UNIVERSIDADE DE ÉVORA

ESCOLA DE CIÊNCIA E TECNOLOGIAS

Mestrado em Gestão e Conservação de Recursos Naturais

Dissertação

Caracterização da contaminação por metais da lampreia-marinha *Petromyzon marinus* nas principais bacias hidrográficas nacionais

Sílvia Susana Ferreira Pedro

Orientador:
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Nota prévia

Da elaboração da presente dissertação resultou um artigo científico que corresponde, naturalmente de forma mais condensada, ao conteúdo integral da mesma. O mesmo encontra-se submetido para publicação numa revista científica internacional, sob o título *"Trace elements accumulation in anadromous sea lamprey spawners*", com autoria de S. Pedro, I. Caçador, B. Quintella, M.J. Lança e P.R. Almeida. Considerando que o trabalho apresentado foi realizado em colaboração com os autores mencionados, a candidata esclarece que participou activamente na obtenção, análise e discussão de todos os resultados e na elaboração dos manuscritos resultantes.

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RESUMO

A lampreia-marinha, *Petromyzon marinus*, é um ciclóstomo e migrador anádromo que ocorre nas principais bacias hidrográficas nacionais. Em Portugal, e em alguns outros países da Europa (*e.g.* Espanha e França), é considerada uma iguaria, atingindo um valor comercial bastante elevado, mas tendo ainda assim uma elevada procura pelos consumidores durante a época autorizada para a sua captura. Em Portugal esse período ocorre entre Janeiro e Abril, o que corresponde ao final da migração reprodutora desta espécie. Apesar de existir uma extensa bibliografia que aborda diferentes aspectos da biologia e ecologia da lampreia-marinha, a informação relativamente à caracterização da concentração de metais nos diferentes tecidos desta espécie é reduzida, e analogamente desconhece-se o seu perfil de contaminação; consequentemente, a segurança patente no consumo humano desta espécie tem sido relegada das considerações das autoridades nacionais e internacionais.

Determinou-se a concentração de sete elementos em amostras de músculo e fígado de exemplares adultos de lampreia-marinha, nomeadamente As, Cd, Cu, Hg, Ni, Se e Zn, por espectrometria de emissão atómica com indução de plasma acoplada (ICP-AES). Os espécimes utilizados para esta determinação foram provenientes de oito bacias hidrográficas nacionais que correspondem à sua distribuição em Portugal: de norte para sul, Minho, Lima, Cávado, Douro, Vouga, Mondego, Tejo e Guadiana.

Este estudo teve com objectivos principais: 1) avaliar o perfil da concentração de metais essenciais e não essenciais no músculo e fígado de lampreia-marinha; 2) avaliar a segurança relacionada com o consumo humano de lampreia-marinha, com base na concentração de metais de músculo; 3) investigar possíveis diferenças na acumulação de elementos nos exemplares adultos que entram nas bacias hidrográficas.

Os principais resultados deste estudo mostraram que, de uma forma generalizada, as fêmeas apresentam maior concentração de metais que os machos, tanto no músculo como no fígado, apesar de apenas para o fígado essas diferenças possuírem significância estatística. A concentração de metais no músculo foi, em geral, baixa, à excepção do Hg. Este metal apresentou

níveis de concentração no músculo acima do limite legal estabelecido para o consumo humano, pelo que deve ser dada especial relevância e atenção a este aspecto futuramente. O perfil estabelecido através da análise conjunta dos três elementos não-essenciais em análise no músculo evidenciou uma segregação das amostras em dois grupos principais, influenciada principalmente pela concentração de mercúrio. A justificar esta separação estarão possivelmente questões relacionadas com a ecologia trófica das presas parasitadas pela lampreia-marinha – nível trófico e/ou contaminação distintos – ou ainda diferenças na duração da fase parasítica desta espécie.

Trace elements accumulation in the sea lamprey, *Petromyzon marinus*, L., along the major hydrographic basins of Portugal

SUMMARY

The sea-lamprey, *Petromyzon marinus*, is an anadromous cyclostome that occurs in the main river basins of Portugal, where it is considered a gastronomic delicacy, as it is in other European countries (*e.g*. Spain and France). Sea lampreys have a high demand in restaurants (notwithstanding the high commercial value) during the period of authorized capture, which coincides approximately to the final period of the spawning run in Portugal (January to April).

Despite the extended literature addressing different aspects of the biology and ecology of the sea lamprey, the information regarding trace metals concentration in this species is sparse and reduced, and likewise, its contamination profile is poorly known; consequently, the safety for the human consumption of sea lamprey has been neglected by the national and international health authorities.

Trace metals' concentration (As, Cd, Cu, Hg, Ni, Se and Zn) was analyzed by ICP-AES (Induced Coupled Plasma Atomic Emission Spectrometry) in the liver and muscle of adult specimens of sea lamprey from eight national river basins. The river basins correspond to the distribution of the sea lamprey in Portugal: from north to south: Minho, Lima, Cávado, Douro, Vouga, Mondego, Tagus and Guadiana.

This study aimed: 1) to assess the concentration profile of essential and non-essential elements in the muscle and liver of sea lampreys that spawn on the Portuguese river basins; 2) to determine the safety of sea lamprey for human consumption regarding metal content; and 3) to investigate possible differences in the trace elements accumulation in adult sea lampreys entering Portuguese river basins.

The main results of this study revealed that females accumulated higher levels of metals than males, in muscle and liver, but only differences in the liver were statistically significant. In a general overview, the concentration of most elements analyzed was low, except for Hg in the muscle. This metal exhibited

an average concentration in the muscle that exceeded the statutory limits for fish muscle, and for that reason special attention must be paid to this issue in the future. The concentration profile based on non-essential elements (As, Cd and Hg) in the muscle evidenced a segregation of the samples into two groups, mostly based on Hg concentration. Distinct trophic and contamination levels of sea lamprey's preys, and/or different duration of the parasitic phase, may be in the origin of this separation.

Trace elements accumulation in the sea lamprey, *Petromyzon marinus*, L., along the major hydrographic basins of Portugal

ABSTRACT

The sea lamprey, *Petromyzon marinus*, is an anadromous cyclostome that occurs in the main Western Europe river basins draining to the Atlantic Ocean, and considered a gastronomic delicacy in Portugal, Spain and France. The contamination profile of this species is fairly unknown as far as trace metals are concerned, with only a few studies dedicated to the subject. Trace elements concentration was analyzed in muscle and liver samples of adult specimens from eight Portuguese river basins. This study aimed: 1) to assess the profile of essential and non-essential elements accumulation in the muscle and liver of sea lampreys spawners; 2) to determine the safety of sea lamprey for human consumption regarding elements content; and 3) to investigate possible differences in the trace elements accumulation in adult sea lampreys entering Portuguese river basins. Females accumulated higher levels of elements than males, but only differences in the liver were significant. In a general overview, the accumulation of most elements analyzed was low, except for Hg in the muscle, which exceeded the statutory limits for fish concentration. The muscle accumulation profile based on non-essential elements (As, Cd and Hg) evidenced a segregation of the samples into two groups, mostly based on Hg concentration. Distinct trophic levels and contamination of preys and different time duration of the parasitic phase may be in the origin of this separation.

