Universidade de Aveiro Departamento de Química
2012

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Bioacumulação do mercúrio no sacarrabos (*Herpestes ichneumon*)

Mercury bioaccumulation in the Egyptian mongoose (*Herpestes ichneumon*)

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Química Analítica e Qualidade, realizada sob a orientação científica do Doutor João Pedro Martins Coelho, Investigador de Pós-Doutoramento do Departamento de Química da Universidade de Aveiro e co-orientação da Doutora Maria Eduarda da Cunha Pereira, Professora Auxiliar do Departamento de Química da Universidade de Aveiro

Dedico este trabalho aos meus pais pelo apoio incondicional ao longo da minha vida e formação académica.

o júri

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palavras-chave

Herpestes ichneumon, Sacarrabos, Mercúrio total, Mercúrio orgânico, Bioacumulação, Tecidos, Idade, Género, Localização geográfica.

resumo

O sacarrabos (*Herpestes ichneumon*) é um predador que desempenha um papel essencial na cadeia alimentar terrestre. A sua introdução e rápida proliferação pelo território português levaram à necessidade da sua caça como controlo de predadores.

Estudos em espécies predadoras terrestres ainda são escassos, pelo que o presente trabalho representa uma mais-valia para um melhor entendimento da acumulação de contaminantes em níveis tróficos superiores. Existem vários contaminantes que representam uma constante preocupação para o ambiente; entre eles, o mercúrio tem tido uma atenção acrescida devido à sua persistência e toxicidade. Estudos no meio terrestre são importantes para um melhor entendimento da forma como se acumula neste meio, para a preservação da vida selvagem mas também para prevenir a exposição humana ao mercúrio.

Este estudo teve como principal objectivo avaliar os padrões de acumulação de mercúrio nos tecidos do *H. ichneumon*, tendo em atenção factores como o género e a idade.

H. ichneumon de localizações diferentes foram analisados de forma a avaliar a variação geográfica dos níveis de mercúrio em território Português.

Músculo, fígado, pulmão, coração, baço, rins, sangue, cérebro, gordura e pêlo de 29 indivíduos provenientes de 14 localizações foram analisados (Évora, Soure, Ferreira do Zêzere, Castelo Branco, Mértola, Torres Novas, Tondela, Lousã, Coimbra, Montemor-o-Novo, Castro Daire, Olhão, Moura e Coruche). Além disso, de forma a estudar diferenças entre machos e fêmeas ao longo do ciclo de vida da espécie, 25 indivíduos provenientes de Serpa foram analisados.

Os níveis de mercúrio nos diferentes órgãos variaram entre 0.01 e 12.7 µg g^{-1} peso seco, e seguiram geralmente a seguinte ordem, do menos para o mais contaminado: Gordura <Cérebro <Pulmão <Coração <Baço <Músculo <Rins <Fígado <Pêlo <Sangue.

Diferenças entre machos e fêmeas foram apenas significativas para dois tecidos, o sangue e o cérebro. Diferenças entre idades apenas foram verificadas para crias machos, sendo que estas se distinguem dos juvenis, subadultos e adultos.

Nos machos, todos os tecidos (exceto a gordura) evidenciaram ter uma regressão linear entre as concentrações de mercúrio e as idades, com coeficientes de correlação elevados (> 0.74). Verificou-se que os níveis de mercúrio no sangue e no fígado estão fortemente correlacionados (r=0.998).

O mercúrio orgânico presente no tecido muscular dos machos e das fêmeas foi analisado e a sua percentagem variou entre 77 e 98%. A correlação entre mercúrio orgânico e total nos machos foi de 1.000 e de 0.986 nas fêmeas.

Nenhum dos níveis de mercúrio atingiu valores considerados letais ou tóxicos para predadores terrestres (20 a 100 µg g^{-1} peso seco).

keywords

Herpestes ichneumon, Egyptian mongoose, Total mercury, Organic mercury, Bioaccumulation, Tissues, Age, Gender, Geographical location.

abstract

The Egyptian mongoose (*Herpestes ichneumon*), a terrestrial predatory species, has an essential role in the terrestrial food chain. Their introduction in Portugal and rapid proliferation throughout Portuguese territory led to the necessity of their hunt as predator control measure.

Studies in terrestrial predatory species are sparse; thereby, the present study is an asset for a better understanding of contaminants accumulation in higher trophic levels. Many contaminants are of environmental concern; mercury has had increased attention due to its persistence and toxicity. Studies have been mostly directed to aquatic wildlife due to the fact that fish consumption is considered to be the principal route of human exposure to mercury. However, terrestrial wildlife studies are also important for a better understanding of mercury accumulation, wildlife preservation and also for preventing human exposure to mercury.

The main purpose of this study was to evaluate the different tissue accumulation in *H. ichneumon*, as well as differences between males and females, throughout the lifespan of the species. *H. ichneumon*s from different locations were also analyzed in order to compare levels throughout Portuguese territory.

Muscle, liver, lungs, heart, spleen, kidneys, blood, brain, fat and pelage were analyzed for 29 *H. ichneumon* from 14 locations (Évora, Soure, Ferreira do Zêzere, Castelo Branco, Mértola, Torres Novas, Tondela, Lousã, Coimbra, Montemor-o-Novo, Castro Daire, Olhão, Moura and Coruche). In order to study differences between ages, males and females, 25 individuals from Serpa were analyzed.

Total mercury concentrations in *H. ichneumon* tissue samples ranged between 0.01 to 12.7 μg g^{-1} dw, and followed the order, from least to most contaminated: Fat <Brain <Lungs <Heart <Spleen <Muscle <Kidneys <Liver <Pelage <Blood. Differences between males and females were only significant for blood and brain mercury levels. Differences between ages only were significant for males, in cubs towards juveniles, subadults and adults.

With the exception of fat, all the tissues of *H. ichneumon* males revealed a linear regression between mercury concentrations and age, with high correlation coefficients (> 0.74), not visible in females. Moreover, correlations between organs showed that blood and liver were highly correlated (r=0.999).

Organic mercury in muscle tissue of males and females was analyzed and its percentage ranged from 77% to 98%. Correlation between organic and total mercury in males was 1.000 and 0.995 for females.

None of the mercury levels reached the lethal or toxic values established for terrestrial predators (20 to 100 μg g^{-1} ww).

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1 - Introduction and aims of the study

Metal pollution in air, water and soil has been generated for decades by anthropogenic activities. The production of mercury through mining activities increased over time, and its subsequent release into the environment has been of some concern, given their well-known toxic effects on biological systems. Ecosystems act as a huge and effective filter, retaining contaminants in aquatic and/or terrestrial systems. However, such exposure increases its bioavailability to wildlife and humans, bringing poisoning risks to both (Sánchez-Chardi *et al.*, 2007).

Most investigations related with mercury bioaccumulation and biomagnification have focused on freshwater ecosystems, where methylation conditions are highly promoted and Hg concentrations in upper trophic levels are generally high (Rimmer *et al.*, 2010). The mercury biogeochemical cycle and bioaccumulation processes in terrestrial ecosystems are not as thoroughly studied, justifying further research to assess the risk of mercury toxicity to biota and humans.

1.1 - Mercury

Mercury (Hg) is a naturally occurring element. Also called quicksilver, it is a heavy, odorless metal belonging to group 12 of the Periodic Table (Kirk and Othmer, 1998). It is one of the most toxic metals found in the global environment, whose main sources of emissions may be natural, anthropogenic and re-emitted (Zhang and Wong, 2007).

It is naturally found dispersed at very low concentrations (parts per billion – ppb) in soils, sediments, natural waters and atmosphere, as well as in plants and animals (Chrystall and Rumsby, 2008). A small amount of mercury is naturally contained in rocks, sediments and soils, however, some local mineral occurrences and thermal springs are naturally rich in mercury (USGS, 2000). The chemical forms of mercury in the environment include sorbed mercury species, organic (carbon-containing) and inorganic compounds, and mercury vapor (Chrystall and Rumsby, 2008).

As mercury is an element, it cannot be broken down or degraded to harmless substances. It may change between different states and chemical species in its cycle, but its simplest form is elemental mercury, which itself is harmful to humans and the environment. Therefore, it won't "disappear" again in time spans comparable to human lifetime, and will be recycled permanently through physical, chemical and biological processes in the environment (UNEP, 2010).

1.1.1 - Main properties, uses and environmental contamination sources

Many uses of mercury were discovered by mankind over time, being used throughout the millennia dating back to some of the earliest recorded civilization (Clarkson and Magos, 2006). The first recorded mention of mercury was by Aristotle in the fourth century BC, at a time when it was used in religious ceremonies. Samples of mercury have been found in graves dating to the fifteenth or sixteenth century BC, when cinnabar (HgS) was used as a pigment for cave and body decoration (Kirk and Othmer, 1998). In the late fifteenth century AD, the idea that

mercury might be useful for the treatment of syphilis emerged, and that's when the dictum "The dose makes the poison" surged, as they noted that a little mercury might be useful, but too much was fatal (Clarkson and Magos, 2006).

Well known by alchemists of the ancient world because of its high propensity to form stable amalgams with other metals (especially gold and silver), it was widely used during the gold rush (Schuster *et al.*, 2002).

Mostly in the new world, tons of liquid mercury were shipped in order to obtain pure gold, evaporating it by heating with an open flame. The gold extraction from river sediments in the Amazon basin and elsewhere still continues to these days (Clarkson and Magos, 2006).

About 150 years ago, a new kind of dental amalgam was introduced in France, which by weight comprised 50% mercury along with a number of other metals, mainly silver and copper. Literally billions of people today have such fillings (Clarkson *et al.*, 2003). From time to time, concern has been raised about the possibility of mercury poisoning from amalgams. The debate has now reached a critical stage, with some countries starting to restrict their use on the basis of potential health risks (Clarkson and Magos, 2006).

Since the 1950s, metallic mercury's use as an electrode in the chlor-alkali industry has spread worldwide. Over 100 tons of mercury are needed as an electrode in a single plant producing chlorine gas and caustic soda from brine. Such intensive mercury use is reflected in the environment, since this industry produces nearly 90% of European anthropogenic mercury emissions to the atmosphere (Hylander, 2001). Mercury electrode technology is now gradually being phased out in Europe and in the USA and replaced by cleaner technologies. However, emissions from existing Hg-cell plants remain significant in less developed countries where stringent environmental controls are lacking, and decommissioned plants typically leave behind widespread contamination (Brooks and Matos, 2005; Hylander, 2001; Reis *et al.*, 2009; Ullrich *et al.*, 2007).

One of the most terrible examples of mercury accumulation and toxicity was the outbreak of contamination related diseases and death in Minamata Bay in the 1950's. Epidemiological research revealed poisoning to be a result of trophic transfer of mercury into marine food webs, leading to very high metal concentrations in fish and shellfish (Boudou and Ribeyre, 1997; Ekino *et al.*, 2007). Similar poisoning cases derived from the use of mercury as fungicides in agriculture, namely alkylmercury, to treat seeds. (Brooks and Matos, 2005).

Since the beginning of the industrialization era, the release of mercury into the environment has spread worldwide. Anthropogenic sources of mercury pollution (as mentioned above) have led to an increase of about ten-fold in the amount of mercury in the atmosphere since the beginning of the industrial revolution. Annually, the natural emission of mercury into the atmosphere (volcanoes, forests, soils, lakes and open oceans) was about 2000 tons. While the amount of anthropogenic mercury released each year due to combustion and waste incineration was about 2000-2200 tons, and the amount of previously deposited mercury re-emitted was about 2000 tons (Zhang and Wong, 2007).

Despite the many attractive and useful uses of mercury, it presents a high risk of toxic effects. The challenge faced today is to take advantage of these useful applications while at the same time assuring no adverse health effects occur. A key role for the toxicologist is to develop an understanding of its toxic properties so as to give advice to users and regulatory agencies to ensure that safe levels of exposure are not exceeded (Clarkson and Magos, 2006).

1.1.2 - Biogeochemical cycle

Mercury can be found in three different oxidation states: elemental mercury (Hg^0 or mercury (0)), mercurous mercury (Hg^+ or mercury (I)) and mercuric mercury (Hg^{2+} or mercury (II)), which can be interconverted in the environment. The last two can form several chemical compounds: organic and inorganic. Although, compounds

formed from mercuric mercury (Hg^{2+}) are much more common than those formed from mercurous mercury (Hg^{+}) (Nascimento and Chasin, 2001) (Figure 1).

