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RECEIVED 11 September 2024

ACCEPTED 04 November 2024

PUBLISHED 20 November 2024

CITATION

Cabral AE, Calado R, Palma J, Ricardo F,
Ferreira da Silva E, Patinha C and Planas M
(2024) Use of isotopic and elemental
fingerprints for seahorse species
discrimination and traceability of
geographic origin.
Front. Mar. Sci. 11:1494320.
doi: 10.3389/fmars.2024.1494320

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Use of isotopic and elemental fingerprints for seahorse species discrimination and traceability of geographic origin

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Seahorses (*Hippocampus* spp.; Family Syngnathidae) are mainly targeted by Traditional Chinese Medicine, curio and ornamental trade, as dried or live specimens. Traceability tools may help fill the gaps on supply chains, securing information on geographic origin and identification of traded specimens. Fin-clipping, a non-lethal and well-established method in seahorse research, offers a potential approach to trace the geographic origin and certify the aquaculture of these flagship species. As such, this study aimed to investigate the existence of differences in isotopic profiles of four seahorse species cultured at research centers located in southern Portugal and northern Spain, as well as between cultured *Hippocampus guttulatus* sourced from two research centers, and between wild and cultured specimens of this species. This research also evaluated the potential of combining isotopic and elemental fingerprints for seahorse species discrimination, through inductively continuous-flow isotope ratio mass spectrometry (IR-MS) and plasma mass spectrometry (ICP-MS). Species cultured at the same research centers exhibited similar stable isotope composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), except in the case of temperate *H. guttulatus* from northern Spain, which differed significantly from tropical species *H. kuda* and *H. reidi*. These differences could be due to phylogenetic dissimilarities and differences in seawater temperature. The $\delta^{15}\text{N}$ composition allowed to discriminate between cultured *H. guttulatus* from the two research centers and between cultured and wild specimens. While dorsal fin isotopes alone did not prove to be a reliable tool for the discrimination of different cultured species, combining them with elemental profiles from seahorses' whole-body allowed to successfully discriminate between

H. kuda and *H. reidi*. This preliminary research demonstrates the potential of stable isotope and elemental analyses for tracing seahorses' geographic origin and species identification. However, further research should be performed to validate these findings for wild specimens, particularly those from illegal, unreported and unregulated (IUU) fisheries and trade.

KEYWORDS

syngnathids, *Hippocampus* spp., trophic niche, stable isotopes, fin clipping, ICP-MS, geochemical tools

1 Introduction

Seahorses (*Hippocampus* spp.) are marine teleosts that belong to family Syngnathidae, alongside pipefishes, pipehorses and seadragons (Foster and Vincent, 2004). These flagship species, whose appealing looks may contribute to their conservation, along with that of habitats they occupy (Cohen et al., 2017), are particularly vulnerable to anthropogenic pressures (Correia et al., 2015), such as habitat degradation (Vivas et al., 2023; Watchorn et al., 2022), overfishing (Cohen et al., 2017) and bycatch (Vaidyanathan and Vincent, 2021). Features such as reduced mobility, patchy distribution, habitat fidelity, monogamy and low fecundity exacerbate their vulnerability (Foster and Vincent, 2004). *Hippocampus* spp. were included, in 1996, in the Red List of Threatened Species from International Union for Conservation of Nature (IUCN) (Vincent et al., 2011). Two seahorse species, from the forty-two already described, are listed as endangered (EN), twelve listed as vulnerable (VU) and seventeen species, for which available information is scarce, are classified as Data Deficient (DD) (IUCN, 2023). Seahorses were also formally added in 2004, to Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), due to high pressure exerted on wild populations. Species included in Appendix II of CITES may be traded, with national government's approval, as long as these practices do not add pressure to wild populations (Kuo et al., 2018). Seahorses are extensively traded, supplying demand in the form of dried specimens, targeted by Traditional Chinese Medicine (TCM) and curio trade (Boehm et al., 2023), as well as live specimens destined to the marine aquarium trade (Kuo and Vincent, 2018).

Traceability tools may contribute to fill gaps, along supply chains, and potentially provide origin certification, allowing consumers to make informed and sustainable choices (Cohen et al., 2013; Duarte et al., 2022; Leal et al., 2015). Initial studies explored the use of bacterial communities from live seahorse skin mucus to trace their geographic origin (Cohen et al., 2018). Additionally, elemental analysis of bony structures (vertebrae and bony plates) showed promise for tracing the geographic origin of dried cultured *H. guttulatus* specimens (Cabral et al., 2021).

Species identification in syngnathids can be challenging (Curtis et al., 2017), as some species lack distinctive characteristics, and intraspecific variability, such as in color or number of cirri, may hamper species discrimination (Curtis, 2006; Woodall et al., 2018). Genetic studies have already been performed for seahorse species differentiation, which is of major importance for understanding seahorse life-history and ecology, and the application of effective conservation measures (Luo et al., 2015; Woodall et al., 2018). Metal and element contents have also revealed species-specific differences in two closely related fish species, *Sardina pilchardus* and *Engraulis encrasicolus*, sampled at the same geographic locations, indicating that elemental fingerprints could be strongly influenced by species factor (Sofoulaki et al., 2018).

Geochemical tools offer a relatively fast and cost-effective approach compared to other molecular and biochemical tools (Leal et al., 2015), being potentially valuable for the traceability of geographic origin and discrimination of seahorse species apprehended from illegal, unreported, and unregulated (IUU) fisheries and trade. Stable isotope analysis (SIA) is a helpful tool for studying food-webs (Fredriksen, 2003; Fry, 1988; Post, 2002), including seahorse trophic niches and ecological features (Piñeiro-Corbeira et al., 2021; Valladares and Planas, 2020; Valladares et al., 2016). Carbon and nitrogen stable isotope ratios ($^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$, respectively) are frequently employed in ecological studies, with carbon indicating food sources and nitrogen reflecting trophic position (Valladares and Planas, 2012). However, consumers typically exhibit higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values than their prey due to the fractionation of lighter isotopes (DeNiro and Epstein (1978, 1981). Given the conservation status of many *Hippocampus* species (IUCN, 2023), non-lethal techniques such as partial fin-clipping (Piñeiro-Corbeira et al., 2021; Planas, 2021; Planas et al., 2020a; Valladares et al., 2016; Woodall et al., 2012), have been employed for SIA in seahorses, providing a suitable matrix with similar isotopic composition to muscle tissues (Valladares and Planas, 2012).