Keywords

Petromyzon marinus; metal concentration; muscle; liver; Portuguese basins; tolerable weekly intake

INTRODUCTION

The sea lamprey

The sea lamprey, *Petromyzon marinus* Linnaeus, 1758, is the sole representative of its genus. It is not a true fish, but a cyclostome – a jawless, aquatic vertebrate, belonging to Agnatha, a group that flourished during the Paleozoic. Sea lampreys have a slender, rounded, eel-like body, with a fibrous and cartilaginous skeleton, no scales, no paired appendages, two dorsal fins and an elongated caudal fin. One of the most distinguished characteristics of sea lampreys is the sucker-like oral disk and tongue with well-developed teeth (Fig. 1). They have seven pairs of gills, each with branquial aperture to the exterior, and their digestive system lacks a stomach. The name of the genus *Petromyzon* (Gr. *Petros*, stone, + *myzon*, sucking) refers to the species habit to grasp stones with the oral disk to maintain their position in a current (Hyckman *et al*., 1997).

Figure 1 – Detail of the oral disk of *Petromyzon marinus*

Petromyzon marinus occurs on both sides of the North Atlantic with anadromous populations, and in the Great Lakes of North America as a landlocked form. This landlocked form was firmly established in the Great Lakes by the late 1940's, and it is considered a pest that has been affecting dramatically fisheries and the ecological balance of the region (Hyckman *et al*., 1997). The anadromous form, on the other hand, has a varying occurrence throughout European waters – rare in the northern and central Europe and with the largest populations in rivers that flow into the Atlantic; in Portugal it occurs in the main river basins. Listed as "Least Concern" by the IUCN Red List of Threatened Species (Freyhof & Kottelat, 2008), *P. marinus* populations in Europe have been decreasing, and in Portugal the sea lamprey is classified as "Vulnerable" in the national Red List of Threatened Vertebrate Species (Rogado *et al.*, 2005). Gravel extraction, impassable dams and weirs (Assis, 1990, Almeida *et al.*, 2000), dredging, habitat loss (Quintella *et al.*, 2007), pollution (Maitland, 1980; Rogado *et al.*, 2005), poaching and intense fisheries (Andrade *et al.*, 2007) are among the main reasons for the decrease of *P. marinus* populations in Portugal.

The life cycle of the anadromous populations of sea lamprey in the east coast of the Atlantic is represented in Figure 2; adults may reach 1.20 m total length and about 2.3 kg total weight (Hardisty, 1986).

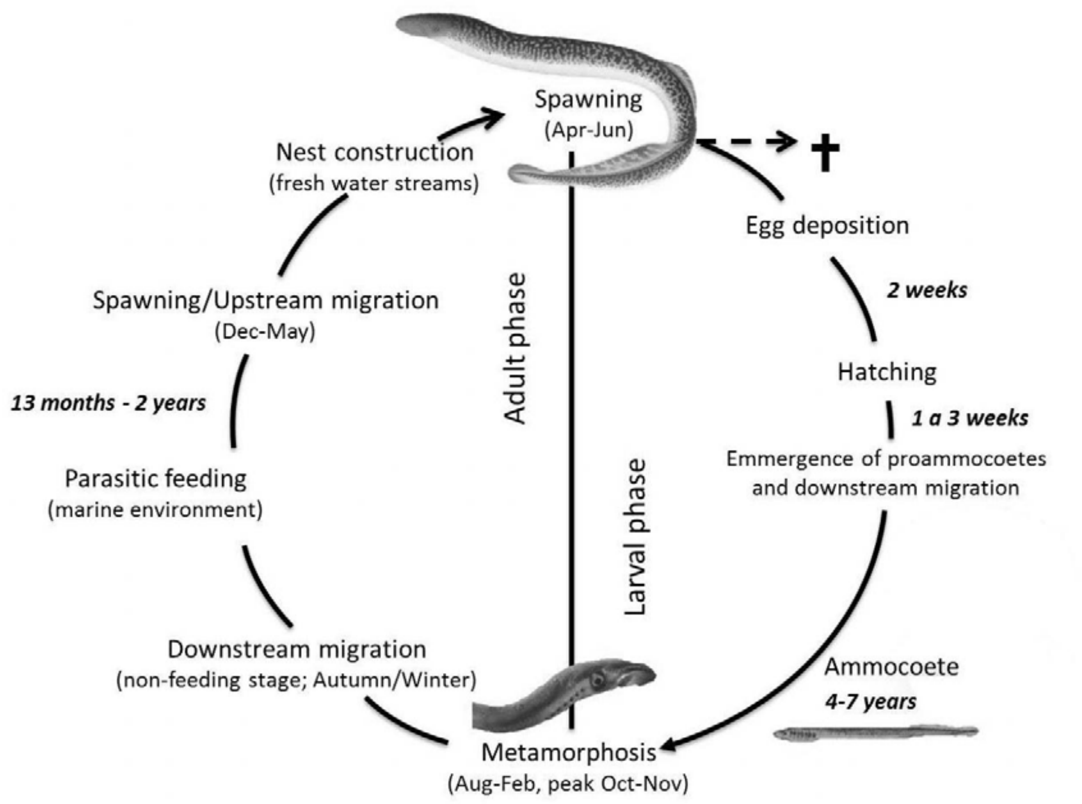

Figure 2 – Life cycle of *P. marinus*.

The microphagous filter feeding ammocoetes (larvae) live 4 to 7 years burrowed in freshwater sediments and then undergo a metamorphosis (macrophtalmia) that ends with the ongoing of the juvenile trophic migration. Adults adopt a parasitic strategy. They attach to the preys with their sucker-like mouth and feed primarily on blood and flesh of the hosts, remaining in the marine environment for a period that may last from 13 months (F. Cobo Cardín and S. Silva-Bautista, pers. comm.) to about 2 years (Beamish, 1980). After that period, sea lampreys return to freshwater environment to spawn, entering the Portuguese river basins from the late fall and early winter, until late spring (May), with a peak between February and April (Almeida *et al*., 2000; 2002). It is during the spawning run that sea lampreys are captured by fishermen. Capture is allowed between January and April, and occurs mostly in the estuaries and lower reaches of the rivers. Nevertheless, poachers capture spawning animals in their nests upstream, representing an elevated number of captured lampreys (Andrade *et al*., 2007). Sea lamprey is highly appreciated in the Portuguese gastronomy, and the price of a single specimen may reach 30-80 euros in peak season (Suissas, 2010), depending on the abundance of lampreys on each year.

Trace metals

Factors like fish species, feeding habits, ontogenic development, or the physical and chemical characteristics of the surrounding environment play an important role in the intake and accumulation of trace elements (Dallinger *et al*., 1987; Pourang 1995; Farkas *et al*., 2002, 2003; Türkmen *et al.*, 2005).

