Rúben Miguel Rodrigues Mina

Bioaccumulation of metals in bats: is there a potential risk?

Tese de Mestrado em Ecologia, orientada pelo Professor José Paulo Sousa e pela Doutora Joana Silva Alves (Universidade de Coimbra) e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Resumo

Durante as últimas décadas, tem sido observado em quase todo o mundo o declínio das populações de morcegos. A exposição a metais pesados pode ser um fator importante, estando a contribuir para esses declínios. Assim sendo, a bioacumulação de metais pode representar um potencial risco para as populações de morcegos. Dado o estatuto de conservação de muitas espécies de morcegos, e a necessidade de estudos efetuados a larga escala, existe uma necessidade crescente de desenvolver ferramentas não invasivas para avaliar se a acumulação de metais é, de facto, um dos fatores associados ao declínio das populações de morcegos. O objetivo deste estudo foi validar o uso de amostras não-letais para determinar a bioacumulação de metais em morcegos. Para isso, foi medida a concentração de 10 metais essenciais e não essenciais (As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se e Zn) nos órgãos internos (osso, cérebro, coração e fígado) e em tecidos externos (pelo e membrana da asa) de quatro espécies de morcegos insectívoros (*Hypsugo savii*, *Nyctalus leisleri*, *Pipistrellus pipistrellus*, *Pipistrellus pygmaeus*). Foram estabelecidas correlações entre as concentrações de metais nos tecidos externos (amostras não-letais) e as concentrações de metais nos órgãos internos (amostras letais). A significância das correlações foi avaliada individualmente para cada metal, e foram consideradas todas as combinações possíveis entre os tecidos amostrados. Em geral, para os diferentes metais analisados, o pelo e a membrana da asa foram as amostras que apresentaram maiores concentrações, enquanto que o osso foi o tecido que apresentou as menores concentrações. Poucas correlações foram encontradas entre as concentrações de metais nos tecidos externos e a concentração de metais nos órgãos internos. No entanto, todas as amostras biológicas apresentaram padrões de resposta semelhantes em termos de acumulação de metais, exceto o osso para alguns metais. Concluindo, o pelo e a membrana da asa demonstraram ser matrizes biológicas adequadas para avaliar a exposição de metais em morcegos, e podem ser úteis para prever as concentrações endógenas de metais nestas espécies.

Palavras-chave: Morcegos; metais pesados; bioacumulação; amostras não-letais; pelo; membrana da asa

Abstract

During the last decades, have been observed almost everywhere in the world the decline of bat populations. Heavy metal exposure may be one important factor contributing to these declines. Therefore, bioaccumulation of metals may be a potential risk to bat populations. Given the conservation status of many bat species, and the need for large-scale studies, there is an increasing need for developing non-invasive tools to assess whether metal accumulation is, indeed, one of the factors associated with the declining of the bat populations. Thus, the aim of this study was to validate the use of non-lethal samples to determine the bioaccumulation of metals in bats. For that, the concentration of 10 essential and non-essential metals (As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se and Zn) was measured in internal organs (bone, brain, heart and liver) and in external tissues (fur and wing membrane skin) of four insectivorous bat species (*Hypsugo savii, Nyctalus leisleri, Pipistrellus pipistrellus, Pipistrellus pygmaeus*). Correlations between metal concentrations in external tissues (non-lethal samples) and metal concentrations in internal organs (lethal samples) were established. The significance of the correlations was evaluated individually for each metal, and all the possible combinations between the tissues sampled were considered. In general, for the different metals analyzed, fur and wing membrane were the samples that showed the highest concentrations, while the bone was the tissue that presented the lowest concentrations. Few correlations were found between the metal concentrations in external tissues and the metal concentration in internal organs. However, all the biological samples showed similar response patterns in terms of metal accumulation, except the bone for some metals. In conclusion, fur and wing membrane demonstrated to be suitable biological matrices to evaluate metal exposure in bats, and may be useful to predict endogenous metal concentrations in these species.

Key words: Bats; heavy metals; bioaccumulation; non-lethal samples; fur; wing membrane

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Chapter I – General Introduction

Ecology and behavior of bats

Bats are mammals of the order Chiroptera that are traditionally separated in two sub-orders: Megachiroptera (commonly known as Megabats) and Microchiroptera (commonly known as Microbats).

Bats occur in all continents, except in Antarctica, and represent the second largest mammal order, comprising around 20% of all mammal species (S. P. Mickleburgh, Hutson, and Racey 2002; Zukal, Pikula, and Bandouchova 2015). Over 1200 species of bats have been described in the World (Dietz & Kiefer 2016). In Europe, according to the Agreement on the Conservation of Populations of European Bats – EUROBATS – there are 53 species of bats. In Portugal are known 27 species of bats (25 in Portugal Continental and 2 in the islands), constituting 40% of the total fauna of terrestrial mammals in the country. All these species are insectivorous, and half of them are cavernicolous (Palmeirim, JM & Rodrigues L, 1992).

The fact that bats have real wings put them apart from the other mammals, and contribute to their widespread distribution and diversity (Jones et al. 2009). Another particular feature of bats is their rich dietary diversity. Although the majority of bats are insectivores, their dietary may include insects, fruits, leaves, flowers, nectar, pollen, seeds, fish, frogs, other vertebrates and blood (Jones et al. 2009).

Bats are long-lived species (some of them can live up to 30 years) with the highest known longevity of 41 years for a Brandt´s bat (*Myotis brandtii*) found in Siberia (Seim et al. 2013; Béatrice V. Hernout et al. 2013). They consume a large amount of preys per night, between 40% to 100% of their body mass, and have high metabolic rates (Hickey et al. 2001; Jones et al. 2009; Hernout et al. 2013). Some species can fly for several kilometers to find food during their nightly journeys (Jones et al. 2009). Most of the species reproduce only once a year and have very small litter sizes, usually singletons (T J O'Shea 2009). Some of these mammals are known for using echolocation. Echolocating bats emit tonal signs produced at the larynx and analyze the returning echoes to detect, localize and characterize the reflecting targets. This technique is used to obtain food, to localize a perch, to avoid obstacles and to navigate from one place to another (Moss and Schnitzler 1995; Schnitzler, Moss, and Denzinger 2003).

To survive the winter, some species of bats migrate, others hibernate and yet

others go into torpor (where the metabolic rate is reduced, body temperature is decreased, and breathing and heart rates are slowed down) that can last from a few hours to a few months, and can save up to 99% of their daily energy requirements (O'Farrell and Bradley 1970; Willis and Brigham 2003).

Ecosystem services provided by bats

Ecosystem services are commonly referred as the set of natural processes provided by the ecosystems that directly or indirectly benefit human well-being. These natural processes can be categorized in regulating and maintaining services (e.g., carbon sequestration and climate regulation, waste decomposition and detoxication, purification of water and air, pest and disease control); provisioning services (e.g., food, raw materials, genetic resources, water, energy, fiber, and medicinal resources); and cultural services (e.g., spiritual, educational and recreational) (Kunz et al. 2011).

Bats play an important role in some processes related to regulatory services through their contributions to pollination, seed dispersion and insect suppression (Jones et al. 2009; Kunz et al. 2011; Bayat et al. 2014).

Insectivorous bats are predators on several economically important insects, including cucumber beetles, June bugs, corn earworm moths, cotton bollworm moths, tobacco budworm moths and Jerusalem crickets, which are important agricultural pests on crops like corn, cotton and potatoes (Jones et al. 2009; Kunz et al. 2011). Frugivorous bats disperse seeds across different ecosystems, often introducing novel plant species into previously disturbed landscapes and to oceanic islands, contributing to maintain the diversity of forests (Kunz et al. 2011). Nectarivorous bats by visiting flowers are active pollinators, and disperse pollen, having an important role in the maintenance of genetic diversity of flowering plants (Kunz et al. 2011).

Besides the role of the bats in the natural processes described above, these mammals may redistribute nutrients and energy through their guano to sustain terrestrial, aquatic and cave ecosystems.

In conclusion, bats are key-organisms in the maintenance of the ecosystem stability.

Bats as bioindicators

Bioindicators are living organisms such as plants, animals and microbes, which are used to assess the health of the environment, constituting an important tool for detecting changes in the environment, either positive or negative (Parmar, Rawtani, and Agrawal 2016).

Bats are excellent indicator taxa and thus have been used as ecological indicators of habitat quality (Jones et al. 2009). The main reasons why bats are excellent bioindicators are: taxonomy relatively stable; can be sampled at several levels (e.g. populations, feeding rates of individuals); wide geographic range; graded responses to habitat degradation correlated with responses of other taxa (e.g. insects); rich trophic diversity; slow reproductive rates (mean population declines can be rapid); provide relevant contribution to key ecosystem services; constitute reservoirs of a wide range of emerging infectious diseases, whose epidemiology may reflect environmental stress (Jones et al. 2009). Besides these reasons, their high mobility can be a disadvantage on the use of bats as bioindicator because the long distances usually traveled to foraging areas (several kilometers every night) often result in low geographical accuracy for detection of specific polluting sites (Zukal, Pikula, and Bandouchova 2015).

Conservation status of bats at global and local scale

Bat populations are declining all around the World, (S. P. Mickleburgh, Hutson, and Racey 2002; Jones et al. 2009; Thomas J. O'Shea et al. 2016). According to the IUCN Red List of Threatened Species, 15.8% of all the species that belong to the order Chiroptera are threatened (i.e. their conservation status is Vulnerable, Endangered or Critically Endangered). This number may be higher since the number of species whose data was considered *Data Deficient* is high (204 species). Currently, some species are already considered extinct, including the *Pteropus brunneus* from Australia, *P. pilosus* from Palau, *P. subniger* from the Mascarene Islands, *P. tokudae* from Guam, *Mystacina robusta* from New Zealand and *Nyctophilus howensis* from Lord Howe Island (Jones et al. 2009).

In Portugal, according to the Portuguese Red Book of Vertebrates, nine species are considered threatened: *Rhinolophus ferrumequinum* (Vulnerable), *Rhinolophus*

hipposideros (Vulnerable), *Rhinolophus mehelyi* (Critically Endangered), *Rhiniopholus euryale* (Critically Endangered), *Myotis myotis* (Vulnerable), *Myotis blythii* (Critically Endangered), *Myotis escalerai* (Vulnerable), *Myotis bechsteinii* (Endangered), and *Miniopterus schreibersii* (Vulnerable).

Reasons for the decline of bat populations

Bat populations are declining in response to a series of environmental stresses, many of which are induced by humans. The increase of human populations, that bring extra demands for land, food and other natural resources, resulting in the degradation or destruction of habitat, can be directly related to this decline (S. P. Mickleburgh, Hutson, and Racey 2002; Jones et al. 2009).

Global climate change

Global climate change is likely to have multiple impacts on bats, namely extreme drought and cold events, cyclones and extreme heat seasons are some factors that can affect directly or indirectly bat populations (Jones et al. 2009).

While drought, heat or cold extreme events may reduce insect food supply available for bat populations, the increased frequency of hurricanes and typhoons, have a deleterious effect, particularly on populations of bats that roost in trees of islands, due to tree losses and the increased hunting by inhabitants experiencing food shortages (Jones et al. 2009).

Bat populations may also suffer from indirect effects of global climate changes. These indirect effects may result from changes in the means of energy production occurred in response to the need for carbon emission reduction by humans. One of these changes is the increasing number of wind energy facilities, which has led to a large number of unexpectedly death bats (Baerwald et al. 2008; Jones et al. 2009; Thomas J. O'Shea et al. 2016).

Habitat loss or modification

One of the most important, but universally threatened habitat for bats, is forest or woodland, frequently used for bats roosting and feeding. Tree lines, hedgerows, canals and other linear structures are used by chiropterans during flight, and may provide vital connections between roosts and feeding areas. Thus, their loss or disruption affects bats in many ways (S. P. Mickleburgh, Hutson, and Racey 2002).

Beyond changes caused by nature, anthropogenic factors like changes in water quality, urbanization, agricultural intensification, forest disturbance and loss of roosting sites affect bat populations (Jones et al. 2009; Béatrice V. Hernout et al. 2013; Zukal, Pikula, and Bandouchova 2015).

Insectivorous bats use riparian areas for foraging, once rivers and lakes support a large number of insects (Fukui et al. 2006). However, deterioration of water quality may occur because of agrochemicals runoff and industrial pollution. High input of organic matter and toxins into water courses may lead to lake's eutrophication that can, in turn, affect the biomass and diversity of insects emerging (Jones et al. 2009).

Urbanization can also affect bats in different ways. One of the factors is light pollution, which can bring advantages or disadvantages to bats. For example, emergence may be extended and infant growth retarded by house lights in some slow-flight bat species mainly adapted to forested habitats (Boldogh, Dobrosi, and Samu 2007). In contrast, some populations adapted to foraging in open spaces may benefit from feeding on insects attracted to streetlights. The increasing number of bridges and buildings in urban areas has led to changes in geographic distributions and local population densities of some bat species (Jones et al. 2009). Bats may also be negatively affected by increases in road traffic. One study performed with the greater mouse-eared bat *Myotis myotis,* concluded that this species spends less time foraging when subjected to traffic noise in laboratory conditions, presumably because the noise masks rustling noises made by moving insects that these bats normally detect by passive listening (Schaub, Ostwald, and Siemers 2008). Although less often, bats may also be killed by collisions with motor vehicles on busy roads.

Changes in agricultural practices are occurring worldwide, and intensification is ongoing as the human population increases. "Slash and burn" agriculture, where forest

is burned to plant crops is one of the problems, since it destroys vegetation cover and may also kill individual bats that use tree crevices as roosts (S. P. Mickleburgh, Hutson, and Racey 2002). The removal of hedgerows and field margins is another problem because it contributes to eliminate valuable foraging and commuting habitats, reducing also the availability of important habitats for bat prey (Jones et al. 2009). The major problem with agriculture is the use of pesticides. Increased pesticide use not only reduces food available for insectivorous bats, but also affects them directly, when they feed on pesticide contaminated insects. Several studies have mentioned the effects of pesticides in bats, evidencing that pesticides may be lethal, sub-lethal and may provoke chronic effects such as immune suppression to bats (e.g. (T. J. O'Shea, Everette, and Ellison 2001; S. P. Mickleburgh, Hutson, and Racey 2002; Allinson et al. 2006; T J O'Shea 2009; Jones et al. 2009; Bayat et al. 2014)).

