ESCOLA UNIVERSITÁRIA VASCO DA GAMA

Mestrado Integrado em Medicina Veterinária
Artigo de Investigação

BIOMONITORING OF CONTAMINANTS IN SCAVENGING BIRDS OF PORTUGAL: PESTICIDES AND HEAVY METALS

Beatriz Carvalho Oliveira das Neves

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	Professor Doutor André Monteiro Pais Teixeira Pereira

"If I have seen further, it is by standing on the shoulders of giants."

— ***Isaac Newton***

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General Index

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List of acronyms, symbols, and abbreviations.

AChE - Acetylcholinesterase

As - Arsenic

BChE - Butyrylcholinesterase

CB - Carbamate

CERVAS – Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens

CRAS-HVUTAD – Centro de Recuperação de Animais Selvagens do Hospital Veterinário da Universidade de Trás-os-Montes e Alto Douro

Cd - Cadmium

CbE - Carboxylesterase

ChE - Cholinesterase

D.w. – Dry weight

DDW – Double distilled water

DTNB – 5,5-dithio-bis-(2-nitrobenzoic acid)

EU - European Union

GC/MS - Gas chromatography–mass spectrometry

HNO3 - Nitric acid

HPLC - High-performance liquid chromatography

ICP-MS - Inductively coupled plasma mass spectrometry

IQR – Interquartile range

IUCN - International Union for Conservation of Nature

kg - kilogram

M - Molarity

mg - Milligram

ml - Milliliter

mM - Millimolar

nm – Nanometers

n.º - number

OP – Organophosphates

PBS - Phosphate-buffered saline

rpm - Revolutions per minute

SD - Standard deviation

SE - Standard error

U - μmoles of substrate hydrolysed per minute

w.w. – Wet weight

WRC – Wildlife Rehabilitation Centers

μmoles - Micromoles

% - Percentage

© - Copyright

ºC - Degree Celsius

p - statistical significance

Os resultados relativos à análise da atividade da enzima acetilcolinesterase apresentados na presente Dissertação de Mestrado culminaram na elaboração de um *poster* científico apresentado nas IV Jornadas Ibéricas de Toxicologia.

- Neves, B., Carneiro, M., Almeida, A., Pereira, A., Sousa, L. and Sargo, R. (2024). *Atividade da colinesterase plasmática como biomarcador de exposição a pesticidas no Grifo euroasiático (Gyps fulvus)* [Poster]

Biomonitoring of contaminants in scavenging birds of Portugal: Pesticides and Heavy Metals

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1. ABSTRACT

Biomonitoring is a vital tool for assessing environmental pollutants, acting as an early warning system for contaminants that affect both human and animal health. Vultures, as top scavengers in the food chain, are particularly important bioindicators of environmental health due to their feeding habits and ecological position. These animals play a crucial role in maintaining ecosystems by removing carcasses and reducing the risk of disease transmission. However, vultures face significant threats from human activities, including illegal poisoning through pesticide exposure, and heavy metal contamination. This study aimed to investigate the exposure of wild vultures admitted to Wildlife Rehabilitation Centres in Portugal to organophosphate (OP) and carbamate (CB) pesticides, as well as heavy metals. Acetylcholinesterase (AChE) activity was measured in plasma samples to assess pesticide exposure, while lead (Pb), cadmium (Cd), and arsenic (As) concentrations were quantified in liver and kidney samples using inductively coupled plasma mass spectrometry (ICP-MS). This study represents the first application of the AChE assay in blood samples from vultures in Portugal. The results revealed that lead was the most frequently detected metal in the samples from Eurasian Griffon Vultures (*Gyps fulvus*), with one case exceeding safe exposure levels, suggesting subclinical exposure. Cadmium levels were higher in renal tissues compared to the liver, but overall exposure appeared low, possibly due to the young age of the animals studied. However, the detection of 1.691 mg/kg w.w. of cadmium and 2.45 mg/kg w.w. of lead in the kidneys of juvenile vultures is a noteworthy finding. In contrast, arsenic concentrations were low, posing minimal ecotoxicological risk. AChE activity was lower than reference values from other countries, indicating potential subclinical pesticide exposure.

These findings highlight the ongoing risks these birds face and underscore the need for continued biomonitoring and conservation efforts, which are crucial for protecting these species and preserving ecosystem integrity within the framework of the "One Health" concept.

Key words: Cadmium; Carbamates; Contaminants; Conservation; Lead; Organophosphates; Wildlife; Toxicology; Vultures

RESUMO

A biomonitorização é uma ferramenta essencial para a avaliação de poluentes ambientais, atuando como um sistema de alerta precoce para a presença de contaminantes que afetam tanto a saúde humana, como a animal. Os abutres, como necrófagos de topo da cadeia alimentar, são particularmente importantes como bioindicadores da saúde ambiental, devido aos seus hábitos alimentares e à sua posição ecológica. Estes animais desempenham um papel crucial na manutenção dos ecossistemas, ao eliminar carcaças e reduzir o risco de transmissão de doenças. Contudo, estas aves enfrentam ameaças preocupantes resultantes das atividades humanas, como a exposição a pesticidas e a contaminação por metais pesados.

O presente estudo teve como objetivo investigar a exposição de abutres selvagens, admitidos em Centros de Recuperação de Animais Selvagens em Portugal, a pesticidas organofosforados (OP) e carbamatos (CB), bem como metais pesados. A atividade da enzima acetilcolinesterase (AChE) foi medida em amostras de plasma, permitindo avaliar a exposição a pesticidas, enquanto as concentrações de chumbo (Pb), cádmio (Cd) e arsénio (As) foram quantificadas em amostras de fígado e rins, utilizando a técnica de espectrometria de massa com plasma acoplado indutivamente (ICP-MS).

O presente estudo foi pioneiro na aplicação do ensaio de AChE em amostras de sangue de abutres em Portugal. Os resultados revelaram que o chumbo foi o metal mais frequentemente detetado nas amostras de Grifo-euroasiático (*Gyps fulvus*), com um dos casos excedendo os níveis seguros de exposição, o que sugere uma exposição subclínica. Os níveis de cádmio foram mais elevados nos tecidos renais em comparação com o fígado, mas a exposição global revelou-se baixa, possivelmente atribuído à jovem idade dos animais estudados. A deteção de concentrações de 1,691 mg/kg w.w. de cádmio e 2,45 mg/kg w.w. de chumbo em amostras de rins de indivíduos juvenis é, contudo, um achado notável.

Em contraste, as concentrações de arsénio revelaram-se baixas, apresentando este um risco ecotoxicológico mínimo. A atividade da AChE mostrou-se inferior aos valores de referência de outros países, sugerindo uma possível exposição subclínica a pesticidas. Estes achados demonstram os riscos que estas aves enfrentam e evidenciam a necessidade de que os esforços contínuos de biomonitorização e conservação, essenciais para a proteção dessas espécies e para a preservação da integridade dos ecossistemas, sejam realizados dentro do conceito "Uma Só Saúde".

Palavras-Chave: Abutres; Cádmio; Carbamatos; Contaminantes; Conservação; Chumbo; Organofosforados; Vida-selvagem; Toxicologia

2. INTRODUCTION

Biomonitoring refers to a set of scientific techniques that assess environmental pollution based on the sampling and analysis of tissues and fluids from individual organisms (Market *et al*., 2003). Wildlife biomonitoring serves as an early warning system for potential impacts of contaminants on humans and animals health and can also be used as a tool for monitoring the effectiveness of mitigation strategies (Gómez-Ramírez *et al.,* 2014). Many of these studies are carried out in birds, which are particularly sensitive to anthropogenic contamination and other environmental changes. The most striking examples of the value of birds as biomonitors originate from their use as qualitative and quantitative accumulative indicators of pesticides and heavy metals (Becker, 2003). Scavenging bird species are especially effective for monitoring bioaccumulated and biomagnified pollutants due to their position at the top of the trophic chain and their wide feeding range, which allows them to reflect pollutant levels over large areas with fewer samples needed (Carneiro *et al*., 2016; Kruger *et al*., 2022).

Monitoring pesticides and metals in scavengers 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 (Gómez-Ramírez *et al.*, 2014). Additionally, 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 (Furness *et al*., 1993; Golden *et al*., 2003; Smiths *et al.,* 2013).

Obligate and facultative scavenger birds are among the faunal group most vulnerable to the illegal use of poisons, which is considered one of the main threats to these species worldwide (Margalida and Colomer, 2012). Vultures are the only group of birds classified as obligate scavengers and they are expertly adapted to take advantage of a diverse array of wild and domestic animal carcasses in their habitat (Ogada, Keesing, and Virani, 2012).

According to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, Portugal's mainland is home to three vulture species, each with varying conservation statuses: the Griffon vulture (*Gyps fulvus*) (Least Concern), the Cinereous vulture (*Aegypius monachus*) (Critically Endangered), and the Egyptian vulture (*Neophron percnopteru*s) (Endangered) (IUCN, 2016) (Figure 1). Despite being protected under Law n. º 140/99, April 24, most scavenger bird species face a concerning conservation status in Portugal.

Figure 1. Cinereous, Egyptian and Griffon Vulture © Bruno Berthemy

Vultures provide crucial ecosystem services by decomposing carrion, preventing dead biomass accumulation, aiding in waste removal, disease control, and nutrient recycling (Margalida and Colomer, 2012), mitigating the transmission of infectious diseases to humans, domestic animals, and other wildlife (Ives *et al.*, 2022). Furthermore, due to their position at the top of the food chain, vultures are historically impacted by human activities. A striking clear example of this vulnerability and crucial role in the ecosystem is the sharp decline of vulture populations in India, caused by ingesting diclofenac-treated cattle carcasses. Though useful for livestock, diclofenac is fatal to vultures, leading to mass deaths and its eventual ban by the Indian government (Green *et al*., 2016). This mortality led to an increase in scavengers like stray dogs and rats, potential carriers of zoonoses, highlighting the crucial role vultures play in protecting human health (Jalihal *et al*., 2022).Their dependence on these activities - such as livestock management or hunting practices that provide food sources - put them at risk of exposure to heavy metals and pesticides, making them sensitive indicators of environmental contamination (Angliester *et al*., 2023; Carneiro *et al*., 2015).