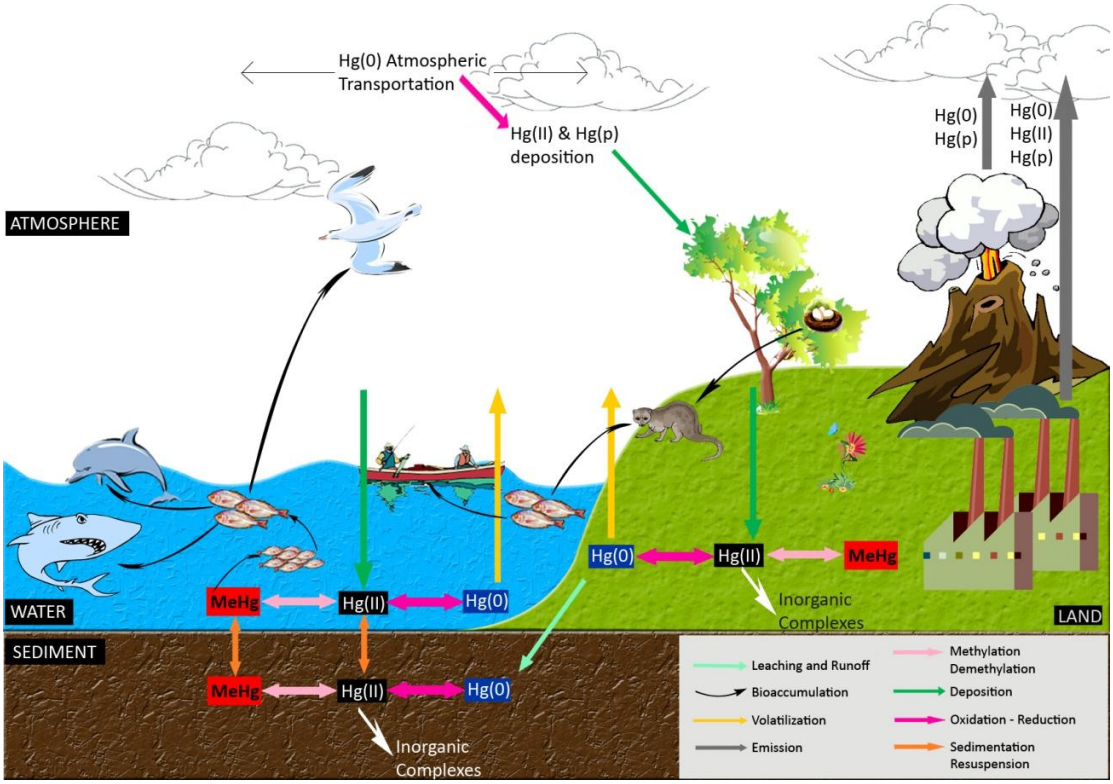

Figure 1 - Overall biogeochemical mercury cycle (Adapted from Sacramento (2011))

Elemental mercury is the most volatile mercurial compound. It is liquid at ambient temperature and pressure, although it is not common for mercury to be in its pure liquid form in nature. Elemental mercury can evaporate (volatilize) forming mercury vapor when it is exposed to air. Rates of evaporation increase with increasing temperature. Mercury vapor is colorless, odorless, and very toxic (Chrystall and Rumsby, 2008).

Due to its limited solubility in water, some of the Hg^{0} will be lost from the atmosphere by dry deposition, but the vast majority will be transported over long distances and incorporated in the global atmospheric pool because of its lifetime being 0.5 to 1 year. For the same reason, Hg^{0} is the most abundant form of mercury in the atmosphere. This form of mercury is less available to fish and other living organisms. Yet, it can be transformed to other, highly toxic forms of mercury, including the reactive form (Hg^{2+}) (Alley *et al.*, 2004; Selin, 2009).

Oxidized mercury (Hg^{2+}) is much more soluble in water and reactive than Hg^0 (Table 1), therefore its lifetime in the atmosphere is much shorter (days or weeks) being mostly deposited by wet deposition from a few miles to a few hundred miles from its source (Baeyens *et al.*, 1996).

Table 1 - Some examples of different compounds that may be formed from Hg^{2+}

Fluorides	Chlorides	Bromides	Iodides	Oxides	Sulfides	Nitrates
HgF_2;	$HgCl_2$;	$HgBr_2$;	HgI_2;	HgO;	HgS;	$Hg_2(NO_3)_2$
Hg_2F_2	Hg_2Cl_2	Hg_2Br_2	Hg_2I_2	Hg_2O		

Organomercurials are also an important class of compounds formed from Hg^{2+} bonded to at least one carbon atom. The resulting compounds may be RHgX and RHgR', being R and R' a representation for organic radicals and X a variety of anions. Organomercurials are the most toxic compounds, although the most concerning are those who have a short-chain radical (methyl, ethyl) (Nascimento and Chasin, 2001). Methylmercury (MeHg) is the most common and most concerning in terms of toxicity due to its bioaccumulative properties (Chrystall and Rumsby, 2008; EC, 2011).

Usually, organomercurials are formed via bacteria in lakes, streams, oceans but also in land, converting elemental mercury to its organic compounds (Tchounwou *et al.*, 2003). Of all the mercury forms, organomercurials present the greatest risk to living species, having the capacity to penetrate through cell membranes and react with essential proteins, amino acids and nucleic acids within the cells (Chrystall and Rumsby, 2008; SRWP, 2012). Along the food chain MeHg bioaccumulates and biomagnifies, thus compromising the health of higher organisms, posing a serious threat to humans as well as wildlife (SRWP, 2012).

Once in the environment, elemental, inorganic and organic mercury compounds become part of the biogeochemical cycle of mercury. Biogeochemical cycles are pathways for the transport and transformation of matter within four categorical

areas that make up the planet Earth (biosphere, lithosphere, hydrosphere and atmosphere). These transformations between forms may occur by natural or anthropogenic processes (Figure 2) (Chrystall and Rumsby, 2008; Muhumuza and Hogan, 2011). For example, in the presence of organic matter, elemental mercury (Hg^0) may be oxidized to mercuric mercury (Hg^{2+}) which in turn, may be reduced to Hg^0, being released into the atmosphere. Another natural transformation is methylation of Hg^{2+}, performed anaerobically by bacteria (Boening, 2000).

Generally, the most common form present in the atmosphere is elemental mercury. Organic and inorganic mercury are mostly present in terrestrial and aquatic environments. Even if mercury cannot be decomposed, it can be transformed into non-bioavailable, non-water soluble chemical species (HgS or HgO) allowing mercury removal from the biosphere by deposition in soils or sediments (Chrystall and Rumsby, 2008).

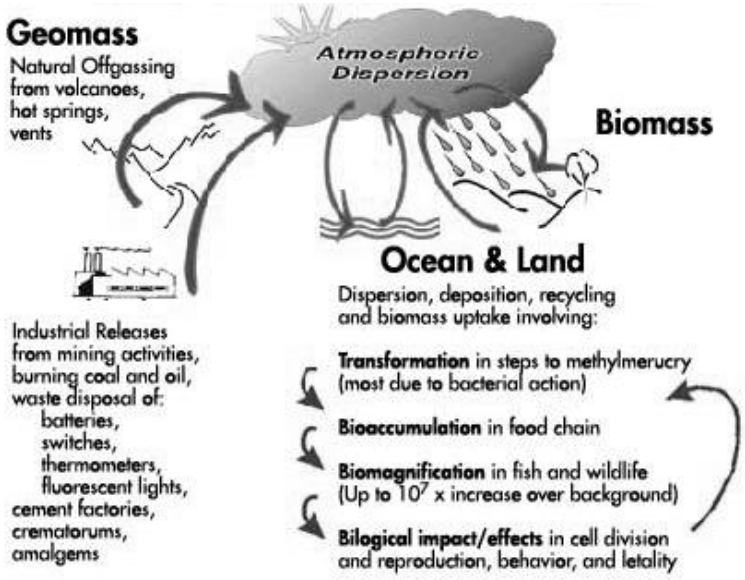

Figure 2 - Subsurface to surface distribution and biomass uptake of mercury in the environment (adapted from Sacramento (2011)).

In the terrestrial environment (similarly to the aquatic) mercury comes mostly from dry and wet deposition/removal processes of mercury emitted from natural sources and/or re-emission of originally released mercury from anthropogenic sources (Chrystall and Rumsby, 2008; Schroeder and Munthe, 1998).

Once incorporated into the soil, mercury can persist for a long time even after sources have been removed (Cachada *et al.*, 2009; Chrystall and Rumsby, 2008). Therefore, soils play an important role in the biological cycle of mercury (Reis *et al.*, 2010).

The different species of mercury have different interactions with the soil matrix, causing changes in solubility, toxicity and bioavailability of the metal (Biester *et al.*, 2002). Han *et al.* (2003), studied the mobility of mercury species in soils being MeHg and EtHg (Ethylmercury) highly mobile, thus more available and toxic than other chemically stable species, such as HgS (cinnabar) which is non-mobile, therefore much less toxic.

Vegetables are a part of the human diet, providing carbohydrates, vitamins, minerals but also trace elements. Leafy vegetables are classified as high elemental uptake crops, and as a part of the human diet, therefore they can affect human health through mercury intake of the mobile fraction by the roots (Reis *et al.*, 2009; Reis *et al.*, 2010).

Animal feeding on plants may also affect them, and as in aquatic environment, mercury concentrations may increase along the food chain (up to predators), thus affecting the entire terrestrial ecosystem.

1.1.3 – Routes of exposure and health effects

Despite the known mutagenic and teratogenic effects of mercury, data on the mechanisms of such effects are very sparse and controversial in the available literature (Tchounwou *et al.*, 2003).

All forms of mercury are toxic to almost all the biosphere, although its adverse effects in an organism may vary depending on its form at the time of exposure and the route of exposure. The toxicity of mercury also depends on its transport and intracellular bioavailability. Usually, mercurials are attracted to thiol groups (R-SH),

thus binding to proteins on membranes and to enzymes (Graeme and Pollack, 1998; Hodgson, 2011).

Inhalation is the primary route for elemental mercury (Hg0) to enter the body. Inhaled vapor easily crosses the pulmonary alveolar membranes to enter the bloodstream, and 80% is absorbed through the lungs. Primarily, red blood cells are invaded, then the Central Nervous System (CNS) and kidneys; once converted to $HgCl_2$ it may be retained in the CNS and kidneys for years (Tchounwou *et al.*, 2003).

However, only less than 0.1% of elemental mercury is absorbed from the gastrointestinal tract when ingested, being almost completely eliminated by the urine and feces. Therefore, the ingestion of elemental mercury is harmless (Tchounwou *et al.*, 2003). Dermal absorption of elemental mercury is also limited and estimated to be approximately 2.6%, while the other 97.4% occurs through inhalation. In addition to the excretion mentioned above, the expired air, sweat and saliva are also routes of mercury elimination from the organism, though at a much lower extent (Risher, 2003).

When mercury salt is ingested, only about 10% is absorbed because it is very corrosive for the gastrointestinal tract. In the body, inorganic mercury accumulates in the kidneys causing renal damage. Usually a very small amount enters the body via dermal absorption. Its excretion is mostly fecal, being urinary excretion about 10% or less of its total excretion from the body (Tchounwou *et al.*, 2003).

Methylmercury has been known to be the most toxic. For humans, the primary route of exposure for methylmercury is by consumption of contaminated fish. Although organic mercury isn't significantly transformed in the body, it may be oxidized in the liver and also in the intestinal flora to its inorganic form. In vitro studies have shown that methyl mercury inhibits microtubule formation, as well as protein synthesis in neurons, membrane activity is altered and DNA synthesis is disrupted. Animal studies have shown that intoxication with methylmercury during fetal period may lead to skeletal malformation (Tchounwou *et al.*, 2003).

Researchers have been demonstrating that MeHg affects reproductive performance, lifetime, growth and development behavior, motor skills and survivorship in aquatic birds and other wildlife. Besides, it may suppress the immune system, disrupt endocrine responses to stress, and interact with other contaminants leading to more adverse effects (Reis *et al.*, 2009).

Due to the higher risk of mercury accumulation in aquatic wildlife and piscivorous species than on terrestrial predators, most studies have focused on aquatic ecosystems. Little attention has been given to carnivorous mammals that feed on terrestrial invertebrates and vertebrates, despite their high trophic position and potential exposure risk (Kalisińska *et al.*, 2009; Lavoie *et al.*, 2010). However, consumers at higher trophic levels in terrestrial systems might be useful to predict risks to human health, as a proxy of the human trophic position in terrestrial food webs. Moreover, such studies are needed in view of the high level of global environmental mercury pollution (Kalisińska *et al.*, 2009; Rimmer *et al.*, 2010; Sánchez-Chardi *et al.*, 2007).

1.1.4 - Legislation for mercury control in the environment

Due to the high toxicity of the different chemical forms of mercury, and their high potential to bioaccumulate in terrestrial and aquatic organisms, development of legislation is a key step to control emissions of mercury to the environment.

It thus became essential for public health to maintain the concentration of mercury at levels that are toxicologically acceptable and should therefore be reduced as much as possible. This goal can be achieved through good manufacturing or agricultural practices, in order to attain a high level of health protection, particularly with regard to population groups considered at risk (CE regulation nº 629/2008 of 2 July).

In an effort to strictly control mercury emissions into the environment, particularly into water resources, prevention on its main sources of pollution has

been of constant concern. For this reason the regulation nº 84/156/CEE of 8 March establishes threshold values for mercury discharges in industries other than chlor-alkali industries, in order to minimize the discharge of this hazardous substance in both aquatic and terrestrial resources.

