# Sympatric *Globicephala* species: feeding ecology and contamination status based on stable isotopes and trace elements

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ABSTRACT: Recent legal frameworks and the limited availability of information highlight the need to understand the ecological status and impact of anthropogenic threats on marine populations. In this study, a multi-tracer approach combining stable isotopes and 14 trace elements was used to infer information concerning the feeding ecology, contamination status and ecological segregation of 2 sympatric species of the *Globicephala* genus which stranded off the northern and western Iberian Peninsula (NWIP): the long-finned pilot whale *Globicephala melas* (LFPW) and the short-finned pilot whale *Globicephala macrorhynchus* (SFPW). Additionally, the potential influence of biological variables was investigated. Although both species presented similar stable isotope values, there were inter-specific differences in non-essential trace elements, with higher levels reported for SFPW. There was evidence of the bioaccumulative behaviour of some elements (Ag, Hg, Se, Fe, Co). Results of this study suggest that when both SFPW and LFPW are present in the NWIP, they show similar feeding patterns over at least a short timescale, but over a long timescale different feeding and habitat preferences may occur. This study provides useful information on *Globicephala*. In particular, it represents the first report on the ecology and contamination status of SFPW in the northern limits of their distribution range, i.e. in the NWIP.

KEY WORDS: Stable isotopes · Trace elements · Multi-tracer approach · Feeding ecology · Ecological segregation · Toxicology · Bioaccumulation · North-west Iberian Peninsula

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## INTRODUCTION

The absence of ecological niche partitioning between similar sympatric species may result in competitive behaviors for the same resources (Roughgarden 1976). Within cetaceans, there are several examples of niche differentiation based on resource or habitat partitioning, which minimizes the overlap between sympatric predators (e.g. Foote et al. 2009, Méndez-Fernandez et al. 2013).

Within the *Globicephala* genus, the ecological niche seems to be defined, among other drivers, by

water temperature. This genus comprises 2 species with allopatric distributions: the antitropical long-finned pilot whale *G. melas* (LFPW) and the tropical short-finned pilot whale *G. macrorhynchus* (SFPW) (Olson 2009). However, there are overlaps in the distribution range of both species, comprising the northern limit of SFPW (Nores & Pérez 1988, González et al. 2000) and the southern limit of LFPW distribution (Robineau & Vely 1998). In the north-east Atlantic, the sympatric region is roughly located between the Bay of Biscay and the Mauritanian coast. Extensive knowledge exists on LFPW from higher latitudes of the north-east Atlantic (e.g. Amos et al. 1993, Desportes & Mouritsen 1993, Fullard et al. 2000), but information was only recently acquired about this species in Atlantic waters off southern Europe (e.g. Portugal and northwest Spain), coincident with the convergence region for the *Globicephala* genus (e.g. Spyrakos et al. 2011, Méndez-Fernandez et al. 2014, Santos et al. 2014, Monteiro et al. 2015a,b). Regarding SFPW, most studies in the north-east Atlantic focused on the dietary ecology and distribution of animals from the Canary islands (e.g. Hernández-Garcia & Martín 1994, Fernández et al. 2009, Carrillo et al. 2010), while no information is available about this species in northern waters, except for some rare stranding reports (Nores & Pérez 1988, González et al. 2000).

The study of ecological niches and segregation in sympatric species is essential to understand ecosystem functioning and the response to potential changes in a local ecosystem (Leibold 1995), either related with the distribution of other species or with environmental changes due to natural or anthropogenic causes. Since it may be challenging to understand the drivers for ecological niche and segregation in complex marine environments, renewed interest has arisen in the use of biogeochemical tracers (e.g. stable isotopes, trace elements, fatty acids; Evans & Teilmann 2009, Newsome et al. 2010).

Regarding stable isotopes, $\delta^{13}C$ indicates consumer foraging areas or latitudinal variation in the contribution to food intake (Hobson et al. 1994, Newsome et al. 2010, Pajuelo et al. 2012, McMahon et al. 2013), while $\delta^{15}N$ is used as an indicator of consumers' trophic positions (Vanderklift & Ponsard 2003) and feeding habitat (Chouvelon et al. 2012, Ruiz-Cooley et al. 2012). Trace elements, especially the non-essential elements, may also be used as dietary biotracers since ingestion is the main pathway for these elements, which may be biomagnified through marine food webs (Das et al. 2003a, Chouvelon et al. 2012). In fact, trace elements may be a proxy for

ingested prey groups, considering, as an example, the known bioaccumulation of Cd in cephalopods (Bustamante et al. 1998). Additionally, although information is still scarce in physical marine environments regarding variation in trace element concentrations, these elements may also represent habitat proxies considering their variation relative to ecosystems (coastal vs. oceanic), oceanographic conditions (depth vs. surface) or oceanic water masses (e.g. Fitzgerald et al. 2007, Bowman et al. 2015, Wu & Roshan 2015). Further, considering the persistence, and sometimes biomagnification through the food web and toxicity of chemical contaminants (Braune et al. 2005), trace elements may represent a threat for marine organisms. As long-lived species and top predators, cetaceans may function as sentinels of ecosystem health (e.g. Gaskin et al. 1979, Das et al. 2003b, Bossart 2011). This is particularly relevant considering that human activities are leading to escalating pressures on marine and coastal ecosystems. In fact, recent European legal frameworks (e.g. Marine Strategy Framework Directive, 2008/56/EC) have emphasized the need to understand the impact of anthropogenic contaminants in marine populations.

In addition to the multitude of information gathered, depending on the tissue turnover and the half-life of the analysed compounds or elements, multi-tracer approaches are able to provide data over timescales spanning from weeks to years (e.g. Kjellstrøm & Nordberg 1978, Abend & Smith 1995). However, it is important to consider that signatures of biogeochemical tracers may be influenced by individual physiological and biological features, such as age, sex, metabolism or reproductive state (e.g. Aguilar et al. 1999, Das et al. 2003b, Vanderklift & Ponsard 2003, Newland et al. 2009). In essence, variability in natural or anthropogenic elements provide independent but complimentary insights into marine mammal distribution, feeding ecology and contaminant status and hopefully clarifies the ecological processes involved in intra- and inter-specific diversity structure over different timescales, as evidenced in several cetacean species (Evans & Teilmann 2009, Giménez et al. 2013, Méndez-Fernandez et al. 2014, Monteiro et al. 2015b).