All metals[1] are toxic, but only some are essential. As a general rule of thumb, essential metals are those that play important roles in the biota) and non-essential metals are those with no biological function recognized, and for that reason are simply and strictly toxic when present (Brown & Depledge, 1998. Examples of essential metals are cobalt, copper, chromium, iron, selenium, zinc and manganese, among others. These elements are vital components of enzymes, several other proteins and structural elements in organisms (Eisler, 1987; Brown & Depledge, 1998; Eisler, 1998a). Zinc (Zn) is a component of

[1] In order to simplify the reading of the present text, the terms “trace elements”, “trace metals” or “metals” may be used to refer all elements analyzed, notwithstanding the fact that As and Se are not metals, but a metalloid and a non-metal, respectively.

several enzymes, like carbonic anhydrase, and several hydrogenases; Zn also assures stability of biological molecules such as DNA and structures like membranes and ribosomes (Eisler, 1993; Brown & Depledge, 1998). Selenium (Se) constitutes an integral part of glutathione peroxidase and may have a role in other biological active compounds, like vitamin E (Eisler, 1985b; Brown & Depledge, 1998). Copper (Cu) is present in cytochrome *c* oxidase and the respiratory pigment haemocyanin and is also part of the enzymes responsible for the production of melanin (tyrosinase) and catecholamine (dopamine beta hydroxylase), among many others (Brown & Depledge, 1998; Eisler, 1998a). Nickel (Ni) is essential for the normal growth of several organisms, from microorganisms to plants and vertebrates (Eisler, 1998b). As referred initially, all essential metals are also toxic when the concentration needed to their biological role is exceeded, or when certain interactions occur. Some examples of essential elements toxicity in aquatic environments are high concentrations of Zn that promote physical damage to the gills of fishes (Eisler, 1993); reproductive impairment of aquatic birds and teleosts has been reported after exposure to toxic concentrations of Se (Eisler, 1985b); excess Cu is known to cause a variety of toxic effects, including altering membrane permeability (Eisler, 1998a); retarded growth, central nervous system disorders, carcinogenic and mutagenic effects are some of the toxic effects attributed to Ni (Eisler, 1998b).

Arsenic (As), cadmium (Cd) and mercury (Hg) are examples of non-essential metals, and their effects in the biota will go from 'negligible' to 'extremely toxic' depending on the concentrations (Eisler, 1985a, Eisler, 1987, Sorensen, 1991; EFSA, 2004, 2009, 2011). Arsenic is extremely poisonous for fishes, depending on the chemical form, and bizarre morphological alterations are induced by As in these organisms (Sorensen, 1991); among other effects, the inorganic form is highly carcinogenic in humans (EFSA, 2011). Cadmium, like excess Zn, can disrupt or terminate enzymatic activity (Eisler, 1985a; Sorensen, 1991); direct Cd-induced injury of gills in fishes is also reported, with the subsequent alteration of respiratory function (Sorensen, 1991); in humans, Cd is toxic primarily to the kidney, leading to renal dysfunction (Eisler, 1985a, EFSA, 2009). Mercury is extremely poisonous and its presence in the cells of living organisms is undesirable and potentially hazardous; it binds strongly to sulfhydryl groups, promoting strong cell division inhibitions (Sorensen, 1991). Organic mercury forms, like methylmercury (Me-Hg), are the ones of most concern (Eisler, 1987; EFSA, 2004). The widely documented disaster of Minamata Bay (1950's) caused by Me-Hg poisoning is a clear statement of the biological implications of Hg acute and chronic exposure. Erratic behavior was observed in several species of mammals and large numbers of dead fish appeared floating on the sea surface. Humans that consumed contaminated fish and shellfish suffered from severe neurological damages and sensory impairment, among many other disturbances. The congenital cases of physical and mental development disturb reached abnormally high levels (Eisler, 1987; Sorensen, 1991).

Objectives

With respect to trace metals, the contamination of P. marinus is poorly known, with only a few studies addressing the subject (*e.g*. Drevnick *et al.*, 2006). This work aimed 1) to assess the profile of essential and non-essential trace elements accumulation in the muscle and liver of sea lampreys that spawn on the Portuguese river basins; 2) to determine the safety of sea lamprey for human consumption regarding its trace elements content; and 3) to investigate possible differences in the trace elements accumulation in adult sea lampreys entering Portuguese river basins.

MATERIALS AND METHODS

Sampling

A total of 80 specimens from eight Portuguese river basins (Minho, Lima, Cávado, Douro, Vouga, Mondego, Tagus and Guadiana, 10 from each basin (Fig. 3), sex ratio 1:1) were collected by professional fishermen with trammel nets during the peak of the sea lamprey spawning seasons of 2008 and 2009, at the lower reaches of each river. Specimens were transported to the laboratory and kept alive in tanks equipped with basic life support systems (*i.e.* water aeration and filtration) until being processed. Transportation time varied between 3 and 5 hours, animals were processed until maximum 24 hours of arrival to the laboratory.

Figure 3 – Geographical distribution of *P. marinus* in the main Portuguese rivers; bold line: available habitat, broken line: unavailable habitat due to impassable barriers (white blocks).

Tissue preparation and collection

Data on body total mass (M_T, nearest g) and total length (L_T, nearest mm – length between the beginning of the oral disk to the end of the caudal fin) was registered for each sea lamprey. Whole liver, gonads and trunk muscle between the posterior edge of the last branchial opening to the anterior edge of the cloacal slit in the left flank of the animal were collected; samples were washed with physiologic saline solution and immediately stored at -20°C until further processing in the laboratory, where samples (muscle and liver) were then freeze-dried and homogenized to a fine powder material. Gonads were only used to differentiate genders and to determine gonad somatic index (GSI).

Muscle lipid extraction

Muscle total lipids were extracted using a Dionex 100 accelerated solvent extractor (ASE). To prepare for extraction, the tissue samples were removed from liquid nitrogen, weighed and lyophilized until constant mass to determine the percentage of water loss. Aliquots of liver tissue with 1 g of dry weight were then pulverized in an aluminum mortar with a stainless steel pestle, both cooled in liquid nitrogen. The tissue powder was combined with 1 g of hydromatrix drying agent (Diatomaceous Earth, hydromatrix Varian, P/N 049458) and hydromatrix mixture were transferred to a 11-mL stainless steel extraction cell fitted with two cellulose filters, and additional hydromatrix was added to fill the cell. The total lipid sample was then extracted with a mixture of 60% chloroform and 40% methanol (Merck, Darmstadt, Germany) at 100°C and 13.8 MPa. Both extraction solvents were residue-analysis grade and were treated with 100 mg/L BHT (3,5-Di-tert-butyl-4-hydroxytoluene, Merck, Darmstadt, Germany) as an antioxidant. Two static extraction cycles were carried out during a 5 min period each. The crude extract was then concentrated under a stream of nitrogen and vacuum using a TurboVap apparatus (Zymark; Hopkinton, MA) set at a bath temperature of 50 °C and the dry mass of recovered material was measured to the nearest 0.01 mg to determine muscle total lipids (TL_M).

Trace elements determination

All labware was soaked in 0.25M HNO3 for 24h and 0.25M HCl for 48h, and rinsed three times with deionized water to avoid contamination.

To extract elements from the samples, ca. 0.1g of freeze-dried, homogenized material was acid digested with 2ml of HNO_3 and $HClO_4$ (v/v, 9:1), during 2 hours at 110°C (Julshamn *et al.*, 1982). Cadmium (Cd), copper (Cu), nickel (Ni), zinc (Zn) were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Horiba Jobin-Yvon, France, model Ultima equipped with a RF generator of 40.68 MHz and a type Czerny-Turner monochromator with 1.00 m (sequential); a Concomitant Metals Analyzer (CMA) was used to simultaneously determine arsenic (As), mercury (Hg) and selenium (Se) in the samples. The accuracy and precision of the analytical methodology for elemental determinations were assessed by replicate analysis of certified reference materials (CRM), namely TORT-2 (lobster hepatopancreas). Blanks and the concurrent analysis of the standard reference material were used to normalize sample data.

Statistical analysis of data

The statistical packages Statistica v.10 (Stat Soft. Inc., 2011) and Primer v.6 & PERMANOVA (Clarke & Gorley, 2006) were used for data treatment and statistical analysis.

MANOVA was used to see the main and interaction effects of categorical variables (gender and river basins) on total mass (MT,) total length (LT,) gonads weight (Gonads), liver weight (Liver), hepatosomatic index (HSI) and gonad somatic index (GSI).