Stable isotope analysis of dorsal fins could be considered an interesting non-lethal technique to trace and certify the origin of seahorse specimens. As such, to assess the reliability of this tool, this study aimed to: i) describe the dorsal fins' isotopic fingerprints

($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) in wild and cultured *Hippocampus* spp. (*H. guttulatus*, *H. hippocampus*, *H. reidi* and *H. kuda*); ii) assess the existence of isotopic differences among seahorse species cultured at the same research centers (Instituto de Investigaciones Marinas - IIM, Vigo, Spain or Centro de Ciências do Mar - CCMAR, Faro, Portugal), in cultured *H. guttulatus* originating from two different research centers (IIM or CCMAR), and between wild *H. guttulatus* (Ria Formosa coastal lagoon, Portugal) and cultured *H. guttulatus*; and iii) evaluate the potential of combining dorsal fin isotopes and whole-body elemental fingerprints for species discrimination when isotopic fingerprints alone may not be sufficient.

2 Materials and methods

2.1 Bioethics

Animal capture, handling and sampling followed all bioethical standards of the Spanish Government (Real Decreto 1201/2005, 10th October 2005) and the Regional Government Xunta de Galicia (REGA ES360570202001/15/FUN/BIOL.AN/MPO01). Additionally, the captive breeding program for *H. hippocampus* and *H. guttulatus* (Project HIPPONUTRE, reference 16-02-01-FMP-54) was approved by the ethics committee from the Portuguese Veterinary Medicines Directorate for the Ministry of Agriculture, Rural Development and Fisheries. Under this approval, the program was performed in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. Frozen samples of wild *H. guttulatus*, were donated by CCMAR. The protocol for fish sacrifice allowed to immediately and rapidly euthanize *H. guttulatus* specimens with an excess of anesthetic (2-phenoxyethanol solution, 0.40 mg/L). Individuals were placed in the anesthetic solution for at least 20 minutes and removed not less than 10 minutes after ventilation stopped.

2.2 Seahorse samples

Seven wild *H. guttulatus* were sampled from Ria Formosa coastal lagoon (36°59' N; 7°51' W; southern Atlantic coast; Portugal), in 2001. Seven *H. hippocampus* and six *H. guttulatus* were cultured at CCMAR (University of Algarve, Faro, Portugal) and died, from natural causes, in the year 2020. These seahorses were maintained in seawater from Ria Formosa coastal lagoon and physicochemical parameters, temperature and salinity, followed seasonal patterns with annual mean values of 20°C and 35.7, respectively. In the present study, the Practical Salinity Scale (PSS) was adopted to report salinity (IAPSO, 1985). Therefore, salinity is expressed as a conductivity ratio and has no units. *Hippocampus guttulatus* and *H. hippocampus* were fed on wild mysids *Mesopodopsis slabberi* and/or *Diamysis lagunaris*., depending on natural availability. Eight *H. guttulatus* and nine *H. reidi* and *H. kuda* (five replicates of *H. reidi* and four replicates of *H. kuda*), were cultured at IIM (CSIC, Vigo, Spain), presenting death

dates between 2013 and 2019. These seahorses were cultured in seawater from Ria de Vigo (42°21' N; 8°36' and 8°54' W; southwestern Atlantic coast; Galicia, Spain), being maintained at seasonal temperatures of 15°C (winter) and 19°C (summer), in the case of *H. guttulatus* and at a constant temperature of 26 ± 0.5°C, in the case of *H. reidi* and *H. kuda*. The salinity mean value (37 ± 1) was the same for the three species. Specimens cultured at IIM were fed on mixtures of adult enriched *Artemia* (Iberfrost, Spain), commercially frozen mysids *Neomysis* sp. (Ocean Nutrition, Spain) and mysids collected from the wild (*Siriella armata* and *Leptomysis* sp.).

In the laboratory, seahorse samples frozen at -20°C, were rapidly rinsed with distilled water and freeze-dried (CoolSafe 55-9L Pro, Labogene, Lillerød, Denmark). The dorsal fins were separated from the rest of the body (Figure 1) with the help of stainless-steel tweezers and cut into smaller portions with ceramic blades, before performing isotopic analysis. Tweezers and ceramic blades were cleaned with 99% (w/v) ethanol, between replicates of each species, to avoid cross contamination.

