**Population diversity and structure of long-finned pilot whale (*Globicephala melas*) in Atlantic waters assessed through biogeochemical and genetic markers**

Sílvia S. Monteiro\*1,2; Paula Méndez-Fernandez3‡; Stuart Piertney4; Colin F. Moffat5; Marisa Ferreira1,2; José V. Vingada1,2,6; Alfredo López7; Andrew Brownlow8; Paul Jepson9; Bjarni Mikkelsen10; Misty Niemeyer11; José Carlos Carvalho1,2; Graham J. Pierce6,12

(1) Centro de Biologia Molecular e Ambiental (CBMA) & Departamento de Biologia, Universidade do Minho, Campus de Gualtar, 4710-047 Braga, Portugal

(2) Sociedade Portuguesa de Vida Selvagem, Departamento de Biologia, Universidade do Minho, Campus de Gualtar, 4720-057 Braga, Portugal

(3) Littoral Environnement et Sociétés (LIENSs), UMR 7266 CNRS-ULR, 2 Rue Olympe de Gouges, 17042 La Rochelle Cedex 01, France

(4) School of Biological Sciences (Zoology), University of Aberdeen, Tillydrone Avenue, Aberdeen AB242TZ, UK

(5) Marine Scotland Science, Marine Laboratory, 375 Victoria Road, Aberdeen, AB11 9DB, UK

(6) Centro de Estudos do Ambiente e do Mar (CESAM) & Departamento de Biologia, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal

(7) Coordinadora para o Estudio dos Mamíferos Mariños (CEMMA). P.O. Box 15, 36380 Gondomar, Pontevedra, Spain

(8) Wildlife Unit, SAC Veterinary Science Division, Drummond Hill, Stratherrick Road, Inverness, IV2 4JZ, UK

(9) UK Cetacean Strandings Investigation Programme, The Wellcome Building, Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY

(10) Museum of Natural History, V. U. Hammersheimsgøta 13, FO-100 Tórshavn, Faroe Islands

(11) International Fund for Animal Welfare (IFAW) Marine Mammal Rescue & Research Program, World headquarters, 290 Summer Street, Yarmouth Port, MA 02675, USA

(12) Oceanlab, University of Aberdeen, Main Street, Newburgh, Aberdeenshire, AB41 6AA,UK

\* Corresponding author: [s.monteiro@](mailto:s.monteiro@)ua.pt. Current address: CESAM & Departamento de Biologia, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal

‡ Current address:Oceanographic Institute of the University of São Paulo, Praça do Oceanográfico, 191, Cidade Universitária, São Paulo 05508-120, SP, Brazil

**Running page head:** Population diversity and structure of pilot whales

**Abstract**

Integration of ecological and genetic approaches is a particularly powerful strategy to identify natural population diversity and structure, over different timescales. In order to investigate the potential occurrence of population differentiation in long-finned pilot whales (*Globicephala melas*) in the north Atlantic, both biogeochemical (fatty acids and stable isotopes) and genetic (mitochondrial DNA) markers were analyzed in animals from four regions within north Atlantic: northwestern Iberian Peninsula, United Kingdom, Faroe Islands and United States of America. Genetic data revealed strong regional levels of divergence, although AMOVA revealed no differentiation between eastern and western Atlantic. Results from biogeochemical tracers supported previous dietary studies, revealing geographic and ontogenetic dietary variation in pilot whales. Fatty acids revealed ecological differentiation between all regions analyzed, while stable isotopes showed an overlap between some sampling regions. These results suggest that both ecological and genetic factors may drive the levels of pilot whale differentiation in the north Atlantic. The ecological differentiation observed may be related to the exploitation of different foraging niches (e.g. oceanic *vs*. coastal), which can be highly influenced by prey distributions or oceanographic phenomena. Genetic differentiation may result from historical or contemporary processes, or even limited dispersal mediated through the social structure displayed by this species and potential foraging specialization. These results highlight some problems when assessing population structure across multiple markers and the ecological versus evolutionary timescales over which differences may accumulate. Notwithstanding, the data provide preliminary information about pilot whale diversity and stocks in north Atlantic giving essential baseline information for conservation plans.

**Keywords:** stable isotopes; fatty acids; mitochondrial DNA; marine mammal; stock structure

**Introduction**

Determining the spatial distribution of intra-specific genetic and ecological diversity is essential for identifying evolutionarily and/or ecologically independent populations that may require specific management or conservation actions (Witteveen et al. 2011). However, it may be a challenge to understand the genetic or ecological processes that drive population differentiation, especially in complex environments, such as marine habitats where several factors may be important either independently or in combination.

Most scientific studies aim to define wildlife populations based on their evolutionary traits, with genetic stocks representing reproductively isolated units (Coyle 1998), even if the management units finally used may be modified by geopolitical considerations. Neutral molecular markers have been extensively applied to identify demographically and evolutionarily independent units driven by microevolutionary forces such as gene flow, drift and selection (Ballard & Whitlock 2004, Selkoe et al. 2006, Xu et al. 2010). However, the adaptation to environmental or ecological variables may also lead to discontinuous ecological or phenotypic stocks, representing isolated adapted units (Coyle 1998). These ecological units may occur even in the absence of genetic differentiation (Coyle 1998) and the difficulty may be to understand whether intra-specific ecological differences may ultimately result in genetic structuring. Those variables include currents and other oceanographic characteristics (e.g. Fullard et al. 2000, Fontaine et al. 2010), habitat discontinuities (e.g. Wiszniewski et al. 2010), geographic barrier (Fontaine et al. 2007), social organization (Lyrholm et al. 1999) and dietary specializations (e.g. Foote et al. 2009).

Although it is evident that genetically isolated units should normally be recognized as separate management units (Moritz 2002, ICES 2009) for conservation purposes, it has been suggested that for some species (e.g. *Delphinus delphis*), the finer scale represented by an ecological time-scale may be more relevant to management issues than the evolutionary time-scale (Evans & Teilmann 2009). In addition, ecological stocks may become reproductively isolated in the future (Funk et al. 2006) or hold unique characteristics that justify their separate conservation. This is particularly relevant considering that demographic responses to external stressors can only be meaningfully interpreted at the population level (Hoelzel 1998, ICES 2009, 2013). The combination of genetic knowledge with data from biogeochemical markers, in a multi-approach strategy, provides more complete insight into marine mammal distribution, trophic ecology and social structure and hopefully clarifies the genetic and ecological processes involved in intra-specific diversity structure (Frankham et al 2002, Evans & Teilmann 2009).

Biogeochemical markers, such as fatty acids (FA) and stable isotopes (SI) are bioavailable environmental compounds and elements which are incorporated into marine mammal tissues mainly through food and may be considered as proxies of foraging habitat and habits (DeNiro & Epstein 1978,1980). In addition to providing qualitative and quantitative information about trophic ecology of wild animals, several studies have also used stable isotopes and fatty acid analyses to reveal population or ecotype differentiation in marine top predators, based on the idea that consistent differences in trophic ecology would be sufficient to delimit “ecological stocks”, possibly even in the presence of gene flow (e.g. Foote et al. 2009, Quérouil et al. 2013). Depending on the tissue turnover and the half-life of the compounds or elements analyzed, these tracer signatures can reveal differences between groups of animals over time-scales spanning from weeks to years (e.g. Hobson & Clark 1992, Nordstrom et al. 2008). This highlights the usefulness of biogeochemical markers for the understanding of wildlife foraging ecology, over a wide range of time-scales. However, it is important to consider that signatures of biogeochemical markers in tissues may be influenced by individual physiological and biological features, such as age, sex, metabolism or reproductive state (e.g. Vanderklift & Ponsard 2003, Newland et al. 2009). As such, there is a need to distinguish individual-level and stock-level variability.