Forest or woodland management practices can also negatively affect bats. Removal of dead trees or decaying branches from living trees can reduce the availability of potential roosting sites (S. P. Mickleburgh, Hutson, and Racey 2002; Jones et al. 2009).

Underground sites such as caves and mines are crucial for the survival of many bat species worldwide. Consequently, when abandoned mines are sealed, usually for safety reasons, a dramatic impact often occurs on bats (S. P. Mickleburgh, Hutson, and Racey 2002).

Deforestation is another problem that bats face. As mentioned before, since most bats eat insects, fruit, nectar and pollen, they depend upon the forests to survive. Therefore, rapid rates of deforestation lead to the decline of bat populations.

Diseases and hunting

Bats may also be victims of several diseases. The fungal disease white-nose syndrome is one of the diseases affecting this group of mammals (Blehert et al. 2009; Jones et al. 2009; Thomas J. O'Shea et al. 2016). According to O´Shea et al. (2016), white-nose syndrome was the second largest cause of multiple mortality events on bats between 1790 and 2015.

In Indian and Pacific Ocean islands, human hunting and bat's consumption remains a major factor affecting bat populations (S. Mickleburgh, Waylen, and Racey

2009). Overhunting has resulted in the extinction of *Pteropus subniger* in Mauritius and Réunion (Jones et al. 2009).

HEAVY METALS

Definition

The term “heavy metals” refers to any metallic element that has a relatively high density, and is toxic or poisonous even at low concentrations (Duruibe, Ogwuegbu, and Egwurugwu 2007). Although there is no clear definition of what a heavy metal is, in most cases, density of the element is taken as the property of the element to be included within this chemicals group. Heavy metals are thus commonly defined as the elements that have a specific density higher than 5 g/cm³ (Järup 2003; Duruibe, Ogwuegbu, and Egwurugwu 2007; Zukal, Pikula, and Bandouchova 2015).

Heavy metals are separated in essential elements, those who play a physiological role in living organisms, such as iron (Fe), cobalt (Co), copper (Cu), manganese (Mn) and zinc (Zn), and non-essential elements, those who do not play any physiological role in living organisms, such as mercury (Hg), arsenic (As), lead (Pb), vanadium (V) and cadmium (Cd). These late can be toxic to the living organisms at low concentrations (Johri, Jacquillet, and Unwin 2010; Zukal, Pikula, and Bandouchova 2015). Essential elements can also be toxic when occurring in concentrations higher than normal.

Eleven elements are recognized as being of greatest wildlife concern: arsenic, cadmium, cobalt, chromium, copper, mercury, manganese, nickel, lead, tin and thallium (Beyersmann and Hartwig 2008). The heavier metals, such as lead, mercury, arsenic and cadmium are among the most hazardous.

Main sources and pathways

Heavy metals can be released to the environment by both natural and anthropogenic causes. These elements occur naturally in the environment, and there is always a natural background concentration in soils, rocks, sediments, water and living

organisms. Anthropogenic pollution results in higher concentrations of these metals relative to the normal background values (Callender 2003; Zukal, Pikula, and Bandouchova 2015).

The principal natural source of heavy metals in the environment derives from crustal material that is either weathered on (dissolved) and eroded from (particulate) the Earth´s surface or transferred to the Earth´s atmosphere by volcanic activity. Other natural sources are forest fires and biogenic source. Particles released by erosion appear in the atmosphere as windblown dust. In addition, some particles are released by vegetation (Callender 2013).

The major sources of heavy metals are from mining and smelting. Mining releases metals to the fluvial environment as tailings, and to the atmosphere as metal-enriched dust whereas smelting releases metals into the atmosphere as a result of high-temperature refining processes (Callender 2003). In several cases, even after mining activities have ceased, large contaminated areas remain highly polluted by heavy metals that persist in the soil and atmosphere (through dust dispersion) over extended periods of time affecting the terrestrial systems (Duruibe, Ogwuegbu, and Egwurugwu 2007).

Other important sources of metals include fossil-fuel combustion, municipal waste incineration, cement production, discharge of sewage sludges, use of commercial fertilizers and animal waste (Nriagu and Pacyna 1988).

Mammals can be contaminated with metal by drinking metal contaminated water, by eating contaminated prey, by contact with contaminated soil or through inhalation. For example, when agricultural soils are polluted, the metals are taken up by plants and consequently, accumulate metals in their tissues. Animals that graze on such contaminated plants also accumulate such metals in their tissues, and milk, if lactating. In summary, all living organisms within a given ecosystem somehow exposed to metal contaminated areas are prone to be contaminated along the food chain (Duruibe, Ogwuegbu, and Egwurugwu 2007).

Effects and biotoxicity

Heavy metals may provoke lethal and sub-lethal effects to living organisms. The biotoxic effects of heavy metals refer to the harmful effects of heavy metals on the body when consumed above the bio-recommended limits. Toxic effects (e.g. neurotoxic, carcinogenic, mutagenic or teratogenic) may be divided into acute (causes a rapid or immediate death of individuals) or chronic (the effects manifest themselves slowly) (Duruibe, Ogwuegbu, and Egwurugwu 2007).

As mentioned before, cadmium, lead and arsenic are three of the most hazardous heavy metals, and three of the most well studied in terms of effects on living animals. Thus, they will be used as an example to describe some concrete effects on animals, including humans.

Cadmium is toxic at extremely low levels. Exposure to cadmium can cause both acute and chronic tissue injury, and can damage various organs, including liver and kidney (Gaurav, Preet, and Dua 2010). Accumulation in kidneys leads to nephropathy (damage to or disease of a kidney) and proteinuria (excess of proteins in the urine) (Martelli et al. 2006; Johri, Jacquillet, and Unwin 2010). Central nervous system is also affected by cadmium exposure. Minami et al. 2001 found that cadmium released from the amygdalar neuron terminals affects the degree and balance of excitation–inhibition in synaptic neurotransmission. Inhalation of cadmium causes respiratory stress and injures the respiratory tract. Emphysema, anosmia and chronic rhinitis have been linked to high cadmium concentrations in polluted air. Cadmium has been classified as a human pulmonary carcinogen because of the large incidence of lung cancers in occupationally exposed populations (Martelli et al. 2006). This metal is also associated with bone defects, namely, osteoporosis and spontaneous fractures (Duruibe, Ogwuegbu, and Egwurugwu 2007).

Lead is known to induce a broad range of physiological, biochemical, and behavioral dysfunctions in laboratory animals and humans, including central and peripheral nervous systems, hematopoietic system, cardiovascular system, kidneys, liver and reproductive systems (Hsu and Guo 2002). Other effects include damage to the gastrointestinal tract (GIT) and urinary tract resulting in bloody urine and neurological disorder that can cause severe and permanent brain damages (Duruibe, Ogwuegbu, and

Egwurugwu 2007).

Arsenic, which is found in several different chemical forms and oxidation states, causes acute and chronic adverse health effects (Hughes 2002). Intake of large quantities leads to gastrointestinal symptoms, severe disturbances of the cardiovascular and central nervous systems, diabetes, cirrhosis, peripheral neuropathy and eventually death (Hughes 2002; Järup 2003). Arsenic exposure via drinking water is also causally related to cancer in the lungs, kidney, bladder and skin (Järup 2003).

Bioaccumulation and biomagnification

Species vary greatly in the degree to which they accumulate dietary contaminants. Bioenergetic processes play a key role in chemical uptake and elimination, and interspecific variation in bioaccumulation can be attributed in large part to variation in how species feed, digest, and allocate energy (Adrian and Gobas 2006).

Bioaccumulation is a process that occurs when a chemical element or a chemical compound accumulates at high concentrations within the organisms, wherein the concentration tends to be higher in a consumer than in its food or environment (Adrian and Gobas 2006; Bärlocher and Rennenberg 2014; Gobas and Haffner 2015). This process can occur directly, done directly from the environment, or indirectly, through feeding.

Chemical concentrations in consumers arise from a complex interplay of processes that promote (e.g., gastrointestinal magnification) and counteract (e.g., growth dilution, respiratory elimination, metabolic transformation) bioaccumulation (Adrian and Gobas 2006).

After bioaccumulation of a chemical occurs in an organism, it can lead to another process called biomagnification. This is the process where chemical concentrations in organisms increase with each step in the food chain causing concentrations of contaminants in organisms at the top of food chains to be many times greater than those in organisms at the bottom of food chains (Adrian and Gobas 2006; Bärlocher and Rennenberg 2014; Gobas and Haffner 2015).

AIMS

The main goal of this study is to evaluate if there is a potential risk of heavy metal contamination in bat species occurring in Portugal. In addition, it is intended to validate the use of non-lethal samples to determine the bioaccumulation of metals in bats. To achieve this goal, the concentration of different heavy metals will be analyzed in different types of organs/tissues of some bat species. More specifically, we expect to establish correlations between the metal concentration in external tissues and metal concentration in internal organs. Thus, we intend to determine the most suitable tissue for non-lethal samplings to evaluate metal bioaccumulation in vital organs.

THESIS FRAMEWORK

This thesis is divided into three chapters. Chapter I consists in a general introduction regarding the two main topics of this thesis, bats and heavy metals. Ecology and behavior of bats, ecosystem services provided by them, bats as bioindicators, conservation status and reasons for their decline are the topics addressed in this chapter. The definition, main sources and pathways, and effects and biotoxicity of heavy metals are also tackled. In addition, this chapter includes the definition of two important concepts, which are bioaccumulation and biomagnification.

Chapter II addresses the bioaccumulation of metals in bats and attempts to approach the main goal of this thesis. This chapter is written on the form of a scientific manuscript and can be divided in two parts. In the first part, the differences in the concentration of the metals between the species, organs and sampling locations are presented and discussed. In the second part, are presented and discussed the correlations between the metal concentration in external tissues and metal concentration in internal organs.

Chapter III includes the main conclusions from the work concerning bioaccumulation of heavy metals in bats, as well as guidelines for future research.

Chapter II – Bioaccumulation of metals in Bats: is there a potential risk?

Introduction

Bats are spread worldwide, except in Antarctica, and include around 20% of all the known mammal species (S. P. Mickleburgh, Hutson, and Racey 2002; Zukal, Pikula, and Bandouchova 2015). Given their widespread distribution, large taxonomic and functional diversity, and their important role in the provision of specific ecosystem services, bats are crucial organisms in the maintenance of ecosystem's functionality. Pollination, seed dispersal and insect suppression are key ecosystem services provided by bats (Jones et al. 2009; Kunz et al. 2011; Bayat et al. 2014). In addition, their guano contributes to the redistribution of nutrients and energy to sustain terrestrial, aquatic, and cave ecosystems (Kunz et al. 2011).

During the last decades, declines in bat populations have been observed almost everywhere in the world. Several stressors including changes in water and food quantity and quality, roost's availability, urbanization and agricultural intensification, exposure to chemicals, increase of wind turbines, the pressure of diseases such as white-nose syndrome, and climate change have contributed to these declines (Mickleburgh et al. 2002; Blehert et al. 2009; Jones et al. 2009; Pikula et al. 2010; Hernout et al. 2013; Hernout et al. 2015; Zukal et al. 2015; O'Shea et al. 2016; Hernout et al. 2016;). Due to their relatively long life and high daily food intake, bats can be particularly prone to chemical exposure, especially to contaminants such as metals that accumulate through the food chain (Hernout et al. 2016). The coexistence of bats with humans in urban, industrial and agricultural landscapes (Zukal, Pikula, and Bandouchova 2015), and the fact that some bat species feed on emerging insects that spend their larval stages in sediments where contaminants may have accumulated (Hickey et al. 2001), are other features which make bats particularly susceptible to bioaccumulate metals. Bats are usually at high trophic levels, and this can contribute to the high accumulation of metals through biomagnification (Yates et al. 2014).

Bats can be exposed to toxic elements originated from air pollution through industrial processes, mining activities, and to a series of other anthropogenic and natural sources (T J O'Shea 2009; Zukal, Pikula, and Bandouchova 2015). The pathways of contamination may include consumption of contaminated water, inhalation of polluted air, ingestion of contaminated preys and contact with contaminated soils, sediments or

rocks (Zocche et al. 2010; Béatrice V. Hernout et al. 2013).

Metals may be categorized in essential or non-essential. Non-essential metals do not play any physiological role in living organisms, and are usually toxic at low concentrations. Essential metals are vital for normal functioning of life processes, and are usually toxic at relatively high concentrations. Exposure to these chemicals can be manifested by acute effects, but sub-lethal effects, such as immune suppression, are also of concern for the long-term survival of bat populations (Bayat et al. 2014).

The effects that metals can have on bats is an issue that started to be addressed few decades ago. Clark (1979), raised the question "What effect, if any, might the observed lead concentrations have on these mammal populations?". Until date, however, few studies have investigated the effects of metals on bat populations. These few studies have reported effects of metals on bats such as hepatopathy, DNA damage, hemochromatosis, renal inclusion bodies, ascending paralysis, tremors, spasms, general slowness, lack of control in body movement and mortality (Sutton and Wilson 1983; Hariono, Ng, and Sutton 1993; Skerratt et al. 1998; Hoenerhoff and Williams 2004; Farina et al. 2005; T J O'Shea 2009; Zocche et al. 2010; Nam et al. 2012). More recently, Lovett & McBee (2016) conducted a study that shows behavioral effects on bats caused by metal contamination. This study found a possible alteration on circadian rhythms of bats, wherein bats from a contaminated site exhibited a different pattern of emergence when compared with bats from uncontaminated locations.

Effects of contaminants on bats are often difficult to understand, particularly the sub-lethal effects, due to the difficulties usually associated to population samplings, exposure monitoring and detection of relationships between exposure and effects (Bayat et al. 2014).