The limited information regarding *Globicephala* species—particularly SFPW—in a sympatric region of the north-east Atlantic, together with the fact that both species are currently categorized as 'Data Deficient' (IUCN 2015), highlights the need to reinforce knowledge about these species. The main objectives of the present study were, there-

fore, to combine biogeochemical tracers (i.e. stable isotopes and trace elements) in a multi-tracer approach to assess feeding ecology, contamination status and ecological segregation of 2 sympatric species of the *Globicephala* genus in waters off the northern and western Iberian Peninsula (NWIP): *G. melas* and *G. macrorhynchus.* Additionally, the potential influence of biological variables on trace element concentrations was also investigated in each species.

## MATERIALS AND METHODS

### Sample collection

A total of 11 LFPW and 13 SFPW samples were collected from animals stranded off the NWIP from 2004 to 2013 (Table 1, Fig. 1). While the 11 LFPW samples originated from individually stranded animals, the 13 SFPW samples were collected from

one mass stranding in Galicia, involving a total of 19 animals. Strandings were attended in all cases by experienced personnel from 2 stranding networks operating in the study area: Sociedade Portuguesa de Vida Selvagem (SPVS) in collaboration with the National Institute for Nature Conservation (ICNF) in Portugal and Coordinadora para o Estudio dos Mamíferos Mariños (CEMMA) in Galicia (north-west Spain).

When the condition of the animal permitted, detailed necropsies were performed. Otherwise, basic measurements/information (i.e. length, sex, decomposition state) and samples were collected. Skin samples were preserved frozen (−20°C) for stable isotope analysis. Additionally, kidney and liver samples were stored in glass vials and frozen (−20°C) for posterior trace element analysis. To prevent biases associated with the decomposition state of the animals, only recently dead animals (decomposition state ≤ 3, moderate decomposition; Kuiken & Hartmann 1991) were used in these analyses.

Table 1. Whale ID, location and date of stranding, sex, total length and physical and sexual maturity status of long-finned pilot whales *Globicephala melas* (LFPW, Gme) and short-finned pilot whales *G. macrorhynchus* (SFPW, Gma) analysed in the present study. M = male; F = female. Physical maturity status based on Bloch et al. (1993) for LFPW; sexual maturity status based on Bloch et al. (1993) for LFPW and Kasuya & Marsh (1984) for SFPW (Pacific)

| Species/ ID | Location | Date | Sex | Length (cm) | Physical maturity | Sexual maturity |
|---|---|---|---|---|---|---|
| **LFPW** | | | | | | |
| Gme 1 | Portugal | 11/09/2008 | M | 190 | Immature | Immature |
| Gme 2 | Portugal | 07/09/2008 | F | 414 | Immature | Mature |
| Gme 3 | Portugal | 01/04/2004 | M | 267 | Immature | Immature |
| Gme 4 | Portugal | 08/06/2009 | F | 400 | Immature | Mature |
| Gme 5 | NW Spain | 16/01/2006 | M | 268 | Immature | Immature |
| Gme 6 | NW Spain | 22/05/2007 | F | 281 | Immature | Immature |
| Gme 7 | NW Spain | 07/06/2008 | F | 265 | Immature | Immature |
| Gme 8 | NW Spain | 24/09/2008 | F | 214 | Immature | Immature |
| Gme 9 | NW Spain | 07/09/2008 | M | 515 | Mature | Mature |
| Gme 10 | NW Spain | 02/02/2004 | F | 414 | Immature | Mature |
| Gme 11 | NW Spain | 22/05/2007 | F | 400 | Immature | Mature |
| **SFPW** | | | | | | |
| Gma 1 | NW Spain | 07/10/2013 | F | 390 | – | Mature |
| Gma 2 | NW Spain | 07/10/2013 | M | 397 | – | Immature |
| Gma 4 | NW Spain | 07/10/2013 | F | 247 | – | Immature |
| Gma 5 | NW Spain | 07/10/2013 | F | 365 | – | Immature |
| Gma 6 | NW Spain | 07/10/2013 | M | 457 | – | Immature |
| Gma 7 | NW Spain | 08/10/2013 | F | 376 | – | Mature |
| Gma 8 | NW Spain | 08/10/2013 | F | 251 | – | Immature |
| Gma 10 | NW Spain | 08/10/2013 | F | 284 | – | Immature |
| Gma 11 | NW Spain | 08/10/2013 | F | 366 | – | Immature |
| Gma 13 | NW Spain | 08/10/2013 | F | 325 | – | Immature |
| Gma 15 | NW Spain | 08/10/2013 | F | 317 | – | Immature |
| Gma 17 | NW Spain | 08/10/2013 | F | 381 | – | Mature |
| Gma 18 | NW Spain | 08/10/2013 | M | 267 | – | Immature |

### Stable isotope analysis

Skin samples were dried in an oven at 50°C for 48 h and then ground into a fine powder. Afterwards, a lipid extraction was performed following Méndez-Fernandez et al. (2012) by agitating approximately 50 mg of powder with 4 ml of cyclohexane for 1 h, followed by a centrifugation at $4000 \times g$ for 5 min, and the supernatant containing lipids was discarded. Samples were then dried in an oven at 45°C for 48 h and subsamples of lipid-free powder were weighed in tin cups for stable isotope analyses.