Sediment contamination by mercury came to be ranked by classes in the legislation published in the official Portuguese gazette, Series II nº141 of 21 June 1995 concerning the classification of dredged materials in accordance with the degree of mercury contamination (mg g^{-1}). Class 1 sediments ([Hg] < 0.5 μg g^{-1}) may be used without stringent regulations (e.g. beaches supply), while class 2 (0.5 < [Hg] < 1.5 μg g^{-1}), class 3 (1.5 < [Hg] < 3.0 μg g^{-1}) and class 4 (3.0 < [Hg] < 10 μg g^{-1}) presents a successive increase of its restricting dredging rules and uses. Class 5 are highly contaminated sediment presenting concentrations above 10 μg g^{-1} and cannot be dredged; if imperative it should be treated as industrial waste, and shall not be deposited on land.

The regulation 98/83/EC concerning the quality of water intended for human consumption established threshold values for various chemical and microbiological parameters, being mercury one of the chemical parameters that should not exceed 1.0 μg L^{-1}. The environmental quality criteria from the European Water Framework Directive is now altering this value to 70 ng L^{-1} as threshold for internal waters.

Also, animal feeding stuffs should not present any danger to animal health and in order to provide such health-safety, regulation nº139/2010 of 29 December provides maximum tolerable thresholds of undesirable substances, being the maximum level 0.5 mg/kg for total mercury in animal feeding stuffs obtained from fish or another aquatic animals processing.

On February 24, 2004, the European Food Safety Authority (EFSA) emitted a report of the opinion of the scientific panel related to MeHg in food altering the limits of the Provisional Tolerable Weekly Intake (PTWI) for MeHg. First established to be 3.3 μg kg^{-1} bw (body weight), this limit was then altered to 1.6 μg kg^{-1} bw.

In this report, the opinion of the scientific panel is that mercury is mostly present in fish and seafood products in the form of MeHg (90%) and that in other products it is mostly present as the inorganic form, which is not as toxic as MeHg, thus not being considered sources with a relevant impact. For this reason, and acknowledging fish as an important constituent in a healthy diet, a priority has been made for establishing limits on fish and seafood products as in the environment where it belongs.

According to those statements, a new regulation was introduced (nº1881/2006/EC of 19 December) fixing maximum thresholds for certain contaminants in food stuffs. Moreover, the maximum admissible value for mercury is 1.0 mg kg^{-1} wet weight for predatory fish (EC466/2001). However, there is no applicable legislation for mercury in meat and terrestrial animals.

However, it is also mentioned that other possible sources for human consumption, such as meat and meat products of animals fed with MeHg containing fishmeal, has not been taken into account as it should in order to assess MeHg ingestion. Not only for humans, but also in order to protect terrestrial ecosystems more studies should be done in order to establish maximum thresholds in terrestrial animals.

1.1.5 – Mercury in mammals

During the 1950's and 1960's the widespread of organomercurials fungicides lead to mercury intoxication of wild mammals due to their dietary exposure to high concentrations of mercury. Other contamination events also occurred in lakes close to chlor-alkali plants, causing the death of minks and otters as a result of methylmercury bioaccumulation (Kruuk and Conroy, 1991; Wobester and Swift, 1976).

Although no legislation has been established for terrestrial organisms, studies have been carried out during the past years in order to understand how mercury

bioaccumulates in terrestrial mammals, and what are the levels considered being toxic. Those studies have focused mainly in predatory mammals with an aquatic feeding habit.

Controlled dietary-dosing experiments on mink indicated that total mercury concentrations between the range of 20 to 100 μg g^{-1} ww (wet weight) in liver and over 10 μg g^{-1} ww in brain, were potentially lethal (Aulerich *et al.*, 1974; Wren *et al.*, 1987). Borg *et al.* (1969), have reported values within those ranges (30 μg g^{-1} ww and 40 μg g^{-1} ww in liver and kidneys respectively), in a fox and a marten showing symptoms of methylmercury intoxication. Roelke *et al.* (1991) also reported a suspected mercury poisoning on a Florida panther which presented 110 μg g^{-1} ww of total mercury in liver tissue.

Gamberg and Braune (1999) analyzed total mercury in liver and kidneys of arctic wolves. Liver had concentrations ranging from 0.04 to 0.24 μg g^{-1} ww and kidneys from 0.11 to 0.46 μg g^{-1} ww of total mercury. Dietz *et al.* (2000) found that mercury levels in polar bears from Greenland were lower in muscle tissue (0.034 – 0.191 μg g^{-1} ww) and higher for liver and kidneys (liver: 2.13 – 22.0 μg g^{-1} ww; kidneys: 2.87 – 32.0 μg g^{-1} ww). Rush *et al.* (2008) also analyzed liver tissue of polar bears from Greenland, detecting levels of total mercury ranging from 7.34 to 62.5 μg g^{-1} ww.

Liver, kidneys, bones, testis and muscle of two non-predatory mammals: red deer and wild boar were analyzed by Berzas Nevado *et al.* (2012). Higher mercury concentrations were found in kidneys (red deer: 0.092 μg g^{-1} dw; wild boar: 0.103 μg g^{-1} dw) and also in liver (red deer: 0.013 μg g^{-1} dw; wild boar: 0.023 μg g^{-1} dw).

According to the mercury levels reported in the various studies, only polar bears showed to have levels high enough to be of concern for wildlife. However, none of the studies referred any evidences of mercury poisoning for the polar bears, suggesting that mercury may be present in less toxic forms.

1.2 – *H.ichneumon* – history, morphology, habitat, dietary and protection status

Paintings of mongooses dating from 2800 BC were found on temple walls, as they were considered sacred to Egyptians. European fossil records were unknown in its area of habitat, the Iberian Peninsula. Due to this fact, some authors suggested that the mongoose was introduced, among other animals (e.g., the genet, *Genetta Genetta*) during historical times to Portugal and southern Spain, by the Arabs, who used it as a domestic animal in order to control the excess rodents and reptiles.

Figure 3 - Scientific classification of the studied species.

Estimated date showed that mongooses came from between the XI and XIII century, ^{14}C age: 885±40 years, emphasizing the hypothesis that Arabs introduced this species to the Iberian Peninsula (Macdonald and Barrett, 1993; Riquelme-Cantal *et al.*, 2008; Vários, 2005).

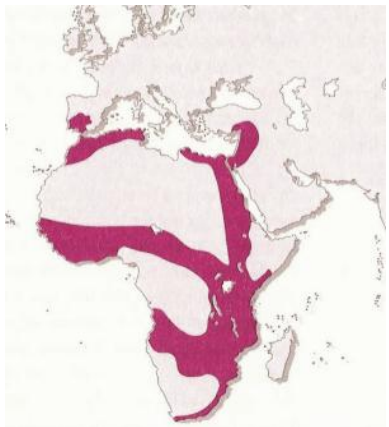

Figure 4 - World distribution of *Herpestes ichneumon* (Barros, 2008).

H. ichneumon is a middle-sized mammal carnivore of the *Herpestidae* family (Figure 3), characterized by their long and slim bodies, very well adapted for the pursuit of their prey right up to their burrows. Generally, its fur is greyish-brown and rough. Adult's head-body length ranges from 50 to 55 cm and have a broad pointy tail of about 30 to 45 cm length.

Although not being an endangered species, its presence is highly conditioned by the existence and

preservation of its typical habitat, which is characterized by a large coverage of trees and bushes, rivers and wetland areas. *H. ichneumon* is widely distributed in Africa and is one of the most abundant carnivorous species in Portugal (Figure 4). Mostly distributed through the southwest of the Iberian Peninsula, actually this species has been expanding from south to north on portuguese territory. This extension has been related with the significant decrease of one of its predator, the Iberian Lynx (*Lynx pardinus*) (Barros, 2008; Horai *et al.*, 2006).

H. ichneumon is a largely diurnal species mostly adapted to hunt on the ground, although some of them have adapted to wetlands. Despite its diurnal activity, it also may be active by night. This species has an carnivore diet that includes rabbits, rodents, birds, reptiles, eggs and carrion, with insects also being an important component of its food habits. Due to its proportionally short legs, this carnivore is a typical animal digger. Each limb has five fingers with non-retractable claws, thus being unable to climb up and hunt in trees (Barros, 2008).

Males are slightly bigger and heavier than females. Sexual maturity is reached between 18 to 24 months, and gestational time ranges between 72 and 88 days, giving birth to 2-8 cubs. Furthermore, the offspring of previous litter remains with the mother until the birth of the subsequent litter. In the wild, its lifespan is about 12 years, while in captivity it may be extended to 20 years. Due to its high trophic level, the toxic effects of bioaccumulative metals, such as mercury, through biomagnification on the *H. ichneumon* are more severe (Horai *et al.*, 2006; Riquelme-Cantal *et al.*, 2008).

Presently, *H. ichneumon* is not considered an endangered species in Portugal, and its hunt may be authorized in the months of October to February inclusive (Regulation nº136/96 of 14 August). It can also be slaughtered as predatory control (article nº94-97 of the hunting act, chapter XI). The hunt of this species emphasizes the importance of the study, given that if consumed by humans, mercury bioaccumulation in this species may constitute a risk to human health. Also, studying a terrestrial mammal may give additional information in the contamination level in the terrestrial ecosystem in Portugal.

Very few studies have been performed regarding the mercury cumulative patterns of mongooses. In the study of Horai *et al.* (2006), the concentration of 22 elements were examined in the liver (n=54), kidney (n=47) and brain (n=10) of 54 Javan mongooses (*Herpestes Javanicus*) from the Amamioshima Island (Japan). Total and organic mercury were analysed and are refered as T-Hg and O-Hg respectively. Almost all of the elements had higher concentrations in the liver, with a mean T-Hg concentration of 12.7 μg g^{-1} wet wt (1.75 to 55.5 μg g^{-1} wet wt, n=53).

Significant correlations of T-Hg with O-Hg and selenium (Se) were observed in liver, being almost 1 with Se ($r=0.916$, $p < 0.001$). Those results reinforced the hypothesis of Se being involved in the detoxification mechanism of mercury in the liver, allowing Javan mongooses to tolerate mercury toxicity. Also, 10 Javan mongooses from Okinawa Island were used to compare mercury levels with those from Amamioshima Island, in order to clarify wether the high T-Hg accumulation in the Javan mongoose is affected by their habitat. No significant area difference was observed ($p=0.679$). Also, kidney/liver ratio showed to be higher than brain/liver ratio, suggesting that the accumulation is higher in the kidneys than in the brain.

Excrement analysis was performed to Javan mongooses, and showed that a rare species of rabbit (Amami rabit) is an important food item for this species in Amamioshima islands. Liver analyses of 2 Amami rabit for T-Hg (mean=0.032 μg g^{-1} wet wt) and comparison with other species proved mercury levels to be similar to other herbivourous, but lower than omnivores and carnivores. The higher T-Hg concentrations in the Javan mongooses livers, however, support the idea that those higher values were due to species-specific accumulation. Besides, the dietary preferences of the Javan mongoose (insects, non-insect vertebrates, avian preys and rodents) do not justify such high levels. The authors conclude that the Javan mongoose may therefore have a high mercury absorption and accumulation ability, or/and low mercury excretion, leading to such high biomagnification.

The history of mining activities in Spain are known to have resulted in high metal contamination in two specific areas – Doñana and Sierra Morena. The Iberian Lynx, the most endangered felid in the world (200 remaining specimens) inhabit those two

areas. The main aim of the work of Millán *et al.* (2008) was to fill an information gap regarding the bioaccumulation of metals by carnivores inhabiting these areas, with special focus on the critically endangered Iberian Lynx. In this study, the concentrations of 7 elements were determined in the liver, bones and muscle of 9 lynx (*Lynx pardinus*), 17 red foxes (*Vulpes vulpes*), 11 Egyptian mongooses (*H. ichneumon*), 4 common genets (*Genetta genetta*) and 1 Eurasian badger (*Meles meles*). The lynx samples came from Sierra Morena (n=3) and Doñana, all of the remaining species came from Doñana. All *H. ichneumon* were adults and the T-Hg average in liver, bones and muscle was 1.753, 0.011 and 0.601 μg g^{-1} dw (dry weight). In all studied species, females had higher concentrations of selenium (Se), cadmium (Cd) and mercury (Hg) in the liver than males. No differences were found between males and females for bones and muscle. Muscle and liver showed significant correlations for As ($r=0.534$, $p=0.001$), Se ($r=0.434$, $p=0.009$), Cd ($r=0.463$, $p=0.004$), Zn ($r=0.388$, $p=0.021$), and Hg ($r=0.822$, $p < 0.001$). Correlations between elements were similar for liver and muscle, being stronger for Hg and Se, especially in the *H. ichneumon* ($r = 0.899$, $p < 0.001$ and $r=0.449$, $p=0.003$, respectively). Regardless of its higher level in the food chain, the Iberian lynx showed much lower T-Hg levels in liver (0.233 μg g^{-1} dw – Doñana; 0.135 μg g^{-1} dw – S.Morena) and muscle (0.024 μg g^{-1} dw – Doñana; 0.601 μg g^{-1} dw – S.Morena) than in the *H. ichneumon*.