While several studies have been conducted to investigate the effect of organic contaminants (mainly pesticides) on bats (Jones et al. 2009; T J O'Shea 2009; Bayat et al. 2014), few studies have evaluated the effect of inorganic contaminants like metals. Recently, Zukal et al. (2015) conducted a review of the published articles on metal effects to bat species, where they considered 52 articles. The first article was published in 1970 (Zook et al. 1970) but the increase of published articles began only since the year 2000, which reflects the increasing interest of scientific community to investigate the effect of heavy metal pollution on bat populations. In the same review (Zukal, Pikula,

and Bandouchova 2015), it was reported that only sixty-five bat species (i.e. approximately 5% of all known bat diversity) have been included in heavy metal studies, and from those only two species from North American (*Eptesicus fuscus* and *Myotis grisescens*) and two from European insectivorous bat species (*Myotis myotis* and *Pipistrellus pipistrellus*) have been used in metal researches more than five times. The four sampling strategies mostly used include the use of the whole body/carcass, and the use of samples collected from the kidney, liver or guano. In fact, wildlife exposure assessments are traditionally done by analyzing internal organs, for which the animals have to be sacrificed. Given the conservation and protection status of bats in many countries, the preparation of experimental in-vivo bat models to obtain standard toxicological data is unfeasible. Thus, the use of non-lethal samples, such as hair and wing membranes, must be considered. Several advantages were listed specifically for the use of hair in monitoring studies. Firstly, hair sampling is easy and involves minimal stress to individuals (Schramm 1997). Secondly, concentrations of metals in hair are usually higher than those in biological fluids like blood and urine, and sometimes even higher than organ concentrations (e.g. Hariono et al. 1993; Halbrook et al. 1994; Liu 2003). Finally, some studies confirmed that hair may be an indicator of internal organ concentrations for a number of metals in other mammals (Hariono, Ng, and Sutton 1993; Halbrook et al. 1994; Nolet, Dijkstra, and Heidecke 1994).

Concerning bats, fur has been used in ecotoxicology studies. In 1978, Miura et al. (1978) quantified the mercury content in tissues of internal organs and in the fur of some insectivorous bats from Japan. With few exceptions (e.g. Hariono et al. 1993; Hickey et al. 2001), only recently the fur was integrated again in studies of metal contamination in bats (Yates et al. 2014; Flache, Becker, et al. 2015; Flache, Czarnecki, et al. 2015; Hernout et al. 2015; Hernout et al. 2016).

Regarding the use of the wing membrane, usually, biologists punch the wing membrane of bats to collect tissue for molecular analyses or to mark animals in the field. To our knowledge, the wing membrane was never used to quantify the concentration of heavy metals. We hypothesize that this tissue is a good sample to be used as a non-lethal sample for two main reasons. First, because of its great exposure to the external environment, which possibly leads to the direct accumulation of contaminants through skin absorption. In second, with this structure having a high regenerative capacity, bats

may possibly use it to excrete the contaminants, which leads to an intentional accumulation of metals in this tissue. The greatest difficulty may be the collection of the membrane in live animals, since the membrane sample must be as small as possible, to cause the least damage to the bats, but still large enough to provide metal concentrations sufficiently high to be measured.

Faure et al. (2009) conducted a study to evaluate which membrane, wing membrane (chiropatagium) or tail membrane (uropatagium), is most recommended (less injurious) to take samples. They concluded that regardless of the size of the wound inflicted, tail membranes healed significantly faster than wing membranes for wounds of the same size. On the other hand, they found that tail membrane wounds bled more and for longer than wing membrane wounds, which happens because the uropatagium has a higher density of blood vessels than the chiropatagium.

The major aim of this study is to validate the use of non-lethal samples to determine the bioaccumulation of metals in bats. For that, we will measure the concentration of 10 essential and non-essential metals in internal organs and in external tissues of four insectivorous bat species. We expect to find significant correlations between metal concentrations in external tissues (non-lethal samples) and metal concentrations in internal organs (lethal samples). The significance of correlations will be evaluated individually for each metal and all the possible combinations between tissues of lethal and non-lethal samplings will be considered. The information gathered in this study will allow to determine the most suitable tissue for non-lethal samplings to evaluate metal bioaccumulation in vital organs. Our working hypothesis assumes that in bats, metal concentrations in vital organs can be estimated through metal measurements in non-lethal samples, which ends with the need to sacrifice living animals. Furthermore, we also expect to understand if the metal contamination constitutes a potential risk for bat populations in Portugal, being one of the reasons for their decline.

Materials and methods

Study area and bat collection

Bat carcasses used for metal analysis were collected in North and Central Portugal (Fig.1). These carcasses were collected between 2006 and 2014 by the project team from the University of Trás-os-Montes e Alto Douro, during monitoring programs on the impact of windfarms in bat species. A total of 56 individuals of four different species (*Hypsugo savii, Nyctalus leisleri, Pipistrellus pipistrellus, Pipistrellus pygmaeus*) were collected. All the bat species studied belong to the family Vespertilionidae, are insectivorous, and some of them, use urban areas as habitat (see Table 1).

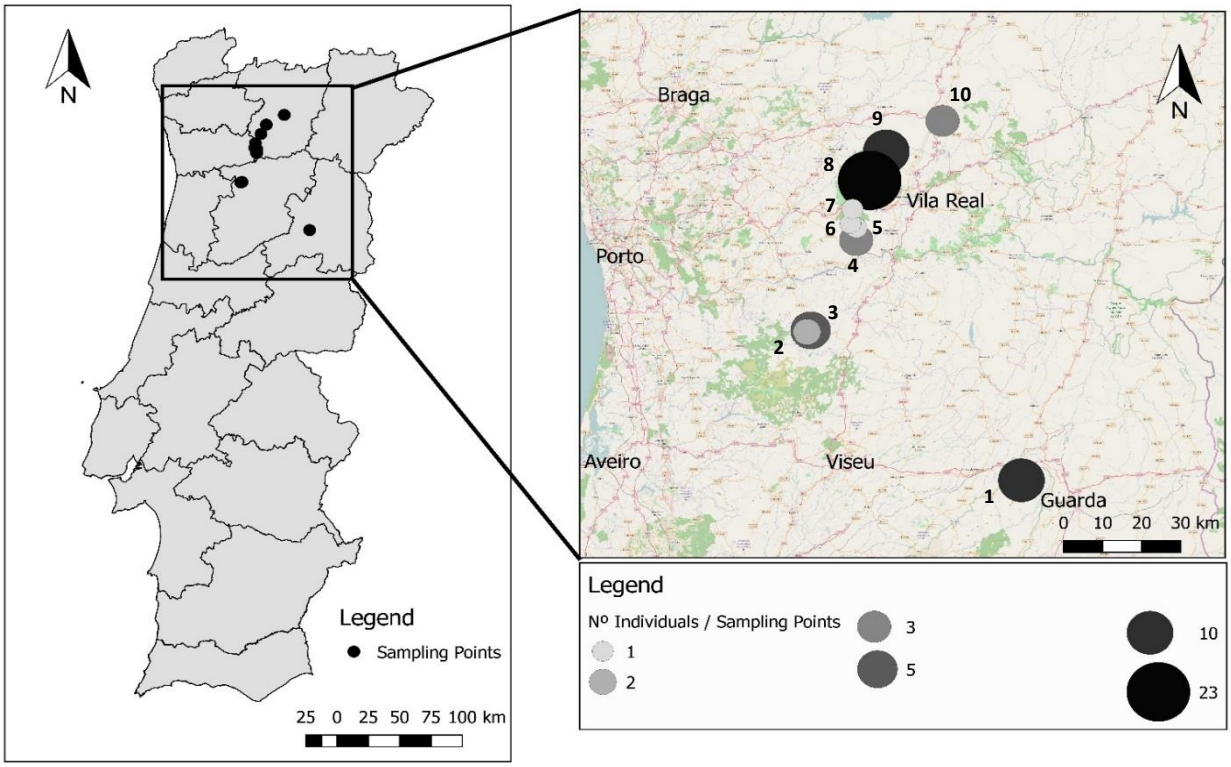

Fig. 1. Map of the study area and sampling points located in the North and Center of Portugal.
1 – Serra do Ralo; 2 – Sobrado; 3 – Lameira; 4 – Teixeiró; 5 – Seixinhos; 6 – Penedo Ruivo; 7 – Portal da Freita; 8 – Outeiro; 9 – Alto do Marco; 10 – Negrelo e Guilhado

Metal concentrations in the topsoil of the sampling sites were obtained using the maps on the distribution of heavy metals in the topsoil of Europe from the FOREGS website and using the maps produced by Lado et al. (2008) (Table 2).

Table 1 – Ecological and morphological characteristics of the species studied.

Species	Family	Conservation status[a]	Head-Body length (mm)	Biomass (g)	Roosting site	Diet	Longevity (years)	Home range (Km)	Habitat
Hypsugo savii [1]	Vespertilionidae	Data Deficient	44 - 51	7 - 10	Rock crevices; Occasionally in fissures in buildings;	Insectivorous	n.i.	n.i.	Woodlands, pasture, wetlands; Rural and urban areas
Nyctalus leisleri [2]	Vespertilionidae	Data Deficient	48 - 68	8 - 20	Mainly in Trees; Occasionally in buildings;	Insectivorous	9	18 km^2	Woodlands, pasture, river valeys
Pipistrellus pipistrellus [3]	Vespertilionidae	Least Concern	35 - 50	3 - 9	Mainly crevices in buildings and trees;	Insectivorous	5	15.26 km^2	Woodlands, farmland, rural gardens and urban areas
Pipistrellus pygmaeus [4]	Vespertilionidae	Least Concern	35 - 45	3 - 7	Trees, rock crevices and buildings;	Insectivorous	5	4.87 km^2	Woodland, wetlands and urban areas

n.i. – No information available [a] – Conservation status in Portugal; References: [1] Russo & Jones 2003; Juste & Paunović 2016a; [2] Kaňuch et al. 2005; Juste & Paunović 2016b; JNCC 2007; [3] Swift 1980; Nicholls & Racey 2006; Hutson et al. 2008; [4] Nicholls & Racey 2006; Benda et al. 2016.

Table 2 - Metal concentration (µg/g) in the topsoil of the sampling sites.

	Alto do Marco	Serra do Ralo	Outeiro	Seixinhos	Negrelo e Guilhado	Lameira	Penedo Ruivo	Portal da Freita	Teixeiró	Sobrado
As	23.96	17.43	30.10	27.81	19.31	33.83	27.81	24.39	28.28	33.83
Cd	0.27	0.20	0.33	0.31	0.23	0.33	0.31	0.30	0.36	0.33
Co	6.50	4.50	6.50	6.50	6.50	4.50	6.50	6.50	6.50	4.50
Cr	30.27	25.34	32.86	41.17	29.18	36.49	41.17	33.52	32.12	36.49
Cu	25.86	23.24	31.21	32.02	25.11	31.98	32.02	30.96	34.02	31.98
Mn	315.00	315.00	315.00	315.00	315.00	315.00	315.00	315.00	315.00	315.00
Ni	0.21	0.21	0.34	0.31	0.24	0.31	0.31	0.30	0.37	0.31
Pb	48.04	43.20	66.97	61.38	62.39	64.09	61.38	64.09	61.38	64.09
Zn	105.71	85.01	121.27	118.16	112.93	130.85	118.16	119.54	116.48	130.85

Collection of biological samples

Biological samples collected from the bat carcasses were divided into two categories: lethal-samples, that comprise the collection of liver, heart, bone and brain; and non-lethal samples, that comprise the collection of wing membrane skin and fur. Due to the advanced state of decomposition of some carcasses, some organs could not be collected in some individuals and, for that reason the number of samples collected was not the same for each organ.

For the dissection of the bats, stainless steel dissection tools were used, including, scissors, tweezers, scalpel and dissecting pins. Between each sampling, all tools were rinsed in acetone to avoid contaminations. Lethal samples were always composed by the entire organ. The bone used for this study was the forearm, which was measured during the dissection of the individuals. Small samples of fur were clipped from the mid-dorsal region of each bat, about 1-2mm above the skin, corresponding to an area of approximately 1 cm^2. Sampling of the wing membrane skin consisted in the collection of four punches of 4 mm from each wing, between the 4^{th} and the 5^{th} fingers (dactylopatagium major).

All the samples collected were placed in clean Eppendorf's, which were labeled with a unique identification. Non-lethal tissues were washed one time with detergent (Triton.X-100), two times with acetone and three times with distilled water. This process was done to eliminate external contaminations and, thus, to ensure that the concentration of metals obtained comes only from bioaccumulation.

After dissection, the samples were oven dried at 45ºC for 72h and then weighed (±0.0001g).

Metal extraction and quantification

Once dried, the biological samples were mixed with 1 mL, except for the wing membrane that was mixed with 0.5 mL, of 65% nitric acid and left under pressure in PDS-6 systems (Loftfields analytical solutions, Neu Eichenberg, Germany) at 150ºC for 10 hours. The resulting solutions were diluted with ultrapure water to a final volume of 6.5 mL (for lethal samples and fur samples) or 3.25 mL for wing membrane, to obtain a final extract within the calibration range and with an acid concentration of about 10%.

In each extract, a group of 10 elements (As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se and Zn) was measured in a ICP-MS spectrophotometer (Model iCAP Q, Thermo Fisher Scientific, Bremen, Germany). Accuracy and precision of the extraction and analytical methods were evaluated by analyzing a certified reference material (DOLT-3 - Dogfish Liver Certified Reference Material for Trace Metals certified by National Research Council Canada) and blanks. Standard solutions were prepared by appropriate dilutions of a multielement standard (92091, Periodic table mix 1 for ICP, Sigma-Aldrich). The calibration of ICP-MS measurements was ensured by using a 5-point calibration curve per each element. The detection limits obtained were 0.015, 0.002, 0.003, 0.007, 0.242, 0.045, 0.086, 0.077, 0.207 and 0.534 µg/g for As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se, and Zn, respectively. The average recoveries reached were 91.6, 87.8, 94.4, 86.7, 98.7, 153, 103 and 111% for As, Cd, Cr, Cu, Ni, Pb, Se, and Zn, respectively. Average recoveries of Co and Mn could not be calculated since we did not have the reference values for these elements. For statistical analyses, metal concentrations below the detection limit were replaced by the value of the detection limit.

All metal concentrations are expressed in µg of metal/g of tissue's dry weight.

Data analysis

Error distributions were often not normal and we transformed the concentration of metals in each organ/tissue using a log transformation to achieve an approximation of a normal distribution and to reduce heterogeneity. We tested the effect of organ, species and sampling sites (independent variables) on the different metal concentrations (dependent variables) using general linear mixed models (GLMM), where the individuals were added as a random effect. Model validation was performed by inspecting the residuals for normality, homogeneity, and independence.

The correlations between the metal concentrations in the different sampling tissues were measured using Pearson's correlations (r). This analysis was performed individually for each metal and for all the possible tissue's combinations (i.e. bone vs brain; bone vs heart; bone vs liver; bone vs wing; bone vs fur; brain vs heart; brain vs liver; brain vs wing; brain vs fur; heart vs liver; heart vs wing; heart vs fur; liver vs wing; liver vs fur; wing vs fur).