In the last 30 years, obligate scavenger populations have been rapidly declining globally, consequently increasing the risk of disease transmission to other animal species and humans (Jalihal, Rana, and Sharma, 2022; Ogada, Keesing and Virani, 2012). The impact of this loss is devastating, resulting in higher biomass near human settlements, longer carcass decomposition times, and an increase in zoonotic diseases (Ives *et al.,* 2022). The most impactful anthropogenic cause contributing to this decline all over the world are emerging contaminants (Krüger *et al.*, 2022). Ives *et al.* (2022) analysed global reports on morbidity and mortality in free-living vulture species, concluding that toxins were the leading cause of death (60%), particularly heavy metals and pesticides. Besides mortality events, pesticide or heavy metal exposure may also interfere with vultures by their sublethal effects on reproductive success, behaviour, immune response and physiology, posing a long-term risk to their populations (Ogada, Keesing and Virani, 2012; Krüger *et al.,* 2022).

Heavy metals, including arsenic (As), cadmium (Cd) and lead (Pb) are considered the most hazardous from both environmental and toxicological standpoints for humans and animals. This is attributed to their non-biodegradable nature, persisting in the environment for prolonged periods (Krüger *et al*., 2022). Furthermore, they also present the ability to undergo biomagnification throughout the food chain, thereby intensifying the threat to vultures, other wildlife and humans (Krüger *et al.,* 2022).

Among various toxic metals, lead is the primary cause of poisoning in bird species and poses a significant threat to all raptors, particularly long-lived species that can bioaccumulate this compound. (Krüger *et al.*, 2022). Chronic exposure may result in long-term detrimental effects on reproduction, immune competence, and behavioural patterns, ultimately decreasing the survival and fitness of exposed bird populations. In cases of acute lead poisoning, rapid accumulation can lead to sudden mortality, even in birds that appear to be in good physical condition, due to its direct impacts on the cardiovascular and nervous systems (Krone, 2018).

Vultures can develop lead poisoning either from being directly shot at when persecuted or, more commonly, by ingesting carcasses of game animals shot with lead-based ammunition (Gangoso *et al.,* 2009; Ogada, Keesing, & Virani, 2012). As obligate scavengers, Griffon vultures are particularly vulnerable to ingesting lead fragments embedded in the remains of hunted animals, which is the primary source of lead contamination in raptors (Fisher *et al.*, 2006; Mateo, 2019). Occurrences of intoxication in Griffon vultures have been documented in the Iberian Peninsula as a result of this practice (Carneiro *et al*., 2016; Garcia-Fernandez, 2005). Additionally, widespread environmental lead contamination from waste dumping, along with mining and smelting activities, has impacted a variety of bird species through the food chain (Carneiro *et al.,* 2015).

Pesticides are widely used chemicals with unique properties designed to control pests and prevent plant diseases. While they protect agricultural crops globally, their extensive use poses significant risks to wildlife (Anglister *et al.,* 2023). Additionally, these compounds can severely impact humans, with pesticide exposure linked to higher incidences of various cancers, as well as Parkinson's and Alzheimer's diseases (Kapka-Skrzypczak *et al.*, 2011). Pesticides include insecticides, herbicides, fungicides, and rodenticides. The widespread use of different pesticide groups leads to global cross-contamination and unintentional exposure for both humans and other non-target species (Kapka-Skrzypczak *et al.,* 2011).

When it comes to pesticide poisoning, vultures can either directly ingest the bait or feed on the carcasses of an already poisoned animal (Krüger *et al.,* 2022). These poisoning incidents can either be deliberate or accidental when avian scavengers are not the target of these actions (Oropesa *et al.,* 2017). Even so, this illicit activity poses a significant conservation challenge in Europe, frequently emerging as the primary cause of non-natural fatalities (Grilo *et al.,* 2021).

The ecology of vultures heightens their susceptibility to toxin exposure and poisoning (Ives *et al.*, 2022). Being amongst the world's largest bird species, vultures take advantage of air thermals that enable them a soaring flight (Pirastru *et al.,* 2021), outcompeting other scavenger species and being one the first to locate and feed on fresh carcasses (Ogada, Keesing, and Virani, 2012). Because vultures typically feed communally, a single carcass can poison many individuals in a single feeding event (Ogada, Keesing, and Virani, 2012). Furthermore, with lifespans of up to 50 years, vultures exhibit delayed maturity and low productivity, characteristics that make their populations susceptible to mass mortality events from non-natural causes (Green *et al*., 2016).

It seems that most exposures in vultures are unintended consequences of either environmental pollution or efforts to eradicate other wildlife species that prey on livestock (Ives *et al.,* 2022). As climate change continues to escalate, Abrahms (2021) suggests that rising human-wildlife conflicts will likely increase predator persecution and the indiscriminate use of poisons for livestock protection, a trend linked to higher vulture fatalities (Krüger *et al.,* 2022). In Portugal, the Antidote project reflects growing awareness, with 1534 cases of poisoned animals documented in the last decade and with the Griffon vulture notably affected (Garcês *et al*., 2023). These data reveal the profound danger posed by the illegal use of poisons, representing merely the tip of the iceberg of a significantly larger problem (Sentinelas, 2019).

Regarding to pesticides biomonitoring studies, it has been shown that plasma cholinesterase (ChE) activity may serve as a reliable biomarker for assessing exposure to OP and CM pesticides in live vultures (Angliester *et al.*, 2023; Oropesa *et al.,* 2017). Cholinesterases, including acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterase (CbE), are serine hydrolases found in blood plasma. These enzymes are inhibited by OP and CB insecticides, which are used as biomarkers to detect exposure to anticholinesterase pesticides. These composts inhibit ChE in the nervous system by covalently binding to the active-site serine residue, leading to irreversible inhibition (Angliester *et al.,* 2023). This results in the accumulation of acetylcholine, which disrupts cholinergic transmission and can ultimately lead to respiratory failure and death (Roy *et al.,* 2005). Angliester *et al.* (2023) reports that in Griffon vultures, plasma is predominantly characterised by AChE activity, with minimal to no response response to butyrylthiocholine substrate and avian erythrocytes show low AChE activity relative to most mammalian erythrocytes (Thompson *et al.*, 1991).

Direct techniques such as gas chromatography–mass spectrometry (GC/MS) or high-performance liquid chromatography (HPLC) face limitations due to the rapid breakdown of these composts, as well as the possibility of dermal exposure or even in cases concentrations levels are below detection limits (Angliester *et al.*, 2023). However, given the high sensitivity of ChEs to OP/CB inhibition, measuring AChE activity can serve as a rapid, sensitive, and non-destructive biomarker for anti-ChE pesticide exposure across various species (Angliester *et al.,* 2023). Additionally, Oropesa *et al.* (2017) asserts that plasma AChE activity serves as the most effective biomarker for detecting low-level exposure to anti-ChE pesticides, as it is inhibited more swiftly and extensively compared to brain ChE. Consequently, plasma is a valuable tissue for monitoring, as it allows for repeated, minimally invasive assessments in the same animal. This technique also provides a rapid and efficient diagnostic method, allowing veterinarians to identify pesticide exposure promptly and implement suitable treatment protocols (Angliester *et al*., 2023). This, in turn, supports conservation efforts for these endangered species.

Given the substantial threats facing vultures, it is imperative to address and identify their sources, uncovering the detrimental effects of environmental contaminants and human activities to ensure the sustainability of these species' populations (Krüger *et al*., 2022). It is crucial to gather more accurate and comprehensive data on the frequency and distribution of poisoning incidents. The low detectability of these events—estimated at around 10% in Spain—significantly hampers efforts to address this issue effectively (Sentinelas, 2019).

A significant research gap exists regarding contaminant exposure in avian scavengers in Portugal. This is particularly concerning because the Iberian Peninsula—especially Spain, which shares several vulture populations with Portugal—hosts the majority of Europe's vulture populations (Margalida and Colomer, 2012). Only a single biomonitoring study on vultures has been conducted in Portugal, in which blood heavy metals concentrations were measured and the results were similar to those found in other European studies (Carneiro *et al.,* 2015). To the best of our knowledge, no other biomonitoring studies on pesticide exposure in vultures have been published in Portugal. Additionally, the only study on avian ChE activity was conducted by Santos *et al.* (2013) on waterbirds in this country.

The current study aimed to assess the exposure of free-living vultures admitted to Wildlife Rehabilitation Centres (WRC) in Portugal to pesticides and heavy metals. Pesticide exposure was indirectly monitored through the determination of plasma AChE activity levels, while heavy metal exposure was quantified through the analysis of liver and kidney samples. This study is the first to apply the AChE assay technique to vulture blood samples in Portugal, as well as to identify heavy metal concentrations in vulture tissues within the country.

3. MATERIALS AND METHODS

3.1 SAMPLE COLLECTION AND HANDLING

For the indirect analysis of pesticides, plasma was obtained from blood samples taken from ten Griffon vultures and one Cinerous vulture (n=11) admitted to the WRC - *Centro de Recuperação de Animais Selvagens do Hospital Veterinário da Universidade de Trás-os-Montes e Alto Douro (CRAS HVUTAD) - in Vila Real, Portugal.*

Blood samples were collected by veterinarians, from either the medial metatarsal vein or the ulnar vein, upon arrival and during routine clinical practice and then transferred to 1 ml Lithium Heparin blood collection tubes. Plasma samples were then obtained by centrifugation at 2000 rpm for 5 minutes. Following biochemical analysis, samples were stored at -20ºC. Time between collection and freezing never exceeded 5 hours. Details regarding the reasons for admission to the WRC and the origin of these animals are provided in Table 1 (Appendices), as well as Figures 2 and 3.