Authors concluded that although carnivores are at the top of the food chain, and thus potentially exposed to any biomagnification processes that may occur in a food web, the individuals studied had low levels of toxic metals in their tissues, suggesting that metals do not appear to represent a major threat for the critically endangered Iberian lynx, or other carnivores.

Higher mercury levels in the *H. ichneumon* of this study may reinforce the conclusion of the first study which suggested that such high levels were due to a species-specific accumulation pattern. Those two studies enhance the importance of further studies in this area, as it is a hunted species and such high mercury levels may be concerning for human health.

1.3 – Objectives of this study

The present study focussed on the impact of anthropogenic pollution in the terrestrial fauna of Portugal, analysing mercury (total and organic) in several tissues of *H. ichneumon*. Considering the lack of information regarding mercury accumulation in terrestrial organisms, especially predator mammals, the main aim of this study was to provide new data on the concentration, distribution and speciation of mercury in *H. ichneumon* in order to determine if mercury levels pose a threat for the studied species.

The results will provide a better understanding of mercury bioaccumulation in a predator species. The overall objectives of this study were established according to the following topics:

- ✓ Geographical differences of mercury levels in *H. ichneumon*;
- ✓ Distribution and speciation of mercury in different tissues (muscle, liver, lungs, kidneys, brain, blood, pelage, spleen, fat and heart);
- ✓ Differences between genders;
- ✓ Lifespan bioaccumulation pattern.

2 - Materials and methods

2.1 - Sampling and sample treatment

This study is included in a broader project about the population dynamics, genetics and diet of the *H. ichneumon*. The samples were provided by the project leaders and were obtained from road kill and predator control activities on Portuguese territory.

Licensed veterinarians performed the necropsies, and all possible tissues harvested (muscle, liver, lungs, kidneys, brain, blood, pelage, spleen, fat and heart), depending on the condition of the animal. All tissues were preserved frozen until further processing. All of the tissue samples were then freeze-dried at -50ºC and 0.06 bars, and homogenized in an electrical mill prior to analysis.

2.2 - Analytical methods

2.2.1 - Mercury determinations

There are several methods for mercury analyses, such as Cold Vapor Atomic Absorption Spectrometry (CV-AAS) and Cold Vapor Atomic Fluorescence Spectrometry (CV-AFS). In order to analyze solid matrices using the referred methodologies, a conversion to aqueous form is necessary. Usually, concentrated acids are used, either at atmospheric or elevated pressure, using open or sealed vessels. Sealed vessels are preferred, as they prevent the loss of analyte. However, physical treatment of the sample may induce losses of volatile mercury components, thus leading to a low recovery factor.

Taking advantage of mercury's volatility was a requirement to develop a new system for mercury analysis, allowing also a direct analysis of solid matrices. LECO AMA-254 (Advanced Mercury Analyser) surged as a new instrument, evolved from the previous non-commercial instrument TMA-254, allowing the direct determination of mercury from both liquid and solid matrices (no previous sample treatment needed).

Atomic Absorption Spectroscopy is the quantification method of LECO AMA-254, after thermal decomposition of the sample. The system consists of a nickel boat in a quartz combustion catalytic tube in which the sample (solid or liquid, up to 1000 mg/ 1 mL) is initially dried at 120ºC prior to combustion at 750ºC in an oxygen atmosphere. The mercury vapor produced is then trapped on the surface of a gold amalgamator. After a pre-specified time interval (120-150 s), the amalgamator is heated to 900ºC in order to release the mercury and to transport it to a heated cuvette (120ºC). AAS analysis is performed using a silicon UV diode detector at 253.6 nm (Figure 5). The total analysis time is about 5 minutes (Costley *et al.*, 2000).

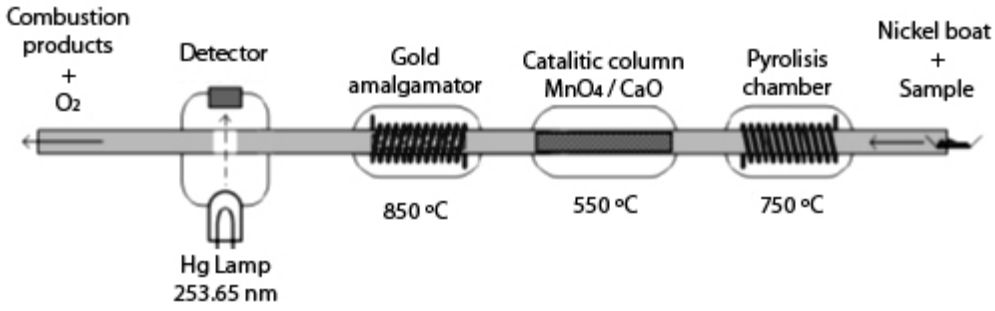

Figure 5 - Scheme of the LECO AMA 254 (Rosa, 2006).

2.2.2 - Organic mercury extraction

About 50–200 mg of sample are weighed into 50 mL polypropylene centrifuge tubes and then 5 mL of KBr (18%) in H_2SO_4 (5%) and 1 mL of $CuSO_4$ (1 mol L^{-1}) are added to the tube. The tubes are held at room temperature for 15 minutes and then treated with 5 mL of toluene (C_7H_8), followed by vigorous agitation for 15 minutes, in order to extract organic mercury (O-Hg). The organic phase is then separated by centrifugation (4000 rpm for 15 minutes) and 3 mL of the organic extract are decanted to glass vessels and stored. The extraction process is repeated once more and again, the organic extract (5 ml) is retained. The O-Hg compounds retained in the toluene are back-extracted into an aqueous sodium thiosulphate solution 0.002

mol L^{-1} (5 mL) (Figure 6). Procedural blanks are carried out for quality assurance purposes, and certified reference materials extracted parallel to the samples to assure extraction efficiency (Válega *et al.*, 2006).

Quantification of liquid aliquots is carried out in the aqueous sodium thiosulphate fraction, using the LECO AMA-254.

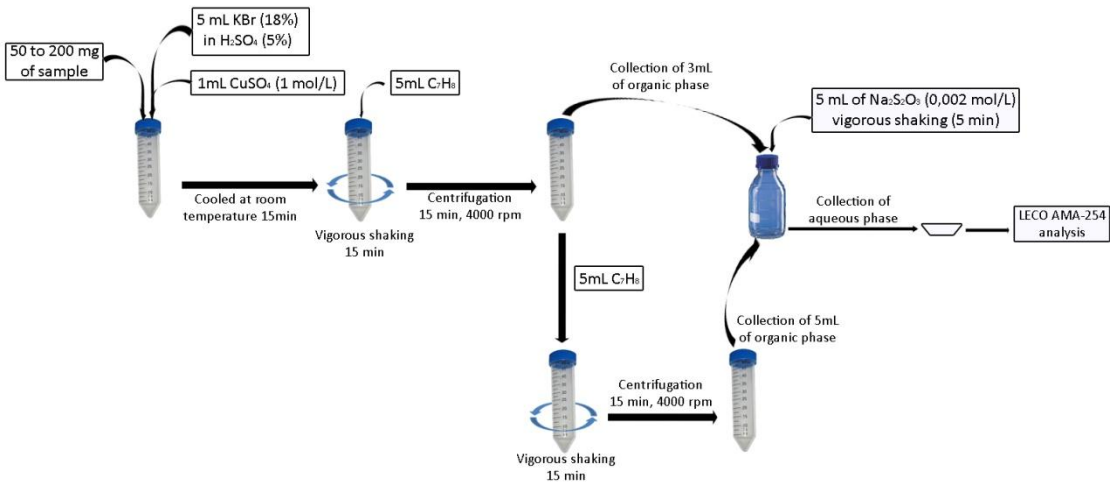

Figure 6 - Schematic procedure for the extraction of O-Hg compounds.

2.3 - Quality control

All analytical methods are susceptible to errors, from the sampling procedures, storage, sample handling and treatment, to the analysis procedure itself and data evaluation. Every effort was made to prevent and minimize errors in each of these steps, in order to assure accurate and reliable results. Precision, accuracy and analytical detection limits were continuously monitored as means of accessing analytical performance, and hence the validity of results.

Precision describes random error; one way of assessing precision is analyzing the dispersion between replicate analyses (repeatability) (Miller and Miller, 2005). Three replicates were analyzed and the acceptance criterion established was for the relative standard deviation to be below 10%. Above this value (in cases of

heterogeneous solid matrices), the samples were re-analyzed (at least 10 replicates) in order to assure a reliable result.

T-Hg in solid matrices presented an average relative standard deviation of 4.16±0.23 % (95% confidence, n=481), and was even lower for O-Hg presenting an average relative standard deviation of 3.04±0.74 % (95% confidence, n=50).

The tool used to measure analytical accuracy was parallel analysis of certified reference material (CRM) of a similar matrix to the real samples. Considering the limited available choices of CRMs for terrestrial organisms, the CRM used was TORT-2, which is lobster hepatopancreas Reference Material (from Research Council Canada). Table 2 summarizes the overall analytical performance of CRM analyses for T-Hg and O-Hg.

Table 2 – Analytical accuracy of Certified Reference Material (CRM) determinations for T-Hg and O-Hg.

		Laboratory concentration (mg kg^{-1})			Certified concentration (mg kg^{-1})	
		Mean	95% confidence	n	Mean	95% confidence
TORT-2	T-Hg	0.30	0.30–0.31	144	0.27	0.21–0.33
	O-Hg	0.115	0.103–0.126	9	0.152	0.139–0.165

No significant differences ($p<0.05$) were found between the certified concentration (± confidence limits) and the laboratory concentration (± confidence limits), except for O-Hg extraction. However, and given that this is a multiple step methodology, the results were still considered to be acceptable.

Limit of detection (LOD) is considered to be the lowest amount of analyte that can be statistically distinguished from the noise signal, the baseline or the blank level (Miller and Miller, 2005). Depending on the analytical method, the LOD may be determined differently. For the methods used, LOD was calculated as the blank

signal (procedural blanks which underwent the entire methodology) plus three times its standard deviation. The LOD of the method used (Thermal decomposition AAS) was 0.0091 ng for T-Hg and $1.04*10^{-04}$ ng g^{-1} for O-Hg.

3 - Results

3.1 – Total Hg in the tissues of *H. ichneumon* and geographical differences

Aiming to evaluate the importance of mercury bioaccumulation in various tissues of *H. ichneumon*, 10 different tissues of 29 specimens from various areas of Portugal (14 locations) were analyzed. Gender and age range were unknown, so this part of the study was focused on the Hg distribution throughout the different tissues, as well as the evaluation of contamination levels of the areas concerned. The results are presented in annex I, and in order to obtain a better visualization of the data bellow a chart was drawn and is presented in figure 8. Figure 7 also provides a better visualization of the mercury levels for each location. The larger the circles are, the higher the mercury levels.

T-Hg ranged from 0.010 to 12.7 μg g^{-1} dw (dry weight), with fat being the tissue with the lowest mercury levels (0.010 to 0.11 μg g^{-1} dw); on the opposite side, the tissue with the highest mercury concentrations was blood (0.76 to 12.7 μg g^{-1} dw). The lungs, heart and spleen present similar levels of T-Hg (0.050 to 0.98, 0.045 to 1.3 and 0.047 to 1.0 μg g^{-1} dw respectively. Blood and pelage showed a greater variation in T-Hg concentrations than the other tissues.

As the data did not follow a normal distribution, non-parametric tests were performed in order to compare T-Hg concentrations regarding location and tissues. To test for differences between tissues, Kruskal-Wallis test was performed and showed that the differences were statistically significant ($p<0.001$). In order to isolate the tissues that differ from the others, Mann-Whitney U-test was performed as multiple comparison procedure. Almost all the tissues presented statistical significant differences; the exceptions are indicated in table 3.

Table 3 - **Mann-Whitney U-test performed for the various tissues of H*. ichneumon*.**

		Muscle	Liver	Lungs	Heart	Spleen	Kidneys	Blood	Brain	Fat	Pelage
Muscle	**T**		153 (*)	260 (*)	245	254 (*)	153 (*)	105 (*)	127	55 (*)	138 (*)
	p		0,023	0,009	0,057	0,02	0,023	< 0,001	0,076	< 0,001	0,003
Liver	**T**			284 (*)	277 (*)	282 (*)	211	271 (*)	101 (*)	55 (*)	186
	p			< 0,001	< 0,001	< 0,001	0,73	0,002	0,002	< 0,001	0,448
Lungs	**T**				176	187	123 (*)	105 (*)	164	60 (*)	113 (*)
	p				0,223	0,476	< 0,001	< 0,001	0,939	< 0,001	< 0,001
Heart	**T**					215	130 (*)	105 (*)	152	57 (*)	115 (*)
	p					0,597	< 0,001	< 0,001	0,625	< 0,001	< 0,001
Spleen	**T**						126 (*)	105 (*)	154	61 (*)	112 (*)
	p						< 0,001	< 0,001	0,700	< 0,001	< 0,001
Kidneys	**T**							283 (*)	101 (*)	55 (*)	175
	p							< 0,001	0,002	< 0,001	0,206
Blood	**T**								78 (*)	55 (*)	248 (*)
	p								< 0,001	< 0,001	0,041
Brain	**T**									59 (*)	91 (*)
	p									< 0,001	< 0,001
Fat	**T**										55 (*)
	p										< 0,001
Pelage	**T**										
	p										

(*) Statistical significant differences.

