only 1.1% had a potentially lethal blood-Pb concentration. Taking hepatic-Pb concentrations into consideration, several researchers determined higher concentrations in the same species than those we quantified [1, 4, 33, 35, 40, 43, 45]. In contrast, García-Fernández et al. [24, 26] quantified lower hepatic- and renal-Pb concentrations. According to the ranges established by Pain et al. [43], none of the studied animals exceeded the calculated dry weight threshold for massive exposure and most of them had very low hepatic and renal Pb concentrations of <2 µg/g, with many <1 µg/g d.w., indicating a safe environmental exposure. These results suggest that Common buzzards in

Portugal are exposed to relatively low levels of Pb. However, some studies provide evidence that low-level Pb exposure, although not causing the clinical symptoms of classical Pb poisoning, may nevertheless have subtle detrimental effects on normal behaviour and cognitive function [8, 47], while Pb poisoning has been recognized as one of the most significant causes of mortality in raptors [42].

There are very few studies on Hg concentrations in birds of prey and, as far as we know, this is the first report on Hg blood concentrations in the Common buzzard and, in Portugal, the first in raptors. Tartu et al. [50] and Goutte et al. [28] evaluated the effect of Hg in seabirds predators and they conclude that Hg exposure could affect the ability of modulate their reproductive effort. As threshold-effects levels for Hg have yet to be established for bird blood, it is unclear whether Hg levels were high enough to pose a risk to any of these birds. Considering the hepatic and renal Hg concentrations, other authors have quantified higher Hg concentrations in the Common buzzard [1, 9]. Hg concentrations observed in our birds suggested that a source of Hg does exist. According to the previous information and to Scheuhammer [47], the Common buzzards studied are chronically exposed to normal background levels.

It is perhaps because Portugal is a small country (with a maximum extension in length of 561 Km and 218 Km in width) that we did not observe significant differences between areas of origin in the concentrations of various elements analyzed.

Age was the only factor explaining Cd accumulation in both the liver and kidney, as observed in other studies with wild birds [25, 40, 46]. With continued exposure, even at low levels, this non-essential element is accumulated throughout the life span of birds, due to its extremely long biological half-life once bound to metallothionein in tissues and its slow elimination from these tissues [23, 47]. Age also had an influence on blood Pb and Hg concentrations, but this influence was not verified in hepatic and renal concentrations. Differences in blood Hg levels between age classes seems to be related with prey-size selection and stage of juvenile feather moult [19]. Knowing that blood Hg is strongly influenced by dietary uptake, these age-related differences could be due to: adults and young eat different foods or eat different proportions of the same foods [7]. Apart from the dietary intake, the Hg concentration in blood reflects physiological influences, such as mobilization and storage in feathers and eggs [12, 13, 32]. The amount of Hg eliminated into eggs is usually small compared to the amount transferred into feathers during the molt [22]. Feather moult and growth is the main Hg excretion pathway [6, 31, 39]. The ability to rapidly transfer blood Hg into growing feathers partly accounts for the significant difference in blood Hg levels between adults and juveniles prior to fledging [19]. Although the Pb excretion into growing feathers occurs to a lesser extent compared with Hg [14, 22] the differences between adults and juveniles in blood Pb concentrations could also be related to the stage of juvenile feather moult and growth. Once blood Pb concentrations reflect immediate dietary intake [22], the differences for blood Pb concentrations between adults and juveniles could also be explained by considerations of feeding behavior [7].

Only Pb- and Hg-blood concentrations were influenced by season. Pb poisoning in raptors is likely to be more significant in autumn and winter, since the proportion of carrion taken by certain species may be higher in these seasons. In addition, since waterfowl and other game species are generally hunted during autumn and winter, killed, crippled and poisoned individuals provide a readily available, Pb-contaminated food source [41]. Common buzzards often act as scavengers and, in this way, are more likely to be exposed to the Pb shot prevalent in small game species [4]. This fact could help to understand the generally higher blood Pb concentration quantified in autumn and winter. In the case of Hg, possible explanations for the significant differences found between the different seasons are migration, diet [15] and molt. It is during the molting that Hg is incorporated in the keratin structure of the feathers, thus reducing the Hg levels in blood [6, 32, 39].

Regarding correlations, we found that Pb- and Hg-blood concentrations were statistically related to their corresponding concentrations in the liver or kidney - which suggests that blood concentrations of these metals may be a useful indicator of the degree of recent exposure. García-Fernández et al. [25] showed that blood-Cd concentration may also be a useful indicator of the degree of exposure to this metal. In this study, due to the large number of samples in which it was not detected, it was not possible to show whether blood could be a useful indicator of the degree of exposure to Cd. Kidney samples could be used to assess chronic exposure to As, Cd, Pb and Hg, a working hypothesis substantiated by the significant correlations between liver and kidney concentrations of these trace elements.

4.5. Conclusion

In general, Hg was the element studied present in the highest concentrations in the three types of samples, and the kidney was the sample with the highest concentrations of each element. If possible, in future studies it would be important to exclude birds that had migrated from Northern Europe and to measure Hg concentrations in feathers, in order to further examine the causes of the higher blood-Hg concentrations in winter and autumn. The generally higher blood-Pb concentrations quantified in autumn and winter are possibly due to birds' higher consumption of individuals crippled and poisoned through the hunting of small game species, which indicates that future measures regarding hunting practices are necessary in order to avoid high Pb exposure and/or Pb poisoning in wild birds. Although raptors are at the top of the food chain, and thus potentially exposed to any biomagnification processes that may occur in a food web, the individuals studied in this study generally had low levels of heavy metals in blood and tissues, compared with other authors. However, there are unknown sources of exposure to the trace elements studied, so further studies are needed to determine their origin.

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Chapter 5

Assessment of the exposure to heavy metals and arsenic in captive and free-living Black kites (*Milvus migrans*) nesting in Portugal

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5. ASSESSMENT OF THE EXPOSURE TO HEAVY METALS AND ARSENIC IN CAPTIVE AND FREE-LIVING BLACK KITES (*Milvus migrans*) NESTING IN PORTUGAL

5.1. Introduction

Heavy metals have always been natural components of our environment and under natural circumstances they are found in very low concentrations. However, in the past and nowadays considerable quantities of these compounds have been released into the environment through a variety of industrial, urban, hunting, mining and smelting activities, combustion of fossil fuels, solid-waste incinerators, agriculture and waste disposal [5, 23, 35, 42].

Exposure of terrestrial vertebrates to environmental contaminants occurs through ingestion of contaminated biotic or abiotic matter, absorption through skin, inhalation of volatile, aerosolized or particle bound contaminants and maternal transfer. Ingestion is the main pathway of contaminant exposure in birds and it can be classified as primary and secondary exposure [26, 43]. Primary exposure occurs directly through the consumption of free chemical and usually involves the consumption of Pb gunshot, fishing tackle or pesticide granules that are intentionally ingested by birds as grit or mistakenly as food [6]. Secondary exposure occurs indirectly through consumption of contaminated food, water, or environmental and anthropogenic substrates. The most common routes of secondary exposure in birds are the ingestion of prey items that have previously accumulated contaminants in their tissues (trophic transfer, biomagnification) [1, 13, 39] or the consumption of recently poisoned prey species, including debilitated, dying and dead animals as a result of contaminant exposure [27], contaminated carrion [36], animal body parts and organs containing Pb shot or bullet fragments [22, 33, 37], or baits set out to intentionally kill birds or other wildlife [47]. Secondary exposure occurs more frequently than primary one and it has resulted in large-scale avian exposures and in some cases mass mortalities.

The recognition of the occurrence, importance and the effects of heavy metals on food chains and ecosystems has led to the development of biomonitoring schemes aimed at directly measuring the levels of these toxic elements in various organisms [15]. Therefore, there has been a tendency to evaluate pollutants in long-living species that are high on the food chain, such as birds of prey [14]. Black kites are appropriate bird species for evaluating these persistent contaminants, because they are long-lived species that occupy a high position in the terrestrial webs and they are commonly found over a wide geographical range [38]. Furthermore, some studies have shown that Black kites are susceptible to metal toxicity [2, 7] and the contamination has been suspected as an important contributing factor to the decline of their populations [46].

Metal levels in blood reflect the input of metals through immediate dietary intake and mobilization of metals from internal tissues, as well as, the output of metals through excretion *via* the digestive tract and sequestration in feathers, eggs and internal tissues [9, 17], being the dietary uptake the main contributor to the metal levels in blood [1]. The measurement of metal concentrations in blood is a good indicator of recent exposure and for this reason it has been increasingly used in wildlife programs as a monitoring unit in the measurement of the actual contamination and its short-term variation [4, 21, 30].

This study was conducted to determine if the habitat of the Black kite in Portugal is contaminated by heavy metals and As and to assess the degree and type of exposure that they may be subjected. For this purpose, we determine the concentrations of As, Cd, Hg and Pb in whole blood samples taken from captive and free-living birds from different areas of Portugal.

5.2. Materials and methods

5.2.1. Sample collection

The present investigation was conducted on 43 Black kites, a medium-sized bird of prey that belongs to the Accipitridae family. European birds are migratory, they move to tropical and sub-tropical Africa in winter and they are present in the Portuguese territory from March to August. They are opportunistic hunters and their ability to exploit carrion plays an important role in the distribution of the species, which is often associated with dung heaps, farms, landfills and trash dumps; they are also especially prone to feed on road kills and, dead fish along shorelines.

Thirty-one birds were found injured and referred at two Portuguese WRCs (CERVAS and WRC of the Gaia Biological Park) and twelve birds lived in captivity in Gaia Biological Park for at least two years, for the environmental education purpose due to the impossibility of being returned to wildlife.

The group of captive birds was considered as a reference group for birds living in an uncontaminated environment. They were periodically evaluated by a veterinarian and they exhibited a good body condition and health status. They were housed in wire-mesh cages elevated over a concrete slab. Their diet consisted of frozen day-old chicks and fresh chicken carcass and they were fasted on Tuesday and Wednesday.

Blood samples were collected from the brachial vein of all birds to Eppendorf tubes without anticoagulant, when they were admitted at the WRCs (between 2009 and 2012) and in September 2010 for captive birds. All samples were stored at -20 ºC until analysis.

5.2.2. Blood metal analysis

Sample analysis was performed in the laboratories of the SCT-UB, Spain.

A sample of blood was placed in a Teflon reactor and HNO_3 at 65% and H_2O_2 at 30% were added. Thermal digestion was done in an oven at 90 ºC. The quantity of HNO_3 and H_2O_2 that were used for blood samples digestion and the time (24h to 48h) that samples were left in the oven until the digestion was completed varied according to the volume of blood contained in the tubes. The proportion of 1:5:5 was applied to the mixture of blood, HNO_3 and H_2O_2 respectively. After digestion, the sample was allowed to cool and brought up to a volume of 20, 30, or 40 ml with tetra-distilled purified water according to the blood volume to be analyzed, and then transferred to the measuring vessel. The Teflon reactors used for the wet digestion were previously washed with 3 ml of HNO_3, left in the oven for two hours and then rinsed twice with tetra-distilled water and finally dried in an oven at 100 ºC. Blood concentrations of As, Cd, Hg and Pb were measured by ICP-MS (Perkin Elmer Model Elan 6000, Perkin Elmer, Waltham, USA).

