Universidade de
Aveiro

Ano 2020

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GARCIA CABRAL**

UTILIZAÇÃO DE FERRAMENTAS GEOQUÍMICAS NA RASTREABILIDADE DA ORIGEM GEOGRÁFICA DE *HIPPOCAMPUS GUTTULATUS*

USE OF GEOCHEMICAL TOOLS FOR TRACING THE GEOGRAPHIC ORIGIN OF *HIPPOCAMPUS GUTTULATUS*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha Aplicada, realizada sob a orientação científica do Doutor Ricardo Jorge Guerra Calado, Investigador Principal do Departamento de Biologia da Universidade de Aveiro e sob a coorientação do Doutor Fernando António Francisco Ricardo, Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro.

o júri

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palavras-chave

Elementos traço, perfil geoquímico, placas ósseas, pesca ilegal, conservação, cavalo-marinho.

resumo

Os cavalos-marinhos do género *Hippocampus* encontram-se atualmente expostos a diversas pressões antropogénicas, tais como a degradação dos seus habitats, pesca direcionada e pesca acidental. De facto, milhões de indivíduos são capturados todos os anos, de modo a dar resposta à intensa procura para a Medicina Tradicional Chinesa, aquariofilia e venda de *souvenirs*. Apesar das restrições implementadas por vários países na sua extração, importação e exportação, verifica-se que a captura e comércio ilegal de cavalos-marinhos ainda ocorre. Uma das ferramentas utilizadas para rastrear a origem geográfica de recursos vivos marinhos é a análise dos perfis geoquímicos das suas estruturas mineralizadas. A assinatura elementar da estrutura mineralizada de cavalos-marinhos, que inclui um exosqueleto de placas ósseas, foi determinada em organismos produzidos em cativeiro da espécie *Hippocampus guttulatus*, oriundos de duas localizações distintas (Galiza e Ria Formosa). Foi igualmente aferida a adequabilidade da utilização destas assinaturas para a rastreabilidade da origem geográfica desta espécie. Foram analisados os seguintes elementos químicos, expressos em rácios referentes à quantidade de Ca (cálcio) detetada: Al (alumínio), Ba (bário), Ce (cério), Cr (crómio), Cu (cobre), Fe (ferro), K (potássio), Mg (magnésio), Mn (manganês), Na (sódio), Ni (níquel), P (fósforo), Sr (estrôncio) e Zn (zinco). Os perfis geoquímicos da estrutura óssea da cabeça, tronco, barbatana dorsal e cauda de cavalos-marinhos da Galiza foram comparados com o perfil geoquímico de conspecíficos do mesmo local processados como um todo, tendo estes apresentado sempre diferenças significativas nas suas assinaturas geoquímicas (ANOSIM, p=0,001). Desta forma, nenhuma destas partes do corpo é uma variável sub-rogada perfeita para a assinatura geoquímica de um organismo inteiro. A análise das partes do corpo de cavalos-marinhos da Galiza e da Ria Formosa revelou que a barbatana dorsal apresentava os rácios mais elevados de Al/Ca, Ba/Ca, Ce/Ca, Cr/Ca, Cu/Ca, Fe/Ca e Mn/Ca. A análise CAP, que incluiu as quatro partes do corpo de cavalos-marinhos de ambos os locais, classificou corretamente todas as réplicas dos troncos. Uma análise ANOVA, que confrontou cada rácio elementar individualmente, das mesmas partes do corpo e de ambos os locais, revelou que os perfis geoquímicos eram de uma maneira geral significativamente diferentes. Um teste CAP adicional, para a validação do tronco como modelo para a rastreabilidade da origem geográfica, resultou na correta classificação de todas as réplicas dos organismos processados inteiros da Galiza. Uma análise CAP que incluiu seis porções do corpo de cavalos-marinhos (sem uma ou duas partes do corpo), de forma a simular a apreensão de organismos que possam não apresentar a integridade da sua forma, resultou na atribuição correta de todas as porções corporais à respetiva origem (Galiza ou Ria Formosa). A análise das assinaturas geoquímicas da estrutura óssea de *Hippocampus guttulatus* apresenta o potencial de constituir uma ferramenta importante para a rastreabilidade da origem geográfica de cavalos-marinhos apreendidos de pesca e comércio ilegais. Os resultados deste primeiro estudo guiarão assim os próximos ensaios experimentais que visem aferir a origem geográfica de organismos selvagens capturados ilegalmente.

keywords

Trace elements, geochemical profile, bony plates, illegal fishing, conservation, seahorse.

abstract

Seahorses within genus *Hippocampus* are currently exposed to a wide range of anthropogenic pressures, such as habitat loss, bycatch and fisheries targeting seahorses. In fact, millions of individuals are sold every year to overcome the demand, mainly for Traditional Chinese Medicine (TCM), curios and aquarium purposes. In spite the restrictions imposed by several countries in the extraction, exportation and importation of seahorses, the illegal capture and trade still occur. A tool used for the traceability of geographic origin of marine living resources, is the analysis of elemental fingerprints (EF) from mineral structures. The EF of the mineral structure, that includes an armor composed by bony plates, was determined in cultured seahorses of the species *Hippocampus gutullatus* from two different origins (Galicia and Ria Formosa). It was also evaluated the suitability of these EF for the traceability of the geographic origin of this species. The following elements, Al (aluminium), Ba (barium), Ce (cerium), Cr (chromium), Cu (copper), Fe (iron), K (potassium), Mg (magnesium), Mn (manganese), Na (sodium), Ni (nickel), P (phosphorus), Sr (strontium) and Zn (zinc), were considered and expressed in ratios referent to the quantity of detected Ca (calcium). The EF from the bones, belonging to the head, trunk, dorsal fin and tail, of seahorses from Galicia, were confronted to the EF of conspecifics processed as a whole from the same location, presenting always significant differences in their EF (ANOSIM, p=0.001). Thus, none of these body parts is a perfect proxy to the whole body. The analysis of body parts of seahorses from Galicia and Ria Formosa revealed that the dorsal fin was the one exhibiting the higher ratios of Al/Ca, Ba/Ca, Ce/Ca, Cr/Ca, Cu/Ca, Fe/Ca and Mn/Ca. A CAP analysis that included the four body parts, from both locations, correctly classified all trunk replicates. An ANOVA analysis, for each individual elemental ratio and that included the same body parts, from both locations, indicated that, in general, the EF were significantly different. An additional CAP analysis to validate the trunk as a model for the traceability of the geographic origin, resulted in the correct classification of all whole body replicates from Galicia. A CAP analysis, which included six seahorse body portions (without one or two body parts), to simulate the apprehension of individuals with body parts missing, resulted in the correct attribution of all body portions to the respective origin (Galicia or Ria Formosa). The EF from the bony structure of *Hippocampus guttulatus*, has the potential of being an important tool for the traceability of the geographic origin of organisms, apprehended from illegal fishing and trade. The results from this preliminary work will guide the following experimental essays that intend to assess the geographic origin of wild organisms captured illegally.

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1 Introduction

1.1 Seahorse Biology

Hippocampus spp. are marine teleost fishes, belonging to family Syngnathidae, which also integrates seadragons, pipehorses and pipefishes (Zhang *et al*., 2017). Seahorses have an unusual body shape, presenting horse-shaped heads that are bent, at almost right angles relatively to their body axis, their trunk is curved and they do not have a caudal fin, exhibiting instead a prehensile tail that allows them to grasp holdfast. They also present distinctive characteristics (Figure 1) as the absence of scales and ribs, a single dorsal fin for propulsion and two pectoral fins for maneuvering. Furthermore, they exhibit facultative growth of cirri (skin filaments), that confer camouflage, and a body covered by subdermal bony plates, which are overlapped and organized as series of ridges (sliding anterior-posteriorly) and rings (sliding dorsally-ventrally and laterally) (Curtis *et al*., 2017; Lourie, 2003a; Lourie *et al*., 2004; Neutens *et al*., 2017; Porter *et al*., 2013; Praet *et al*., 2012).

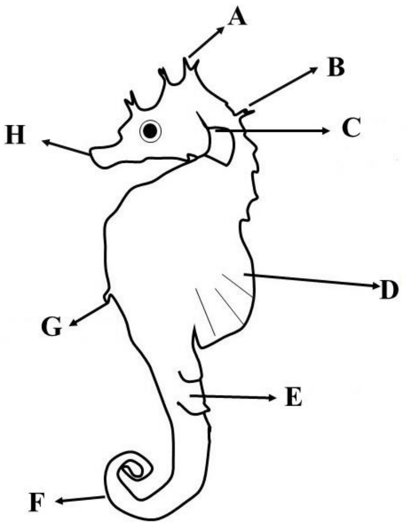

Figure 1-Illustration of a *Hippocampus* sp., being A-coronet, B-skin filament, C-pectoral fin, D-dorsal fin, E-bony plates, F-tail, G-anal fin and H-snout.

These bony plates act as a flexible, subdermal armor, that allows axial bending and the prehensility of the tails (Figure 2), as well as the protection of spinal column from compressive forces, since several of their predators capture their preys through impact and crushing. The bony plates present mineral (calcium phosphate), organic and water fractions. The fish skeletal system is formed by bones and cartilage, being the vertebrae composed mainly by calcium, phosphate and carbonate, and at a smaller extent, by magnesium, sodium, strontium, lead, citrate, fluoride, hydroxide and sulfate. Three types of cells have a pivotal role in the bone formation and in its remodeling process. The osteoblasts are bone forming cells, the osteocytes, which are inside the bone matrix, may be related to the preservation of bone substances and exchanges of ions present in body fluids, and the osteoclasts, which are involved in bone resorption (Lall and Lewis McCrea, 2007; Porter *et al*., 2013).

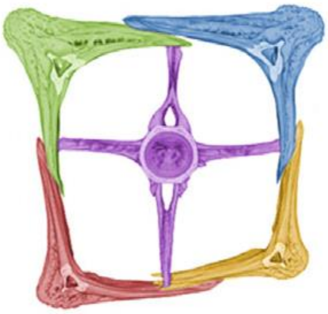

Figure 2-Scheme of a tail cross-section, with the bony plates in the periphery and the vertebra (purple) in the center (Source: Porter et al. (2013)).

Fish, in opposite to the other vertebrates, have two routes for elemental uptake, from diet and water (Bury *et al*., 2003; Kerr and Campana, 2014). Calcium and phosphorus have a significant importance in the development and maintenance of the aquatic organisms' skeletal structure. Essential trace elements, such as zinc, manganese and copper are necessary for the normal growth and development of fish bones. In the ichthyofauna, the calcium homeostatic regulation does not occur mainly in the bone structure, being this process a primary function of the gills, and, at a smaller extent, of the intestinal and opercular epithelia. The control of calcium influx and efflux occurs at the gills, fins and buccal epithelia. Phosphorus is also absorbed from the water, however, because the concentration of this element in seawater is low, the diet constitutes the main source for bone mineralization (Lall and Lewis-McCrea, 2007; Loewen *et al.*, 2016). According to Lin et al. (2008), seahorses are also, generally, rich in sodium, potassium, zinc and manganese.

Seahorses, along with the other syngnathids, have fused jaws, allowing suction, as a feeding strategy. The buccopharingeal expansion causes a water current that makes difficult for prey to escape. Thus, these organisms present a very characteristic cranial morphology, since suction through a narrow snout encompasses specific adaptations in the cranium. Additionally, the near 90-degree angle between their head and the trunk, allows them to enhance the strike distance during feeding (Blanco, 2014; Leysen *et al.*, 2010). These organisms, are usually diurnal ambush predators that feed on small prey, as mysids, amphipods, decapods, copepods, isopods and fish larvae (Blanco, 2014; Curtis *et al.*, 2017; Manning *et al.*, 2019).

Hippocampus spp. have a wide distribution, from 50º N to 50º S (Figure 3), in temperate and tropical coastal waters, not occurring in cold-temperate and polar seas. They may be associated to threatened biogenic habitats, as, for instance, mangroves, seagrasses and coral reefs, but also to macro algae, roots and even artificial structures. The habitat use may also vary, depending on the life stage. In fact, the juvenile individuals of some species are planktonic, inhabiting the water column (Blanco, 2014; Laurie, 2004; Manning *et al.*, 2019).

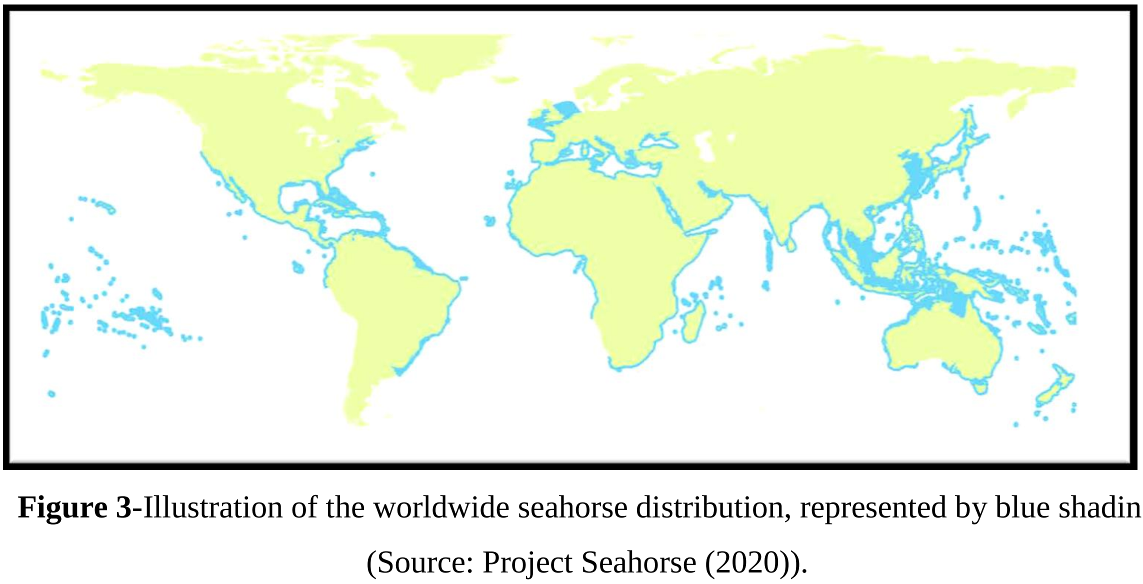

Figure 3-Illustration of the worldwide seahorse distribution, represented by blue shading (Source: Project Seahorse (2020)).

Most seahorse species form monogamous pair bonds, encompassing a long and elaborate courtship ritual (Faleiro *et al.*, 2008; Masonjones and Lewis, 1996). Seahorses are batch spawners, producing several clutches of eggs during the breeding season that are released periodically. In females, the oogenesis is synchronized with the males' brooding phase. The female transfers the unfertilized eggs to the male's brooding pouch (Figure 4), where they are fertilized. During the embryonic development, which occurs in the male's pouch, most of the energy available is derived from the female, since the embryos rely on the egg's yolk lipid reserves. The male, however, also contributes with some nutrient transfer (Saavedra *et al.*, 2016; Zhang *et al.*, 2017).

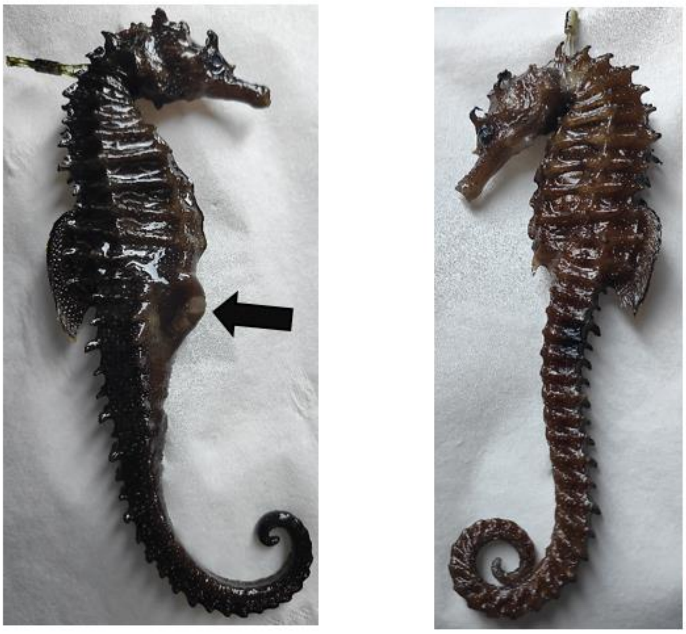

Figure 4-*Hippocampus guttulatus*: male (left), exhibiting a brood pouch, indicated by the black arrow and female on the right.

1.2 **Hippocampus guttulatus** ***as a study case***

The benthic long-snouted seahorse is associated with biogenic habitats, as macrophytes and macroalgae, in shallow inshore waters of the Mediterranean Sea and north-eastern Atlantic Ocean. Juveniles can spend nearly 8 weeks in the water column before recruiting to benthic habitats (Curtis and Vincent., 2006; Curtis *et al.*, 2017). *Hippocampus guttulatus* may be found in deeper depths in the winter, being the maximum reported depth of 12 meters (Lourie *et al.*, 2004).

Hippocampus guttulatus is sometimes mistaken with *H. hippocampus* (Figure 5), having this last one a smaller size, different shape of the head and cirri, as well as a shorter snout. Although these two species are sympatric, *H. guttulatus* inhabits more complex habitats than *H. hippocampus*, which is usually seen in less structured areas, as dispersed rocks and algae (Curtis *et al.*, 2017; Gristina *et al.*, 2015).

Figure 5-*Hippocampus guttulatus* on the left and *Hippocampus hippocampus* on the right.

In Ria Formosa coastal lagoon, where the two species co-occur, there has been a visible decrease in the largest seahorse populations ever recorded worldwide. Recent studies, have pointed to an abundance decrease of about 94% in the case of the long-snout seahorse (*H.guttulatus*) and 73% for short-snout seahorse (*H. hippocampus*) (Correia *et al*., 2018).

The long-snout seahorse exhibits sexual dimorphism, being the females larger than males (Woodall *et al*., 2018). The breeding season of *H. guttulatus,* may extend from March to September and even to November in some latitudes. Factors as photo regime or temperature may impact female maturation and the characteristics of the egg clutches (Planas *et al*., 2013). The gestation, in males, longs for about 3 to 5 weeks and, usually, nearly half of the population reaches sexual maturity at a height of 100 mm (Lourie *et al*., 2004). This species is serially monogamous across a breeding season, presenting only one partner at each time (Woodall *et al*., 2018). The long-snout seahorse is, however, socially polygamous, greeting and courting individuals indiscriminately from the same population (Curtis *et al*., 2017).