Friedman's test was performed in order to confirm that the differences between locations were statistically significant. As the differences were in fact statistically significant ($p<0.001$), then Tukey's test was performed in order to isolate the locations that differed from each other. The locations with statistical differences are indicated in table 4.

Coruche presented the highest levels of T-Hg, while Moura and Mértola showed to have the lowest levels from the 14 studied locations, having both similar levels of T-Hg, except for blood.

Table 4 – Tukey's test performed for the various studied locations.

	Évora	Soure	Ferreira do Zêzere	Castelo Branco	Mértola	Torres Novas	Tondela	Lousã	Coimbra	Montemor-o-Novo	Castro Daire	Olhão	Moura	Coruche
Évora		1,69	0,761	2,197	4,479	3,127	0,169	0,423	1,775	3,803	0,676	2,789	4,902(*)	2,282
Soure			2,451	3,888	2,789	1,437		2,113	3,465	2,113	1,014	1,099	3,212	3,972
Ferreira do Zêzere				1,437	5,24(*)	3,888	0,93	0,338	1,014	4,564	1,437	3,55	5,663(*)	1,521
Castelo Branco					6,677(*)	5,324(*)	2,366	1,775	0,437	6,001(*)	2,874	4,986(*)	7,099(*)	0,0845
Mértola						1,352	4,31	4,902(*)	6,254(*)	0,676	3,803	1,69	0,423	6,761 (*)
Torres Novas							2,958	3,55	4,902(*)	0,676	2,451	0,338	1,775	5,409 (*)
Tondela								0,592	1,944	3,634	0,507	2,62	4,733	2,451
Lousã									1,352	4,226	1,099	3,212	5,324(*)	1,859
Coimbra										5,578(*)	2,451	4,564	6,677 (*)	0,507
Montemor-o-Novo											3,127	1,014	1,099	6,085 (*)
Castro Daire												2,113	4,226	2,958
Olhão													2,113	5,071 (*)
Moura														7,184 (*)
Coruche														

(*) Statistical significant difference.

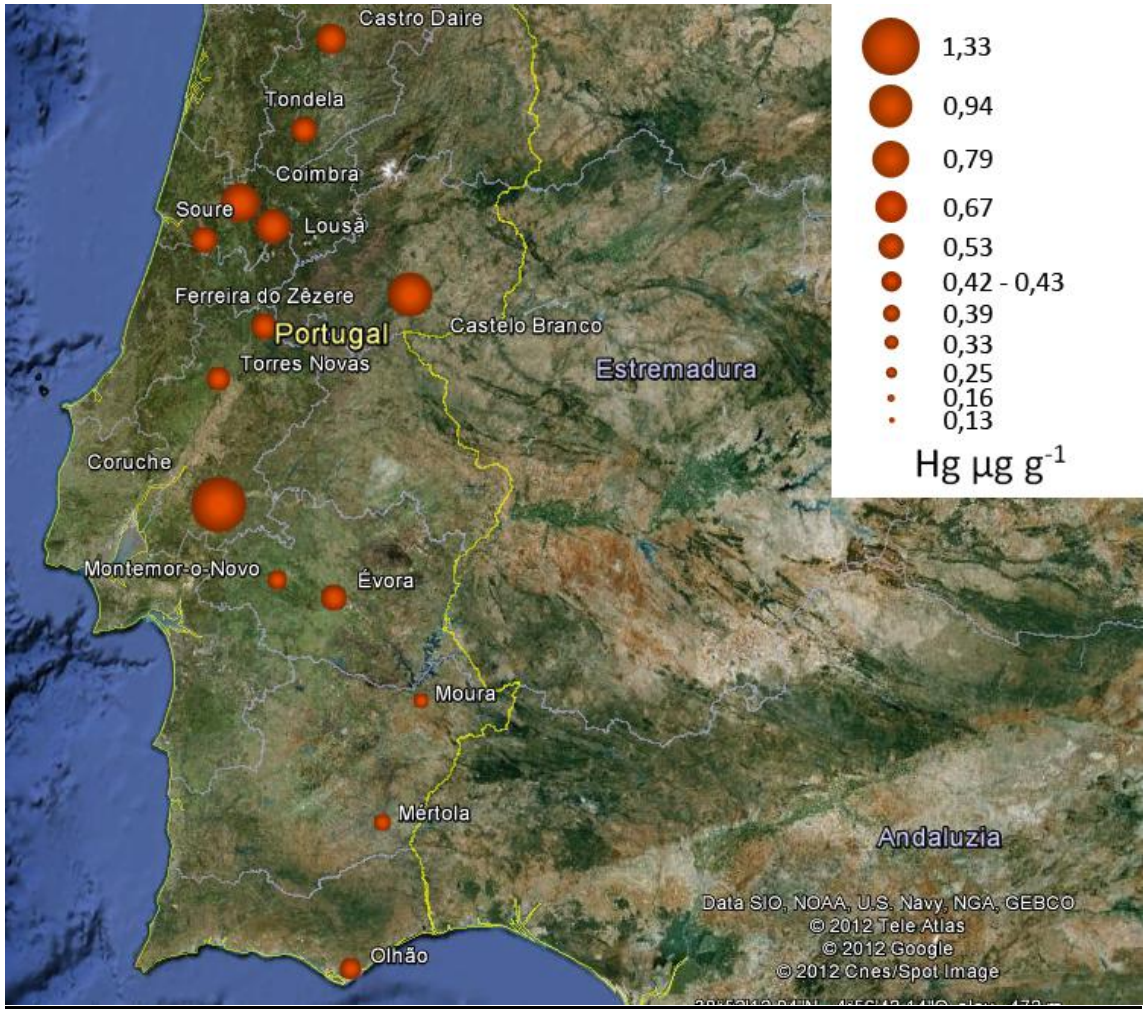

Figure 7 – Map showing the sampling locations and its corresponding mercury levels in muscle tissue.

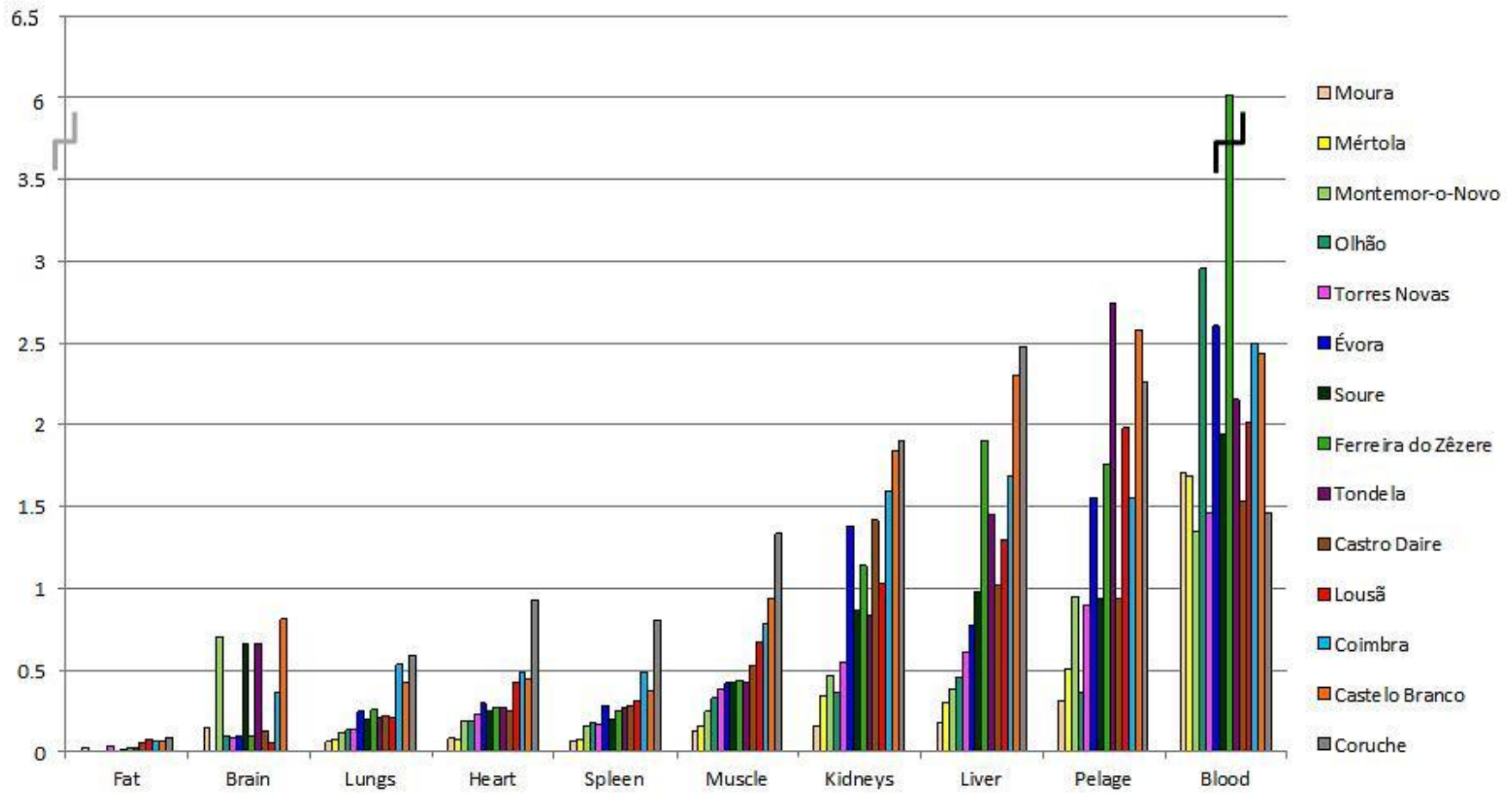

Figure 8 – T-Hg (µg g^{-1} dw) in selected tissues of *H. ichneumon* from different locations in Portugal

3.2 – Lifespan mercury accumulation pattern in several tissues of *H. ichneumon* from Serpa

3.2.1 – Effects of gender and age range

The goal of this part of the study was to evaluate the effect of gender and age in the mercury accumulation pattern in several tissues of *H. ichneumon*. 25 specimens (15 females and 10 males) from Serpa were analyzed. As the exact ages were unknown, 4 development stages intervals were defined: cubs, juveniles, sub-adults and adults.

T-Hg concentrations in all the 10 tissues of *H. ichneumon* collected from Serpa are presented in annex II. In order to obtain a better visualization of the data from annex II, two charts were drawn and are presented in figure 9.

Concentrations of T-Hg for males and females ranged from 0.007 to 1.6 and 0.012 to 7.2 μg g^{-1} dw respectively. For both, the lowest values were observed for fat and the highest for blood, similar to previous findings from the geographical study.

Figure 9 show that females undergo greater variation depending on the life stage. For all tissues, T-Hg concentrations increase from cubs to juveniles, decrease when reaching the sub-adult stage and then increase again in adults. A different behavior is observed in males, in which the concentration of T-Hg linearly increases throughout the growth of the individuals.

As the data for gender concentrations of T-Hg follows a normal distribution, two-sided F-test was performed in order to compare tissues between males and females. There only were statistically significant differences for blood and brain ($F_{3,3}$=35.67 and $F_{3,2}$=15.54, p=0.05).

In order to compare between age groups, a normality test was performed and as the data did not follow normal distribution, the Kruskal-Wallis test was performed. No significant statistical differences were observed for female age groups (p=0.213.

As for male mongooses, the difference between groups was statistically significant ($p<0.001$). Therefore, a Tukey's test was performed in order to verify which of the groups significantly differed from each other. The result of the test was that there were only differences between cubs and juveniles, sub-adults and adults ($q=3.895$, $q=4.599$ and $q=5.464$, $p<0.05$). Mann-Whitney U-test also was performed in order to compare age groups between males and females, where no significant differences were observed for juveniles, sub adults or adults ($p=0.273$, $p=0.345$ and $p=0.089$ respectively), while significant differences exist for cubs ($p=0.003$).

Only males present an increase of T-Hg concentrations with increasing age, so linear regressions were carried out for each one of the tissues (Figure 10). The equations and correlation coefficients are presented in table 5, the lowest correlation coefficient stands for fat tissue ($r=0.742$) and the highest for liver ($r=0.991$). The tissues with the higher accumulation rates (extracted from the regression equations) were pelage, liver and kidney, while fat and brain were the tissues with the lowest rates of accumulation.

Pearson correlation was performed in order to see which tissues mercury burdens co-vary. Positive correlation coefficients with values of $p<0.050$ implied that the pair of tissues concerned tend to increase together. For pairs with p values greater than 0.050, the relationship between tissues was not statistically significant (table 6). All tissues were observed to significantly increase together, with the highest correlation found between blood and liver ($r=0.999$, $p=0.00347$) and the lowest for pelage and lungs ($r=0.979$, $p=0.0413$).