Mann-Whitney U test was used to compare trace elements accumulation in the muscle and the liver samples between genders, whereas Kruskal-Wallis H test, followed by Simultaneous Test Procedures (STP, Siegel & Castellan, 1988), was used to compare trace elements accumulation in the muscle and liver of individuals among river basins. Spearman correlation analysis was applied to test the relationship between elements concentrations and i) body total mass, ii) body total length, and iii) muscle total lipids. Previous results revealed that neutral lipids contents of sea lamprey muscle represents between 22-29% of the total muscle dry weight against 7-8% in liver. For this reason, only correlation between elements concentration and muscle total lipids were done (Lança *et al*., 2011).

A principal component analysis (PCA) and a group average cluster analysis performed based on Spearman rank correlation were used to investigate the existence of groups of samples with similar trace elements' profile. A distance-based permutational multivariate analysis of variance (PERMANOVA) based on the previously obtained Spearman rank correlation resemblance matrix was used to compare muscle non-essential elements accumulation profile between the groups that corresponded to clusters obtained by the cluster analysis. Liver data and essential elements were not used to avoid misinterpretations arising from metabolic issues. The analysis was done using 999 random permutations of the appropriate units (Anderson & ter Braak, 2003). The data set comprised 74 observations x 3 variables (*i.e.,* trace elements) and the design included one factor (group (two levels fixed)). SIMPER test (similar percentages) was used to determine which specific variables contribute to overall differences, *i.e*., which elements had more influence on dissimilarities among groups (Warwick *et al*., 1990; Clarke, 1993).

RESULTS

The biometric parameters total length (L_T), total mass (M_T), liver and gonads mass, muscle total lipids (TL_M), hepatosomatic and gonadosomatic indexes by gender for the sea lampreys collected in the eight river basins are shown in Table 1.

Multivariate tests for individual effects overall revealed that gender (GLM test, F=163.209; df=1; p=0.001) had significant effect on gonads mass (GLM test, F=1035.83; df=1; p=0.001), hepatosomatic index (GLM test, F=18.283; df=1; p=0.001) and on gonadosomatic index (GLM test, F=603.408; df=1; p=0.001), whereas river basin (GLM test, F=1.644; df=7; p=0.009) had significant effect on gonads mass (GLM test, F=4.155 df=7; p=0.001), liver mass (GLM test, F=2.249; df=7; p=0.043), total mass (GLM test, F=4.822; df=7; p=0.001) and total length (GLM test, F=3.798; df=7; p=0.002). Males presented slightly higher values of L_T, whereas females had higher M_T values (males: $\overline{L_T}$= 877±43 mm, $\overline{M_T}$= 1196± 175 g; females: $\overline{L_T}$= 868±52 mm; $\overline{M_T}$= 1246±205 g); nonetheless, L_T and M_T were not significantly affected by gender. The smallest lampreys were captured in the river Douro ($\overline{L_T}$= 851.5±34.8 mm; $\overline{M_T}$= 1094±112.4 g) and river Guadiana ($\overline{L_T}$= 852.1±45.8 mm; $\overline{M_T}$= 1079.7±143.6 g), and the largest ones were captured in river Tagus ($\overline{L_T}$= 908.6±24.3 mm; $\overline{M_T}$= 1350.4±89 g) and river Cávado ($\overline{L_T}$= 903.3±41.7 mm; $\overline{M_T}$= 1394.1±190.9 g).

Zn was the most abundant element in the muscle ($\bar{x}$ =11.3 ± 3.6 µg/g wet weight), and Cu was the most abundant element in the liver ($\bar{x}$ =172.8 ± 56.7 µg/g wet weight). In muscle, the average concentrations for the trace metals analyzed showed the following decreasing order: Zn (11.3±3.6 µg/g)> Cu (1.2±0.4 µg/g)> As (1.2±0.4 µg/g)> Hg (1.0±0.6 µg/g)> Se (1.0±0.3 µg/g)> Ni (0.06±0.1 µg/g)> Cd (4.0±0.4 ng/g) and in the liver, the ordination was Cu (172.8±56.7 µg/g)> Zn (25.6±11.4 µg/g)> Se (4.5±1.8 µg/g)> As (2.0±0.8 µg/g)> Cd (0.5±0.3 µg/g)> Hg (0.3±0.3 µg/g)> Ni (0.06±0.05 µg/g).

Table 1 – Mean (± standard deviation) total length (L_T), total mass (M_T), muscle total lipids (TL_M), liver mass (Liver), gonads mass (Gonads), hepatosomatic index (HSI) by genders and gonadosomatic index (GSI) by genders of sea lampreys collected in the eight river basins

	Minho (N = 10)	Lima (N = 10)	Cávado (N= 10)	Douro (N = 10)	Vouga (N = 10)	Mondego (N = 10)	Tagus (N = 10)	Guadiana (N=10)
L_T, mm	899.7±21.4	889.2±45.3	903.3±41.7	851.5±34.8	844.3±50.3	878.4±50	908.6±24.3	852.1±45.8
M_T, g	1336±50	1255±173.4	1394.1±190.9	1094±112.4	1157.4±161	1267.5±193.8	1350.4±88.9	1079.7±143.6
Liver, g	23.6±2.3	21±3.1	24±4	18.4±2.9	21.6±3.2	22.1±5.3	23.7±2.8	19.7±1.5
Gonads, g - males	19.8±2.4	19.9±2.9	23.5±7.6	16.7±7.8	21.9±3.2	21.4±4	25.4±3.8	16.1.1±7.8
Gonads, g - females	106.4±11.6	98.5±16.8	109.9±13.4	90.9±2.3	93.7±12.1	128.3±19.9	116.3±10.4	131.1±25.5
TL_M - males	327±97	519.9±152.9	490.5±34	420.1±99.48	302.6±31.7	336.7±25	264.9±13.1	400.9±26.8
TL_M - females	352.6±18	400.5±132.5	523.2±67.5	332.4±100.3	266.2±49.9	283.9±43	292.9±28.2	376±118.3
HSI (%) - males	1.87	1.94	1.90	1.74	2.02	1.78	1.94	1.96
HSI (%) - females	1.61	1.73	1.62	1.71	1.75	1.77	1.59	1.68
GSI (%) - males	1.51	1.47	1.80	1.45	1.93	1.70	1.90	1,60
GSI (%) - females	7.99	9.20	7.63	8,57	8.07	9.90	8.45	10.78

Comparing both tissues (Fig. 4), and for all metals but Hg and Ni, concentrations found in the liver were significantly higher than in the muscle ($p<0.05$) whereas the opposite situation was observed for Hg concentrations with significantly higher concentrations in the muscle ($p<0.05$).