The stable isotope analyses were performed on an elemental analyser coupled to an Isoprime (Micromass) continuous-flow isotope-ratio mass spectrometer (CF IR-MS). The results are presented in the usual δ notation relative to Vienna PeeDee Belemnite Standard for $\delta^{13}C$ and atmospheric $N_2$ for $\delta^{15}N$, in parts per thousand (‰) (Bond & Hobson 2012). Replicate measurements of internal laboratory standards (acetanilide) indicated that measurement errors were ±0.15 and ±0.20‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

Fig. 1. Stranding locations of long-finned pilot whales *Globi-cephala melas*, (grey dots) and short-finned pilot whales *G. macrorhynchus* (black dots) used in the present study

## Trace element analysis

Frozen liver and kidney samples were freeze-dried and ground to powder using a mortar and pestle. Afterwards, the trace element analyses and quality control were performed as described in Méndez-Fernandez et al. (2014). Briefly, 2 replicates for each sample were prepared and 14 trace elements [silver (Ag), arsenic (As), cadmium (Cd), cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb), selenium (Se), vanadium (V) and zinc (Zn)] were analysed. Excepting Hg, all elements were analysed with a Varian Vista-Pro for inductively coupled plasma-atomic emission spectrophotometry (ICP-AES) and a Thermofisher Scientific XSeries 2 for inductively coupled plasma-mass spectrometry (ICP-MS). Total Hg was determined using an Advanced Mercury Analyser (Altec AMA 254).

For ICP-AES and ICP-MS measurements, aliquots of dried samples, from 0.1 to 0.3 g, were digested

with 6 ml 67−70 % $HNO_3$ and 2 ml 34−37 % HCl (both from Merck and of Suprapur® quality). Acid digestion of the samples was carried out overnight at room temperature, and then in a Milestone microwave oven. After digestion, each sample was made up to a final volume of 50 ml with Milli-Q water. For samples with weight <0.1 g, the mixture used was 3 ml 67−70 % $HNO_3$/1 ml 34−37 % HCl and the samples were diluted to a final volume of 25 ml Milli-Q water. Mercury analysis does not require acid-digestion of the samples. Thus, aliquots of 5 ± 0.5 mg dried sample were directly analysed after being inserted in the oven of the apparatus (see Méndez-Fernandez et al. 2014 for details).

All trace element concentrations in tissues are reported in µg g$^{−1}$ dry weight (dw).

## Statistical analysis

### Univariate analysis

All data series were explored for outliers, collinearity, heterogeneity of variance and for visualization of potential relationships between response and explanatory variables, following Zuur et al. (2010). Generalized linear models (GLMs) were used to determine the occurrence of specific differences in stable isotopes and trace element concentrations (species as categorical variable, LFPW vs. SFPW). Afterwards, GLMs were also used to test the effect of biological variables, such as animal length (as a proxy for age) on stable isotopes and trace element concentrations. It is noteworthy that there were no significant differences in animal length between species (Wilcoxon test, $W = 71.5$, p > 0.05). The effect of sex was not tested due to highly unbalanced data (Table 1).

Since response variables (stable isotopes and concentration of each trace element) were continuous, a Gaussian distribution was applied. Validation of the model involved checking the assumptions of normality, homogeneity and independence of residuals, together with the lack of highly influential data points ('hat' values) (Zuur et al. 2007). If a model was not validated due to lack of normality or homogeneity of the residuals, data were log10 transformed to achieve an approximate Gaussian distribution. Highly influential data points were removed from the analysis.

In situations where model validation showed heterogeneity in the residuals after transformation, such as hepatic Pb and renal Ag when testing specific differences, generalized least squares (GLS)

were applied, using the package nlme (Pinheiro et al. 2014). GLS allow the incorporation of variable heterogeneity into the models (Zuur et al. 2009). Since the 2 response variables (hepatic Pb and renal Ag) were continuous and appeared to have an approximately normal distribution, a Gaussian probability distribution was applied. The models fitted for hepatic Pb and renal Ag included a variance structure (i.e. VarIdent) related to the variable species in the error term, to account for the heteroscedasticity observed in the residuals in relation to this variable. No random factor was defined, since the variable species does not have a sufficient number of levels (Zuur et al. 2009). All models were estimated using restricted maximum likelihood (REML). The best-fitting model was selected using a likelihood ratio test (L). Validation of the final model involved checking the assumptions of homogeneity and independence of residuals, together with the lack of highly influential data points ('hat' values) (see Zuur et al. 2007).

### Multivariate analysis

Discriminant analysis was selected to examine inter-specific differences based on non-essential elements which are not submitted to homeostasis (Ag, Cd, Hg, Pb) and stable isotope profiles. In order to avoid problems due to potential collinearity of variables (i.e. concentrations of different trace elements), the discriminant analysis used principal component analysis (PCA) scores rather than raw data. Since the number of samples should exceed the number of variables, 2 different disriminant analyses were performed, one using renal trace element concentrations and one using hepatic concentrations. Also, analytes that had values below the limits of detection of quantitation were excluded for PCAs.

All the statistical analyses were performed in R v.3.1.1 (R Core Team 2014).

## RESULTS

### Intra-specific variation of biogeochemical tracers

Concerning the effect of biological variables such as animal length (as a proxy for age) on the biogeochemical tracers, all individuals were considered for stable isotope analysis since no inter-specific difference occurred. There was no influence of length on $\delta^{13}C$ (*t*-test, $t = 1.76$, $p > 0.05$) or $\delta^{15}N$ ($t = -0.84$, $p >$ 0.05). In contrast, linear models revealed the influence of pilot whale length in some trace element concentrations. In SFPW, a significant influence of pilot whale length occurred in hepatic levels of Hg and Se and in renal levels of Fe and Mn (Table 2). With the exception of renal Mn, trace element concentrations increased with animal length (Fig. 2). In LFPW, a significant influence of pilot whale length occurred in hepatic levels of Co, Hg and Se and in renal levels of Ag, Co and Hg (Table 2). In all situations, trace element concentrations increased with animal length (Fig. 2).

### Inter-specific variation of biogeochemical tracers and ecological segregation

For skin isotopic composition, the mean ± SD values of $\delta^{13}C$ and $\delta^{15}N$ in LFPW stranded in the NWIP were −17.9 ± 0.7 and 12.1 ± 1.0‰, respectively, while in SFPW they were −17.5 ± 0.3 and 11.9 ± 0.3‰, respectively (Fig. 3). Although there was no evidence of significant differences between species in $\delta^{13}C$ ($t = -1.89$, $p > 0.05$) and $\delta^{15}N$ ($t = 0.45$, $p > 0.05$), a higher variation was observed in LFPW when compared with SFPW (Fig. 3).