All the statistical tests were considered significant when $P < 0.05$. The statistical analysis was performed using IBM.SPSS®, version 23, and R 3.3.2 (R Development core team 2017).

Results

There were no significant differences between the concentrations obtained in the different sampling locations ($F_{(9,45)}$ = 1.241; P = 0.295). Concerning the metal concentration obtained in each sampling tissue, significant differences were found between the concentrations obtained in each species for all the metals ($P < 0.05$), except for Zinc ($F_{(3, 64)}$ = 1.501; P = 0.223). In general, *Nyctalus leisleri* presented lower concentrations in all the organs/tissues than the other species (Fig. 2). Post hoc tests with Bonferroni correction confirmed this trend, demonstrating that the most significant differences were between the *N. leisleri* and the other three bat species. Despite these differences, the pattern of concentrations in the different organs for each species is similar (Fig. 2), as evidenced by the lack of interaction between species and organ for most of the metals analyzed ($P > 0.05$). Yet, significant interactions between species and organs were found for As ($F_{(15,175)}$ = 4.062; $P < 0.001$), Cd ($F_{(15, 184)}$ = 1.883; P = 0.027), Cr ($F_{(15, 181)}$ = 1.846; P = 0.032), Cu ($F_{(15, 178)}$ = 2.904; $P < 0.001$) and Zn ($F_{(15, 178)}$ = 1.891; P = 0.027).

Significant differences were also found between organs ($F_{(5,2371)}$ = 536.125; $P < 0.001$), metals ($F_{(9,2346)}$ = 1521.095; $P < 0.001$) and the interaction between organs and metals ($F_{(45,2346)}$ = 27.519; $P < 0.001$).

Depending on the metal, the organ/tissue that showed higher concentrations varies, but, in general, for the different metals, fur and wing showed the highest concentrations, while bone was the tissue that presented the lowest concentrations (Table 3). The highest metal concentrations were recorded for the essential elements Cu (Wing), Mn (Fur) and Zn (Fur), while the lowest concentrations were recorded for the essential element Co (Bone) and the non-essential elements As (Bone) and Cd (Bone) (Table 3). Comparing the accumulation of metals in the internal organs, the highest concentrations of As, Cd, Mn, Se and Zn were obtained in the liver; while Co and Cu where highest in the heart and Cr, Ni and Pb in the brain. In all the tissues, there was a greater accumulation of the metals Cu, Mn and Zn, following the sequence Zn > Cu > Mn, except for the bone, in which the sequence was Zn > Mn > Cu (Table 3). As, Cd and Co were the metals that registered the lowest concentrations for all organs, except in the liver, in which the metals with the lowest concentrations were As, Co and Pb. It is noteworthy the concentration of Cd found in the liver and of Cr and Ni in the wing since

the accumulation of these metals was much higher than for the rest of the tissues analyzed.

Additionally, no correlations were found between the concentrations of the different metals in the different organs and the concentrations of metals in the topsoil of each sampling point of the bat carcasses ($P > 0.05$).

Table 3 - Metal concentration (µg/g dry weight) in organs/tissues of the bats analyzed from North and Centre of Portugal.

	Bone (N=54)		Brain (N=42)		Heart (N=36)		Liver (N=14)		Fur (N=51)		Wing (N=50)		F (df)	*P* value
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range		
As	0.05	(0.01-0.26)	0.2	(0.05-1.61)	0.17	(0.01-3.05)	0.3	(0.07-6.85)	0.88	(0.2-7.62)	0.44	(0.04-2.1)	116.124 (5, 175)	<0.001
Cd	0.01	(0-0.06)	0.04	(0.01-0.1)	0.16	(0.03-1.04)	0.58	(0.17-1.77)	0.06	(0.02-0.57)	0.08	(0.02-0.24)	200.991 (5, 185)	<0.001
Co	0.05	(0.01-2.66)	0.09	(0.03-8.62)	0.18	(0.03-7.78)	0.12	(0.05-1.05)	0.34	(0.07-7.82)	0.33	(0.1-3.24)	25.803 (5, 175)	<0.001
Cr	0.56	(0.12-4.1)	1.04	(0.23-4.05)	0.95	(0.07-5.63)	0.39	(0.12-0.79)	2.52	(0.82-8.67)	6.87	(1.29-43.99)	96.233 (5, 182)	<0.001
Cu	1.23	(0.07-4.33)	13.42	(7.84-28.75)	21.83	(8.7-50.49)	18.23	(11.37-33.17)	12.56	(6.86-49.63)	18.03	(2.41-54.76)	281.050 (5, 179)	<0.001
Mn	1.65	(0.63-5.81)	2.15	(1-5.25)	6.49	(2.12-15.66)	8.25	(1.34-22.1)	11.37	(3.7-110.99)	7.84	(2.49-27.09)	139.036 (5, 177)	<0.001
Ni	0.37	(0.05-38.3)	0.76	(0.11-8.68)	0.58	(0.03-179.74)	0.51	(0.16-3.45)	2.6	(0.58-44.86)	7.76	(1.44-323.29)	40.989 (5, 184)	<0.001
Pb	0.46	(0.13-14.87)	0.65	(0.15-2.2)	0.5	(0.07-2.99)	0.27	(0.15-0.98)	2.5	(1.18-57.56)	3.51	(0.82-21.87)	68.164 (5, 183)	<0.001
Se	0.23	(0.1-0.45)	1.25	(0.77-2.05)	1.45	(1-3.38)	2.72	(1.32-4.15)	3.42	(0.95-21.91)	2.85	(0.72-10.95)	289.136 (5, 183)	<0.001
Zn	67.86	(27.87-106.86)	56.5	(33.66-131.16)	59.1	(32.92-104.19)	77.14	(39.28-212.45)	239.33	(149.21-827.16)	92.82	(37.07-428.97)	125.955 (5, 179)	<0.001

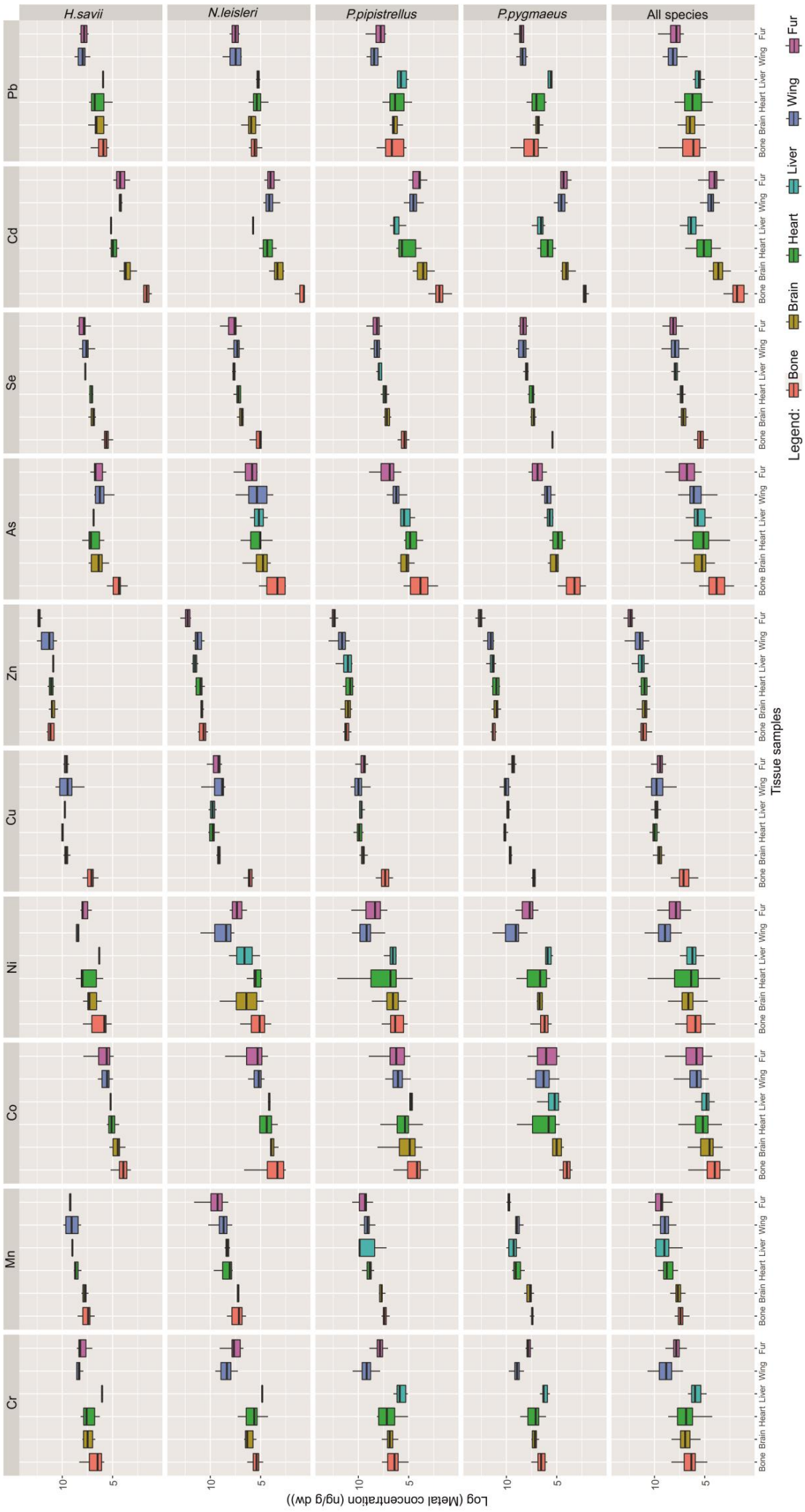

Fig. 2. Boxplots of the log-transformed metal concentrations (ng/g dw) in the different organs/tissues for the four bat species studied.

Depending on the metal analyzed, the accumulation pattern in the different organs varies, and consequently, the correlations between the organs/tissues also change depending on the metal.

Correlations between the liver and all the other tissues were found for As (liver vs bone: r=0.76; liver vs brain: r=0.87; liver vs heart: r=0.86; liver vs wing: r=0.54; and liver vs fur: r=0.61) (Fig. 3). The liver was the only organ that showed correlations with, at least, one tissue for all metals, except Ni (Fig. 3 to 12). In general, strong correlations were found between the metal concentration in the liver and the concentration in the other internal organs, varying the correlations according to the metal analyzed. Few correlations were found between the liver and the external tissues.

Several positive correlations were obtained between the metal concentration in heart and in the other internal organs, especially with the liver (As: r=0.86; Cd: r=0.93; Co: r=0.87; Cr: r=0.65; Cu: r=0.74; Mn: r=0.88; Se: r=0.60 and Zn: r=0.88) and with the brain (As: r=0.88; Cd: r=0.77; Co: r=0.79; Cr: r=0.51; Cu: r=0.74 and Se: r=0.77). Only two correlations were found between the heart and the external tissues, one with fur (Co: r=0.60) and one with wing (Se: r=0.62). Liver and heart presented consistently stronger and significant correlations for all metals analyzed.

Results of metal concentration in the brain were correlated to the results from other internal organs, being the majority of them with liver, as mentioned previously. Only one correlation was found with the fur (Co: r=0.69) and two with wing (As: r=0.56 and Se: r=0.76).

The bone was the tissue that presented the lowest correlations with the other organs/tissues. Correlations were only found for As, Cd, Co, Cr and Pb. The majority of the correlations were obtained between the bone and the heart and/or the liver. Two correlations were obtained between bone and fur (Co: r=0.58 and Pb: r=0.54), as well as, two correlations were found between bone and wing (As: r=0.57 and Cd: r=0.50).

For most metals, the concentration in the wing was not correlated with the concentration in the other organs, and for the few metals where a positive correlation was found, it was a moderate correlation (As: wing vs bone (r=0.57); wing vs brain (r=0.56); wing vs liver (0.54); Cd: wing vs bone (r=0.50); Mn: wing vs liver (r=0.59); Se: wing vs brain (r=0.76); wing vs heart (r=0.61)). A correlation between the concentrations in fur and in wing was found only for As (r=0.64), Se (r=0.63) and Zn (r=0.70). A moderate

correlation between fur and some of the internal organs was obtained for As (fur vs liver: r=0.61), Co (fur vs bone: r=0.58; fur vs brain: r=0.69; fur vs heart: r=0.60; fur vs liver: r=0.62), Cu (fur vs liver: r=0.64), and Pb (fur vs bone: r=0.54; fur vs liver: r=0.62). Cobalt was the only metal where correlations were found between the fur and all internal organs (Fig. 5).

As shown, the correlations found between the two non-lethal samples (wing and fur) and the internal organs were not consistent for the different metals, with the exception of arsenic.