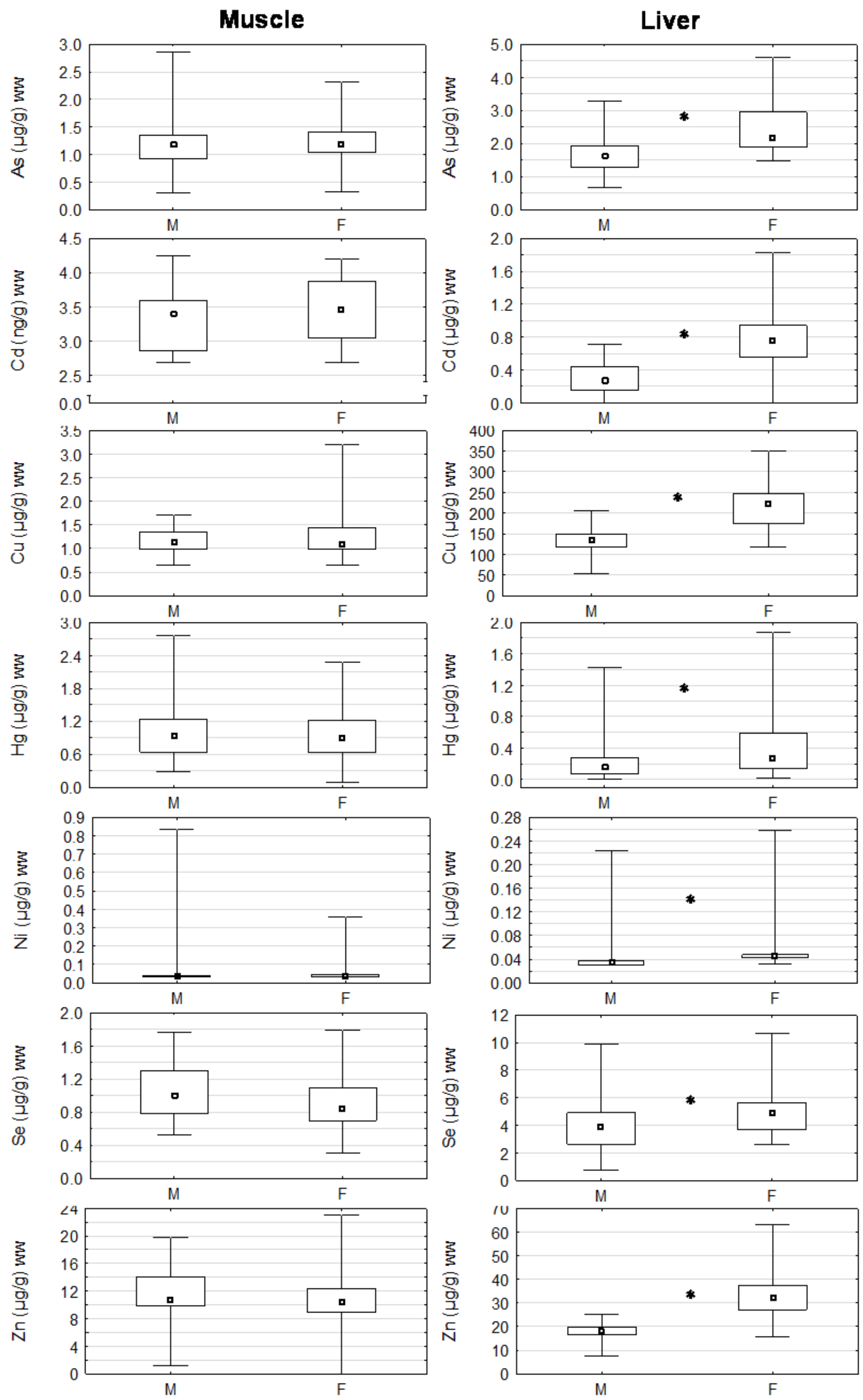

Figure 4 - Trace metals in muscle and liver of *P. marinus*; boxes: median and interquartile range; whiskers: minimum and maximum values; M: males, F: females; ww: wet weight; * significant differences between genders ($p<0.05$).

Nickel concentrations did not show significant differences between liver and muscle. In muscle samples, the concentration of trace elements did not differ between gender, as opposed to the situation observed in liver, with consistently higher values ($p<0.05$) for females (Fig. 4).

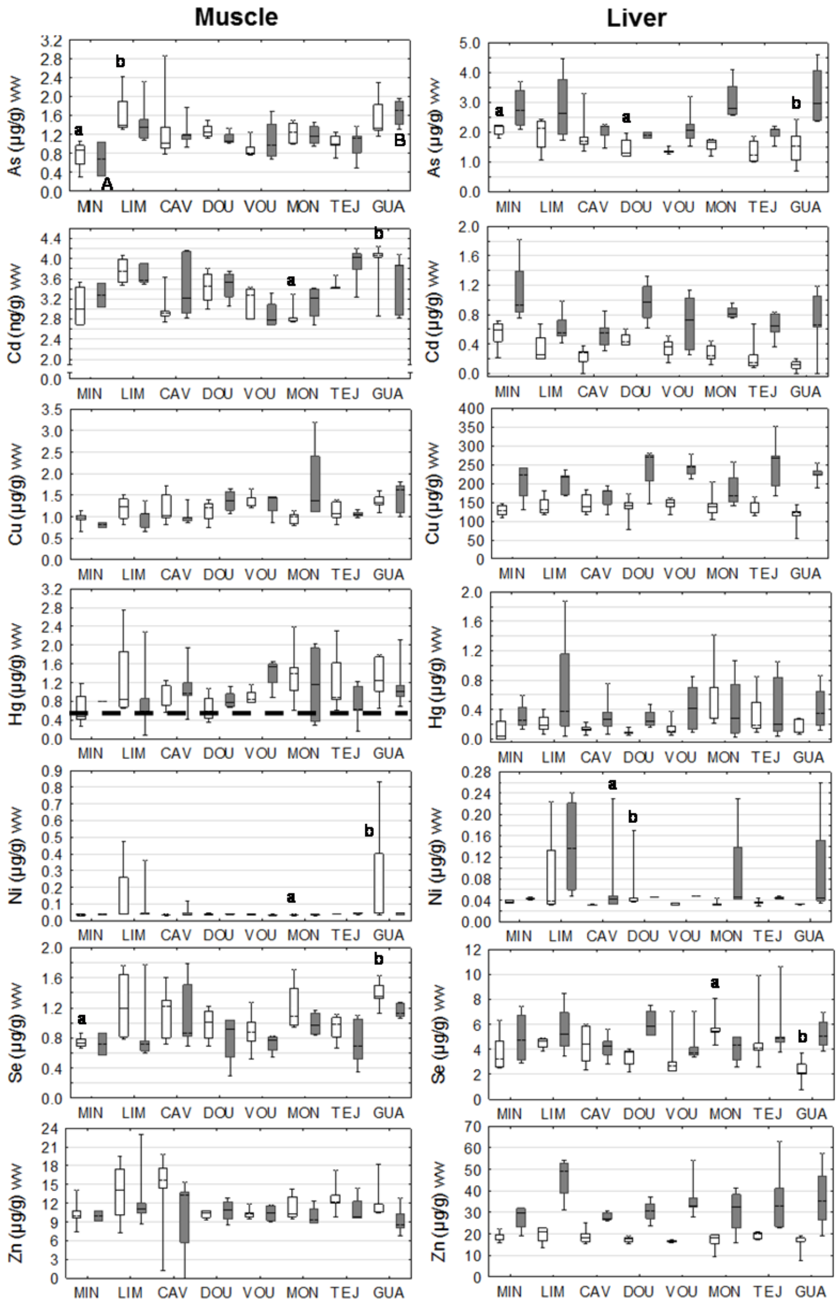

Figure 5 – Trace metals in muscle and liver of male (white) and female (grey) *P. marinus* in the main Portuguese river basins (see text for acronyms); boxes: median and interquartile range; whiskers: minimum and maximum values; ww: wet weight; different letters next to the boxes: significant differences between basins for males (lower case letters) and females (upper case letters) ($p<0.05$); dashed line: statutory limits for Hg concentration in fish.

Some variation was found among individuals from river basins (Fig. 5); Minho (MIN) individuals presented in general the lowest median values for all the elements analyzed in the muscle (except for Cd), while Cávado (CAV), Mondego (MON), Vouga (VOU) and Lima (LIM) individuals displayed the highest median accumulation values (CAV: Se and Zn; MON: Hg; VOU: Cu; LIM: As).