The conservation status of the long-finned pilot whale (*Globicephala melas*), hereafter referred to as pilot whale, is currently categorized as “Data Deficient” (IUCN 2013). Previous genetic evidence (mtDNA and microsatellites) showed nonexistent or low levels of genetic structure in the north Atlantic (Siemann 1994, Fullard et al. 2000). However, analysis of biogeochemical markers, such as stable isotopes (based solely on three animals, Abend & Smith 1995), parasites (IWC 1990) and morphometric differences (Bloch & Lastein 1993) suggested an eastern vs. western differentiation of pilot whales in the north Atlantic. Additionally, stomach contents analysis in the northeast Atlantic reported the occurrence of dietary variation in pilot whales, related to geographical location, sex and length of the animal (Santos et al. 2014). The main aims of the present study are to combine ecological and genetic approaches to provide new insights into pilot whale diversity in the north Atlantic and to provide a model for whether different approaches can be used in combination, to obtain a clearer picture of population structure. This study will integrate data from both mitochondrial DNA and biogeochemical markers (fatty acids and stable isotopes) to: i) assess trophic and genetic characteristics of pilot whales in different regions of the north Atlantic; ii) investigate the putative population structure of this species, using different time-scales and iii) investigate whether genetic and ecological (mainly trophic) processes are responsible for the spatial distribution of intra-specific diversity.

**Methodology**

**Sample collection**

Samples were collected from pilot whales stranded in the north Atlantic (the northwestern Iberian Peninsula (NWIP), United Kingdom (UK) and the United States of America (USA)), from 1992 to 2012. In addition, this study used samples collected from animals taken in drive fisheries in 2010 and archived at the tissue bank of the Museum of Natural History of the Faroe Islands (FI) (Table 1). Detailed necropsies were performed, by experienced stranding network personnel, if the condition of the animal permitted. Otherwise, basic information (i.e., length, sex, decomposition state) and skin and blubber samples were collected. Only samples collected from fresh or moderately decomposed animals (decomposition state ≤ 3; Kuiken & Hartmann 1991) were used for stable isotope and fatty acid analyses, to prevent sampling biases associated with tissue decomposition. The sex of the animals was assessed either during the necropsy or through genetic analysis. Skin samples were preserved in 70% ethanol or frozen (-20ºC) to be used in genetic and stable isotope analysis (SI were analyzed only in frozen samples), while full-depth blubber samples were collected from the mid-region of the body, wrapped in aluminum foil and frozen (-20ºC) prior to fatty acid analysis.

**Genetic analysis**

Skin samples were digested in cetyl trimethylammonium bromide (CTAB) extraction buffer and DNA was purified by a standard phenol–chloroform-isoamyl alcohol procedure (modified from Sambrook et al. 1989). Sex determination was performed using specific primers for introns within the Zfx and Zfy genes, namely LGL331 (5’- CAAATCATGCAAGGATAGAC - 3’) and LGL335 (5’ – AGACCTGATTCCAGACAGTACCA - 3’) (Shaw et al. 2003). PCR conditions followed De Stephanis et al. (2008a). Afterwards, electrophoresis on 1% agarose gel allowed discrimination between males (2 bands: 930 bp for the X-specific fragment and 1000 bp for the Y-specific fragment) and females (1 band).

A 400 base pair (bp) fragment of the mtDNA control region was sequenced in a total of 102 samples, from the north Atlantic, using primers L15926 (5’- ACA CCA GTC TTG TAA ACC-3’) in the tRNA-Thr-region (Eggert et al*.* 1998) and H16498 (5’-CCT GAA GTA AGA ACC AGA TG-3’) (Rosel et al. 1995). PCR reactions were carried out in a 10 µl final volume reaction containing 1x PCR Buffer, 2 mM MgCl2, 0.2 mM DNTPs, 0.5 units of BIOTAQ DNA Polymerase (Bioline) and 0.4 µM of each primer. Cycling conditions were: 2 min at 95º C, 20 cycles of 30 sec at 92ºC, 30 sec at 60 - 50ºC (decreasing 0.5ºC per cycle) and 45 sec at 72ºC, 19 cycles of 30 sec at 92ºC, 30 sec at 50ºC and 45 sec at 72ºC, followed by a final extension at 72ºC for 2.5 min. PCR products were purified using QIAquick PCR purification columns (Qiagen) according to the manufacturer’s protocol. DNA sequencing was performed using the primer L15926 on an ABI3700 automated DNA sequencer (Applied Biosystems, CA, USA), according to the manufacturer’s instructions.Ambiguous sequences were re-sequenced using the reverse primer H16498. All haplotypes were confirmed both in forward and reverse directions.

Data obtained from this study were augmented with previously published mtDNA control region haplotypes of pilot whales from the north Atlantic (n = 66, U20926 and U20927; Siemann 1994).

**Biogeochemical analyses**

**Fatty acids**

Inner blubber (i.e. portion of blubber situated approximately 0 – 1 cm above the muscle, Samuel & Worthy 2004) was collected from thawed tissue samples, for fatty acid analysis. Lipids were extracted from the inner blubber of 56 cetaceans, using a modified Folch method (Folch et al. 1957).

Before esterification, lipid classes were measured in blubber samples to test for indications of decomposition, such as the presence of a high level of free fatty acids. For this purpose, High Performance Thin-Layer Chromatography (HPTLC) using hexane:diethyl ether (8:2, v/v) was performed. Although most of the samples showed a very high percentage of triacylglycerols, a small number of blubber samples presented high levels of free fatty acids, a potential sign of degradation, and these were excluded from further analysis.

Fatty acid methyl esters (FAMEs) were prepared directly from 10 mg of extracted lipid, using 1% (v/v) sulphuric acid in methanol, at 50ºC for a minimum of 12 h. FAMEs were analyzed by gas chromatography using a Hewlett-Packard 6890 gas chromatograph, equipped with a flame-ionization detector (GC-FID) and fitted with a fused silica capillary column (30 m x 0.25 mm internal diameter, J & W Scientific Inc. California, USA). Quality assurance procedures for the fatty acid analysis included the use of standard reference materials (LRM 144 and LRM 145), calibration and method standard (EO23) and solvent blanks. The FAMEs were identified by comparison with standard reference materials, and the normalized area percentage (NA%) was calculated for each fatty acid as a percentage of the total area, for all identified fatty acids. For the monounsaturated acids 18:1, 20:1 and 22:1, the chromatographic area used includes two structural isomers, due to difficulty of separating the isomers in some samples. Fatty acid names used here follow the standard nomenclature of carbon chain length:number of double bonds, with (n-x) indicating the location of the double bonds relative to the terminal methyl group.

**Stable isotopes**

Following the methodology used by Méndez-Fernandez et al. (2012), skin samples of 115 cetaceans were dried in an oven at 50ºC for 48 h and ground to powder. Lipid extraction was performed to avoid biases associated to lipid variation in the animals analyzed, considering that the known depletion of 13C in lipids (e.g. DeNiro & Epstein 1977) would introduce bias to the analysis. Hence, lipid was extracted by agitating approximately 100 mg of powder with 4 ml of cyclohexane, for 1 h, followed by a centrifugation at 4000 g for 5 min. The supernatant was discarded. Samples were then dried in an oven at 45ºC for 48h, and subsamples of the lipid-free powder were weighed in tin cups for stable isotope analyses.

The stable isotope analyses were performed on an elemental analyzer 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 δ13C and atmospheric N2 for δ15N, in parts per thousand (‰). Replicate measurements of internal laboratory standards (acetanilide) indicated that measurement errors were ± 0.15 and ± 0.2 ‰ for δ13C and δ15N, respectively.

**Statistical analyses**

**Genetic analysis**

Sequence variation and alignment was performed using Clustal W (Thompson et al. 1997) and MEGA 6.0 (Tamura et al*.* 2013). Sequences were confirmed as mitochondrial control region by the National Center for Biotechnology Information (NCBI) BLAST comparison. In order to allow for direct comparisons with sequences available in GenBank, the size of the sequences obtained for the north Atlantic samples were truncated to 347 bp. All the variable sites detected within the 400 bp amplicon were also within the shorter fragment. Nucleotide(π) and haplotypic (h) diversities (Nei 1987) were estimated for each region and for the entire set of the north Atlantic samples, using ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010).

Genetic structure in the north Atlantic was tested through pairwise comparisons and an Analysis of Molecular Variance (AMOVA, Excoffier et al*.* 1992), using ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010). In pairwise analysis, FST (Weir & Cockerham 1984) was estimated to assess the divergence between the sequences. Statistical significance of FST was calculated using 20000 permutations of haplotypes among regions (Fisher’s exact test). Given that sample sizes vary considerably among populations, which can introduce bias into the analysis, FST estimates were recalculated after randomly sampling equal numbers of individuals (n = 23, set to the smallest sample size) from the original populations. This process was repeated 100 times and the 95% confidence intervals around the mean pairwise FST values were obtained.

