Universidade de Aveiro Departamento de Biologia
Ano 2019

SANDRA RAQUEL OLIVEIRA ASCENÇÃO

MERCURY CONCENTRATIONS IN DAYTIME BIRDS OF PREY IN PORTUGAL

CONCENTRAÇÕES DE MERCÚRIO EM AVES DE RAPINA DIURNAS EM PORTUGAL

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica do Doutor António Manuel da Silva Luís Professor Auxiliar do Departamento de Biologia e coorientação da Doutora Maria Eduarda da Cunha Pereira, Professora Associada do Departamento de Química da Universidade de Aveiro

Dedico este trabalho à minha família por todo o apoio que me deram

o júri

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palavras-chave Metal vestigial, contaminação, biomonitorização, rapinas, teia alimentar

resumo

O mercúrio é um dos elementos não-essenciais mais prejudiciais à vida selvagem, pela sua capacidade de bioacumular e bioamplificar ao longo da cadeia alimentar. Devido ao contínuo aumento de concentração deste elemento, as aves de rapina são frequentemente utilizadas como biomonitores devido à sua vasta diversidade de habitats e elevada probabilidade de acumulação de contaminantes por serem predadores de topo.

No presente trabalho foi analisada a concentração de mercúrio em 10 tecidos (unhas, bico, penas, fígado, rins, pulmões, coração, músculo, cérebro e pele) de quatro aves de rapina: águia-d'asa-redonda (*Buteo buteo*), gavião-europeu (*Accipiter nisus*), peneireiro-comum (*Falco tinnunculus*) e mocho-galego (*Athene noctua*) e avaliada a influência de parâmetros como género e idade na acumulação deste elemento.

Os resultados obtidos evidenciaram diferenças significativas entre os tecidos, pela ordem de concentrações: pele < cérebro < músculo < coração < pulmões < fígado < rins < penas < unhas < bico.

Foram observadas diferenças significativas entre as diferentes espécies, em que o gavião-europeu e a águia-d'asa-redonda apresentaram os valores mais elevados. A faixa etária influenciou na acumulação no peneireiro-comum, em que subadultos apresentaram valores superiores aos demonstrados nos adultos, não sendo observadas diferenças em relação ao género. Para as restantes espécies de aves não foram identificadas diferenças significativas para a variação da acumulação com o género ou idade.

keywords Trace metal, contamination, biomonitoring, raptors, food web

abstract

Mercury is one of the most harmful non-essential elements to wildlife for its ability to bioaccumulate and bioamplify throughout the food chain. Due to the continuous increase in concentration of this element, birds of prey are often used as biomonitors due to their wide habitat diversity and high probability of contaminant accumulation as top predators.

In the present work, the concentration of mercury in 10 tissues (nails, beak, feathers, liver, kidneys, lungs, heart, muscle, brain and skin) of four birds of prey was analyzed: common buzzard (*Buteo buteo*) , eurasian sparrowhawk (*Accipiter nisus*), common kestrel (*Falco tinnunculus*) and little owl (*Athene noctua*) and evaluated the influence of parameters such as gender and age on the accumulation of this element.

The results showed significant differences between tissues in the order of concentrations: skin < brain< muscle < heart < lungs < liver < kidneys < feathers < nails < beak.

Significant differences were observed between the different species, in which the eurasian sparrowhawk and the common buzzard presented the highest values. The age group influenced the accumulation in the common kestrel, where subadults presented higher values than those shown in adults, with no differences regarding gender. For the remaining bird species no significant differences were identified for variation of accumulation with gender or age.

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1. Introduction

Several trace metals are essential for metabolism and are naturally found in the environment at low concentrations. When these concentrations reach a limit, however, some metals can become toxic (Burger & Gochfeld, 2009; Kitowski et al., 2017; Naccari et.al., 2009; Patlar et al., 2014; Zolfaghari et al., 2007). Others do not have a known metabolic role, and therefore are considered non-essential, such as Hg, Pb or Cd.

The high retention capacity of contaminants by ecosystems makes susceptible the contamination of aquatic and/or terrestrial biological systems, becoming a risk to wildlife, humans and the ecosystem itself (Sánchez-Chard et al., 2007).

Anthropogenic activities such as industry and urban settlements, mining, smelting activities, fossil waste combustion, incineration, waste disposal or agriculture have led to pollution of systems by metals such as mercury. Mercury mining and uses has doubled its environmental concentration in recent centuries, becoming a problem and a subject of concern for its possible negative effects on exposed biota (Driscoll et al., 2007; Grúz et al., 2018; Hernández et al., 1999; Ochoa-acuña et al., 2002; Pacyna et al., 2006).

1.1. Mercury and its biogeochemical cycle

Mercury (Hg) is an element that occurs naturally, being considered a trace metal, without scent and one of the most toxic present in the environment for human and wildlife. Its emission may occur naturally or due to anthropogenic factors (such as those mentioned above) and by re-emission (Zhang & Wong, 2007).

In nature, this element is found at low concentrations in sediments, rocks or soils, volcanos, natural and thermal waters or even in plants and animals, among others (Chrystall & Rumsby, 2008).

Mercury can be found as elemental mercury (Hg^0 or mercury (0)), mercurous mercury (Hg^+ or mercury (I)), resulting from the reaction: $Hg_2^{2+} <-> Hg^0 + Hg^{2+}$, but this species is less common in the environment due to its instability when compared with

mercuric mercury (Hg^{2+} or mercury (II)) (Figure 1). The last two states, when subjected to specific conditions, can suffer several changes and convert into highly toxic compounds, occurring in organic or inorganic form (Boening, 2000; Burger & Gochfeld, 1997).

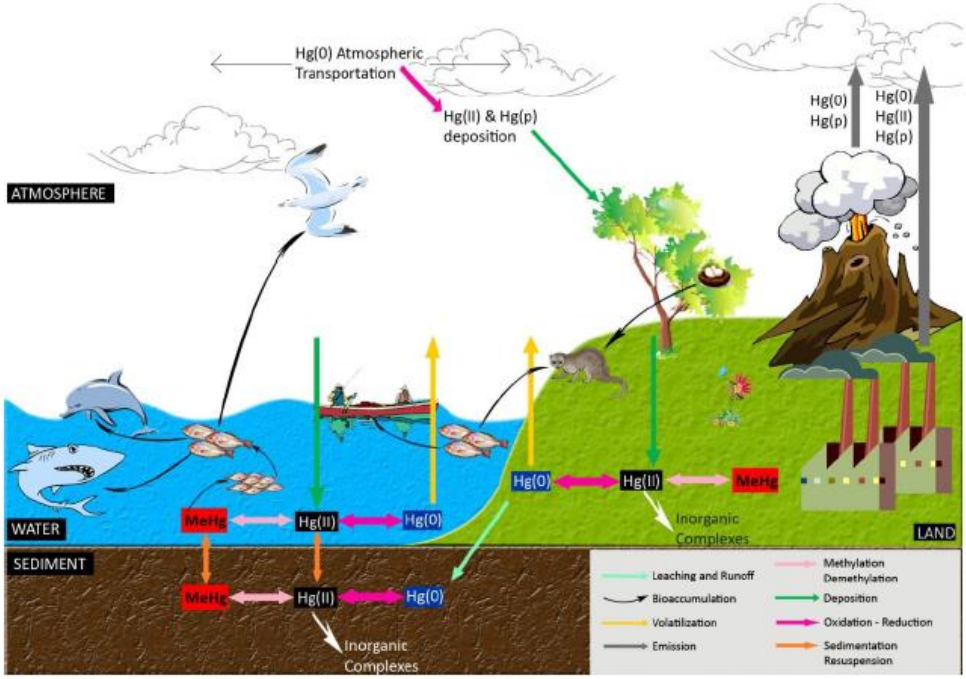

Figure 1: Biogeochemical mercury cycle (Taken from Rodrigues, (2012)).