Adult *H. guttulatus* phenotype may vary (Figure 6), presenting colors as dark green, brown and blackish or yellowish tones, along with a white dotted pattern. However, the coloration may change due to stress, for camouflage purposes or during the breeding season (Blanco, 2014).

Figure 6-Two color variations in *Hippocampus guttulatus* individuals from aquaculture. On the left, is visible a blackish color and on the right a yellowish tone.

1.3 Conservation status

Nowadays, the structure of marine fish communities, in coastal ecosystems, is negatively impacted by anthropogenic pressures, such as illegal exploitation and habitat destruction, which results from high levels of human occupation in these areas (Correia, 2014). The genus *Hippocampus*, from the family Syngnathidae, is exposed to the previously mentioned anthropogenic pressures, as well as to bycatch and fisheries targeting seahorses (Cohen *et al*., 2017). These organisms are found in close association with some of the estuarine and costal marine habitats, most susceptible to climate change and to human occupation (Aurélio *et al*., 2013; Correia, 2014; Curtis *et al*., 2017). Although it has been described that adult seahorses are relatively resilient to changes in the oceans' temperatures, juveniles are more sensitive to the rise of water temperature, and may have to overcome metabolic challenges in the future (Aurélio *et al*., 2013). Seahorses also present characteristics of their life cycle and ecology that make them especially vulnerable to habitat destruction and to over-exploitation. Some examples include the heterogeneous spatial distribution and fidelity to certain habitats, poor swimming skills, monogamy, which can represent an obstacle when pair bonds are broken, low fecundity, relatively small broods and dependency on the male for the embryos' success (Curtis and Vincent., 2006; Faleiro *et al*., 2015). Thus, in 1996, after the execution of a worldwide study, which focused on the main threat factors to seahorses, as well as the health of their populations, all the species were included in the first version of the "Red List of Endangered Species" of the International Union for Conservation of Nature (IUCN) (Blanco, 2014; Vincent *et al*., 2011a; Vincent, 1996). Still in 1996, the Project Seahorse was born, as a way of assessing the trade of syngnathids and constructing the first conservation projects for these organisms (Vincent *et al*., 2011a). Of the 42 seahorse species considered, nowadays, by IUCN, 17 are categorized as Data Deficient (DD), that is, without sufficient data to evaluate their conservation status, 10 as Least Concern (LC), 1 as Near Threatened (NT), 2 as Endangered (EN) and 12 as Vulnerable (VU). A significant percentage of the *Hippocampus* species, approximately 40%, are Data Deficient, meaning that the existent data did not provide the information required to assess the extinction risk for these species (IUCN, 2019; Lawson *et al*., 2015). In 2002, due to the high trade of individuals of wild populations per year, all the species that integrate the genus *Hippocampus* were included in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which became effective in 2004 (Cohen *et al*., 2018; Kuo *et al*., 2018). The species attributed to Appendix II can be traded, in a regulated way, as their over-exploitation can compromise the health of wild populations (Foster *et al*., 2016). In fact, seahorses were among the first marine fishes with commercial value to be listed by CITES (Lourie *et al*., 2004). Nowadays, CITES comprises 183 member parties, which are “States or regional economic integration organizations for which the Convention has entered into force” (CITES, 2020).

Seahorses may be important promoters of their conservation and sustainable use of their habitats, as they are flagship species (Cohen *et al*., 2017). This nomination is attributed to species that, because of particular characteristics of their ecology or appealing aspect, can be the representatives of conservation campaigns (Cohen *et al*., 2017; Zhang and Vincent, 2019). Furthermore, additional methods for seahorses’ conservation have been proposed. Commercial aquaculture, for example, has been pointed as a way of fighting the overexploitation of individuals destined to trade. Thus, this practice may represent a way of preserving wild populations and still enable a sustainable trade, as well as benefit, economically, smaller coastal communities (Blanco, 2014; Faleiro *et al*., 2008; Koldewey and Martin-Smith, 2010; Zhang *et al*., 2017). For the accomplishment of the mentioned goals, it is of main importance the development of low-technology and small scale protocols for seahorse rearing. Nevertheless, the benefits from aquaculture will only be noticeable, if the demand for organisms from aquaculture stays relatively constant and exceeds the preference for wild ones. This will be possible if cultured organisms are cheaper, but, in developed countries, wild specimens are still being traded at a more affordable price, due to the low expenses on their capture (Blanco, 2014). Moreover, seahorse aquaculture can provide additional knowledge, which still has its limitations, about their behaviors and biology, as feeding behaviors and nutritional requirements (Segade-Botella, 2016). In fact, the aquaculture of seahorses presented some challenges throughout the times. Prior to 1990s this aquaculture was ineffective, showing issues related to the maintenance of the organisms, which reflected in nutritional deficiencies, diseases and high death rates (Koldewey and Martin-Smith, 2010). Skeletal disorders, for example, are still a problem related to marine fish culture, which can result from phosphorus and other micronutrients deficiencies or excesses, during the larval and juvenile stages (Lall and Lewis-McCrea, 2007). During the period between 1990s and 2000s many research efforts, for a number of seahorse species, were made, resulting in the publication of valuable information. This allowed, to a certain degree, the expansion and development of seahorse aquaculture, as well as an increase in species cultured (Koldewey and Martin-Smith, 2010). It has been suggested that organisms obtained from aquaculture may reinforce the wild populations. However, the release of captive seahorses requires an assessment of the biological risks to the wild populations, as well as to coastal and marine ecosystems (Blanco, 2014; Segade-Botella, 2016).

1.4 Seahorse trade

Hippocampus spp. are exposed to a wide range of fishing pressures, being captured as target species by smaller and subsistence fisheries and incidentally by gears from industrial fleets (Vincent and Foster, 2017). Millions of individuals are collected every year to overcome the demand, especially for the Traditional Chinese Medicine (TCM) and, to a smaller extent, for marine aquariums and the curio trade (Cohen *et al.*, 2017). The commercialization of seahorses occurs, essentially in two distinct ways, as these organisms can be sold dry (whole organism or powder) (Figure 7), for TCM and curios, or, with a smaller contribution, live for aquarium uses (Kuo and Vincent, 2018).

Figure 7 - Photo of dried seahorses for trade (Source: Project Seahorse (2014)).

Some of the species that most contribute for TCM are *H. barbouri*, *H. borboniensis*, *H. camelopardalis*, *H. comes*, *H. erectus*, *H. fuscus*, *H. histrix*, *H. ingens*, *H. kelloggi*, *H. kuda*, *H. reidi*, *H. spinosissimus* and *H. trimaculatus* (Lourie *et al*., 2004; Nijman, 2010). The species *H. algiricus*, was also pointed to be heavily exported and explored by artisanal fisheries in West Africa (Cisneros-Montemayor *et al*., 2016). Between 1998 and 2001, prior to CITES implementation, *H. subelongatus* and *H. zosterae*, along with the species mentioned, were referred to be traded dry and/ or alive, imported, exported and used domestically, by non-Asian countries (Vincent *et al*., 2011b). Seahorses represent a high portion of heavily traded species, expressed in number of individuals, under CITES legislation (Lam *et al*., 2016). Nearly 37 million seahorses are caught incidentally by non-selective fisheries annually (Table 1), and more than 20 million individuals are estimated to be traded, worldwide, during that period (Blanco, 2014; Cisneros-Montemayor *et al*., 2016; Kuo and Vincent, 2018; Vincent and Foster, 2017). However, according to Lawson et al. (2017), a more reduced annual average of 5.7 million dried, wild caught seahorses, were recorded from international trade exports, between 2004 and 2011, by 61 CITES member parties.

Table 1 - Nine main categories of gear types (dredges, falling gear, gill/entangling net, hook and line, scoop net, seine net, surround net, trap and trawl net) indicated by Food and Agriculture Organization of the United Nations (FAO) and the 31 countries that reported using these gear types, when bycatch of seahorses occurred. The data regarding the gear types, ranged from 1989 to 2013 (Source: Lawson et al. (2017)).

FAO gear type category	**Reported gear type**	**Countries reporting**
Dredges	Dredge	Malaysia, Mexico
Falling gear	Cast nets	Brazil, India, Kenya, Panama, Senegal, Nicaragua, Tanzania, Thailand
Gill/entangling net	Drift nets	India, Malaysia, Senegal
	Tidal shrimp nets	Indonesia, Malaysia, Senegal
	Trammel nets	Bangladesh, India, Malaysia, Portugal, Senegal
	Gill nets	Bangladesh, Costa Rica, Ecuador, Guatemala, Honduras, India, Indonesia, Malaysia, Mexico, New Zealand, Nicaragua, Pakistan, Panama, Peru, the Philippines, Portugal, Tanzania, Turkey
	Tangle nets	Senegal
Hook and line	Longline	Malaysia, Senegal
Scoop net	Bag nets	Bangladesh
	Push nets	The Philippines, Portugal, Thailand
Seine net	Beach seine	Bangladesh, Brazil, Costa Rica, Ecuador, Honduras, India, Indonesia, Kenya, Mexico, Mozambique, the Philippines, Portugal, Senegal, Tanzania
	Shore seine	India
	Dragnets	India
Surround net	Purse seine	Ecuador, India, Indonesia, Kenya, Malaysia, Peru, Senegal, South Korea, Tanzania, Thailand
	Surround net	Tanzania
Trap	Fish traps	Indonesia, New Zealand
	Crab traps	India, Indonesia, New Zealand
	Basket traps	Tanzania
	Enclosure pens	The Philippines
	Trap nets	India, Tanzania
Trawl net	Trawl	Australia, Bangladesh, Belize, Brazil, China, Costa Rica, Ecuador, France, Guatemala, Honduras, India, Indonesia, Kenya, Malaysia, Mexico, Mozambique, New Zealand, Nicaragua, Nigeria, Pakistan, Panama, Peru, the Philippines, Portugal, Senegal, Tanzania, Thailand, Turkey, United States, Vietnam
Gear not specified		Argentina, Croatia, Japan, Spain, Taiwan

In several locations, the incidental capture during nonselective bottom trawling is a major treat to seahorse populations. The majority of seahorses traded results from incidental catch during shrimp trawling, since many syngnathids occupy the same habitats as shrimps. The countries with significant expression on bycatch of seahorses in shrimp trawling include Vietnam, Thailand, Philippines, India, Malaysia, Indonesia and Mexico (Kuo *et al*., 2018; Lourie *et al*., 2004; Vincent *et al*., 2011a). A study led by Aylesworth et al. (2018), in which small scale and commercial fishers that operate along the Thailand coast, were interviewed, showed that two commercial gears, bottom otter trawls and pair trawls, as well as gillnets, used in small scale fisheries, were the ones that caught more seahorses. Gillnets were responsible for the majority of the catches of juvenile individuals from the species *H.kuda* and *H. trimaculatus* and, these two species, along with *H. kelloggi*, were the three most susceptible to fishing.

It is estimated that more than 80 countries are involved in the commercialization of millions of dried specimens (300 dried seahorses correspond to nearly 2 kilograms), and, according to data from 2004 to 2011, hundreds of thousands of live individuals, belonging to about 30 species were traded (Foster *et al*., 2016; Kuo and Vincent, 2018; Vincent *et al*., 2011b; Vincent and Foster, 2017). Between 2004 and 2011, period in which CITES restrictions were already in place, 98% of the reported trade was in the form of dried individuals, being 93% of this trade imported by Hong Kong Specialist Administrative Region (SAR) (Figure 8), the largest known importer of dried seahorses, Taiwan and mainland China. Thailand was considered the source of nearly 75% of all wild dried seahorses, and, as result, suspended all exports in January 2016 (Foster *et al*., 2019). A number of other countries also present restrictions in live and dried syngnathid trade and extraction (Foster *et al*., 2019; Vincent *et al*., 2011a).

Figure 8 - Dried seahorses for sale in a market located in Hong Kong SAR (Source: Project Seahorse (2020)).

However, a study that occurred between 2016 and 2017, in which 220 interviews to traders of Hong Kong SAR were conducted, showed that dried seahorses were being sourced by many countries with bans on exports (Table 2) (Foster *et al*., 2019). The two most evident cases were Thailand, being reported as the greatest source of dry individuals, by volume, and Philippines, which banned the capture and trade of seahorses in 2004. Mainland China, Australia, Indonesia, India, Malaysia and Vietnam were also pointed as important sources of seahorses by Hong Kong SAR traders. This study highlights the illegal and unreported international trade of dried seahorses, by countries that imposed restrictions and bans on exports (Foster *et al*., 2019). In some countries, seahorses are under targeted fisheries, mainly by hand, as in India, which banned all seahorse captures and exports in 2001, Brazil, where is illegal to fish these species, Malaysia, Philippines and Thailand (Stocks *et al*., 2017; Vincent *et al*., 2011a). Regarding the live trade, the 6 CITES Parties pointed as top consumers, between 2004 and 2011, were the United States of America, France, Canada, United Kingdom, Netherlands and Germany (Foster *et al*., 2016). The top 6 member Parties that were mentioned to be the main sources of live specimens, during that period, were Vietnam, Sri Lanka, Indonesia, Australia, Brazil and Mexico (Foster *et al*., 2016). Fishers worldwide have reported significant decreases in seahorse populations, being of extreme importance the assessment and management of an, otherwise, unsustainable exploitation.

Seasonal closures on fisheries and minimum size limits, may be important measures tools to insure sustainable seahorse fisheries (Stocks *et al.*, 2017).

Table 2 - Countries with bans on exports of dry wild seahorses, reported by traders as sources of dried specimens for Hong Kong SAR, between 2016 and 2017 (adapted from: Foster et al. (2019)).

Countries	**Year of bans on exports of wild dry seahorses**	**Species**
Mexico	1994	All species that are intentionally captured
India	2001	All species
Philippines	2004	All species
Indonesia	2009	All species
Malaysia	2009	All species
Mainland China	2011	All species
Vietnam	2013	*H. kuda*
Senegal	2016	*H. algiricus*
Thailand	2016	All species

Two seahorse species *H. hippocampus* and *H. guttulatus*, are sympatric, meaning that they co-occur on inshore habitats throughout Europe. They have been described as data deficient (DD) and are not widely traded (Curtis *et al.*, 2017). However, captures of *H. guttulatus*, either directly or incidentally, have been reported for most Mediterranean countries and Portugal, mostly for aquariums and curiosities (Blanco, 2014). In south of Portugal, more precisely in Ria Formosa coastal lagoon, *H. guttulatus* and *H. hippocampus* populations have shown significant decreases. Although the reasons for this loss are still unclear, human activities, as fisheries, including illegal practices, and natural changes in the dynamics of the lagoon, have been pointed as possible reasons (Curtis *et al.*, 2007). According to a study effectuated in 2012 and described by Correia (2014), there has been an increase of illegal bottom trawling fisheries in Ria Formosa coastal lagoon, which seriously affects seahorse species that are associated with benthic habitats. Moreover, the use of trammel nets was observed in this ecosystem (Table 1), which affects seahorse populations, in the sense that the specimens get tangled in these nets and are not restored back to their habitats. In Galicia, the main threats to *H. guttulatus* are habitat loss and bycatch. Thus, to overcome these challenges, Proyeto *Hippocampus*, a captive breeding program for *H. guttulatus*, was initiated in 2006. The main goal of this project, developed in Spain, was to recover the wild populations of *H. guttulatus* in Galicia (Blanco, 2014).

1.5 Problems in controlling the seahorse trade

The traceability and the application of legislative measures on wildlife trade, in general, is challenging, as it usually implicates large transaction volumes, high intervention of diverse shipments across international borders and distinct trade routes (Loh *et al.*, 2016). Moreover, because many *Hippocampus* spp. are Data Deficient, meaning that there is a gap in the knowledge about traits regarding their ecology and biology, and about their exploitation, as information on catches, fishing effort, species and fishing gears involved, it is difficult to manage fisheries and trade in a sustainable manner (Aylesworth *et al*., 2018). One of the most evident problems that CITES faces is the significant mismatch in species and volumes between export and import data, indicating that the reports are not totally reliable and complete. CITES database includes data submitted by the member parties, being the units, in number of individuals or kilograms, often absent, the required export and voluntary import data do not match and the species are not correctly identified. Another challenge that CITES encounters is tracing the dried seahorse trade, in comparison with the smaller and more easily-tracked trade of live seahorses (Foster *et al*., 2016; Vincent *et al*., 2011a). Nearly a decade ago, reports from a confiscation in Poland, of a single shipment of dried seahorses from Indonesia, accounted for between 1 and 2 million dried seahorses, suggesting that the veridical levels of exports may be higher than reported (Nijman, 2010). To overcome these obstacles, the countries that are CITES member parties should provide information regarding all shipments involved in the seahorse trade, export units, for example in kilograms or number of individuals, and identify the exported species. Previous field surveys, used to document the seahorse trade, were, however, time consuming, costly and unsustainable (Foster *et al*., 2016). According to Vincent et al. (2011b), a problem with the collection of data at species level is the fact that frequently fishers and traders do not know how to identify the species, making, several times, assumptions based on coloring, marks or size. It has previously been described that the inability to identify the seahorse species may undermine the researches on their geographic range, characteristics of the populations and prevents the understanding of trade routes (Lourie *et al.*, 1999). Furthermore, the large number of names attributed to the species in the early literature meant that nomenclature given to seahorses was often unreliable (Foster and Vincent, 2004). The species *H. guttulatus*, for instance, is also known as *H. filamentosus*, *H. bicuspis*, *H. longirostris*, *H. hippocampus microcoronatus*, among others, being these synonyms not accepted (CITES, 2013; WoRMS, 2020). Thus, further research on seahorses' life history, behavior and ecology, is extremely important, due to the existent challenges associated with species differentiation (Curtis *et al.*, 2017).

1.6 Methods used to overcome the problems in controlling the seahorse trade

Accurate species identification and information about the habitats where they occur, is of major importance to construct effective conservation measures (Woodall *et al.*, 2018). However, the parts involved in the seahorse trade, as fishers and traders, make several times assumptions based on coloration, skin patterns or size, parameters that are not entirely reliable for species identification (Vincent *et al.*, 2011b). Even custom officers often experience serious difficulties when identifying seahorse species (Vincent *et al.*, 2017). Moreover, it has been demonstrated that for the two sympatric species *H. guttulatus* and *H. hippocampus*, size variation and presence or number of cirri, are not reliable for species differentiation. Thus, genetic approaches are useful to clarify aspects about taxonomy and analyze the characteristics of the populations, as DNA sequencing from the cytochrome b region of the mitochondrial genome (Lourie *et al.*, 1999; Woodall *et al.*, 2018).