For the Analysis of Molecular Variance (AMOVA), a hierarchical assessment of structure was examined, partitioning variance between western and eastern Atlantic, between regions within each side of the Atlantic (western: USA; eastern: FI, UK, NWIP) and among individuals within regions. In addition, pairwise genetic distances among sampling regions were computed in MEGA 6.0 (Tamura et al. 2013), using Tamura-Nei model (Tamura & Nei 1993).

A Median Joining Network was constructed for the mitochondrial haplotypes, using NETWORK 4.6 (Bandelt et al. 1999). The transition:transversion ratio was set to 1:3, deletions weighted the same as transversions and epsilon was set to 10.

**Fatty acids**

A total of 24 FAs was routinely identified in all pilot whales. However, the number of FAs identified exceeded the number of individual animals present in the smallest group used in the analysis (number of animals ranged from 12 to 26). Therefore, two criteria were applied to reduce the number of FAs to be used in the multivariate analysis: 1) only FAs with proportions > 0.4% were selected, to avoid misidentification of fatty acids found at low or trace levels (Iverson et al. 2004) and 2) if normalized areas of two FAs were highly correlated (Pearson’s r > 0.8, Zuur et al. 2007), one of them was discarded. Thus, the 12 FAs selected for use in the statistical analysis were: 14:0, 16:0, 16:1 (n-7), 16:2(n-6), 18:0, 18:1, 18:2(n-6), 18:4(n-3), 20:4(n-6), 20:5(n-3), 22:1 and 22:6(n-3). This subset of FAs comprised 82.7% of the normalized area of the total FAs.

Redundancy Analysis (RDA) was used to visualize any relationships between the set of response variables (FAs) and sampling region (categorical), sex (categorical) and length of pilot whales (continuous), using the function rda in the package *vegan* (Oksanen et al. 2011) with 9999 permutations (see Zuur et al. 2007; Legendre & Legendre 2012). No interaction of explanatory variables was tested, due to small sample size within categories.

For the independent categorical variables presenting significant values in the RDA, a forward-stepwise LDA was performed to assess which FA subset optimally separated the pilot whales by group. Assumptions of LDA were tested: multivariate normality (Dagniele test = 0.982, p > 0.1; Legendre & Legendre 2012) and homogeneous covariance matrices between groups (F = 0.58, p > 0.1 for region; Anderson 2006; package vegan*,* Oksanen et al. 2011). Results indicated there was no need to transform the variables. For the LDA, the forward selection algorithm selects, at each step, the variable that minimizes the overall Wilk’s lambda. This was carried out using the package klaR(Weihs et al*.* 2005). The prediction accuracy of the final model was evaluated by a jack-knifing procedure (leave-one-out cross-validation) using the function lda of the R package MASS (Venables & Ripley 2002). All the analyses were performed using R v.3.1.1 (R Core Team 2014).

**Stable isotopes**

The mean isotopic composition in skin, its standard deviation (SD) and 95% confidence intervals were calculated for pilot whales.

To determine which explanatory variables influence δ13C and δ15N values in the skin of pilot whales, Generalized Least Squares (GLS) were applied, using the package nlme (Pinheiro et al. 2014). GLS allows for the incorporation of variable heterogeneity into the models (Zuur et al. 2009). Since the two response variables were continuous and appeared to have an approximately normal distribution, a Gaussian probability distribution was applied. The explanatory variables included as fixed factors were pilot whale sex (categorical), length (continuous) and sampling region (categorical). If the final model included categorical variables, contrasts were constructed to perform pairwise tests and a Bonferroni correction was applied as an adjustment of critical p-values due to multiple comparisons, using the package multcomp (Hothorn et al. 2008). The model fitted for δ13C included a variance structure related to sampling region (VarIdent) in the error term, to account for the heteroscedasticity observed in the residuals in relation to this variable, while the model fitted for δ15N included variance structures for both region (VarIdent) and length (VarComb). No random factor was defined, since the variable sampling region does not have a sufficient number of levels (Zuur et al. 2009). Before entering the explanatory variables, we used generalized additive models (GAM), restricting smoother complexity by limiting number of knots to five, to visually check the linearity assumption of the variables, using the package mgcv (Wood 2014). Non-linear variables, which improved the fitness of the model, were included as quadratic terms in the GLS model. All models were estimated using restricted maximum likelihood (REML). The best fitting model was selected using a likelihood ratio test (L) in combination with the Akaike Information Criterion value (AIC), using a backward selection of nested models. 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). All the analyses were performed using R v.3.1.1 (R Core Team 2014).

**Results**

**Genetic analysis**

A total of five polymorphic sites (2 deletions and 3 transitions) defined six haplotypes across the four regions in the north Atlantic (Table 2, Fig. 1). The haplotypes E and G had not been previously described in pilot whales (Genbank accession numbers: KC934933-34), but A, B, C and F have already been identified in previous studies in the north Atlantic (A, B and C correspond to GenBank GMU20926, GMU20928 and GMU20927, respectively; Siemann 1994) and Pacific (F corresponds to GenBank FJ513345, Oremus et al. 2009) (Table 2).

Overall, haplotype and nucleotide diversities were 0.47 ± 0.04 and 0.17% ± 0.15%, respectively (Table 3). Within the north Atlantic, the UK presented the highest nucleotide diversity (π = 0.16% ± 0.15%), followed by NWIP and the FI, while the highest haplotype diversity was seen in the NWIP (h = 0.42 ± 0.08) (Table 3). The USA showed the lowest values for both nucleotide and haplotype diversities (h = 0.03 ± 0.03; π = 0.02 ± 0.04) (Table 3).

There is evidence of genetic differentiation within the north Atlantic (FST = 0.63, p < 0.001). The AMOVA showed no differentiation, either using haplotype or nucleotide differences, between the eastern and western Atlantic (FST = 0.16, p > 0.05), highlighting that most of the genetic variance occurred among regions within east Atlantic (45.3%; FI, UK, NWIP) rather than between eastern and western Atlantic (16%). This result is in agreement with pairwise regional comparisons, that show high and significant levels of differentiation among regions, except between the United Kingdom and the United States of America (FST = 0.09, p < 0.05,Table 4). When accounting for the potential bias associated to different sample sizes, FST recalculated after sample randomization showed no qualitative and little quantitative effect on the overall patterns of divergence, with all pairwise estimates remaining high and significant, except UK-USA (Table 4). Pairwise genetic distances ranged from 0% for USA to UK, to 0.4% for NWIP – FI, with the remaining pairwise comparisons showing similar values (0.2%) (Table 4).

The network of mtDNA haplotype differences supports the results from AMOVA, since no clear separation occurs between the west and east Atlantic, mainly due to haplotype A (n = 118), the most common control region haplotype found (Table 2, Fig. 1). Haplotype A is the only haplotype shared between both sides of the Atlantic, and by all the sampling regions analyzed (Table 2, Fig. 1), which together with its position in the network, suggests that it corresponds to the ancestral haplotype. There is a high variation in haplotype frequencies distribution among sampling regions, especially in the east Atlantic. Haplotypes C and F are almost exclusive to FI and NWIP, respectively, with these regions sharing only haplotype A among them (Table 2 and Fig. 1). Additionally, haplotype E is almost exclusive to the UK, which shares haplotypes with all the remaining sampling regions (Table 2 and Fig. 1). At last, haplotype B is exclusive to USA.

**Fatty acids**

Overall, the FA profiles of the pilot whales were generally high in monounsaturated FA (MUFA, 55.72% ± 7.29%), with saturated FA (SFA) and polyunsatured FA (PUFA) showing lower contributions (22.92% ± 2.59% and 20.78% ± 7.04%, respectively) (Table S1, Supplementary Material). The predominant FAs were 18:1, 16:0, 22:6(n-3), 22:1 and 20:1, with clear variation between the different regions, notably for 20:1 and 22:1, which showed low values for the NWIP compared to other regions, and for 20:4(n-3) which showed high values in the NWIP. In addition, several FAs showed high variability within regions (Table S1, Supplementary Material).