Elementar mercury is the most volatile and abundant form of this metal. At environmental temperatures and pressure it is in its liquid state and can evaporate, forming mercury vapor that could contaminate air and be quite toxic, although its exposure to living organisms is low (Chrystall & Rumsby, 2008).

Approximately 95% of the mercury in the atmosphere is in the elemental state, where it is slowly oxidized to the mercury ion (Hg^{2+}) which then returns to the earth's surface via precipitation. However, this compound can persist in the atmosphere, having a lifetime of 0.5 to 1 year, and be transported over long distances polluting regions where there is no local source (Pacyna et al., 2006).

Mercuric mercury is more soluble in water, having a lifetime in the atmosphere shorter than Hg^0. It can persist only for days or weeks being deposited by wet deposition (Bisinoti & Jardim, 2004).

In oceans, after several chemical and biological transformations, part of Hg^{2+} is reduced to Hg^{0} and returns to the atmosphere. Similar processes occur in the earth's crust, with some of the reduced mercury returning to the atmosphere and the rest remaining in the soil and sediment.

Organomercurials formed from Hg^{2+} are the most toxic compounds, with methylmercury (MeHg) being the most concerning (Nascimento & Chasin, 2001).

MeHg is the most common organic mercury form and results from Hg^{2+} methylation through the action of anaerobic bacteria and fungi in lakes, oceans or land. Its faster incorporation and assimilation into the food chain and slow elimination make this form the most dangerous to environmental and human health. (Boening, 2000; Jensen & Jernelow, 1969). It is considered the chemical form of the most process-resistant mercury environmental changes and may be in the form of methylmercury chloride (CH_3HgCl) or methylmercury hydroxide (CH_3HgOH).

Mercury is one of the few known metals to accumulate through food webs to concentrations that are much higher in upper trophic level than those in primary producers or consumers (Rimmer et al., 2010). Its transfer occurs directly from the environment to organisms and is conditioned by the bioavailability of this element. When the concentration of Hg^{2+} or MeHg exceeds the concentration present in the surrounding environment, this process is called bioaccumulation. This is the basis of incorporation into food chains, being triggered essentially by water and food.

Mercury still exhibits biomagnification properties, mainly of the organic form CH_3Hg^{+}, i.e. when mercury concentration transfer occurs through at least two trophic levels in the food chain and is higher in the consumer than in the prey (Drouillard, 2008; Kidd et al., 2011).

Due to higher concentrations of this element in aquatic ecosystems, studies of terrestrial systems are not as common for demonstrate low concentrations which do not indicate a need for greater focus (Kalisiñska et al., 2009).

However, the transfer of this trace metal also occurs at different trophic levels in terrestrial webs, affecting terrestrial biota. Cristol et al., (2008) conducted a study trying to determine the biomagnification of MeHg in terrestrial biota near a Hg-contaminated river in Virginia. The results showed an increase of total Hg in different bird prey belonging to the orders Orthoptera, Lepidoptera and Aranea.

The highest concentration of Hg was observed in the form of methylmercury in spiders, which correspond to approximately 30% of diet in three local songbird species.

Of 13 bird species, twelve were observed with blood Hg concentration higher than the contaminated site. Thus, the authors concluded that the Hg present in water has moved to and through the terrestrial trophic chain, where avian consumption of predatory invertebrates increased the food chain and MeHg biomagnification.

1.2. Routes of exposure

Biosphere exposure to this contaminant occurs in several ways and in possible different states. Despite all forms of mercury are toxic, the adverse effects may depend on factors such as time and route of exposure and biochemical, physiological or ecological factors (Esselink et al., 1995; Graeme & Pollack, 1998; Horai et al., 2007; Kitowski et al., 2017).

Exposure to Hg^0 may occur by: inhalation of elemental mercury, in the form of steam, traversing alveolar membranes, in which 80% is absorbed into the lungs and may spread and penetrate the bloodstream invading red blood cells, central nervous system and kidneys; absorbed through the dermis, which accounts for a low percentage of exposure and is mostly excreted in urine or faeces; or ingestion.

However, only 0.1% of the amount ingested is absorbed by the gastrointestinal tract, most of which is eliminated and is not an extreme danger (Tchounwou et al., 2003).

Mercuric salt ingestion is an exposure route where only 10% of the ingested amount can be absorbed into the tract due to its corrosiveness. As inorganic mercury, it can lead to internal damage such as stomach, mouth and throat ulcers and corrosive stomach damages. This form also tends to accumulate in the kidneys, causing severe damages which can lead to acute renal failure. Similarly, only a small amount is absorbed by the dermis with the excretion being mostly faecal (Tchounwou et al., 2003).

Methylmercury is considered the most toxic chemical form for both humans and the rest of the biosphere, being quickly absorbed and slowly eliminated compared to other mercurial forms.

Its main route of exposure is ingestion, which in humans occurs mainly through the consumption of fish. However, depending on the level of concentration, air and water may contribute to a higher concentration of mercury in the body.

MeHg has the capacity to penetrate through cell membranes and react with essential proteins, amino acids and nucleic acids (Chrystall & Rumsby, 2008), being demonstrated through research that can impair reproductive performance, growth and development, behavior, motor skills, and survival in wildlife and humans (Scheuhammer et al., 2007; Wolfe et al., 1998).

One of the most significant cases that demonstrated the potential danger of mercury accumulation was the incident that occurred in Minamata, Japan. This event became known as “Minamata disease” in which several people died. One of the largest chemical fertilizer industries, in Japan, obtained methylmercury as a by-product of acetaldehyde production. Waste was discharged into the region's basins, contaminating water resources, wildlife and the population of Minamata, that fed on fish from this bay and were exposed to high methylmercury concentrations (liver from 22.0 to 70.5 $mg.kg^{-1}$, brain from 2.6 to 24.8 $mg.kg^{-1}$ and kidneys from 21.2 to 140 $mg.kg^{-1}$) (Santos, 2008).

1.3. Mercury concentrations in birds

Studies have shown that wild bird populations are extremely sensitive to changes in ecosystems and more susceptible to poisoning, being found several times as victims (Denneman & Douben, 1993; Guitart et al., 2010).