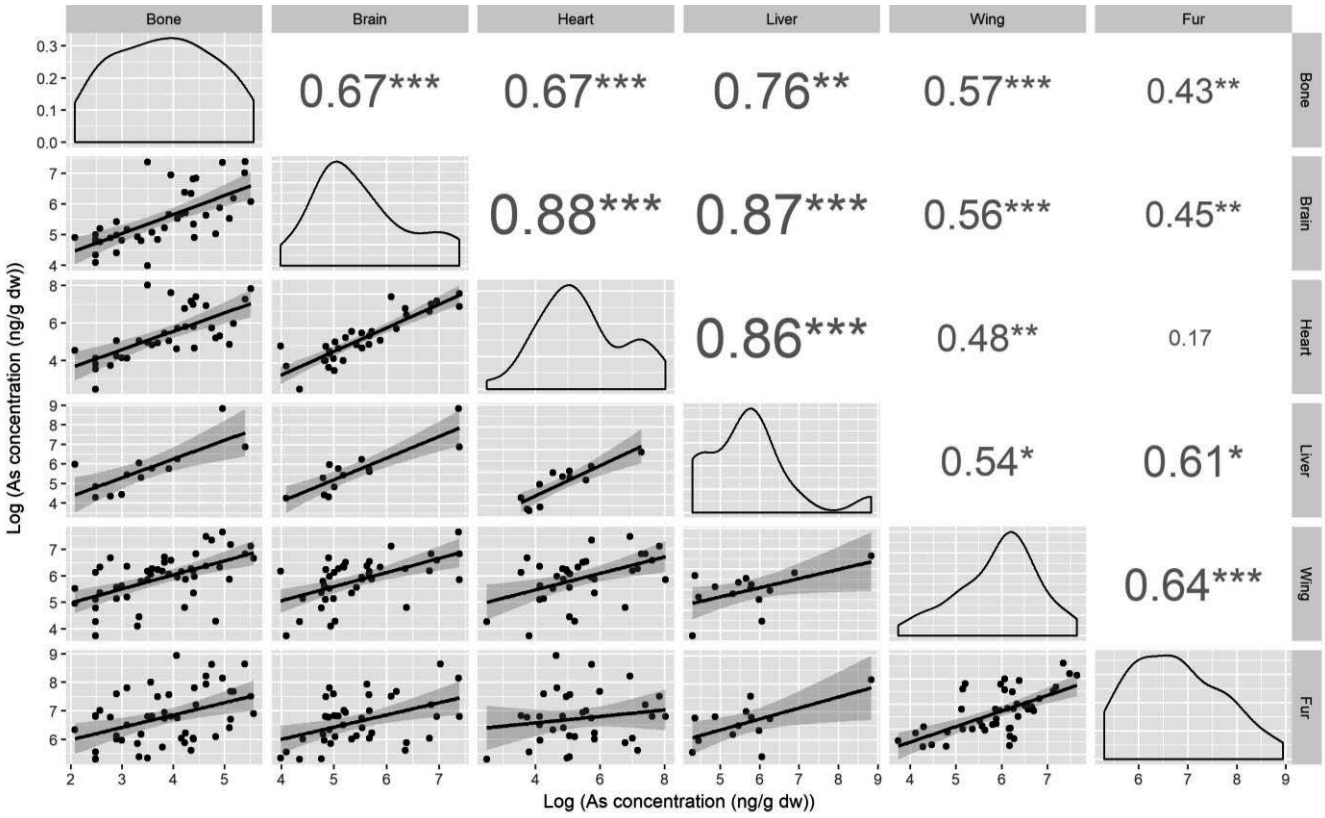

Fig. 3. Relationships between log-transformed As concentration (ng/g dw) in the different tissues analyzed. The trend line indicates the linear regression. Values presented corresponding to the Pearson correlation coefficient (r). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

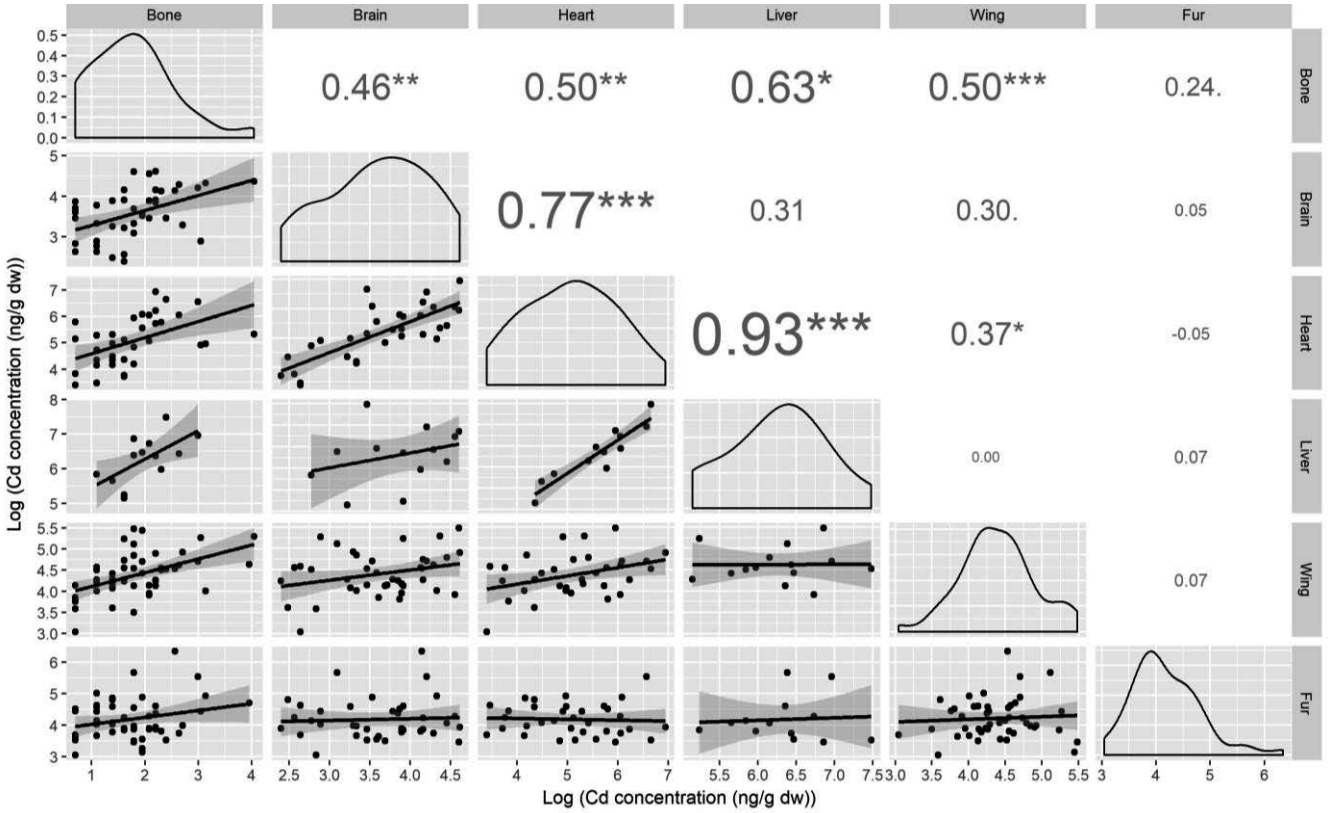

Fig. 4. Relationships between log-transformed Cd concentration (ng/g dw) in the different tissues analyzed. The trend line indicates the linear regression. Values presented corresponding to the Pearson correlation coefficient (r). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

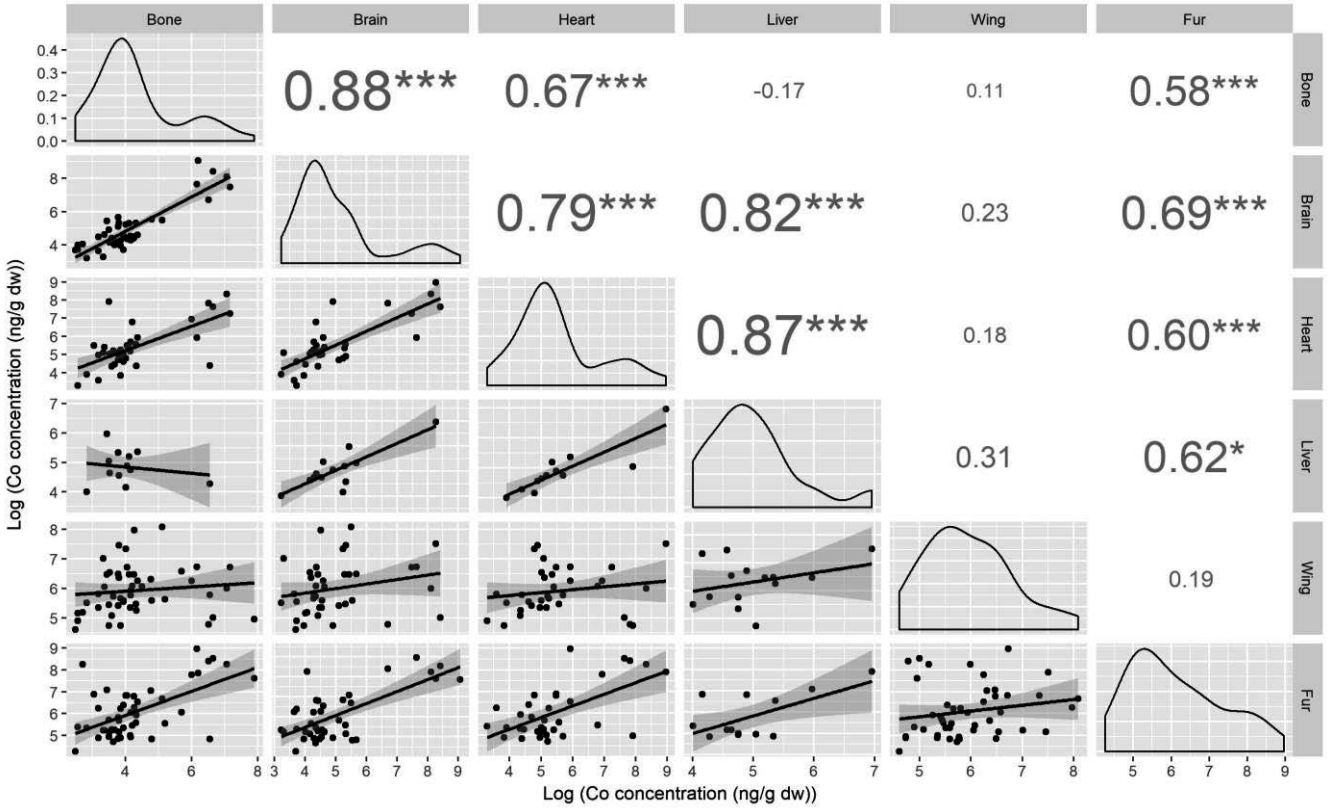

Fig. 5. Relationships between log-transformed Co concentration (ng/g dw) in the different tissues analyzed. The trend line indicates the linear regression. Values presented corresponding to the Pearson correlation coefficient (r). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

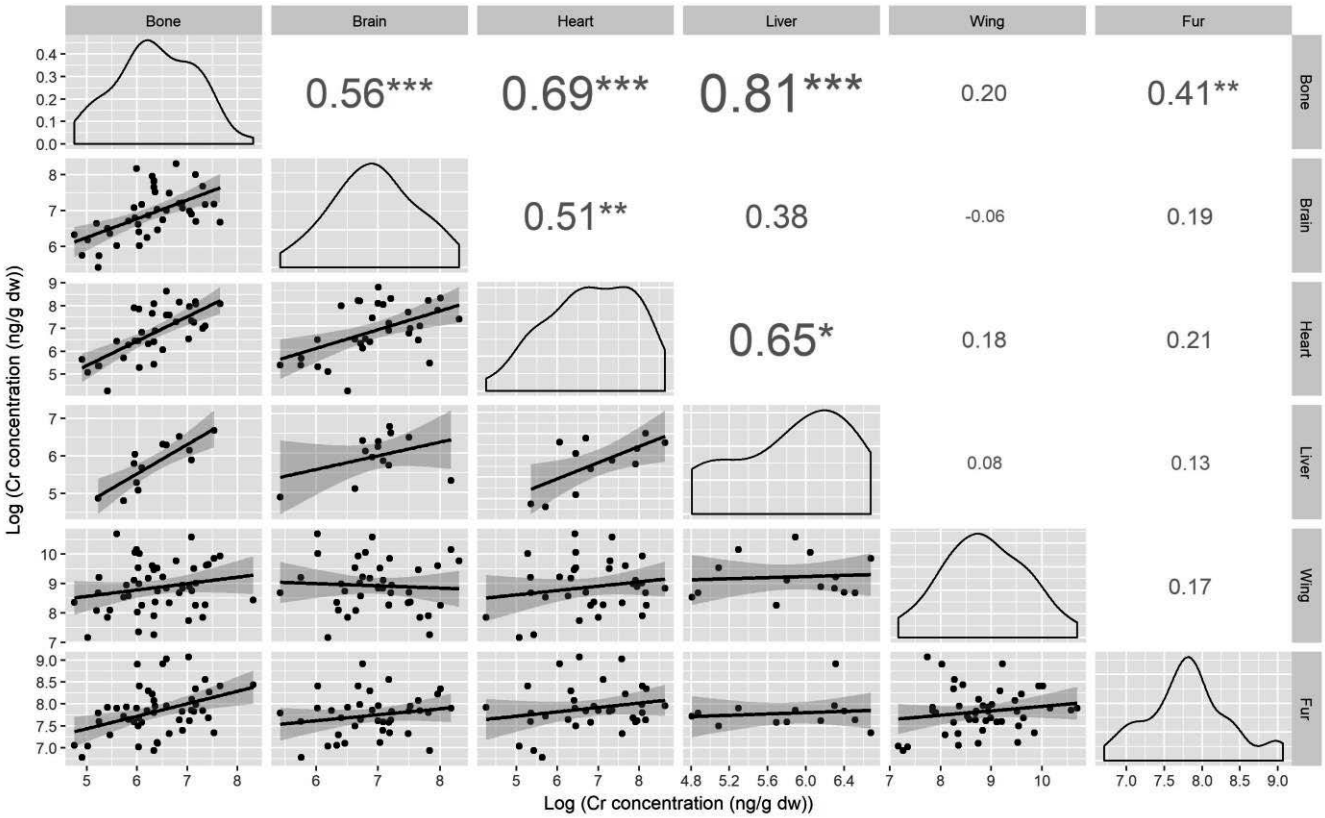

Fig. 6. Relationships between log-transformed Cr concentration (ng/g dw) in the different tissues analyzed. The trend line indicates the linear regression. Values presented corresponding to the Pearson correlation coefficient (r). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