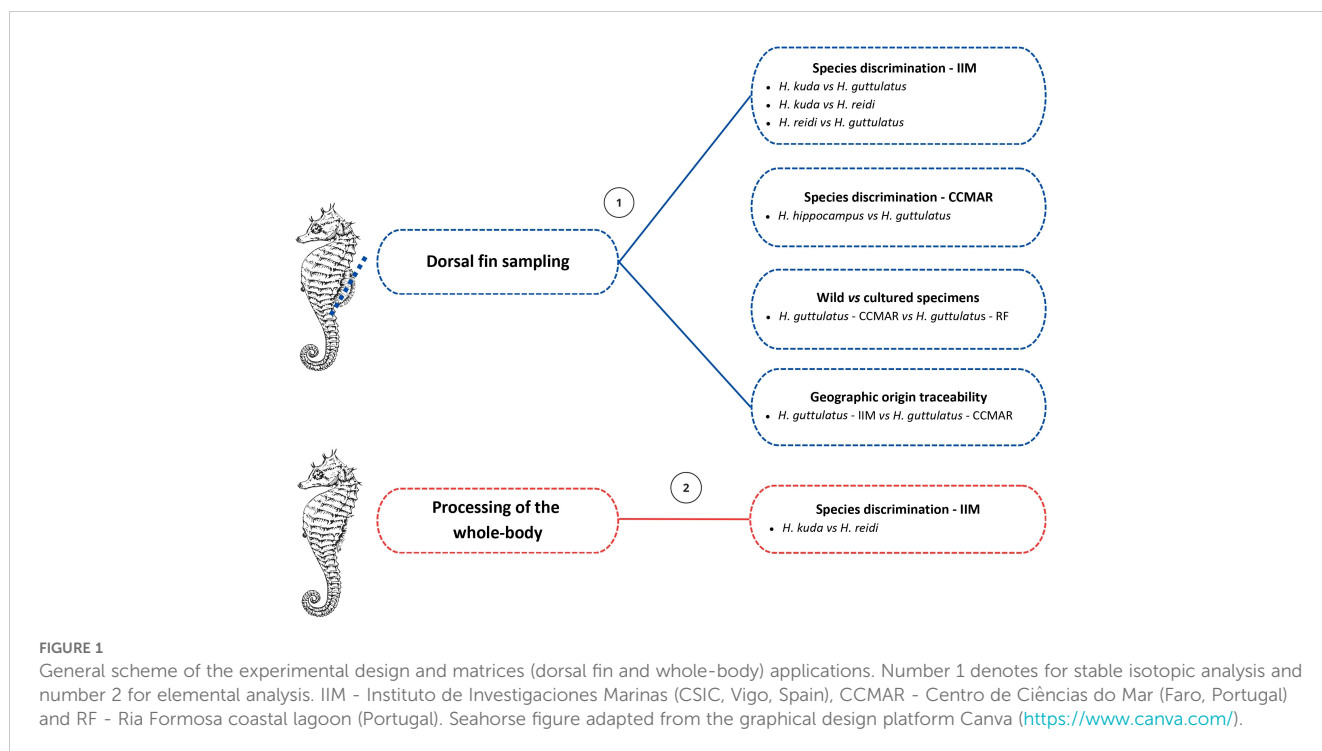
The whole-body replicates from species *H. kuda* and *H. reidi* (n = 9) (Figure 1), were cut into smaller portions and manually macerated with liquid nitrogen, using a ceramic mortar and pestle. Samples were then placed in a mortar grinder (RM 200, Retsch, Hann, Germany) and homogenized. The mortar grinder and the ceramic mortar and pestle were cleaned with quartz powder and alcohol (70%), between samples, to avoid cross contamination (Ricardo et al., 2020). For acidic digestion, a subsample from the whole-body, of approximately 0.5 g was employed.

2.3 Stable isotope analysis

Fin samples, used for determining $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, were transferred to tin capsules and weighed (2.22 ± 0.66 mg). Sample analysis was performed at Servizos de Apoio á Investigación (SAI) from University of A Coruña, by a continuous-flow isotope ratio mass spectrometry equipment (FlashEA1112 elemental analyzer, Thermo Finnigan, Italy) coupled to a Delta Plus mass spectrometer (FinniganMat, Germany) through a ConFlo II interface. Carbon and nitrogen isotopic values were expressed as permil (‰), relative to Vienna Pee Dee Belemnite (VPDB) and atmospheric air, as described in the following equation:

$$\delta X = \left(\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right) \times 10^3$$

X corresponds to ^{13}C or ^{15}N and R to ratios $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. International reference materials for ^{13}C (NBS 22, IAEA-CH-6 and USGS24) and ^{15}N (IAEA-N-1, IAEA-N-2 and USGS25) were employed in analytical sample batch runs. To evaluate the precision in the analysis of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, acetanilide was used, resulting in a standard deviation of ± 0.15‰ (1-sigma, n = 10). Standards were run in every 10 samples. The isotopic analysis procedure fulfils the requirements of the ISO 9001 standard. The laboratory is submitted to annual intercalibration exercises (e.g., Forensic isotope ratio mass spectrometry scheme - FIRMS, LGC Standards, UK).



2.4 Elemental extraction and ICP-MS analysis

The acidic digestion of the samples consisted in the addition of 3 mL of concentrated nitric acid (HNO₃) and 1 mL of hydrochloric acid. After overnight digestion (14 - 16 h), the solutions were placed in a digestion block (DigiPrep, SCP Science; Montreal, QC, Canada) and three heating cycles were performed. These consisted in the increase, for 10 minutes, from room temperature to 50°C, which was kept stable during a period of 15 minutes. Temperature was then increased, in 15 minutes, from 50°C to 85°C and stabilized for another period of 15 minutes. The three heating cycles were interspersed with the addition of 2 mL of hydrogen peroxide. The drying process was performed in the digestion block at a temperature of 50°C. After evaporation, samples were diluted with Millipore water (Milli-Q) to a final concentration of 1-2% HNO₃, to reduce acid concentration and avoid damaging the inductively coupled plasma mass spectrometry (ICP-MS) equipment.

Total concentrations of 21 elements, aluminum (Al), arsenic (As), boron (B), barium (Ba), calcium (Ca), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), potassium (K), lithium (Li), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), phosphorus (P), rubidium (Rb), selenium (Se), strontium (Sr), vanadium (V) and zinc (Zn), were determined in an Agilent 7700 ICP-MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an octopole collision cell and autosampler. Calibrations with standard solutions of each analyte were performed for element quantification. A quality program, with reagent blanks, replicate samples and two certified reference materials, Febs-1 (NRC Canada) and ERM-BB422 (JRC - European Commission), was

employed in order to evaluate the accuracy of the ICP-MS method. Precision was estimated by the relative standard deviation (RSD) of replicate samples, being ≤ 10%. The elements' concentrations were expressed as ratios of Ca (mmol/mol Ca) for posterior statistical analyses (Ricardo et al., 2017).

2.5 Data analysis

Statistical analyses were performed using the software R v.4.3.2 (R Core Team, 2019) and PRIMER v6 (Primer-e, Auckland, New Zealand) with the add-on PERMANOVA+. Niche regions and niche overlap of seahorses cultured in the same research centers (IIM or CCMAR), cultured *H. guttulatus* from different origins and wild and cultured *H. guttulatus*, were analyzed through a multidimensional δ¹³C and δ¹⁵N bi-plot. The region of the niche was considered the probability of the density function, of both isotopes, at a confidence interval of 95%. The bi-plot accounts for species-specific distribution and bivariate projections of the niche regions (Swanson et al., 2015). The package Siber v.2.1.4, from R software, was used to obtain total convex hull areas (TA), which consist in a polygon that is drawn around the outermost points in the data, as well as trophic niche areas, corrected for small sample sizes (SEAc) (Jackson, 2023). The probability of trophic overlap, at a confidence interval of 95%, was estimated through NicheRover v.1.1.0 package, which uses Bayesian corrections (Swanson et al., 2015).