It is also important that stakeholders are well aware of the countries' policies. CITES authorities should inform importers and exporters about the bans implemented, so they may contribute to the minimization of illegal trade (Foster *et al.*, 2019). National management authorities also play an important role, as they are required to authorize exports through permits, confirm if the individuals were obtained accordingly to the current laws and submit annual trade reports to CITES. However, the reliability of the data collected by management authorities varies, as there are many stakeholders from different countries working with different laws. Also, the unwillingness of some people involved in the trade to collaborate with CITES, may result in incomplete or dubious trade records (Lam *et al*., 2016).

1.7 Traceability of geographic origin

Obtaining information on the habitat where a species occurs is extremely important for the evaluation of populations' status, as well as the application of effective conservation measures (Woodall *et al*., 2018). The determination of geographic origin for seafood destined for human consumption has deserved a growing attention to ensure that consumers are receiving a reliable description of the product and that its consumption is safe (Ricardo *et al*., 2015). The fatty acid composition, for example, may constitute a useful tool for traceability purposes, as it varies with intrinsic factors, as sex or age, and extrinsic factors, as salinity, temperature, diet or depth (Leal *et al*., 2015). In the case of seahorses, it has been suggested the use of the whole dorsal fin for stable isotope analysis employed in feeding ecology studies, as it is a non-lethal method (Valladares and Planas, 2012). Phelps et al. (2012), conducted a laboratory study, in which they assessed the relationship between the water and pectoral fin ray chemistry of the fresh water species *Scaphirhynchus sturgeons* and verified the utility of Sr:Ca values, present in the pectoral fin rays, in the traceability of wild fish origin. Furthermore, accordingly to Cohen et al. (2018), the bacterial fingerprints of live seahorses' skin mucus, hold the potential to reveal local signatures and, consequently, trace the seahorses' geographic origin.

Biomineral chemistry is frequently used as a way of inferring about the habitat use and aspects of the life history of fish. Biominerals are primarily composed by calcium based compounds, as calcium phosphates, existent in fins, scales and bones, and calcium carbonate found in otoliths. Strontium and barium, for example, are non-essential elements and divalent cations in solution, as calcium, substituting this last one in biominerals. These two elements have been frequently used to infer aspects about life history, habitat and biological characteristics of marine fishes. Calcium homeostasis has been pointed to be the factor that influences the presence of some non-essential elements, for example strontium and barium, as some of these elements enter the organism via calcium uptake mechanisms. Thus, competition for calcium transport channels is likely dependent on calcium and trace elements concentration on the environment, in relation to the fish's internal calcium concentration. Also the use of strontium isotopic ratios in otholits, bones or teeth, for the determination of organisms' geographic origin, has been mentioned to have a high rate of success (Lowene *et al.*, 2016). The use of otoliths' geochemical properties allows the assessment of the movement and habitat use of marine fish (Longmore *et al.*, 2011). There has also been a growing interest in the analysis of the chemical properties of other hard parts, for example, fish spines, scales and vertebrae, as a way of tagging fish stocks (Kerr and Campana, 2014). Fish populations that inhabit physicochemically distinct environments can be identified through the analysis of otolith's trace elements. Otoliths incorporate trace elements that become permanent, indicating the chemical characteristics of the environment experienced by the fish. Furthermore, temperature has been pointed as an influencing factor on the concentration of many elements and isotopes in fish structures. Thus the isotopic composition of oxygen and carbon in the aragonite of otoliths, may provide information regarding the water temperature and depth (Longmore *et al.*, 2011). However, more homogeneous environments, as the deep sea, may evidence less clear geographic differences, expressed in otoliths' composition. The coastal environments are, by contrast, more dynamic in terms of their chemical and physical nature, which usually results in distinct chemical profiles in otoliths (Thorrold *et al.*, 2002). Trace element fingerprinting, as a way to discriminate the geographic origin of marine organisms, through the elemental profiling of their mineral structures, has also proven to be an accurate and reliable method for bivalve shells (Leal *et al.*, 2015; Ricardo *et al.*, 2015; Ricardo *et al.*, 2017). Furthermore, the use of bone and scale collagen isotopic data from fish, has also been proposed as a way of studying long term changes in ecosystems (Guiry and Hunt, 2020).

1.8 Objectives

This study aimed to: **a)** evaluate if the processing of only a segment of the bony structure (namely the head, trunk, dorsal fin or tail), of the species *Hippocampus guttulatus*, originating from Galicia, yields comparable geochemical fingerprints to those achieved when processing the whole animal; **b)** to perform a geochemical comparison of the bony structures of seahorses from the species *H. guttulatus*, originating from two distinctive locations (Galicia and Ria Formosa) to evaluate if their geochemical fingerprints differ significantly with their geographic origin; and **c)** to determine the geographic origin of six seahorse portions (with one or two of the considered body parts missing), from Galicia and Ria Formosa, since the organisms apprehended in illegal fisheries and trade may be lacking their structural integrity. Thus, for these purposes, four hypothesis were formulated: 1) there is a reliable proxy (head, trunk, dorsal fin or tail) to the whole animal's geochemical fingerprints; 2) the use of a segment of the bony structure allows to trace the geographic origin of seahorses; 3) the trunk is a valid alternative to the organisms processed as a whole for the traceability of the geographic origin; and 4) when the whole body is lacking part of its structural integrity, it is still possible to correctly trace the geographic origin of that specimen. By pursuing these objectives, it is aimed to verify the prevalence of geographic origin on the elemental fingerprints of seahorse bony structures to enable the traceability of the place of origin of apprehended specimens. The processing of a smaller sample of seahorse bony structure, has also the potential to speed-up analysis, decrease processing costs and minimize the production of chemical residues that must be properly processed and discarded. By contributing to the development of reliable traceability tools to pinpoint the geographic origin of seahorses, it is aimed to resource the fight against the illegal, unreported and unregulated (IUU) fisheries and trade of this flagship species.

2 Materials and methods

2.1 Biological material

A total of 15 seahorse samples (*Hippocampus guttulatus*) cultured in captivity were supplied by the Instituto de Investigaciones Marinas (CSIC, Vigo, Spain) (n = 10) and by the Centro de Ciências do Mar (CCMAR) (University of the Algarve, Portugal) (n = 5).

Five specimens from Galicia were subdivided, as described by Lourie (2003a), into four body parts: the head, trunk, dorsal fin and tail (1 species X 4 body parts X 5 specimens = 20 samples). Briefly, the head was perceived as the extension from the extremity of the snout until the cleithral ring, the incomplete bony-ring that underlies the operculum (bone arrangement that confers gill coverage) (Figures 9 and 10A). Seahorse head was isolated by making a cut proximate to the posterior side of the cleithral ring (Figure 9). The trunk was defined as the part that extends from the cleithral ring until the last trunk ring (Figures 9 and 10B), which is immediately anterior to the anal fin and, in the case of *H. guttulatus*, corresponds to the eleventh trunk ring. This body part was separated from the tail, by cutting close to the posterior margin of the last trunk ring (Figure 9). The tail was considered the remaining part of the seahorse, starting after the last trunk ring (Figure 9). Five whole seahorses from Galicia, were not subdivided in order to test on which way the elemental fingerprints (EF) of a seahorse body part could be used as a proxy to the whole body (1 species X 5 whole seahorse = 5 samples).

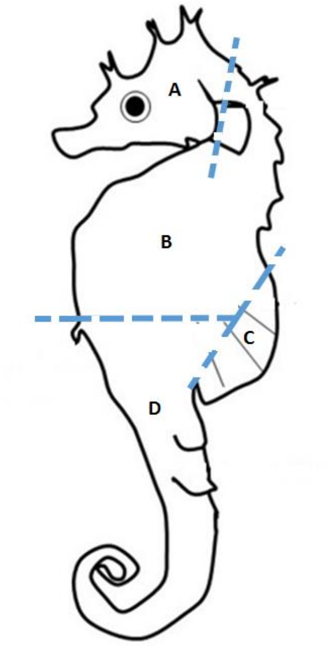

Figure 9 - Scheme of the four body parts (A- head, B- trunk, C- dorsal fin and D- tail), from a *Hippocampus guttulatus* specimen, being the cuts represented by the dotted blue lines.

The EF in seahorse bones, of the different body parts, were compared between the samples supplied by CSIC and the samples provided by CCMAR. Five specimens were supplied from each centre and divided into the four body parts (2 locations X 4 subdivisions X 5 replicates =40 samples). All specimens were provided frozen (died from natural causes) and maintained at -20 ºC until further analysis.

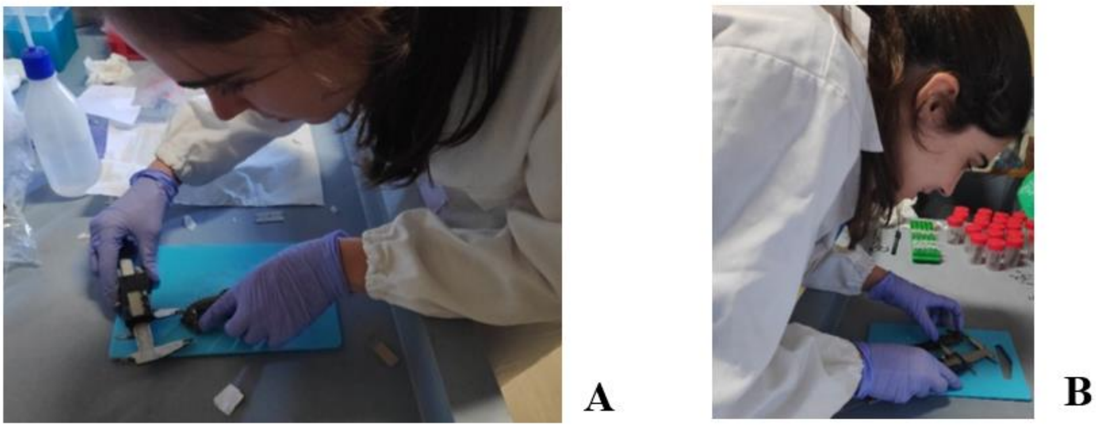

Figure 10 - Measurement of two body parts under study: A - measurement from the extremity of the snout until the proximity of the cleithral ring; B- measurement from the cleithral ring until the posterior extremity of the eleventh trunk ring.

2.1.1 Instituto de Investigaciones Marinas (CSIC)

The individuals from Vigo (Figure 11) were cultured and maintained at the Instituto de Investigaciones Marinas (Figure 12) from CSIC, Vigo, Spain.

Figure 11 - *Hippocampus guttulatus* from Instituto de Investigaciones Marinas (Source: Fishbase (2020)).

The seawater was provided from Ría de Vigo, located on the Atlantic margin of south western Galicia (located between 42º 09´ and 42º 21´ N; 8º 36´ and 8º 54´ W). Seawater was filtered using a customized system, which was also equipped with a cooling unit with an electronic thermostat control. Seawater temperature was maintained stable between 15 ºC in the winter and 17.5 ºC in the summer. Salinity and pH presented levels of 37 ± 2 and 8.0 ± 0.2, respectively (Planas *et al.*, 2008).

Figure 12 - Instituto de Investigaciones Marinas (Source: CSIC (2020)).

2.1.2 Ramalhete Aquaculture Field Station

The specimens from CCMAR at the University of the Algarve (Figure 13) were maintained at Ramalhete Aquaculture Field Station (Figure 14).

Figure 13 - *Hippocampus guttulatus* from the Ramalhete Aquaculture Field Station (Source: UAlg (2018)).

Natural seawater, from Ria Formosa coastal lagoon (mesotidal coastal lagoon, south Portugal, 36º 59´ N; 7º 51´ W), was provided to the tanks at a constant flow, in a close circuit recirculation system. The seawater was previously sterilized with UV radiation and aerated. The seawater temperature, salinity and dissolved oxygen were posteriorly kept at 21.4 ± 0.5 °C, 37.6 ± 0.1 and 6.9 ± 0.1 mg L^{-1}, respectively (Palma *et al.*, 2011).

Figure 14 - Ramalhete Aquaculture Field Station (Source: UAlg (2020)).

2.2 Samples preparation

Prior to elemental analysis, all organic matter of seahorse body parts was removed using ceramic coated blades and tweezers, as a way to avoid metal contamination. Followed by the removal of the remaining organic matter (e.g. soft tissues and skin), with the purpose of obtaining only the skeletal structure, all samples were freeze-dried for ≈24 h in a lyophilizer (Figure 15).

Figure 15 - Lyophilizer (Labogene CoolSafe 55-9L PRO).

The bones composing the head, trunk, tail and whole body, were individually homogenized using a mortar grinder (RM 200, Retsch, Hann, Germany; Figure 16 and 17), which was carefully cleaned between samples, to avoid cross-contamination, with silicate followed by alcohol (70%). In the case of the dorsal fin, the homogenized powder was obtained by using a manual ceramic mortar and pestle. For the posterior digestion of samples, a subsample weighting proximately 0.045 g was used for the body, head, trunk and tail, while the whole dorsal fin was employed.

Figure 16 - Mortar grinder (RM 200, Retsch, Hann, Germany).

Figure 17 - Homogeneous powder, obtained from the mortar grinder.

2.3 Elements extraction and ICP-MS analysis

The digestion of the samples was performed through the addition of 2 ml of high-purity concentrated nitric acid (HNO_3), 0.75 ml of hydrochloric acid (HCl) and 2 ml of hydrogen peroxide (H_2O_2) (3:1:3 v/v/v), in covered flat bottom graduated tubes (Figure 18).

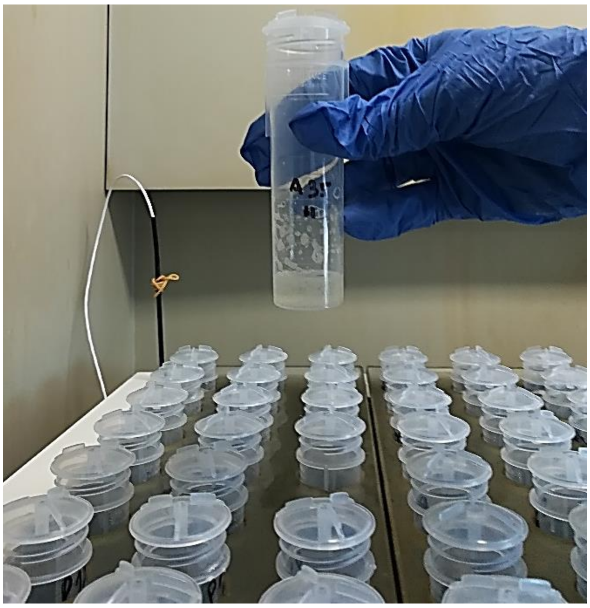

Figure 18 - Flat bottom graduated tubes for the digestion of the samples.

After digestion (overnight 14-16h), the solutions were heated on a digestion block (DigiPrep, SCP Science) (Figure 19) according to the following program, in which the samples were exposed, for 10 min, to an increase from room temperature to 50 °C, that were stabilized during 15 min. The temperature was then increased from 50 °C to 85 °C during a period of 15 min. When the temperature was stabilized at 85 °C, it was maintained for about 15 min (Figure 20), until the end of the cycle. After the first warm digestion, 2 ml of H_2O_2 were added, with the finality of removing the remaining organic matter. The action of the H_2O_2 was coupled with a second heating cycle, with the characteristics described above.

Figure 19 - Digestion block (DigiPrep, SCP Science).

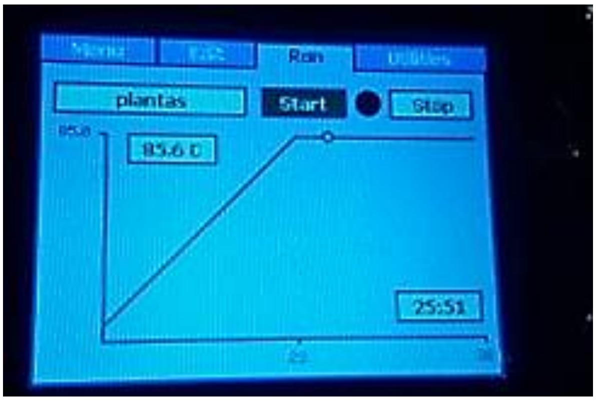

Figure 20 - Curve of the heating cycle, being registered the maintenance of the temperature 85 ºC, during 15 min.

The drying process of the samples, which consists in the evaporation of the liquid phase, was performed subsequently, at a temperature of 45 °C, in the digestion block. This process occurred with the tubes uncovered, being selected a program with the duration of proximately 1000 min. For the samples that still presented evidences of organic matter, 2 ml of H_2O_2 were posteriorly applied, and a new cycle of evaporation was effectuated. This procedure was repeated until the remaining organic matter was eliminated. After the total evaporation, 25 ml of a solution of HNO_3, at a concentration of 1% were added to the samples. Initially, 10 ml of the solution were applied to the samples for about 5 min, at a temperature of proximately 45 ºC to maximize the dissolution. Afterwards, the remaining 15 ml were added. The solution of HNO_3 at 1% was obtained by adding 10 ml of HNO_3 to

990 ml of water Milli-Q (Millipore). Moreover, a dilution consisting in the addition of 3 ml of HNO_3 at 1% to 1.5 ml of the samples' solution, was prepared and placed in plastic vial tubes for the posterior analysis of the bones' elemental composition in the a ICP-MS equipment.

Total concentrations of 14 elements, Al (aluminium), Ba (barium), Ce (cerium), Cr (chromium), Cu (copper), Fe (iron), K (potassium), Mg (magnesium), Mn (manganese), Na (sodium), Ni (nickel), P (phosphorus), Sr (strontium) and Zn (zinc), were determined through inductively coupled plasma spectrometry (ICP-MS), using an Agilent 7700 ICP-MS equipped with an octopole collision cell and autosampler (Figure 21). The analyses performed were validated trough a quality control program for the determination of each element under study, including method blanks, replicate samples, and a certified reference material. Accuracy of the ICP-MS method was evaluated by the analysis of certified reference material BCS-CRM-513 (SGT Limestone 1). Precision was estimated by the relative standard deviation (RSD) of five replicate samples, being ≤ 10%.

Figure 21 - Inductively coupled plasma mass spectrometry (ICP-MS), on an Agilent 7700 ICP-MS.

2.4 Statistical analysis

The concentration values of the elements were standardized to Ca (mmol/mol Ca) and the statistical analysis was carried out on the element ratios (X: Ca) (Ricardo *et al.*, 2015). Bootstrap resample was carried out based on 15 theoretical samples (n = 5 from Ria Formosa and n = 10 from Galicia) of the original set and presented 6 times the size of the original matrix (5 replicates X 6 = 30 replicates per group) (Horowitz, 2001). The resemblance matrix among samples was obtained from a normalized Euclidian distance.