The set of explanatory variables used in the RDA explained 41.5% of the total variation in pilot whale fatty acids, with axes 1 and 2 accounting for 32.4% and 7.9% of the variation, respectively. Although some caution is needed, since the first two RDA axes only explain 40.3% of the variation in fatty acid profiles, the first axis of the RDA contrasts 22:1 against 16:1 (n-7), 18:1 and 22:6(n-3), while the second axis opposes 16:1(n-7) against 18:1 (Fig. 2). The three regions analyzed in this study are well separated in the RDA, suggesting differences in FA profiles in these regions, especially with respect to the higher values of 16:1(n-7) present in animals from USA (Fig. 2). Length of the animal is negatively correlated with16:1 (n-7) (Fig. 2).Significance tests confirm effects of sampling region (F = 13, p < 0.001) and length of the animal (F = 7.2, p < 0.001) but no influence of sex (F = 2.3, p > 0.05) on fatty acid signatures.

Linear Discriminant Analysis (LDA) was used to determine which fatty acids best defined each region and a clear separation was obtained with a model based on the proportions of 16:0, 16:1(n-7), 16:2(n-6), 18:1, 18:2(n-6), 18:4(n-3), 20:4(n-6) and 20:5(n-3) (overall p < 0.001; Fig. 3). The 1st discriminant function mostly separated NWIP profiles from those in other regions, mainly because of the higher proportion of 20:4(n-6) in Iberian samples and 18:4(n-3) in the UK/USA group, while the 2nd discriminant function separated UK and USA, based on the proportions of 16:2(n-6) (higher in UK samples) and 16:1(n-7) and 20:5(n-3) (higher proportions in whales from the USA) (Table S2, Supplementary Material). A slight overlap occurred between individuals from the UK and the USA.

The ability of the model to predict sampling regions based on these eight FAs was tested using cross-validation, which achieved a correct assignment of 96.5% of blubber samples to their respective region. Results indicated a correct assignment of 100%, 92.3% and 100% for NWIP, UK and USA samples, respectively. The low misclassification rate (two UK samples incorrectly classified as USA), demonstrates that, among the north Atlantic samples included in the current study, pilot whale location can be determined with acceptable reliability from fatty acid analysis of inner blubber.

**Stable isotopes**

Overall, mean values of δ13C and δ15N in the skin of pilot whales from the north Atlantic were -18.3 ± 0.8‰ and 12.0 ± 1.0‰, respectively. The specimens from NWIP showed the highest values of δ13C (-17.7 ± 0.7‰), while both USA and FI animals presented intermediate values, and low values were seen in the UK whales (-18.7 ± 0.7‰) (Fig. 4). The lowest variability in both isotopes was found in animals from the Faroe Islands.

The best performing GLS model for δ13C revealed a significant effect of sampling region, when the heterogeneity of this explanatory variable was taken into account (F = 11.30, p < 0.001), since adding the variance structure related to sampling region to this model yielded significant improvement (likelihood ratio p = 0.001). This model supports differences among the studied regions of the north Atlantic. Pairwise analyses showed significant differences among some of the regions, except between NWIP and FI (Tukey test = -2.39, p > 0.05), NWIP and USA (Tukey test = -2.63, p = 0.05) and USA and FI (Tukey test = 0.88, p > 0.05). There were no significant effects of either sex or length of pilot whales on δ13C values. The inclusion of these variables increased the AIC and decreased the significance of the likelihood ratio; therefore they were excluded from the final model (model 3, Table 5).

Regarding δ15N, high levels of intra-specific variability were observed, with the highest values presented by USA animals (13.3 ± 0.7‰) and the lowest again exhibited by UK whales (11.3 ± 0.6‰) (Fig. 4). The best performing GLS model of δ15N revealed significant effects of sampling region (F = 70.53, p < 0.001) and length (as a quadratic term, F = 24.71, p < 0.001), when the heterogeneity of variance for these explanatory variables was taken into account. Adding the variance structure related to region and length to this model yielded a significant improvement of the model fit (likelihood ratio p = 0.001). 71% of pilot whales with body length less than 219 cm were N-enriched compared to larger animals, although a slight increase was also verified for animals measuring around 400 cm. As for the effect of sampling region on δ15N, pairwise analyses showed significant differences among most of the regions, except UK and FI (Tukey test = 1.74, p > 0.05). There was no significant effect of sex of pilot whales on δ15N values and this variable was, therefore, excluded from the final model (model 2, Table 5).

**Discussion**

Understanding population structure within wild species is crucial for identifying their behavioural, ecological and genetic diversity (Coyle 1998), as well as supporting informed conservation management. Although the combination of different methodologies produces no clear definition of management units for pilot whales in the north Atlantic, this study showed that both genetic and biogeochemical markers support the occurrence of differentiated units of pilot whales in this oceanic basin.

**Genetic markers**

The detection of three new mitochondrial haplotypes in the north Atlantic increased haplotype and nucleotide diversity values when compared to those described by Siemann (1994) and Oremus et al. (2009). The striking differences in haplotype distribution observed, together with genetic distances and FST values suggest considerable levels of differentiation between most of the regions analyzed. In particular, genetic differences were evident in the northeast Atlantic, where some constraints to gene flow seem to occur, especially between northern (FI) and southern (NWIP) regions. Patterns of mitochondrial genetic differentiation in pilot whales from the north Atlantic are difficult to resolve, since it would be expected that neighbouring regions would be genetically more similar, based on the “isolation by distance” (IBD) mechanism (Wright 1943). Instead, in the present study, the strongest genetic similarity occurred between USA and UK. The main challenge is to associate the observed spatial patterns of genetic divergence to levels of gene flow between populations, since several sources of bias may prevent simply equating lower differentiation with higher dispersal.

The first source of bias relates to sex-biased dispersal. There is compelling evidence for the occurrence of natal group philopatry in pilot whale (e.g. Amos et al*.* 1993, Caurant et al. 1993, Fullard et al. 2000). However, males do not father offspring from the same pod, being able to mate only when two pods meet or when males perform short-term dispersal in order to reproduce (Amos et al. 1993, Andersen & Siegismund 1994), resulting in groups of “multiple matrilines” (e.g De Stephanis et al. 2008b, Oremus et al. 2013). Therefore, sex-biased dispersal and high levels of female philopatry may influence the high levels of genetic divergence seen in the maternally inherited haploid marker for pilot whales.

Another source of bias relates to the influence of historical vs. contemporary processes involved in the patterns of gene flow and genetic divergence. Historical processes, such as past global climate changes can lead to genetic splits between populations that currently do not seem to have obstacles to gene flow (Avise 2009). While the similarity between western and eastern north Atlantic revealed by AMOVA may reflect retained common ancestry after glacial ages (e.g Last Glacial Maximum), the results from northeast Atlantic may reflect ancestral movement limitations due to past climate changes, followed by local adaptation, as previously described for harbour porpoise in this oceanic basin (Fontaine et al. 2010, 2014).

Concerning contemporary processes, genetic differentiation may also be biased by behavioural traits or ecological processes. Recent studies have suggested the occurrence of “isolation by environmental distance” (IBED), in which the environmental distance between populations correlates with their genetic separation (Mendez et al. 2010). In fact, distinct environmental conditions have been suggested as barriers to gene flow between populations of cetaceans (e.g. Fullard et al. 2000, Rosenbaum et al. 2009, Mendez et al. 2010). Both FI and NWIP are influenced by distinctive oceanographic phenomena, such as the convergence of warm and subpolar waters in the former (reviewed in Hatún et al. 2009) and the occurrence of upwelling in the later (Figueiras et al. 2002). These features may influence prey and pilot whales movements (Hatún et al. 2009) and lead to potential discontinuities in gene flow, particularly when associated with social structure and resource specialization which may be key determinants of levels of contemporary population structure in cetaceans (Foote et al. 2009). Thus, specialized strategies for the exploitation of local resources, such as benthic prey in NWIP vs. pelagic prey in FI and UK (present study, Santos et al. 2014, Monteiro et al. 2015) may involve some social learning (e.g. Krutzen et al. 2005, Hoelzel et al. 2007,Pilot et al. 2010), leading to a potential reduction in an individual’s fitness if it disperses from a natal habitat, and therefore, also reducing the gene flow between sampling regions.