Moreover, muscle accumulation of metals among males of river basins yielded significant differences ($p<0.05$) for As, Cd, Ni and Se, whereas for liver significant differences ($p<0.05$) was also found for Cd, Ni and Se (Fig. 5). For females, only As in muscle revealed significant ($p<0.05$) differences among river basins.

In muscle, As, Cu and Hg were negatively correlated with M_T and L_T whereas Zn was only negatively correlated with L_T (Table 2). The correlation between trace elements and TLM was not significant ($p>0.05$). Regarding to liver, the only significant correlation found was between Cd and MT ($R=0.271$, $p<0.05$) (Table 2).

Table 2 – Spearman rank correlations between trace elements in the muscle and liver and total mass (M_T) and length (L_T) of *P. marinus* and between trace elements and muscle total lipids (LT_M) in the muscle

Muscle	M_T	L_T	TL_M	Liver	M_T	L_T
As	-0.379**	-0.262*	*n.s.*	As	*n.s.*	*n.s.*
Cd	*n.s.*	*n.s.*	*n.s.*	Cd	0.271*	*n.s.*
Cu	-0.298*	-0.312**	*n.s.*	Cu	*n.s.*	*n.s.*
Hg	-0.354**	-0.336**	*n.s.*	Hg	*n.s.*	*n.s.*
Ni	*n.s.*	*n.s.*	*n.s.*	Ni	*n.s.*	*n.s.*
Se	*n.s.*	*n.s.*	*n.s.*	Se	*n.s.*	*n.s.*
Zn	-0.249*	*n.s.*	*n.s.*	Zn	*n.s.*	*n.s.*

n.s. – non-significant; * $p<0.05$, ** $p<0.01$

The principal components analysis based on the concentration of the non-essential elements under study in the muscle (As, Cd and Hg), and the superimposed group average cluster analysis using Spearman rank correlation (R= 0.81), evidenced a separation of the individuals into two groups (Fig.6).

Group I included 0% to 44% and Group II included 56% to 100% of the specimens from each river basin.

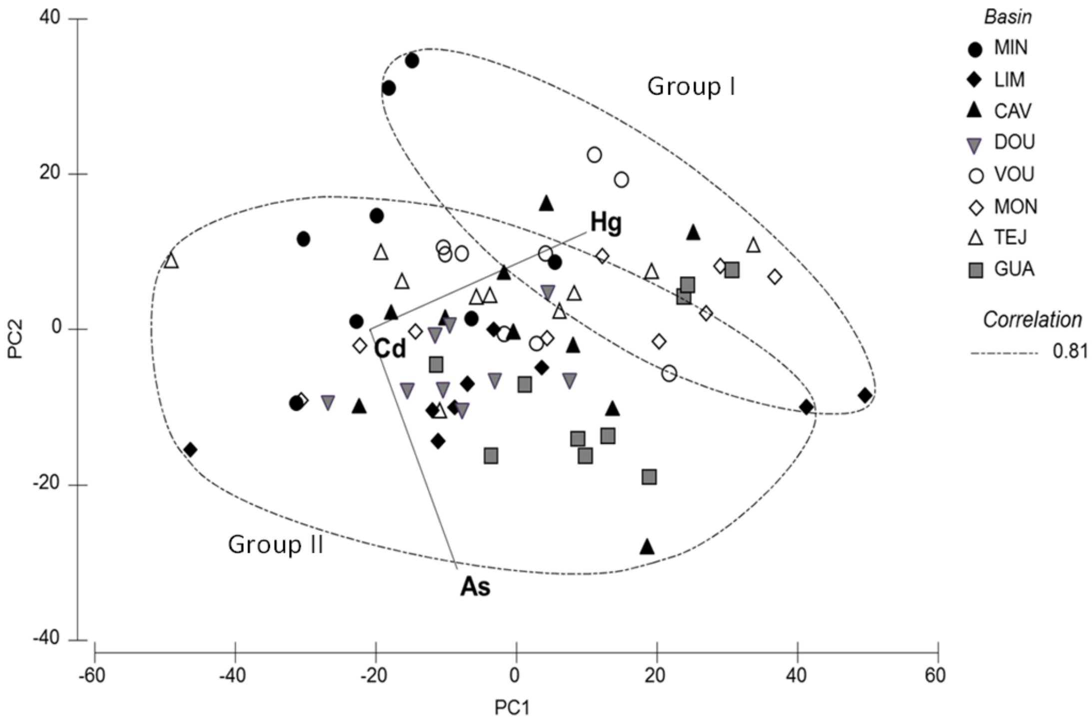

Figure 6 – PCA diagram with overlaid cluster analysis (Spearman correlation, R=0.81) representing the contamination profile of *P. marinus* muscle based on As, Cd and Hg concentrations; PC1=77.2% variation, PC2=24.8% variation.

The PERMANOVA results showed that the "Group" factor (group based on cluster analysis) exhibited significant differences ($p<0.05$) in the muscle contamination profile (Table 3).

Table 3. Results of PERMANOVA analysis testing changes with group for muscle trace elements accumulation profile

Source	df	SS	MS	Pseudo-F	P (perm)	Perm
Group	1	0.49	0.49	455.04	0.001	11
Residual	72	0.07	1.07×10^{-3}			
Total	73	0.56				

df, degrees of freedom; SS, sum of squares; MS, mean square; Pseudo-F, pseudo-F statistic, P (perm), P-value (permutations); Perm, number of permutations.

SIMPER analysis indicated that Hg concentration was the main differentiating factor, due to its high contribution to the dissimilarity between Groups I and II (Table 4).

Table 4. – Summary of the results from the SIMPER procedure for muscle trace elements accumulation

	Variables	Contribution to dissimilarity (%)
1. Dissimilarities within groups		
Group 1		
Average Squared Distance = 452.97	As	50.58
	Cd	0.05
	Hg	49.37
Group 2		
Average Squared Distance = 355.84	As	38.24
	Cd	0.04
	Hg	61.72
2. Dissimilarities among groups		
Group 1 vs. Group 2		
Average squared distance = 1454.82	As	25.45
	Cd	0.03
	Hg	74.53

DISCUSSION

A high variability concerning As, Hg and Ni and Cd, Hg, Ni and Zn accumulation was found in muscle and liver, respectively. The present work's results were compared to the few available data for *P. marinus* and it was observed that, in most cases, the values obtained are in the same order of magnitude. Nevertheless, the average of Hg concentration in the muscle was twice that of determined by Drevnick (pers. comm.). Results for high and variable Hg accumulation were also referred by Drevnick *et al.* (2006) in whole body of adult specimens of P. marinus, with a skeletal muscle coefficient of variation identical to the one found in this work (Drevnick, pers. comm.). The high variability in concentrations of Hg in adult sea lamprey is uncommon among fishes and is likely due to the diversity of habitats used by the species and the multitude of potential preys selected during their parasitic marine phase (Drevnick *et al*., 2006).

Trace metals can reach the marine environment through several routes depending on the form and structure in which the metal exists. When an element reaches the aquatic environment, the distribution within the aquatic environment is in accordance with its characteristics and those of the ecosystem (Landrum & Fisher, 1999). Metals may dissolve in the aqueous phase and remain as such in the water column or otherwise become adsorbed on some suspended solids of organic or inorganic nature (Landrum & Fisher, 1999).