To assess the existence of significant differences (p -value ≤ 0.05) in the isotopic profiles (δ¹³C and δ¹⁵N) of fin samples from the four cultured seahorse species (*H. reidi*, *H. kuda*, *H. guttulatus*, and *H. hippocampus*), the groups originating from the same center (IIM or

CCMAR), were compared through non-parametric analysis of similarities (ANOSIM). This analysis was performed on a resemblance matrix, which used normalized Euclidean distances. Additionally, isotopic fingerprints of wild and cultured *H. guttulatus*, cultured *H. guttulatus* specimens from CCMAR and IIM, as well as combined isotopic and elemental profiles of two cultured seahorse species (*H. kuda* and *H. reidi*), were analyzed using ANOSIM. The R statistic of this test can range from 0 to 1, which allows to measure differences between groups (Anderson et al., 2008). The ANOSIM tests were run with 9999 permutations. Due to the low number of replicates and after performing a Shapiro-Wilk test, for normality assessment, a non-parametric version of the *t*-test for unpaired samples (Mann-Whitney U) was performed in the raw data. This statistical analysis allowed to compare pairs of sample groups, in terms of stable isotopes of cultured seahorse species and cultured and wild *H. guttulatus*, as well as in terms of element composition of two cultured species. The Benjamini - Hochberg correction was applied whenever multiple comparisons were performed.

To evaluate the potential of using combined elemental and isotopic profiles for species discrimination, Principal Component Analysis (PCA) was performed on normalized data. Both stable isotopes ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), as well as elements that differed significantly (p -value ≤ 0.05 , Mann-Whitney U) between two cultured seahorse species (*H. kuda* and *H. reidi*) were selected for PCA. The packages factoMineR (Husson et al., 2020), factoextra v1.0.7 (Kassambara, 2020) and corrplot v0.8.4 (Wei et al., 2017), from R software, were used for PCA.

All graphical representations were constructed with ggplot2 v3.3.0 (Wickham, 2016) package for R.

3 Results

3.1 Isotopic profile and trophic niche

The $\delta^{13}\text{C}$ mean values of dorsal fin samples from four seahorse species ranged between $-20.38 \pm 0.71\text{‰}$ (*H. kuda* from IIM) and $-14.37 \pm 0.34\text{‰}$ (*H. hippocampus* from CCMAR) (Table 1). In the case of $\delta^{15}\text{N}$, the mean values ranged from $8.81 \pm 0.31\text{‰}$ (*H. hippocampus* from CCMAR) to $16.34 \pm 0.82\text{‰}$ (*H. kuda* from IIM) (Table 1).

Hippocampus guttulatus from CCMAR presented the largest standard ellipse, corrected for small sample sizes, SEAc (2.98), while *H. hippocampus* from CCMAR occupied the smallest SEAc (0.39) (Table 1; Figure 2). The centroids of the ellipses corresponding to species cultured at the same research center were positioned closer to each other, except in the case of *H. guttulatus* from IIM (Figure 2).

Niche overlap estimates of seahorse species from IIM showed the highest value (55.65%) for the probability of *H. kuda* being found within the niche of *H. reidi*, while the lowest probability (0.12%) was registered for *H. kuda* being found within the niche of *H. guttulatus* (Table 2). In the case of seahorse species from CCMAR, *H. hippocampus*' niche almost fully overlapped (95.49%) with the one of *H. guttulatus*. When comparing *H. guttulatus*, cultured at different research centers (IIM and

TABLE 1 Isotopic values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, mean \pm standard deviation) from dorsal fin samples of four seahorse species (*Hippocampus guttulatus*, *H. kuda*, *H. reidi* and *H. hippocampus*) cultured at Instituto de Investigaciones Marinas (IIM - CSIC, Vigo, Spain) and Centro de Ciências do Mar (CCMAR, Faro, Portugal), as well as wild *H. guttulatus* from Ria Formosa coastal lagoon (Portugal).

Seahorse species	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	TA	SEA	SEAc
IIM					
<i>H. guttulatus</i>	-15.67 ± 1.20	11.90 ± 0.52	2.84	1.92	2.23
<i>H. kuda</i>	-20.38 ± 0.71	16.34 ± 0.82	1.00	1.30	1.95
<i>H. reidi</i>	-19.52 ± 0.68	14.89 ± 1.00	1.48	1.36	1.81
CCMAR					
<i>H. guttulatus</i>	-14.73 ± 1.39	9.17 ± 0.55	2.67	2.38	2.98
<i>H. hippocampus</i>	-14.37 ± 0.34	8.81 ± 0.31	0.52	0.32	0.39
Ria Formosa					
<i>H. guttulatus</i>	-15.62 ± 1.07	9.99 ± 0.40	1.54	1.00	1.20

Estimated niche areas for cultured species and wild *H. guttulatus*. TA, total convex hull area; SEA, standard ellipse area and SEAc, standard ellipse area with a correction for small sample sizes.

CCMAR), the highest probability value, even if low (2.05%), was registered for niche overlap of specimens from IIM and specimens from CCMAR. Probability of wild *H. guttulatus* niche overlaying the one from *H. guttulatus*, cultured at CCMAR, was higher than the other way around, with a value of 59.18% (Table 2).