**Biogeochemical markers**

The differentiation of pilot whales in the north Atlantic revealed by mtDNA was only partially mirrored in biogeochemical markers. These markers are relevant at a shorter timescale and primarily provide information about ecological and trophic processes underlining intra-specific diversity distribution, which may ultimately result in population structuring.

Although fatty acids were more in accordance to mtDNA results on population differentiation, both stable isotope and fatty acid signatures indicated the occurrence of geographical differences in pilot whales from the north Atlantic. Previous studies examining stomach contents, stable isotopes and habitat use provided insights regarding preferences of pilot whales in different regions. In NWIP, pilot whales seem to inhabit mainly neritic habitats and exhibit a preference for benthic octopus (e.g. Pierce et al*.* 2010, Santos et al. 2014, Monteiro et al. 2015). In USA they perform seasonal inshore-offshore movements (e.g. Payne & Heinemann 1993) and prefer demersal squids(*Loligo pealei*) and epipelagic fish (*Scomber scombrus*) (Gannon et al. 1997). Off the UK and FI, pilot whales exhibit oceanic preferences in terms of habitat and prey species, since they mostly occur off the continental shelf in both locations (Bloch et al. 2003, MacLeod et al. 2007) and oceanic pelagic squids (*Todarodes sagitattus*, *Gonatus sp.*)are the predominant prey (Desportes & Mouritsen 1993, Santos et al. 2014).

The geographical differences in prey species and habitat use revealed by both biogeochemical markers in the present study, support the trophic and habitat preferences previously described for this species. Most of the FAs responsible for separation of different regions of the north Atlantic were of dietary origin, although some could also be biosynthesized by the predator (Iverson et al. 2004). A higher proportion of the dietary FA arachidonic acid (AA, 20:4(n-6), Iverson et al. 2004) was observed in Iberian animals compared to remaining regions. AA is proposed as a marker of benthic and coastal feeding (Piché et al. 2010) and an inherent characteristic in octopus (e.g. Navarro & Villanueva 2000), confirming the preference for octopus by whales in Iberia, as verified in previous dietary analysis (Gannon et al. 1997, Santos et al. 2014), while they may be feeding on other prey species in UK and USA.

Stable isotope analysis indicates that pilot whales exhibit some degree of dietary plasticity in their foraging areas and prey consumed, evident in the variability shown by δ13C and δ15N. The only exception refers to animals from FI, whose variability most likely reflects within-pod rather than within-region SI variability since animals analyzed were all captured together and may, therefore, have belonged to the same pod. Generally, stable isotope results are in agreement with the habitat and dietary preferences of pilot whales described above. Significant 13C-depletion was observed in animals from the UK relative to other regions, which may be attributed to the exploitation of oceanic habitats or ingestion of relatively 13C-depleted resources. Contrastingly, Iberian pilot whales seem to be feeding on more coastal habitats and/or benthic prey. Similarities between NWIP-USA may relate to inshore movements performed at least occasionally by pilot whales in those locations. This could also be the explanation for the similar values of δ13C between NWIP-FI and FI-USA, since Desportes and Mouritsen (1993) suggested that in FI this species may feed between 100 - 500m depths. However, δ13C values in FI may also be masked by the low variability detected in that region, since most previous studies describe pilot whales from FI as oceanic animals. Theδ15N values indicate that different trophic levels of prey are being targeted in most study regions, except in FI and UK, which is not surprising considering the similar diet reported for whales in both locations (Desportes & Mouritsen 1993, Santos et al. 2014, Monteiro et al. 2015).

The differences detected between regions, in terms of prey consumed and foraging habitats reflect trophic regime differences across the geographic range analyzed and some level of feeding niche separation may be associated with coastal vs. oceanic feeding habits where prey movements and oceanographic features (gyres, upwellings, topography) may play an important role. Some studies showed the influence of oceanographic phenomena on pilot whale distribution, which may be mediated by environmental effects on the abundance of target prey species. Thus, there seem to be links between the abundance of pilot whales in FI and NWIP, their main preyand the marine climate in those regions (e.g. subpolar and subtropical gyres in FI and upwelling in NWIP) in a bottom-up process (Hátún et al. 2009, Monteiro 2014).

Oceanographic phenomena, such as the upwelling occurring in NWIP or the convergence of warm and cold currents around FI, may also influence the isotopic baseline through increased phytoplankton growth rates (and higher δ13C values) (Pancost et al. 1997). Differences in nutrient cycling at the base of food web may produce spatial and temporal isotopic baseline variation, at oceanic scales (McMahon et al. 2013). Such variation presents a challenge for stable isotope studies on consumers since it makes it hard to distinguish differences due to natal habitats with different baseline isotopic values from those due to shifts in foraging ecology (Post 2002, McMahon et al. 2013). Based on isoscapes, the δ15N and δ13C baseline geographic gradients observed in north Atlantic (McMahon et al. 2013) could be responsible for an increment in the difference of stable isotope values between the regions analyzed in the present study, leading to a stronger ecological differentiation. However, this would not explain the similarity between FI and NWIP or NWIP and USA isotopic niches, especially when the zooplankton organic δ13C seems to vary around 2‰ between those pairs of locations (McMahon et al. 2013). In addition, fatty acids are not influenced by isotopic baselines, so there may be other reasons, such as the ones described above, for the ecological differentiation found in pilot whales.

In addition to geographic sources of variation in feeding habits of pilot whales, intrinsic factors such as the sex or length of the animal may also influence biogeochemical signatures.As a size-dimorphic species (Bloch et al. 1993), differences in diet could be expected in pilot whale, in order to fulfil the higher energy requirements of the larger sex.However, in the present study, no evidence of sex differences in foraging habits was found. This is in agreement with previous stomach contents and stable isotope analyses (De Stephanis et al.2008a, Santos et al*.* 2014). In contrast, there was a significant effect of the length of pilot whales on fatty acid profiles and δ15N values. Individuals smaller than 239cm (weaning stage, Sergeant 1962) showed the expected higher than average δ15N isotopic values of un-weaned individuals (Hobson et al. 1997). However, separation of un-weaned and weaned pilot whales was not so evident in fatty acid analysis. The length of pilot whales was negatively correlated with the relative abundance of a FA originated either from diet or biosynthesis (16:1(n-7), Iverson et al. 2004) and a dietary FA (20:5(n-3), Iverson et al. 2004), suggesting an effect of animal size on fatty acid biosynthesis or dietary variation with animal length, confirming previous dietary analysis (Desportes & Mouritsen 1993, Santos et al. 2014).

**Multi - approach strategy**

The importance of defining genetic and ecological diversity and stocks has been the subject of much debate (ICES 2014). The impact of localized anthropogenic threats depends on population dynamics and, as such, reproductive isolation is the fundamental basis for identification of true “stocks” or “populations”. Neither genetic nor biogeochemical markers are foolproof in this sense: reproductive isolation may have occurred too recently to be reflected in some genetic markers, while several ecological stocks may occur within a population (ICES 2014). In the present study, although there is clear evidence of population structure, based on genetic and biogeochemical markers in pilot whales from the north Atlantic, it may be difficult to define robust stocks to be used in a management context.

While combining results from different methodologies may provide a more complete picture of the population ecology and structure of wild species, it may also reveal the difficulty of objectively defining stocks, since different types of approaches do not necessarily return identical patterns of structure. First, the biases associated to each methodology may preclude the achievement of congruent results. Additionally, the understanding of biogeochemical tracers may be hampered by bioavailability, spatio-temporal variations in the food webs (Newsome et al. 2010, McMahon et al. 2013) or biological factors such as sex, growth, dietary shifts, metabolism and physiology of the individuals (e.g. Vanderklift & Ponsard 2003). Furthermore, mtDNA may be influenced by historical or contemporary processes influencing the levels of gene flow and the occurrence of sex-biased dispersal, which may difficult the combination with contemporary ecological results. Another bias relates with the occurrence of local social structure that may confound population genetic and ecological structure if the diversity of local samples does not reflect the one from the underlying population, which may be the case of whales from FI where variability described most likely reflects within-pod rather than within-region variability.