Mercury effects on birds may lead to changes in behavior, resulting in lethargy, loss of appetite and lack of motivation (Seewagen, 2015; Sepúlveda et al.,1999); neurological effects, causing severe damage to nervous system, which affects their coordination (Spalding et al., 2006; Wolfe et. al., 1998) and physiological damage, leading to a change in hemoglobin and consequently difficulty in transporting oxygen to tissues, an abnormality in feather growth, decreased reproductive success and ability to sustain long-distance flights (Evers et al., 2007; Olsen et al., 2000).

Some of these effects were observed and confirmed in a study conducted by Frederick & Jayasena (2011), exposing white ibisis over a period of 3 years, where they observed a decrease in male courtship and female productivity.

Ingestion is the main route of exposure, being considered primary when results from direct ingestion of the chemical; or secondary, when exposure occurs through the consumption of contaminated food, water or substrate (Best & Fischer 1992; Rattner et al. 2000; Smith et al. 2007; Malik & Zed 2009). However, as mentioned above, concentration may depend on biochemical, physiological, ecological and exposure factors (Esselink et al., 1995; Horai et al., 2007; Kitowski et al., 2017).

Mercury bioaccumulation and bioamplification capacity makes birds of prey one of the most affected groups due to their position as top predators. The same conclusions were observed in a study by Rimmer et al., (2010) where carnivorous species had higher Hg concentrations, with an increase in raptors compared to their prey.

Top predators play an important role in ecosystems, contributing to maintain the abundance and richness of several species. The loss of these species could result in negative ecological effects on both ecosystems and the rest of the chain (Prugh et al., 2015; Ripple et al., 2014).

Awareness of the increase in heavy metals and their effects has led to monitoring measures that provide information about species health and ecosystem contamination through analysis of several tissues, assisting in early detection and possible extrapolation to other ecosystems.

Feathers, eggs and blood are usually more commonly used, as they allow for easy collection and storage, without sacrificing the individual (Goede & Bruin, 1986).

During feather growth, the keratin structure is linked to blood flow. As mercury forms bonds with keratin sulfridyl groups, its concentration increases depending on the condition and nutrition of the species (Ackerman et al., 2008; Costa et al., 2012). When the feathers reach their maximum growth, blood flow is interrupted and the feathers of adult individuals may possess up to 70% of the mercury present in the body (Boening, 2000; Costa et al., 2012; Ochoa-acuña et al., 2002).

In females, eggs are an extra alternative for mercury excretion, providing recent information on individual contamination during the reproductive cycle (Peakall & Lovett, 1972).

As explained, although heavy metals are a widely studied topic, the current literature shows a greater richness in waterfowl studies, as values are higher in these ecosystems.

However, several studies also show a large accumulation of this contaminant in terrestrial bird species (Driscoll et al., 2007; Finley & Stendell, 1978; Palma et al., 2005; Rodríguez- Jorquera et al., 2017).

Raptors' wide range of habitat diversity, high probability of contaminant accumulation and biological habits makes this group a good bioindicator candidate, enabling greater and more complete data collection (Battaglia et al., 2005; Jager et al., 1996).

Different species of birds of prey like common buzzard (*Buteo buteo*), common kestrel (*Falco tinnunculus*), golden eagle (*Aquila chrysaetos*), white-tailed sea eagle (*Haliaeetus albicilla*), peregrine falcon (*Falco peregrinus*), tawny owl (*Strix aluco*) and barn owl (*Tyto alba*) are the most commonly used in monitoring processes, as they show visible responses to contaminants (Gómez-ramírez et al., 2014).

1.4. Birds of prey in Portugal

In Europe, the common buzzard (*Buteo buteo*) and the eurasian sparrowhawk (*Accipiter nisus*) are considered the most abundant birds of prey (Svensson et al., 2017).

In Portugal, about 32 birds of prey are registered. Within these species, the common buzzard, eurasian sparrowhawk, common kestrel and the little owl are the diurnal raptors that most often enter animal recovery centers (Martins et al., 2016).

The common buzzard (*Buteo buteo*) (Fig.2), is a common resident species, with longevity in the wild state of 28 years, of medium size with broad wings and neck and fan tail. It has a brown plumage ranging from dark to light, a very striped gray-white tail and a light bar at the bottom of the chest.

It nests in trees and breeds in forests or small woods with access to open fields, meadows or swamps, feeding on birds, rabbits, insects, amphibians or reptiles.

Juveniles do not have bars on the edge of the tail and the lower parts have coarse stripes. Females are identical to males but slightly larger (Naccari et al., 2009; Svensson et al., 2017).

Figure 2: *Buteo buteo* specimen
Source: https://badoca.com/butio-comum-ou-aguia-de-asa-redonda/

The eurasian sparrowhawk (*Accipiter nisus*) (Fig.3) is a short, wide-winged bird of prey with rounded tips. Like *Buteo buteo* it is a resident species, feeding on small birds through surprise attacks and fast flights. Breeds in forests and near villages and may opt for large parks or gardens and can live for at least 16 years. Males have gray upper parts with bluish matrices and reddish-brown face.

On the other hand, females have gray upper and brownish gray lower parts. Their dimensions are larger than males and sometimes confused with juveniles (Svensson et al., 2017).

Figure 3: *Accipiter nisus* specimen
Source:https://www.shutterstock.com/video/clip-16174147-eurasian-sparrowhawk-accipiter-nisus-sitting-on-branch

The common kestrel (*Falco tinnunculus*) ((Fig.4) is a medium-sized hawk with long wings and a longevity of 16 years. Its food consists on mongoose and insects and is found in open fields, plains, urban or flooded areas. Males have grey-blue rump and upper face, tail without stripes and broad tail with dark terminal bar. Females have brown rump and face with tail terminal bar. Juveniles are similar to females with orange colour (Svensson et al., 2017).

Figure 4: Male *F.tinnuculus* specimen
Source: https://www.flickr.com/photos/faraon06271/30796311657

Finally, the little owl (*Athene noctua*) (Fig.5) is a fast-flying sedentary bird of prey that breeds in low, open areas mixed with meadows, fields and vineyards and a longevity in the wild up to 15 years. Usually nests in tree holes or buildings and feeds on insects, birds, small amphibians and leftovers. It has a small, compact, brown upper body with white specks, thin at the crown of the head and more evident at the back. Males and females don't have visible differences and juveniles have a more vivid plumage pattern without white spots on the crown (Svensson et al., 2017).

Figure 5: *Athene noctua* adult specimen
Source: https://badoca.com/mocho-galego/

All species are considered diurnal, exhibiting a more active behavior during the day, except for the little owl that may be partially nocturnal.

According to Svensson et al., (2017), the common buzzard, little owl and common kestrel are distributed throughout the Portuguese territory, while the eurasian sparrowhawk is mostly in the north and center of the country.

1.5. Objectives

The present study focussed on analysing mercury concentrations in several tissues of four different birds of prey: *Buteo buteo*, *Accipiter nisus*, *Falco tinnunculus* and *Athene noctua* and determine if there are significant differences between organs and species. The main aim of this study was to provide new data on the mercuy concentration of terrestrial birds and evaluate if there's a connection with parameters such as age and gender.