For trace element analyses, hepatic and renal concentrations detected in LFPW and SFPW are presented in Table 3. Concentrations of V and Cr in SFPW were 100 and 50%, respectively, below the limit of detection, and therefore not further consid-

Table 2. Influence of animal length in hepatic and renal trace element concentrations of long-finned pilot whales *Globicephala melas* (LFPW) and short-finned pilot whales *G. macrorhynchus* (SFPW) stranded off the northern and western Iberian Peninsula. −: no relationship detected

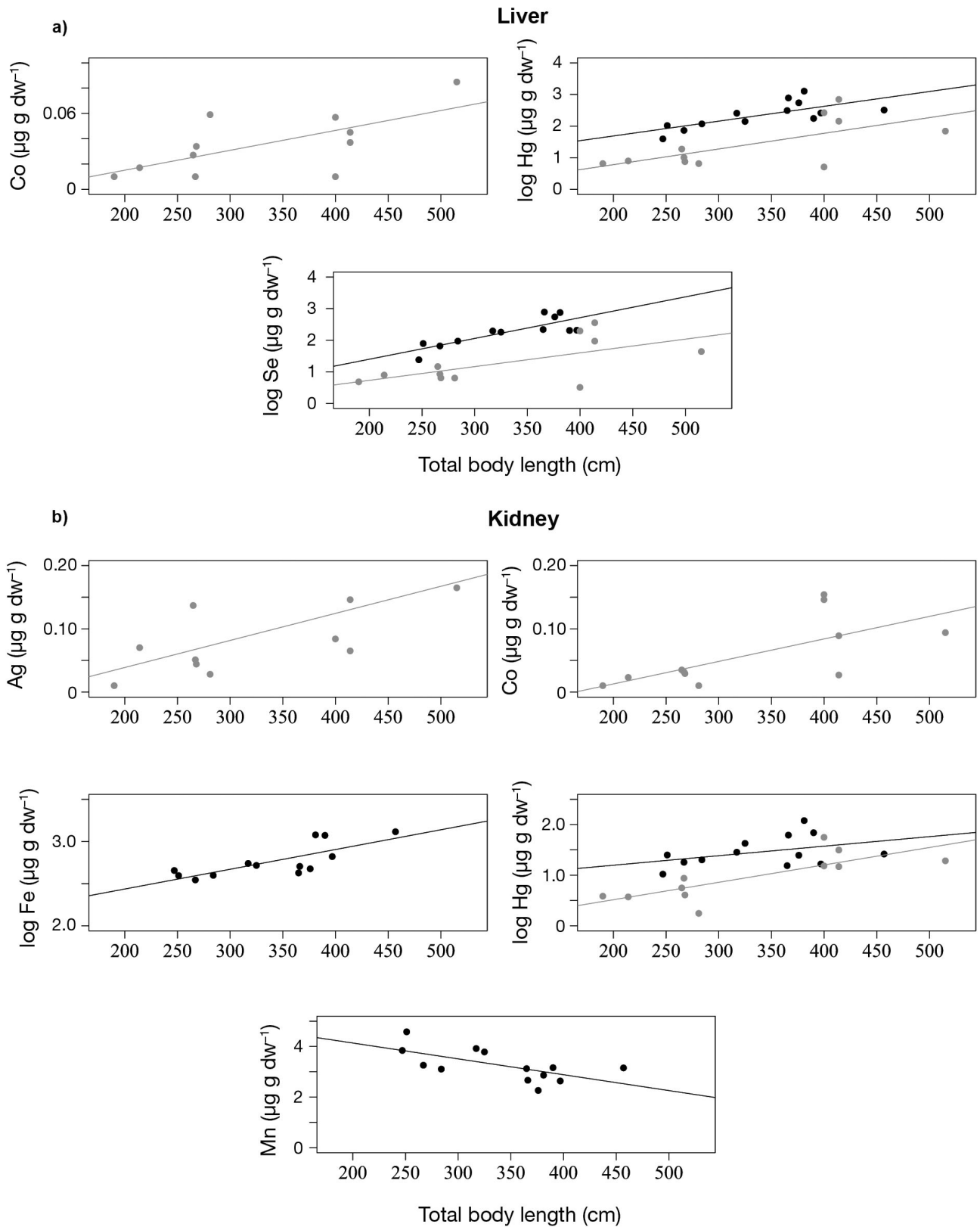| | | Liver | | Kidney | |
|---|---|---|---|---|---|
| | | *t* | p | *t* | p |
| Ag | LFPW | – | – | 2.48 | 0.035 |
| As | – | | | | |
| Cd | – | | | | |
| Co | LFPW | 2.67 | 0.025 | 2.87 | 0.019 |
| Cu | – | | | | |
| Fe[a] | SFPW | – | – | 3.90 | 0.002 |
| Hg[a] | LFPW | 2.72 | 0.023 | 3.89 | 0.005 |
| | SFPW | 3.42 | 0.006 | – | – |
| Mn | SFPW | – | – | −2.76 | 0.019 |
| Ni | – | | | | |
| Pb | – | | | | |
| Se[a] | LFPW | 2.47 | 0.036 | – | – |
| | SFPW | 4.59 | 0.001 | – | – |
| Zn | – | | | | |
| [a]log-transformed | | | | | |

Fig. 2. Trace element concentration in (a) livers and (b) kidneys of long-finned pilot whales *Globicephala melas* (grey) and short-finned pilot whales *G. macrorhynchus* (black) stranded off the northern and western Iberian Peninsula, plotted against total body length. Fitted regression lines are included. dw: dry weight

ered. Except for Cd, Co and Ni in both species, as well as Cu in LFPW and As in SFPW, all remaining trace elements presented higher mean concentrations in the liver (Table 3, Fig. 4). In both species,
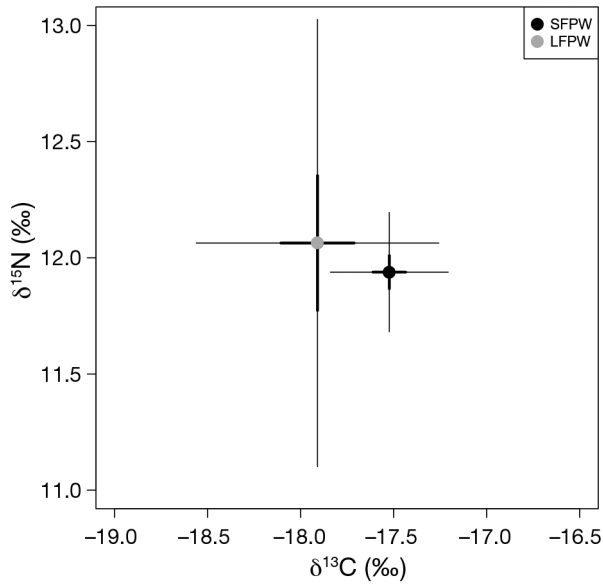