Integration of ecological and genetic methodologies allowed the evaluation of pilot whale diversity and differentiation in the north Atlantic. Biogeochemical markers (SI) showed higher similarity between most sampling regions, when compared to genetic analysis indicating that, at least partially, the observed genetic differences are not mirrored by ecological differences. Both genetic and biogeochemical markers (SI) suggest similarities between the western and eastern sides of north Atlantic. The differences between genetic and biogeochemical markers highlight the importance of using complementary tools to detect putative differentiation (Coyle 1998), but also highlight to the difficulties associated to the interpretation of data different approaches.

Further studies including additional genetic markers (nuclear and adaptive markers) would be helpful to determine processes involved in contemporary population structure and understand the role of natural selection in the adaptation to environmental gradients and potential inhibition of haplotypes exchange between regions. Additionally, the inclusion of more samples and intermediate sampling regions such as Bay of Biscay, English Channel, Greenland would increase the accuracy of statistics, provide a more complete knowledge of ecological and contemporary genetic diversities and differentiation of pilot whales in north Atlantic, while helping to detect potential migratory routes and define stock boundaries.

**Acknowledgments**

Cetacean samples were collected under the auspices of strandings monitoring programs run by Sociedade Portuguesa de Vida Selvagem, Coordinadora para o Estudio dos Mamíferos Mariños (supported by the regional government Xunta de Galicia), the UK Cetacean Strandings Investigation Programme and the Scottish Agriculture College Veterinary Science Division (jointly funded by Defra and the Devolved Governments of Scotland and Wales), the Marine Mammals Research Group of the Institute of Marine Research (Norway), the Museum of Natural History of the Faroe Islands and the International Fund for Animal Welfare Marine Mammal Rescue & Research Program (USA). The authors thank all the members of these institutions/organisations for their assistance with data and sample collection. SSM, PMF and MF were supported by Ph.D. grants from Fundação para a Ciência e Tecnologia (POPH/FSE ref SFRH/BD/38735/2007, SFRH/BD/36766/2007 and SFRH/BD/30240/2006, respectively). AL was supported by a postdoctoral grant from Fundação para a Ciência e Tecnologia (ref SFRH/BPD/82407/2011). The work related with strandings and tissue collection in Portugal was partially supported by the SafeSea Project EEAGrants PT 0039 (supported by Iceland, Liechtenstein and Norway through the EEA Financial Mechanism), by the Project MarPro–Life09 NAT/PT/000038 (funded by the European Union–Program Life+) and by the Project CetSenti FCT RECI/AAG-GLO/0470/2012; FCOMP-01-0124-FEDER-027472 (Funded by the Program COMPETE and Fundação para a Ciência e Tecnologia). GJP thanks the University of Aveiro and Caixa Geral de Depósitos (Portugal) for financial support. The authors would like to acknowledge the assistance of the chemical analysts at Marine Scotland Science with the fatty acid analysis

**References**

Abend AG, Smith TD (1995) Differences in ratios of stable isotopes of nitrogen in long-finned pilot whales (*Globicephala melas*) in the western and eastern North Atlantic. ICES J Mar Sci 52: 837–841

Amos B, Schlotterer C, Tautz D (1993) Social-Structure of Pilot Whales Revealed by Analytical DNA Profiling. Science 260:670-672

Andersen LW, Siegismund HR (1994) Genetic evidence for migration of males between schools of the long-finned pilot whale *Globicephala melas*. Mar Ecol Prog Ser 105:1-7

Anderson MJ (2006) Distance-Based Tests for Homogeneity of Multivariate Dispersions. Biometrics 62:245–253

Avise JC (2009) Phylogeography: retrospect and prospect. J Biogeogr 36:3–15

Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. Mol Ecol 13:729-744

Bandelt H-J, Forster P, Rohl A (1999) Median-Joining Networks for Inferring Intraspecific Phylogenies. Mol Biol Evol 16:37-48

Bloch D, Heide-Jorgensen MP, Stefansson E, Mikkelsen B, Ofstad LH, Dietz R, Andersen LW (2003) Short-term movements of long-finned pilot whales *Globicephala melas* around the Faroe Islands. Wildl Biol 9:47-58

Bloch D, Lastein L (1993) Morphometric segregation of long-finned pilot whales in eastern and western North Atlantic. Ophelia 38:55-68

Bloch D, Lockyer C, Zachariassen M (1993) Age and growth parameters of the long-finned pilot whale off the Faroe Islands. Report of International Whaling Commision Special Issue 4:163-207

Caurant F, Amiard-Triquet C, Amiard JC (1993) Factors influencing the accumulation of metals in pilot whales (*Globicephala melas*) off te Faroe Islands. Reports of the International Whaling Commission Special Issue 14:369-390

Coyle T (1998) Stock identification and fisheries management: the importance of using several methods in a stock identification study. In: Hancock DA (ed) Taking Stock: defining and managing shared resources. Australian Society for Fishery Biology, Sydney

De Stephanis R, Garcia-Tiscar S, Verborgh P, Esteban-Pavo R, Perez S, Minvielle-Sebastia L, Guinet C (2008a) Diet of the social groups of long-finned pilot whales (*Globicephala melas*) in the Strait of Gibraltar. Mar Biol 154:603-612

De Stephanis R, Verborgh P, Perez S, Esteban R, Minvielle-Sebastia L, Guinet C (2008b) Long-term social structure of long-finned pilot whales (*Globicephala melas*) in the Strait of Gibraltar. Acta Ethologica 11:81-94

DeNiro M, Epstein S (1980) Influence of diet on the distribution of nitrogen isotopes in animals. Geochimica et Cosmochimica Acta 45:343-351

DeNiro MJ, Epstein S (1977) Mechanism of carbon isotope fractionation associated with lipid synthesis. Science 197:261-263

DeNiro MJ, Epstein S (1978) Influence of diet on the distribution of carbon isotopes in animals. Geochim Cosmochim Acta 42:495-506

Desportes G, Mouritsen R (1993) Preliminary results on the diet of long-finned pilot whales off the Faroe Islands. Report of International Whaling Commision:305-324

Eggert LS, Lux CA, O'corry-Crowe GM, Dizon AE (1998) Dried dolphin blood on fishery observer records provides DNA for genetic analyses. Mar Mamm Sci 14:136–143

Evans PGH, Teilmann J (2009) Report of ASCOBANS/HELCOM Small Cetacean Population Structure Workshop. ASCOBANS, Bonn, Germany

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564-567

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of Molecular Variance Inferred From Metric Distances Among DNA Haplotypes: Application to Human Mitochondrial DNA Restriction Data. Genetics 131:479-491

Figueiras FG, Labarta U, Reiriz MJF (2002) Coastal upwelling, primary production and mussel growth in the Rías Baixas of Galicia. Hydrobiologia 484:121–131

Folch J, Lees M, Stanley GHS (1957) A Simple Method For the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497-509

Fontaine MC, Baird SJ, Piry S, Ray N, Tolley KA, Duke S, Jr AB, Ferreira M, Jauniaux T, Llavona Á, Öztürk B, Öztürk AA, Ridoux V, Rogan E, Sequeira M, Siebert U, Vikingsson GA, Bouquegneau J-M, Michaux JR (2007) Rise of oceanographic barriers in continuous populations of a cetacean: the genetic structure of harbour porpoises in Old World waters. BMC Biology 5:doi:10.1186/1741-7007-1185-1130

Fontaine MC, Tolley KA, Michaux JR, Birkun A, Ferreira M, Jauniaux T, Llavona Á, Öztürk B, Öztürk AA, Ridoux V, Rogan E, Sequeira M, Bouquegneau J-M, Baird SJE (2010) Genetic and historic evidence for climate-driven population fragmentation in a top cetacean predator: the harbour porpoises in European water. Proc R Soc B 277: 2829–2837