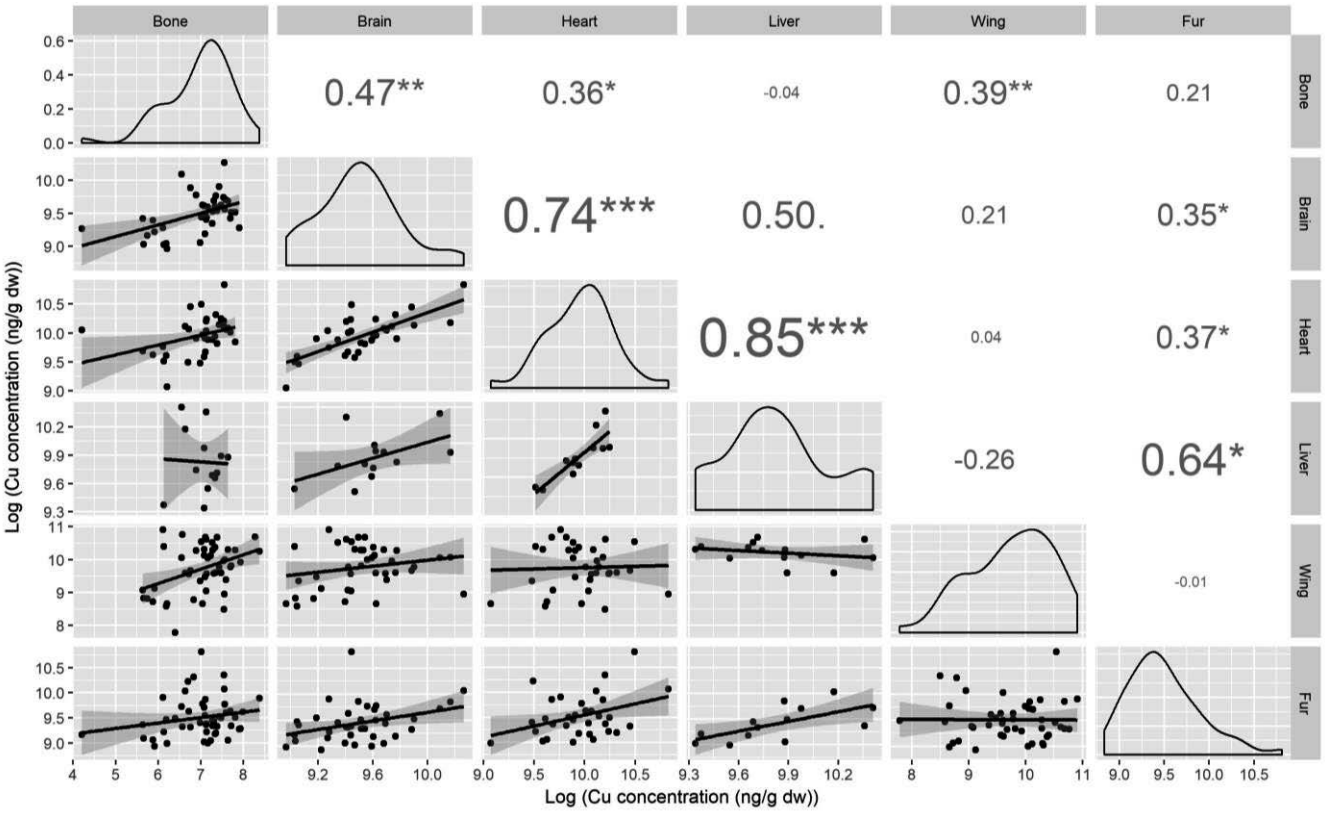

Fig. 7. Relationships between log-transformed Cu concentration (ng/g dw) in the different tissues analyzed. The trend line indicates the linear regression. Values presented corresponding to the Pearson correlation coefficient (r). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

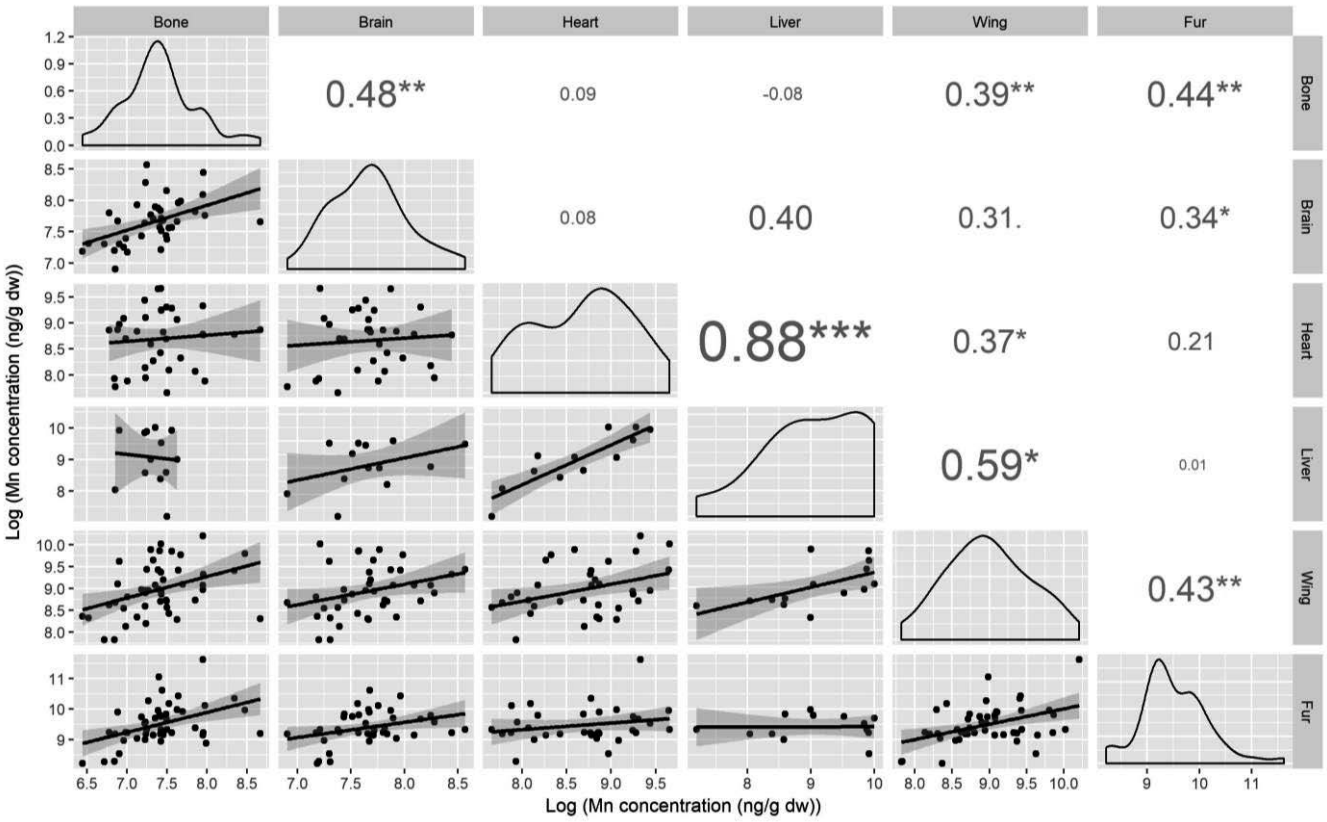

Fig. 8. Relationships between log-transformed Mn concentration (ng/g dw) in the different tissues analyzed. The trend line indicates the linear regression. Values presented corresponding to the Pearson correlation coefficient (r). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

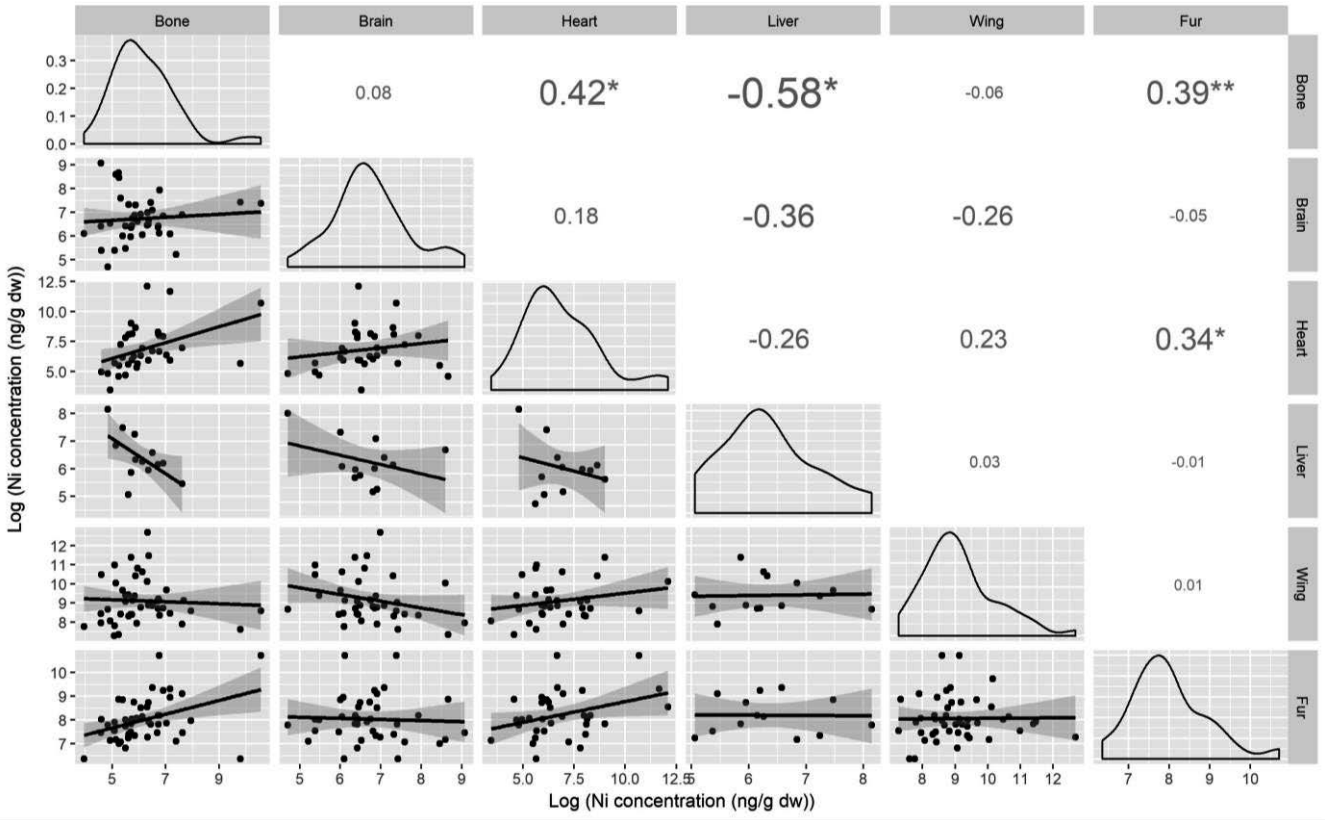

Fig. 9. Relationships between log-transformed Ni concentration (ng/g dw) in the different tissues analyzed. The trend line indicates the linear regression. Values presented corresponding to the Pearson correlation coefficient (r). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

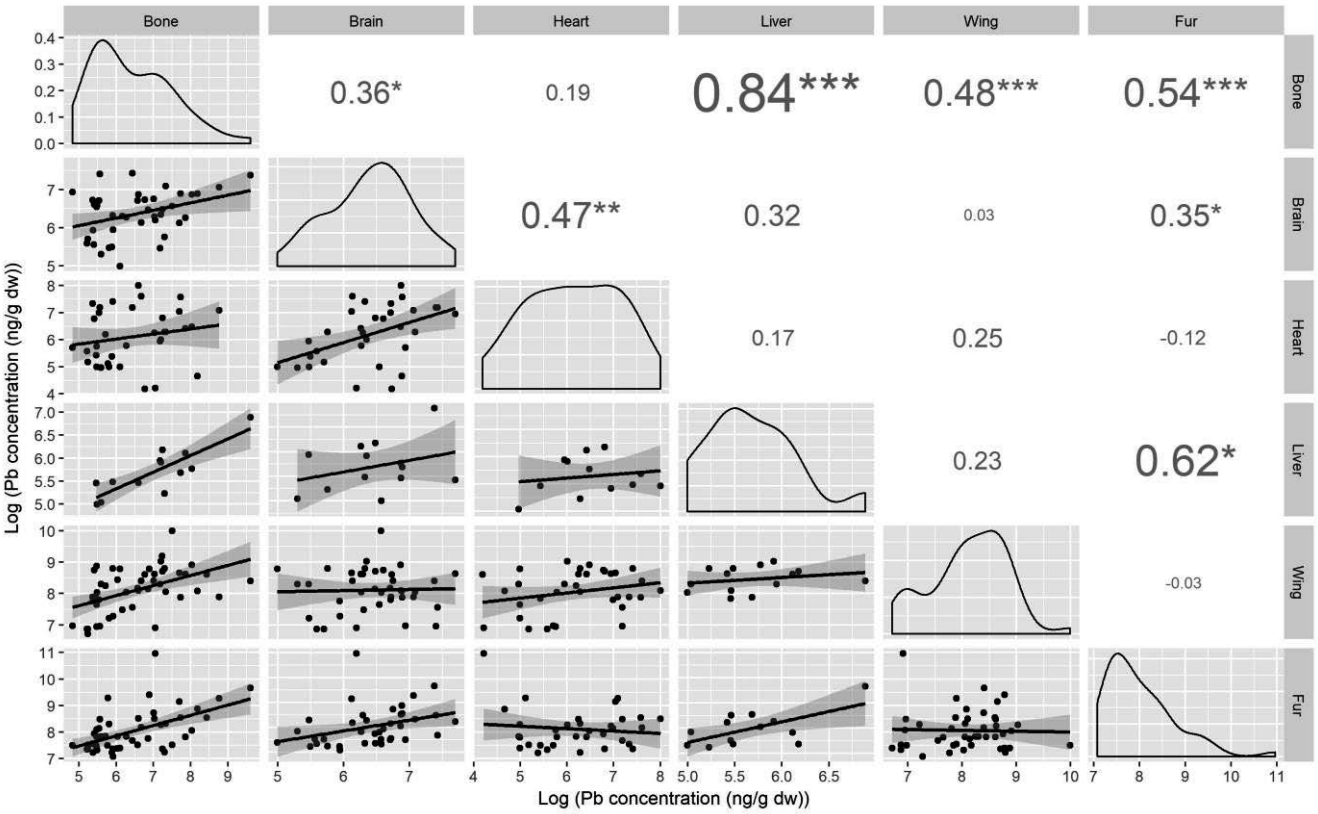

Fig. 10. Relationships between log-transformed Pb concentration (ng/g dw) in the different tissues analyzed. The trend line indicates the linear regression. Values presented corresponding to the Pearson correlation coefficient (r). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