Fig. 3. Carbon (δ¹³C) and nitrogen (δ¹⁵N) isotope values (mean ± SD [thin lines] and ± SE [thick lines]) in long-finned pilot whales *Globicephala melas* (LFPW, grey point) and short-finned pilot whales *G. macrorhynchus* (SFPW, black point) stranded off the northern and western Iberian Peninsula

Table 3. Trace element concentrations (mean ± SD, μg g⁻¹ dry weight) in kidneys and livers of long-finned pilot whales *Globicephala melas* (LFPW) and short-finned pilot whales *G. macrorhynchus* (SFPW) stranded off the northern and western Iberian Peninsula. Length: mean (± SD) of analysed animals

| | LFPW (n = 11, length = 329 ± 103 cm) | | SFPW (n = 13, length = 340 ± 64.2 cm) | |
|---|---|---|---|---|
| | Liver | Kidney | Liver | Kidney |
| Ag | 0.39 ± 0.73 | 0.09 ± 0.07 | 4.05 ± 3.74 | 0.36 ± 0.25 |
| As | 4.65 ± 5.39 | 4.54 ± 4.20 | 3.46 ± 1.95 | 3.90 ± 3.53 |
| Cd | 24.4 ± 29.2 | 122 ± 138 | 127 ± 116 | 141 ± 41.6 |
| Co | 0.04 ± 0.02 | 0.06 ± 0.05 | 0.07 ± 0.02 | 0.10 ± 0.04 |
| Cu | 12.7 ± 4.87 | 15.6 ± 5.49 | 25.3 ± 26.1 | 11.6 ± 3.16 |
| Cr | 0.24 ± 0.10 | 0.44 ± 0.52 | 0.08 ± 0.02[a] | 0.63 ± 0.20[a] |
| Fe | 1631 ± 1322 | 533 ± 329 | 1646 ± 790 | 646 ± 341 |
| Hg | 112 ± 212 | 15.0 ± 16.3 | 337 ± 349 | 36.8 ± 30.6 |
| Mn | 10.3 ± 4.4 | 2.96 ± 0.65 | 9.49 ± 4.48 | 3.26 ± 0.63 |
| Ni | 0.16 ± 0.17 | 0.43 ± 0.55 | 0.15 ± 0.06 | 0.76 ± 1.47 |
| Pb | 0.20 ± 0.40 | 0.03 ± 0.03 | 0.31 ± 0.29 | 0.02 ± 0.02 |
| Se | 67.4 ± 112 | 16.7 ± 11.7 | 270 ± 255 | 42.6 ± 31.7 |
| V | 0.86 ± 0.09 | 0.34 ± 0.05 | 0.50 ± 0.000[b] | 0.50 ± 0.000[b] |
| Zn | 192 ± 85.4 | 112 ± 36.8 | 135 ± 36.4 | 90.5 ± 20.3 |

[a]<50% of individuals with values < limit of detection (LOD); [b]100% of individuals with values < LOD

considering the essential elements analysed in the present study, Fe showed the highest concentrations in all tissues, while hepatic Hg and renal Cd presented the highest concentrations among non-essential elements (Table 3, Fig. 4).

Inter-specific differences were more evident in trace elements compared to stable isotopes. In particular, SFPW exhibited significantly higher concentrations of hepatic Cd, Co and Pb, as well as Ag, Hg and Se in both tissues (Table 3, Fig. 4). In turn, although LFPW exhibited higher concentrations of renal Pb and Cu, as well as As and Zn in both tissues, only renal Cu showed significant differences between species (Table 3, Fig. 4).

In an attempt to detect ecological segregation within the *Globicephala* genus, a multi-tracer approach was applied using the combination of stable isotopes with hepatic or renal non-essential elements in a discriminant analysis on the principal components of a PCA (i.e. to avoid collinearity among variables). Discriminant analysis revealed a strong ability to assign *Globicephala* individuals to their respective species. In particular, based on stable isotopes and hepatic trace elements, a correct assignment of 90.9 and 61.5% of LFPW and SFPW individuals to their respective species was observed, with one LFWP and 5 SFPW animals being misclassified (Table 4). In turn, based on stable isotopes and renal trace elements, discriminant analysis indicated a correct assignment of 81.9 and 92.3% of LFPW and SFPW individuals to their respective species, with only 2 LFPW and 1 SFPW misclassified individuals (Table 4). Therefore, these results suggest a lower misclassification rate and overlap between LFPW and SFPW when using the combination of stable isotopes and renal non-essential elements.

## DISCUSSION

### Intra-specific variation of biochemical tracers

In the present study, there was no influence of animal length (as a proxy of age) on stable isotopes. Previous dietary studies in LFPW revealed ontogenetic variation in this species' diet to be mainly associated with dietary differences between calves
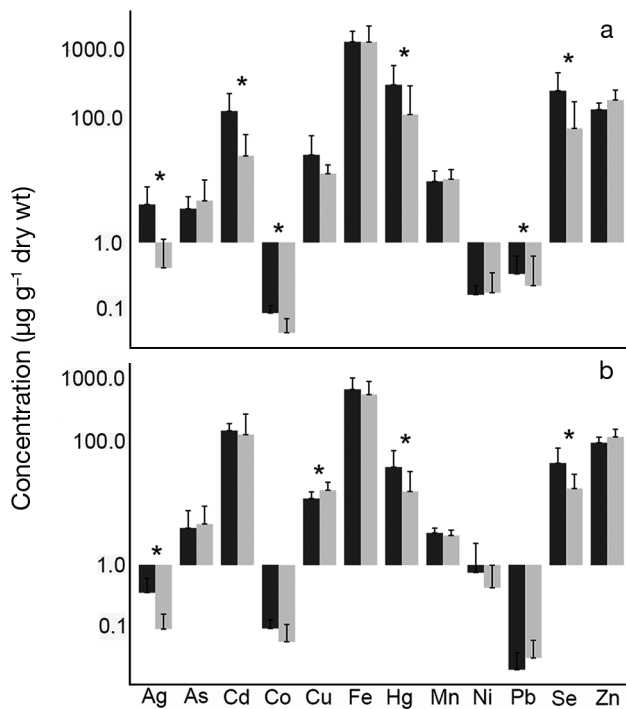
Fig. 4. Concentrations (mean ± SD) of trace elements analysed in (a) livers and (b) kidneys of long-finned pilot whales *Globicephala melas* (LFPW, grey) and short-finned pilot whales *G. macrorhynchus* (SFPW, black) stranded off the northern and western Iberian Peninsula. The *y*-axis is on a logarithmic scale. *Significant differences between species (p < 0.05)