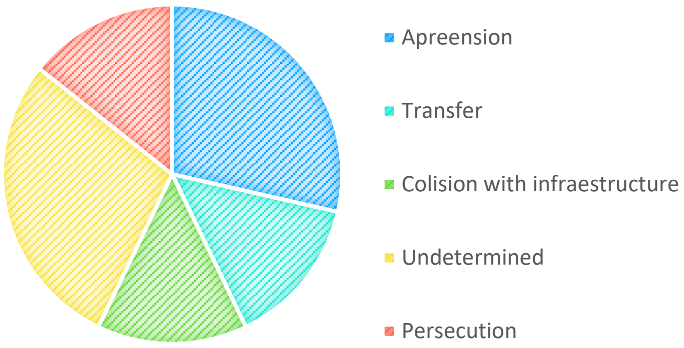

Figure 2. Reasons for admission to the WRC of sampled vultures for the AChE assay.

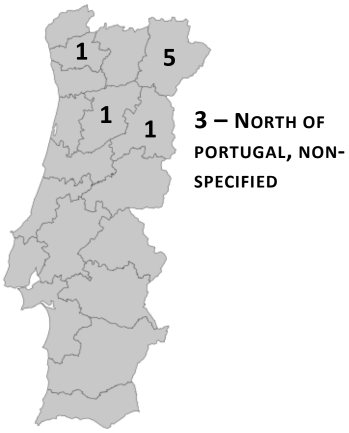

Fig. 3. Map illustrating the number of sampled vultures by origin for the AChE assay.

Regarding the direct analysis of heavy metals, liver (n=21) and kidney (n=19) samples were collected, between 2009 and 2022, during necropsies of 25 Griffon vultures and 1 Egyptian vulture (n=26) admitted to the WRC - *Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens* (CERVAS) in Gouveia, Portugal. Paired liver and kidney samples were successfully obtained from only 15 individuals. The analysis included five additional kidney samples and six liver samples from different animals, resulting in a total sample size of 26 individuals. Among these, only three were identified as adults, while the remainder were juveniles, and the age of one animal could not be determined. Additionally, 14 individuals were found dead in the wild, 9 did not survive treatment, and 3 were euthanized. All samples were stored at −20ºC until analysis.

The reasons for admission to these WRC and the origin are detailed in Table 2 (Appendices), Figure 4 and 5.

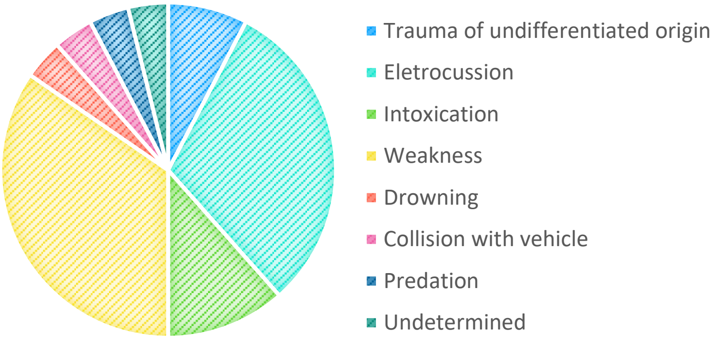

Fig. 4. Reasons for admission of sampled vultures to the WRC for the heavy metal assay

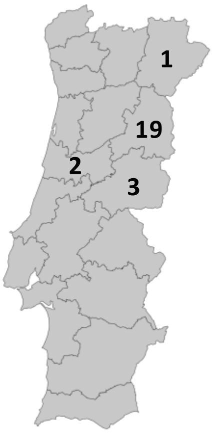

Fig. 5. Map illustrating the origin of vultures sampled for the heavy metal assay (*n*= number of sampled animals)

3.2 ACETYLCHOLINESTERASE ACTIVITY ASSAY IN PLASMA

Following the method described by Angliester *et al.* (2023), plasma samples were diluted 1:4 in phosphate-buffered saline (PBS). The AChE assay was conducted in triplicates, performed at 25ºC in a 96-well microplate. Each well contained 0.01 ml of the diluted plasma, 0,01 ml of Double Distilled Water (DDW) and 0.180 ml of the reaction mixture, which consisted of 1 mM 5,5'-dithiol-bis (2-nitrobenzoic acid, DTNB) in 0.1 M Tris-Cl buffer at pH 7.6, and 0.33-mM substrate acetylthiocholine iodide. AChE activity was quantified by measuring the optical density changes of the reaction product, at 415 nm wavelength, for 10 minutes (1-minute intervals) using a spectrophotometer (Thermoscientific multiskan FC with incubator). Enzymatic activity was expressed as *µ*moles of substrate hydrolyzed per minute (U) and per milliliter of plasma (U/ml), using a molar extinction coefficient calculated according to wavelength and temperature used in the assay (Eyer *et al.*, 2003). Blanks were analysed with the same procedure as that used for the samples and the values obtained were subtracted from the readings before the results were calculated. The precision and accuracy of the method were tested by analysing samples of reconstituted lyophilized Acetylcholinesterase from *Electrophorus electricus* (Sigma-Aldrich), together with the samples, used as controls.

This analysis was performed at the Toxicology Laboratory of *Escola Universitária Vasco da Gama (EUVG), Coimbra, Portugal.*

3.3 HEAVY METALS QUANTIFICATION IN TISSUE SAMPLES

For the direct arsenic (As), cadmium (Cd) and lead (Pb) detection in tissues, samples were submitted to an extraction technique as described by Carneiro *et al.* (2014). Liver and kidney subsamples [250–350 mg wet weight (w.w.)] were digested in Teflon reactors with 3ml of 65% concentrated nitric acid (HNO3) and 2ml of 30% hydrogenperoxide (H2O2) at 90ºC in an oven and left overnight. After digestion, each sample was brought up to a volume of 40 ml with milli-Q water. All samples were transferred to the measuring vessel and then analysed for As, Cd and Pb in an inductively coupled plasma-mass spectrometer (ICP-MS) (Perkin Elmer Model Elan 6000, Perkin Elmer, Waltham, USA). All material used in the digestion process was thoroughly acid-rinsed. An analytical quality-control program was applied throughout the study, according to López-Alonso *et al.* (2007). Blank absorbance values were monitored throughout the survey and subtracted from the readings before the results were calculated.

This analysis was performed at the Department of Geosciences, University of Aveiro.

3.4 DATA ANALYSIS

The dataset was firstly analysed using descriptive statistics, calculating the mean, median, minimum, maximum, and standard deviation. Outliers were defined as values that significantly deviated from the overall distribution of the data and identified using a threshold of 1.5 times the interquartile range (IQR). The results above the third quartile or below the first quartile were excluded prior to these calculations. Statistical analysis of the data was performed using IBM SPSS V.25.0 statistical software for Windows. A statistical significance level of $p<0.05$ was applied to determine rejection of the null hypothesis.

The Kolmogorov-Smirnov test was used to assess whether the data adhered to a normal distribution, which the majority of the variables did not exhibit. Therefore, a non-parametric approach was required for data analysis. The Mann-Whitney U test was used to test the statistical significance of sample type in the concentrations of each metal. Additionally, comparisons between the cause of admission and metal concentrations across both sample types were analysed using the Kruskal-Wallis test followed by Dunn's post-hoc test. A Wilcoxon test was used to compare paired corresponding liver and kidney samples from the same animal. Samples from birds with an unknown cause of admission were excluded from the analysis when testing these variables.

To investigate the relationship between cause of admission and metal concentration, animals were divided into three groups: Weakness, Trauma, and Intoxication. Each animal's cause of admission was assigned to one of these groups. The Trauma group included animals affected by trauma of undifferentiated origin (n=2), vehicle collisions (n=1), electrocution (n=8) and drowning (n=1).

Given that some studies on heavy metal concentration report in mg/kg dry weight (d.w.), it was necessary to convert our results, initially expressed in mg/kg wet weight (w.w.), to d.w. equivalents. To achieve this, we first determined the moisture content by comparing the mass of each tissue sample before and after lyophilization. This method ensures complete removal of water while preserving the structural integrity of the tissue. After obtaining the dry and wet mass values, the percentage of humidity for each sample was calculated, and an average humidity value was derived for both liver and kidney. The following formula was then applied to each sample:

Concentration (dry weight) = Concentration (wet weight) / [1− (Humidity (%) / 100)]

resulting in concentrations expressed in mg/kg d.w.

4. RESULTS

4.1 ACETYLCHOLINESTERASE ACTIVITY IN PLASMA

The average AChE activity among the surveyed Griffon vultures was 0.194 ± 0.048 U/ml of plasma (mean ± standard deviation), with a median value of 0.195 U/ml. The highest value obtained corresponded to 0.289 and the lowest 0.127. In the Cinereous vulture sample, AChE activity was measured at 0.008 U/ml of plasma. Additionally, two plasma samples from the same Griffon vulture, taken thirteen days apart, showed AChE activity levels of 0.127 U/ml and 0.235 U/ml, respectively. These results are presented in Figure 6.