Fontaine MLC, Roland K, Calves I, Austerlitz F, Palstra FP, Tolley KA, Ryan s, Ferreira M, Jauniaux T, Llavona A, Ozturk B, Ozturk A, Ridoux V, Rogan E, Sequeira M, Siebert U, Vikingsson GA, Borrel A, Michaux JR, Aguilar A (2014) Postglacial climate changes and rise of three ecotypes of harbour porpoises, *Phocoena phocoena*, in western Palearctic waters. Mol Ecol 23:3306–3321

Foote AD, Newton J, Piertney SB, Willerslev E, Gilbert MTP (2009) Ecological, morphological and genetic divergence of sympatric North Atlantic killer whale populations. Mol Ecol 18:5207–5217

Frankham R, Ballou J, Briscoe D (eds) (2002) Introduction to conservation genetics. Cambridge University, Cambridge

Fullard KJ, Early G, Heide-Jorgensen MP, Bloch D, Rosing-Asvid A, Amos W (2000) Population structure of long-finned pilot whales in the North Atlantic: a correlation with sea surface temperature? Mol Ecol 9:949–958

Funk DJ, Nosil P, Etges WJ (2006) Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. PNAS 103: 3209–3213

Gannon DP, Read AJ, Craddock JE, Fristrup KM, Nicolas JR (1997) Feeding ecology of long-finned pilot whales *Globicephala melas* in the western north Atlantic. Mar Ecol Prog Ser 148:1-10

Hátún H, Payne MR, Beaugrand G, Reid PC, Sandø AB, Drange H, Hansen B, Jacobsen JA, Bloch D (2009) Large bio-geographical shifts in the north-eastern Atlantic Ocean: From the subpolar gyre, via plankton, to blue whiting and pilot whales. Progress in Oceanography 80:149–162

Hobson KA, Clark RG (1992) Assessing Avian Diets Using Stable Isotopes I: Turnover of 13C in Tissues. The Condor 94:181-188

Hobson KA, Sease JL, Merrick RL, Piatt JF (1997) Investigating trophic relationships of pinnipeds in Alaska and Washington using stable isotope ratios of nitrogen and carbon. Mar Mamm Sci 13:114-132

Hoelzel AR (1998) Genetic structure of cetacean populations in sympatry, parapatry, and mixed assemblages: Implications for conservation policy. J Hered 89:451-458

Hoelzel AR, Hey J, Dahlheim ME, Nicholson C, Burkanov V, Black N (2007) Evolution of Population Structure in a Highly Social Top Predator, the Killer Whale. Mol Biol Evol 24:1407–1415

Hothorn T, Bretz F, Westfall P (2008) Simultaneous Inference in General Parametric Models. Biometrical Journal 50:346–363

Ices (2014) Report of the Working Group on Marine Mammal Ecology (WGMME). International Council for the Exploration of the Sea. CM 2014/ACOM 27. 234 pp.

Ices (2013) Report of the Working Group on Marine Mammal Ecology (WGMME). International Council for the Exploration of the Sea. CM 2013/ACOM 26. 117 pp.

Ices (2009) Report of the Working Group on Marine Mammal Ecology (WGMME). International Council for the Exploration of the Sea. CM 2009/ACOM 21. 129 pp.

Iucn (2013) Red List of threatened species. available online : [www.iucnredlist.org](http://www.iucnredlist.org)

Iverson SJ, Field C, Bowen WD, Blanchard W (2004) Quantitative fatty acids signature analysis: a new method of estimating predator diets. Ecol Monogr 74:211-235

IWC (1990) Report of the Scientific Committee. Annex H (small cetaceans). Report of the International Whaling Commission 40, 144–157

Krutzen M, Mann J, Heithaus MR, Connor RC, Bejder L, Sherwin WB (2005) Cultural transmission of tool use in bottlenose dolphins. PNAS 102:8939–8943

Kuiken T, Hartmann MG (1991) Cetacean pathology:dissection techniques and tissue sampling. European Cetacean Society Newsletter Nº 17:43

Legendre P, Legendre P (2012) Numerical Ecology, Vol 24. Elsevier, Oxford

Lyrholm T, Leimar O, Johanneson B, Gyllensten U (1999) Sex-biased dispersal in sperm whales: contrasting mitochondrial and nuclear genetic structure of global populations. Proc R Soc Lond B 266:347-354

MacLeod CD, Weir CR, Pierpoint C, Harland EJ (2007) The habitat preferences of marine mammals west of Scotland (UK). J Mar Biol Assoc U K 87:157-164

McMahon KW, Hamady LL, Thorrold SR (2013) A review of ecogeochemistry approaches to estimating movements of marine animals. Limnol Oceanogr 58:697–714

Méndez-Fernandez P, Bustamante P, Bode A, Chouvelon T, Ferreira M, López A, Pierce GJ, Santos MB, Spitz J, Vingada JV, Caurant F (2012) Foraging ecology of five toothed whale species in the Northwest Iberian Peninsula, inferred using carbon and nitrogen isotope ratios. J Exp Mar Biol Ecol 413: 150–158

Mendez M, Rosenbaum HC, Subramaniam A, Yackulic C, Bordino P (2010) Isolation by environmental distance in mobile marine species: molecular ecology of franciscana dolphins at their southern range. Mol Ecol 19:2212-2228

Monteiro S (2014) Population Ecology of Long-finned Pilot Whale (*Globicephala melas*) off the Western Coast of the Iberian Peninsula. PhD thesis, Universidade do Minho, Braga

Monteiro S, Ferreira M, Vingada JV, López A, Brownlow A, Méndez-Fernandez P (2015) Application of stable isotopes to assess the feeding ecology of long-finned pilot whale (*Globicephala melas*) in the Northeast Atlantic Ocean. J Exp Mar Biol Ecol 465:56–63

Moritz C (2002) Strategies to Protect Biological Diversity and the Evolutionary Processes That Sustain It. Syst Biol 51:238–254

Navarro JC, Villanueva R (2000) Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. Aquaculture 183:161–177

Nei M (1987) Molecular Evolutionary Genetics. Columbia University Press, New York

Newland C, Field IC, Nichols PD, Bradshaw CJA, Hindell MA (2009) Blubber fatty acid profiles indicate dietary resource partitioning between adult and juvenile southern elephant seals. Mar Ecol Prog Ser 384:303–312

Newsome SD, Clementz MT, Koch PL (2010) Using stable isotope biogeochemistry to study marine mammal ecology. Mar Mamm Sci 26:509–572

Nordstrom CA, Wilson LJ, Iverson SJ, Tollit DJ (2008) Evaluating quantitative fatty acid signature analysis (QFASA) using harbour seals *Phoca vitulina richardsi* in captive feeding studies. Mar Ecol Prog Ser 360:245-263

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Henry M, Stevens H, Wagner H (2011) Vegan: Community Ecology Package. R package version 2.0-2. <http://CRANR-projectorg/package=vegan>

Oremus M, Gales R, Dalebout ML, Funahashi N, Endo T, Kage T, Steel D, Baker SC (2009) Worldwide mitochondrial DNA diversity and phylogeography of pilot whales (*Globicephala* spp.). Biol J Linn Soc 98:729–744

Oremus M, Gales R, Kettles H, Baker CS (2013) Genetic Evidence of Multiple Matrilines and Spatial Disruption of Kinship Bonds in Mass Strandings of Long-finned Pilot Whales, *Globicephala melas*. J Hered 104:301-311

Pancost RD, Freeman KH, Wakeham SG, Robertson CY (1997) Controls on carbon isotope fractionation by diatoms in the Peru upwelling region. Geochimica et Cosmochimica Acta 61:4983-4991

Payne PM, Heinemann DW (1993) The distribution of pilot whales (*Globicephala* spp.) in shelf/shelf-edge and slope waters of the northeastern United States, 1978-1988. Report of International Whaling Commision Special Issue 14:51-68

Piché J, Iverson SJ, Parrish FA, Dollar R (2010) Characterization of forage fish and invertebrates in the Northwestern Hawaiian Islands using fatty acid signatures: species and ecological groups. Mar Ecol Prog Ser 418:1–15

Pierce GJ, Caldas M, Cedeira J, Santos MB, Llavona Á, Covelo P, Martinez G, Torres J, Sacau M, López A (2010) Trends in cetacean sightings along the Galician coast, north-west Spain, 2003–2007, and inferences about cetacean habitat preferences. J Mar Biol Assoc U K 90:1547–1560