An analytical quality control programme was applied throughout the study, according to López-Alonso et al. [32]. The LOD for each metal were calculated analyzing repeated blanks with the same procedure that was used for the samples, determining the S.D. of the values and multiplying by 3 times. The LOQ, expressed as the concentration in the blood, were calculated on the basis of the mean volume of the analyzed samples. The LOD and LOQ calculated for each metal were, respectively, 0.009 µg/L and 0.121 µg/dl for As; 0.042 µg/L and 0.312 µg/dl for Cd; 0.163 µg/L and 0.949 µg/dl for Hg; 0.025 µg/L and 0.269 µg/dl for Pb. Blank absorbance values were subtracted from the readings before the calculation of the results.

The method was validated by the analysis of reconstituted lyophilized blood from the certified reference material Seronorm, Trace Element, Whole Blood 2 - ref. 201605 and Whole Blood 3 - ref. 102405 from SERO AS, Norway. Lyophilized blood was reconstituted in accordance with the accompanying instructions. The recovery rates (in view of the concentrations in the reference material) were 106.2% for As, 94.5% for Cd, 99.3% for Hg and 101.8% for Pb.

The density of bird blood samples was calculated to express the concentrations in µg/dl. For this purpose, duplicated blood samples (n=10) stored in lithium-heparinized vials were used and with a pipette of viscous liquids, the weight of 100 µl of blood was determined. The mean density of the Black kites' blood was 1.03.

5.2.3. Data analysis

Statistical analysis of the data was performed using IBM SPSS v.20.0 statistical software for Windows.

The distribution of data was highly skewed and the variables did not fit a normal distribution even after log-transformation the data sets. So, it was necessary a non-parametric approach to the analysis of the data.

For statistical comparison, the non detectable concentrations were taken as half the LOQ [12]. Differences between blood metal concentrations were tested using the Kruskal Wallis test followed by the Dunn's post-hoc test. The Mann-Whitney U test was used to determine significant differences on As, Cd, Pb and Hg blood concentrations between captive and free-living birds admitted to the WRCs. Differences with $p<0.05$ were considered statistically significant.

5.3. Results

Table 5.1 shows the arithmetic mean and ranges of As, Hg and Pb blood concentrations (expressed in µg/dl and µg/g w.w.) of the Black kites sampled in the present study. Since Cd was only detected in one free living bird and thus 97.7% of the values are a constant (1/2 LOQ=0.156 µg/dl), it was not calculated the mean, S.D. and range for this metal. The blood As concentrations found in the studied birds varied between not detected and 22.57 µg/dl, the blood Hg varied between not detected and 31.34 µg/dl and the Pb concentrations varied between 1.67 and 184.48 µg/dl.

The highest blood concentrations were found for Pb in free-living birds, they were statistically significant different from blood concentrations of As and Cd in both groups and from blood concentrations of Hg in captive birds ($p<0.05$). Hg was not detected in any bird living in captivity, while in the free-living birds group it was detected in 77.4% of birds. The blood concentrations of Hg in free-living birds were the second highest concentrations presented in the sampled Black kites (Table 5.1 and Fig. 5.1).

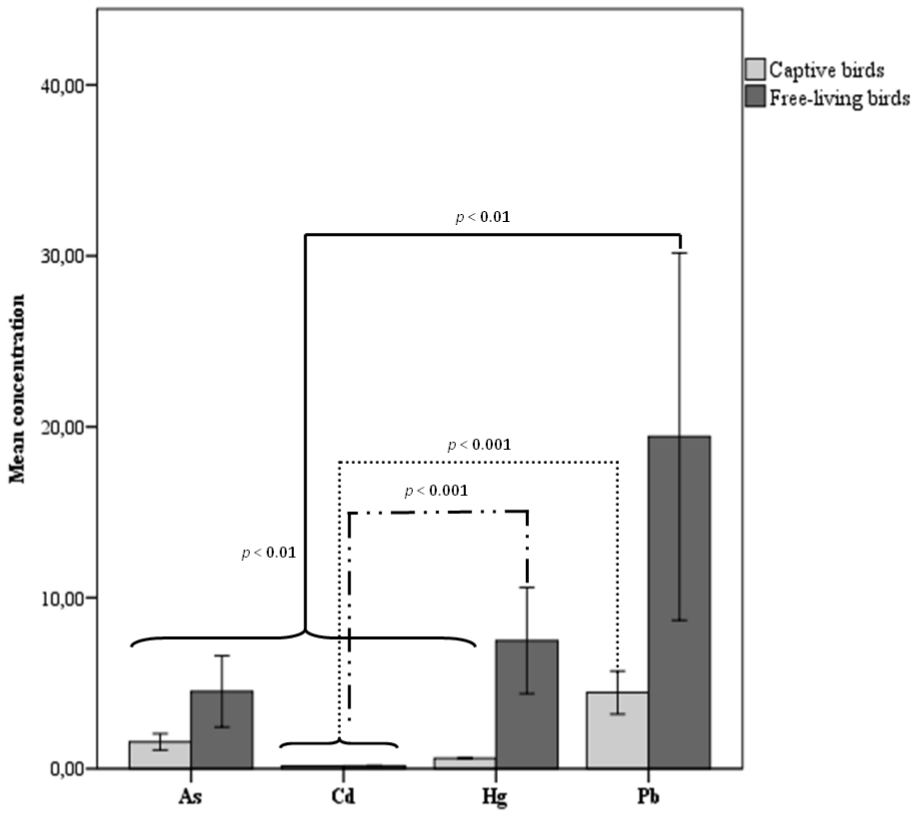
The captive birds group had the lowest blood concentrations for all the toxic elements analyzed but significant differences with the free-living birds group were only observed for Hg and Pb (p<0.01) (Table 5.1).

Table 5.1. Blood concentrations of arsenic (As), lead (Pb) and mercury (Hg) observed in the Black kites analyzed in the present study.

		***Captive birds* (n=12)**		***Free-living birds* (n=31)**	
		µg/dl	**µg/g w.w.**	**µg/dl**	**µg/g w.w.**
As					
	Mean ± S.D.	1.566 ± 0.753	0.0151 ± 0.007	4.521 ± 5.695	0.044 ± 0.055
	Median	1.931	0.188	2.440	0.023
	range	n.d. – 2.22	n.d. – 0.021	n.d. – 22.57	n.d. – 0.218
	% n.d. (n)	16.7 (2)	16.7 (2)	12.9 (4)	12.9 (4)
Hg					
	Mean ± S.D.	n.d.[a]	n.d.[a]	7.493 ± 8.464	0.072 ± 0.082
	Median	-	-	4.127	0.040
	range	-	-	n.d. – 31.34	n.d. – 0.302
	% n.d. (n)	100 (12)	100 (12)	22.6 (7)	22.6 (7)
Pb					
	Mean ± S.D.	4.449 ± 1.987[b]	0.043 ± 0.019[b]	19.430 ± 29.294	0.187 ± 0.282
	Median	4.346	0.042	10.406	0.100
	range	1.68 – 8.16	0.016 – 0.079	1.67 – 184.48	0.016 – 1.432
	% n.d. (n)	0	0	0	0

S.D.: standard deviation, n.d.: not detected, n: number of samples
a (p < 0.01 *versus* group of free-living birds for Hg)
b (p < 0.01 *versus* group of free-living birds for Pb)

Figure 5.1. Bar chart showing the blood concentrations (µg/dl) of As, Cd, Hg and Pb in the 43 Black kites considered in this study (only p values <0.05 are referred).



5.4. Discussion

This present study is the first that assesses the exposure to toxic metals and As in Black kites nesting in Portugal, being also the first. It is also the first to our knowledge that compares metal levels between captive and free-living raptors.

Despite the absence of significant differences, the free-living birds presented higher blood concentrations of As than the captive ones. As blood concentrations found in free-living birds were similar to those found in nestling Black kites exposed to the emissions from a solid-waste incinerator in Madrid, Spain [7] and in nestling Black kites sampled four years after a mining accident at Doñana, Spain [3]. In contrast, As blood concentrations in the two groups were much lower than the values found in the nestling Black kites sampled a year later in the area where the mining accident occurred [2]. Comparing the mean concentrations found in our study with those obtained in another raptor species of the same family (Common buzzard) that resides in Portugal, the free-living Black kites had higher concentrations while the captive birds had similar concentrations [11]. Differences between species in accumulation of As have been explained by diet, with predatory and piscivorous birds accumulating the highest residues [18, 31]. Taking into account this information and knowing that the fish is part of the diet of Black kites, higher concentrations of As in free-living birds of this species might be expected. However, there is limited information on threshold values of As in blood that may cause detrimental effects in raptors and the mean value of As found in free-living Black kites was higher than the reference level (2µg/dl) found in uncontaminated areas [10]. Although this element could not be considered a threat to the birds' survival, it could possibly be involved in sub-lethal effects; we are of the opinion that further investigation into possible sources is necessary, as well as, in the possible biological effects of this metalloid in raptors from Portugal.

Regarding Cd results, the high percentage of blood samples with not detected levels of Cd (97.7%) may be associated with the LOD of our analytical technique and/or can suggest that the local terrestrial environment that provides food and water to Black kites presents low levels of Cd. Once the blood concentrations obtained in our study were lower than the calculated LOQ, it is difficult to compare our results with those obtained by other authors. However, the mean Cd blood concentrations found in nestling Black kites living near potential sources of metals [2, 3, 7] were also lower than the LOQ calculated in our study and the authors considered that most individuals were exposed to low levels. However, Cd have some measurable physiological effects negatively affecting metabolism even at very low concentrations, especially during growth [44]. These physiological effects may not cause visible damage but they may be associated with the inactivation of enzymes involved in major metabolic pathways of many physiological systems, leading to nutritional, developmental and immunological stress [8, 25].

There are few studies on blood Hg concentrations in birds of prey [1, 11, 19, 20, 41]. Only one report has documented blood Hg concentrations in Black kites [1], with concentrations similar than those found in the free-living birds of this study. Comparing with other raptors species from Portugal [11], were reported blood Hg concentrations two fold higher in Common buzzard. Differences in Hg exposure among species are primarily correlated with trophic position and dietary habits, wherein the piscivorous species present higher concentrations [16, 21]. Although Black kite is not considered a piscivorous bird, the fish is part of their diet and therefore would be expected higher concentrations in this species when compared with the Common buzzard that shares the same geographical area. However, this difference may be due to the mobilization and storage of Hg in feathers. The free-living Black kites were sampled during the molting period and it is during this period that Hg is incorporated in the keratin structure of feathers, reducing its level in blood [29, 34]. In turn, the statistical significant differences of Hg levels between captive and free-living birds and the non detection in captive birds could be strongly related to the differences in the diet between these two birds groups, once the blood Hg levels are also strongly influenced by the dietary uptake [21, 30]. These results suggest that there is an available source of Hg and that Black kites are exposed to this metal. However, it is unclear whether Hg levels are high enough to pose a risk to the studied birds, because there is not a commonly accepted toxicity threshold for Hg in the blood of birds of prey and reproductive effects occur at lower doses than those required to produce other pathological effects [28, 40, 45]. Espín et al. [19, 20] provide new data on blood Hg concentrations related to the effects in the antioxidant system of Griffon vulture and Eagle owl from Spain. According to their results, blood Hg concentrations higher than 3µg/dl induced the activity of an antioxidant enzyme in these species. Therefore, some free-living Black kites (64.5%) from this study could have physiological effects related to Hg exposure.