2. Methods

2.1. Sample Preparation

Samples were obtained from the Ecology, Monitoring and Recovery Center of Wild Animals (CERVAS) and came from individuals found dead or later euthanized due to irreversible injury or disease.

A total of 56 birds (21 *Falco tinnunculus*, 17 *Athene noctua*, 13 *Buteo buteo* and 5 *Accipiter nisus*) were provided by CERVAS and RIAS (Ria Formosa's Wildlife Research and Recovery Center).

For a thorough evaluation of contaminant distribution, samples were taken from 10 different tissues: nails, beak, feathers, liver, kidneys, lungs, heart, muscle, brain and skin (Figure 6) and measures, gender, age and body condition (analyzing the muscle condition around the keel, according to the scale of Coles, (1997)) determined (Attachment I). For feather analysis, flight feathers were collected.

The samples were separated and placed in aluminum foil, individually, stored in bags and then frozen. Samples were freeze-dried using a Unicryo MC 4L -60 ° C freeze dryer (Uniequip, Germany) through dry ice and manually homogenized prior to analyses. As hard tissues, nails and beaks were ground with an electric mill, before further analysis.

Figure 6: Demonstrative image of skin collection in *Buteo buteo*

2.2. Mercury quantification in bird tissues

Total Hg concentration was determined in all samples by thermal decomposition, followed by atomic absorption spectroscopy, using an AMA 254 Advanced Mercury Analyzer (Altec, Czech Republic) with the software WinAMA.

The samples were weighed directly into nickel boats and inserted into the equipment.

Weighed values ranged from 0.5 to 6 mg to work within the areas covered by the internal calibration curves (1st curve between 0.1 and 30 ng Hg and the second calibration curve from 100 to 500 ng Hg).

In analysis process, the samples are subjected to a drying period at 120 ºC followed by thermal decomposition at 750 ºC under oxygen flow. Decomposition products are then carried by an oxygen stream and retained in a gold amalgamator. Mercury is subsequently released upon being subjected to brief heating to 950 °C and finally transported through a cuvette heated to 120 °C. Quantitation is performed on a UV radiation detector at 253.6 nm by atomic absorption spectroscopy.

The accuracy of the method was monitored by analyzing a certified reference material: TORT-3 lobster hepatopancreas (lobster hepatopancreas) purchased from NRCC-IAEA, Canada, whose certified Hg concentration is 0,29 ± 0.06 mg / kg.

Daily measured values for this material were always within a range of less than 10% from the reference value.

Mostly 2 repetitions were performed per sample, but in some more heterogenous samples 5 repetitions occurred to obtain the average values for each tissue presented in Attachment II.

2.3. Statistical analysis of data

Statistical analysis were performed by SPSS V.25 software.

Data was tested for normality with Shapiro Wilk test, and nonparametric tests used whenever this assumption was not met.

During the descriptive analysis of the data, outliers were identified and eliminated in two species, reducing the number of individuals of *B. buteo* (11), *F. tinnunculus* (20) and *A. noctua* (15).

Correlations between tissues were calculated using Spearman's test, except for *A. nisus* due to insufficient data.

Friedman´s test was used for comparisons between tissues in each species, and differences between species were tested using the Kruskal-Wallis test for independent samples.

To analyze the effects of gender and age on tissue mercury concentrations, individuals were initially classified as male/female, adult/subadult or undetermined. However, for a correct and objective analysis, individuals classified as undetermined were removed. The effects of these parameters were performed by the Mann-Whitney test for independent samples. This parameters could not be analyzed in *A. nisus* due to insufficient number of individuals

3. Results and Discussion

3.1 Mercury concentration in tissues

Mercury concentration (dry weight, dwt) in tissues ranged from 0.06 µg/g to 8.8µg/g in *Buteo buteo*; 0.32 µg/g and 10 µg/g in *Accipiter nisus*; 0 µg/g and 4.7 µg/g in *Falco tinnunculus* and 0.02 µg/g and 1.7 µg/g in *Athene noctua,* where the extreme values for each tissue are highlighted in tables 1 to 4.

High intra-specific variation was observed for all species, with up to as high as 500x difference between the least and most contaminated individual of each species, which may hamper comparisons between tissues and species.

Table 1: Total Hg concentrations in *Buteo buteo* tissues (µg/g, dry weight) - Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

N	B	F	L	H	M	K	Br	S	Lv
1.4	2.7	0.71	0.47	0.30	0.22	0.79	0.43	0.10	0.70
0.73	2.3	0.63	0.60	0.64	0.35	1.0	0.45	0.19	0.90
0.66	**0.17**	0.66	**0.26**	0.31	0.25	0.43	**0.24**	0.22	0.46
2.2	2.1	1.9	1.2	1.7	1.2	2.6	0.85	0.62	3.3
3.4	3.4	2.3	0.54	0.71	0.47	1.6	0.52	0.26	0.87
5.3	**8.2**	0.75	**1.9**	**2.0**	**1.5**	**8.8**	**1.9**	0.71	2.3
1.4	0.68	**4.5**	0.35	**0.18**	**0.16**	**0.42**	0.27	**0.06**	**0.44**
2.5	2.4	2.3	1.8	1.8	**1.5**	4.2	1.5	**0.81**	**4.3**
4.0	4.9	2.1	0.42	0.68	0.49	2.5	0.66	0.33	0.74
2.3	5.7	2.1	1.4	1.4	0.98	3.3	0.98	0.64	1.6
3.7	4.2	**0.30**	1.2	1.2	0.82	1.4	0.92	0.36	1.7

Table 2: Total Hg concentrations in *Accipiter nisus* tissues (µg/g, dry weight) - Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

N	B	F	L	H	M	K	Br	S	Lv
6.2	**10**	**6.3**	**5.7**	**4.4**	**3.1**	**7.7**	**3.0**	**2.5**	**9.2**
4.8	3.5	2.1	1.9	2.1	1.2	3.5	1.1	1.1	4.0

4.7	6.9	3.0	2.5	2.8	1.3	4.0	1.2	0.85	4.5
2.0	**2.4**	3.0	**0.57**	**0.55**	**0.36**	**0.99**	**0.32**	**0.60**	**0.74**
5.6	3.9	**1.4**	1.5	1.1	1.0	1.8	0.95	0.82	1.7

Table 3: Total Hg concentrations in *Falco tinnunculus* tissues (µg/g, dry weight) - Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