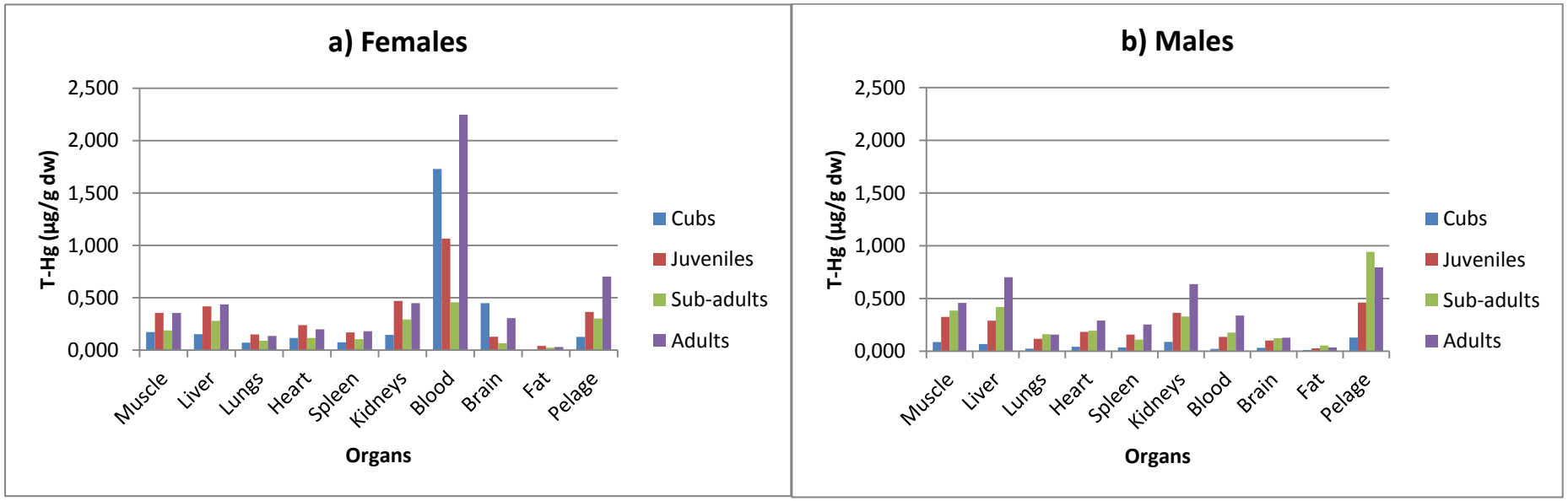

Figure 9 – T-Hg concentrations (µg g^{-1} dw) of females and males from Serpa, showing age variation for each tissue.

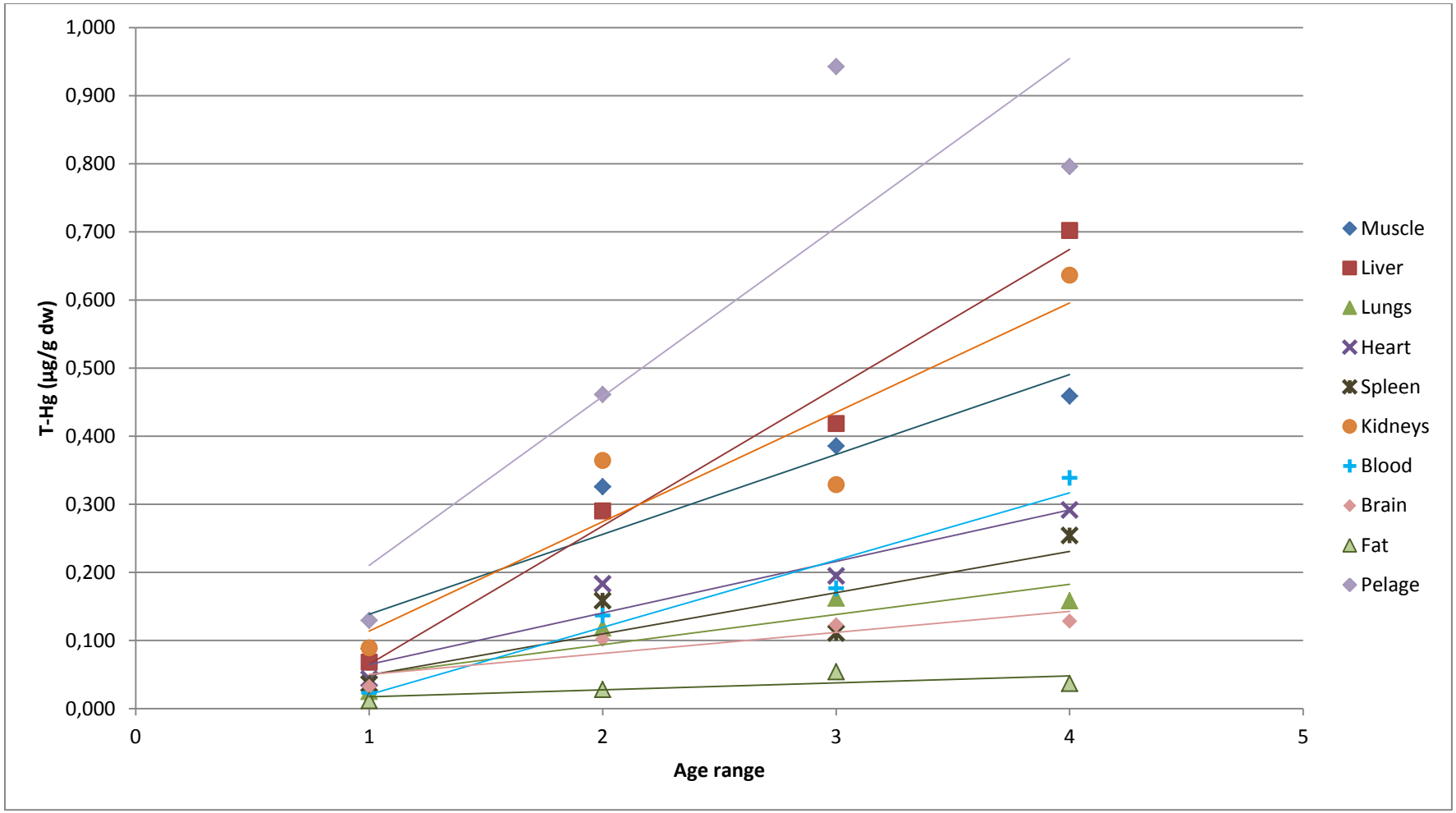

Figure 10 – Linear regressions for each tissue of males from Serpa (1 – Cubs, 2 – Juveniles, 3 – Subadults and 4 – Adults).

Table 5 – Equations and correlation coefficients (r) of the linear regressions from figure 10.

	Equation (y)	Correlation coefficient (r)
Muscle	0.1174x + 0.0211	0.943
Liver	0.203x – 0.1379	0.991
Lungs	0.0443x + 0.0051	0.898
Heart	0.0756x – 0.0108	0.955
Spleen	0.0606x – 0.0115	0.857
Kidneys	0.1606x – 0.0469	0.925
Blood	0.0987x – 0.0781	0.975
Brain	0.0309x + 0.0191	0.904
Fat	0.0102x + 0.0072	0.742
Pelage	0.2481x – 0.0379	0.883

Table 6 – Pearson correlation values for the statistically significant correlated tissues.

Tissues			
Muscle	Lungs	r	0.988
		p	0.0226
	Heart	r	0.989
		p	0.022
	Brain	r	0.995
		p	0.00995
Liver	Heart	r	0.985
		p	0.0294
	Kidneys	r	0.982
		p	0.035
	Blood	r	0.999
		p	0.00347
Lungs	Brain	r	0.998
		p	0.00426
	Pelage	r	0.979
		p	0.0413
Heart	Kidneys	r	0.991
		p	0.0193
	Blood	r	0.985
		p	0.0291
Kidneys	Blood	r	0.990
		p	0.0202
	Spleen	r	0.994
		p	0.0115

3.2.2 – Organic mercury in muscle of *H.icnheumon* from Serpa

In order to understand if mercury concentration in the *H. ichneumon* is mostly organic, extractions of O-Hg were performed in muscle tissue of all the specimens from Serpa. The results for O-Hg concentrations, as well as its percentage in the tissue relatively to T-Hg concentrations, are presented in annex III.

For a better visualization of the results, figure 11 shows the percentages of O-Hg for each age range of males and females. We can observe that the same tendency of T-Hg concentrations applies for O-Hg concentrations, that is, for females the concentration rises from cubs (76.8%) to juveniles (95.9%) and then decreases for sub-adults (78.7%) increasing again for adults (94.5%). For males, O-Hg concentrations increase with age (such as for T-Hg), ranging from 79.3% in cubs to 96.8% in adults. Figure 12 represents the relation between O-Hg and T-Hg concentrations for males and females. Significant correlations were found in muscle between T-Hg and O-Hg for males ($r=1.000$, $p=0.0000002$) and females ($r=0.995$, $p=0.0000002$).

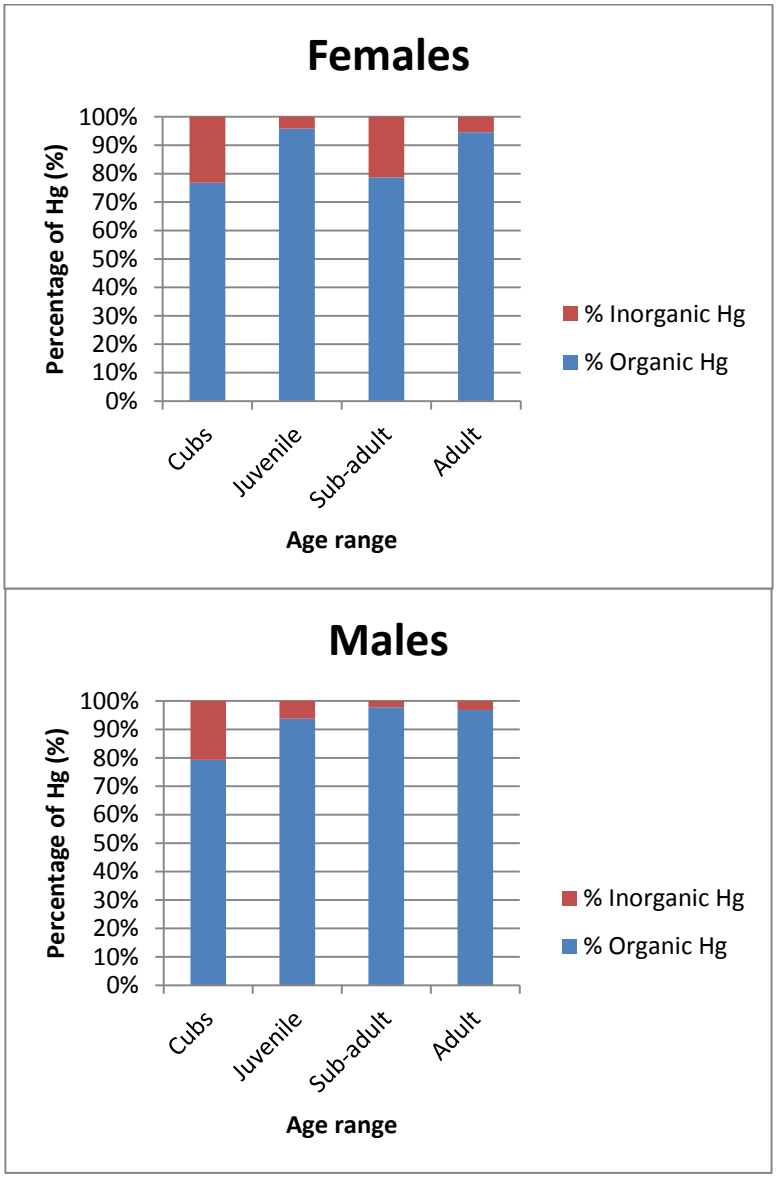

Figure 11 – O-Hg and inorganic-Hg percentages in muscle for each age.