Taking into account all analyzed elements, the highest concentrations were found for Pb in the free-living Black kites. The statistical significant difference observed with the captive group (p=0.001) suggests that the Black kites nesting in Portugal are exposed to Pb. Considering the free-living birds group of our study and according to Franson [24], 77.4% of the birds presented a background exposure to Pb (blood Pb <20 µg/dl); Pb levels causing sub-lethal effects (20 – 100 µg/dl) were only detected in 19.4% of the birds and only one Black kite presented blood Pb concentrations compatible with clinical toxicity. The blood Pb concentrations obtained in the studied free-living birds are higher than those reported by Benito et al. [4] and Baos et al. [2, 3] in Black kites living in a area affected by a mining accident (Doñana, Spain) and by Blanco et al. [7] in nestling Black kites exposed to the emissions from a solid-waste incinerator in Madrid (Spain). In turn, comparing with Common buzzards studied in Portugal [11], we obtained similar concentrations for free-living birds. However, despite a background exposure which can be associated to industrial and urban areas, our results can be of concern once even at very low concentrations Pb can negatively affect metabolism, especially during growth [44]. Furthermore, Espin et al. [19, 20] showed that Pb at lower concentrations than those typically accepted of causing physiological effects in Falconiformes (20 µg/dl) may produce negative effects on oxidative stress biomarkers.

5.5. Conclusion

In this study, the captive birds group showed the lowest concentrations of all studied elements, compatible with a background exposure. Despite the free-living birds admitted at WRCs showed higher blood concentrations, they seemed to be present in concentrations associated to a no-observed adverse effects exposure. However, it is necessary to taking into account that all elements, even at low concentrations, can negatively affect the reproductive performance of the birds and their metabolism, especially during growth.

It should be especially considered the toxic potential of Pb, once it was observed the occurrence of a sub-clinical exposure to this metal in some individuals. Since Hg is incorporated in the keratin structure of feathers during the molting period reducing the Hg levels in blood, we recommend further analysis to determine its concentrations in feathers in order to better understand the degree and type of exposure to this metal. The statistical significant differences between captive and free-living birds for blood Hg and Pb concentrations indicate the existence of an available source of these metals.

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CHAPTER 6

ASSESSMENT OF THE EXPOSURE TO HEAVY METALS IN GRIFFON VULTURES (*Gyps fulvus*) FROM THE IBERIAN PENINSULA

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6. ASSESSMENT OF THE EXPOSURE TO HEAVY METALS IN GRIFFON VULTURES (*Gyps fulvus*) FROM THE IBERIAN PENINSULA

6.1. Introduction

Wild bird populations are susceptible to dangers derived from the environmental presence of toxic elements, especially those that are non-degradable and that in many occasions tend to concentrate through the food chain, such as heavy metals [28]. Cd, Pb and Hg are the most dangerous metals, from environmental and toxicological standpoints, both for humans and for animals [3, 13, 25].

The toxic effects of the various heavy metals on birds have been documented. Pb can cause bird mortality or can indirectly affect populations through its effects on the food base, avian behaviour, reproductive success and immune response, depending on the dose [3]. The use of Pb pellets for shooting has resulted in their release into the environment over many years, with serious repercussions for many bird-species populations, which have ingested them either directly or indirectly. Galliforms and doves probably ingest spent shot as grit, which is retained in their gizzards to aid digestion, while raptors are usually poisoned through ingesting pellets in dead or injured prey or in gut piles [18, 27, 35]. Diffuse environmental Pb contamination resulting from waste dumping, as well as mining and smelting activities, has also affected a wide range of bird species via the food chain [1, 2, 30]. There are few reports of Cd-induced injury to terrestrial wildlife in nature [4]. In a laboratory setting, Cd can cause kidney toxicity, disrupted calcium metabolism, decreased food intake, decreased growth rate, altered avoidance behaviour, reduced egg production and thin egg shells [4, 20]. Hg is considered to be very toxic for wild animals [13] and has been shown to adversely affect birds particularly through its neurotoxicity and negative effects on reproduction. Some of those effects are neurobehavioral underdevelopment, induction of eggshell thinning and malformations, inhibition of egg production and embryotoxic effects [16, 29, 38]. Reproductive effects occur at lower doses than those required to produce other pathological effects [52, 55].

Vultures, by virtue of their position at the top of the food chain and their millenary dependence on human activities, which provide food resources through livestock management or hunting practices, are at risk of accumulating and concentrating heavy metals in their tissues and thus serve as sensitive indicators of the level of environmental contamination [28, 48, 57]. Several papers about metal concentrations in vultures have been published [5, 12, 21, 31, 51]. However, few studies have monitored heavy metals in the blood of the Griffon vulture [14, 23, 26, 54].

The Griffon vulture is a large bird of prey which is exclusively or near-exclusively a scavenger in its feeding habits as it feeds mainly on the muscles and viscera of ungulates. In the 20th century there was a proven widespread decline, mainly due to poisoned baits set for carnivores and in some areas a reduction in available food supplies, due to changes in livestock management and sanitary legislation [8, 11, 33, 39, 45, 49]. When feeding on wild-game carcasses, free-ranging scavengers such as Griffon vultures are thus more predisposed to the ingestion of illegal poison baits intended to kill predators or the Pb particles stemming from ammunition used for hunting. Illegal poisoning is probably the most significant cause of non-natural mortality among large birds of prey [36, 39, 40]. The decline in food availability following the introduction of European Union (EU) regulations (Regulation CE 1774/2002) prohibiting the disposal of carcasses in the field - due to the outbreak of BSE in 2001 - had harmful effects on vulture populations. These effects included a halt in population growth, a decrease in breeding success and an apparent increase in mortality in younger age classes of vultures [11, 43, 45]. What is more, behavioral changes and dietary shifts are also related to food shortages. The dietary range of the Griffon vulture has broadened and now includes significant amounts of wild rabbits (*Oryctolagus cuniculus*) and rubbish while there has also been an increase in the number of cases of vultures attacking and killing cattle [10, 41, 42]. In Portugal the conservation status of the species is “near-threatened” [6], whereas in Spain the Griffon vulture is classified as “least concern”, due to a positive population trend in this country [9].

In this report, we attempt to investigate the blood heavy metal (Cd, Pb and Hg) concentrations in Griffon vultures from different areas of Portugal and from Catalonia, Spain and also to determine if heavy metals in the blood of weak and/or injured Griffon vultures admitted to WRCs reflect profiles of contamination in the local, free-living and outwardly healthy Griffon vulture population. Differences between sampling areas were also investigated.

6.2. Materials and methods

6.2.1. Sample collection

Blood samples were taken by veterinarians by puncturing the radial or metatarsal vein from a total of 121 Griffon vultures, which came from catches (n=54) performed in Portugal and Spain and from animals referred to three different WRCs in Portugal (n=47) and one in Catalonia, Spain (n=20) (Fig. 6.1).

All blood samples were stored in Eppendorf tubes without anticoagulant at -20 ºC until analysis.

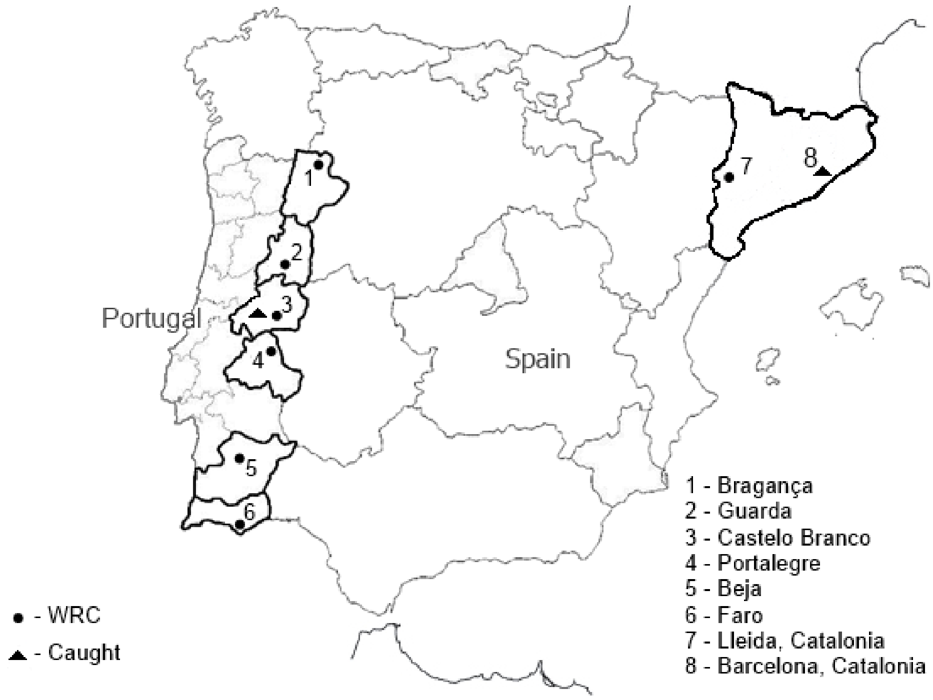
Thirty Griffon vultures were caught in May of 2011 by means of a cage-trap at a feeding station placed near a municipal rubbish dump in Barcelona, Catalonia, Spain and twenty-four Griffon vultures were caught in July of 2012 using a cage-trap placed inside the “Monte Barata” feeding station for scavenger birds in Monforte da Beira, Castelo Branco, Portugal. Both in Portugal and Catalonia, Spain the catches were performed outside the hunting season. The hunting season occurs mainly between the months of October to February in both areas. Feeding stations are places where supplementary food is provided for vultures. In the feeding station in Barcelona, Spain, lungs and carcasses of pigs purchased from slaughterhouses are provided once a week throughout the year. The rubbish dump near the feeding station also provides a significant fraction of the trophic needs of Griffon vultures. In the area of Castelo Branco, Portugal, between October and February, the food provided mainly comes from hunting activities (*Cervus elaphus* and *Sus scrofa*) and is supplied once a week. During the rest of the year, on a monthly basis, food consisting mainly of domestic rabbits supplied. The health status of the captured vultures was evaluated by veterinarians. The clinical examination preformed by the veterinarians included an evaluation of general body conformation, posture, attitude, stimulus response, type of respiration and examination of the feathers, skin, beak, eyes, ears, cere, nares, oral cavity, bones, breast muscle, wings, faeces, abdomen and vent. After the proceedings, all birds were freed.

Regarding animals referred to WRCs (n=67), all of them were found alive but injured (24%) or weak/underfed (76%) and then taken to WRCs where veterinarians collected blood samples at the time of the birds’ arrival. The animals from Portugal came from six municipalities (Beja n=5, Bragança n=6, Castelo Branco n=10, Faro n=12, Guarda n=9 and Portalegre n=5) between September 2007 and October 2012. The animals from Spain came from the Lleida municipality between March 2010 and October 2012 (Fig. 6.1). The birds were brought to the WRCs for care in the months of August to November. All municipalities (Portuguese and Spanish) can be considered as having a low population density and with agriculture and livestock as their primary economic sector.