To assess if the head, trunk, dorsal fin or tail are a reliable proxy of seahorses whole body, a one-way analysis of similarity (ANOSIM) was performed to detect significant differences in EF of seahorses' body parts when compared to the whole body. Briefly, ANOSIM calculates a global R, ranging from 0 to 1, that analyses the variability between groups and uses permutation tests, as a way of obtaining the significance of R (Ricardo, 2017). One-way analysis of variance test (ANOVA) was applied to assess differences ($p < 0.05$) between the samples from Galicia for each individual elemental ratio. A Principal Coordinates Analysis (PCO) was performed to visualize inter-individual spatial differences in EF among the 5 groups of samples (head, trunk, dorsal fin, tail and whole body).

Differences in EF of the same body parts (head, trunk, dorsal fin or tail) belonging to seahorses from the two locations (Galicia and Ria Formosa) were analyzed through ANOSIM. ANOVA was used to detect differences on each individual elemental ratio between the same body parts, from both locations. A PCO analysis was applied to assess the existence of inter-individual spatial differences in EF among the groups of the four body parts, from both locations.

Six seahorse portions (1-head+trunk, 2-head+trunk+fin, 3-head+trunk+tail, 4-trunk+fin, 5-trunk+fin+tail, 6-trunk+tail) were selected to assess the reliability of using organisms that are apprehended with body parts missing, to trace their geographic origin. These were compared, along with the equivalent to the whole body (head+trunk+dorsal fin+tail), by adding the elemental concentration determined on the four original body parts. Differences in EF of the same body portions and belonging to individuals from the two locations were analyzed through ANOSIM. An ANOVA test was applied to detect significant differences between each individual elemental ratio on the same body portions from specimens originating from the two locations.

A Canonical Analysis of Principal Coordinates (CAP) was performed to assess the reliability of using EF displayed by seahorse bones to infer their geographic origin. The samples from the four body parts, as well as the six seahorse portions, were considered to be blind samples, while the equivalents to the whole bodies, from both locations, were marked with their respective origin. For each blind sample, the CAP analysis attributed a location, regardless their effective origin. Moreover, a CAP prediction model using samples from the two locations, (30 replicates per location= 60 samples) was built and samples of the whole body from Galicia, considered as blind samples, were evaluated by classification on this model.

ANOSIM, PCO and CAP were performed using PRIMER v6 with the add-on PERMANOVA+, while ANOVA (Kruskall-Wallis test), after confirming normality with the Shapiro test and homogeneity of variance with the Bartlett, was performed using R.

3 Results

3.1 Assessment of a proxy for the whole body

The EF of seahorse bones from Galicia are summarized in Figure 22 and Table 4. Regarding the ratios Al/Ca, Ce/Ca, Cr/Ca, Cu/Ca and Fe/Ca, the samples that exhibited the highest concentrations of these elements were the ones from the dorsal fin (Figure 22; Table 4; Appendix A). The ANOVA test showed that the previously mentioned ratios were all significantly different between the dorsal fin and the whole body ($p < 0.001$) (Table 4). Ba/Ca, K/Ca, Mg/Ca, Mn/Ca, Na/Ca, Ni/Ca, P/Ca and Zn/Ca were recorded in higher concentrations in the whole body (Figure 22; Table 4; Appendix A). The Sr/Ca was recorded in higher concentrations in the head (Figure 22; Table 4; Appendix A), being significantly different between this body part and whole body ($p < 0.001$) (Table 4). Al/Ca, Ba/Ca, Ce/Ca and Zn/Ca exhibited lower concentrations in the tail (Table 4; Appendix A), being significantly different between the tail and the whole body ($p < 0.001$, with the exception of Ce with $p < 0.01$) (Table 4). The trunk recorded lower concentrations of Cr/Ca and Cu/Ca (Table 4; Appendix A), being the two ratios significantly different between the trunk and whole body ($p < 0.001$) (Table 4). The head registered the lowest concentrations of K/Ca, Mg/Ca, Mn/Ca, Na/Ca, Ni/Ca and P/Ca (Table 4; Appendix A). K/Ca, Na/Ca, Ni/Ca and P/Ca were significantly different between the head and whole body ($p < 0.001$), while Mg/Ca and Mn/Ca were not significantly different between these two groups ($p = 0.1$; $p = 0.06$) (Table 4). The concentrations of Fe/Ca and Sr/Ca were lower in the whole body (Table 4; Appendix A).

The ANOVA analysis between the four body parts and the whole body, showed that the majority of the elements/Ca were significantly different (Table 4). In the case of the head, in addition to Mg/Ca and Mn/Ca, the Cu/Ca was not significantly different, in comparison to the whole body ($p = 0.5$) (Table 4). The ratios Mg/Ca and Mn/Ca, did not differ significantly between the trunk and the whole body ($p = 1$; $p = 0.2$) (Table 4), the ratios Ba/Ca, Mn/Ca and P/Ca did not differ significantly between the dorsal fin and the whole body ($p = 0.2$; $p = 0.7$; $p = 0.1$) (Table 4) and the ratios Mg/Ca and Mn/Ca were not significantly different in the tail and whole body ($p = 0.3$; $p = 0.9$) (Table 4).

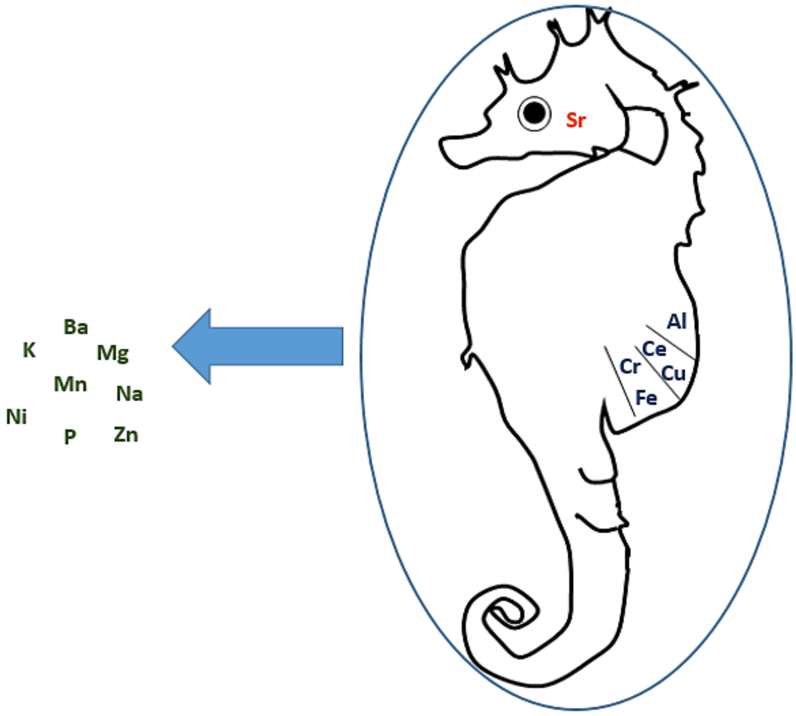

Figure 22 - Representation of the distribution of the higher EF concentrations (mmol /mol Ca), belonging to *Hippocampus guttulatus* specimens from Galicia.

The ANOSIM revealed significant differences among the EF of the four body parts (head, trunk, dorsal fin and tail) and the whole body (p = 0.001) (Table 3).

Table 3 - Analysis of the similarity (ANOSIM) between the EF of the bones that integrate the four body parts and the whole body, of the *Hippocampus guttulatus* individuals from Galicia.

	ANOSIM	
Samples	**R**	***p***
Head vs Whole-body	0.752	0.001
Trunk vs Whole-body	0.817	0.001
Dorsal fin vs Whole-body	0.999	0.001
Tail vs Whole-body	0.742	0.001

The first two axes of the PCO analysis explained 76.7% of the EF variation in the data set (PCO axis 1: 65.9%, PCO axis 2: 10.8%) (Figure 23). The dorsal fin appears as a distinctive group, as shown in Figure 23.

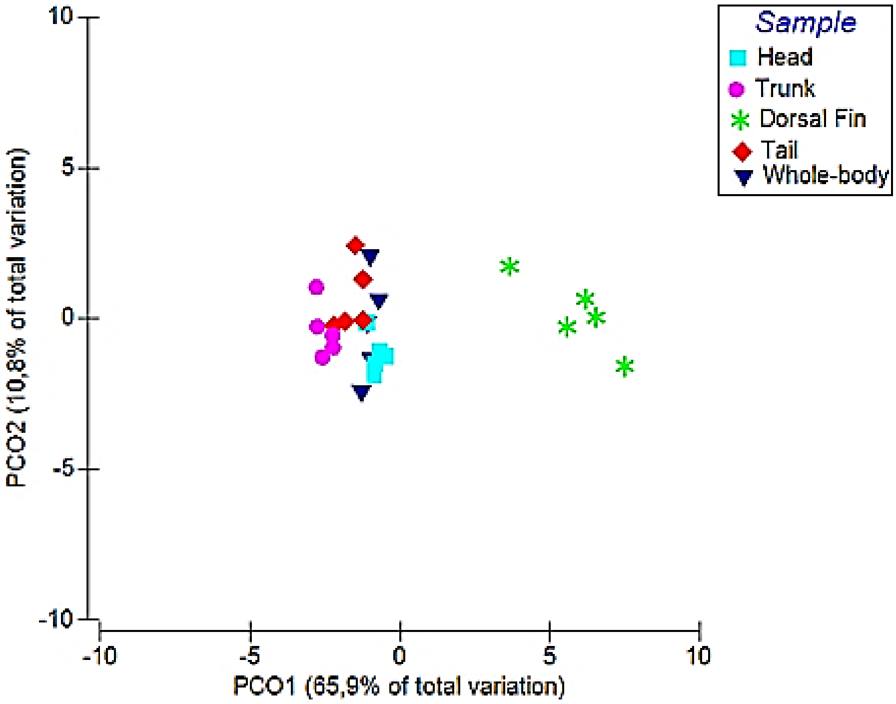

Figure 23 - Principal coordinates analysis of the EF of the bony structure of the four body parts (head, trunk, dorsal fin and tail) and the whole body, belonging to the *Hippocampus guttulatus* specimens from Galicia.

Table 4 - Ratios of elements to Calcium (Ca) concentrations (mmol to mol Ca) (average ± SD; n=30) of the five groups of samples (head, trunk, dorsal fin, tail and whole body), from Galicia long-snouted seahorses and representation of the *p*-values indicating significant differences between each individual elemental ratio of the four body parts and whole body, when * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and n.s., when the values are not significant (ANOVA, Kruskall-Wallis test).

						Kruskall-Wallis test (p-value)			
Elements	**Head**	**Trunk**	**Dorsal Fin**	**Tail**	**Whole body**	**Head vs Whole body**	**Trunk vs Whole body**	**Dorsal fin vs Whole body**	**Tail vs Whole body**
Al	0.18 ± 0.03	0.07 ± 0.01	0.51 ± 0.12	0.07 ± 0.02	0.11 ± 0.03	***	***	***	***
Ba	0.02 ± 0.002	0.02 ± 0.002	0.02 ± 0.003	0.01 ± 0.002	0.02 ± 0.001	***	***	n.s.	***
Ce	$6.12\ e^{-05} \pm 8.22\ e^{-06}$	$4.67\ e^{-05} \pm 5.55\ e^{-06}$	0.0004 ± 0.0001	$4.52\ e^{-05} \pm 5.28\ e^{-06}$	$4.1\ e^{-05} \pm 5.5\ e^{-06}$	***	***	***	**
Cr	0.02 ± 0.004	0.005 ± 0.0008	0.03 ± 0.007	0.01 ± 0.002	0.009 ± 0.0005	***	***	***	***
Cu	0.004 ± 0.0005	0.002 ± 0.0002	0.02 ± 0.004	0.003 ± 0.0003	0.004 ± 0.0007	n.s.	***	***	***
Fe	0.52 ± 0.07	0.26 ± 0.04	2.04 ± 0.58	0.39 ± 0.06	0.25 ± 0.02	***	**	***	***
K	6.03 ± 0.44	7.53 ± 0.88	8.25 ± 0.82	8.29 ± 0.81	44.44 ± 7.02	***	***	***	**
Mg	27.66 ± 2.70	28.12 ± 3.48	30.06 ± 5.39	30.87 ± 3.37	45.84 ± 6.42	n.s.	n.s.	***	n.s.
Mn	0.22 ± 0.03	0.23 ± 0.07	0.24 ± 0.04	0.22 ± 0.03	0.25 ± 0.03	n.s.	n.s.	n.s.	n.s.
Na	75.67 ± 9.69	92.52 ± 10.96	81.08 ± 7.18	126.54 ± 12.33	304.85 ± 39	***	***	***	***
Ni	0.008 ± 0.0005	0.01 ± 0.0006	0.009 ± 0.001	0.009 ± 0.0006	0.01 ± 0.001	***	***	***	***
P	426.61 ± 12.53	475.48 ± 5.97	439.18 ± 10.23	457.65 ± 2.66	477.98 ± 13.01	***	***	n.s.	***
Sr	7.18 ± 1.16	6.36 ± 0.38	5.32 ± 0.71	5.81 ± 0.23	3.49 ± 0.35	***	*	***	***
Zn	0.60 ± 0.07	0.67 ± 0.09	0.76 ± 0.07	0.55 ± 0.05	0.78 ± 0.11	***	*	**	***

3.2 Traceability of geographic origin

3.2.1 Elemental composition of the samples from Galicia and Ria Formosa

The distribution of the higher concentrations of the 14 elements to Ca present in the samples analyzed varied between the Galicia and Ria Formosa seahorses (Figure 24, Table 5 and Appendix A). In similitude with Galicia, the ratios Al/Ca, Ba/Ca, Ce/Ca, Cr/Ca, Cu/Ca, Fe/Ca and Mn/Ca were recorded at higher concentrations in the dorsal fin from Ria Formosa samples (Figure 24; Table 5; Appendix B). In the case of specimens from Ria Formosa, the highest concentrations of K/Ca and Ni/Ca were recorded in the dorsal fin, while the head recorded higher concentrations of Mg/Ca, Na/Ca, P/Ca and Sr/Ca (Figure 24; Table 5; Appendix B). The tail displayed the highest concentration of Zn/Ca (Figure 24, Table 5 and Appendix B). Both Galicia and Ria Formosa exhibited the lowest concentrations of Cr/Ca, Cu/Ca, Fe/Ca in the trunk (Table 5; Appendix B). The dorsal fin recorded the lowest concentrations of Sr/Ca in both locations (Table 5; Appendix B). Regarding the remaining elemental ratios from the Ria Formosa samples, the lowest concentrations of Al/Ca and Ba/Ca were recorded in the trunk (Table 5; Appendix B). Ce/Ca and Zn/Ca were recorded at lower concentrations in the head. The tail recorded the lowest concentrations of K/Ca, Mg/Ca, Mn/Ca, Na/Ca and Ni/Ca, while P/Ca was recorded at lower concentrations in the dorsal fin (Table 5; Appendix B).

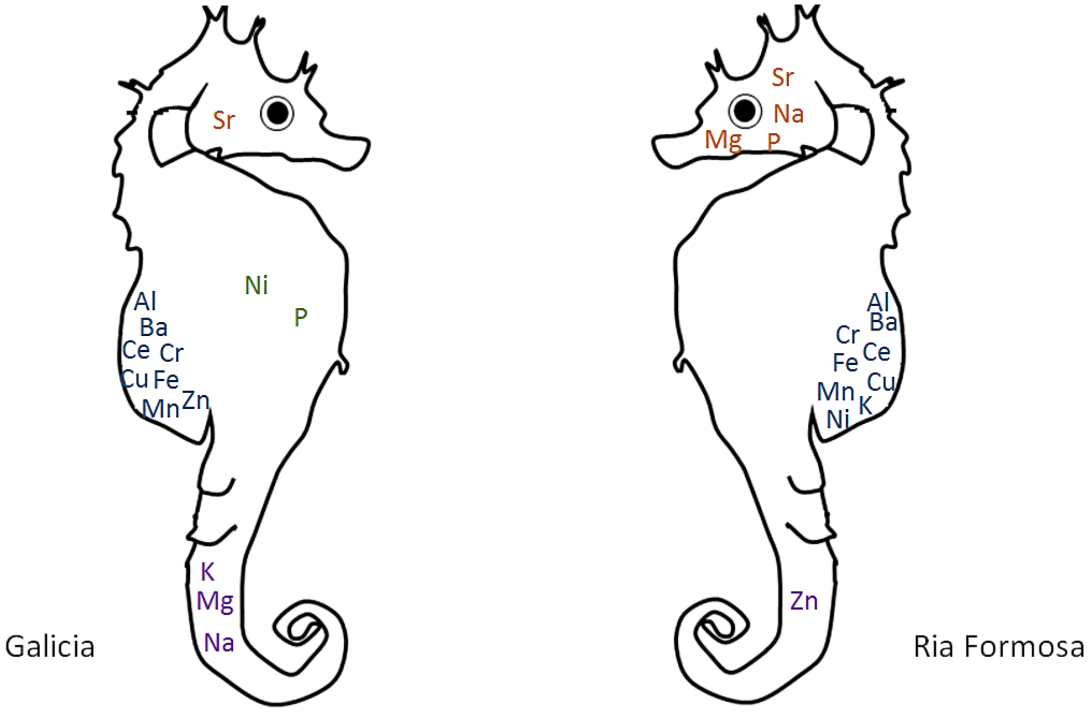

Figure 24 - Distribution of the higher ratios of elements to Ca concentrations (mmol to mol) in the four body parts (head, trunk, dorsal fin and tail), belonging to *Hippocampus guttulatus* specimens from Galicia (left) and Ria Formosa (right).

The ANOVA analysis between the same body parts of specimens originating from different locations showed that the majority of elements/Ca were significantly different (Table 5). The ratio Cr/Ca was not significantly different between the head groups (p = 0.9) (Table 5), the ratios K/Ca and Mn/Ca were not significantly different between the dorsal fin groups (p = 0.2; p = 0.1) (Table 5) and the ratios Ba/Ca, Mg/Ca and P/Ca, were not significantly different between the tail groups (p = 0.2; p = 0.3; p = 0.8) (Table 5).

Table 5 - a) Ratios of elements to Calcium (Ca) concentrations (mmol to mol Ca) (average ± SD; n=30) of the five groups of samples (head, trunk, dorsal fin, tail and whole body), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa and b) representation of the *p*-values indicating significant differences between the individual elemental ratios of the same body parts, from the seahorses of both locations, when * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and n.s., when the values are not significant (ANOVA, Kruskall-Wallis test).