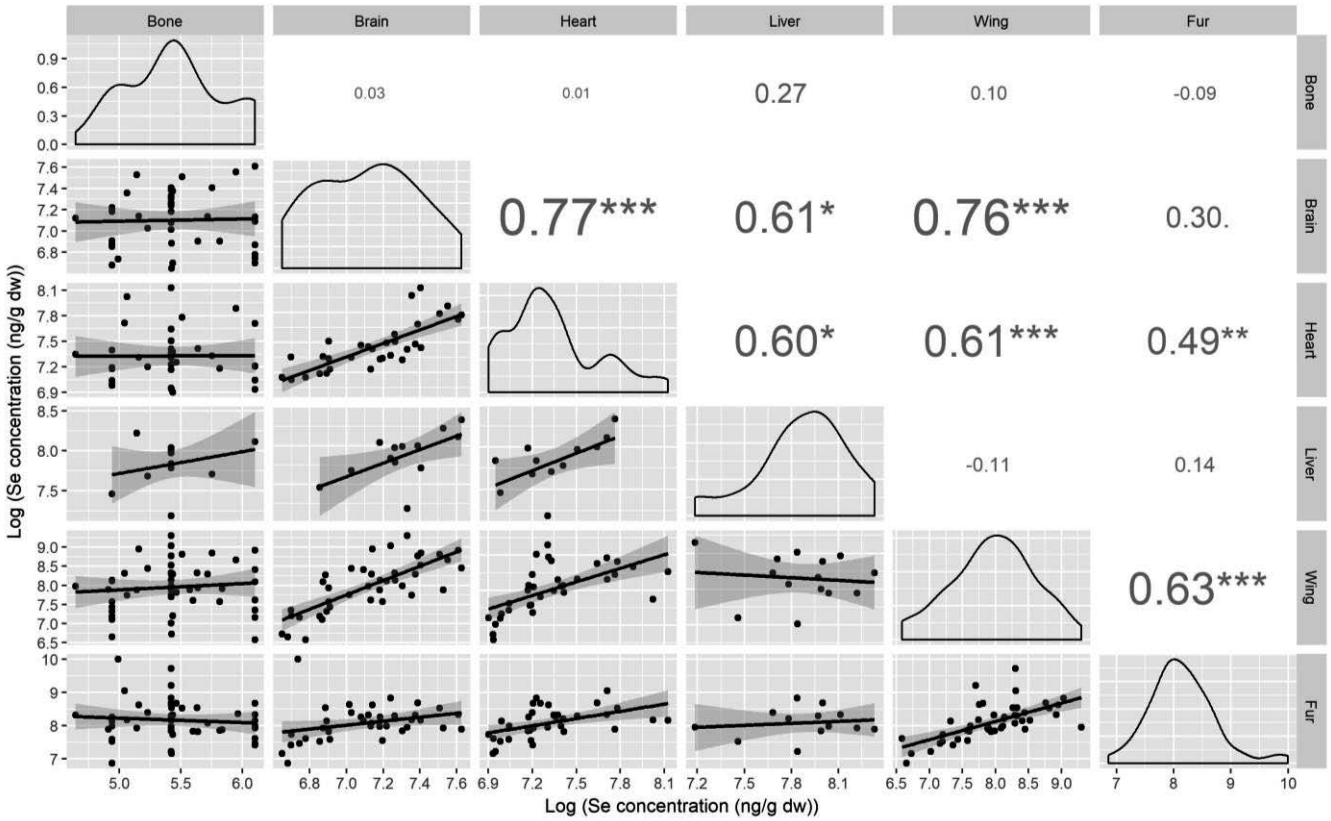

Fig. 11. Relationships between log-transformed Se concentration (ng/g dw) in the different tissues analyzed. The trend line indicates the linear regression. Values presented corresponding to the Pearson correlation coefficient (r). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

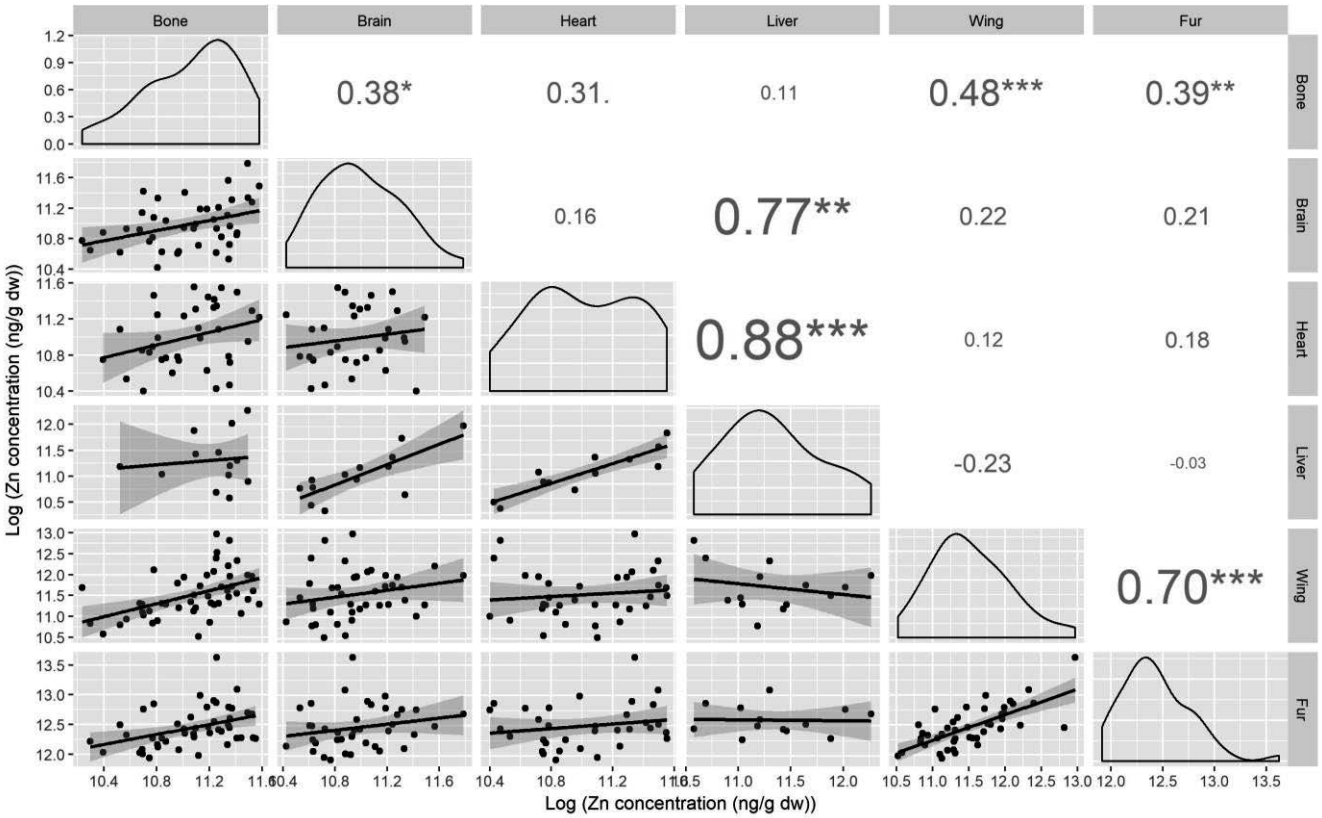

Fig. 12. Relationships between log-transformed Zn concentration (ng/g dw) in the different tissues analyzed. The trend line indicates the linear regression. Values presented corresponding to the Pearson correlation coefficient (r). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Discussion

Is the bioaccumulation of metals a potential risk for bat populations?

Interspecies differences in the bioaccumulation of metals like the ones obtained in this study were also observed in other studies (Miura et al. 1978; Hariono et al. 1993; Hickey et al. 2001; Flache et al. 2014; Flache et al. 2015; Hernout et al. 2016; Racero-casarrubia et al. 2017). These differences can be explained by physiological differences, differential foraging behavior, diet composition and patterns of habitat use (Hickey et al. 2001; Pereira et al. 2006; Béatrice V. Hernout et al. 2015). Diet composition is one important factor that leads to the interspecies differences in the bioaccumulation of metals. Coleoptera, Diptera and Lepidoptera are the most important food items in bat's diets, and the proportion of these insect orders in the overall diet varies greatly across the different bat species (Flache et al. 2014; Béatrice V. Hernout et al. 2015). The exposure of different bat species to metals is likely to vary due to differences in dietary composition. For example, bat species that, usually, feed on insects that have a large accumulation of metals, might be expected to have higher exposure than others (Walker et al. 2007; Béatrice V. Hernout et al. 2015).

A possible explanation for the lower levels of metals recorded in *Nyctalus leisleri* can be the fact that this species, usually, do not use urban areas as habitat and thus, the level of exposure to metals is lower than for the other three species that use urban areas. The roosting site may be another possible explanation, since *N. leisleri* mainly uses trees, unlike the other species that use mines, rock crevices and fissures in buildings, which are generally associated with higher levels of metal contamination. Finally, the same exposure levels to metals may not express the same concentrations/effects depending on the biomass of the species. Thus, as *N. leisleri,* on average, have higher biomass than the other three species, the same exposure levels may lead to lower concentrations in this species, compared to the others.

The significant interaction between species and organs/tissues, obtained for some metals, was an unexpected result. Metabolic pathways of metal accumulation are expected to be similar within the studied group of bats (Vespertilionidae family), so these results must be carefully interpreted, since a larger number of samples may be

needed to fully understand the reason behind it. The significant interaction is due to a dissimilar pattern of metal concentrations in the different organs of *Nyctalus leisleri*, more specifically in heart and liver for Mn, Ni and Zn, which may be a consequence of a reduced number of samples of these organs.

The absence of correlation between the metal concentration in the biological samples and the topsoil was an expected result because the accumulation of metals in bats may result from the contribution of several factors, not just contamination from the soil. The type of prey consumed, the possible higher contact or not with soil, the air contamination and the sources of water used for consumption, are some of the factors that may constitute exposure pathways. In addition, the high mobility and the large home range of our species are other factors that may explain this lack of correlation.

Similar results were obtained by Hernout et al. (2016) in their study with *Pipistrellus pipistrellus*, where no significant correlations were found between the Cd concentration in fur and in the soil of the area where the bats were collected. On the other hand, a study on insectivorous marsupials and rodents has shown significant positive correlations between non-essential metals (Cd and Pb) concentrations in hair and soil samples in the trapping areas (McLean et al. 2009).

Furthermore, metal concentrations in soils may not reflect accurately the environmental exposure and bioavailability of metals for bats, as many environmental and biological factors play a role in the exposure of contaminants through the food chain, such as: uptake from soils into prey items; environmental factors affecting this uptake (e.g. pH, organic matter, cation exchange capacity); oral exposure of the target species; foraging behavior, age and reproductive stage of the target species (Hernout et al., 2011).

Regarding the bioaccumulation of metals in the organs/tissues, our results demonstrated that external tissues, such as fur and wing membrane, may accumulate substantial concentrations of certain metals, since these were the samples that presented the highest concentrations for the majority of the metals analyzed. Fur sampling is highly suited as a non-invasive sampling technique as hair is easily accessible, easily transportable, its storage does not require restricted conditions, and their collection does not require the sacrifice of the animals (Pereira et al. 2006; Hernout et al. 2016). Mammalian hair is predominantly composed of keratin, a protein rich in

cysteine sulfhydryl (thiol) containing amino acids that avidly bind certain metals (Burger, Marquez, and Gochfeld 1994; McLean et al. 2009). Each hair shaft is continuously in contact with the bloodstream at the hair root, and thus may incorporate metals circulating through the blood during growth.

Mammalian hair of species including that of Racoons *Procyon lotor* (Clark et al. 1989); Opossum *Didelphis virginiana* (Burger, Marquez, and Gochfeld 1994); Ringed seals *Phoca hispida ladogensis,* ringed seals *Phoca hispida hispida* and bearded seals *Erignathus barbatus* (N. Medvedev, Panichev, and Hyvärinen 1997); Rodents (wood mice *Apodemus sylvaticus*, bankvoles *Clethrionomys glareolus*, black rats *Rattus rattus* and Algerian mice *Mus spretus*) (Erry et al. 2005; Pereira et al. 2006; Tête et al. 2014); European hedgehog *Erinaceus europaeus* (D'Havé et al. 2006; Vermeulen et al. 2009); Sled dogs *Canis lupus familiaris* (Dunlap et al. 2007) and Flying foxes *Pteropus* sp. (Hariono, Ng, and Sutton 1993) has been successfully utilized as indicator of the exposure to a range of metals and metalloids including As, Cd, Cu, Pb and Se.

When fur is used to measure metal exposure, molting effects should be taken into consideration. As fur incorporates metals while growing, older hair is presumed to contain higher metal levels due to a longer growing time than newer hair. Consequently, right before molting, the fur probably contains higher amounts of metals than during, or just after molt (Beernaert et al. 2007; Hernout et al. 2016). In general, bat species grow new fur once a year (usually in late summer-fall) and males tend to grow new fur before females. The timing and progression of the molt cycle vary substantially among bat species, as well as among sex and age classes (Fraser, Longstaffe, and Fenton 2013).

The bats analyzed in this study were collected between August and October, the period of greatest activity of bats in Europe, so some of them were collected during molt.

The high number of punches used in this study to collect the wing membrane skin was due to the need to ensure that we would have measurable metal concentrations that allowed to assess possible correlations between metal concentrations in wing membranes and metal concentrations in other tissues sampled. The results obtained showed that is possible to use only half of the punches that were used for our study, whenever the animals have quantifiable metal concentrations accumulated.

The high concentration of some metals found in the wing membrane supports our hypothesis that this tissue can be a good indicator and can be used as a non-lethal sample to monitoring metal exposure on bats. Given the high number of blood vessels and the high regenerative capacity of wing membrane (Faure, Re, and Clare 2009; Weaver et al. 2009), bats may possibly use it to excrete the contaminants, which leads to an accumulation of metals in this tissue. To support this hypothesis, further studies are needed to find out if the wing membrane has a continuous regeneration cycle, and if it is used to intentionally excrete contaminants, or if it is a consequence of it continuous wear and regeneration.

It is well documented that some metals and pesticides can be absorbed by the skin in humans and rats (Wester et al. 1992; Sandt et al. 2000; Hostynek 2003; Larese et al. 2007; Modjtahedi and Maibach 2008; Ngo, O'Malley, and Maibach 2010). The level of absorption varies according to the different skin regions and according to the different metals (Hostynek 2003; Ngo, O'Malley, and Maibach 2010). Based on this information, another hypothesis that can explain the high metal concentration in the wing membrane is the great exposure to the external environment, which possibly leads to the direct accumulation of contaminants through skin absorption. To our knowledge, no study has investigated this possibility in bats.

Depending on the metal, the organ that showed higher concentrations varies. However, in general for the various metals, fur and wing showed the highest concentrations, while bone was the tissue that presented the lowest concentrations.

Regarding arsenic, our results showed that the highest concentrations were recorded in fur and liver. These findings are consistent with the literature, that conclude that high arsenic intake was associated with elevated arsenic concentrations in the liver, kidneys and fur which are involved in the detoxification, excretion or sequestration of arsenic (Marie Vahter 1981; Foa 1981; Erry et al. 2005). The toxicity of As in mammals was found to be related with levels above 3 μg/g in the liver (Gupta and Gupta 1998; Pereira et al. 2006). In our study, we collected bats with a concentration of arsenic in the liver well above this threshold (range 0.07-6.85 μg/g). The predominance of arsenic in fur, is in accordance with Vahter (1994), who stated that early distribution of arsenic is to the liver and kidneys, while after 24h little arsenic would remain in these organs,

and the majority of arsenic is excreted into the urine and external tissues, such as hair, skin and nails.