3.2 Potential of isotopic profiles for species and geographic origin discrimination

The stable isotopic profiles ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of seahorse's (*H. kuda*, *H. reidi* and *H. guttulatus*) dorsal fins from IIM were significantly different (ANOSIM, p -value < 0.001). However, *H. kuda* and *H. reidi* did not differ significantly in terms of isotopic composition (ANOSIM, p -value = 0.095), while *H. guttulatus* and *H. reidi*, as well as *H. guttulatus* and *H. kuda* were significantly different (ANOSIM, p -value = 0.002 and 0.003, respectively) (Table 3). Moreover, *H. guttulatus* and *H. reidi* differed significantly in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ compositions (Mann-Whitney U, p -value = 0.003 for both isotopes), as was the case of *H. guttulatus* and *H. kuda* (Mann-Whitney U, p -value = 0.007 for both isotopes) (Table 3). The two species cultured at CCMAR (*H. hippocampus* and *H. guttulatus*) were significantly different in terms of the isotopic profile (ANOSIM, p -value = 0.011), even though there were no significant differences for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (Mann-Whitney U, p -value = 0.317 and 0.306, respectively) (Table 3). Cultured *H. guttulatus* sourced from CCMAR and IIM differed significantly in the isotopic profile (ANOSIM, p -value < 0.001), with $\delta^{15}\text{N}$ as the variable contributing for significant differences (Mann-Whitney U, p -value = 0.004). Wild *H. guttulatus* from Ria Formosa and cultured *H. guttulatus* from CCMAR, revealed significant differences in the isotopic profiles (ANOSIM, p -value = 0.006), with $\delta^{15}\text{N}$ as the

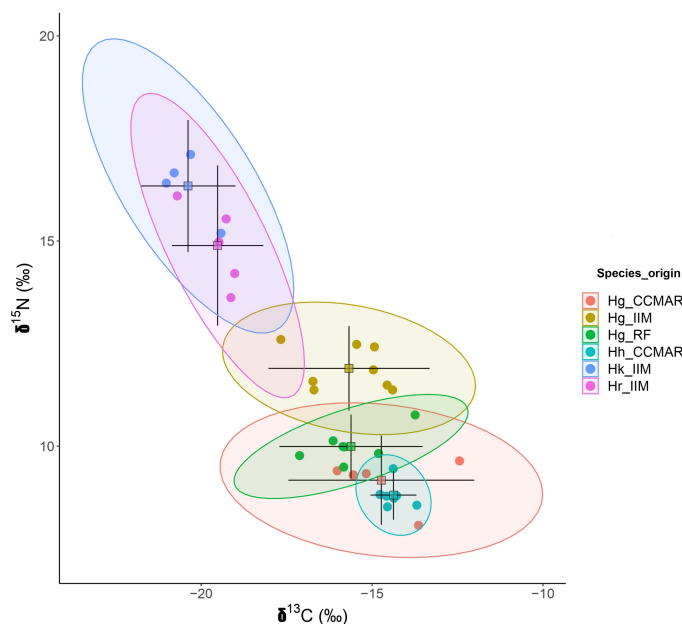


FIGURE 2
Isotopic bi-plot relative to average $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of dorsal fin samples from four *Hippocampus* species (*H. guttulatus*, *H. kuda*, *H. reidi* and *H. hippocampus*), cultured at Instituto de Investigaciones Marinas (IIM - CSIC, Vigo, Spain) and Centro de Ciências do Mar (CCMAR, Faro, Portugal), as well as wild *H. guttulatus* from Ria Formosa coastal lagoon (RF, Portugal). Standard ellipses with 95% credible intervals for the means. Hg, *H. guttulatus*; Hk, *H. kuda*; Hr, *H. reidi* and Hh, *H. hippocampus*.

variable significantly different (Mann-Whitney U, p -value = 0.008) between the two groups (Table 3).

3.3 Combined elemental and isotopic fingerprints for species discrimination

Hippocampus reidi and *H. kuda* were not significantly different concerning their stable isotope profile of dorsal fin samples (ANOSIM, p -value = 0.095). Thus, the potential of combining elemental fingerprints from the whole-body and isotopes from dorsal fins, was assessed. Phosphorous (P) was the element that revealed higher mean ratio values (mmol/mol Ca) in the whole-

body of both species (771.782 ± 54.188 and 657.125 ± 17.534 mmol/mol Ca for *H. kuda* and *H. reidi*, respectively) (Table 4). Arsenic and V were the elements with lower mean Ca ratios in both *H. kuda* ($0.002 \pm 0.004 e^{-1}$ and $0.001 \pm 0.009 e^{-2}$ mmol/mol Ca, respectively) and *H. reidi* ($0.001 \pm 0.004 e^{-1}$ and $0.001 \pm 0.968 e^{-4}$ mmol/mol Ca, respectively) (Table 4). The combined isotopic and elemental profiles of *H. kuda* and *H. reidi* were significantly different (ANOSIM, p -value = 0.008). The elements that most contributed to the significant differences recorded between the two species were P, K, Na, Mg, Rb, Co, Sr and Ba (p -value = 0.035, Mann-Whitney U for all elements) (Table 4).

The elements that revealed significant differences in the whole-body of *H. kuda* and *H. reidi*, as well as the stable isotopes from

TABLE 2 Niche overlap estimates, expressing the probability (%) at a $\alpha = 0.95$ of species in the rows being found within the niche of species in the columns.

Species - origin	Hg - IIM	Hk - IIM	Hr - IIM	Hg - CCMAR
Hg - IIM	-	0.18	1.58	2.05
Hk - IIM	0.12	-	55.65	-
Hr - IIM	1.69	46.61	-	-
Species - origin	Hg - CCMAR	Hh - CCMAR	Hg - RF	Hg - IIM
Hg - CCMAR	-	23.3	20.95	1.26
Hh - CCMAR	95.49	-	-	-
Hg - RF	59.18	-	-	-

IIM, Instituto de Investigaciones Marinas (CSIC, Vigo, Spain); CCMAR, Centro de Ciências do Mar (Faro, Portugal); RF, Ria Formosa coastal lagoon (Portugal). Hg, *Hippocampus guttulatus*; Hk, *H. kuda*; Hr, *H. reidi*; Hh, *H. hippocampus*.

TABLE 3 Pair-wise comparisons between seahorse species cultured at different research centers, Instituto de Investigaciones Marinas (IIM - CSIC, Vigo, Spain) or Centro de Ciências do Mar (CCMAR, Faro, Portugal), between cultured *Hippocampus guttulatus* from different locations (IIM or CCMAR) and between wild *H. guttulatus* from Ria Formosa coastal lagoon (RF, Portugal) and cultured *H. guttulatus* from CCMAR.