									Kruskall-Wallis test (p-value)			
Elements	**Head-G**	**Head-RF**	**Trunk-G**	**Trunk-RF**	**Dorsal Fin-G**	**Dorsal Fin-RF**	**Tail-G**	**Tail-RF**	**Head vs Head**	**Trunk vs Trunk**	**Dorsal fin vs Dorsal fin**	**Tail vs Tail**
Al	0.18 ± 0.03	0.70 ± 0.13	0.07 ± 0.01	0.26 ± 0.02	0.51 ± 0.12	3.43 ± 0.62	0.07 ± 0.02	0.32 ± 0.04	***	***	***	***
Ba	0.02 ± 0.002	0.01 ± 0.001	0.02 ± 0.002	0.008 ± 0.0007	0.02 ± 0.003	0.02 ± 0.002	0.01 ± 0.002	0.01 ± 0.001	***	***	***	n.s.
Ce	6.12 e^{-05} ± 8.22 e^{-06}	2.54 e^{-05} ± 1.29 e^{-06}	4.67 e^{-05} ± 5.55 e^{-06}	2.86 e^{-05} ± 5.95 e^{-06}	0.0004 ± 0.0001	0.001 ± 0.0003	4.52 e^{-05} ± 5.28 e^{-06}	2.83 e^{-05} ± 2 e^{-06}	***	***	***	***
Cr	0.02 ± 0.004	0,02 ± 0.004	0.005 ± 0.0008	0.01 ± 0.002	0.03 ± 0.007	0.05 ± 0.007	0.01 ± 0.002	0.02 ± 0.002	n.s.	***	***	***
Cu	0.004 ± 0.0005	0.009 ± 0.001	0.002 ± 0.0002	0.008 ± 0.001	0.02 ± 0.004	0.03 ± 0.004	0.003 ± 0.0003	0.009 ± 0.0009	***	***	***	***
Fe	0.52 ± 0.07	0.57 ± 0.09	0.26 ± 0.04	0.25 ± 0.03	2.04 ± 0.58	1.04 ± 0.11	0.39 ± 0.06	0.43 ± 0.06	**	*	***	***
K	6.03 ± 0.44	26.39 ± 2.1	7.53 ± 0.88	29.95 ± 4.98	8.25 ± 0.82	48.49 ± 2.47	8.29 ± 0.81	21.97 ± 0.68	***	***	n.s.	***
Mg	27.66 ± 2.70	45.12 ± 3.68	28.12 ± 3.48	35.38 ± 1.19	30.06 ± 5.39	33.95 ± 4.47	30.87 ± 3.37	30.39 ± 3.23	***	***	***	n.s.
Mn	0.22 ± 0.03	0.25 ± 0.028	0.23 ± 0.07	0.19 ± 0.028	0.24 ± 0.04	0.26 ± 0.002	0.22 ± 0.03	0.17 ± 0.02	***	*	n.s.	***
Na	75.67 ± 9.69	183.30 ± 17.30	92.52 ± 10.96	127.50 ± 14.37	81.08 ± 7.18	129.28 ± 11.21	126.54 ± 12.33	97.73 ± 14.45	***	***	***	**
Ni	0.008 ± 0.0005	0.01 ± 0.0007	0.01 ± 0.0006	0.01 ± 0.002	0.009 ± 0.001	0.03 ± 0.009	0.009 ± 0.0006	0.01 ± 0.001	***	***	***	**
P	426.61 ± 12.53	594.89 ± 18.23	475.48 ± 5.97	583.18 ± 49.54	439.18 ± 10.23	432.54 ± 32.54	457.65 ± 2.66	451.54 ± 34.97	***	***	***	n.s.
Sr	7.18 ± 1.16	5.12 ± 0.31	6.36 ± 0.38	4.59 ± 0.51	5.32 ± 0.71	2.59 ± 0.43	5.81 ± 0.23	3.65 ± 0.36	***	***	***	***
Zn	0.60 ± 0.07	0.48 ± 0.02	0.67 ± 0.09	0.48 ± 0.01	0.76 ± 0.07	0.56 ± 0.06	0.55 ± 0.05	0.66 ± 0.03	***	***	***	***

The ANOSIM analysis revealed significant differences between the EF of the bony structures of the same body parts (head, trunk, dorsal fin or tail) from the two locations (*p* = 0.001) (Table 6).

Table 6 - Similarity values (ANOSIM) between the EF of the bones that integrate the same body parts, from Galicia and Ria Formosa long-snouted seahorses.

	ANOSIM	
Samples	R	*p*
Head Galicia vs Head Ria Formosa	1	0.001
Trunk Galicia vs Trunk Ria Formosa	0.928	0.001
Dorsal Fin Galicia vs Dorsal Fin Ria Formosa	0.996	0.001
Tail Galicia vs Tail Ria Formosa	0.786	0.001

The first two axes of the PCO analysis explained 64.5% of the EF variation of the four body parts and the equivalent to whole bodies, from Ria Formosa and Galicia (PCO axis 1: 44.9%, PCO axis 2: 19.6%) (Figure 25). The dorsal fins of seahorses from the two locations appear as distinctive groups, as shown in Figure 25.

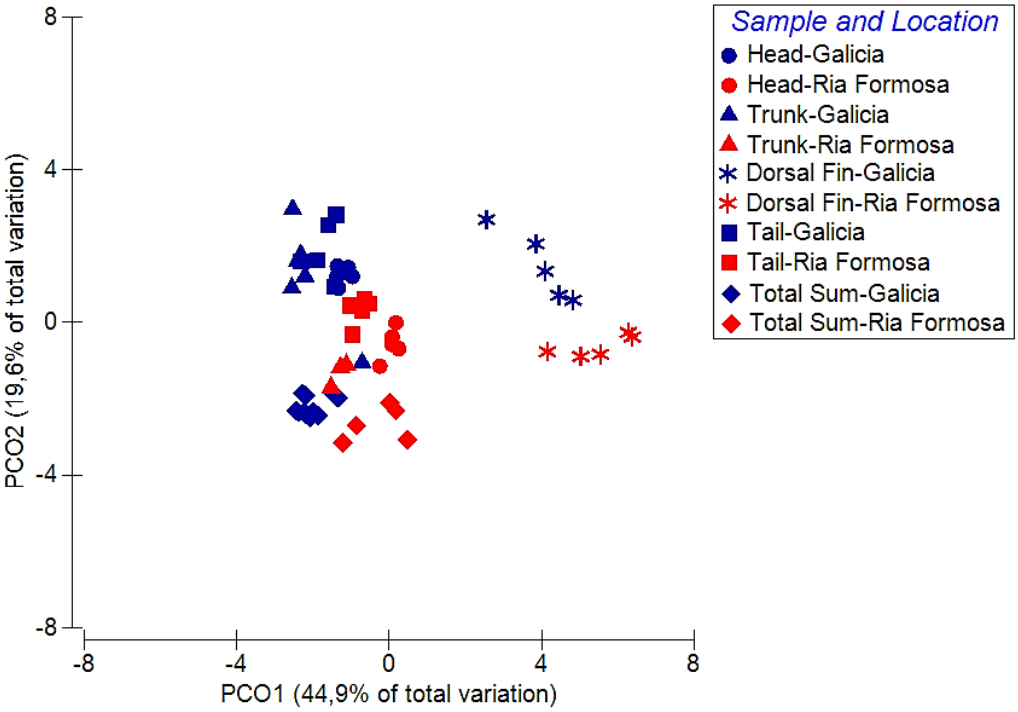

Figure 25 - Principal coordinates analysis of the EF, belonging to the four body parts (head, trunk, dorsal fin and tail) and the equivalent to whole bodies, from Galicia (blue) and Ria Formosa long-snouted seahorses (red).

3.2.2 Body parts as blind samples

A CAP analysis, with only the equivalent to the whole bodies marked with known origin, resulted in the correct allocation of the place of origin of all trunk samples from seahorses originating from the two locations (Table 7). The head, dorsal fin and tail did not exhibit a success rate of allocation of 100%. The head replicates of seahorses from Ria Formosa were all correctly classified, while 43.3% of the replicates from Galicia were placed in Ria Formosa. In the case of the dorsal fin, the replicates from both locations were placed in Ria Formosa. The tail from Galicia seahorses exhibited 60% of its replicates classified as Ria Formosa, while 30% of the replicates from Ria Formosa were attributed to Galicia.

Table 7 - Classification success by location (Galicia or Ria Formosa) of a Canonical analysis of principal coordinates based on the EF from the seahorses' body parts.

Location-sample	Galicia (%)	Ria Formosa (%)
Galicia-Head	56.7	43.3
Galicia-Trunk	100.0	0.0
Galicia-Dorsal Fin	0.0	100.0
Galicia-Tail	40.0	60.0
Ria Formosa-Head	0.0	100.0
Ria Formosa-Trunk	0.0	100.0
Ria Formosa-Dorsal Fin	0.0	100.0
Ria Formosa-Tail	30.0	70.0

3.2.3 Validation of the use of trunk EF as a model to trace the geographic origin of seahorses

The CAP analysis for the trunk, from Galicia and Ria Formosa, and the organisms processed as a whole, from Galicia (blind samples), exhibited a successful classification rate of 100%, being all samples correctly allocated to Galicia (Table 8).

Table 8 - Canonical analysis of principal coordinates for the trunk replicates (n=60), from seahorses of the two locations, and the whole body replicates from Galicia (n=30), considered as the blind samples.

Possible origin	Predicted origin	% Correct classification
Galicia	30	100
Ria Formosa	0	

3.2.4 Elemental composition of the portions of the body

The ANOSIM analysis revealed significant differences between the EF of the same seahorse body portions, originating from Galicia and Ria Formosa (*p* = 0.001) (Table 9).

Table 9 - Similarity values (ANOSIM) between the EF of the same portions, belonging to long-snouted seahorses from Galicia and Ria Formosa.

	ANOSIM	
Samples	R	*p*
H+T+B Galicia vs **H+T+B** Ria Formosa	0.862	0.001
T+B Galicia vs **T+B** Ria Formosa	0.728	0.001
T+C Galicia vs **T+C** Ria Formosa	0.83	0.001
T+C+B Galicia vs **T+C+B** Ria Formosa	0.781	0.001
H+T Galicia vs **H+T** Ria Formosa	0.847	0.001
H+T+C Galicia vs **H+T+C** Ria Formosa	0.77	0.001

The ANOVA analysis between the same body portions, of seahorses from different locations, resulted in the majority of the elements/Ca, with significant differences (Appendixes C, D, E, F, G and H). The ratios Ba/Ca, Fe/Ca and Zn/Ca, were the ones that least differ significantly between the portions considered. The ratios Fe/Ca and Zn/Ca, were not significantly different between the groups H+T+B (p = 0.6; *p* = 0.1) (Appendix C), Ba/Ca, Fe/Ca and Zn/Ca, were not significantly different between the groups T+B (*p* = 0.7; *p* = 0.5; *p* = 0.3) (Appendix D), Ba/Ca did not differ significantly between the groups T+C (*p* = 0.07) (Appendix E), Ba/Ca and Fe/Ca, were not significantly different between the groups T+C+B (*p* = 0.8; *p* = 0.2) (Appendix F) and Zn/Ca did not differ significantly between the groups H+T (*p* = 0.7) (Appendix G).

3.2.5 Body portions as blind samples

The CAP analysis, with only the equivalent to the whole body marked with known origin, resulted in the correct attribution of all the replicates of the six seahorse body portions considered (1-head+trunk, 2-head+trunk+fin, 3-head+trunk+tail, 4-trunk+fin, 5-trunk+fin+tail, 6-trunk+tail), to Galicia and Ria Formosa.

4 Discussion

The combat to the seahorse illegal trade remains a challenging task, due to the number of people involved and the submission of incomplete or ambiguous reports to CITES (Foster *et al*., 2016). The analysis of EF may allow to combat the fraudulent trade of seahorses through the traceability of their geographic origin, being a relatively fast and less expensive approach when compared with other traceability methods (e.g. biochemical and molecular tools) (Leal *et al*., 2015).

Previous studies addressing seahorse bony structure have mainly focused its development and ossification processes (Novelli *et al*., 2017; Silveira, 2000), the morphological variation in seahorse vertebral system (Bruner and Bartolino, 2008; Neutens *et al*., 2017) and deformation mechanisms of the bony armor (Porter *et al*., 2013; Praet *et al*., 2012; Van Cauter *et al*., 2010). The use of the geochemical composition of seahorse vertebrae and bony plates to trace their geographic origin has never been previously explored. Thus, this study may be a starting point for the development of important traceability tools to fight illegal, unregulated and unreported seahorse fisheries, by tracing the origin of specimens being traded and apprehended.

From the chemical elements considered in both locations, P was the one that exhibited the higher mean concentration values to Ca, ranging from 594.89 mmol/mol Ca, value of the head from the seahorses of Ria Formosa (Table 5; Appendix B), to 426.61 mmol/mol Ca, value of the head from the seahorses of Galicia (Table 4; Appendix A). This element plays an important role on the bony structure of fishes, with diet being its main source. P, in similarity to Ca, is involved in the maintenance and development of the bony structure, and the stability of the vertebrae is achieved through a solid phase of calcium phosphate (Lall and McCrea, 2007). Na followed P in terms of higher mean concentrations, in both locations, ranging from 304.85 mmol/mol Ca, mean value of the whole body from Galicia seahorses, to 75.67 mmol/mol Ca, value of the head from Galicia seahorses (Table 4; Table 5; Appendix A; Appendix B). According to Lin et al. (2008), seahorses are rich in P, Na and K, ranging the concentrations of the third one from 48.49 mmol/mol Ca, in the dorsal fin from Ria Formosa, to 6.03 mmol/mol Ca, in the head of seahorses from Galicia (Table 4; Table 5; Appendix A; Appendix B). These three elements were pointed to potentially benefit humans' health through the consumption of this marine resource (Lin *et al*., 2008).

Furthermore, the main components of fish vertebrae are calcium, phosphate and carbonate and, to a smaller extent, magnesium, sodium, strontium, lead, citrate, fluoride, hydroxide and sulfate (Lall and McCrea, 2007). In fact, the elements Mg and Sr followed the previously three mentioned elements (P, Na and K) in terms of higher mean concentrations. Transition metals, such as Cu, Zn, Fe and Mn, are essential, as these are part of important proteins, being, however, toxic when present in excess (Bury *et al*., 2003). In the case of ictyophauna, essential metals can be acquired through the diet or from the water (Bury *et al*., 2003). In the present analysis, these elements presented lower mean concentrations to Ca, in comparison with the elements previously mentioned (Table 4; Table 5; Appendix A; Appendix B). Furthermore, the trace elements Cu, Zn and Mn are needed for the development of fish bones (Lall and McCrea, 2007). The remaining elements, Al, Ba, Ce, Cr and Ni, in similitude to Cu, Zn, Fe and Mn, presented lower mean concentrations, in comparison with P, Na, K, Mg and Sr (Table 4; Table 5; Appendix A; Appendix B). The element Ba is a non-essential element that, in similitude with Sr, is able to replace calcium in the biomineralization of bony structures (Loewen *et al*., 2016). In the other hand, it has been pointed that high intakes of Al and Sr, have deleterious effects on skeletal tissue metabolism (Lall and McCrea, 2007). The dorsal fin presented significantly higher concentrations of Al/Ca, Ba/Ca, Ce/Ca, Cr/Ca, Cu/Ca, Fe/Ca and Mn/Ca, in comparison to other samples from both locations (Tables 4 and 5; Appendix A and B). The study developed by Rubio et al. (2000), which considered the geochemistry of subtidal sediments from Ría de Vigo, showed that this ecosystem is slightly to moderately polluted, when considering Cr, Cu, Fe, Pb (lead) and Zn. Furthermore, in a study developed between 2007 and 2008, metals such as Cd, Cu, Ni, Pb and Zn were reported to accumulate in *Ruditapes decussatus* from Ria Formosa coastal lagoon, although in lower concentrations than previously reported (Cravo *et al*., 2012). According to the research developed by Avigliano et al. (2020), ratios of metal/Ca, as Cu/Ca, Fe/Ca, Na/Ca and Zn/Ca, of the edge of dorsal fin spines, belonging to the species *Genidens barbus*, showed to be potentially useful for the discrimination of habitat use and fish stocks. Additionally, natural element/Ca, as Ba/Ca, Mg/Ca, Mn/Ca and Sr/Ca, present in the dorsal fin spine edges of *G. barbus*, were also reported to be effective in stock identification (Avigliano *et al*., 2019). Otolith chemistry has been preferentially used for aspects related with fishes' life cycle, since the fin spines can alter their composition, due to resorption. Nevertheless, fin edges present lower rates of resorption, being able to retain chemical compositions obtained during the development (Avigliano *et al*., 2020). Thus, when present in higher concentrations than in otoliths, metals from anthropogenic origin, such as Cu, Pb and Zn, present in fin spines can complement the classification of fish populations that inhabit contaminated ecosystems (Avigliano *et al*., 2020).

The ANOSIM analysis that integrated the four body parts and the whole body of seahorses from Galicia, evidenced that none of the body parts were a proxy to the whole body, in terms of the EF of the bony structures (Table 3). According to Kerr and Campana (2014), the incorporation of some elements in fish vertebrae can occur through absorption across the skin and gills or through diet, whose contribution to the chemical composition of fish hard parts, varies with element and type of structure. Nevertheless, the relative contribution of water and diet, as well as the proportion of elemental incorporation into the vertebrae, are not extensively studied (Kerr and Campana, 2014). The ANOVA analysis showed that the four body parts differed from the whole body, in the majority of the elements/Ca (Table 4), which indicated (as the ANOSIM test did) that there is no perfect proxy for the whole body. The ratios Mg/Ca and Mn/Ca, were the ones that least varied between the whole body and the body parts, being Mg one of the main constitutes of fish vertebrae (Lall and McCrea, 2007). The PCO analysis exhibited a more distinguishable group (Figure 23), composed by the dorsal fin replicates, indicating a higher variability between the EF present in the bones of this structure than in the bones of other samples.

The ANOSIM test, for the assessment of the potential of using a body part, in alternative to the whole homogenized seahorse, evidenced that the same body parts, from the two locations, were all significantly different. This result indicates the possibility of using just a body part for tracing the geographic origin of different populations from the same species (Table 6). In the case of the ANOVA analysis, the majority of the elements/Ca were significantly different between the same body parts, of both locations (Table 5). Thus, this analysis indicated that the EF of the same body parts, belonging to the same species but from distinct origins were in general significantly different. This finding sets the starting point to pursue the traceability of seahorses' geographic origin. The PCO analysis showed that the dorsal fins from seahorses belonging to both locations appeared as distinctive groups in comparison to the other samples (Figure 25), thus indicating a higher EF variability.