The diet of Griffon vultures is based on the cadavers of wild and domestic animals, who have died from natural causes, hunting or whose remains have been supplied at supplementary feeding stations. In Portugal, the cadavers are mainly the *Cervus elaphus*, *Sus scrofa*, *Dama dama*, *Capreolus capreolus*, *Sus scrofa* var. dom., *Orictolagus cuniculus*, *Equus asinus*, *Ovis aries*, *Capra hircus* and in Catalonia, Spain they are mainly the *Rupicapra pyrenaica*, *Cervus elaphus*, *Sus scrofa*, *Capra pyrenaica*, *Capreolus capreolus*, *Sus scrofa* var. dom., *Ovis aries*, *Capra hircus*, *Bos Taurus* and *Equus caballus*.

The ages of all Griffon vultures sampled were known. The individuals admitted to WRCs were mostly juveniles in their 1st calendar year, both in the cases of Portugal (n=46) and Spain (n=18). Regarding the birds that were trapped, in Portugal mostly were juveniles in their 2nd calendar year (n=22) while in Spain all of those caught were adult birds.

Figure. 6.1. Map showing the geographical location of the studied areas in the Iberian Peninsula.



6.2.2. Blood metal analysis

Sample analysis was performed in the laboratories of the SCT-UB, Spain.

A sample of blood was placed in a Teflon reactor, with HNO_3 at 65% and H_2O_2 at 30% then added. Thermal digestion was carried out in an oven at 90ºC. Depending on the volume of blood contained in the tubes, different amounts of HNO_3 and H_2O_2 were used until the digestion of blood samples was complete, in addition to the time (24h to 48h) that samples were left in the oven. The proportion of 1:5:5 was applied to the mixture of blood, HNO_3 and H_2O_2 respectively. After digestion, the sample was allowed to cool, brought up to a volume of 20, 30, or 40 ml with tetra-distilled purified water (depending on the volume of blood to be analysed), and transferred to the measuring vessel. The Teflon reactors used for the wet digestion were previously washed with 3 ml of HNO_3, left in the oven for two hours and then rinsed twice with tetra-distilled water and finally dried in an oven at 100ºC. Blood concentrations of Cd, Hg and Pb were measured by ICP-MS (Perkin Elmer Model Elan 6000, Perkin Elmer, Waltham, USA).

An analytical quality control programme was applied throughout the study, according to López-Alonso et al. [37]. Blank absorbance values were monitored throughout the survey with the same procedure as that used for the samples and were subtracted from the readings before the results were calculated. The LOD in the acid digest (set at three times the S.D. of the reagent blanks) were 0.042 (Cd), 0.163 (Hg) and 0.025 (Pb) µg/L. The LOQ, expressed as a concentration in the blood, were calculated on the basis of the mean sample volume analysed and were 0.312 (Cd), 0.949 (Hg) and 0.269 (Pb) µg/dl. The precision and accuracy of the method were tested by analysing samples of reconstituted lyophilized blood from the certified standard reference materials (CRM) (Whole-blood Seronorm, Trace Element, Whole Blood 2 - ref. 201605 and Whole Blood 3 - ref. 102405 from SERO AS, Norway). Lyophilized blood was reconstituted in accordance with the accompanying instructions. The recovery rates (in view of the concentrations in the reference material) were 94.5% for Cd, 99.3% for Hg and 101.8% for Pb.

6.2.3. Data analysis

Statistical analysis of the data was performed using IBM SPSS v.20.0 statistical software for Windows. The reported concentrations of metals are represented by the mean ± S.D., median and range.

For interpretation of blood-Pb concentrations, different categories of Pb exposure were applied according to Franson [19]: a level <20 µg/dl was considered to be indicative of “background” Pb exposure and individuals were considered to be “Pb exposed” when concentrations exceeded 20 µg/dl. Between 20 and 100 µg/dl was considered a subclinical exposure, when concentrations exceeded 100 µg/dl clinical toxicity was evident and concentrations greater than 500 µg/dl were compatible with death.

Previous confirmation of normal distribution was done using the Kolmogorov-Smirnov test. As the normal distribution assumption was violated, data sets were log-transformed before analysis, which also did not follow a normal distribution pattern. So a non-parametric approach to the analysis of the data was necessary.

Mean comparisons tests did not show significant differences between the different Portuguese municipalities, therefore they were considered to be one single population with respect to sampling area.

The Kruskal Wallis test followed by the Dunn test was used to test statistical significance across all subgroups (separated according to sampling method and sampling area) in the blood Pb concentrations. A Mann-Whitney U test was used to test statistical significance in the blood Pb concentrations for the sampling method (WRCs *vs.* caught) with the same sampling area and for sampling areas (Portugal *vs.* Catalonia, Spain) with the same sampling method. Hg and Cd were excluded from the statistical analysis because the concentrations obtained were below the LOQ in most of the individuals analysed.

No comparison was made between the different age classes, because each of the groups studied (WRCs Portugal, WRCs Spain, caught Portugal, caught Spain) were comprised of only one age class.

A statistical significance level of $p<0.05$ was used for null hypothesis rejection.

6.3. Results

Cd and Hg were not detected in 119 (98.3 %) and 115 (95%) respectively of the total blood samples and, when detected, the blood concentrations were near the LOQ and were therefore excluded from statistical analysis. In contrast, Pb was detected in all blood samples: 41 Griffon vultures (33.9%) had Pb concentrations below 20 µg/dl; 79 (65.3%) had Pb concentrations between 20 and 100 µg/dl; and one vulture exhibited 300.23 µg/dl of Pb in blood, a concentration that is associated with clinical toxicity.

The blood concentrations of the three elements analysed ranged from not detected to 12.18 µg/dl for Cd, not detected to 4.39 µg/dl for Hg and from 4.97 µg/dl to 300.23 µg/dl for Pb. Regarding the blood samples with detected Cd (n=2) and Hg (n=6), the mean and S.D. were 7.28±6.93 and 2.31±1.25 µg/dl respectively. The results for the blood Pb of the studied animals were presented in two groups according to the sampling method (WRCs *vs*. caught) and in different subgroups according to sampling area (Portugal *vs.* Catalonia, Spain) (Table 6.1).

The population of vultures captured in Catalonia, Spain had higher mean blood Pb concentrations (42.22±50.08 µg/dl) than the other populations, these concentrations were statistically significantly different from the population of vultures admitted at the WRCs in Portugal (p=0.001) and the ones in Spain (Catalonia) (p=0.038) (Fig. 6.2). The interindividual variability (comparison between mean and median) was also higher in the birds captured in Catalonia, Spain, as was the highest maximum value. For each sampling method (WRCs and caught), the mean blood Pb concentrations were not significantly different between Portugal and Catalonia, Spain (Table 6.1). When the same sampling area was considered, the animals admitted to WRCs had significantly lower Pb concentrations than those animals caught in the wild (Table 6.1).

6.4. Discussion

This is the first study involving the biomonitoring of heavy metals performed on vultures from Portugal and from the autonomous community of Catalonia, Spain. It is also the first study which compares blood Pb concentrations of weak and/or injured animals referred to WRCs against those of outwardly healthy animals caught in the wild.

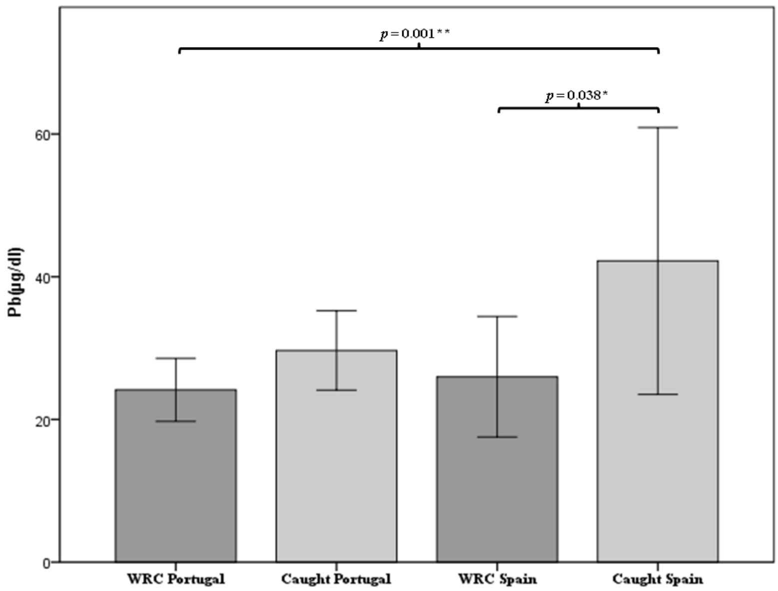
It is known that blood concentrations of Cd, Hg and Pb are good indicators of contamination and can be considered appropriate indicators of recent exposure [7, 17, 24, 26]. However, few papers have studied Cd and Hg concentrations in the blood of terrestrial birds of prey and no commonly accepted toxicity threshold exists for Cd and Hg in the blood of these birds [22, 34, 46, 53, 56]. Recently, Espín et al. [14, 15] provided threshold concentrations at which Cd and Hg can affect the antioxidant system in Griffon vultures and Eagle owls from Spain.

Table 6.1. Blood Pb concentrations (µg/dl) of the studied Griffon vultures.

	Wildlife Rehabilitation Centre		*Caught*	
	Portugal (n=47)	**Catalonia, Spain** (n=20)	**Portugal** (n=24)	**Catalonia, Spain** (n=30)
Pb Mean ± S.D.	24.15 ± 15.07^{a,c}	25.98 ± 18.04^{a,d}	29.67 ± 13.19^{b,c}	42.22 ± 50.08^{b,d}
Median	21.78	21.87	24.42	32.51
Minimum	4.97	6.02	15.44	15.32
Maximum	71.86	82.48	66.23	300.23
% *n*				
≤ 20 µg/dl	46.8	45	33.3	6.7
20-100 µg/dl	53.2	55	66.7	90
>100 µg/dl	0	0	0	3.3

n: number of samples, S.D.: standard deviation
a ($\chi2$ = 0.301, *p* = 0.763) (comparison between animals from WRCs in Portugal and from WRCs in Catalonia, Spain);
b ($\chi2$ = 1.81, *p* = 0.07) (comparison between animals caught in Portugal and caught in Catalonia, Spain);
c ($\chi2$ = 2.042, *p* = 0.041) (comparison between animals from WRCs and those caught, both from Portugal);
d ($\chi2$ = 2.535, *p* = 0.011) (comparison between animals from WRCs and those caught, both from Catalonia, Spain).

Figure. 6.2. Bar chart showing the blood-Pb concentrations (expressed as arithmetic means, µg/dl) in the 121 Griffon vultures considered in this study (only the *p* values <0.05 are referred).



The high percentage (>90%) of birds' blood samples with non-detected levels of Cd and Hg may be associated with the LOD of our analytical method and/or could suggest that the local terrestrial environment that provides food and water to Griffon vultures may be relatively free of Cd and Hg. The method described by García-Fernández et al. [26], with a LOD for Cd that is lower than that calculated in our study, enabled the measurement of Cd concentrations in blood of all six Griffon vultures studied, however these concentrations were lower than our LOQ. In turn, Espín et al. [14], who used the same method described by García-Fernández et al. [26], also detected Cd in all the Griffon vultures whose blood was sampled, even though their LOD exceeded ours. However, the average concentrations obtained continued to be below our LOQ. Shlosberg et al. [54] quantified Cd in blood spots of Griffon vultures and did not find concentrations above the LOQ in any birds. Although there is no well-established toxicity threshold for Cd in birds' blood, our results appear to be lower than the levels expected detrimental effects and reflect low Cd exposure. According to the Cd threshold level of 0.05 µg/dl associated with alterations in antioxidant enzymes' activity in Griffon vulture [14], only two individuals in this study with detectable levels of Cd concentrations could end up being susceptible to suffering sub-lethal effects related to oxidative stress. Further analysis to evaluate liver and kidney Cd concentrations are necessary to determine if Griffon vultures are chronically and continuously exposed to low levels of this toxic metal.