Table 4. Specific assignment (%) using discriminant analysis based on stable isotopes and trace element concentrations analysed in livers and kidneys of long-finned pilot whales *Globicephala melas* (LFPW) and short-finned pilot whales *G. macrorhynchus* (SFPW) stranded off the northern and western Iberian Peninsula

| | —— Liver —— | | —— Kidney —— | |
| | LFPW | SFPW | LFPW | SFPW |
| --- | --- | --- | --- | --- |
| LFPW | 90.91 | 9.091 | 81.82 | 18.18 |
| SFPW | 38.46 | 61.54 | 7.69 | 92.31 |

1997) already reported in this species (Monteiro et al. 2015b).

Long-term exposure to contaminants associated with a process of elimination of long half-life pollutants in marine mammals may lead to increasing non-essential trace element bioaccumulation in older cetaceans (e.g. Gaskin et al. 1979, Aguilar et al. 1999). Hence, it is not surprising to observe a positive relationship between pilot whale length and some non-essential elements (Ag, Hg) in the present study. Similar results were already reported in other cetacean species and also in LFPW (e.g. Caurant et al. 1993, Agusa et al. 2008, Mendéz-Fernandez et al. 2014).

For some essential elements (Co, Se, Fe, Mn), a relationship between concentration and pilot whale length has been shown in the present study. With respect to Se, the higher hepatic concentration in larger animals (in both SFPW and LFPW) may be related to Se complex formation as a detoxification strategy for some non-essential elements, such as Hg and Ag (e.g. Caurant et al. 1996, Ikemoto et al. 2004, Lailson-Brito et al. 2012). Ontogeny effects of Fe levels, with higher Fe concentrations in larger animals, have been reported in cetaceans (e.g. Honda et al. 1987, Agusa et al. 2008) and may be associated with cumulative oxidative stress with age and the important role of Fe as a pro-oxidant in ageing (e.g. Cook & Yu 1998, Arruda et al. 2013). The previously described interaction between age and gender in the accumulation of Fe (e.g. Hahn et al. 2009), with females showing higher Fe levels with increasing age, may explain the higher Fe levels in older SFPW in this study, since most animals were females. However, the unbalanced dataset prevented the analysis of a gender and age interaction in Fe accumulation. Regarding Mn, considering its essential role in growth in mammals (e.g. Hansen et al. 2006) and the high level of Mn absorption in younger mammals (Keen et al. 1986), the negative relationship between Mn concentration and LFPW length in the present study is not surprising. In fact, similar results were observed in other cetacean species (e.g. Agusa et al. 2008, Méndez-Fernandez et al. 2014, Monteiro et al. 2016).

The high variability in stable isotope concentrations in LFPW suggest a higher degree of dietary plasticity in their foraging habits and habitats compared to SFPW. This variation was also reported in previous dietary and distribution studies (Pierce et al. 2010, Spyrakos et al. 2011, Santos et al. 2014) and may ultimately influence trace element concentrations and enhance inter-specific variability. However,

and adult animals, with a higher variation presented by the calves (Desportes & Mouritsen 1993, Santos et al. 2014, Monteiro et al. 2015b). Several factors may explain the similarity between stable isotope values. It may be due to the fact that most analysed animals were immature, probably showing a more similar diet among them compared to adults. Additionally, the presence of only 2 unweaned individuals in the LFPW dataset (<239 cm, Sergeant 1962) probably prevented the detection of the usually high $\delta^{15}N$ isotopic values of unweaned individuals (Hobson et al.

there have been no dietary or distribution studies to date of SFPW in their northern range limits of the Atlantic which would help understand whether diversity in foraging ecology is common to the *Globicephala* genus.

Additionally, some factors may also influence the inter-specific differences observed in the present study, such as the higher temporal coverage of LFPW in comparison to SFPW data, which may bias the results. Moreover, $\delta^{13}C$ and $\delta^{15}N$ values of the mass-stranded SFPW suggest a potential unique pod in light of the very low variability obtained. Likewise, on a longer timescale, the lower variability in some trace elements (e.g. renal Cd) observed for SPFW compared to LFPW, coupled with the fact that the SFPW mass stranding comprised 10 females (out of 13 individuals), seem to also support the scenario of a matrilineal social structure belonging to a unique pod. Hence, although a recent genetic study on LFPW suggested the occurrence of multiple maternal lineages and, consequently, different pods in mass strandings (Oremus et al. 2013), the observed variation in SFPW may reflect within-pod rather than within-region variability. Hence, the single vs. mass stranding sample collection method may have also been a source of sampling bias. The analyses of more biogeochemical tracers in different tissues with different turnover are needed to better understand the history of a pod before stranding.