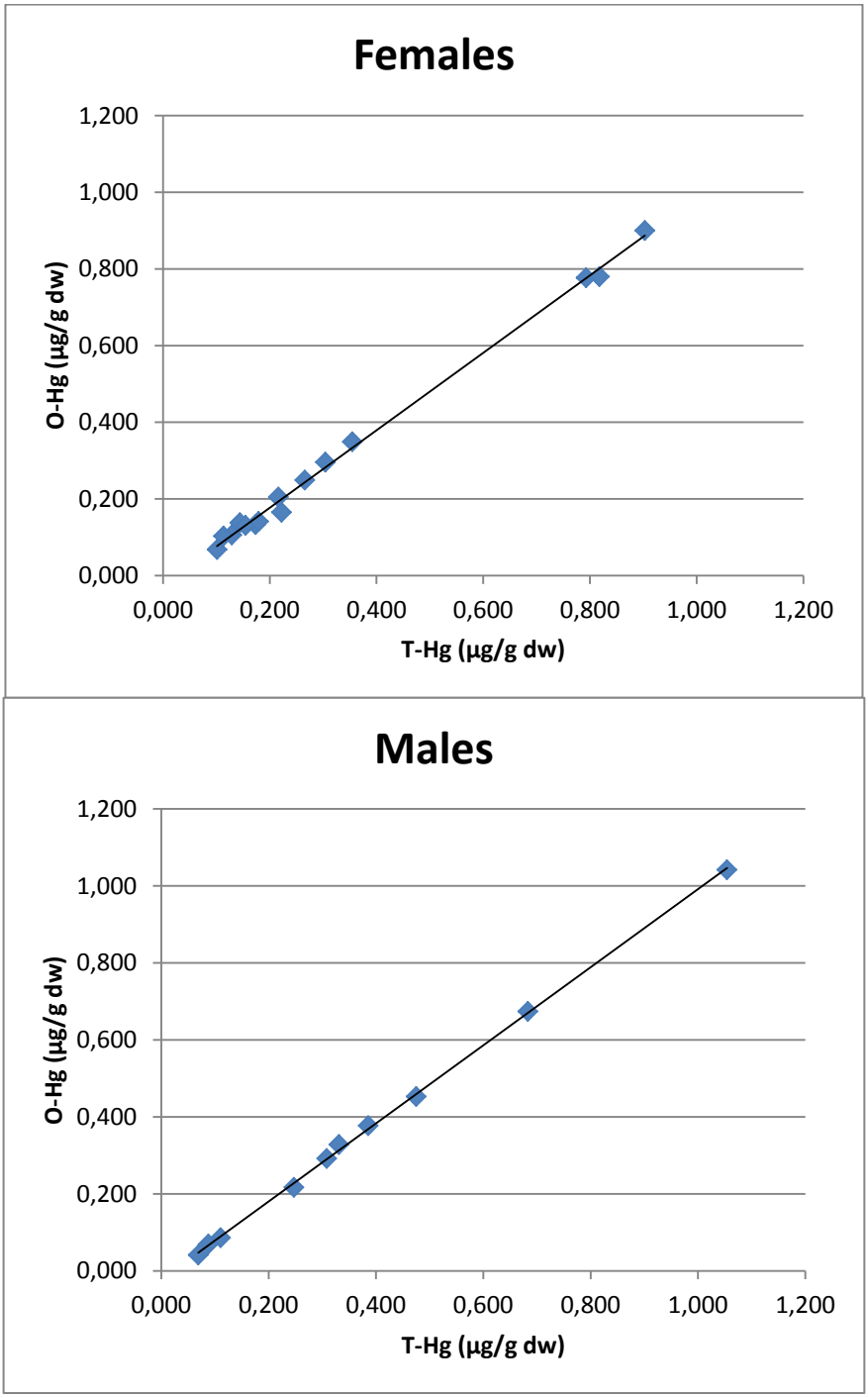

Figure 12 – Relation between O-Hg and T-Hg for muscle of females and males from Serpa.

4 - Discussion

As far as we know, no studies regarding mercury concentrations in wild carnivores have previously been performed in Portugal. The only study found regarding the *H. ichneumon* was performed in Spain. Another, regarding a different species from the same genus, the *Herpestes javanicus* was performed in Japan. According to the Handbook of Ecotoxicology, T-Hg concentrations in the range of 20 to 100 µg g^{-1} wet weight (ww) in liver, or more than 10 µg g^{-1} ww in brain, indicates potentially lethal exposure to methylmercury (in wild otter and mink that died exhibiting signs of methylmercury poisoning). Other poisoned predatory mammals had similar reported values, such as a fox (*Vulpes vulpes*) with 30 µg g^{-1} ww of T-Hg in its liver and kidneys (Beyer *et al.*, 1996).

Mercury concentrations detected in this study are below those associated with mercury intoxication for non-marine mammals. However, and considering that toxicity is highly species-specific, further studies would be necessary, namely related with enzymatic stress response and oxidative stress, to assure the observed mercury levels are not having negative effects on individuals.

4.1 – Mercury concentrations in tissues

Published data regarding mercury accumulation in terrestrial predatory mammals as the *H. ichneumon* is sparse. The two studies mentioned above where mercury concentrations in liver, kidney and muscle of Javan mongooses and *H. ichneumon* were determined showed to have significantly different concentrations. The Javan mongooses from Japan had much higher liver concentrations than the *H. ichneumon* from Spain. Despite the fact that concentrations for Javan mongooses were expressed in wet weight, the conversion factor proposed by Froslie et al., was used to convert those concentrations to dry weight (table 7).

Table 7 – Conversion ratios for liver and kidneys (Shore and Rattner, 2001).

Tissues	Wet/Dry ratio	% Dry matter
Liver	2.76	36
Kidneys	3.66	27

Table 8 shows that even though slightly lower than the ones from Spain and even lower than the ones from Japan, the data from this study shows a similar tendency for T-Hg accumulation. Liver is the highest accumulating tissue, followed by kidney and muscle.

Table 8 – Comparison between liver, kidney and muscle for T-Hg concentrations (µg g^{-1} dw).

Location	Tissue	Minimum	Maximum	Source
Japan	**Liver**	4.83	153.18	**(Horai *et al.*, 2006)**
	Kidney	4.61	46.11	
Spain	**Liver**	0.242	9.686	**(Millán *et al.*, 2008)**
	Muscle	-	2.634	
Portugal	**Liver**	0.10	3.7	**Present Study**
	Kidney	0.075	2.8	
	Muscle	0.085	1.6	

These results highlight the importance of liver as a detoxifying organ for mercury contamination, as highlighted in literature (Berzas Nevado *et al.*, 2012).

Dietz *et al.* (2000), also analyzed livers, kidneys and muscle of polar bears for T-Hg and verified that concentrations in liver and kidney were similar but also higher than in muscle tissue.

Results from the Mann-Whitney U-test showed that such as in polar bears, the differences between liver and kidneys aren't significant, but both are significantly different from muscle tissue. As for pelage and blood concentrations, they showed significant differences, suggesting pelage contamination not to be a reliable predictor of blood or whole-body concentrations.

Hargreaves *et al.* (2011), also observed the same in feathers and blood of shorebirds with the concentrations of 17 elements. Species with the highest blood levels of a given element didn't always have the highest feather level as well. The reason why pelage or feathers are more difficult to compare with blood levels is because they reflect multiple pathways of contaminations: exogenous, endogenous or both (Burger *et al.*, 2008; Veerle *et al.*, 2004).

Despite the fact that brain, lungs, heart, spleen and muscle did not show significant differences between them, they tend to accumulate with increasing levels from brain to muscle. Capelli *et al.* (2008), studied the distribution of mercury in muscle, liver, kidney, heart, lung, brain and spleen of cetaceans. Liver and kidney, as already mentioned, showed the highest levels, as for the remaining tissues and depending on the species, brain was the lowest accumulating tissue and spleen the highest, being lungs, heart and muscle in between in this same order.

The explanation for higher levels in liver, kidney, lungs and spleen is that they all are tissues involved in detoxification and elimination processes. Liver tends to reflect short-term exposure as it metabolizes and excretes mercury. As for kidney, it is a target tissue for inorganic mercury, being excreted by urine (Berzas Nevado *et al.*, 2012). Mercury concentrations for fat and brain were very low (except for four locations). Such low mercury levels in brain when blood levels is much higher, may be explained by the blood-brain barrier that protects the central nervous system (CNS) against chemical threats, by different complementary mechanisms (Zheng *et al.*, 2003). It is possible that for the *H. ichneumon* the brain-blood barrier is the reason why even with high mercury levels in the other tissues, brain remains protected from accumulation.

No studies were found for fat tissue in terrestrial mammals, only a study of mercury levels in muktuk (skin and fat) of beluga whales that showed lower levels than liver, kidneys and muscle, suggesting that fat tissue does not seem to be a target for mercury sequestration and storage (Lockhart *et al.*, 2005).

4.2 – Tissue correlation for mercury levels

Correlation in mercury levels between tissues showed that blood was highly correlated with liver, kidneys and heart. In fact, these correlations enable the assumption that blood is the carrier of mercury throughout the organism, redirect it into the liver for detoxification and then excreting it via the kidneys. Heart is responsible for pumping blood, so it may be a justification for its high correlation.

Pelage was found to have a correlation with lungs. Born *et al.* (1991) also observed correlations between pelage of polar bears and internal tissues. Being pelage seen as an excretion route for mercury, they considered reasonable to assume that the concentrations in pelage generally reflected the mercury levels in the internal tissues of an individual. Although they did not find a correlation between pelage and lungs but between pelage, kidney, liver and muscle, the same assumption may be applied here.

The fact that pelage did not accurately reflect the mercury loads in other internal organs may be related with the time of exposure to contamination. Pelage will integrate the contamination over time, while internal tissues will respond faster to recent mercury intakes. Taking into account the considerable mobility of this species, migrations to or from more contaminated areas will be reflected on internal mercury levels prior to pelage, which will still reflect ancient contamination conditions until pelage shedding.

Kidney and spleen also showed a high correlation, which makes sense as they are involved in excretion and detoxification processes respectively (Capelli *et al.*, 2008; Wolfe *et al.*, 1998).

As for T-Hg and O-Hg, high levels of T-Hg in muscle correspond to high levels of O-Hg. Capelli *et al.* (2008) also found high correlation for O-Hg and T-Hg in muscle of cetaceans ($r=0.825$, $p<0.05$). For both males and females, the accumulation of O-Hg in the muscle followed the same pattern of T-Hg (Figure 11). The high O-Hg content will probably result from the high trophic position of this carnivore in terrestrial food

webs, reflecting the biomagnification potential of organic mercury species. Also, it suggests that diet is the main pathway for mercury incorporation in this species.

4.3 – Geographical comparisons

Total mercury concentrations in all tissues were highest for Coruche and lowest in Moura. Although a clear tendency between locations can be seen (Figure 7), they were not all significantly different. Blood and pelage were not found to be good predictors for geographical distinction, as blood reflects a short-term exposure and pelage reflects multiple pathways of contaminations (Burger and Gochfeld, 1993; Veerle *et al.*, 2004). We can see an example for blood concentration of one individual from Ferreira do Zêzere which presents a much higher mercury level than the other two individuals from the same location and yet, presented similar mercury levels in other tissues.

Not much data is available in order to elucidate the reason of such differences between locations. The Geochemical Atlas of Europe reports data on mercury in topsoils across Europe, including Portugal (Salminen *et al.*, 2005). According to figure 13, the mercury levels in topsoil do not comply with bioaccumulation data. For example, *H. ichneumon* samples from Coruche presented the higher levels mercury, yet similar with Castelo Branco, corresponds to the area of lower mercury levels in topsoil. Samples from Moura and Mértola had the lowest mercury bioaccumulation record, despite being located in an area of higher mercury level in topsoil than Coruche.

Dietz *et al.* (2000) considered feeding habits as being a possible reason for differences between levels of mercury in polar bears from different locations. Considering the dietary plasticity of the *H. ichneumon*, the diet may be considerably different between areas, depending on the available food items. Therefore, dietary contribution to the mercury body burden will rely on food availability, abundance and its contaminant load. Different genetic patterns also may be a factor to be considered.

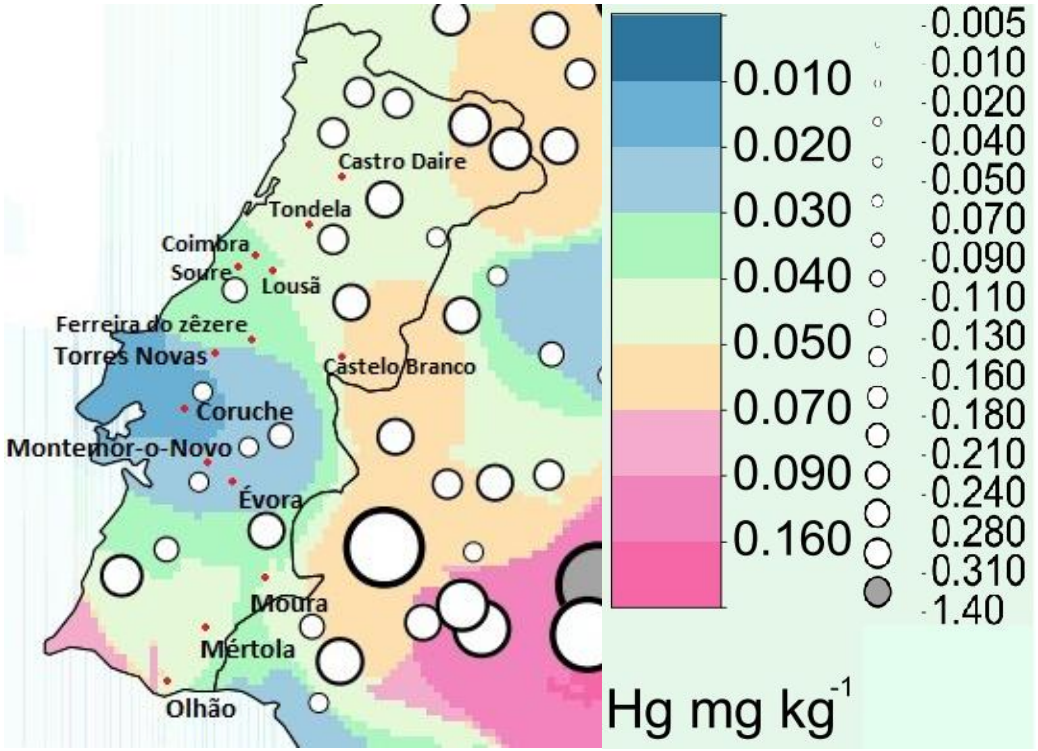

Figure 13 – Map of mercury levels in Portuguese topsoil (Salminen *et al.*, 2005).