Only two reports have documented blood Hg concentrations in vulture species [14, 54] with similar concentrations to those found in this study. These researchers concluded that the Hg concentrations found seem to be too low to cause adverse effects on vultures. According to the Hg threshold level of 3µg/dl associated with alterations in antioxidant enzymes' activity in Griffon vultures [14], only one individual in this study could end up being susceptible to suffering sub-lethal effects related to oxidative stress.

References to blood Pb levels in Griffon vultures are scarce [14, 23, 26, 54]. The association of blood-Pb concentrations with several physiological and pathological effects in Falconiformes was established by Franson [19], but is not well established in vultures [5, 23, 51]. Recent studies have found that blood Pb concentrations lower than 20 µg/dl, which is the threshold level commonly accepted for taking into consideration physiological effects in Falconiformes, may have an effect on oxidative stress biomarkers in birds of prey, including Griffon vultures [14, 15]. However, according to García-Fernández et al. [23] and Espín et al. [14], it would appear that Griffon vultures are relatively tolerant to Pb. Therefore, more studies are needed in order to establish clear threshold levels related to the physiological and pathological effects in Griffon vulture.

Pb was identified in all analysed samples and, according to Franson [19], most birds have suffered subclinical exposure – with one individual even enduring clinical exposure. However, the bird with 300.23 µg/dl of blood Pb, which came from a catch preformed in Barcelona, did not exhibit any difference in behaviour to the other birds sampled. Given that Griffon vultures are obligate scavengers, they are potentially susceptible to the ingestion of Pb shot embedded in dead game animals, which is the most common source of Pb contamination in raptors [18, 47, 50].

The blood Pb concentrations in Griffon vultures caught in Catalonia, Spain were similar to the results published by García-Fernández et al. [23, 26] in Southern Spain, while the other groups evaluated had lower blood Pb concentrations. The researchers concluded that the birds are likely to have suffered subclinical exposure to Pb. A recent study carried out in two areas of the Autonomous Community of Valencia, in the East of Spain (Alicante and Castellón) also reported blood Pb concentrations in caught Griffon vultures [14]. The group of Castellón exhibited blood Pb concentrations similar to those found in Griffon vultures caught in Catalonia. In contrast the group of Alicante exhibited lower Pb concentrations than those found in any of the groups of our study and below levels expected to cause any detrimental effects. Concentrations were even lower than in Griffon vultures sampled in the deserts of Israel [54]. This result could possibly be explained by the fact that in Alicante food of mainly pork origin is provided once a week throughout the year, while in Castellón food is only provided 6 weeks every year and therefore it is possible that Griffon vultures need to consume hunting residues more frequently to meet their dietary needs. In Israel the diet of Griffon vultures is mainly based on carcasses of domestic origin provided in feeding stations (75%), animals that were originally destined to supply meat and milk for human consumption [54]. On the other hand in Europe, particularly in Portugal and Spain where the biggest population of scavenger birds are, the exposure of vultures to Pb may have increased after the ban on abandoning carcasses of domestic ruminants in the field, due to the BSE crisis (Regulation CE 1774/2002), because since the ban vultures may be obliged to consume hunting residues more frequently and have been known to scavenge in waste dumps [10, 45]. Compared to the results from other vulture species [12, 21, 31], the Griffon vultures in our study had higher blood-Pb concentrations.

The significant differences in blood Pb between the Griffon vultures admitted at WRCs and those caught in the wild can be explained by the rate of food intake. Taking into account that ingestion has been described as the most important source of Pb exposure in raptors [18, 23, 35], it is probable that a low ingestion of food can subsequently reduce recent exposure to Pb. The caught Griffon vultures exhibited generally good body condition while those admitted to WRCs did not (76% of birds were admitted to WRCs due to malnutrition and 24% due to injury), it is probable that the caught birds had ingested more contaminated food, since less Pb-uncontaminated food has been available following the EU sanitary legislation. In Europe, from 2000 onwards, the bovine spongiform encephalopathy (BSE) crisis led to the application of sanitary legislation (Regulation CE 1774 ⁄ 2002) that greatly restricted the disposal of animal by-products not intended for human consumption and deprived bird populations of the resources they depended on to survive. The decline in food availability due to EU regulations prohibiting the disposal of livestock carcasses in the field has triggered significant changes in the dietary composition of the Griffon vulture. In Portugal and Spain, the consumption of more Pb-contaminated food such as that found in rubbish dumps and the carcasses of wild mammals that have died from by natural causes or through hunting practices has become an important food resource for vertebrate scavengers [10, 43-45]. Therefore, Pb concentrations in the blood of Griffon vultures admitted at WRCs do not reflect the Pb levels of the local, free-living populations.

Despite the fact that Griffon vultures caught in Catalonia were sampled outside the hunting season and their diet is mainly based on carcasses of pork origin provided at feeding stations all year around, perhaps the ecological context of this bird’s habitat in Barcelona, Catalonia (an urban and industrial area, where rubbish dumps provide a significant fraction of the trophic needs of Griffon vultures) predisposes them to a greater exposition to Pb, compared to other studied groups. The levels found in the Griffon vultures from WRCs in Portugal and Spain and in the Griffon vultures caught in Portugal may be considered relatively low and normal for vultures sampled outside or in the beginning of the hunting season and which live in relatively unpolluted areas.

Although all vultures were sampled outside the hunting season, the hunting of some species is permitted all the year both in Portugal and Catalonia, Spain, and for this reason the ingestion of game meat with bullet fragments in carcasses or with Pb shots embedded in their flesh could be the cause of the higher blood Pb concentrations found in some vultures (71.86, 74.24, 82.48, 300.23 µg/dl).

Espín et al. [14] were the first to provide threshold concentrations at which Pb may produce physiological effects related to the antioxidant system in erythrocytes of free-living Griffon vultures from Spain. In their findings, Pb concentrations above 15 µg/dl in blood inhibited antioxidant enzymes `activity, and inducted lipid peroxidation in erythrocytes. According to these findings, most individuals sampled in the current study could be at risk of suffering Pb-related sub-lethal effects.

Although García-Fernández et al. [23] and Espín et al. [14] found very high blood-Pb levels in some healthy Griffon vultures, suggesting that this species may be more tolerant to Pb and that the levels required to cause clinical toxicity might be higher compared to other Falconiformes, subclinical exposure cannot be overlooked, since sub-lethal toxic effects may result in physiological, biochemical and behavioural changes [14, 15, 32, 52]. Most of the Griffon vultures analysed in this study exhibit higher concentrations than the threshold level established by Espín et al. [14] at which Pb can affect the antioxidant system in this species. As a result of these changes, birds may become increasingly susceptible to starvation and infection by disease, increasing the probability of death from other causes. Reproductive success can also be affected at subclinical levels [3, 18, 32]. So, we must consider the potential risks of Pb exposure in the vulture populations studied and we recommend further studies in order to evaluate the sub-lethal toxic effects which may occur following exposure to Pb and could indirectly affect Griffon vulture populations.

6.5. Conclusion

In conclusion, the data from this study indicates the Griffon vultures were recently exposed to low levels of Hg and Cd and most of the studied birds exhibited subclinical exposure to Pb. However some individuals in the present study could be at risk of suffering sub-lethal effects related with Cd, Hg and Pb exposure.

The Pb levels found in the Griffon vultures from WRCs and those caught in Portugal may be considered relatively low and normal for vultures sampled outside or in the beginning of the hunting season and for vultures living in unpolluted areas. In Barcelona, Spain the Griffon vultures are exposed to higher Pb-levels due to the municipal rubbish dump located near the feeding station. The ingestion of game meat with bullet fragments in carcasses or with Pb shots embedded in their flesh could also be the cause of the blood Pb concentrations found in some vultures.

According to our results, Griffon vultures admitted at WRCs do not seem to be representative of the local, free-living populations in terms of recent Pb exposure, thus it remains necessary to make catches when we want to monitor recent Pb exposure in the Griffon vulture.

The potential risks of Pb toxicity to vulture populations must be taken into consideration. We would recommend the prohibition of Pb ammunition in big-game hunting activities in order to preserve the population of vultures, or begin programmes that promote a voluntary switch from Pb ammunition - particularly in big-game hunting. Moreover, we feel a change in the management of animal by-products not intended for human consumption would be appropriate, with the approval of new regulations that will enable the abandonment of fallen stock in appropriate places or, as has been carried out until now in extensive husbandry, leaving parts of dead animals in natural areas where it is known they are sure to be rapidly consumed by scavenger species. This measure may reduce the numbers of immature Griffon vultures being admitted WRCs with malnutrition, as well as, potentially reduce the consumption of game carcasses and food from waste dumps – thus producing a consequential reduction in Pb exposure.

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Chapter 6.1

Lead poisoning subsequent to lead-pellet ingestion in Griffon vultures (*Gyps fulvus*) from the Iberian Peninsula

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6.1. Lead poisoning subsequent to lead-pellet ingestion in Griffon vultures (*Gyps fulvus*) from the Iberian Peninsula

6.1.1. Clinical report

This paper documents the Pb toxicosis exhibited in three adult female Griffon vultures living in the wild in the Iberian Peninsula. There have been previous reports on Pb poisoning and mortality in raptors that feed on dead or wounded animals over the past decades [8, 15, 20-22]. The distribution of Pb shot in the environment continues to this day through its use in hunting activities. Avian scavengers that typically include game birds and mammals in their diets are at risk of Pb poisoning, due to the ingestion of carcasses with fragments or residual Pb ammunition that are used in hunting [3, 9, 19, 30].

The Griffon vulture is a large bird of prey that belongs to the Old World vulture group and can be found over a wide geographic range (NW Africa, Iberian Peninsula, Balkans, Middle East and India) [11]. Within the Iberian Peninsula, specifically in Portugal, the conservation status of this species is near-threatened [7]. Among the various threats to this species, such as poisoned baits set for carnivores, direct persecution and a reduction in available food supplies [11], Pb poisoning must also be considered as a potential threat, since the carcasses of animals shot by hunters serve as food for these birds [14, 22].

Although Pb poisoning has been documented in all four of the species of vultures living in Spain: Griffon vulture [22], Cinereous vulture [17], Bearded vulture [18] and Egyptian vulture [28], we did not found any reports of Pb poisoning in the vulture species living in Portugal. Therefore, this paper documents the first case of Pb poisoning in a Griffon vulture living in Portugal and, given its integrative approach, it is as far as we know the most complete study of Pb poisoning in Griffon vultures. We evaluated metal concentrations in the birds' blood, liver and kidney, and complete post-mortem examinations and histopathological analysis were also performed.

Two Griffon vultures were found in the Northeast of Spain (Lleida, Catalonia, Spain) and the other was found in the Northeast of Portugal (Freixo de Espada à Cinta, Bragança, Portugal). The Griffon vultures were taken to be cared for at two different WRCs (the WRC in Vallcalent, Spain and the WRC in Gouveia, Portugal respectively) on 11 November 2011, 4 January 2012 and on 15 March 2012. No external injuries were identified and, due to the impossibility of establishing a definitive diagnosis, supportive care (i.e. intravenous fluids, B-complex vitamins, atropine and oxigenotherapy) was performed on the birds, depending on their clinical signs. However, the birds died within 24h of their admittance.