Pair - wise comparisons	ANOSIM (p-value)	Mann - Whitney U (p-value)	
		$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
<i>H. kuda</i> - IIM vs <i>H. guttulatus</i> - IIM	0.003	0.007	0.007
<i>H. reidi</i> - IIM vs <i>H. guttulatus</i> - IIM	0.002	0.003	0.003
<i>H. guttulatus</i> - CCMAR vs <i>H. hippocampus</i> - CCMAR	0.011	0.317	0.306
<i>H. guttulatus</i> - CCMAR vs <i>H. guttulatus</i> - IIM	< 0.001	0.519	0.004
<i>H. guttulatus</i> - CCMAR vs <i>H. guttulatus</i> - RF	0.006	0.153	0.008

Analysis of similarities (ANOSIM) for assessing significant differences (p -value ≤ 0.05) between the isotopic profiles ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of seahorses' dorsal fins. Mann - Whitney U resulting p -values denote significant differences, in individual isotopes ($\delta^{13}\text{C}$ or $\delta^{15}\text{N}$) from seahorse's dorsal fins, when p -value ≤ 0.05 . Benjamini - Hochberg correction was applied for multiple comparisons.

dorsal fins, were included in the PCA. The first two components explained 90.2% of group variability (Figure 3). The centroids for species were distanced from each other and groups were well discriminated. The variables Mg and Na were strongly correlated since the corresponding lines were overlapped (Figure 3). These two variables also revealed a strong relationship with K, Rb, P and Ba appearing, however, less correlated to the remaining variables. The two elements Sr and Co were also positively correlated, while the stable isotopes revealed a negative correlation (Figure 3). *Hippocampus kuda* replicates presented higher values for K, Rb, Mg, P, Na and Ba than those of *H. reidi*, with the opposite being true for Co and Sr, which revealed higher values for *H. reidi* replicates (Figure 3).

4 Discussion

4.1 Potential of isotopic profiles for species and geographic origin discrimination

Isotopic profiles in animals' whole bodies reflect dietary intake and composition (DeNiro and Epstein, 1978, 1981; Hobson and Clark, 1992; Fry, 2006; Martínez del Rio et al., 2009). However, isotopic profiles ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) can vary depending on tissue type and diet (DeNiro and Epstein, 1978, 1981). Valladares and Planas (2012) reported similar $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in dorsal fins and muscle tissues of *H. guttulatus*, validating both matrices for SIA. This analysis has already been applied to fish migration studies, using matrices such as otoliths (Huijbers et al., 2013) and muscle tissues (Davidsen et al., 2017). However, to the authors best knowledge, there are no studies addressing the use of dorsal fins'

TABLE 4 Elemental ratios (mmol/mol Ca) from the whole-body of *Hippocampus kuda* (n = 4) and *H. reidi* (n = 5), cultured at Instituto de Investigaciones Marinas (IIM - CSIC, Vigo, Spain).

Elements	Elements ratios (mmol/mol Ca)		p-value
	Seahorse species		
	<i>H. kuda</i>	<i>H. reidi</i>	
P	771.782 ± 54.188	657.125 ± 17.534	0.035
K	59.276 ± 15.151	28.695 ± 3.193	0.035
Na	597.422 ± 29.438	248.744 ± 63.736	0.035
Mg	115.765 ± 8.005	77.085 ± 8.094	0.035
Mn	0.293 ± 0.089	0.353 ± 0.058	0.203
Cu	0.004 ± 0.001	0.002 ± 0.009 e ⁻¹	0.132
Zn	0.845 ± 0.128	0.621 ± 0.111	0.091
As	0.002 ± 0.004 e ⁻¹	0.001 ± 0.004 e ⁻¹	0.260
Se	0.009 ± 0.002	0.007 ± 0.001	0.132
Rb	0.007 ± 0.001	0.003 ± 0.003 e ⁻¹	0.035
Sr	4.323 ± 0.506	5.953 ± 0.423	0.035
Li	0.075 ± 0.007	0.072 ± 0.009	0.624
B	1.016 ± 0.073	0.641 ± 0.242	0.091
Al	3.777 ± 3.350	1.012 ± 1.215	0.091
V	0.001 ± 0.009 e ⁻²	0.001 ± 0.968 e ⁻⁴	0.624
Cr	0.040 ± 0.069	0.007 ± 0.008	0.624
Fe	0.511 ± 0.489	0.445 ± 0.070	0.260
Co	0.002 ± 0.001 e ⁻¹	0.003 ± 0.001 e ⁻¹	0.035
Ni	0.014 ± 0.016	0.007 ± 0.004 e ⁻¹	0.260
Ba	0.093 ± 0.036	0.035 ± 0.008	0.035

P -values (significant differences when p -value ≤ 0.05) from Mann-Whitney U, with Benjamini - Hochberg corrections for multiple comparisons. Values are expressed as mean ± standard deviation.

isotopic fingerprints for tracing *Hippocampus* spp. geographic origin or for species discrimination.

The dorsal fins of *H. kuda* and *H. reidi* from IIM exhibited similar mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, as was the case for *H. guttulatus* and *H. hippocampus* from CCMAR (Tables 1, 3), expressing the dietary sources of both sites. Nevertheless, *H. guttulatus* from IIM displayed significantly different $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values to the other two cultured species, *H. kuda* and *H. reidi* (Tables 1, 3). The results of a study performed with freshwater fish species, revealed that phylogeny, as well as morphology, could be associated with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, even though the correlation between isotopic data and phylogeny was not considered very strong (Keppeler and Winemiller, 2020). *Hippocampus kuda* and *H. reidi* are phylogenetically closer than *H. guttulatus* (Teske et al., 2004), which may partially explain the similarities in isotopic fingerprints recorded in *H. kuda* and *H. reidi*. Additionally, it has been described that temperature may affect $\delta^{13}\text{C}$ values in *H. guttulatus* juveniles (Valladares and Planas, 2020), which could also be related with the differences reported, as *H. kuda* and *H. reidi*