4.4 – Gender and age differences

In the present study, differences between genders were only significant for blood and brain tissues, where females showed to have higher values. Differences between genders were only observed for cubs.

Although not significantly different, there is a tendency for females having higher mercury levels than males when at cub and juvenile stages. However, when reaching the subadult and adult stages, levels in males tend to be higher than females.

Gamberg and Braune (1999) studied contaminant residue levels in arctic wolves in Canada and the levels of mercury in both liver and kidneys showed that females under 18 months have higher mercury levels than males and, when they reach 19 months, levels in females decreases and increases in males. Although no explanation was given by the authors, this behavior may be related with the reproductive cycle of females. The energy requirements and physiological changes in females reaching reproductive maturity may have an important role in the balance between accumulation, detoxification and excretion mechanisms, therefore leading to lower levels of mercury.

Information regarding gender and age effects on metal concentrations in terrestrial wildlife tissues is limited and also contradictory. While Millán *et al.* (2008) found that for Iberian lynx and other wild carnivores, females had higher mercury levels in liver than males, Hoekstra *et al.* (2003) studied arctic foxes and did not find any significant influence of gender on mercury levels in kidney and liver. Berzas Nevado *et al.* (2012), studied mercury concentrations in red deers and wild boars and found higher levels of mercury in male muscle than in female. Gender differential bioaccumulation therefore seems to be a species-specific trait, and further research would be necessary to determine the underlying reasons responsible for the observed differences.

In this study, only males showed to have increasing levels of mercury with increasing age, although differences were only significant for cubs relatively to

juveniles, subadults and adults. Results from Rush *et al.* (2008), Dietz *et al.* (2000), Braune *et al.* (1991), indicate that mercury levels increases with the age of polar bears sampled. Capelli *et al.* (2008), also verified that young individuals (in cetaceans) had much lower levels of mercury than adults, showing a strong effect of age on mercury accumulation.

The strong effect on age in males from this study was confirmed by linearization of mercury concentrations of each tissue with age, where all the tissues except for fat tissue showed high correlation coefficients. The fact that it only occurred in males will most certainly be related with physiological and feeding behavior differences related with reproduction.

5 - Conclusions

In this study, variation of mercury levels in several tissues of the *H. ichneumon* was observed. T-Hg levels were higher for blood and pelage, followed by liver, kidneys, muscle, spleen, heart, lungs, brain and fat.

T-Hg levels from all samples varied between 0.010 to 12.7 μg g^{-1} dw and although the *H. ichneumon* is considered to be at the top of the food chain, thus being potentially exposed to biomagnification processes throughout the food web, the mercury levels were below the ones considered to be lethal by the Handbook of Ecotoxicology (20 to 100 μg g^{-1} ww in liver).

Increased mercury levels with age was observed for males, although females presented a great decrease during the transition from juvenile to subadult, that may be related with an alteration in the metabolic rate due to reproduction cycle. Alterations in this stage may involve also an alteration in feeding habits along with hormonal changes of females.

For males, correlation coefficients for each tissue with age were higher than 0.74, except for fat (0.55) and correlation between tissues was also observed. Correlations between blood, liver, kidneys, heart and spleen are easily understood, as they all are involved in detoxification or excretion processes.

Both males and females showed high correlation coefficients ($r>0.99$) for O-Hg vs. T-Hg, meaning that to high levels of T-Hg also corresponds high O-Hg levels, being O-Hg percentage between 77% and 98%.

Differences between locations may be justified by several factors, such as industries or mines, different food item availability or even different genetic patterns, although it is not known which of those variables really leads to differences between locations.

As further studies, gastric contents of *H. ichneumon* could be analyzed for T-Hg and O-Hg in order to understand the extent to which feeding affects mercury levels in the individuals. Also, studies for mercury contamination in soils, plants, insects and small mammals from the different studied locations could help understand if in fact mercury biomagnifies through the food web in terrestrial environment.

Finally, and to assure that the recorded mercury levels are in fact not affecting the health of the *Herpestes ichneumon* population, the analysis of the stress response and oxidative stress of organisms could be evaluated, through enzymatic biomarkers.

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Annexes

Annex I – Locations, numbers of samples (n) and mercury in several tissues expressed as arithmetic mean in µg g^{-1} dry weight of *H. ichneumon*.

Locations	n	Tissues									
		Mean (µg g^{-1} dw)									
		Muscle	Liver	Lungs	Heart	Spleen	Kidneys	Blood	Brain	Fat	Pelage
Évora	2	0.42 (0.19-0.65)	0.77 (0.36-1.2)	0.25 (0.13-0.37)	0.30 (0.13-0.46)	0.28	1.4	2.6 (2.4-2.8)	0.10 (0.068-0.13)	-	1.6 (0.39-2.7)
Soure	2	0.43 (0.27-0.58)	0.97 (0.58-1.4)	0.20 (0.12-0.28)	0.25 (0.18-0.32)	0.20 (0.15-0.26)	0.87 (0.46-1.3)	1.9 (1.5-2.3)	0.65 (0.075-1.2)	0.016	0.93 (0.67-1.2)
Ferreira do Zêzere	3	0.44 (0.34-0.55)	1.9 (0.59-3.0)	0.26 (0.15-0.38)	0.28 (0.21-0.32)	0.25 (0.22-0.29)	1.1 (0.50-1.5)	6.0 (1.0-13)	0.10	0.032 (0.014-0.060)	1.8 (1.1-2.2)
Castelo Branco	2	0.94 (0.87-1.0)	2.3 (2.2-2.4)	0.42 (0.35-0.50)	0.45 (0.32-0.45)	0.37 (0.26-0.48)	1.8 (1.8-1.9)	2.4 (1.0-3.8)	0.81 (0.27-1.4)	0.072	2.6 (1.7-3.5)
Mértola	2	0.16 (0.11-0.21)	0.31 (0.16-0.46)	0.079 (0.050-0.11)	0.080 (0.068-0.092)	0.074 (0.073-0.074)	0.35 (0.29-0.40)	1.7	-	-	0.51 (0.31-0.70)
Torres Novas	2	0.39 (0.38-0.40)	0.61 (0.43-0.79)	0.14 (0.12-0.17)	0.23 (0.23-0.24)	0.17 (0.094-0.25)	0.55 (0.41-0.68)	1.5 (1.3-1.6)	0.082	0.035 (0.033-0.037)	0.90 (0.60-1.22)
Tondela	2	0.43 (0.25-0.61)	1.5 (0.61-2.3)	0.21 (0.12-0.30)	0.27 (0.18-0.36)	0.27 (0.15-0.40)	0.83 (0.33-1.3)	2.2 (0.77-3.6)	0.66	0.027 (0.010-0.044)	2.8 (0.60-4.9)

<u>Annex I –</u> Locations, numbers of samples (n) and mercury in several tissues expressed as arithmetic mean in µg g^{-1} dry weight of *H. ichneumon*.

Locations	n	Tissues: Mean (µg g^{-1} dw)									
		Muscle	Liver	Lungs	Heart	Spleen	Kidneys	Blood	Brain	Fat	Pelage
Lousã	2	0.68 (0.20-1.2)	1.3 (0.67-1.9)	0.21 (0.082-0.34)	0.42 (0.14-0.70)	0.31 (0.15-0.48)	1.0 (0.38-1.7)	2.0 (0.76-3.27)	0.053	0.079	2.0 (0.45-3.5)
Coimbra	2	0.79 (0.31-1.3)	1.7 (0.54-2.8)	0.54 (0.099-0.97)	0.49 (0.18-0.80)	0.49 (0.21-0.76)	1.6 (0.66-2.5)	2.5 (1.8-3.2)	0.37	0.073 (0.042-0.10)	1.6 (0.92-2.2)
Montemor-o-Novo	2	0.25 (0.25-0.26)	0.38 (0.36-0.40)	0.11 (0.11-0.12)	0.19 (0.18-0.20)	0.16 (0.14-0.18)	0.47 (0.28-0.66)	1.4 (1.292-1.4)	0.70	-	0.95 (0.38-1.5)
Castro Daire	2	0.53 (0.49-0.56)	1.0 (0.94-1.1)	0.23 (0.16-0.29)	0.25 (0.22-0.28)	0.28 (0.24-0.32)	1.4 (0.76-2.1)	1.5 (1.24-1.8)	0.13 (0.12-0.13)	0.051 (0.029-0.074)	0.94 (0.65-1.2)
Olhão	2	0.33 (0.31-0.34)	0.45 (0.35-0.55)	0.13 (0.12-0.14)	0.19 (0.18-0.20)	0.18 (0.17-0.19)	0.36 (0.30-0.42)	3.0 (2.8-3.2)	0.099 (0.098-0.10)	-	0.37 (0.28-0.45)
Moura	2	0.13 (0.085-0.17)	0.18 (0.10-0.25)	0.065 (0.055-0.075)	0.083 (0.045-0.12)	0.068 (0.047-0.089)	0.16 (0.075-0.24)	1.7 (1.2-2.2)	0.15 (0.022-0.28)	0.024 (0.021-0.027)	0.31 (0.086-0.54)
Coruche	2	1.3 (1.1-1.6)	2.5 (1.2-3.7)	0.59 (0.42-0.76)	0.93 (0.51-1.3)	0.80 (0.60-1.0)	1.9 (1.0-2.8)	1.5 (1.3-1.7)	-	0.084 (0.057-0.11)	2.3 (1.3-3.2)

<u>Annex II –</u> Age range, number of samples (n) and mercury in several tissues expressed as arithmetic mean ± S.D. in µg g^{-1} dw of *H. ichneumon* of Serpa.

Gender	Age Range	n	Tissues (mean ± S.D. (µg g^{-1} dw))									
			Muscle	Liver	Lungs	Heart	Spleen	Kidneys	Blood	Brain	Fat	Pelage
Females	Cubs	1	0.17	0.15	0.071	0.12	0.074	0.15	1.7	0.45	-	0.13
	Juveniles	6	0.36 ± 0.37	0.42 ± 0.49	0.15 ± 0.16	0.24 ± 0.26	0.17 ± 0.18	0.47 ± 0.56	1.1 ± 1.3	0.13 ± 0.1	0.040 ± 0.045	0.37 ± 0.42
	Sub-Adults	2	0.19 (0.15-0.22)	0.28 (0.15-0.41)	0.089 (0.051-0.13)	0.12 (0.084-0.15)	0.11 (0.083-0.13)	0.29 (0.14-0.44)	0.46 (0.30-0.61)	0.066 (0.054-0.078)	0.023	0.30 (0.27-0.33)
	Adults	6	0.35 ± 0.24	0.44 ± 0.26	0.14 ± 0.063	0.20 ± 0.14	0.18 ± 0.13	0.45 ± 0.26	2.3 ± 2.6	0.31 ± 0.48	0.029 ± 0.018	0.70 ± 0.48
Males	Cubs	1	0.088	0.068	0.025	0.043	0.036	0.089	0.023	0.032	0.012	0.129
	Juveniles	4	0.33 ± 0.28	0.29 ± 0.26	0.12 ± 0.097	0.18 ± 0.16	0.16 ± 0.14	0.36 ± 0.36	0.14 ± 0.097	0.10 ± 0.068	0.028 ± 0.018	0.46 ± 0.36
	Sub-Adults	1	0.39	0.42	0.16	0.20	0.11	0.33	0.18	0.12	0.054	0.94
	Adults	4	0.46 ± 0.33	0.70 ± 0.55	0.16 ± 0.094	0.29 ± 0.27	0.25 ± 0.23	0.64 ± 0.59	0.34 ± 0.26	0.13 ± 0.059	0.037 ± 0.022	0.80 ± 0.54

Annex III – O-Hg concentrations (µg g^{-1} dw) and its correspondent percentage in muscle tissue.

Gender	Age Range	N	O-Hg (µg g^{-1} dw)	T-Hg (µg g^{-1} dw)	% O-Hg
Females	Cubs	1	0.13	0.17	76,8
	Juveniles	6	0.35 ± 0.38	0.36 ± 0.37	95,9
	Sub-Adults	2	0.15 (0.13 – 0.17)	0.19 (0.15-0.22)	78,7
	Adults	6	0.34 ± 0.23	0.35 ± 0.24	94,5
Males	Cubs	1	0.070	0.088	79,3
	Juveniles	4	0.31 ± 0.30	0.33 ± 0.28	93,8
	Sub-Adults	1	0.38	0.39	97,8
	Adults	4	0.47 ± 0.38	0.46 ± 0.33	96,8