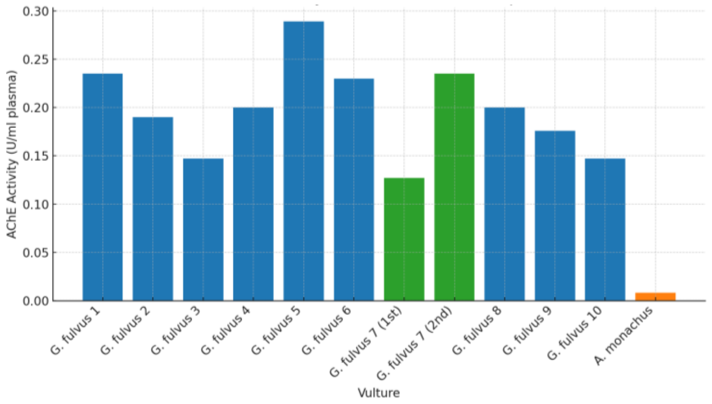

Figure 6. Plasma AChE activity of sampled vultures. Samples from Griffon vulture 7 (highlighted in green) collected 13 days apart; Cinereous vulture (*A. Monachus*) sample highlighted in orange.

4.2 HEAVY METALS QUANTIFICATION IN ORGAN TISSUES

Heavy metal concentrations obtained in the liver and kidney of necropsied vultures are listed in Table 3 and Table 4.

Table 3. Lead, cadmium and arsenic concentration in liver and kidney samples of Griffon vultures (*Gyps fulvus*).

	Liver (mg/kg w.w.)	***Liver (mg/kg d.w.)***	***Kidney (mg/kg w.w)***	***Kidney (mg/kg d.w.)***
Pb				
Mean ± s.d.	0.347 ± 0.307	0.997 ± 0.882	0.408 ± 0.372	1.301 ± 1.150
Median	0.19	0.546	0.215	0.680
Minimum	0.028	0.0805	0.04	0.127
Maximum	0.994	2.857	1.03	3.286
n	20	20	18	18
Cd	0.051 ± 0.04	0.148± 0.125	0.124 ± 0.156	0.3968 ± 0.497
Mean ± s.d.	0.03	0.086	0.06	0.19
Median	0.011	0.0316	0.009	0.028
Minimum	0.14	0.402	0.53	1.69
Maximum	21	21	19	19
n				

As				
Mean ± s.d.	0.0102 ± 0.003	0.029 ± 0.009	0.0133 ± 0.006	0.042 ± 0.037
Median	0.009	0.028	0.012	0.0382
Minimum	0.006	0.017	0.008	0.0255
Maximum	0.018	0.0525	0.029	0.092
n	21	21	18	18

Table 4. - Lead, cadmium and arsenic concentration in kidney samples of Egyptian vulture (*Neophron percnopterus*).

	Kidney (mg/kg w.w)	***Kidney (mg/kg d.w.)***
Pb	1.59	5.073
Cd	0.12	0.382
As	0.058	0.185

Lead was the metal detected at the highest concentration in both sample types, followed by cadmium (Cd), while arsenic (As) was present in the lowest concentration, as illustrated in Figure 7.

Figure 7. Concentrations (mg/Kg w.w.) of Pb, Cd and As in both liver and

Mean lead (Pb) concentrations between liver (0.347 ± 0.307) and kidney (0.408 ± 0.372) showed no significant difference (*p*>0.05) (Figure 6). In the liver, the highest concentration found was 2.45 mg/kg w.w., from an animal with no corresponding kidney sample to compare to, considered to be an outlier. However, the highest concentration found in kidney samples was 2.05 mg/kg w.w., in an animal who showed far lower liver values to this compost (0.994 mg/kg w.w.).

Regarding cadmium (Cd), the mean concentrations were significantly higher in the kidney (0.124 ± 0.156mg/kg w.w.) compared to the liver (0.051 ± 0.04 mg/kg w.w.) (*p* = 0.0225). The highest liver concentration recorded was 0.14 mg/kg w.w., observed in two animals, which had corresponding kidney concentrations of 0.51 mg/kg w.w. and 0.53 mg/kg w.w., the latter being the highest value detected in this tissue.

No significant differences (*p*>0.05) were noted regarding As mean values obtained in the liver (0.0102 ± 0.003 mg/kg w.w.) and kidney (0.0133 ± 0.006 mg/kg w.w.).

These findings are illustrated in Figure 8 and 9.

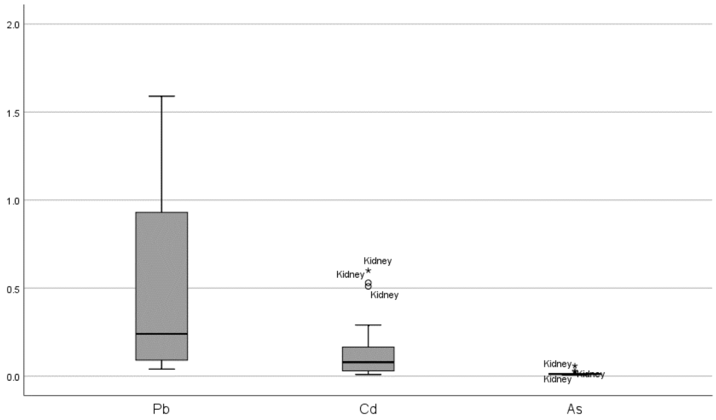

Figure 8. Metal concentrations (mg/Kg w.w.) in the kidney of sampled vultures

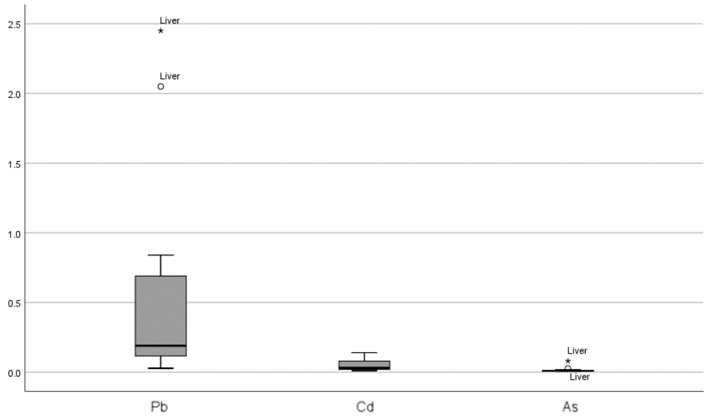

Figure 9. Metal concentrations (mg/Kg w.w.) in the liver of sampled vulture

When comparing liver and kidney samples from the same animal (n=15), 10 animals exhibited higher concentrations of Pb in the kidneys compared to the liver. Similarly, higher concentrations were also for Cd in 13 animals and As in 12 animals.

When comparing metal concentrations based on the cause of admission, our results indicated that liver and kidney levels of Pb, Cd, and As were similar across all the studied groups (p>0.05).

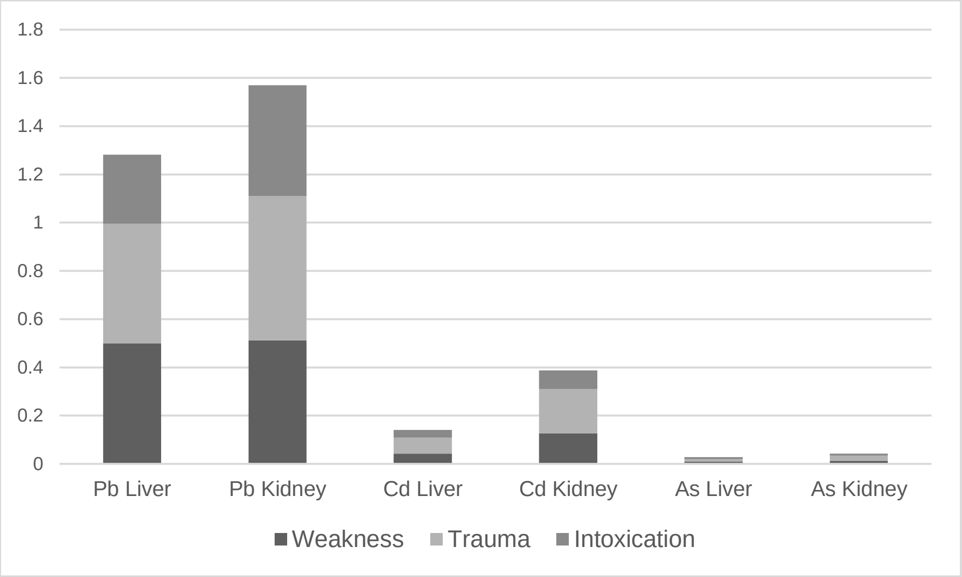

Figure 10. Metal concentrations (mg/Kg w.w.) according to cause of admission

5. DISCUSSION

5.1 PESTICIDES

The assessment of potential exposure and the diagnosis of pesticide intoxication in avian medicine can be particularly challenging due to a variety of factors.

According to Angliester *et al.* (2023), the clinical presentation of pesticide poisoning in birds can vary significantly due to factors such as the animal's pathophysiology, the dosage and rate of pesticide ingestion, and the specific type of ChE inhibitor involved, which can complicate the diagnostic process. Additionally, the effectiveness of chemical detection methods like GC/MS and HPLC can be constrained by their generally slow turnaround times, which can exceed 24 hours, as well as the associated costs. Moreover, these methods require a substantially larger sample volume than the indirect assay used in this study, which poses a major disadvantage for bird species due to limitations related to blood sample collection.