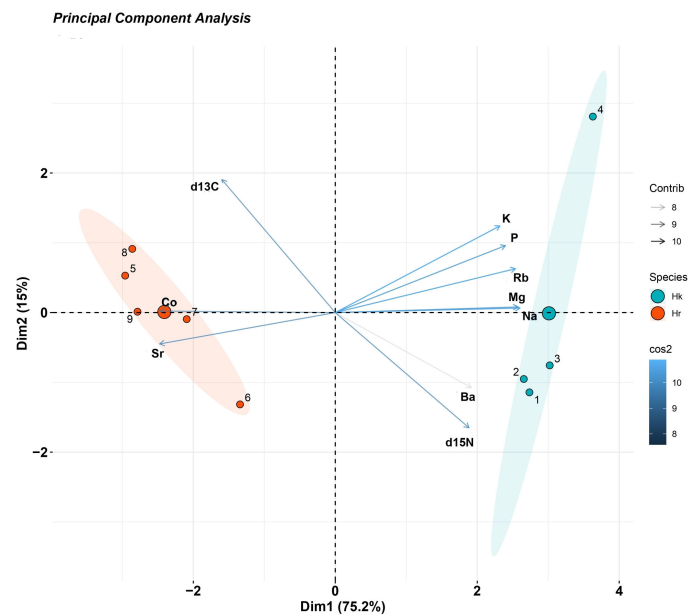


FIGURE 3

Principal Component Analysis (PCA) for *Hippocampus kuda* (HK) ($n = 4$) and *H. reidi* (HR) ($n = 5$) cultured at Instituto de Investigaciones Marinas (IIM - CSIC, Vigo, Spain). Variables: potassium (K), rubidium (Rb), magnesium (Mg), phosphorus (P), sodium (Na), strontium (Sr), cobalt (Co), barium (Ba), $\delta^{13}\text{C}$ (d13C) and $\delta^{15}\text{N}$ (d15N). Elemental profile was obtained from seahorses' whole bodies, while stable isotopes were obtained from dorsal fins. Ellipses correspond to 95% confidence.

are both tropical species and, thus, were cultured at higher water temperatures than temperate seahorse species *H. guttulatus*. The ellipses referring to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ showed a trend for species from the same research center being placed closer to each other, except for *H. guttulatus* from IIM (Figure 2). Trophic overlap evidenced dietary similarities between *H. reidi* and *H. kuda* from IIM and between *H. hippocampus* and *H. guttulatus* from CCMAR (Table 2). Nevertheless, *H. guttulatus* from CCMAR displayed a low probability of niche overlap with *H. hippocampus*, potentially due to intra-specific variability, increasing niche area (Table 1). Factors, such as specimens' size, may contribute to variability in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Davidsen et al., 2017). In seahorses, size can influence $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values due to changes in prey preferences with growth (Valladares et al., 2016).

Hippocampus guttulatus cultured at IIM exhibited significantly higher $\delta^{15}\text{N}$ values than those of conspecifics cultured at CCMAR (Tables 1, 3). These differences likely originated from dissimilarities in the diet provided at each site. A study on the pipefish *Syngnathus acus* revealed that juveniles, with a higher habitat dispersion than adults, exhibited isotopic heterogeneity, mainly at $\delta^{15}\text{N}$ level, due to high dietary variability (Planas, 2022). Moreover, in the study developed by Planas et al. (2020b), dorsal fins from *H. guttulatus* fed on different diets (*Artemia* and/or a mixture of frozen and captured mysidaceans) displayed differences in the stable isotopic profiles. Nonetheless, it is noteworthy that the similarities in $\delta^{13}\text{C}$ of *H. guttulatus* from IIM and CCMAR may be due to a similar assimilation of dietary items in both groups, while differences in $\delta^{15}\text{N}$ likely reflect variations in trophic levels of consumed prey (Planas et al., 2020b).

Wild *H. guttulatus* from Ria Formosa coastal lagoon exhibited significantly higher mean $\delta^{15}\text{N}$ values than those of cultured specimens from CCMAR (Tables 1, 3). This pattern aligns with findings for wild *Sparus aurata*, which also displayed higher $\delta^{15}\text{N}$ values than cultured conspecifics (Guerra-García et al., 2023). These differences could be due to a higher trophic level occupied by fishes that feed in the marine environment (Arechavala-Lopez et al., 2013). Additionally, the relationship between $\delta^{15}\text{N}$ values in estuarine fish and urbanization levels has been documented (Morris et al., 2015). In fact, between 2001 and 2002, when studied *H. guttulatus* were sourced from the wild, Ria Formosa coastal lagoon experienced changes in seagrass meadows due to abnormal wastewater discharges (Cabaço et al., 2008).

4.2 Combined elemental and isotopic fingerprints for species discrimination

Previous studies have resorted to the combination of different analyses to improve geographic origin discrimination (Ricardo et al., 2024). Dorsal fin isotopic profiles, more specifically $\delta^{15}\text{N}$, effectively differentiated cultured *H. guttulatus* from different origins, and distinguished between wild and cultured specimens. However, this approach proved insufficient for discriminating between phylogenetically close species (Table 3). As such, the potential of combining both isotopic profiles from dorsal fins, as well as elemental fingerprints from the whole body, was assessed. The elements that significantly differed between *H. kuda* and *H. reidi* from IIM were P, K, Na, Mg, Rb, Co, Sr and Ba

(Table 4). Seahorses are known to be rich in Na, K, P (Lin et al., 2008) and Mg (Lall and Lewis-McCrea, 2007), a finding in line with the results recorded for cultured *H. kuda* and *H. reidi* (Table 4).

There are several factors that influence elemental composition of fish calcified structures, such as temperature, diet, water chemistry, physiology and genetics (Clarke et al., 2011; Kerr and Campana, 2014; Sturrock et al., 2015). In some cases, environmental factors are assumed to outweigh physiological aspects, when it comes to elemental composition, as is the case of Sr (Sturrock et al., 2014). This element has been pointed out as a marker of calcified structures, influenced by environmental conditions, such as water chemistry (Bath et al., 2000; Campana, 2005), even though physiological aspects can also exert a strong influence on its regulation (Sturrock et al., 2014).