The CAP analysis, for the traceability of seahorses' origin, resulted in the correct allocation of all trunk replicates to their respective place of origin (Table 7). Although the dorsal fin and head replicates from Ria Formosa seahorses were all correctly classified, this was not the case for other samples. In the case of the dorsal fin from Galicia seahorses, none of the samples were correctly attributed to their respective origin (Table 7). Thus, the model body part with most potential, in alternative to the whole body, to trace the geographic origin of seahorses is most likely the trunk (Table 8).

There is a number of factors, that may affect the chemical composition of calcified structures in fish, such as water chemistry, diet, environmental conditions (e.g., temperature), physiology, fish size, and even genetic aspects (Kerr and Campana, 2014; Lall, 2003). Additionally, the bone is not a metabolically inert structure, with the occurrence of elemental mobilization being possible to occur (Campana, 2005). The developmental stage, may also affect elemental fingerprints, since habitat use may differ with life cycle stage (Gillanders and Kingsford, 2003). In the case of seahorses, the juveniles of some species present a planktonic life stage, while adults are mostly demersal (Blanco, 2014). According to Kerr and Campana (2014), initial studies regarding the water chemistry, where the groups of fish under study occur, may give some insights about the possibility of the groups differ in their chemistry. Coastal ecosystems, such as estuaries with lower oceanic influences, present a more variable physical and chemical environment, when compared with the more homogenous nature of the deep ocean. This factor can reflect in the geochemical fingerprints of the populations under study (Longmore *et al*., 2011; Thorrold *et al*., 2002). Water chemistry can vary depending on spatial and temporal scales (e.g., seasonal and interannual), which was reported to reflect on otolith chemistry (Kerr and Campana, 2014). Furthermore, the temporal variability in water chemistry can have different expressions on the fingerprints of individuals from the same population, due to factors as size (Kerr and Campana, 2014). In the present study, although the water supplied was from natural sources, the chemical and physical conditions were maintained stable, which reduced the spatio-temporal variability. Furthermore, according to Kerr and Campana (2014), some of the elements under strict physiological regulation, may not be ideal environmental indicators which, in the case of otoliths, include P, Na and K. However, these elements can be useful biological tracers, if their concentration vary significantly among groups (Kerr and Campana, 2014).

In the present study six body portions were considered, since it is possible that during the apprehension of illegally acquired seahorses, the organism's structure may be missing some of its integrity. The ANOSIM analysis resulted in significant differences between the same body portions of seahorses originating from Galicia and Ria Formosa (Table 9). This result, in similarity to the one of the body parts, indicates the possibility of using seahorses with body parts missing to trace the geographic origin of organisms of the same species and from different populations. The ANOVA analysis indicated that the majority of the elements/Ca, with the exception of Ba/Ca, Fe/Ca and Zn/Ca, were significantly different between the same portions, from the two locations (Appendixes C, D, E, F, G and H). Thus, as EF from the body portions here considered being, in general, significantly different, it can be possible to trace the geographic origin of apprehended seahorses missing part of their body. The CAP analysis, which included the six body portions, revealed that all of them can be successfully employed to trace the geographic origin of seahorses.

5 Conclusions

The results of this preliminary research suggest that the vertebrae and bony armor of seahorses can be successfully used to trace their geographic origin. The trunk may potentially be an alternative to the whole body, with its processing *per se* being an advantage in terms of time and cost to prepare and process samples. Furthermore, the edge of the dorsal fin may potentially be a model that allows the traceability of seahorse populations occupying polluted environments, with fin clipping already being a well-established method that does not promote significant deleterious effects on the growth and survival of several fish species, including seahorses (Lourie, 2003b).

In the case of an apprehension of seahorses with missing body parts, the use of EF from the bones of available body parts, holds the potential to still being used to successfully trace their geographic origin. Nevertheless, it is important to validate the use of EF of seahorses' bones as a tool for traceability using wild specimens, as the present study used cultured seahorses and wild conspecifics are exposed to more dynamic environments (from a biotic and abiotic perspective).

Being the present work a preliminary research, there are aspects that may be approached in future studies, such as employing a larger number of replicates from different geographic origins, the inclusion of more than one species when determining the traceability of seahorses geographic origin and taking into account the size and gender of the specimens being studied.

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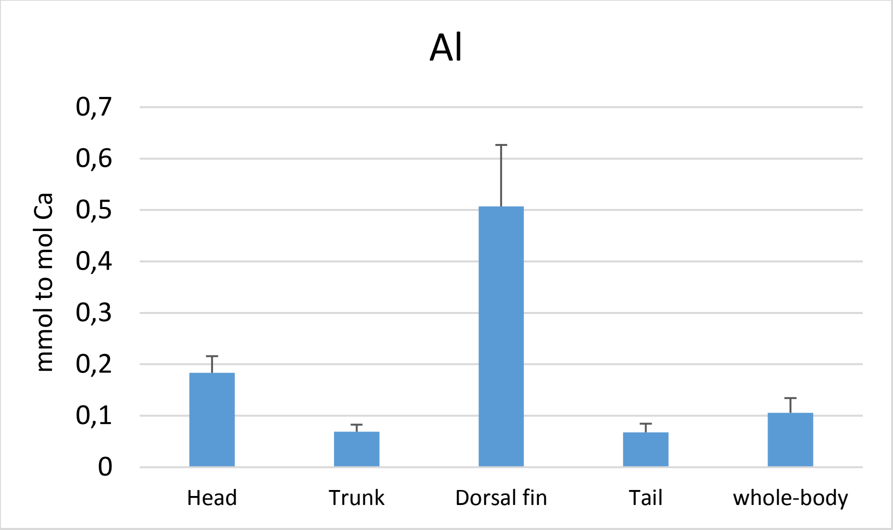
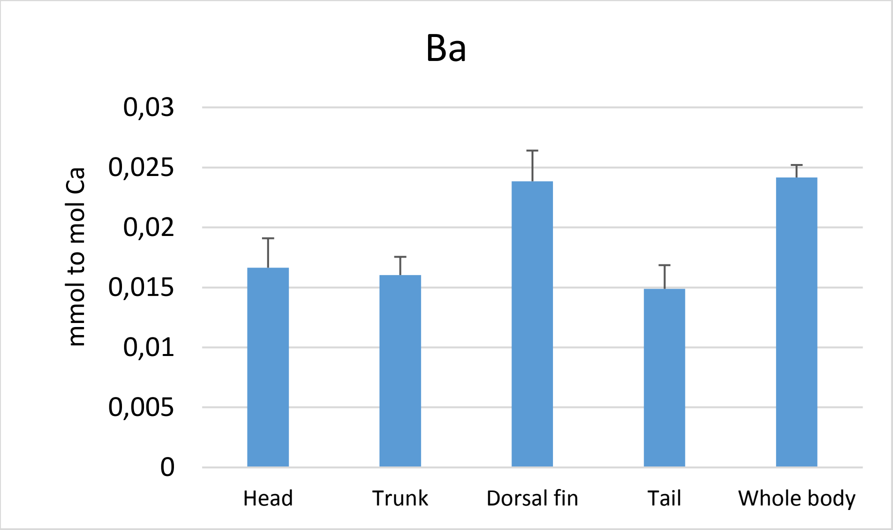
7 Supplementary data

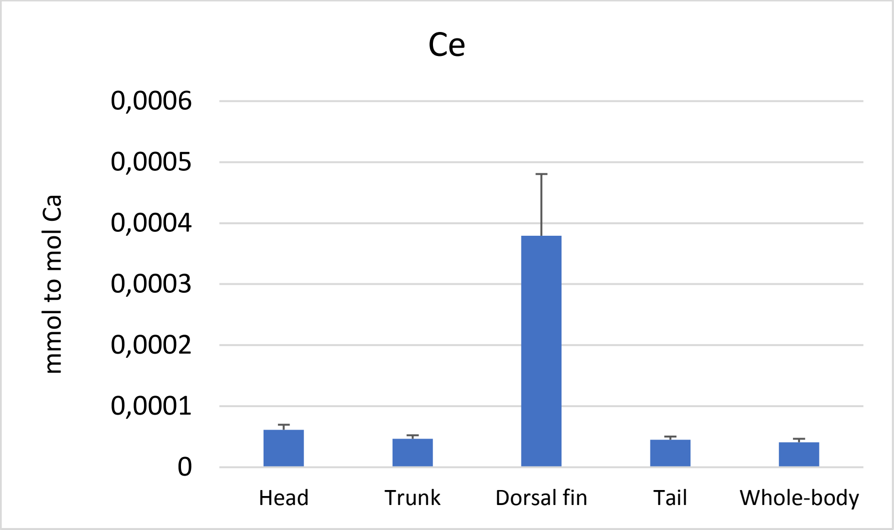
7.1 Appendix A



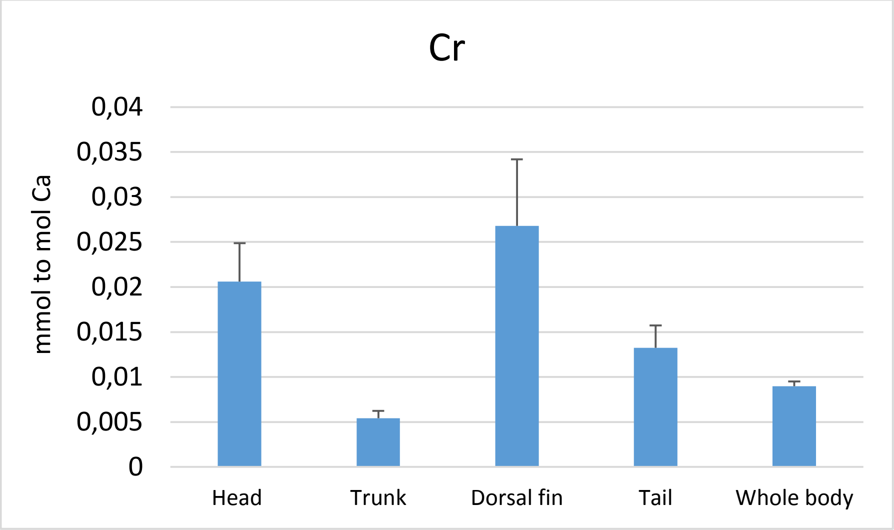
Graphic A1- Ratios of Al to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



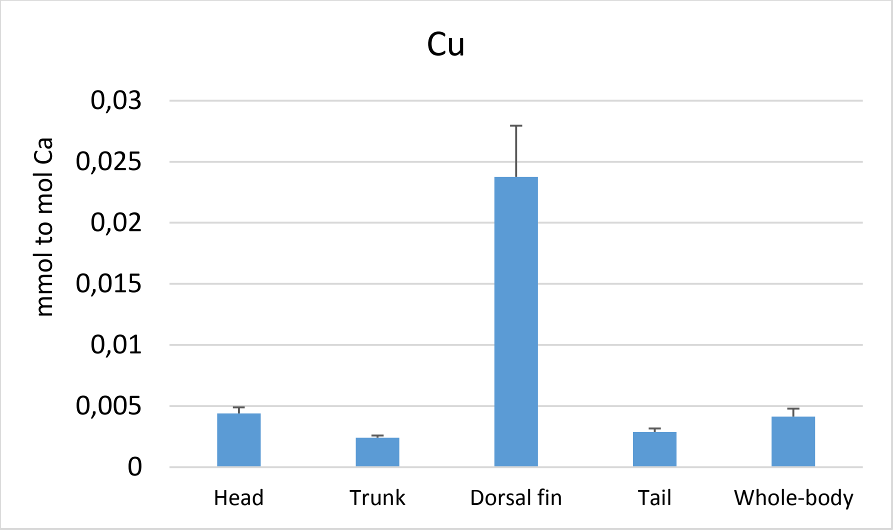
Graphic A2- Ratios of Ba to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



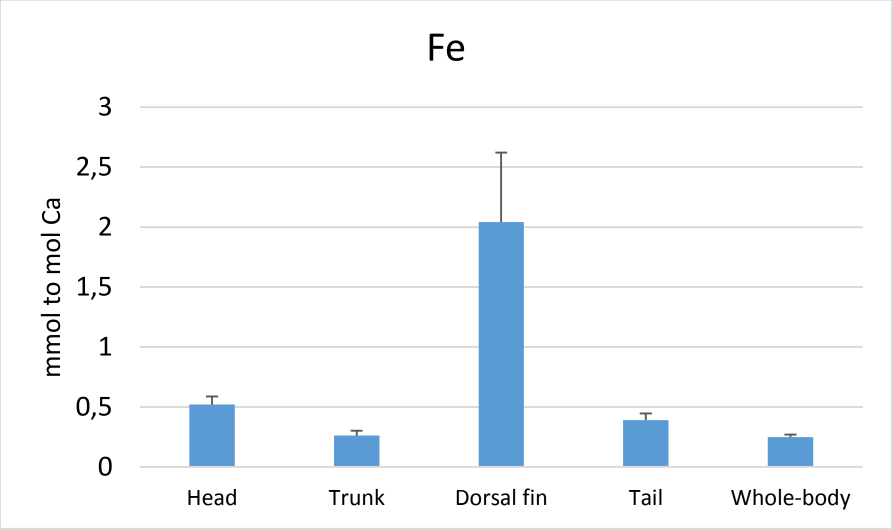
Graphic A3- Ratios of Ce to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



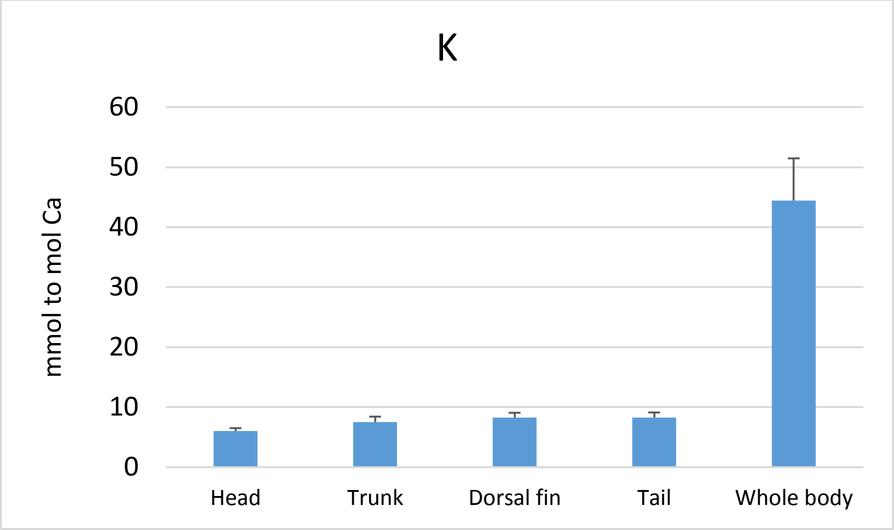
Graphic A4- Ratios of Cr to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



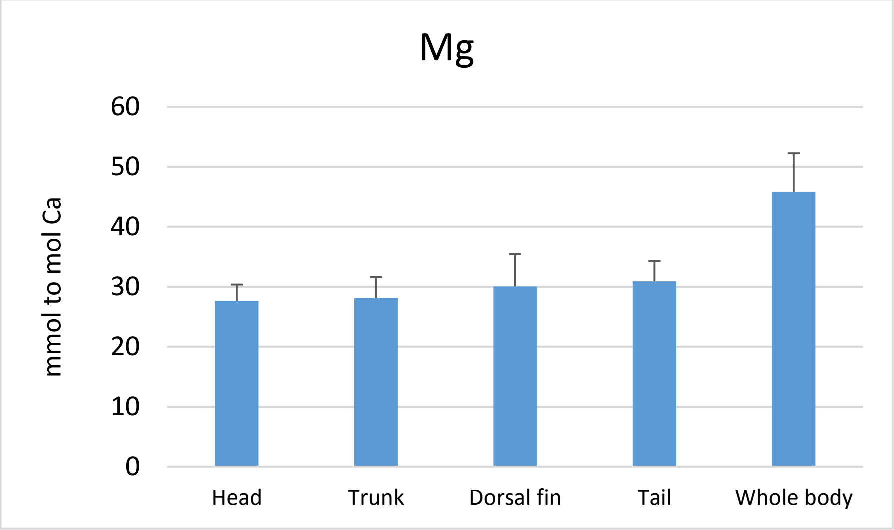
Graphic A5- Ratios of Cu to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



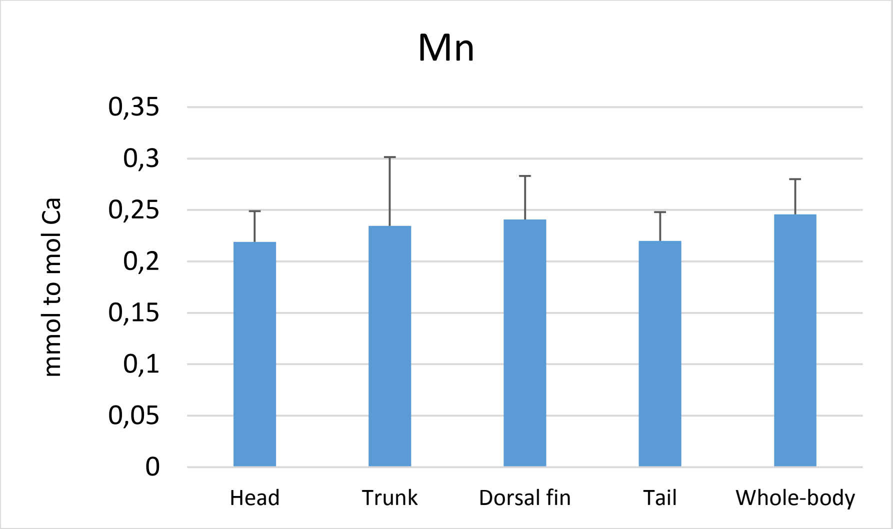
Graphic A6- Ratios of Fe to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