N	B	F	L	H	M	K	Br	S	Lv
4.5	**4.7**	**3.9**	1.8	2.0	**1.4**	2.6	0.79	0.85	3.0
3.4	3.3	0.30	0.81	1.2	0.73	1.6	0.71	0.43	1.6
1.8	0.45	1.6	0.56	1.0	1.1	1.6	0.39	0.51	2.2
1.6	1.6	2.0	0.33	0.43	0.35	0.59	0.27	0.35	0.78
1.2	1.2	2.2	0.33	0.37	0.24	0.64	0.22	0.26	0.89
1.5	1.6	1.8	0.73	0.77	0.64	1.0	0.35	0.33	1.5
0.63	0.16	0.53	0.33	0.29	0.27	0.42	0.16	0.19	0.85
0.36	0.37	0.56	0.16	0.20	0.16	0.33	0.09	0.08	0.43
2.4	1.1	3.3	0.75	0.93	0.71	1.4	0.20	0.59	2.0
1.6	1.6	0.87	0.67	0.80	0.65	1.2	0.58	0.47	1.4
3.5	3.9	**0.29**	**2.3**	1.7	1.3	2.8	**0.95**	**1.0**	2.9
3.7	3.4	4.0	1.3	1.1	1.2	2.4	0.25	0.81	3.0
1.8	2.7	0.30	0.13	0.13	0.32	0.37	0.11	0.06	0.22
0.34	0.51	0.48	0.06	0.07	0.05	0.10	0.07	0.04	0.09
1.6	0.96	1.34	0.71	0.65	0.63	0.81	0.53	0.34	0.99
1.1	1.3	1.4	0.10	0.11	0.09	0.15	0.09	0.09	0.15
2.6	4.1	3.7	1.3	**2.2**	**1.4**	**3.7**	0.89	0.70	**3.6**
0.98	0.99	0.48	0.13	0.15	0.14	0.21	0.09	0.07	0.20
0.15	**0.07**	1.0	**0.03**	**0.02**	**0.03**	**0.04**	**0.01**	**0.03**	**0.08**
0.98	1.17	**0.29**	0.46	0.63	0.51	0.87	0.28	0.35	0.97

Table 4:Total Hg concentrations in *Athene noctua* tissues (µg/g, dry weight) - Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

N	B	F	L	H	M	K	Br	S	Lv
0.58	0.70	0.32	0.24	0.27	0.32	0.38	0.31	0.16	0.35
1.3	**1.5**	**1.7**	0.38	0.34	0.38	0.37	0.30	0.20	0.56
0.26	0.27	0.27	0.14	0.13	0.09	0.11	0.06	0.06	0.24
0.36	0.49	0.21	0.16	0.15	0.11	0.24	0.12	0.04	0.21
0.18	0.22	0.23	0.06	**0.03**	0.05	0.11	0.04	0.02	0.08

0.20	0.09	0.28	0.04	0.05	**0.02**	0.06	0.03	0.03	0.11
0.92	0.54	0.35	0.11	0.14	0.06	0.11	0.10	0.07	0.20
0.87	0.95	0.59	0.31	0.27	0.17	0.49	0.50	0.06	0.48
0.17	0.07	0.62	**0.03**	0.06	**0.02**	0.05	**0.02**	0.04	0.09
0.79	1.0	0.27	0.20	0.16	0.09	0.31	0.16	0.06	0.27
0.71	0.69	0.79	0.42	0.48	**0.39**	0.50	0.28	0.18	0.75
0.19	0.17	0.73	**0.03**	0.03	**0.02**	0.04	0.04	0.02	0.05
1.7	0.81	0.83	**0.64**	**0.67**	0.44	**0.89**	**0.54**	**0.35**	**1.1**
1.3	1.2	0.88	0.25	0.29	0.24	0.32	0.43	0.15	0.43
0.12	**0.04**	**0.20**	**0.03**	**0.03**	**0.02**	**0.04**	**0.02**	**0.00**	**0.02**

Generally, positive correlations were observed between tissues, with internal organs having stronger correlations between them (Tables 5 to 7). Stronger correlations are highlighted in the tables.

Although positive and mostly significant, keratinized structures presented lower correlations coefficients, with emphasis on feathers that represent the structure with the lowest values. In the case of common buzzard, feathers were found to have a non-significant correlation with internal organs.

Table 5: Spearman r correlation between *Buteo buteo* tissues - Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

	N	**B**	**F**	**L**	**H**	**M**	**K**	**Br**	**S**	**Lv**
N	1.000	0.839	0.203	0.615	0.720	0.734	0.755	0.804	0.706	0.587
B		1.000	0.042	0.643	0.615	0.629	0.720	0.769	0.608	0.490
F			1.000	0.028	0.077	0.056	0.196	0.098	0.112	-0.028
L				1.000	0.930	0.916	0.902	0.944	0.881	0.937
H					1.000	0.993	0.958	0.951	0.972	0.944
M						1.000	0.965	0.958	0.979	0.937
K							1.000	0.951	0.951	0.874
Br								1.000	0.944	0.902
S									1.000	0.902
Lv										1.000

Table 6: Spearman r correlation between *Falco tinnunculus* tissues - Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

	N	B	F	L	H	M	K	Br	S	Lv
N	1.000	0.855	0.449	0.874	0.866	0.906	0.878	0.786	0.871	0.856
B		1.000	0.347	0.738	0.708	0.734	0.717	0.722	0.703	0.668
F			1.000	0.427	0.431	0.436	0.435	0.270	0.483	0.515
L				1.000	0.974	0.962	0.966	0.900	0.942	0.962
H					1.000	0.981	0.987	0.913	0.953	0.978
M						1.000	0.988	0.900	0.947	0.978
K							1.000	0.904	0.957	0.988
Br								1.000	0.862	0.857
S									1.000	0.944
Lv										1.000

Table 7: Spearman r correlation between *Athene noctua* tissues - Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

	N	B	F	L	H	M	K	Br	S	Lv
N	1.000	0.896	0.626	0.846	0.850	0.818	0.807	0.879	0.870	0.843
B		1.000	0.542	0.857	0.821	0.825	0.814	0.889	0.803	0.832
F			1.000	0.536	0.602	0.545	0.497	0.502	0.707	0.609
L				1.000	0.939	0.979	0.961	0.936	0.878	0.968
H					1.000	0.936	0.925	0.875	0.935	0.971
M						1.000	0.957	0.914	0.906	0.957
K							1.000	0.932	0.874	0.943
Br								1.000	0.828	0.893
S									1.000	0.928
Lv										1.000

Overall, the highest Hg concentrations were obtained in nails, beak, feathers, liver and kidney, while the lowest concentrations were in muscle, brain and skin (Figures 7 to 10).

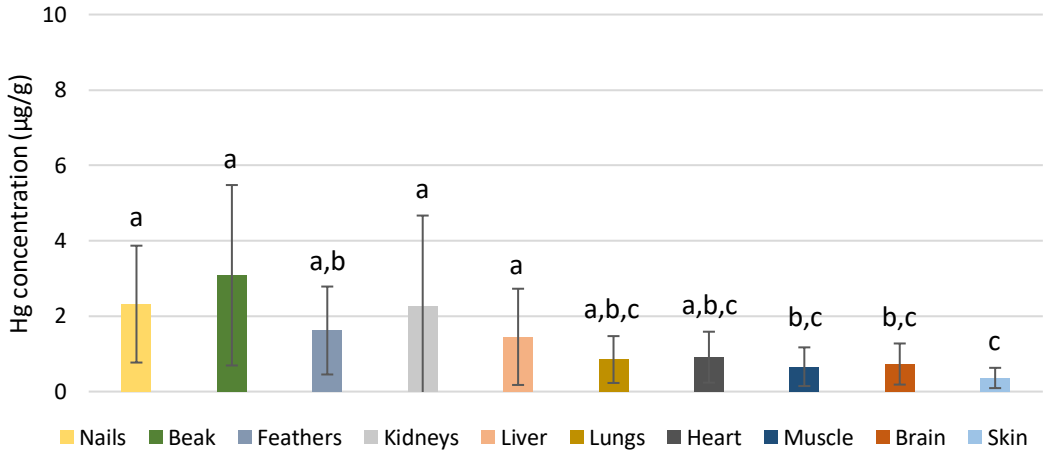

Figure 7: Average concentration of Hg (µg/g) in the tissues of Buteo buteo species.