## Inter-specific variation in biogeochemical tracers and ecological segregation

Beside the biological variables (e.g. length, age, sex of the animals), ecological factors (e.g. dietary and habitat preferences, environmental conditions) may also influence the signatures of biogeochemical tracers (e.g. Aguilar et al. 1999, Ferreira et al. 2016). Previous dietary and distribution studies evidenced the occurrence of geographical differences in LFPW inhabiting the North Atlantic. While LFPW seems to feed on benthic prey species (octopus) and/or inhabits neritic habitats in NWIP (Pierce et al. 2010, Santos et al. 2014, Monteiro et al. 2015a,b), this species either exhibits inshore–offshore movements and preference for demersal squids and epipelagic fish (in USA, e.g. Payne & Heinemann 1993, Gannon et al. 1997) or exhibits oceanic preferences in terms of habitat and ingested prey (in UK and Faroe Islands, e.g. Desportes & Mouritsen 1993, MacLeod et al. 2007, Santos et al. 2014) elsewhere in the North Atlantic. In turn, information about dietary and habitat preferences of SFPW is limited in the North Atlantic. Although ichthyophagous behavior has also been observed in North Carolina (USA) and the Canary islands, SFPW consumed mainly oceanic and pelagic cephalopod species (e.g. Hernández-Garcia & Martín 1994, Mintzer et al. 2008, Fernández et al. 2009), which agrees with the oceanic habitat preference exhibited in both regions (e.g. Carrillo et al. 2010, Wells et al. 2013).

Considering the aforementioned studies, different $\delta^{13}C$ and potentially $\delta^{15}N$ values could be expected between the sympatric *Globicephala* species in NWIP as a consequence of the different habitats and prey preferences. Nevertheless, results from the present study suggest that LFPW and SFPW converging in NWIP seem to be sharing similar foraging habits and habitats, considering the short timescale represented by skin stable isotopes (i.e. 1–3 mo, Abend & Smith 1995, Browning et al. 2014). These results ultimately propose an overlap in the foraging niches and a weak ecological segregation of these species in the stranding area (NWIP). However, it is important to note that the similar $\delta^{15}N$ values in *Globicephala* species do not necessarily imply that they feed on the same prey species, but the lack of complementary information about the diet of SFPW in this region (e.g. stomach contents or fatty acids analyses, feeding behavior information) prevents further conclusions.

Contrasting with stable isotope results, all the non-essential trace elements analysed in the present study presented significantly higher concentrations in SFPW compared to LFPW. Even if an effect of age or sampling bias (individual vs. mass stranding) cannot be excluded, these stronger inter-specific differences in trace element burdens ultimately suggest a stronger ecological segregation between species, at the longer timescale represented by trace element levels (e.g. ≥10 yr for renal Cd and hepatic Hg, 4 to 19 yr for hepatic Cd; Kjellstrøm & Nordberg 1978, Friberg et al. 1979, Stoeppler 1991) when comparing with stable isotope concentrations. Both biotic and abiotic factors can affect trace element concentrations in marine organisms (e.g. Aguilar et al. 1999, Bennett et al. 2001, Das et al. 2004). In particular, the long-term inter-specific differences found in the present study may be due to different reasons, such as differences in feeding preferences or prey availability, differences in habitat or geographical feeding areas (hence exposure to different gradients of trace element concentrations and/or environmental conditions), and metabolic differences between species.

Ingestion is the main pathway for trace element intake by marine mammals (Aguilar et al. 1999) and links have been established between dietary preferences and trace element concentrations in cetaceans (e.g. Fontaine et al. 2007). High Cd levels in *Globicephala* species may result from their typical teuthophagous behavior associated with the Cd bioaccumulation ability in cephalopods (e.g. Bustamante et al. 1998). Likewise, high Hg levels observed in *Globicephala* (especially SFPW) may also be related to diet preferences, since both cephalopods and fish are Hg sources for marine top predators and not only fish prey, as previously thought (e.g. Bustamante et al. 1998, Evers et al. 2008, Chouvelon et al. 2012).

Differences in feeding preferences in terms of prey and habitat may explain the inter-specific differences observed in this study, since a long-term preference for oceanic cephalopod species or feeding habitats of SFPW coupled with the Cd enrichment in deeper waters (e.g. Bruland et al. 1978, Baars et al. 2014), may lead to the enhanced Cd concentrations in SFPW compared to LFPW. Concerning Hg, higher levels would be expected in LFPW stranded off the NWIP, due to potentially higher exposure to anthropogenic Hg when exploiting coastal habitats or prey in this region (Chouvelon et al. 2012, Bowman et al. 2015, Monteiro et al. 2015a). However, similar to Cd, the enhanced Hg concentrations in SFPW may be related to Hg enrichment in deeper oceanic waters due to biogeochemical cycling (Chouvelon et al. 2012, Bowman et al. 2015). Hence, these results could be a consequence of a long-term integration of Cd and Hg by both species in 2 distinct foodwebs (oceanic vs. neritic) in NWIP, as seems to be evident from the aforementioned studies on dietary and habitat preferences (Santos et al. 2014, Monteiro et al. 2015a).

However, it may also be the case that the long-term integrated Cd and Hg levels in *Globicephala* species actually reflect signatures from broader geographical areas, where exposure to different gradients of trace element concentrations may occur. As an example, Cd seawater concentrations (below 300 m) seem to show a latitudinal gradient in the Eastern Atlantic due to biogeochemical processes (e.g. Wu & Roshan 2015), with a peak in the Mauritanian upwelling zone, which represents a potential habitat for SFPW and respective pelagic prey giving the tropical habitat preferences exhibited by SFPW (e.g. Carrillo et al. 2010, Wells et al. 2013, Abecassis et al. 2015). Additionally, studies on marine molluscs and fish revealed a positive temperature-dependent accumulation of

several non-essential trace elements (e.g. Cd, Hg, Pb) and their toxicity effects (e.g. Fischer 1986, Guinot et al. 2012, Abdel-Tawwab & Wafeek 2014). As ectothermic animals, similar temperature-dependent uptake of trace elements may be expected in cephalopods, which may ultimately influence SFPW inhabiting tropical waters and justify their higher non-essential trace element burdens compared to LFPW.