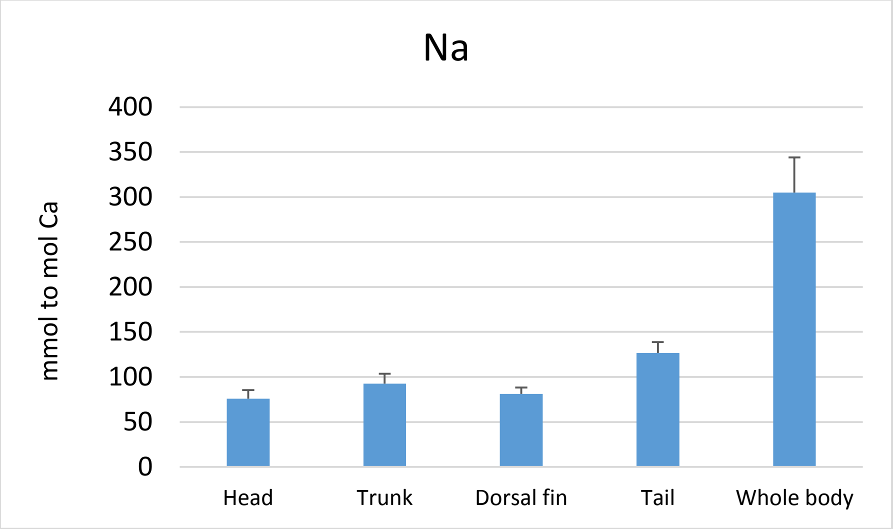
Graphic A7- Ratios of K to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



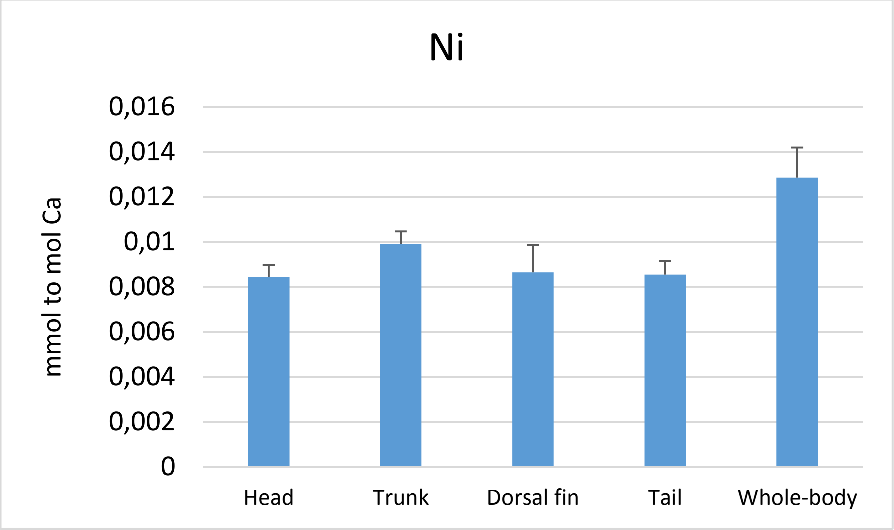
Graphic A8- Ratios of Mg to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



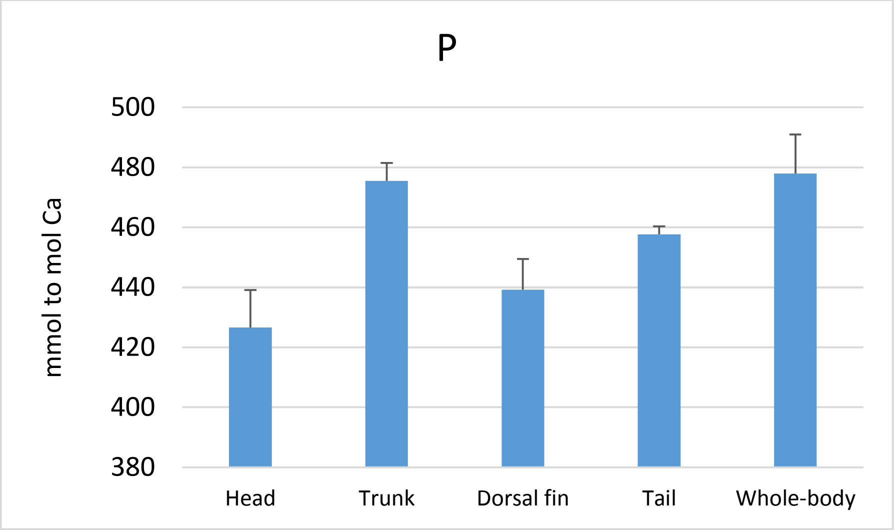
Graphic A9- Ratios of Mn to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



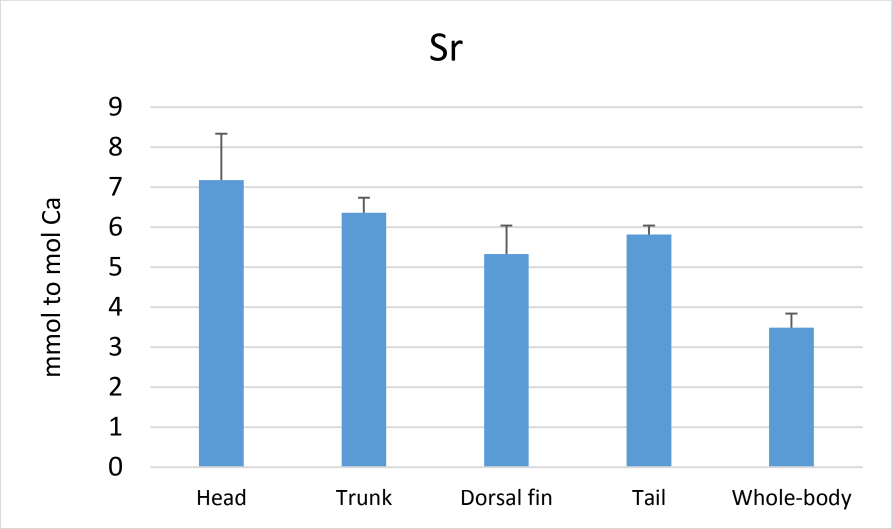
Graphic A10- Ratios of Na to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



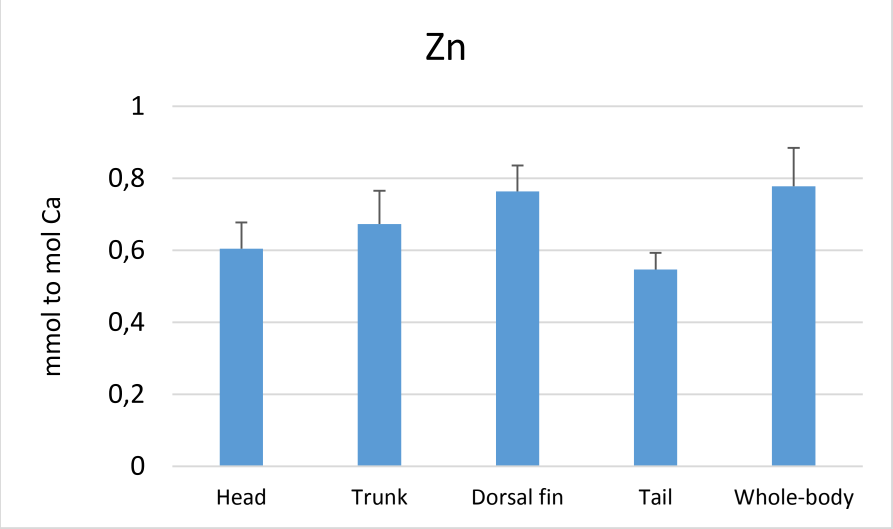
Graphic A11- Ratios of Ni to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.



Graphic A12- Ratios of P to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.

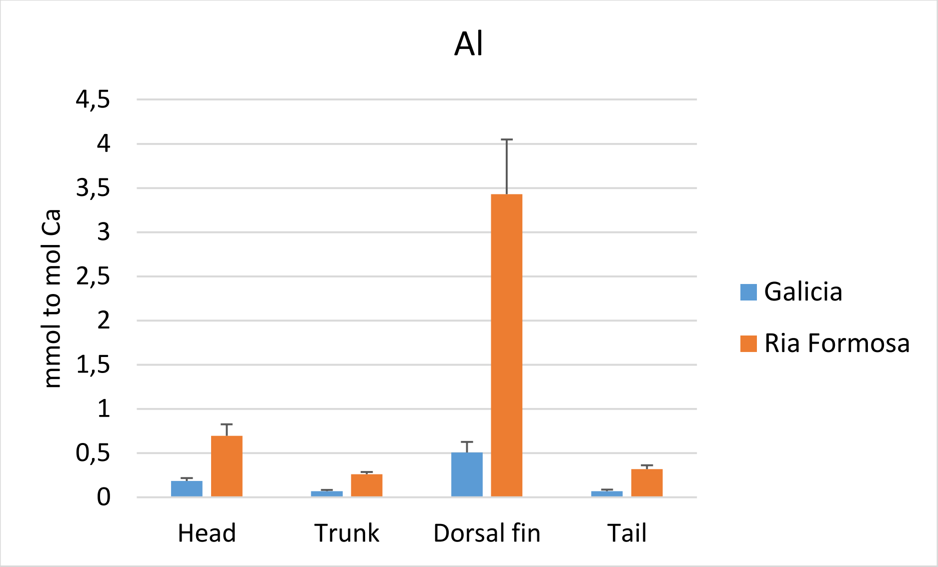


Graphic A13- Ratios of Sr to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.

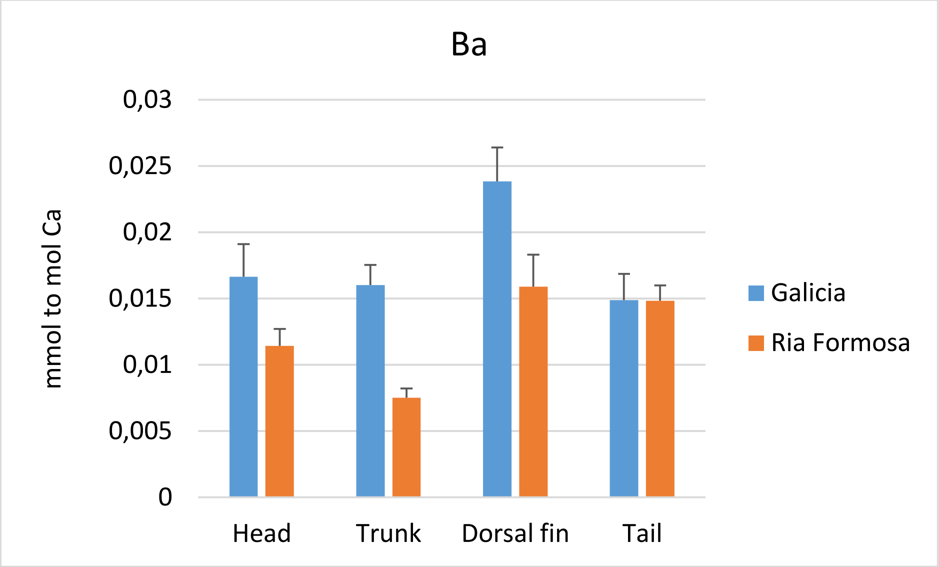


Graphic A14- Ratios of Zn to Ca concentrations (mmol to mol) (average ± SD; n=30) of five sampling groups (head, trunk, dorsal fin, tail and whole-body), belonging to *Hippocampus guttulatus* from Galicia.

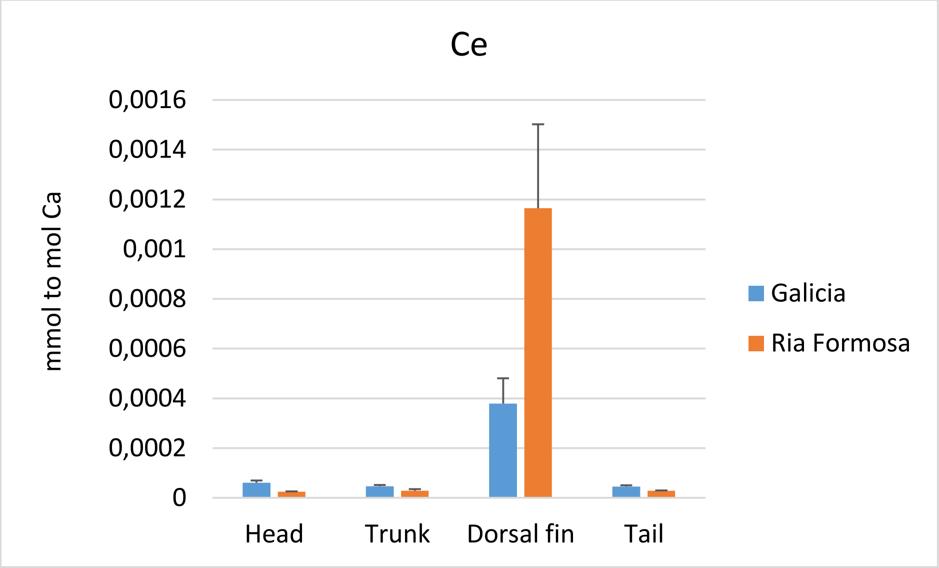
7.2 Appendix B



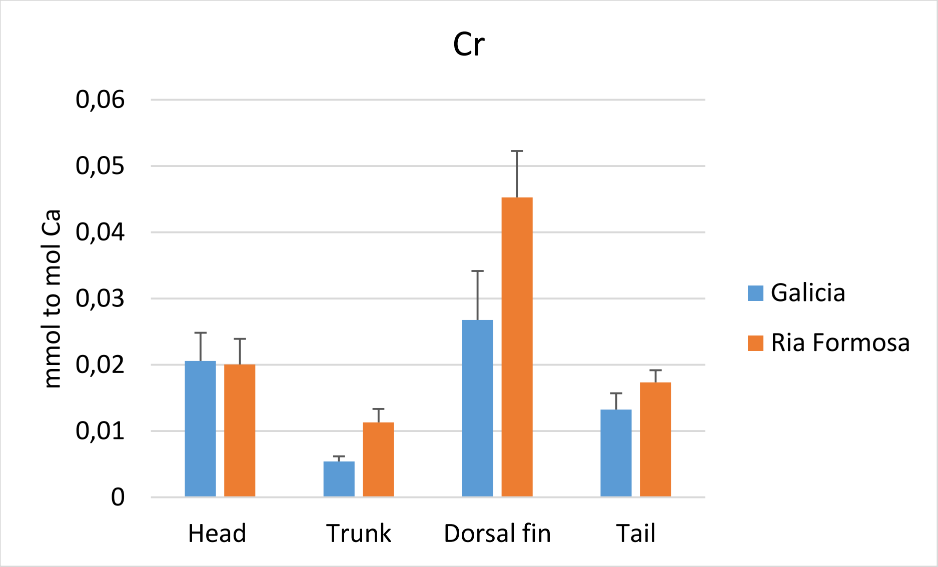
Graphic B1- Ratios of Al to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



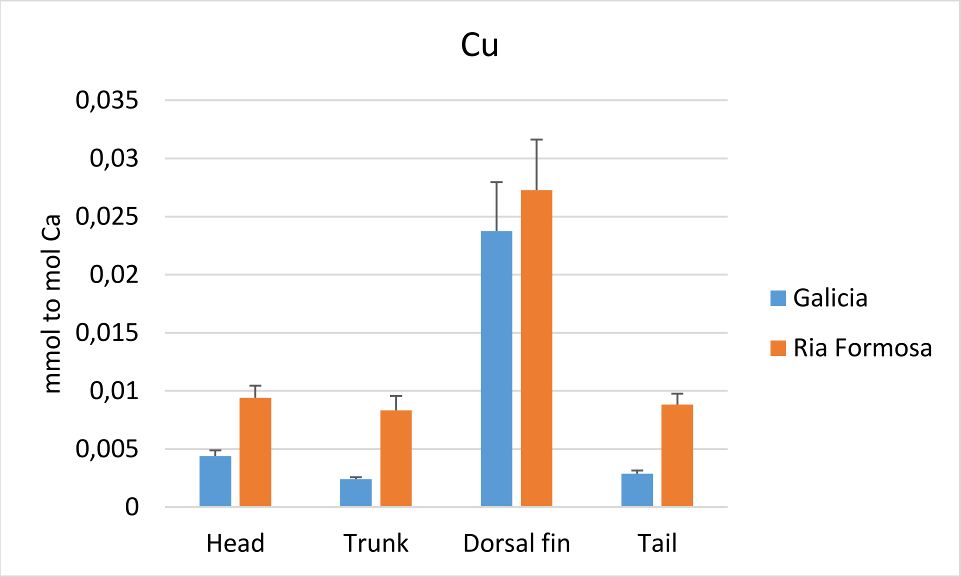
Graphic B2- Ratios of Ba to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



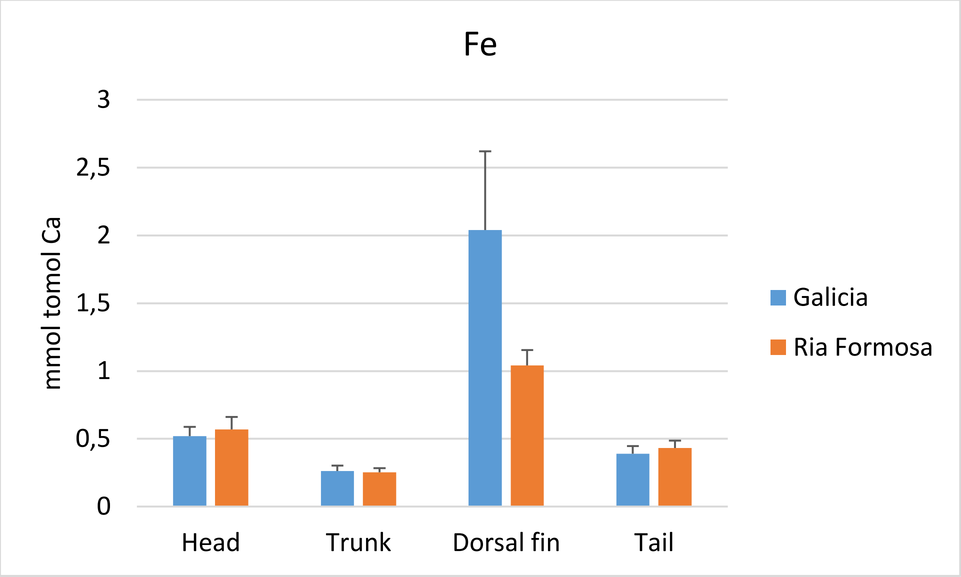
Graphic B3- Ratios of Ce to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