Although the river basins could be the source of trace elements presented in the tissues analyzed, in our opinion, the concentration of trace elements found in the liver and muscle of sea lampreys should have little or no relation at all with the river basins where they were captured. This assumption is supported by some evidences: (i) these animals spent a short period of time (< 1 month) in the estuarine and freshwater environment during the spawning run before their capture (when compared to the time duration of parasitic phase in the sea – ca.1-2 years), during which they do not feed; and (ii) ammocoetes have a high bioaccumulation capacity, but considering that most of the growth of the sea lamprey occurs during the marine phase, this life latter cycle stage should be the main contributor to the accumulation of trace elements through food, and river basins contamination is most likely not reflected in adult specimens.

Sources of elements in aquatic organisms are accumulated from both surrounding water and from food, since whether the metal ends up dissolving in the water column or adsorbed on some particulate matter, it may, further, become ingested and assimilated by marine micro- or macro- flora or fauna, including various filter feeders; or it may eventually settle down into the benthic environment (Landrum & Fisher, 1999). The distribution and accumulation of the contaminant on the organism depends on the characteristics of the interaction between the organism and the phases into which the contaminant is distributed (Landrum & Fisher, 1999). Is also important to refer that direct intake of trace metals from the water by marine fishes, and most likely by sea lampreys, may be of minor importance (Brown & Depledge, 1998). Once an element moves from the environment into living organisms they can move through the food chain. Therefore, based on the aspects stated above, the accumulation of trace elements obtained is most likely a reflex of the feeding ecology of *P. marinus*. Considering that sea lampreys do not feed exclusively at a discrete trophic level (Beamish, 1980; Halliday, 1991; Drevnick, 2006), the feeding strategy should be not only the main source for trace elements but also the main reason for the variation found in the accumulation of those elements in the analyzed tissues of this species.

Overall, it was observed that the females showed the highest values for the majority of the elements, which is a common result in literature (*e.g.* Al-Yousuf *et al*., 2000; Alquezar *et al.*, 2006). Those differences, however, were only statistically significant for liver accumulation of trace elements. In laboratory experiments, females appeared to have grown to greater maximum sizes than males, but then increase shrinkage was shown during sexual maturation (Hardisty & Potter, 1971). Those results were consistent with the slight differences found between length and weight found in the wild specimens used in this study. Slower growth rates lead to a higher accumulation of metals, due to a lower “growth-dilution” effect (Brown & Depledge, 1998). The distinct composition of total lipids and proteins of the liver of migratory males and females (Beamish *et al.*, 1979), may help to explain the significant differences found in trace elements accumulation, since the concentration differ between gender, with consistently higher values for females. These differences may be related to several factors, such as the onset of reproduction and different physiological metabolism in relation to the stage in the reproductive cycle (Nicolletto & Hendricks, 1988; Al-Yousuf *et al*., 2000; Alquezar *et al*., 2006). In fact, the lower size of the females' liver when compared with males' livers and the significantly lower females HSI, could be signs indicative of lipid utilization and mobilization to support gonad development (Adams, 1999). The lower liver mass observed in female lampreys, when comparing with males in early spawning migration, was also observed by Beamish *et al.* (1979) and these authors suggested that the mass loss of the organ was related with the decreased mass of water, protein and lipid contents of liver during this stage, especially in females. Moreover, females showed significantly higher concentrations of elements than males and the concentration augmented with the increase in the gonadosomatic index, as observed by Zyadah (1999).

Our results revealed that for all elements, but Hg and Ni, concentrations found in the liver were significantly higher than in the muscle. Generally, the metal accumulation is higher in the liver than in musculature in most fishes. The higher levels in liver reflect the high metal storage capacity of this organ when compared with musculature tissue and this capacity is associated with the production of metallothioneins which appear as a metal detoxification mechanism within the body (Roesijadi & Robinson, 1994; Peakall & Burger, 2003).

As referred initially, there are only a few published studies addressing metal contamination in adult sea lampreys, namely on Hg concentration on the whole body (Drevnick *et al*., 2006) and Fe in the liver (Youson *et al*., 1983). Araújo (2011) also addressed Cd, Co, Cu, Fe, Mn, Pb and Zn, but only in the muscle of adult migrant females. Values reported by the latter author were in agreement with the present results.

Taking into account that the feeding ecology of the sea lamprey places it in the top of several marine food webs, results for the muscle were also compared to those found in other top marine predators, such as cods, black scabbards, tunas, marlins and sharks. Arsenic in the yellowfin tuna, *Thunnus albacares* (Bonnaterre, 1788), and the black scabbardfish, *Aphanopus carbo* Lowe, 1839, was reported to present similar values to those of the sea lampreys (Mormede & Davies, 2001; Burger & Gochfeld, 2005); the Atlantic cod, *Gadus morhua* Linnaeus, 1758, and the Atlantic bluefin tuna, *Thunnus thynnus* (Linnaeus, 1758), had twice the concentration of As in the muscle (Hellou *et al.,* 1992; Burger & Gochfeld, 2005). Cadmium concentration in the muscle of the skipjack tuna *Katsuwonus pelamis* Linnaeus, 1758, was similar to that of *P. marinus* (Al-Busaidi *et al.,* 2011), very variable in several species of cods (from one order of magnitude lower than the sea lamprey, to one order of magnitude above), and considerably higher in the blue marlin, *Makaira nigricans* Lacepède, 1802, Atlantic bluefin tuna and black scabbardfish (Eisler, 1985a; Hellou *et al*., 1992; Burger & Gochfeld, 2005; Afonso *et al*., 2007). Similar ranges of accumulation of Cu were described in the Atlantic cod, several shark species and swordfish, *Xiphias glaudius* Linnaeus, 1758 (Hellou *et al*., 1992; Eisler, 1998a). Concentrations of Hg in the muscle of the skipjack tuna, yellowfin tuna, and Atlantic cod were lower than in the sea lamprey, but as high as those in *P. marinus*, and higher, in several species of tunas from the NW-Atlantic, in the black scabbardfish and the swordfish (Eisler, 1987; Hellou *et al*., 1992; Afonso *et al.*, 2007). Nickel accumulation in the skipjack tuna largely exceeded that of the sea lampreys, by one and two orders of magnitude (Eisler, 1998b). Selenium levels in the muscle of several shark species and the swordfish were in the same range of those of *P. marinus*, but higher accumulation levels were found in the bluefin tuna, the black marlin, *Istiompax indica* (Cuvier, 1832) and the blue marlin (Eisler, 1985b; Hellou *et al.*, 1992). Finally, Zn accumulation in the muscle of marine predators such as the bluefin tuna was in the same range as that of the sea lamprey (Eisler, 1993; Hellou *et al*., 1992).

The concentrations of As, Cd and Hg in the muscle of *P. marinus* were compared with national and international standards for contaminants in food items (OJ-EU 2006; FAO-WHO, 2011a, 2011b), which is summarized in Table 5.

However, arsenic concentration in fish muscle is not regulated in the European territory.