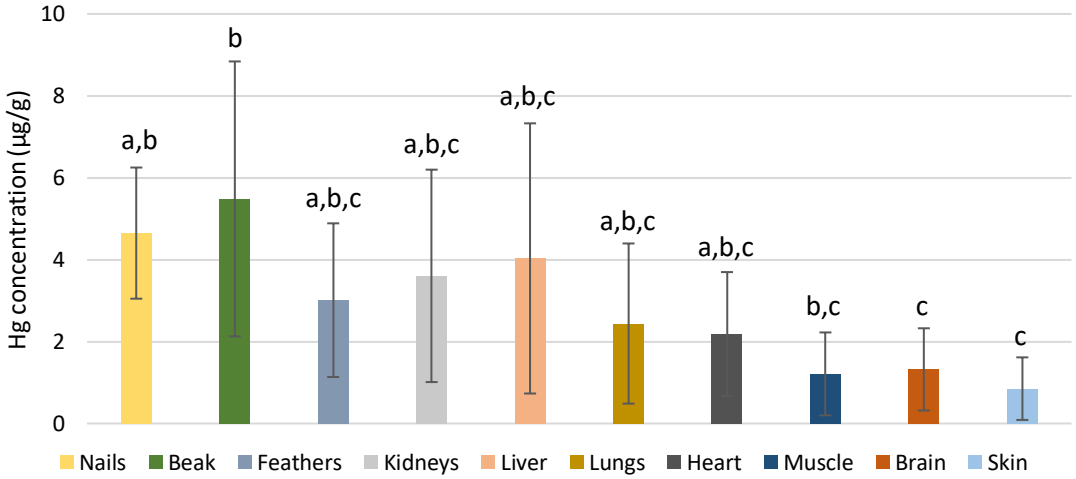

Figure 8: Average concentration of Hg (µg/g) in the tissues of *Accipiter nisus* species.

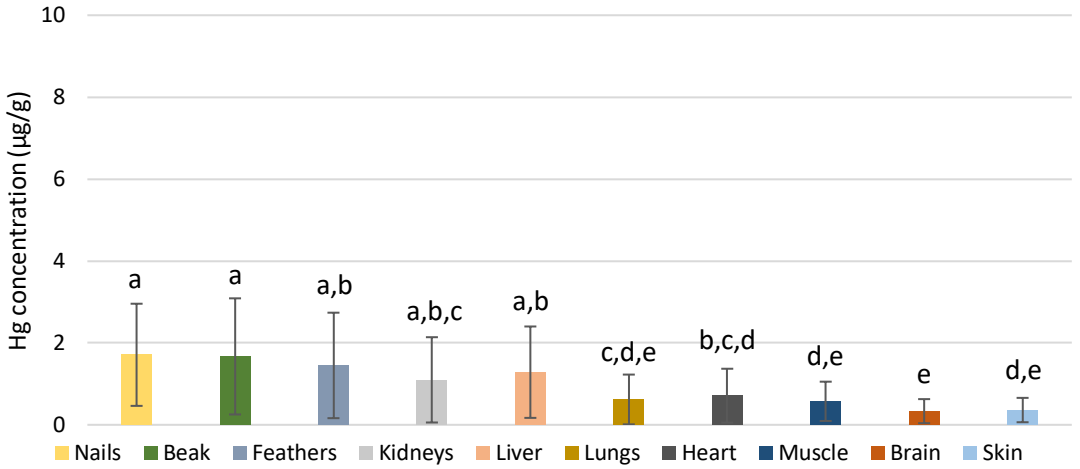

Figure 9: Average concentration of Hg (µg/g) in the tissues of *Falco tinnunculus* species

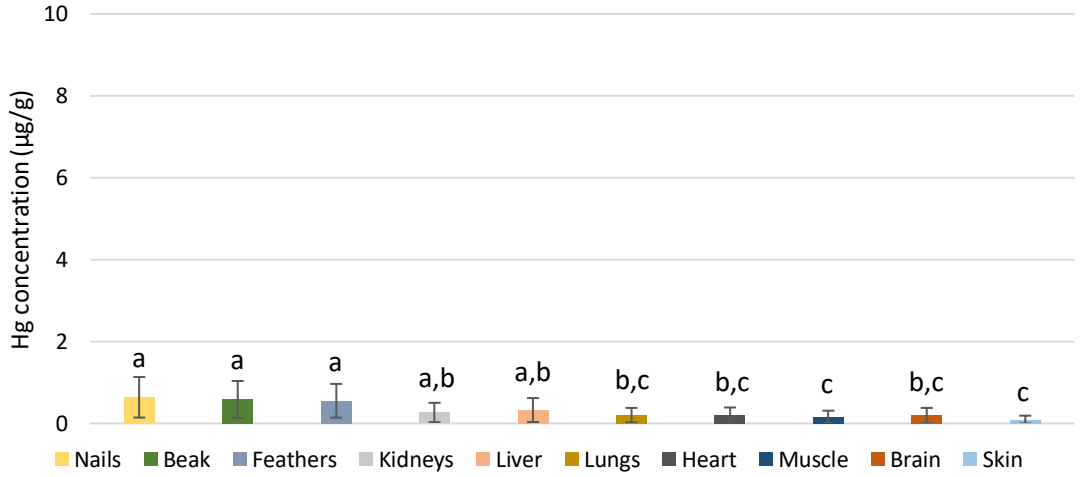

Figure 10: Average concentration of Hg (µg/g) in the tissues of *Athene noctua* species.

Despite differences in overall concentrations between species, a common tendency was observed for the mercury distribution between the different organs, which was supported by Friedman's test for related samples ($p<0.05$). These significant differences were mostly observed between keratinized structures, liver and kidneys and the organs with lower concentrations.

In several studies, feathers and nails have been identified as important Hg elimination pathways. The concentration tends to increase during its formation and

growth, possibly justifying the levels obtained in these matrices (Hopkins et al., 2007; Lewis & Becker, 1993).

However, growth tends to be different between structures. Nails have recently been considered more reliable matrices than feathers as a result of their continuous growth, indicating recent mercury concentrations in a given individual (Low et al., 2019).

These specific traits may influence the total concentrations of Hg and justify the differences observed between the two tissues in some species, such as *B.buteo* where the average amount of Hg in nails is higher than that observed in feathers.