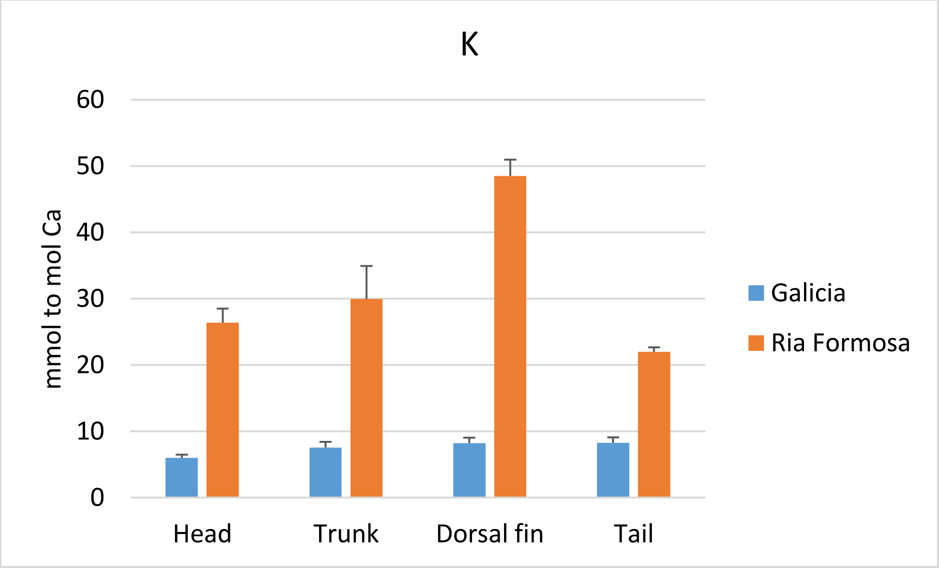
Graphic B4- Ratios of Cr to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



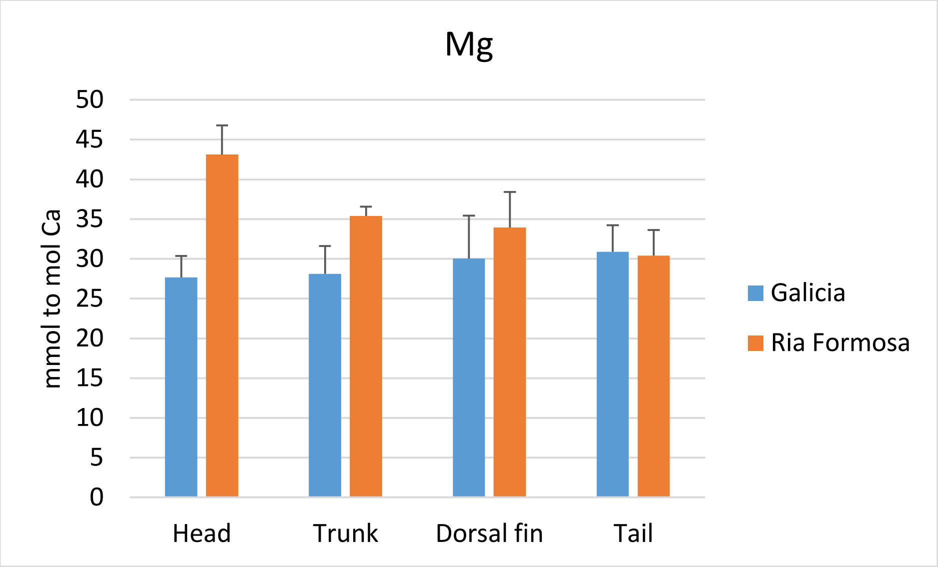
Graphic B5- Ratios of Cu to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



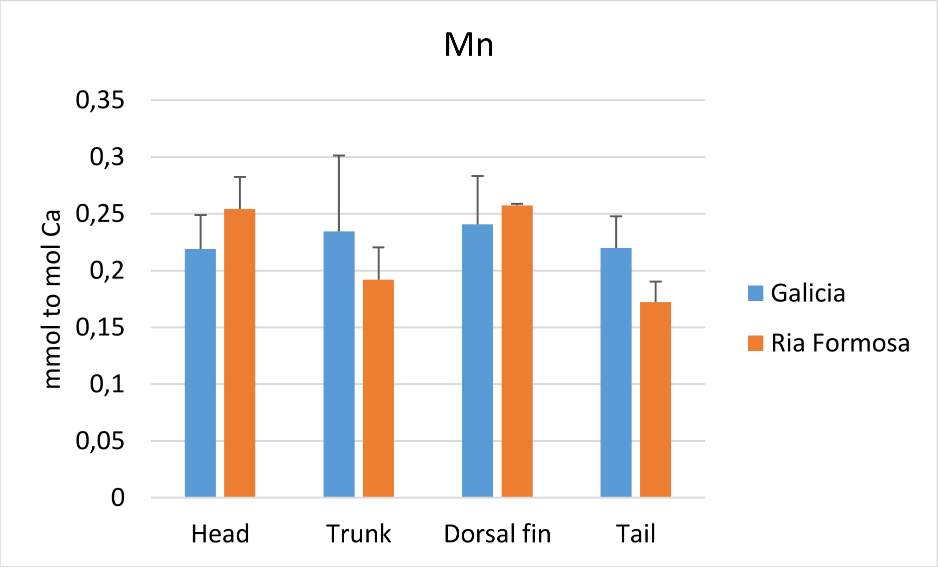
Graphic B6- Ratios of Fe to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



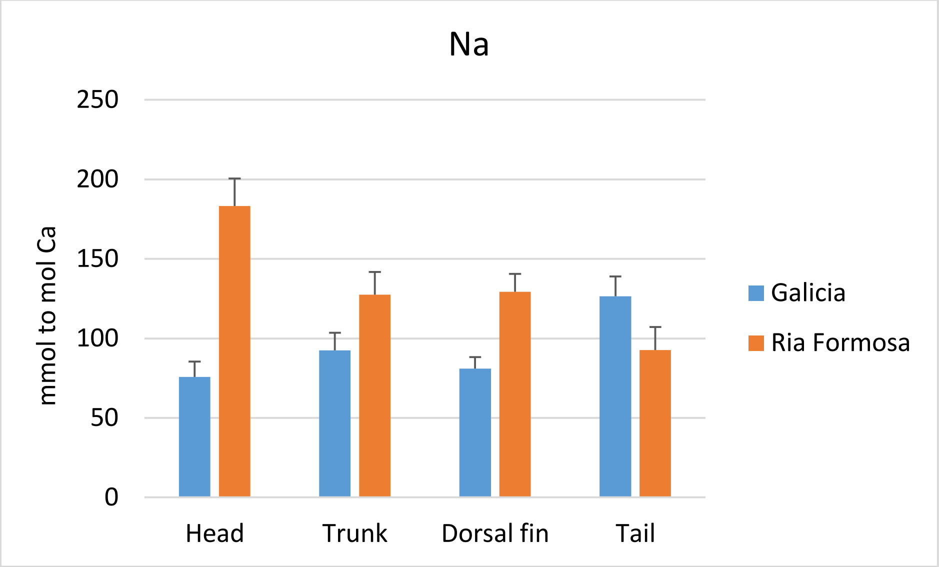
Graphic B7- Ratios of K to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



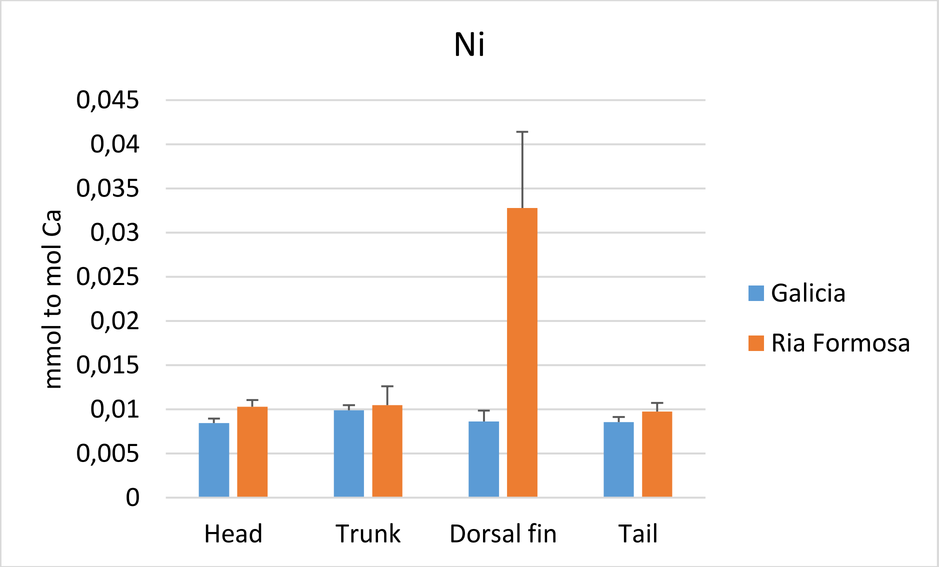
Graphic B8- Ratios of Mg to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



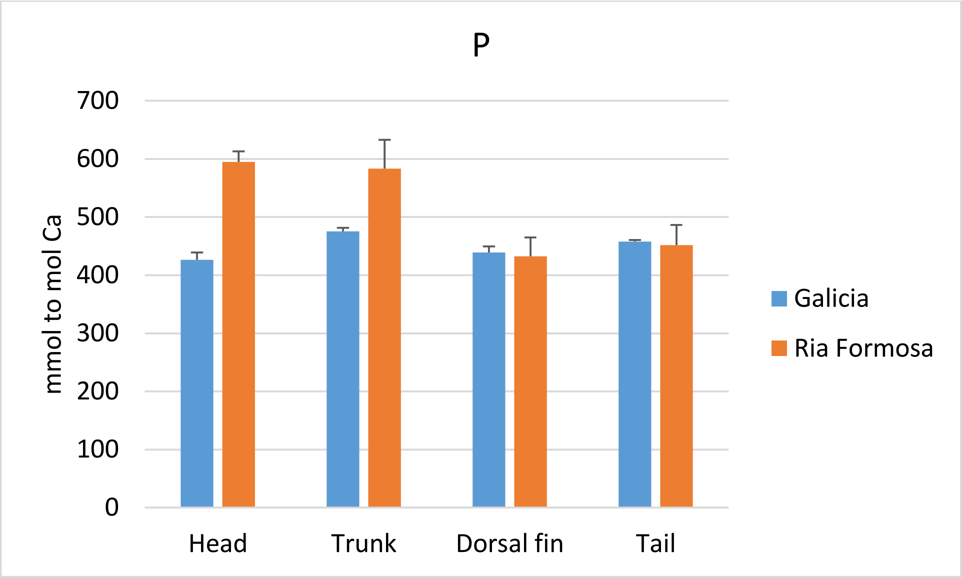
Graphic B9- Ratios of Mn to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



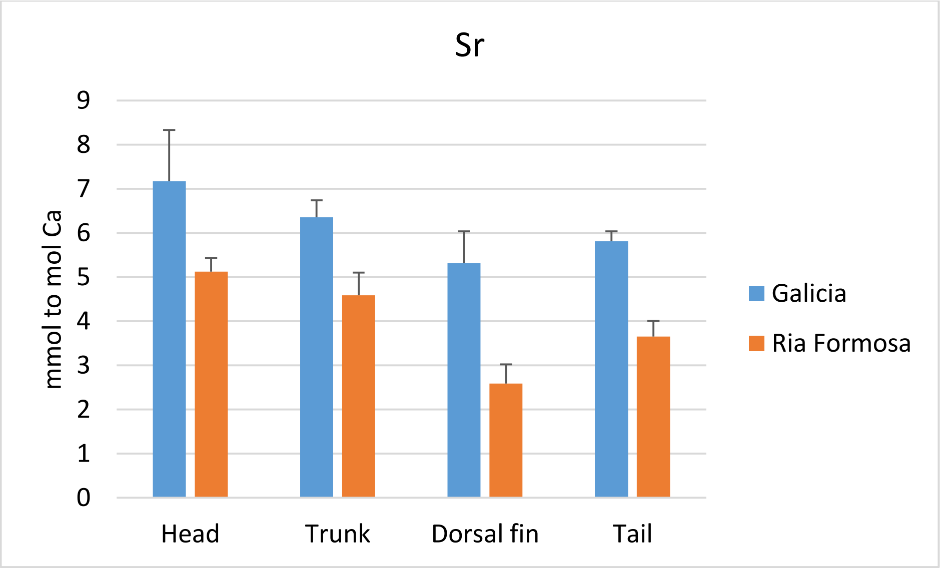
Graphic B10- Ratios of Na to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



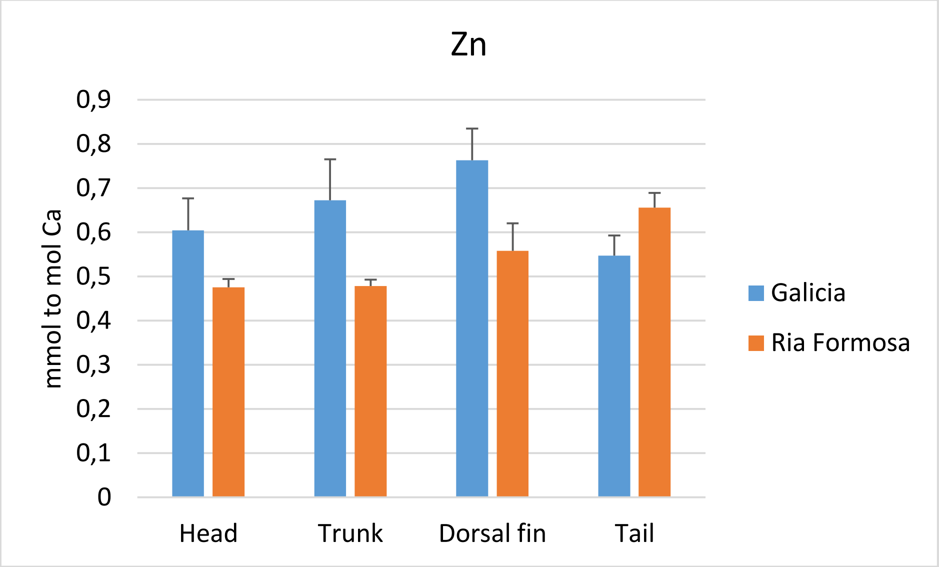
Graphic B11- Ratios of Ni to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



Graphic B12- Ratios of P to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



Graphic B13- Ratios of Sr to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (head, trunk, dorsal fin, tail), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.



Graphic B14- Ratios of Zn to Ca concentrations (mmol to mol) (average ± SD; n=30) of four body parts (fin, tail, head, trunk), belonging to *Hippocampus guttulatus* from Galicia and Ria Formosa.

7.3 Appendix C

Table C1- Values of the Kruskall-Wallis test : chi square, degrees of freedom (df) and p-values indicating significant differences between the EF of the H+T+B from Galicia and the H+T+B from Ria Formosa seahorses, when * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and n.s., when the values are not significant (ANOVA, Kruskall-Wallis test).

Elements	chi-square	df	p-value
Al	45	1	***
Ba	7	1	**
Ce	45	1	***
Cr	33	1	***
Cu	45	1	***
Fe	0.2	1	n.s.
K	45	1	***
Mg	14	1	***
Mn	30	1	***
Na	13	1	***
Ni	45	1	***
P	45	1	***
Sr	10	1	**
Zn	3	1	n.s.

7.4 Appendix D

Table D1- Values of the Kruskall-Wallis test : chi square, degrees of freedom (df) and p-values indicating significant differences between the EF of the T+B from Galicia and the T+B from Ria Formosa seahorses, when * p < 0.05, ** p < 0.01, *** p < 0.001 and n.s., when the values are not significant (ANOVA, Kruskall-Wallis test).

Elements	**chi-square**	**df**	**p-value**
Al	45	1	***
Ba	0.2	1	n.s.
Ce	45	1	***
Cr	23	1	***
Cu	21	1	***
Fe	0.5	1	n.s.
K	35	1	***
Mg	7	1	*
Mn	30	1	***
Na	26	1	***
Ni	45	1	***
P	45	1	***
Sr	6	1	*
Zn	1	1	n.s.

7.5 Appendix E

Table E1- Values of the Kruskall-Wallis test : chi square, degrees of freedom (df) and p-values indicating significant differences between the EF of the T+C from Galicia and the T+C from Ria Formosa seahorses, when * p < 0.05, ** p < 0.01, *** p < 0.001 and n.s., when the values are not significant (ANOVA, Kruskall-Wallis test).

Elements	chi-square	df	p-value
Al	45	1	***
Ba	3	1	n.s.
Ce	6	1	*
Cr	45	1	***
Cu	45	1	***
Fe	25	1	***
K	45	1	***
Mg	18	1	***
Mn	26	1	***
Na	10	1	**
Ni	45	1	***
P	45	1	***
Sr	12	1	***
Zn	11	1	***

7.6 Appendix F

Table F1- Values of the Kruskall-Wallis test : chi square, degrees of freedom (df) and p-values indicating significant differences between the EF of the T+C+B from Galicia and the T+C+B from Ria Formosa seahorses, when * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and n.s., when the values are not significant (ANOVA, Kruskall-Wallis test).

Elements	chi-square	df	p-value
Al	45	1	***
Ba	0.1	1	n.s.
Ce	45	1	***
Cr	45	1	***
Cu	45	1	***
Fe	2	1	n.s.
K	45	1	***
Mg	20	1	***
Mn	30	1	***
Na	13	1	***
Ni	45	1	***
P	45	1	***
Sr	10	1	**
Zn	11	1	***

7.7 Appendix G

Table G1- Values of the Kruskall-Wallis test : chi square, degrees of freedom (df) and p-values indicating significant differences between the EF of the H+T from Galicia and the H+T from Ria Formosa seahorses, when * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and n.s., when the values are not significant (ANOVA, Kruskall-Wallis test).

Elements	chi-square	df	p-value
Al	45	1	***
Ba	24	1	***
Ce	45	1	***
Cr	7	1	**
Cu	45	1	***
Fe	17	1	***
K	45	1	***
Mg	10	1	**
Mn	26	1	***
Ni	45	1	***
Na	33	1	***
P	45	1	***
Sr	10	1	**
Zn	0.2	1	n.s.

7.8 Appendix H

Table H1- Values of the Kruskall-Wallis test : chi square, degrees of freedom (df) and p-values indicating significant differences between the EF of the H+T+C from Galicia and the H+T+C from Ria Formosa seahorses, when * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 and n.s., when the values are not significant (ANOVA, Kruskall-Wallis test).

Elements	**chi-square**	**df**	**p-value**
Al	45	1	***
Ba	17	1	***
Ce	33	1	***
Cr	25	1	***
Cu	45	1	***
Fe	30	1	***
K	45	1	***
Mg	18	1	***
Mn	26	1	***
Ni	30	1	***
Na	45	1	***
P	45	1	***
Sr	12	1	***
Zn	21	1	***