An ante-mortem whole blood sample was taken from the brachial vein of two birds and post-mortem fresh liver and kidney samples were collected from all the birds. The lungs of two of the birds were also collected. All samples were frozen at -20ºC until analysis. Post-mortem examination and histopathological and metal analysis were all performed in order to identify the causes of death. The ingestion of poisons, infectious and/or parasitic diseases were the differential diagnoses.

Samples of tissues (liver, kidney and lung) for histopathology were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4-5µm, and stained with haematoxylin and eosin (H&E) and Perls' Prussian blue. Liver and kidney sections were also stained with Ziehl-Neelsen acid fast.

Metal analysis was performed by ICP-MS (Perkin Elmer Model Elan 6000, Perkin Elmer, Waltham, USA) in the laboratories of the SCT-UB, Spain. Liver and kidney sub-samples (250-350 mg w.w.) and whole blood (0.3ml) were digested in Teflon reactors with 65% concentrated HNO_3 and 30% H_2O_2 at 90 ºC in an oven and left overnight. After digestion, samples were diluted with tetra-distilled purified water, transferred to the measuring vessel and then analysed for metals. A second set of identical liver and kidney samples (1-2g) was oven-dried at 60 ºC until reaching a constant weight, in order to determine the dry weight. An analytical quality-control programme was applied throughout the study. Blank absorbance values were monitored throughout the survey and were subtracted from the readings before the results were calculated. Accuracy of ICP-MS was determined by analysing standard reference materials (Whole-blood Seronorm, Trace Element, Whole Blood 2 - ref. 201605 and Whole Blood 3 - ref. 102405 from SERO AS, Norway and Bovine Liver – 1577b from National Institute of Standards and Technology, Gaithersburg, USA) together with the samples.

The clinical signs exhibited by the Griffon vultures on admission at the WRCs and the results of the physical examination performed on them (also on admission), as well as the results of post-mortem examination and histopathological analysis, are summarized in table 6.1.1. Results of the metal analysis are presented in table 6.1.2.

Table 6.1.1. Clinical signs exhibited by the Griffon vultures, post-mortem examination and histopathological analysis.

	Case 1	***Case 2***	***Case 3***
Body weight (g)	6416	7000	7372
Clinical signs			
Neurological	• Prostrate • Weakness of the wings and legs	• Prostrate • Weakness of the wings and legs	• Ataxia • Obtunded
Cardiorespiratory	• Bradycardia • Slow breathing • Respiratory noise	• Bradycardia • Slow breathing	• Pronounced dyspnoea
Gastrointestinal	• Normal	• Regurgitation • Crop impaction after force feeding (Fig. 5.1.1b) • Greenish diarrhoea (stains on the feathers around the vent) 8Fig. 5.1.1c)	• Regurgitation • Crop stasis • Greenish diarrhoea (stains on the feathers around the vent)
Physical examination	• Dehydrated • Underweight • Poor body condition	• Dehydrated • Underweight • Poor body condition	• Dehydrated • Underweight • Good body condition
Post-mortem examination	• Not performed	• No subcutaneous fat (Fig. 5.1.2a) • Pectoral musculature atrophy (Fig. 5.1.2a) • Fluid in the trachea, airsacculitis (Fig. 5.1.2d) • Right lung pneumonia (Fig. 5.1.2e) • Crop impaction (Fig. 5.1.2b) • Proventricular-ventricular distension with green bile stained mucosa (Fig. 5.1.2c) • Distension of the gall-bladder with dark green viscous bile • Nine non-eroded Pb pellets were recovered from the stomach (Fig. 5.1.2c) • Pale kidneys (Fig. 5.1.2f)	• Rounded pectoral muscle • Mild airsacculitis • Right lung pneumonia • Distension of the gall-bladder with dark green viscous bile
Histopathological analysis	• Not performed	• Bacterial pneumonia in the right lung • Severe hemosiderosis in the liver (Kupffer cells and to a lesser extent in hepatocytes) (Fig. 5.1.3) • Hemosiderosis in lung macrophages • Hemosiderosis in the renal proximal tubular epithelial cells • No intranuclear inclusion bodies were identified with Ziehl-Neelsen acid fast stain	• Bacterial pneumonia in the right lung • Moderate hemosiderosis in the liver (Kupffer cells and to a lesser extent in hepatocytes) • Moderate hemosiderosis in lung macrophages • Moderate hemosiderosis in the renal proximal tubular epithelial cells • No intranuclear inclusion bodies were identified with Ziehl-Neelsen acid fast stain

Figure 6.1.1: Clinical signs **a)** Depression state and weakness of the Griffon vulture **b)** Crop impaction **c)** Dark green-stained feces in feathers around the vent.

Figure 6.1.2: Post-mortem examination **a)** Poor body condition of the animal **b)** Crop impaction **c)** Pellets (red circle) and green bile stained mucosa of the impacted proventriculus and ventriculus **d)** Airsacculitis **e)** Right lung pneumonia (arrow) **f)** Pale kidneys.

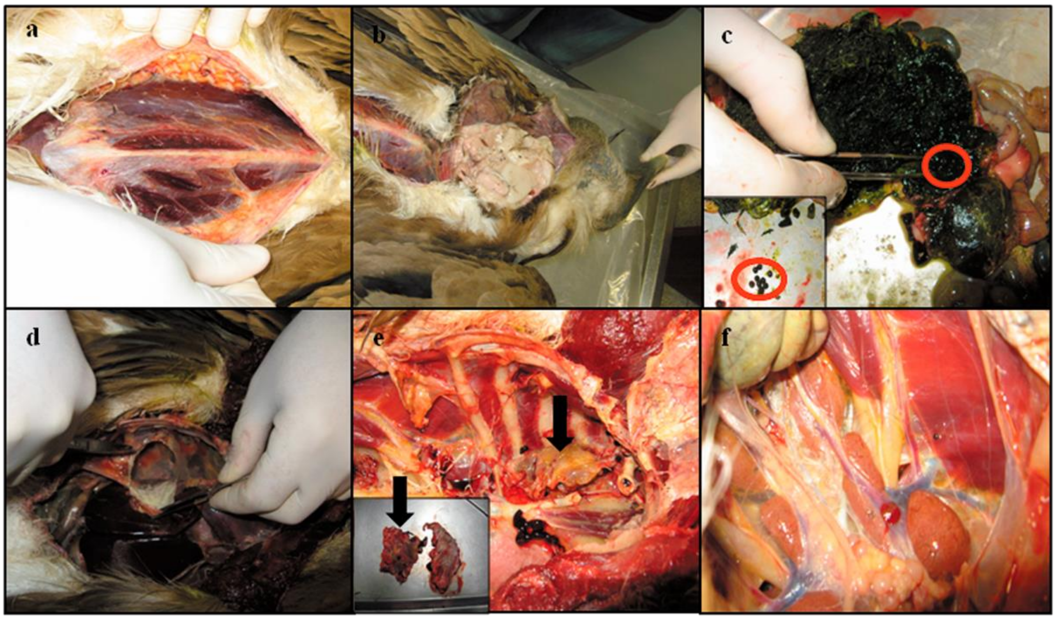

Figure 6.1.3: Liver histopathological analysis (diffuse hemosiderosis). **a)** H&E stain **b)** Perl's Prussian blue stain.

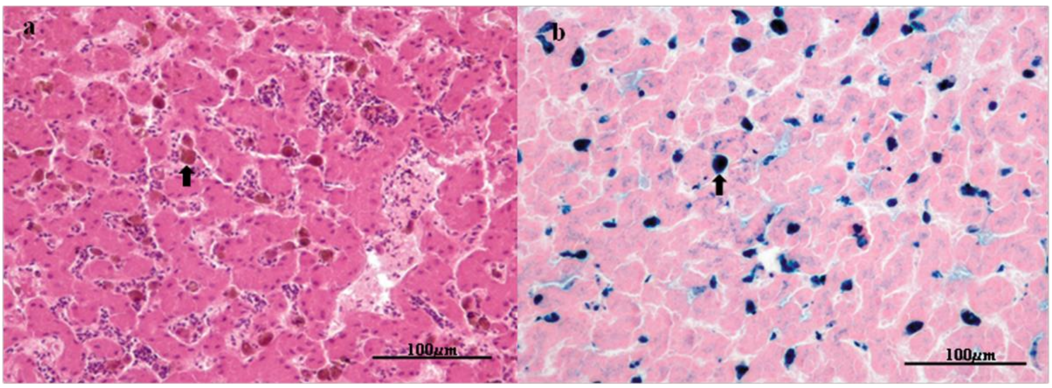

Table 6.1.2. Metal concentrations in the analysed samples taken from the three Griffon vultures.

	Case 1			*Case 2*			*Case 3*		
	Blood (µg/dl)	**Liver (µg/g d.w.)**	**Kidney (µg/g d.w.)**	**Blood (µg/dl)**	**Liver (µg/g d.w.)**	**Kidney (µg/g d.w.)**	**Blood (µg/dl)**	**Liver (µg/g d.w.)**	**Kidney (µg/g d.w.)**
Mn	8.41	13.37	8.43	3.84	13.17	6.11	*	14.57	9.31
Mo	n.d.	0.72	0.72	n.d.	0.58	0.50	*	0.81	0.86
Zn	353.87	140.84	86.01	377.77	255.43	75.08	*	110.43	90.24
Cu	33.93	23.04	30.32	64.26	20.59	19.37	*	44.85	28.24
Co	n.d.	0.13	0.35	n.d.	0.12	0.20	*	0.16	0.46
Se	18.01	3.45	4.74	18.05	1.88	4.18	*	1.75	3.55
Ba	0.61	0.1	0.58	1.40	0.28	0.29	*	n.d.	2.73
As	0.3	n.d.	n.d.	n.d.	n.d.	n.d.	*	n.d.	n.d.
Pb	968.82	1077.38	92.28	1384.44	308.58	34.59	*	308.65	100.46
Cd	n.d.	0.4	1.34	n.d.	0.87	3.92	*	0.36	0.89
Hg	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*	n.d.	n.d.

* Not evaluated; d.w. dry weight; n.d. Not detected, Mn Manganese, Mo Molybdenum, Zn Zinc, Cu Copper, Co Cobalt, Se Selenium, Ba Barium, As Arsenic, Pb Lead, Cd Cadmium, Hg Mercury

6.1.2. Discussion

Taking into account the results of the metal analysis, which revealed extremely high Pb concentrations in all the samples analysed (blood, liver and kidney) and the clinical signs exhibited by the birds, we concluded that the three Griffon vultures suffered from Pb poisoning. The results of post-mortem examination and histopathological analysis carried out on two of the birds support this conclusion.

From a clinical point of view, the triad of central-nervous signs, digestive-tract signs and depression/lethargy/weakness observed in two birds seems to be a constant finding in birds with Pb poisoning [23]. Although digestive-tract signs were not identified in all vultures, neurological disorders and weakness were exhibited by the three vultures. The clinical neurological signs are probably caused by the accumulation of Pb in the microvasculature of the brain, which leads to endothelial cell alterations and results in neurotoxicity and encephalopathy [6]. Cases of Pb poisoning in raptor species are frequently complicated by crop stasis and consequently crop impaction and regurgitation [1, 2, 27]. The poor body condition (the normal weight for healthy females is 8000 to 11000g [22]) observed in two of the birds, which exhibited atrophied skeletal musculature and loss of subcutaneous fat, is commonly observed in Pb-poisoned birds and is probably caused by the paralysis of the digestive tract and consequent inhibition of food digestion [2, 5, 25].