However, the non-significant correlation between feathers and internal tissues observed in the present study is not common in the literature. Since feathers only reflect concentration at the time of their formation, molting is the most viable explanation. According to some authors, after the molt period is finished, the concentration of mercury in feathers remains constant until the next molt and therefore any changes in Hg exposure and accumulation will be more evident and reliable in internal organs (Burger & Gochfeld, 1993; Dauwe et al., 2000). The present results support the idea that blood and internal organs are the best indicators to evaluate the exposure to contaminants in dead animals (García-Fernández et al., 1997).

Due to its nature, the beak is expected to grow and respond in the same way as the nails.

The highest concentrations in the liver and kidneys reflect their important metabolic role in the mercury detoxification and elimination. Hg detoxification and storage by liver and accumulation and excretion in the urine by kidneys explain the values presented and the strong correlation between them ($r_{B.buteo}$ = 0.874; $r_{F.tinnunculus}$= 0.988; $r_{A.noctua}$= 0.943) (Hopkins et al., 2007; Mansouri & Majnoni, 2014; Rodrigues et al., 2014; Szymczyk & Zalewski, 2003).

The lowest mercury concentrations were observed on the skin, brain and muscle. In skin, these values are supported by a study by Honda et al., (1986b) in black-eared kite. However, in another study performed by (Honda et al., 1986a) in eastern great white egret, the skin was reported to have the highest Hg concentrations, suggesting that the role and accumulation ability on the skin will be strongly species-related.

Brain concentrations were low and in accordance with the literature found. The presence of the blood-brain barrier, which separates two brain interfaces, and its selective diffusion, grants protection of the central nervous system (CNS) by blocking

the entry of contaminants such as Hg. However, this barrier cannot block all contaminants or possible dangers and may lead to their partial passage, which leads to the detection of mercury in this tissue (Zheng et al., 2003).

Muscle showed positive correlations with the heart, possibly because of their similar structure ($r_{B.buteo}$= 0.993; $r_{F.tinnunculus}$= 0.981; $r_{A.noctua}$= 0.936) (D'amil, 2017). However, generally the muscle was found to have lower contamination. March et al., (1983) explains these results may occur from Hg dilution during tissue growth or loss, resulting from protein degradation by turnover processes.

In comparison, the heart also has strong correlations with the lungs, where both organs show intermediate concentrations in relation to the other tissues ($r_{B.buteo}$= 0.930 ; $r_{F.tinnunculus}$= 0.974 ; $r_{A.noctua}$= 0.939).

According to the literature found, these organs do not represent the most commonly used matrices for mercury detection, perhaps by requiring lethal sampling techniques or by the observation of relatively low values that may not be representative of the exposure to mercury in the species under study.

In a study by Santos (2018) in waterfowl from the Madeira's river basin, in Brazil, average mercury concentration in these matrices showed values of 0.34 µg/g for lungs and 0.30 µg/g for heart. In this study, average values of 0.88 µg/g and 0.87 µg/g are presented for the same organs of all species, respectively. The higher concentrations observed may probably be explained by the higher trophic level of the species of the present study.

3.2. Mercury concentration between species

Figure 11 shows Hg levels from different organs present in the species under study differ from each other, which was supported by Kruskal-Wallis test for independent samples ($p<0.05$).

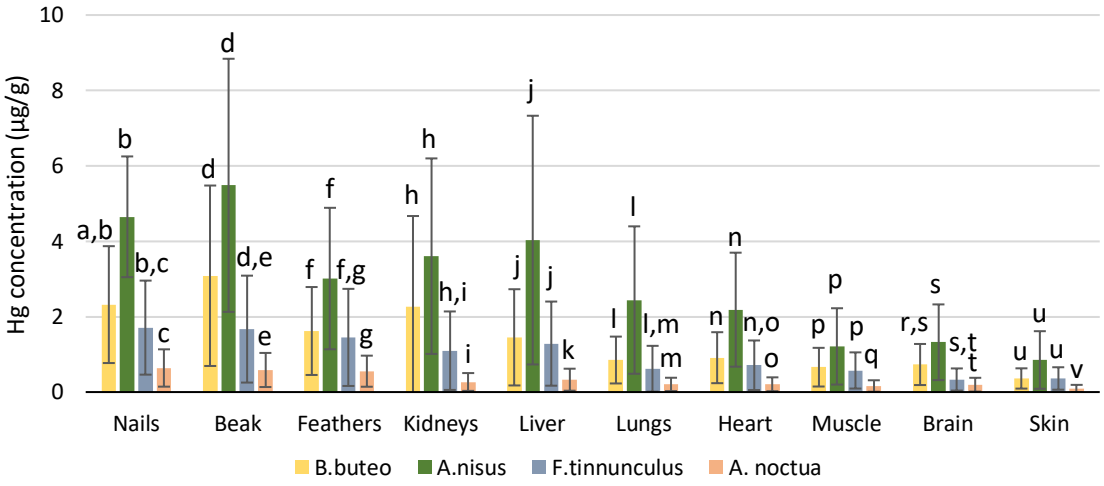

Figure 11: Comparison of mercury between tissues of the species under study

Analyzing the average mercury concentrations, the values are decreasing in the order *A. nisus* > *B. buteo* > *F. tinnunculus* > *A. noctua*, but significant differences were observed only between *A.noctua* and the species *B. buteo* and *A. nisus*.

Higher concentrations were found in eurosian sparrowhawk and common buzzard reflecting possible species-specific differences between species, such as their diet, as a factor that most influences Hg concentration in tissues. *A.nisus* feeds on several small birds and *B.buteo* can feed on mammals, birds and amphibians, that occupy different trophic levels and show different metal concentrations. An explanatory hypothesis for one of the highest concentrations found in *B. buteo* relies on the longevity of this species, considerably higher than the others (up to 28 years, while the life expectancy of the three other species is around 15-16 years). Mercury accumulation is a continuous process, and generally older individuals tend to have higher Hg concentrations (Thompson et al., 1991). However, the possible explanation for the

differences between species (and the high intra-specific variability) and the higher values found in *A. nisus* are the possible geographical differences or regional mercury hotspots, which may influence the results obtained. Similar findings were reported by Grúz et al., (2018) with the highest concentrations found in eurasian sparrowhawk in a study on wild birds in Hungary.

3.3. The effect of gender on mercury concentration

Although several studies report the possibility of contaminant excretion through eggs, which could justify differences in mercury contamination between genders, in the present study this was not observed (Mann-Whitney U test $p>0.05$) (Burger, 2007).

Similar findings were observed by Tavares et al., (2013), Castro et al. (2011) and Martínez et al., (2012) , where the authors did not observe significant differences between male and female feathers, livers or kidneys.

In another studies such as Dietz et al., (2006) and Robinson et al., (2012) were observed that sex may affect feathers Hg accumulation, and obtained different conclusions in relation to gender with higher concentrations. Dietz et al., (2006) concluded that females had higher Hg concentrations than males in peregrine falcon species, possibly related to the larger female dimensions and their ability to prey larger birds. However, Robinson et al., (2012) obtained more expected results and males showed higher values than females, supporting the hypothesis that eggs are an extra Hg excretion mechanism.