Elements under rigorous physiological control, such as P, Na and K, could be interesting for species discrimination, since they are less easily shaped by environmental factors (Campana, 2005; Kerr and Campana, 2014). Magnesium present in calcified structures was previously used as a fish stock marker (Avigliano et al., 2019), even though the effects of environmental factors on Mg/Ca from calcified structures remains unclear (Nishimoto et al., 2010). It has also been described that Ba/Ca in estuarine fish otoliths may be negatively related with salinity, being the opposite true for temperature (Nelson et al., 2018). According to Barnes and Gillanders (2013), stock genetics also affected Ba/Ca incorporation in marine species *Argyrosomus japonicus* fingerlings. The differences recorded in Mg, Sr and Ba composition in *H. reidi* and *H. kuda* might be attributed to physiological regulation, as seawater parameters, such as salinity and temperature, were kept identical and stable for both species. In the case of Co, a study on the marine flatfish *Scophthalmus maximus* has shown that abiotic factors such as pH and temperature, do not have a significant effect on its assimilation (Pouil et al., 2018). Additionally, a study by Sturrock et al. (2014) found that the concentration of Rb in blood plasma of the marine flatfish *Pleuronectes platessa* was not largely influenced by physiological aspects. It has been suggested that this element's uptake by fish is influenced not only by its environmental abundance, but also by concentrations of other metallic and alkali elements in the marine environment (Campbell et al., 2005).

It is important to highlight that the analysis of different biological matrices may result in different elemental profiles, due to aspects related with metabolic pathways (Sadeghi et al., 2020). Indeed, a study led by Uncumusaoğlu et al. (2012), revealed that cadmium (Cd), Cu, lead (Pb) and Zn compositions in the muscle and liver tissues of wild *H. hippocampus* differed significantly. The cultured adults of *H. kuda* and *H. reidi* surveyed in the present study were maintained under the same physicochemical conditions, with whole-body elemental analyses suggesting that inter-specific variability was the primary driver of elemental fingerprint differences. Thus, combining significantly different elements from the whole body and stable isotopes from dorsal fins could enhance seahorse species identification in apprehensions from illegal, unreported and unregulated (IUU) fisheries and trade. As seafood samples may undergo different processing methods, which sometimes may damage DNA quality (Marín et al., 2018),

geochemical tools could offer an alternative for discriminating dry specimens from different seahorse species.

5 Conclusions

The results of this preliminary study suggest that stable isotopes, mainly $\delta^{15}\text{N}$, determined from cultured seahorse dorsal fins, can be successfully used to trace their geographic origin, as well as discriminate between wild and cultured conspecifics. Fin-clipping is already a well established, non-lethal practice, employed on live seahorses (Valladares and Planas, 2012). Thus, resulting stable isotopic profiles may be useful for reconstructing the life history of specimens destined to the marine aquarium trade and originating from the wild or from culture facilities. The analysis of stable isotopes from dorsal fins could therefore be paramount to certify cultured specimens and aquaculture facilities. It is, however, worth further investigating the potential effects of phylogeny on isotopic fingerprints, as the temperate species seahorse *H. guttulatus* significantly differed from tropical species cultured at IIM.

Stable isotopic analysis alone might not be sufficient to discriminate between cultured seahorse species, as *H. kuda* and *H. reidi* from IIM, as well as *H. guttulatus* and *H. hippocampus* from CCMAR, did not differ significantly in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ compositions. However, combining isotopic and elemental fingerprints, from seahorse dorsal fins and whole-body, may prove useful for species discrimination, namely for apprehended dry specimens originating from IUU fisheries and trade. Further research on these topics is still required to evaluate the accuracy of the findings here reported, with an emphasis on specimens sourced from the wild.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the ethics committee from the Portuguese Veterinary Medicines Directorate for the Ministry of Agriculture, Rural Development and Fisheries. Under this approval, the captive breeding program for *Hippocampus hippocampus* and *H. guttulatus* (Project HIPPONUTRE, reference 16-02-01-FMP-54) was performed in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. Animal capture, handling and sampling also followed all bioethical standards of the Spanish Government (Real Decreto 1201/2005, 10th October 2005) and the Regional Government Xunta de Galicia (REGA ES360570202001/15/FUN/BIOL.AN/MPO01). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AC: Conceptualization, Data curation, Formal analysis, Investigation, Software, Visualization, Writing – original draft, Writing – review & editing. RC: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing. JP: Resources, Writing – review & editing. FR: Resources, Writing – review & editing. EF: Resources, Writing – review & editing. CP: Conceptualization, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing. MP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was financially supported by project CITAQUA, “Desenvolvimento do Projeto de Reforço do Polo de Aveiro (H4)”, framed within Measure 10 of Investment TC-C10-i01 -Hub Azul -Rede de Infraestruturas para a Economia Azul, financed by the Recovery and Resilience Plan (RRP) and supported by Fundo Azul of the Portuguese Government. This work was also supported by national funds through Fundação para a Ciência e Tecnologia (FCT) under a PhD grant to AC (UI/BD/153063/2022). This study received Portuguese national funds from FCT through projects UIDB/04326/2020, UIDP/04326/2020 and LA/P/0101/2020 and was financed by the scientific project HIPPONUTRE -”Cultivo do cavalo marinho de focinho comprido, *Hippocampus guttulatus*: Optimização zootécnica e avaliação de requisitos nutricionais” (Ref. proj. 16-02-01-FMP-54), financed by the Programa Operacional MAR2020. Seahorses from IIM-CSIC were cultured under project Hippoeco through Spanish national funds (Ref CGL2015-68110-R, Ministerio de Ciencia, Innovación y Universidades) and co-funding by FEDER. Funding for isotopic

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analysis was provided by project Hippo-DEC: Diagnóstico del estado de conservación de caballitos de mar en el litoral español, áreas críticas y medidas de conservación”, financed by Ministerio para la Transición Ecológica y el Reto Demográfico. We acknowledge FCT/MEC for the financial support to CESAM (UIDP/50017/2020+UIDB/50017/2020) and to GEOBIOTEC (UIDP/04035/2020) through national funds and co-funding by FEDER, within the PT2020 Partnership Agreement and Compete 2020. We also acknowledge the project “Impacto e Consolidação em I&DT da Unidade de Investigação Química Orgânica, Produtos Naturais e Agroalimentares em áreas Agroalimentares e afins ICT_2009_02_005_2034” for financing the ICP-MS.

Acknowledgments

We acknowledge Miguel Cabral for statistical advisement and Pedro Pato Martins for technical support.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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