The birds' gall-bladders were distended and contained dark-green viscous bile, as a consequence of haemolytic anaemia caused by the Pb [27]. The regurgitated bile may stain the proventricular-ventricular mucosa, the faeces, and the pericloacal plumage [2]. The cloacal feathers turn green as a result of the intense green-coloured diarrhoea which is a very characteristic sign of Pb poisoning [10]. Tissues in general may appear pale, also due to anaemia [2].

The cardiorespiratory symptoms identified in the three vultures are justified by the macroscopic and microscopic signs of airsacculitis and pneumonia**.** However, we cannot surmise whether they were present prior to ingestion of Pb shot or as a consequence of Pb exposure.

Deposition of hemosiderin was identified in liver, lungs and kidneys. Hemosiderosis is a common histopathological finding, secondary to intravascular haemolysis or impairment of heme synthesis [24, 27, 31]. Notably absent in these Griffon vultures were acid-fast, intranuclear Pb-inclusion bodies, which are sometimes found in cases of Pb toxicosis within the renal tubule epithelial cells and/or hepatocytes [5, 16, 29]. These inclusions are composed of protein and Pb and it is thought that they have a protective function [6]. Their occurrence, however, may depend on the affected species and the concentration of Pb in tissues [25, 31].

The association of Pb concentrations with several physiological and pathological effects is well established in Falconiformes [12], but is not well established in vultures, except for some data obtained by García-Fernández et al. [14], Carpenter et al. [5], Pattee et al. [24] and Platt et al. [26]. Susceptibility or tolerance levels seem to differ between vulture species. The Turkey vulture (*Cathartes aura*) for example appears to be relatively tolerant to Pb poisoning in comparison to other raptor species, while the Andean condor (*Vultur gryphus*) seems to be quite sensitive [5, 24]. According to the results found by García-Fernández et al. [13, 14] it would appear that Griffon vultures are relatively tolerant to Pb and that the levels required to cause clinical toxicity might be higher than those of other Falconiformes. In another study carried out by Carneiro et al. [4], a vulture that had blood concentrations of 300.23µg/dl - which is compatible with clinical toxicity - was apparently healthy and did not exhibit any clinical symptoms. However, even though Griffon vultures exhibit less susceptibility or a higher tolerance to Pb compared to all other raptor species, the clinical signs that were compatible with Pb poisoning and the high Pb concentrations found in our animals compared with the ranges established for Falconiformes, and with other concentrations previously reported in Pb-poisoned raptors including vultures [1, 8, 12, 15, 21, 22, 25], suggest that the Griffon vultures analysed in this study suffered of Pb poisoning. Furthermore, in the first case of Pb poisoning in a Griffon vulture reported by Mateo et al. [22], the bird showed clinical signs and necropsy findings similar to those described in this paper. However, according to their results, the hepatic Pb concentrations were much lower than the value determined by us.

Liver, kidney and blood Pb concentrations were consistent with acute or sub-acute intoxication and levels responsible for mortality [6, 12, 24, 25]. Syndromes with acute and sub-acute expression are usually manifested in the central nervous and digestive systems [29]. The weight loss and the absence of subcutaneous fat and atrophy of the pectoral musculature identified in two of the Griffon vultures may be indicative of sub-acute exposure, while the vulture exhibiting good body condition could have suffered from acute exposure.

We believe that Griffon vultures admitted to our WRCs represent only a small percentage of the Pb-exposed birds in the Iberian Peninsula. Most vultures suffering from Pb poisoning would never be seen or found alive for diagnosis and medical treatment.

Vultures in the Iberian Peninsula are potentially exposed to secondary Pb poisoning through ingestion of Pb shot in the carcasses of game animals during and/or after the hunting season [28]. This exposure may have increased after the ban on abandoning carcasses of domestic ruminants in the field was introduced due to the bovine spongiform encephalitis crisis. As consequence, vultures have less available food, consume hunting-bag residues more frequently and suffer malnutrition problems due to the consequent mobilization of energy and Pb stores. The approval of new regulations that would enable the abandonment of fallen stock in appropriate places where they are sure to be rapidly consumed by scavenger species may reduce the consumption of game carcasses and consequently reduce the probability of occurrence of Pb poisoning. Moreover, early detection and treatment of Griffon vultures that have ingested Pb pellets can improve their chances of survival. However, to establish a correct diagnosis in these cases, WRCs would require more resources, such as haematology, blood chemistry, blood Pb analysis and radiology.

In Spain and Portugal, the use of Pb shots are only banned in wetlands included in protected areas, due to the risk entailed for waterfowl (Royal Decree 581, 2001 and Ordinance

No. 137/2012). However, Pb ammunition is still used in big-and small-game hunting in both countries. We recommend the prohibition of Pb ammunition in big-game hunting activity in order to prevent vultures of Pb poisoning and preserve their populations.

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CHAPTER 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

Although several studies using raptor species have been carried out in Spain in order to monitor environmental metals pollution [7, 16, 33, 34, 36, 37], the assessment of the exposure to the toxic metals in the Griffon vulture from Northeast of Spain has not been carried so far. Furthermore, previous studies [23, 32, 36] performed with this species in other areas of Spain (Cazorla Natural Park, Murcia and Valencia) have shown elevated blood Pb levels which support the need to continue the monitoring of toxic metals with Griffon vulture. In Portugal, biomonitoring studies with raptors have been scarce, having been previously published three studies about Hg, two of which share the same sampled individuals [27, 44, 54]. Thus, this work contributes with data concerning the abundance of toxic metals, degree of contamination of terrestrial ecosystems, metals' bioavailability in species at the top of food chain and possible impacts on the animals themselves and their populations, in the Northeast of Spain and in Portugal.

The three diurnal raptor species selected to perform this research were Common buzzard, Black kite and Griffon vulture, which belong to the Accipitriformes order and to the Accipitridae family. They were selected as biomonitors because of their characteristics as raptors and opportunistic feeding habits; they are at risk of accumulating and concentrating metals and As in their tissues and serving as sensitive indicators of the level of environmental contamination. In addition, Common buzzards are distributed over almost the entire Iberian Peninsula, being the most frequently observed bird of prey and consequently birds found dead and/or admitted to WRCs can provide a sufficiently large amount of samples. Though not so abundant as Common buzzard, Black kite is widely distributed in various regions of the Iberian Peninsula. It also frequents a wide range of biotopes during its breeding season in the Iberian Peninsula, many of which are highly affected by agricultural, industrial and urban pollution. Therefore, they are important indicators of environmental contamination [7, 8, 50]. Furthermore, some studies [8, 9] have shown that Black kites are susceptible to metal toxicity and contamination has been suspected as an important contributing factor to the decline of their populations. Regarding Griffon vulture, it is important to study this species due to its unfavorable conservation status in Portugal and their millenary dependence from human activities which provide food resources through livestock management or hunting practices predisposing them to an increased exposure to toxic metals.

Taking into account that the measurement of metals concentrations in birds' tissues and blood is one of the best indicator of the degree and type of exposure, and thus a useful procedure to monitor environmental contamination [36], whole blood samples were collected from the species mentioned above. It was also possible to obtain a relatively large number of liver and kidney samples from Common buzzard.

Among the species sampled for this work, the Common buzzard was the most frequently admitted in WRCs and many individuals presented as input reasons injuries by collision with vehicles or power lines and injuries by Pb shot, which influenced their rate of recovery and release. For this reason we collected a considerable number of different sample types of this species. Regarding the Griffon vulture, the main reason of their admission to WRCs was malnutrition of the juveniles, so the birds had a high rate of release and a low frequency of mortality. Therefore the few internal organs collected were from animals that died of suspected poison. The catches of Griffon vultures were also an important method to obtain a relatively large number of blood samples from this species. The migratory habits of the Black kite and consequently relatively low frequency of admission in the WRCs was the main reason for collecting less samples from this species. In Portugal, it was possible to collect samples from these three species, while in the Northeast of Spain only the Griffon vulture was sampled.

As concentrations were measured in samples collected from the Common buzzard and from the Black kite. The lowest mean As concentrations were found in blood of Common buzzards and of captive Black kites which in turn were similar in both groups. The highest mean As concentrations were observed in blood of free-living Black kites and in the kidneys of Common buzzards which were also similar. Considering only free-living birds, there is a clear difference between these two species for blood As levels. Differences between species in As levels can be explained by diet characteristics: predatory and piscivorous birds accumulate the highest residues [22, 43]. Considering this information and knowing that the fish is part of the diet of Black kite, higher concentrations of As in free-living birds of this species were expected. Although there is limited information on threshold values of As in blood that may cause detrimental effects in raptors, the mean value found in free living Black kites was higher than reference level (2µg/dl) published by Burger and Gochfeld [13] in uncontaminated areas. In turn, blood As concentrations found in free-living Black kites were similar to that found in nestling Black kites exposed to the emissions from a solid-waste incinerator in Madrid (Spain) [8] and in nestling Black kites sampled four years after a mining accident at Doñana (Spain) [4], but lower than the values found in the nestling Black kites sampled just a year later in the same area [3, 4]. With respect to As concentrations in the liver and kidneys of Common buzzards, our results are much lower than those observed for the Common buzzard in Galicia (Spain) [55] and in Sicily (Italy) [52] where the exposure to As was considered of no toxicological concern. Also, hepatic and renal As concentrations were below 1 µg/g w.w., which is the lowest range normally found in living beings [11, 21]. Although this metalloid has not been associated with adverse health effects, it can be involved in sub-lethal effects, mainly in the Black kite [46, 47]; and we think that it is necessary further investigation to understand possible biological effects of this metalloid in raptors.