The discrepancies observed in trace elements within the *Globicephala* genus may also result from specific elimination processes of non-essential elements of SFPW and LFPW, reflecting metabolic differences between species. Bustamante et al. (2003) found that despite the high hepatic total Hg values described in SFPW, levels of the hepatic percentage of methyl Hg against total Hg (i.e. 1%) were much lower than in adult LFPW (i.e. 3 to 33%, Julshamn et al. 1987, Caurant et al. 1994) or other cetacean species (1.7 to 100%, Storelli et al. 1998; 3 to 12%, Wagemann et al. 1998). Hence, SFPW may have developed more powerful processes to become resistant to contaminants compared to LFPW, allowing for a higher accumulation of non-essential elements without suffering from their toxicological effects. A protective mechanism against Hg toxicity (and to a lesser extent other elements such as Cd and Ag, e.g. Ikemoto et al. 2004) seems to be associated with a higher accumulation of Se, which is known to form complexes with mercury (HgSe) in the liver to demethylate this non-essential element (e.g. Caurant et al. 1996). In the present study, SFPW showed significantly higher Se levels compared with LFPW, which seems to corroborate this theory.

LFPW stranded off NWIP showed trace element concentrations similar to those previously described in this region (Méndez-Fernandez et al. 2014) and of the same order of magnitude to those reported elsewhere in the Atlantic (e.g. Frodello & Marchand 2001, Sonne et al. 2010, Gajdosechova et al. 2016) (Table 5). Likewise, trace elements in SFPW stranded in NWIP were of the same order of magnitude as those previously described in the Atlantic (e.g. Stoneburner 1978, Bustamante et al. 2003) (Table 5). It is noteworthy that both species presented high Hg and Cd levels compared to other cetacean species, as previously described for this genus (Caurant & Amiard-Triquet 1995, Caurant et al. 1996, Méndez-Fernandez et al. 2014). In fact, 54% of SFPW showed Hg levels above the toxic thresholds previously defined for evidence of liver damage in marine mammals (60 µg g$^{-1}$ wet wt, Rawson et al. 1993). The potential toxic effects of non-essential elements in

Table 5. Mean Hg and Cd concentrations (µg g$^{-1}$ wet wt) of long-finned pilot whales *Globicephala melas* (LFPW) and short-finned pilot whales *G. macrorhynchus* (SFPW) available in the literature. The average or ranges of age (yr) and total length (cm) are given when available

| Species/region | Area | Age/length | | Hg (n) Liver | Hg (n) Kidney | Cd (n) Liver | Cd (n) Kidney | Source |
|---|---|---|---|---|---|---|---|---|
| **LFPW** | | | | | | | | |
| NE Atlantic Ocean | NW Iberia | 190–515 | Length | 30.2 (11)[a] | 4.1 (11)[a] | 5.4 (11)[a] | 26.8 (11)[a] | Present study |
| | NW Iberia | 0–11 | Age | 31 (8) | 2.7 (8) | 8.3 (6) | 30.0 (6) | Méndez-Fernandez et al. (2014) |
| | Faroe Islands | 0–45 | Age | 63.5 (125) | 5.3 (54) | 63.2 (194) | 78 (97) | Caurant et al. (1994) |
| | Faroe Islands | – | – | 61.1 (14) | 4.4 (10) | 45.7 (14) | 72.7 (10) | Julshamn et al. (1987) |
| | Faroe Islands | 435–560 | Length | 139.5 (6) | – | 13.8 (6) | – | Sonne et al. (2010) |
| | England | 502 | Length | 0.7 (1) | – | 0.1 (1) | – | Law et al. (2001) |
| | Scotland | 1–35.5 | Age | 136.2 (21) | 7.0 (21) | 38.2 (18) | 74.7 (19) | Gajdosechova et al. (2016) |
| NW Atlantic Ocean | USA | 274–439 | Length | 40.1 (9) | – | 7.9 (9) | 36.5 (6) | Mackey et al. (1995) |
| | Canada | 1–17 | Age | 16.9 (26)[a] | 1.6 (26)[a] | 9.4 (26)[a] | 17.1 (26)[a] | Muir et al. (1988) |
| Mediterranean Sea | Italy | 6–7 | Age | – | – | 2.9 (3)[a] | 7.3 (3)[a] | Frodello & Marchand (2001) |
| **SFPW** | | | | | | | | |
| NE Atlantic Ocean | NW Iberia | 247–457 | Length | 91.0 (13)[a] | 9.9 (13)[a] | 27.9 (13)[a] | 31.0 (13)[a] | Present study |
| NW Atlantic Ocean | USA | 368–385 | Length | 231 (4) | 24.3 (4) | 13.9 (4) | 31.35 (4) | Stoneburner (1978) |
| Caribbean Sea | St. Lucia | 400–450 | Length | 88.7 (5) | 10.0 (5) | – | – | Gaskin et al. (1974) |
| South Pacific | Caledonia | 12–14 | Age | 386.5 (2)[a] | – | 75.8 (2)[a] | – | Bustamante et al. (2003) |
| | Australia | – | – | 2.19 (1)[b] | 0.51 (1)[b] | – | – | Kemper et al. (1994) |

[a]Converted from dry to wet weight based on conversion factor from Méndez-Fernandez et al. (2014)
[b]*Globicephala* spp.

marine mammals may be mitigated through detoxification strategies, such as the protective action of metallothioneins or the formation of the aforementioned mercury–selenium complex (HgSe) in liver (e.g. Caurant et al. 1996, Lailson-Brito et al. 2012). However, knowledge about the actual toxic effect of non-essential elements in cetaceans is limited and restricted to *in vitro* laboratory assays or extrapolated from small mammals (e.g. Fossi et al. 2008, Frouin et al. 2012) due to the inherent difficulties of performing toxicological studies using wildlife fauna.

## Conclusions

In this study, we increased our knowledge about the foraging niche, contamination status and ecological segregation of 2 sympatric species belonging to the *Globicephala* genus. This is the first study investigating the ecology and contamination status of SFPW in the northern limits (i.e. NWIP) of its distribution range in the northeastern Atlantic, providing valuable baseline information about this species. This is particularly relevant, considering that large marine vertebrates are being used as indicators of

medium and long-term marine environmental change, and their study provides useful information for incorporation into the EU Marine Strategy Framework Directive. Despite the increasing interest in determining long-term oceanic baselines of trace elements (e.g. Geotracers program), the lack of information about temporal and spatial variation in the distribution of natural and anthropogenic trace elements in the open ocean (e.g. Wu & Roshan 2015) hinders a detailed mapping of pilot whale distribution. Finally, a better understanding of the metabolism of trace elements in marine mammals would enable further conclusions.

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