Although practical, confirming OP-CB exposure through the AChE activity method can be challenging due to the absence of documented baseline cholinesterase levels for the species under investigation, along with variability in testing methodologies. To the best of our knowledge, there are limited reference ranges available for plasma AChE activity for vultures. In the Cape vulture (*Gyps coprotheres*), normal plasma enzyme activity has been established as 0.9762 ± 0.002 U/mL (mean ± SD) (Naidoo and Wolter, 2016). Angliester *et al.* (2023) determined benchmarks for Israeli Griffon vultures' AChE activity as 0.601 ± 0.011 U/ml plasma. Based on these published reference values for Griffons, the enzyme activity observed in the current study is markedly low, with the highest value being 0.289 U/mL and the lowest 0.127 U/mL in plasma. However, in a nearby country, Oropesa et al. (2017) reported a ChE activity range of 0.33 ± 0.03 U/mL (mean ± SE) for non-exposed Griffon vultures in Spain, significantly closer to the values observed in this study. Nevertheless, interspecies and intraspecies differences in both the presence and activity levels of acetylcholinesterase have been documented. Although physiological intraspecies variation is typically less pronounced than pesticide-induced inhibition, it can still obscure detection in cases of low organophosphate (OP) or carbamate (CB) exposure or borderline values. For example, an individual with inherently high enzyme levels may retain AChE activity within the normal range despite experiencing 68% inhibition. Furthermore, differences in enzyme levels have been noted between free-roaming and captive birds of the same species (Angliester *et al.*2023; Oropesa *et al.,* 2017; Zwarg *et al*., 2012).

An additional factor contributing to intraspecies variation is the geographical region of these animals, as different stressors are present in different areas, potentially influencing our findings. Angliester *et al.* (2023) demonstrated this by sampling vultures from distinct locations and comparing results. Adult vultures typically remain within a specific geographic region for extended periods, and these geographical areas can exhibit significant variability in climate, food availability, water resources, and potential threats. Such environmental differences can lead to variability in stress levels and overall body condition, thereby influencing the observed variations in AChE activity (Angliester *et al*., 2023). Furthermore, social dynamics within these regions can also impact stress levels and, consequently, AChE activity (Brown *et al.,* 1986). This may account for the differences in the values obtained in the current study. To further validate this hypothesis, it would be beneficial to assess AChE activity in Portuguese vultures from other geographical regions, as this could enhance our understanding and provide more comprehensive insights into their physiological responses.

Other potential contributors to variations in ChE activity include malnutrition, dehydration, stress, infections, anemia, and cardiac disorders —factors known to influence ChE levels in humans but not yet fully studied in avian species (Tafuri and Roberts, 1987; Tryland *et al.* 2006). According to Angliester *et al.* (2023) weight, gender, hematologic parameters and the method sampling, including the time of day, does not significantly influence ChE levels. However, ChE levels are known to vary with age, with adult griffon vultures exhibiting higher mean AChE activity compared to sub-adults and juveniles. This variation can be attributed to a developmental increase in AChE activity as vultures mature, with lower

ChE activity in juveniles potentially making them more vulnerable to pesticide poisoning compared to adults (Angliester *et al.,* 2023).

Given that the majority of vultures sampled in this study are juveniles, and despite the influence of age as noted by Angliester *et al.* (2023) with the expectation of lower values in younger individuals, our results remain lower than those reported by this author, further reinforcing the hypothesis of exposure.

Although none of the sampled vultures were diagnosed with pesticide poisoning at the time of their admission, symptoms of pesticide intoxication can be subtle and non-specific. Furthermore, non-lethal pesticide exposure can impair various avian functions and may lead to mortality or injury through secondary factors like predation, trauma, starvation or disease (Angliester *et al*. 2023; Oropesa *et al.* 2017). Secondary effects resulting from impaired organ function—such as disorientation, anorexia, lethargy, reduced flight capability, and compromised resource acquisition—can also mask the initial signs of poisoning and contribute to under-detection of pesticide exposure (Galindo *et al.,* 1985; Cox, 1991; Fryday *et al.,* 1996; Grue *et al.,* 1997).

We also assessed AChE levels in the same Griffon vulture twice, with one sample taken upon its admission, from an undetermined cause to the WRC and another 13 days later. The recorded values of 0.127 U/mL and 0.235 U/mL, respectively, reveal a 1.8-fold increase in enzyme activity, likely reflecting the animal's recovery during its stay at the WRC. Since the cause of admission is unknown, the increase in enzyme activity can be justified by an improvement in body condition or the recovery from pesticide exposure, facilitated by access to uncontaminated food and water in the WRC environment.

The AChE activity results for the sampled animals were lower than the mean values reported by Oropesa *et al*. (2017), who included birds from a WRC after a minimum stay of 15 days to allow sufficient time for metabolising and eliminating pesticide residues. Our findings suggest that the AChE activity levels observed upon the vultures' arrival are below the baseline values for the species. However, an increase in the number of samples is necessary to confirm this hypothesis.

Lastly, Angliester *et al.* (2023) reported two cases of confirmed diagnosis of pesticide poisoning with methomyl poisoning, in which the values recorded for AChE activity were 0.087 and 0.096 U/mL. Although no reference ChE values exist for the Cinereous vulture, taking these two cases as a reference, the extremely low ChE level (0.008 U/mL plasma) observed in this study can suggest subclinical or even clinical exposure. This study represents the first report of AChE activity in the Cinereous vulture, underscoring the need for further research.

Although the present study has some limitations and may not fully reflect the actual pesticide exposure in wild populations, these individuals can provide direct evidence of the harmful effects of pesticides on wildlife, highlighting their continued presence in Portugal. Furthermore, these findings underscore the importance of expanding our knowledge on this topic and understanding the extent to which pesticides are present in our ecosystems and potentially entering the human food chain

5.2 HEAVY METALS

While research on blood heavy metal levels in raptors is relatively common across Europe, investigations into heavy metal concentrations in organ tissues remain scarce, particularly in vultures.

While blood is a good matrix to evaluate recent exposure to these composts, organs tissues are crucial for analysis due to their propensity to bioaccumulate heavy metals over time. Liver and kidneys serve as primary sites for metal bioaccumulation, making them more reliable indicators of chronic exposure (Carneiro *et al*., 2015). Despite this importance, there are very few biomonitoring studies in Europe focused on vulture organ tissues (Bassi *et al*., 2021; Ganz *et al.,* 2018; Berny *et al.,* 2015).

In the present study, kidney samples demonstrated higher concentrations of all analysed metals than liver samples, though this difference was statistically significant only for Cd concentrations. Thus, the kidney is considered a more suitable biomarker for chronic Cd exposure than the liver. This can be attributed to the kidneys' role in filtering and excreting heavy metals, making them more susceptible to accumulation. Metals such as lead, mercury, and cadmium can bioaccumulate in renal tissue, and their binding to proteins like metallothionein further enhances retention, highlighting the kidneys' increased vulnerability to toxicity compared to the liver (Nordberg et al., 2022).

Our findings also indicate that the cause of admission did not significantly influence the concentrations of metals in the analysed tissues. This may be attributed to the fact that none of the sampled vultures were suspected to be victims of illegal shooting. In cases where vultures were admitted due to general weakness and debilitation, the reduced metal concentrations could be explained by malnutrition, as lower food intake, including contaminated food sources, could have contributed to the diminished metal exposure.

These findings underscore the necessity of more extensive research on heavy metal concentrations in organ tissues, as this remains the most reliable method for assessing long-term toxic metal exposure in vultures. Furthermore, the Griffon vulture is a relatively sedentary species that does not migrate, making its exposure to these compounds more indicative of the local environmental conditions.

Griffon vultures preferentially nest in cliff habitats and rely on open grasslands for foraging, which positions mountainous regions—specifically inland cliffs and peaks—as optimal environments for their survival (IUCN, 2016). As a result, the main resident population of this species is predominantly found in these interior regions, one of which overlaps with our sampling area. The current results may be underrepresented, as many animals that die in the field, particularly those in mountainous habitats like vultures, are not recovered.

5.2.1. Lead

To evaluate our analytical results, we used threshold values from the literature. We followed Franson and Pain (2011) methodology, considering lead concentrations below 2 mg/kg w.w. in liver and kidney as indicative of background contamination. Concentrations above 6 mg/kg w.w. in liver and 4 mg/kg w.w. in kidney were considered indicative of clinical poisoning. Subclinical exposure was indicated by intermediate concentrations. Mortality risk was evaluated using lethal thresholds: liver >18 mg/kg d.w. and kidney >25 mg/kg d.w.

Based on these thresholds, nearly all values obtained in this study are indicative of background contamination, with only one recorded value (2.45 mg/kg w.w.) exceeding the safe range, falling within the subclinical contamination range. However, every sample contained detectable amounts of the compound.

The elevated lead levels in liver and kidney samples are likely due to the extensive environmental contamination caused by lead ammunition (Monclús et al., 2020). Lead is more prevalent than other heavy metals, largely because hunting ammunition and fishing sinkers contribute to pollution in both terrestrial and aquatic ecosystems (Scheuhammer et al., 1996). Additionally, another hypothesis is that Pb may be more bioavailable than other heavy metals, facilitating its absorption through the diet. The foraging behaviour of raptors frequently leads them to areas with higher lead exposure, such as fields associated with hunting activities. As a result, these birds are more likely to encounter lead-laden carcasses, while exposure to other heavy metals may be more localised or dependent on industrial pollution. This underscores the urgency of addressing lead contamination in these environments.

A study by Bassi *et al.* (2021) analysed lead exposure in wild-caught populations of three different European vulture species. Their findings revealed that long and small bones exhibited the highest median lead concentrations (5.56 and 6.8 mg/kg w.w., respectively), the brain had the lowest (0.12 mg/kg w.w.), while the liver and kidney showed intermediate levels (0.47 and 0.284 mg/kg w.w., respectively) and that 44% of individuals had lead concentrations exceeding background thresholds in at least one tissue. However, the study did not assess exposure to other compounds in these birds. As highlighted by Bassi *et al.* (2021), once absorbed, lead swiftly enters the bloodstream, where elevated levels can persist for several weeks. In the subsequent weeks and months, lead primarily accumulates in soft tissues, particularly in the kidneys and liver, which play a vital role in the elimination of foreign substances (De Francisco *et al.*, 2003; Franson and Pain, 2011; Krone, 2018). Large scavengers are frequently subjected to repeated exposure to contaminants, which can lead to a gradual buildup of lead in their bodies (Bassi *et al.,* 2021). The levels of lead in the liver and kidneys vary depending on the degree of absorption at the time the samples are collected (Franson & Pain, 2011; Krone, 2018).