Cd was analyzed in all blood samples collected from the three bird species studied in this work. However, it was below the LOQ of 0.204 - 0.312 µg/dl in more than 90% of total blood samples (94.6% in Common buzzard, 97.7% in Black kite and 98.3% in Griffon vulture). The mean concentrations of Cd in the blood samples, where it was quantified, were: 1.94±1.84 µg/dl (n=5) for Common buzzard, 0.44µg/dl (n=1) for Black kite and 7.28±6.93 µg/dl (n=2) for Griffon Vulture. The high percentage of birds' blood samples with non-detected levels of Cd may be associated with different factors: LOD of our analytical method, LOQ calculation mode and/or very low environmental Cd contamination. The method described by García-Fernández et al. [35, 36] with a LOD for Cd lower than the value calculated in our study, enabled the measurement of Cd concentrations in almost all blood samples of different raptor species, including the species studied in this work. However, García-Fernández et al. [35, 36] does not define the LOQ and the blood Cd concentrations obtained in their studies were lower than our LOQ. Although there is not well-established toxicity threshold for Cd in birds' blood, taking into account that Cd concentrations in blood of raptor species living in an non-polluted Mediterranean forest in the Southeastern Spain range from not detected to 1.5 µg/dl [49], we could consider that the studied raptor species are exposed to background levels of pollution. However, it is necessary to taking into account that even at very low concentrations Cd have some physiological effects that negatively affect the metabolism, especially during growth [59]. These physiological effects may not cause visible damage but they may be associated with the inactivation of enzymes involved in major metabolic pathways, leading to nutritional, developmental and immunological stress [10, 30]. According to Espín et al. [23, 24] that established the Cd threshold level associated with alterations in antioxidant enzymes' activity of 0.05 µg/dl and 0.3 µg/dl in Griffon vulture and Eagle owl, respectively, some birds in our study could end up being susceptible to suffer sub-lethal effects related to oxidative stress. Hepatic and renal Cd concentrations were determined in all Common buzzards and in three Griffon vultures and concentrations were similar between both species. Cd distribution and bioaccumulation showed patterns similar to those previously described in other studies [5, 16, 35, 40, 45, 52]. Kidney, followed by liver, were the main internal organs for Cd accumulation; while the blood Cd content is extremely low. Blood levels of Cd can be adequate as a biomarker of recent exposure [35]. However, in the case of chronic exposure Cd tends to accumulate in soft tissues, being hepatic and renal concentrations good biomarkers of total exposure to this metal [5, 57]. According to Scheuhammer [57], it is preferable to measure Cd concentrations in both liver and kidney since the ratio of Cd concentrations between these two tissues can be informative. According to this author, the ratio between liver and kidney concentration higher than one indicates acute exposure to relatively high doses of Cd, whereas the ratio lower than one is more suggestive of chronic exposure. Of the entire samples of birds studied in this study, all had a liver/kidney Cd ratio lower than one, which means prolonged exposure and accumulation. As far as we know, this work reported the first hepatic and renal Cd concentrations in Griffon vulture. Comparing the Cd concentrations obtained for Common buzzard with those obtained by other studies, our results vary with the geographical area. So, our results are similar with those obtained in Italy by Alleva et al. [1], Battaglia et al [5] and by Naccari et al. [52]; lower than those observed in Galicia (Spain) by Pérez-López et al. [55] and Castro et al. [16], and higher than the results obtained by García-Fernández et al. [35] in Murcia (Spain). According to Scheuhammer [57], the hepatic and renal Cd concentrations obtained in our study for Common buzzard and Griffon vulture are much lower than the threshold values indicative of increased environmental exposure (3 µg/g d.w. in liver and 8 µg/g d.w. in kidney). Furthermore, liver Cd content of healthy adult birds in wild populations is usually between one half and one tenth of the concentration in kidneys of the same bird [30], a situation that was observed in the birds of the present research. However, the birds in this work showed a continuous exposure to low levels. With continued exposure, even at low levels, this non-essential element is accumulated throughout the life span of birds, due to its extremely long biological half-life in kidneys and its slow elimination from these tissues [30, 57].

Concerning to Hg evaluation, the obtained results showed high interspecific differences for blood Hg concentrations. From the analyzed samples of Griffon vulture, Hg levels were only detected in six (5%) blood samples with a mean of 2.31±1.25 µg/dl. Similar findings were obtained in blood samples of the captive Black kites, where none of the analyzed samples had Hg levels above the LOQ. In contrast, 77.4% of the free-living Black kites and 88.2% of Common buzzards had detected levels of blood Hg, with a mean of 7.49±8.46 µg/dl and 20.94±26.73 µg/dl, respectively. The highest levels of Hg were obtained in liver and kidney samples of the Common buzzard, 1.39±1.24 µg/g d.w. and 2.09±1.69 µg/g d.w., respectively. Differences in Hg exposure among species are primarily correlated with trophic position and dietary habits, wherein the piscivorous species generally present higher Hg concentrations [20, 25]. Although Black kite is not considered a piscivorous bird, the fish is part of their diet and therefore would be expected to be found higher concentrations in this species when compared with Common buzzard. However, this difference may be due to the mobilization and storage of Hg in feathers. The free-living Black kites were sampled during the molting period and it is during this phase that Hg is incorporated into keratin structure of feathers, reducing the its levels in blood [18, 31, 39, 51]. Blood Hg concentrations found in the free-living Black kites were similar or lower than those reported for nestling fish-eating raptors, such as Bald eagles (*Haliaeetus leucocephalus*) and Ospreys, while the Common buzzard presented higher or similar concentrations [41, 42]. In turn, the blood Hg identified in Common buzzard are within the range obtained in chicks of Bald eagle from areas considered contaminated by Hg (10-47 µg/dl) [2, 60]. As threshold-effects levels for Hg have yet to be established for bird blood, it is unclear whether Hg levels were high enough to pose a risk to any of these birds. However, according to the Hg threshold level of 3 µg/dl associated with alterations in antioxidant enzymes' activity in Griffon vulture and Eagle owl [23, 24] it is expected that Common buzzard and Black kite presented sub-lethal effects related to oxidative stress. In all cases the concentrations of total Hg were below the threshold (300 µg/dl) reported as high risk to avian piscivorous (i.e. Common loon, Gavia immer) [26]. However, the sensitivity of species to toxic elements vary considerably and it is necessary to establish threshold levels for raptors. The highest Hg concentrations identified in the liver and kidneys of Common buzzard shows that there is an accumulation of this contaminant in birds that feed at the top of terrestrial trophic chains. Since reproductive effects, namely early embryonic mortality, decreased hatchability and increased number of unfertilized eggs were associated with liver concentrations of about 2µg/g w.w. in the adult birds [48, 57], we consider that the Hg concentrations observed in the Common buzzard is related with a source of Hg available without interference in birds breeding or survival.

Amongst the studied species, Griffon vulture had the higher mean blood Pb concentrations, followed by the Black kite and Common buzzard. According to Franson [29] only 33.9% of the studied Griffon vultures had blood Pb concentrations compatible with a background exposure (<20 µg/dl), while 90.3% and 77.4% of the Common buzzards and free-living Black kites studied, respectively presented blood concentrations below 20 µg/dl. One bird of each raptor species presented blood Pb concentrations compatible with clinical toxicity (>100 µg/dl) and Pb poisoning was confirmed in three Griffon vultures. Our results are in accordance with those published by other authors [35, 50] which found the highest Pb levels in raptor species that feed on carrion. Although Common buzzard and Black kite present scavenger feeding habits, Griffon vulture is an obligate scavengers and the carcasses of wild mammals that have died from hunting practices account for a large part of their diet, thus they are more susceptible to the ingestion of Pb shot embedded in dead animals and suffer from Pb poisoning. A greater exposition to Pb was verified in Griffon vultures living near rubbish dumps, which also provides a significant fraction of their trophic needs, mainly after the ban on abandoning carcasses of domestic ruminants in the field, due to the BSE crisis (Regulation CE 1774/2002) [19]. When Griffon vultures live in relatively unpolluted areas and carcasses of domestic origin are provided in feeding stations with a higher frequency, there is a trend to present lower blood Pb concentrations [23, 58].

Although, according to Franson [29], a high percentage of the studied Common buzzards and free-living Black kites presented blood Pb concentrations associated with a background exposure, Pb concentrations above 15 µg/dl in blood inhibits antioxidant enzymes' activity and induces lipid peroxidation in erythrocytes. According to these findings, many individuals sampled in the current study could be at risk of suffering Pb-related sub-lethal effects. Regarding hepatic and renal Pb concentrations obtained for Common buzzards, according to the ranges established by Pain et al. [53], none of the studied animals exceeded the calculated dry weight threshold for massive exposure and most of them had very low hepatic and renal Pb concentrations (<2 µg/g, with many <1 µg/g d.w.). However, some studies provide evidence that low-level Pb exposure, although not responsible for clinical symptoms of Pb poison, can have subtle detrimental effects [14, 57]. As a result of these effects, birds may become susceptible to starvation and infections, increasing their probability of death from other causes. Reproductive success can also be affected at subclinical levels [12, 28]. So, we must consider the potential risk of Pb exposure in the studied bird populations, especially in Griffon vulture populations.

In some countries there is legislation to combat Pb toxicosis at wetlands and in waterbirds. However, Pb ammunition is still employed worldwide, including Iberian

Peninsula, in large and small game in upland hunting even though Pb toxicosis is an important cause of concern for conservation of raptors with scavenger feeding habits [6, 15, 17, 32, 38, 56].

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CHAPTER 8

FINAL CONCLUSIONS

8. FINAL CONCLUSIONS

This work contributes with relevant information on the biomonitoring of toxic metals and arsenic with raptors mainly in Portugal, where studies focusing on this issue have been scarce. Although several studies have been carried out in Spain, this work added new information regarding the exposure to toxic metals in the Griffon vulture population of the Northeast of Spain. The main findings are summarized below, followed by some perspectives for the future.

- The analytical method chosen for the determination of metals concentrations, ICP-MS, is a valid and recently used technique for monitoring metals and metalloids pollution in the environment. It provides sufficiently low detection limits and allows the simultaneous determination of several metals. However, this technique may not be sensitive enough to measure blood Cd at ultra-trace levels.

- In general, the concentrations of metals and As detected in the samples collected from the three raptor species can be considered compatible with a low-risk exposure. However, in certain cases, Pb can be related to toxic side effects and can be a limiting factor for the Griffon vulture conservation. The potential risk of Hg should also be considered and further studies should be conducted to investigate this metal, since Common buzzard presents relatively high concentrations of Hg. The highest Hg concentrations identified in the liver and kidney show that there is an accumulation in birds that feed at the top of the terrestrial trophic chains.

- The interspecific difference observed for blood As, Pb and Hg concentrations seems to be mainly related with the different dietary habits. Carcasses of wild mammals that have died from hunting practices account for a large part of the diet of the Griffon vulture, predisposing this species to increase Pb exposure. High Hg and As concentrations are generally found in predatory birds with dietary habits related to the aquatic biotopes, as the Black kite. In the specific case of Common buzzard, it seems to be unknown sources of exposure to Hg, so further studies are needed to determine its origin.

- In the Common buzzard, blood Hg and Pb levels were mainly influenced by the molting period and hunting season, respectively. The age of birds also influenced blood Hg and Pb concentrations and hepatic and renal Cd concentrations, wherein the adults have higher concentrations. Differences in blood Hg and Pb levels among age classes seem to be related with prey-size selection and stage of juvenile feather moult. The highest Cd concentrations found in adult Common buzzards suggests a continuous exposure and accumulation of this metal over their life. The sampling area, as well as, the reason of admission of the Griffon vultures to the WRCs, mainly malnutrition of the juvenile birds, have a strongly influence on the blood Pb concentrations in this species. The Griffon vultures admitted to WRCs do not seem to be representative of the local free-living populations in terms of recent Pb exposure. The Griffon vultures captured in Catalonia, Spain have the highest blood Pb concentrations possible due to the ecological context of this their habitat: an urban and industrial area, where rubbish dumps provide a significant fraction of their trophic needs.

- We did not observe differences in metals and As concentrations between the Portuguese sampling areas of Common buzzard and Griffon vulture, maybe because Portugal is a small country and raptors forage over a large geographical area.

- Our results suggest that blood concentrations may be a useful indicator of the degree of recent exposure to As, Hg and Pb. In the case of Cd, due to the highest percentage of blood samples of the three raptor species with not detected levels, we suggest that liver and kidney samples are more indicated to evaluate the exposure to this metal.

- Regarding Hg, we recommend further studies to determine its concentrations in feathers or in blood and feathers, once during the molt occur a high mobilization and storage of Hg in feathers reducing its blood levels.