Concerning cadmium, our results showed that the highest accumulation was in the liver, and the accumulation in non-lethal samples (fur and wing) was relatively low. These findings are according to the literature that concludes that liver is the second storage site for cadmium in the body, after the kidney (Świergosz-Kowalewska 2001; Nikolai Medvedev 1995; Pereira et al. 2006; D'Havé et al. 2006; Beernaert et al. 2007). Levels exceeding 10 μg/g of cadmium in the liver may be considered indicative of cadmium contamination in some mammals (Halatek et al. 1989; Thies and Gregory 1994). If our species shows similar toxic responses to cadmium, data obtained from this study do not demonstrate a potentially harmful situation for either species (range 0.17-1.77 μg/g).

In this study, cobalt was mainly recorded in the non-lethal samples (fur and wing). In this case, our results were not in line with previous studies on mammals. D'Havé et al. (2006) obtained higher values of cobalt in the liver of the European hedgehog than in the hair. Our concentration of cobalt in liver was higher than the obtained by Allinson et al. (2006) in their study with *Miniopterus schreibersii bassanii* in Australia.

About chromium concentration, the highest values were recorded in the non-lethal samples, especially in the wing. The predominance of chromium in fur is in line with the results obtained by D'Havé et al. (2006). The concentration of chromium that we obtained in the liver is identical to that obtained by Allinson et al. (2006) in their study with *Miniopterus schreibersii bassanii*. As no other study has analyzed the wing membrane, we cannot make comparisons.

Our results showed a similar distribution of copper concentration between the internal organs and the external tissues (with exception of bone), which indicates that this metal has a uniform accumulation through the body. The hepatic concentrations of copper found in our study (range 11.37-33.17 μg/g) were within the reference range (1.08-99.2 μg/g) determined by Hoenerhoff & Williams (2004) for some frugivorous and hematophagous bat species. In addition, they obtained a copper concentration of 4,500 μg/g in the liver of a Mexican fruit bat (*Artibeus jamaicensis*), which they consider to be a toxic concentration. Once more, our copper concentration in liver is within the range

obtained by Allinson et al. (2006). Flache et al. (2014) and Hernout et al. (2016) carried out studies with insectivorous bat species, where they both analyzed the copper concentration in fur, and where both obtained a copper concentration ranges very similar to the one obtained in the present study. Copper is a well-documented cause of liver toxicity in many domestic species, including sheep, dogs, cats, horses, cattle, goats, pigs and camelids. Excessive gastrointestinal copper absorption may exceed the metabolic capacity of storage in the liver (Hoenerhoff and Williams 2004).

Regarding manganese, fur, liver and wing were the tissues with the highest concentrations of this metal. The concentration of manganese obtained in the liver in this study was lower than that reported by Allinson et al. (2006) for *Miniopterus schreibersii bassanii.* On the other hand, the manganese concentrations in fur are in agreement with the values obtained by Flache et al. (2014) and Flache et al. (2015). Zocche et al. (2010) in their study, carried out with three insectivorous bat species (*Molossus molossus, Tadarida brasiliensis and Eptesicus diminutus*), concluded that the levels of manganese in *Eptesicus diminutus* may offer a contribution to the indices and frequency of DNA damage in the species, although they do not present threshold levels.

Concerning nickel, the highest concentrations were found in the external samples, mainly on the wing. Comparing the concentrations of nickel in liver and fur obtained by Pereira et al. (2006) in black rats, D'Havé et al. (2006) and Vermeulen et al. (2009) both with European hedgehog, our values were significantly higher.

The highest concentrations of lead in this study were recorded in the non-lethal samples. The concentration of lead believed to represent a toxic dose has been determined for many domestic animals and, for most species, values of 10 µg/g in liver are considered to be harmful (Hariono, Ng, and Sutton 1993). Our concentrations of lead in liver were well below this threshold (range 0.15-0.98 µg/g). Hickey et al. (2001) analyzed the fur of two insectivorous bat species (*Myotis lucifugus* and *Eptesicus fuscus*), and obtained a lead concentration range very similar to the one obtained in this study, as well as the results obtained by Allinson et al. (2006) in the liver of *Miniopterus schreibersii bassanii.* Sutton & Wilson (1983) recorded lead concentrations of 18.7 and 59.5 µg/g in liver of two Grey-head fruit bats (*Pteropus poliocephalus).* The bat that presented the highest concentration of lead showed muscle fasciculation, excess

salivation, diarrhea and ataxia, which are signs consistent with lead toxicity (Hariono, Ng, and Sutton 1993; Skerratt et al. 1998).

Our results showed that selenium concentrations were higher in the external tissues and liver. Selenium concentrations obtained in fur were similar to those obtained by Hickey et al. (2001) and Allinson et al. (2006).

Zinc was the metal that showed the highest concentration in all the organs/tissues, with fur and wing presenting the highest accumulation. These results were expected since zinc is an essential trace element with several functions in metabolic processes, and so, subject to homeostatic regulation by the organism (Flache et al. 2015). Zinc concentration in the fur of the bats analyzed varied from 149.21 - 827.16 µg/gm, within the range reported by Hickey et al. (2001), for four insectivorous bats, and by D'Havé et al. (2006) for European hedgehog, both from fur samples. In contrast, in a study made in Portugal, with black rats (*Rattus rattus*) and Algerian mice (*Mus spretus*), the authors found lower levels of zinc than those reported in this study, raising the hypothesis that the low levels of zinc can be explained by the exposure to a mixture of contaminants and the subsequent interactions between them (Pereira et al. 2006). Once more, the zinc concentrations obtained from the liver samples in this study were within the range found by Allinson et al. (2006) in the liver of *Miniopterus schreibersii bassanii.*

Although some effects of metals on bats, such as hepatopathy, DNA damage, hemochromatosis, renal inclusion bodies, ascending paralysis, tremors, spasms, general slowness, lack of control in body movement and mortality, have been reported, the number of studies dedicated to this theme remain very low (Sutton and Wilson 1983; Hariono, Ng, and Sutton 1993; Skerratt et al. 1998; Hoenerhoff and Williams 2004; Farina et al. 2005; T J O'Shea 2009; Zocche et al. 2010; Nam et al. 2012).

Zukal et al. (2015) in their review, reported that there remain many unanswered questions in relation to the metabolism of heavy metals in bats. These include the efficiency of absorption, the level required to show clinical effects, and whether or not excretion occurs. Following these observations, is important to take into account that, even if, high levels of non-essential elements are recorded in some tissues of bats, this may not lead to toxic effects on these. For shrews, for example, it was suggested that high cadmium organ levels may possibly reflect an ability to store cadmium in a non-

toxic form (Shore and Douben 1994). Further studies are needed to check if bats also have this ability.

Concluding, in general the metal concentrations obtained in this study are within the ranges obtained from the literature. Concerning, mainly the non-essential metals, which its toxicity is well documented, some of the bats collected in this study presented arsenic concentrations that exceed toxic thresholds, which suggests that these bats may be affected by metal exposure. The fact that we have obtained readings for all metals analyzed, especially for non-essential metals, supports the hypothesis that bioaccumulation of metals is a potential risk for bat populations occurring in Portugal. In addition, the literature showing direct effects in bats due to contamination by metals reinforces this hypothesis. Thus, we can assume that metal contamination may be a factor that contributes to the decrease of bat populations. Further studies linking the exposure of metals with their direct effects on bats, which should include histological examinations, are needed.

Are the external tissues good indicators of metal accumulation in bats?

Regarding the correlations between the metal concentration in the different organs/tissues, the results obtained were very heterogeneous. Different correlations were found between the different organs/tissues depending on the metal analyzed. These results are due to the fact that the different metals have different accumulation pathways.

In this study, the main interest was to establish correlations between the concentrations found in external tissues and the internal organs. Our results showed few correlations between the wing and the internal organs for the different metals. As already mentioned, to our knowledge, the wing membrane was never used to quantify the concentration of heavy metals, so we cannot compare the results obtained. Further studies using the wing membrane as a non-lethal sample are needed to confirm its usefulness as an indicator of metal bioaccumulation.

Correlations between fur and internal samples were found for some elements. For non-essential metals, Hariono et al. (1993) showed before that lead concentrations

in the fur of fruit bats were significantly correlated with concentrations in the liver (r=0.51). Similar relationships between lead concentration in fur and liver (r=0.53) were observed by D'Havé et al. (2006) in European hedgehogs. Like us, D'Havé et al. (2006) also found a correlation between cobalt and copper concentrations in fur and liver (r=0.63) and (r=0.76) respectively, but, contrary to our results, they also found a correlation between chromium concentration in fur and liver (r=0.62). Several other studies have also reported relationships between hair and internal tissues for some metals. For instance, cadmium and lead concentrations in hair of wood mice were positively correlated to cadmium and lead levels in livers (Beernaert et al. 2007; Tête et al. 2014). Concerning insectivorous bats, Hernout et al. (2016) found positive correlations between the cadmium concentration in fur and bones (r=0.53) and between the lead concentration in fur and bones (r=0.72) in *Pipistrellus pipistrellus and Pipistrellus pygmaeus.*

A possible explanation for the absence of relationships between fur and internal organs, for essential elements, observed in our results and in the literature, may possibly be the result of different exposure levels. Essential elements can, to a certain extent, be regulated in living organisms by homeostatic mechanisms (Talmage and Walton, 1991; D'Havé et al. 2006). As a result, the excretion of essential elements in hair may remain low until the threshold concentration of these elements be reached, and only after the excretion increase. This can suggest an effective regulation process of essential metals for bats, as described for other mammals (Johnson and Roberts 1978; McLean et al. 2009).

Although strong correlations were found between the internal organs, once more, the correlations found varies depending on the metal analyzed, which is expected since the metals have different accumulation pathways, as mentioned previously.

An explanation for the low levels of some essential elements recorded can be the exposure to a mixture of contaminants and the subsequent interactions between them, to which bats are subject. Metals can have additive, synergistic and antagonistic interactions depending on a number of variables, including the type of metals involved and their relative concentrations (Beyer et al. 2004; Hernout et al. 2016). For example, some non-essential metals can out-compete with an essential metal for a key binding

site in a tissue, causing low concentrations of the essential metal in that tissue(Pereira et al. 2006).

Significant positive associations were recorded between non-essential and essential elements in the liver of rats (As / Zn: r =0.692) and mice (Cd / Fe: r =0.651; As / Mg: r =0.543), as well as, significant negative associations were recorded between As / Cr (r =-0.945) and As / Ni (r =-0.876) in the hair of rats (Pereira et al. 2006). These results demonstrated that arsenic may contribute in removing other metals from their binding sites within the hair structure. Other studies reported associations between metals, for example, Flache et al. (2015) found a positive correlation between manganese and copper (r =0.695) in hair samples of *Pipistrellus pipistrellus*, *Myotis daubentonii* and *Nyctalus noctule*. The authors report that the causes underlying the correlation found were not clear. However, they point out that the correlation may be related to a constant exposure of the species to these metals in their foraging habitat over a long period. They support their hypothesis with the fact that the foraging habitat of *Nyctalus noctule* includes agricultural areas, where pesticides and/or fertilizers may be used, potentially resulting in a high exposure to manganese and copper.

The high variability observed in the metal concentration measured in the different organs might be explained by a range of variables such as age, diet and molting, which can impact the levels of metals contained in fur. Bats also display different food ingestion rates and weights depending on their lives-stage (i.e. juvenile, male in spermatogenesis, pregnant female, or lactating female) and cycle (winter torpor or summer active); hence, sensitivity to chemical exposure may vary depending which life stages the bat is in when collected (Hernout et al., 2013; Zukal et al., 2015).

Comparing the metal concentration between the internal organs, it is possible to verify that metal concentration in bones was, in general, lower than in the soft tissues. Despite this, the bone is a good indicator of long-term and chronic metal exposure, since, the half time of some non-essential metals in bones is around 10-30 years, while in soft tissues the half time is measured in months (Kales and Christiani, 2005; Hernout et al. 2016). This is due to the fact that non-essential metals that bind to the bone matrix are not readily accessible into the bloodstream. In contrast to bones, liver, that has detoxification capacity, can regulate their concentrations via level of metallothionein production (Shore and Douben 1994).

So, in general, our results showed that non-lethal samples (fur and wing) are good tools to biomonitoring metal contamination in bats, since, high concentrations of the different metals were recorded in these tissues. The high concentrations obtained for some metals in the wing showed that this may possible be a better indicator of metal accumulation in bats than the fur. Although few correlations were found between the metal concentrations in external tissues and the metal concentrations in internal organs, all the biological samples showed similar response patterns in terms of metal accumulation, except the bone for some metals. Globally, our results indicate that the external tissues are good indicators of metal contamination in bats.

Chapter III – General Conclusions

Conclusion

The fact that we have obtained readings for all metals analyzed, especially non-essential metals, supports the hypothesis that bioaccumulation of metals is a potential risk for the bat populations.

Given the need for large-scale studies to establish whether metal accumulation is indeed one of the factors associated with declining bat populations, non-invasive sampling is the best option to obtain a greater number of samples, more representatives of the existing populations and individuals, without the sacrifice of the animals. The present study has demonstrated that fur and wing membrane are suitable biological matrices to evaluate metals exposure in bats. Accumulation of metals in these tissues was shown to be quite high and equally important compared to the accumulation of metals in the investigated internal tissues. Given the high concentrations of metals obtained in the wing, it is worth noting its use as a good biological sample to assess metal exposure, which has not been used until now, but that may be a better indicator than the fur, for some metals.

Although few relationships were found between the metal concentration in external samples and the metal concentration in internal samples, overall, all the biological samples showed similar response patterns in terms of metal accumulation, except the bone for some metals. Thus, we can conclude that fur and wing membrane may be useful to predict endogenous metal concentration in bats.

Our results can provide a valuable tool in further developing the understanding about the importance of metals as a driver for some of the observed declines in bat populations seen around the globe.

Further studies should integrate more individuals and some individual characteristics that can modulate the accumulation of metals in bats, such as sex, age and molt, to develop more precise predictions. We also encourage the use of the wing membrane in further studies, mainly to improve the method of collection of this tissue in living organisms, but also to reinforce our results.

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