Monclús *et al.* (2020) reviewed lead concentrations across species and found that Griffon vultures had the highest mean levels in both liver and kidney tissues, with a significant correlation showing higher concentrations in the kidney. Our results are consistent with these findings, also showing higher lead levels in the kidney compared to the liver.

According to Carneiro *et al.* (2014), Griffon vultures admitted to WRC tend to have lower blood Pb concentrations than wild-caught individuals, explained by the rate of food intake, once a low ingestion of food can subsequently reduce recent exposure to Pb. On the other hand, the Griffon vultures that were caught and exhibited generally good body condition may have ingested more contaminated food, since less Pb-uncontaminated food has been available following the EU sanitary legislation restricting the disposal of livestock carcasses and leading to an increased consumption of Pb-contaminated food sources such as rubbish dumps and wild mammal carcasses. As ingestion is the main pathway of Pb exposure in raptors, these WRC vultures could not be representative of free-ranging populations.

Several factors may have influenced the results obtained in this study, namely the age and body condition. Given that most of the sampled birds were juveniles, bioaccumulation of lead may not have fully occurred. Furthermore, most animals admitted at the WRC were in a poor body condition which could affect the food intake (including contaminated food intake).

The elevated lead levels observed in Egyptian vultures may be attributed to their migratory behaviour to Africa during the wintering period and their tendency to feed on bones more frequently than Griffon vultures. Being smaller and typically the last to feed on carcasses, Egyptian vultures encounter less competition and consume more bones, which are known to bioaccumulate higher amounts of lead. This dietary habit could explain the increased lead concentrations found in this sample.

5.2.2. Cadmium

Espín *et al*. (2014) identified blood cadmium (Cd) concentrations (0.05 mg/dl) that impair the antioxidant system in Spanish Griffon vultures. On the other hand, Carneiro *et al.* (2015) analysed blood samples from both WRC sources and wild-caught vultures, reporting undetectable Cd levels in most samples (98.3% and 95%, respectively). However, both studies emphasise that blood samples alone may underestimate long-term exposure risks.

Cadmium was the sole metal exhibiting a significantly uneven distribution between the liver and kidney, with elevated concentrations observed in the kidney. This disparity can be attributed to cadmium's well-documented tendency to bioaccumulate preferentially in renal tissue, a finding corroborated by several studies (Nordberg et al., 2022; Rajamani et al., 2015). Furthermore, cadmium's accumulation is linked to its binding with metallothionein (MT), which is filtered by the kidneys and reabsorbed in proximal tubules (Nordberg et al., 2022). Given the kidneys' vital role in metal filtration and excretion through urine, they are more susceptible to metal retention, leading to higher cadmium concentrations compared to the liver.

According to Monclús *et al.* (2021), a hepatic cadmium (Cd) concentration of 3 mg/kg d.w. is considered indicative of significant environmental contamination. Numerous studies also show that Cd levels increase with age, consistently reporting higher liver Cd concentrations in adult birds compared to juveniles (e.g., Battaglia *et al.,* 2005; Carneiro *et al.,* 2014). This pattern is attributed to cadmium's long biological half-life and slow elimination from tissues, resulting in its accumulation over the animal's lifespan (Wayland & Scheuhammer, 2011).

In comparison to these studies, the cadmium levels in our samples are low, with the highest recorded value being 1.691 mg/kg d.w. While this may suggest a lower level of cadmium exposure in our sampled vultures, it is important to note that most of the animals tested were juveniles, and significant Cd accumulation may not yet have occurred. As cadmium should ideally be absent from the environment, the detection of a renal concentration of 1.691 mg/kg d.w. in a juvenile vulture remains noteworthy.

5.2.3 Arsenic

Arsenic (As) originates from both natural and human activities, including fossil fuel combustion, nonferrous metal mining and smelting, and pesticide production and use (Wang & Mulligan, 2006). According to Monclús *et al*. (2021), arsenic levels in the liver range from 2 to 10 mg/kg w.w. are considered elevated, while concentrations exceeding 10 mg/kg w.w. suggest arsenic poisoning (Eisler, 1998; Goede, 1985). Carneiro *et al.* (2016) noted that arsenic is one of the least studied toxic elements in raptors from Portugal and Spain, likely due to its low bioaccumulation and limited biomagnification in the food chain, despite being a harmful compound. In the present study, arsenic levels in the vultures sampled were found to be very low, suggesting low ecotoxicological risk.

6. CONCLUSIONS

Compared to published reference values for healthy, non-exposed Griffon Vultures in other countries, the AChE activity observed in this study is notably low. This could indicate possible subclinical or clinical exposure to cholinesterase-inhibiting pesticides. While AChE activity can be influenced by factors such as age, geographic origin, diet, stress, and illness, the potential risk posed by pesticide exposure in Griffon Vulture populations requires serious attention. Regional differences may also play a role in ChE activity, suggesting that Portuguese vultures could naturally exhibit lower values than those in other countries. However, as no studies on normal AChE levels in Portuguese Griffon Vultures currently exist, further research is essential to establish baseline values. This would aid in developing more reliable diagnostic tools, especially given the significant threat pesticides pose to these birds. Additionally, studies comparing ChE activity before and after rehabilitation would provide valuable insight into the enzyme's recovery time. Notably, this study is the first to report ChE activity in *A. monachus*, which showed a significantly lower value than all other results.

Regarding heavy metal exposure, the concentrations of Pb, Cd, and As in the liver and kidney observed in this study are lower compared to findings from other European studies. This may be attributed to low environmental exposure or the fact that most of the sampled vultures were juveniles, who may not yet have had sufficient time to bioaccumulate these metals. Despite the low obtained values, all samples tested positive for these compounds, with lead showing the highest concentrations, followed by cadmium, and lastly arsenic, which indicated no significant ecotoxicological risk.

Although these findings provide insight into a portion of the reality faced by Portuguese vulture populations, they cannot be extrapolated to these wild populations. The vultures included in this study were sampled from Wildlife Rehabilitation Centres, and thus suffered from conditions that their healthy wild counterparts do not. However, these rehabilitated animals offer a glimpse into the challenges their wild conspecifics endure, reflecting some of the threats vultures face for survival. Considering these ongoing threats, further research is crucial for the conservation of these key species. Such efforts also enhance the broader One Health framework, safeguarding ecosystem integrity and promoting human health.

7. REFERENCES

1. Abrahms, B., Aikens, E. O., Armstrong, J. B., Deacy, W. W., Kauffman, M. J., & Merkle, J. A. (2021). Emerging Perspectives on Resource Tracking and Animal Movement Ecology. In *Trends in Ecology and Evolution* (Vol. 36, Issue 4, pp. 308–320). Elsevier Ltd. https://doi.org/10.1016/j.tree.2020.10.018
2. Anglister, N., Gonen-Shalom, S., Shlanger, P., Blotnick-Rubin, E., Rosenzweig, A., Horowitz, I., Hatzofe, O., King, R., Anglister, L., & Spiegel, O. (2023). Plasma cholinesterase activity: A benchmark for rapid detection of pesticide poisoning in an avian scavenger. *Science of the Total Environment*, *877*. https://doi.org/10.1016/j.scitotenv.2023.162903
3. Battaglia, A., Ghidini, S., Campanini, G., Spaggiari, R. (2005). Heavy metal contamination in little owl (Athene noctua) and common buzzard (Buteo buteo) from northern Italy. Ecotoxicology and Environmental Safety 60, 61-66.
4. Bassi, E., Facoetti, R., Ferloni, M., Pastorino, A., Bianchi, A., Fedrizzi, G., Bertoletti, I., & Andreotti, A. (2021). Lead contamination in tissues of large avian scavengers in south-central Europe. *Science of the Total Environment*, *778*. https://doi.org/10.1016/j.scitotenv.2021.146130
5. Becker, P.B. (2003). Chapter 19 Biomonitoring with birds. pp.677–736. doi:https://doi.org/10.1016/s0927-5215(03)80149-2.
6. Berny, P., Vilagines, L., Cugnasse, J., Mastain, O., Chollet, J., Joncour, G., Razin, M. (2015) 'Vigilance poison: Illegal poisoning and lead intoxication are the main factors affecting avian scavenger survival in the pyrenees (France)', *Ecotoxicology and Environmental Safety*, 118, pp. 71–82. doi:10.1016/j.ecoenv.2015.04.003.
7. Brown, C., Gross, W.B., Ehrich, M. (1986). *Chickens, effects of social stress on the toxicity of malathion in young*. Avian Dis. 30, 679–682.
8. Carneiro, M. A., Oliveira, P. A., Brandão, R., Francisco, O. N., Velarde, R., Lavín, S., & Colaço, B. (2016). Lead Poisoning Due to Lead-Pellet Ingestion in Griffon Vultures (Gyps fulvus) from the Iberian Peninsula. *Journal of Avian Medicine and Surgery*, *30*(3), 274–279. https://doi.org/10.1647/2014-051
9. Carneiro, M., Colaço, B., Brandão, R., Azorín, B., Nicolas, O., Colaço, J., Pires, M. J., Agustí, S., Casas-Díaz, E., Lavin, S., & Oliveira, P. A. (2015). Assessment of the exposure to heavy metals in Griffon vultures (Gyps fulvus) from the Iberian Peninsula. *Ecotoxicology and Environmental Safety*, *113*, 295–301. https://doi.org/10.1016/j.ecoenv.2014.12.016