3.4. The effect of age on mercury concentration

According to the literature found, adult birds may concentrate a higher Hg content than juveniles, which could result from differences in diet, since adult birds have different feeding habits, or bioaccumulation as age increases (Burger, 1994; Frias et al., 2012; Gochfeld et al., 1996).

Mann-Whitney test showed significant differences between ages in common kestrel feathers ($p<0.05$). Contrary to what could be expected, subadults presented higher concentrations than adults, which is in accordance with a study by Gochfeld et al., (1996), where mercury concentration decreased with age. According to the author, birds since first year-old and adult stage, can eliminate higher concentrations by molting. Another explanatory hypothesis relies on the fact that not all samples were collected in the same geographical area, and therefore possible regional differences may hamper the comparison between ages.

Results for the common buzzard and the little owl, showed no significant differences between the age groups, similarly to Castro et al., (2011), Houserova et al., (2005) and Stout & Trust (2002), suggesting that age may not be a significant factor for the accumulation of mercury in these species.

4. Conclusions

In the present study, differential tissue accumulation and a common distribution was observed in the four studied species.

Highest concentrations were observed in keratinized tissues, liver and kidneys and the lowest concentrations in the remaining organs.

Statistical tests showed significant differences between tissues of the same species and between species, where *A.nisus* presented the highest values.

Although strong positive correlations generally occur, keratinized structures had the lowest correlative values compared to soft tissues.

The feathers were the matrix with the lowest correlations and in the case of *B.buteo* considered non-significant. Thus, it is possible to state that in the present study feathers are not considered the best matrices for the determination of internal tissue mercury concentrations and subsequent use for monitoring.

Parameters such as gender did not show significant differences between species, although theres was a possibility of difference in mercury contamination due to contaminant excretion through eggs. In common kestrel, were observed differences related to age in feathers, were subadults showed higher concentrations than adults. Possible confounding factos such as collection site or molting may hamper these findings, and justify further research. For the remaining bird species, no significant differences were identified for the variation of accumulation with age.

Although the internal organs are the most reliable structures for representing the actual concentration of mercury in the organism, their collection would imply the sacrifice of the individual for further analysis. For monitoring purposes, nails or blood are considered most suitable for determining mercury concentration and possible contamination of the food chain and ecosystems, as an alternative to feathers.

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Attachments

Attachment I.1:Information on individuals assigned to the species *Buteo buteo*

Specie	Body condition	Gender	Age
V505/17	3	Male	Adult
V558/18	3	Female	Adult
V327/17	1	Undetermined	Undetermined
V456/18	2	Female	Sub-adult
V199/18	4	Male	Adult
V577/18	3.5	Female	Adult
V007/19	2	Male	Sub-adult
V010/19	1.5	Male	Sub-adult
M021/19	1.5	Male	Sub-adult
V032/18	3	Female	Adult
M030/18	3	Male	Adult
V029/18	4	Male	Adult
V035/19	3	Female	Adut

Attachment I.2:Information on individuals assigned to the species *Accipiter nisus*

Specie	Body condition	Gender	Age
V116/18	1.5	Male	Sub-adult
M498/17	3	Male	Adult
M048/19	2	Male	Sub-adult
M059/19	4	Female	Sub-adult
V051/19	1	Male	Adult

Attachment I.3:Information on individuals assigned to the species *Falco tinnunculus*

Specie	Body condition	Gender	Age
M232/18	2	Undetermined	Undetermined
M003/19	2	Female	Adult
M019/19	1.5	Undetermined	Sub-adult
M020/19	2.5	Undetermined	Sub-adult
V0854/16	4	Undetermined	Sub-adult
V0418/16	5	Female	Adult
V0224/16	1.5	Female	Adult
V0342/14	3	Undetermined	Undetermined
M0432/14	1	Undetermined	Undetermined
V0403/14	2	Undetermined	Sub-adult
M0941/16	1	Undetermined	Undetermined
V0180/16	5	Female	Adult
M1955/16	2.5	Undetermined	Undetermined
V0206/17	1	Undetermined	Undetermined

V0010/15	3	Undetermined	Undetermined
V0541/16	2	Male	Adult
V0421/14	1.5	Undetermined	Undetermined
V0517/14	3.5	Undetermined	Sub-adult
V0374/16	3	Male	Adult
V0587/16	3	Undetermined	Sub-adult
V0634/18	1	Undetermined	Undetermined

Attachment I 4:Information on individuals assigned to the species *Athene noctua*

Specie	Body condition	Gender	Age
V165/18	5	Male	Adult
M414/18	5	Undetermined	Undetermined
V155/14	4	Female	Sub-adult
V328/18	5	Undetermined	Undetermined
V145/17	1	Undetermined	Undetermined
M300/16	1	Undetermined	Sub-adult
V389/18	1	Male	Adult
M209/17	2	Undetermined	Undetermined
M303/18	3	Female	Sub-adult
M012/19	4	Male	Adult
V242/18	4	Undetermined	Sub-adult
V321/18	4	Male	Adult
M143/17	4	Female	Adult
V428/18	3	Undetermined	Undetermined
V233/18	3	Undetermined	Sub-adult
M549/18	2	Undetermined	Sub-adult
V292/16	3	Male	Sub-adult

Attachment II 1: Average concentrations of Hg (µg/g) in *Buteo buteo* tissues – Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

	N	B	F	L	H	M	K	Br	S	Lv
A	2.32	3.09	1.62	0.85	0.91	0.66	2.26	0.73	0.36	1.45
SD	1.55	2.39	1.17	0.62	0.68	0.51	2.41	0.55	0.27	1.28

Attachment II 2: Average concentrations of Hg (µg/g) in *Accipiter nisus* tissues – Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

	N	B	F	L	H	M	K	Br	S	Lv
A	4.65	5.48	3.01	2.44	2.19	1.21	3.61	1.32	0.85	4.03
SD	1.60	3.36	1.88	1.96	1.51	1.01	2.59	1.00	0.76	3.30

Attachment II 3*:* : Average concentrations of Hg (µg/g) in *Falco tinnunculus* tissues – Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

	N	**B**	**F**	**L**	**H**	**M**	**K**	**Br**	**S**	**Lv**
A	1.71	1.67	1.45	0.62	0.71	0.57	1.10	0.33	0.36	1.29
SD	1.25	1.42	1.29	0.61	0.66	0.48	1.04	0.29	0.30	1.12

Attachment II 4: Average concentrations of Hg (µg/g) in *Athene noctua* tissues – Nails (N), Beak (B), Feathers (F), Lungs (L), Heart (H), Muscle (M), Kidneys (K), Brain (Br), Skin (S), Liver (Lv)

	N	**B**	**F**	**L**	**H**	**M**	**K**	**Br**	**S**	**Lv**
A	0.64	0.59	0.55	0.20	0.21	0.16	0.27	0.20	0.10	0.33
SD	0.50	0.45	0.41	0.18	0.18	0.15	0.24	0.18	0.10	0.29