Table 5. Comparison of established levels of As, Cd and Hg as contaminants in food with concentration found on *P. marinus* muscle TWI - Tolerable Weekly Intake; PTWI - Provisional TWI; $BMDL_{01}$ - 95% lower confidence limit of the benchmark dose of 1% extra risk; AWI - Acceptable Weekly Intake based on TWI, PTWI or $BMDL_{01}$; -- not applicable; * 83% of total Hg; ** 10% of total arsenic; *** CONTAM concluded in 2009 that the PTWI established for inorganic As was no longer acceptable; **** indicative value (report to text to further details)

	Cd	Hg_{total}	MeHg*	As_{total}	As_{inorg}**
EC Regulation No. 1881/2006 (µg/g ww)	0.05	0.5	--	--	--
TWI (µg/kg bw)	2.5	--	--	--	--
PTWI (µg/kg bw)	--	5	1.6	--	< 15***
$BMDL_{01}$ (µg/kg bw/day)	--	--	--	--	0.3 - 8
AWI (average adult =60 kg)	150 µg	300 µg	96 µg****	--	180 - 480 µg
100g muscle of *P. marinus*	0.34±0.05 µg	103±56 µg	86±47 µg	122±45 µg	12±5 µg

This metalloid occurs in different organic and inorganic forms, but the latter is of most concern, being associated with several types of cancer (FAO & WHO, 2011a). Since it was not possible to determine the inorganic contribution of As in total As in *P. marinus* muscle, a 10% contribution was assumed, based on a worst case scenario for conversion for marine fish (FAO & WHO, 2011a). That being considered, 100 g of sea lamprey muscle would have 121.7 ± 45 µg of inorganic As. The previously established Provisional Tolerable Weekly Intake (PTWI) for inorganic As, of 15 µg/kg body weight (bw), would correspond to 900 µg of inorganic As intake by an average 60 kg human; this value was considered by the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM) as inappropriate, since data showed adverse effects at that level of intake (*e.g.* cancer of the lung and urinary bladder) (EFSA, 2009). Alternatively, the 95^{th} percent lower confidence limit of the benchmark dose of 1% extra risk of having cancer due to dietary exposure to inorganic As ($BMDL_{01}$) should be used until more information is acquired to establish a safer PTWI (FAO & WHO, 2011a). Considering a $BMDL_{01}$ between 0.3 and 8 µg/kg bw/day, an average 60 kg human would be allowed an intake between 18 and 480 µg of inorganic As/day; 100 g of *P. marinus* muscle was estimated to have 12.2 ± 4.5 µg of inorganic As, which is below the lower limit of the $BMDL_{01}$. Taking these calculations into account, As concentration in sea lamprey muscle should pose no risk for human health when consumed as fillet.

Considering the maximum allowed concentrations for Cd imposed by the Commission Regulation (EC) No 1881/2006 (OJ-EU, 2006), the 0.05 mg/kg wet weight (ww) limit was not exceeded by the samples analyzed in this work; the same conclusion applies to the Tolerable Weekly Intake (TWI) of 2.5 µg/kg bw (EFSA, 2011), which to an average human of 60 kg corresponds to 150 µg of Cd intake – in 100 g of muscle of *P. marinus*, the amount of Cd is 0.34 ± 0.05 µg, which is more than 400-times lower than the TWI.

Regarding Hg concentration, it is apprehensive to verify that 87.5% of the samples were above the maximum level of 0.50 mg/kg ww imposed by the Commission Regulation (OJ-EU, 2006), and over 47% exceeded twice that concentration. A subsample of 10 individuals analyzed for MeHg contribution to total Hg showed that in *P. marinus* this contribution was of 83 ± 13 %, which is in agreement with the accepted value of 80% for marine fishes (FAO & WHO, 2011b); the average amount of MeHg in 100 g of muscle of *P. marinus* is of 86 ± 47 µg, in a total of 103.5 ± 56 µg of Hg. The PTWI established for MeHg (EFSA, 2004; FAO & WHO, 2011a; FAO & WHO, 2011b;) is currently 1.6 µg/kg bw, which corresponds to 96 µg of MeHg intake for an average 60 kg human; the PTWI for total Hg is 5 µg/kg bw, which corresponds to 300 µg of MeHg intake to an average 60 kg human. Both PTWI values are above the level that is present in 100 g of muscle of sea lamprey, but the fact is that more than this amount of sea lamprey is easily consumed in one typical meal in Portugal during the “lamprey season” (from January to April).

The trophic ecology of *P. marinus* and the high lipid content of its muscle tissue in the beginning of the spawning migration period (Lança *et al*., 2011) may help to explain the levels of Hg that were found. In fact, Lança *et al.* (2011) revealed that neutral lipid contents of sea lamprey muscle represents between 22-29% of the total muscle dry weight against 7-8 % in liver. Thus, sea lamprey`s muscle seems to represent a better fat reserve than liver in the beginning of the sea lamprey spawning migration and contrasts also with the lower muscle lipid levels found in early upstream migrants of sea lamprey observed by Beamish *et al.,* (1979). Moreover, MeHg is the predominant form of mercury in teleost fishes, and it is predominantly distributed by the blood to the muscles when it occurs in that form (contrary to Cd, that is primarily distributed to the liver and kidneys) (Olsson *et al*., 1998). It is well known that MeHg is highly lipophilic and contaminants that are highly lipophilic eventually end up in organism lipid stores (like in musculature of sea lamprey during the marine trophic phase) away from their receptor sites for toxic action (Landrum & Fisher, 1999). Since the sea lamprey is reported to attack a wide variety of fish – *e.g*. cod, hake, sturgeons, salmon, swordfish and basking sharks (Beamish, 1980) – some of which are top predators, feeding on blood of the hosts, and because MeHg is the predominant form of mercury in teleost fishes, the element contaminant load increases significantly with each successive trophic level, making MeHg one of the few trace elements that have been proved to biomagnify along the marine trophic levels (Suedel *et al*., 1994). However, MeHg hydrophobicity and the lipid content do not explain the behavior of MeHg in food webs.

By analyzing the accumulation profile of the three non-essential elements under study in the muscle (*i.e* .As, Cd, Hg), it was possible to observe a clear separation within the sampled specimens. A first attempt to use liver data in this analysis showed a complete lack of structure within the samples and liver was eventually discarded from the analysis, as were the essential elements in this study, to avoid misinterpretation arising from metabolic aspects. The two groups evidenced by the multivariate analysis were primarily divided according to the accumulation of Hg. Group I corresponded to an average of about 26% of the specimens and presented the highest Hg contamination levels. This distinction may have two factors on its origin: (i) the groups may be dominated by specimens with different trophic strategies, *e.g.* distinct types of preys, eventually occupying different trophic levels, will probably affect growth rate (Hardisty & Potter, 1971); (ii) the time duration of the marine (parasitic) phase may also differ between the groups; this phase was reported to last from 23 to 28 months (Beamish, 1980), but recent findings (Cobo-Cardín, F. & Silva, S., pers. comm., University of Santiago de Compostela) have evidenced that such period may be as short as 13 months; a greater growth rate, as mentioned earlier in this discussion, could surpass the accumulation rate of Hg, therefore originating dilution of the accumulation by growth, which would not be as evident in specimens spending almost twice that amount of time at sea.

CONCLUSION

Despite the widespread literature on metal accumulation in fishes, it mostly refers to teleosts and in some cases to sharks. Only a few works have addressed this problematic in cyclostomes. The present work represents an important addition to the knowledge of the ecology of *Petromizon marinus*, and an important gap concerning metal concentration in this species has been partially filled. The results have supported other ongoing projects by the same authors that point to the possibility that sea lampreys of the Western Iberia are probably using distinct oceanic regions and/or targeting different groups of hosts during the parasitic feeding phase of their life cycle. However, further and detailed investigation must be pursued regarding this topic, namely on the chemical forms of non-essential metals present in this species, since distinct chemical forms pose different levels of toxicity for human consumption. Although the recommended daily allowance of certain elements was not exceed, concerning values of Hg concentration were found, and a follow-up on this matter is of the most extreme importance in the human health panorama.

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