10. Carneiro, M., Colaço, B., Brandão, R., Ferreira, C., Santos, N., Soeiro, V., Colaço, A., Pires, M. J., Oliveira, P. A., & Lavín, S. (2014). Biomonitoring of heavy metals (Cd, Hg, and Pb) and metalloid (As) with the Portuguese common buzzard (Buteo buteo). *Environmental Monitoring and Assessment*, *186*(11), 7011–7021. https://doi.org/10.1007/s10661-014-3906-3
11. Cox, C., 1991. Pesticides and birds : from DDT to Today's poisons. J. Pestic. Reform 11, 2–6.
12. De Francisco, N., Ruiz Troya, J.D. and Agüera, E.I. (2003). Lead and lead toxicity in domestic and free living birds. *Avian Pathology*, 32(1), pp.3–13. doi: https://doi.org/10.1080/0307945021000070660.
13. Eisler, R. (1988). Lead hazards to fish, wildlife, and invertebrates: a synoptic review. Contaminant Hazard Reviews. U.S. Fish and Wildlife Service Biological Report.
14. Espín, S., Martínez-López, E., Jiménez, P., María-Mojica, P., & García-Fernández, A. J. (2014). Effects of heavy metals on biomarkers for oxidative stress in Griffon vulture (Gyps fulvus). *Environmental Research*, *129*, 59–68. https://doi.org/10.1016/j.envres.2013.11.008
15. Eyer, P., Worek, F., Kiderlen, D., Sinko, G., Stuglin, A., Simeon-Rudolf, V., & Reiner, E. (2003). *Molar absorption coefficients for the reduced Ellman reagent: reassessment q*. www.elsevier.com/locate/yabio
16. Fisher, I.J., Pain, D.J. and Thomas, V.G. (2006). A review of lead poisoning from ammunition sources in terrestrial birds. *Biological Conservation*, [online] 131(3), pp.421–432. doi:https://doi.org/10.1016/j.biocon.2006.02.018.
17. Franson, C., & Pain, D., 2011. Lead in birds. In: *The Toxicology of Birds*. CRC Press, pp. 279-300. Available at: https://www.taylorfrancis.com/chapters/oa-edit/10.1201/b10598-17/lead-birds-christian-franson-deborah-pain [Accessed 19 September 2024].
18. Fryday, Steven L., Hart, A.D.M., Langton, S.D., 1996. Effects of exposure to an organophosphorus pesticide on the behavior and use of cover by captive starlings. Environ. Toxicol. Chem. 15, 1590–1596.
19. Furness RW. (1993). Birds as monitors of pollutants. In: Furness RW, Greenwood JJD (eds). Birds as monitors of environmental change. Chapman and Hall, London, pp 86-143.
20. Galindo, J.C., Kendall, R.J., Driver, C.J., Lacher, T.E., 1985. The effect ofmethyl parathion on susceptibility of bobwhite quail (Colinus virginianus) to domestic cat predation. Behav. Neural Biol. 43, 21–36.
21. Gangoso, L., Mateo, R., Santamaría-Cervantes, C., García-Alfonso, M., Gimeno-Castellano, C., Arrondo, E., Serrano, D., van Overveld, T., de la Riva, M., Cabrera, M. A., & Donázar, J. A. (2024).

Blood lead levels in an endangered vulture decline following changes in hunting activity. *Environmental Research*, *252*. https://doi.org/10.1016/j.envres.2024.118712

22. Ganz, K., Jenni, L., Madry, M. M., Kraemer, T., Jenny, H. and Jenny, D. (2018) Acute and chronic lead exposure in four avian scavenger species in Switzerland. Arch. Environ. Contam. Toxicol. 75: 566–575.
23. Garcia-Fernandez, A.J., Martinez-Lopez, E., Romero, D., Maria-Mojica, P., Godino, A., Jimenez, P. (2005) 'High levels of blood lead in Griffon Vultures (Gyps fulvus) from Cazorla Natural Park (southern Spain)', *Environmental Toxicology*, 20(4), pp. 459–463. doi:10.1002/tox.20132.
24. Garcês, A., Pires, I., Sargo, R., Sousa, L., Prada, J., & Silva, F. (2023). Admission Causes, Morbidity, and Outcomes in Scavenger Birds in the North of Portugal (2005–2022). *Animals*, *13*(13). https://doi.org/10.3390/ani13132093
25. Goede, A.A. (1985) 'Mercury, selenium, arsenic and zinc in waders from the Dutch Wadden Sea', *Ecotoxicology and Environmental Safety*, 9(3), pp. 331-341. Available at: https://doi.org/10.1016/0143-1471(85)90119-9.
26. Golden NH, Rattner BA. (2003). Ranking terrestrial vertebrate species for utility in biomonitoring and vulnerability to environmental contaminants. Rev Environ Contam Toxicol. 176, 67-136.
27. Gómez-Ramírez, P., Shore, R. F., van den Brink, N. W., van Hattum, B., Bustnes, J. O., Duke, G., Fritsch, C., García-Fernández, A. J., Helander, B. O., Jaspers, V., Krone, O., Martínez-López, E., Mateo, R., Movalli, P., & Sonne, C. (2014). An overview of existing raptor contaminant monitoring activities in Europe. *Environment International*, *67*, 12–21. https://doi.org/10.1016/j.envint.2014.02.004
28. Green, R. E., Donázar, J. A., Sánchez-Zapata, J. A., & Margalida, A. (2016). Potential threat to Eurasian griffon vultures in Spain from veterinary use of the drug diclofenac. *Journal of Applied Ecology*, *53*(4), 993–1003. https://doi.org/10.1111/1365-2664.12663
29. Grilo, A., Moreira, A., Carrapiço, B., Belas, A., & Braz, B. S. (2021). Epidemiological study of pesticide poisoning in domestic animals and wildlife in Portugal: 2014-2020. *Frontiers in Veterinary Science*, *7*. https://doi.org/10.3389/fvets.2020.616293
30. Grue, C.E., Gibert, P.L., Seeley, M.E., 1997. Neurophysiological and behavioral changes in non-target wildlife exposed to organophosphate and carbamate pesticides: Thermoregulation, food consumption, and reproduction. Am. Zool. 37, 369–388.
31. IUCN. (2016) Aegypius monachus (Cinereous Vulture). Available at: https://www.iucnredlist.org/fr/species/22695231/154915043 (Acessed: 10 august 2024

32. IUCN. (2016) Gyps fulvus (Griffon Vulture) . Available at: https://www.iucnredlist.org/fr/species/22695219/157719127 (Acessed: 10 august 2024)
33. IUCN. (2016) Neophron percnopterus (Egyptian Vulture). Available at: https://www.iucnredlist.org/fr/species/22695180/205187871 (Acessed: 10 august 2024)
34. Ives, A. M., Brenn-White, M., Buckley, J. Y., Kendall, C. J., Wilton, S., & Deem, S. L. (2022). A Global Review of Causes of Morbidity and Mortality in Free-Living Vultures. In *EcoHealth* (Vol. 19, Issue 1, pp. 40–54). Springer. https://doi.org/10.1007/s10393-021-01573-5
35. Jalihal, S., Rana, S., & Sharma, S. (2022). Systematic mapping on the importance of vultures in the Indian public health discourse. *Environmental Sustainability*, *5*(2), 135–143. https://doi.org/10.1007/s42398-022-00224-x
36. Jenni, L., Madry, M.M., Kraemer, T., Kupper, J., Naegeli, H., Jenny, H. and Jenny, D. (2015). The frequency distribution of lead concentration in feathers, blood, bone, kidney and liver of golden eagles Aquila chrysaetos: insights into the modes of uptake. *Journal of ornithology*, 156(4), pp.1095–1103. doi:https://doi.org/10.1007/s10336-015-1220-7.
37. Kapka-Skrzypczak, L., Cyranka, M., Skrzypczak, M., & Kruszewski, M. (2011). Biomonitoring and biomarkers of organophosphate pesticides exposure-state of the art. In *Annals of Agricultural and Environmental Medicine* (Vol. 18, Issue 2). www.aaem.pl
38. Krone, O. (2018). Lead Poisoning in Birds of Prey. *Birds of Prey*, pp.251–272. doi:https://doi.org/10.1007/978-3-319-73745-4_11.
39. Krüger, S. C., Botha, A., Bowerman, W., Coverdale, B., Gore, M. L., van den Heever, L., Shaffer, L. J., Smit-Robinson, H., Thompson, L. J., & Ottinger, M. A. (2022). Old World Vultures Reflect Effects of Environmental Pollutants Through Human Encroachment. In *Environmental Toxicology and Chemistry* (Vol. 41, Issue 7, pp. 1586–1603). John Wiley and Sons Inc. https://doi.org/10.1002/etc.5358
40. López-Alonso, M., Miranda, M., García-Partida, P., Cantero, F., Hernández, J., Benedito, J.L., 2007. Use of dogs as indicators of metal exposure in rural and urban habitats in NW Spain. Sci. Total Environ. 372 (2–3), 668–675.
41. Margalida, A., & Colomer, M. À. (2012). Modelling the effects of sanitary policies on European vulture conservation. *Scientific Reports*, *2*. https://doi.org/10.1038/srep00753
42. Markert, B. and Wünschmann, S. (1970) *Bioindicators and Biomonitors: Use of organisms to observe the influence of chemicals on the environment*, *SpringerLink*. Available at: https://link.springer.com/chapter/10.1007/978-90-481-9852-8_10 (Accessed: 24 September 2024).

43. Mateo, R. and Kanstrup, N. (2019). Regulations on lead ammunition adopted in Europe and evidence of compliance. *Ambio*, 48(9), pp.989–998. doi:https://doi.org/10.1007/s13280-019-01170-5.
44. Monclús, L., Shore, R. F., & Krone, O. (2020). Lead contamination in raptors in Europe: A systematic review and meta-analysis. In *Science of the Total Environment* (Vol. 748). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2020.141437
45. Nordberg, M. and Nordberg, G.F. (2022). Metallothionein and Cadmium Toxicology—Historical Review and Commentary. *Biomolecules*, 12(3), p.360. doi:https://doi.org/10.3390/biom12030360.
46. Ogada, D. L., Keesing, F., & Virani, M. Z. (2012). Dropping dead: Causes and consequences of vulture population declines worldwide. *Annals of the New York Academy of Sciences*, *1249*(1), 57–71. https://doi.org/10.1111/j.1749-6632.2011.06293.x
47. Oropesa, A. L., Sánchez, S., & Soler, F. (2017). Characterization of plasma cholinesterase activity in the Eurasian Griffon Vulture Gyps fulvus and its in vitro inhibition by carbamate pesticides. *Ibis*, *159*(3), 510–518. https://doi.org/10.1111/ibi.12476
48. Ozaki, S., Movalli, P., Cincinelli, A., Alygizakis, N., Badry, A., Chaplow, J. S., Claßen, D., Dekker, R. W. R. J., Dodd, B., Duke, G., Koschorreck, J., Pereira, M. G., Potter, E., Slobodnik, J., Thacker, S., Thomaidis, N. S., Treu, G., & Walker, L. (2023). The importance of in-year seasonal fluctuations for biomonitoring of apex predators: A case study of 14 essential and non-essential elements in the liver of the common buzzard (Buteo buteo) in the United Kingdom. *Environmental Pollution*, *323*. https://doi.org/10.1016/j.envpol.2023.121308
49. Pirastru, M., Mereu, P., Manca, L., Bebbere, D., Naitana, S., & Leoni, G. G. (2021). Anthropogenic drivers leading to population decline and genetic preservation of the eurasian griffon vulture (Gyps fulvus). In *Life* (Vol. 11, Issue 10). MDPI. https://doi.org/10.3390/life11101038
50. Santos, C.S.A., Monteiro, M.S., Soares, A.M.V.M. and Loureiro, S. (2012). Characterization of Cholinesterases in Plasma of Three Portuguese Native Bird Species: Application to Biomonitoring. *PLoS ONE*, 7(3), p.e33975. https://doi.org/10.1371/journal.pone.0033975.
51. Scheuhammer, A.M. and Norris, S.L. (1996). The ecotoxicology of lead shot and lead fishing weights. Ecotoxicology, 5(5), pp.279–295. doi:https://doi.org/10.1007/bf00119051.
52. Sentinelas (2019) *Projeto - Sentinelas*. Available at: https://www.sentinelas.pt/pt/projeto/. (Acessed: 21 august 2024).

53. Serlin, Y., Shelef, I., Knyazer, B., Friedman, A. (2015). Anatomy and physiology of the blood–brain barrier. Semin. Cell Dev. Biol. 38, 2–6.
54. Smits JE, Fernie KJ. (2013). Avian wildlife as sentinels of ecosystem health. CompImmunol Microbiol Infect Dis. 36(3), 333-342.
55. Taggart, M.A., Shore, R.F., Pain, D.J., Peniche, G., Martinez-Haro, M., Mateo, R., Homann, J., Raab, A., Feldmann, J., Lawlor, A.J., Potter, E.D., Walker, L.A., Braidwood, D.W., French, A.S., Parry-Jones, J., Swift, J.A. and Green, R.E. (2020). Concentration and origin of lead (Pb) in liver and bone of Eurasian buzzards (Buteo buteo) in the United Kingdom. *Environmental Pollution*, 267, p.115629. doi:https://doi.org/10.1016/j.envpol.2020.115629.
56. Tafuri, J., Roberts, J., 1987. Organophosphate poisoning. Ann. Emerg. Med. 16, 193–202. https://doi.org/10.1016/s0196-0644(87)80015-x
57. Thompson, H.M., Walker, C.H. and Hardy, A.R. (1991) 'Changes in activity of avian serum esterases following exposure to organophosphorus insecticides', *Archives of Environmental Contamination and Toxicology*, 20(4), pp. 514–518. doi:10.1007/bf01065841.
58. Tryland, M., 2006. "Normal" serum chemistry values in wild animals. Vet. Rec. 158, 211–212.
59. Rajamani, J. and Subramanian, M. (2015). Toxicity Assessment on the Levels of Select Metals in the Critically Endangered Indian White-backed Vulture, Gyps bengalensis, in India. *Bulletin of Environmental Contamination and Toxicology*, 94(6), pp.722–726. doi:https://doi.org/10.1007/s00128-015-1548-y.
60. Roy, C., Rard Grolleau, G., Chamoulaud, S., & Riviè, J.-L. (2005). PLASMA B-ESTERASE ACTIVITIES IN EUROPEAN RAPTORS. In *Journal of Wildlife Diseases* (Vol. 41, Issue 1). http://meridian.allenpress.com/jwd/article-pdf/41/1/184/2235134/0090-3558-41_1_184.pdf
61. Wang, S., and Mulligan, C.N. (2006) "Occurrence of Arsenic Contamination in Canada: Sources, Behavior and Distribution," Sci. Total Environ., 366, pp 701-721.
62. Wayland, M. and Scheuhammer, A., 2011. Cadmium in birds. In *Environmental Contaminants in Biota: Interpreting Tissue Concentrations*, 2nd ed. Boca Raton, FL: CRC Press, pp.645-666. https://doi.org/10.1201/b10598-21.
63. Zwarg, T., Prioste, F., Vanstreels, R.E.T., Dos Santos, R.J. and Matushima, E.R., 2012. Normal plasma cholinesterase activity of neotropical Falconiformes and Strigiformes. *Journal of Raptor Research*, 46(2), pp.180-187. https://doi.org/10.3356/JRR-11-50.1.

[APPENDICES]

Table 1. Data related to the sample collection and vultures admitted at CRAS HVUTAD

Species	**District**	**Cause of Admission**	**Age**	**Outcome**
Gyps fulvus	Viseu	Apprehension	Juvenile	Released
Gyps fulvus	Bragança	Transfer	Undetermined	In recovery
Gyps fulvus	Bragança	Undetermined	Juvenile	Released
Gyps fulvus	Gerês	Undetermined	Juvenile	Released
Gyps fulvus	Bragança	Collision	Undetermined	Released
Gyps fulvus	Bragança	Apprehension	Undetermined	Released
A. Monachus	Gerês	Shooting	Juvenile	Released
Gyps fulvus	Undetermined	Transfer	Undetermined	In recovery
Gyps fulvus	Undetermined	Transfer	Undetermined	In recovery
Gyps fulvus	Undetermined	Transfer	Undetermined	Euthanasia
Gyps fulvus	Bragança	Undetermined	Undetermined	Euthanasia
Gyps fulvus	Undetermined	Transfer	Undetermined	In recovery

Table 2. Data related to the sample collection and vultures admitted at CERVAS.

Species	Year	District	Cause of Admission	Age	Outcome	Samples
Gyps fulvus	2009	Bragança	Weakness	Juvenile	Died in 2 days	Kidney
Gyps fulvus	2010	Guarda	Weakness	Juvenile	Died in 2 days	Kidney
Gyps fulvus	2010	Guarda	Weakness	Juvenile	Died	Liver
Gyps fulvus	2010	Guarda	Electrocussion	Juvenile	Dead at admission	Kidney
Gyps fulvus	2010	Guarda	Electrocussion	Juvenile	Dead at admission	Liver
Gyps fulvus	2010	Guarda	Poisoning	Juvenile	Dead at admission	Liver + Kidney
Gyps fulvus	2010	Guarda	Intoxication	Juvenile	Dead at admission	Liver
Gyps fulvus	2011	Castelo Branco	Weakness	Juvenile	Euthanasia	Liver + Kidney
Gyps fulvus	2011	Guarda	Electrocussion	Juvenile	Dead at admission	Kidney
Gyps fulvus	2011	Guarda	Electrocussion	Juvenile	Dead at admission	Liver
Gyps fulvus	2011	Castelo Branco	Electrocussion	Juvenile	Died in a month	Liver + Kidney
Gyps fulvus	2012	Guarda	Trauma	Juvenile	Died in a month	Liver
Gyps fulvus	2012	Guarda	Weakness	Juvenile	Died in 2 days	Liver + Kidney

Gyps fulvus	2014	Guarda	Electrocussion	Juvenile	Euthanasia	Liver + Kidney
Gyps fulvus	2014	Guarda	Weakness	Juvenile	Died in 2 days	Liver + Kidney
Gyps fulvus	2014	Castelo Branco	Poisoning	Juvenile	Dead at admission	Liver + Kidney
Gyps fulvus	2014	Coimbra	Electrocussion	Juvenile	Died in 2 days	Liver + Kidney
Gyps fulvus	2015	Guarda	Drowning	Juvenile	Died in 2 days	Liver + Kidney
Gyps fulvus	2016	Guarda	Collision with Car	Adult	Dead at admission	Liver + Kidney
Gyps fulvus	2016	Guarda	Trauma	Juvenile	Euthanasia	Liver + Kidney
Neophron percnopterus	2017	Guarda	Predation	Adult	Dead at admission	Kidney
Gyps fulvus	2017	Coimbra	Weakness	Juvenile	Dead at admission	Liver + Kidney
Gyps fulvus	2018	Guarda	Weakness	Juvenile	Dead at admission	Liver + Kidney
Gyps fulvus	2019	Guarda	Electrocussion	Juvenile	Died in a month	Liver + Kidney
Gyps fulvus	2022	Guarda	Weakness	Juvenile	Dead at admission	Liver + Kidney
Gyps fulvus	Undetermined	Undetermined	Undetermined	Undetermined	Dead at admission	Liver