Pilot M, Dahlheim ME, Hoelzel AR (2010) Social cohesion among kin, gene flow without dispersal and the evolution of population genetic structure in the killer whale (*Orcinus orca*). J Evol Biol 23:20-31

Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RC (2014) nlme: Linear and Nonlinear Mixed Effects Models. R package version 31-117 <http://CRAN>

Post DM (2002) Using Stable Isotopes to Estimate Trophic Position: Models, Methods, and Assumptions. Ecology 83:703-718

Quérouil S, Kiszka J, Cordeiro AR, Cascão I, Freitas L, Dinis A, Alves F, Santos RS, Bandarra NM (2013) Investigating stock structure and trophic relationships among island-associated dolphins in the oceanic waters of the North Atlantic using fatty acid and stable isotope analyses. Mar Biol DOI 10.1007/s00227-013-2184-x

R Core Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. www.R-project.org

Rosel PE, Dizon AE, Haygood MG (1995) Variability of the mitochondrial control region in populations of the harbour porpoise, *Phocoena*, on interoceanic and regional scales. Can J Fish Aquat Sci 52:1210-1219

Rosenbaum HC, Pomilla r, Mendez M, Leslie MS, Best PB, Findlay KP, Minton G, Ersts PJ, Collins T, Engel MH, Bonatto SL, Kotze DPGH, Meyer M, Barendse J, Thornton M, Razafindrakoto Y, Ngouessono S, Vely M, Kiszka J (2009) Population Structure of Humpback Whales from Their Breeding Grounds in the South Atlantic and Indian Oceans. Plos One 4: e7318

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning - A Laboratory Manual, 2nd Edition. Cold Spring Habour Laboratory Press, New York

Samuel AM, Worthy GAJ (2004) Variability in fatty acid composition of bottlenose dolphin (*Tursiops truncatus*) blubber as a function of body site, season, and reproductive state. Cannadian Journal of Zoology 82:1933–1942

Santos MB, Monteiro SS, Vingada JV, Ferreira M, López A, martinez-Cedeira J, Reid RJ, Brownlow A, Pierce G (2014) Patterns and trends in the diet of long-finned pilot whales (*Globicephala melas*) in the northeast Atlantic. Mar Mamm Sci 30:1-19

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecol Lett 9:615-629

Sergeant DE (1962) The biology of the pilot or pothead whale *Globicephala melaena* (Traill) in Newfoundland waters. Bulletin of Fisheries Research Board of Canada 132:1-84

Shaw CN, Wilson PJ, N.White B (2003) A reliable molecular method of gender determination for mammals. J Mammal 84:123-128

Siemann LA (1994) Mitochondrial DNA sequence variation in North Atlantic Long-finned Pilot Whales, *Globicephala melas*. PhD Thesis, Massachusetts Institute of Technology, Massachusetts

Tamura K, Nei M (1993) Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial DNA in Humans and Chimpanzees. Mol Biol Evol 10:5 12-526

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30:2725–2729

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876-4882

Vanderklift MA, Ponsard S (2003) Sources of variation in consumer-diet δ15N enrichment: a meta-analysis. Oecologia 136:169–182

Venables WN, Ripley BD (2002) Modern Applied Statistics with S, Vol Fourth Edition. Springer, New York

Weihs C, Ligges U, Luebke K, Raabe N (2005) klaR: analyzing German business cycles. In: Baier D, Decker R, L LS-T (eds) Data Analysis and Decision Support. Springer-Verlag, Berlin

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358-1370

Wiszniewski J, Beheregaray LB, Allen SJ, Moller LM (2010) Environmental and social influences on the genetic structure of bottlenose dolphins (*Tursiops aduncus*) in Southeastern Australia. Conserv Genet 11:1405–1419

Witteveen BH, Straley JM, Chenoweth E, Baker S, Barlow J, Matkin C, Gabriele CM, Neilson J, Steel D, Ziegesar Ov, Andrews AG, Hirons A (2011) Using movements, genetics and trophic ecology to differentiate inshore from offshore aggregations of humpback whales in the Gulf of Alaska. Endanger Species Res 14: 217–225

Wood S (2014) Mixed GAM Computation Vehicle with GCV/AIC/REML smoothness estimation. R package version 31-117 <http://CRAN>

Wright S (1943) Isolation by distance. Genetics 28:114-138

Xu S, Ren W, Zhou X, Zhou K, Yang G (2010) Sequence Polymorphism and Geographical Variation at a Positively Selected MHC-DRB Gene in the Finless Porpoise (*Neophocaena phocaenoides*): Implication for Recent Differentiation of the Yangtze Finless Porpoise? J Mol Evol 71:6-22

Zuur AF, Ieno EN, Smith GM (2007) Analysing Ecological Data. Springer

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM (eds) (2009) Mixed Effects Models and Extensions in Ecology with R. Springer Science

**Tables and Figures**

**Table 1.** Number of pilot whale samples analyzed in each methodology, along with the length range (in cm) and sex of the animals analyzed using the biogeochemical markers. NWIP: northwestern Iberian Peninsula; UK: United Kingdom; FI: Faroe Islands; USA: United States of America. mtDNA: mitochondrial DNA; SI: stable isotopes;FA: fatty acids.

**Table 2**. Variable nucleotide positions in north Atlantic pilot whale mitochondrial control region sequence (347bp). Hap: Haplotype; Freq: frequency summed across all samples; Dots represents nucleotide identity with haplotype A; within brackets are haplotype frequencies described in Siemann (1994) and included in the analysis. Other abbreviations are described in Table 1.

**Table 3.** Summary of genetic diversity statistics for mitochondrial DNA (mtDNA) of pilot whale analyzed in the present study. Mean values ± SD are shown. n: sample size; h: haplotype diversity; π: nucleotide diversity; s: number of polymorphisms. Other abbreviations are described in Table 1.

**Table 4.** Pairwise comparisons of genetic differentiation between regions based on the mtDNA of pilot whale in the north Atlantic. Above diagonal: pairwise genetic distances (mean values ± SD, %); Below diagonal: FST / FST (mean (95% CI)) recalculated after randomly sampling equivalent numbers of individuals per population. Bold: p-value < 0.05. Abbreviations are described in Table 1.

Table 4. Pairwise comparisons OF GENETIC DIFFERENTIATION BETWEEN REGIONS  based on the mtDNA of pilot whale in the north Atlantic. Above diagonal: pairwise genetic distances (mean values ± SD, %); Bold: p-value < 0.05. Below diagonal: FST / FST (95% CI) RECALCULATED AFTER RANDOMLY SAMPLING EQUIVALENT NUMBERS OF INDIVIDUALS PER POPULATION; Abbreviations are described in Table 1.

**Table 5.** Comparison of the generalized least squares (GLS) models fitted to δ13C and δ15N values of pilot whales. AIC: Akaike Information Criterion; LR: Likelihood ratio. Bold: Final model

**Figure 1.** a) Map with mtDNA haplotype frequencies of north Atlantic pilot whales analyzed in the present study; b) Median Joining network of the haplotypes of north Atlantic pilot whales, with different weights of transitions, transversions and insertions/deletions. Nodes are proportional to haplotype frequencies. All branches between haplotypes represent a single mutational step, unless stated otherwise (numbers). Haplotypes refer to the ones described in Table 2. Abbreviations are described in Table 1.

**Figure 2**. Redundancy analysis results for variables affecting the fatty acid signatures of pilot whales. Explanatory variables (black and bold) and the response variables (grey) are presented. Remaining FA included in the analysis (Table 1) were located under “Female” and “Male” and excluded from the plot to improve the clarity of the figure. Abbreviations are described in Table 1.

**Figure 3**. Geographical differences in the fatty acid (FA) profiles from pilot whales from the north Atlantic, based on linear discriminant analysis (LDA). Each dot represents a pilot whale and ellipses represent 95% data point clouds. Abbreviations are described in Table 1.

**Figure 4.** Carbon (δ13C) and nitrogen (δ15N) isotope values (mean ± SD and ranges, ‰) in pilot whales from different regions of the north Atlantic. Abbreviations are described in Table 1.

**Supplementary Material**

**Table S1.** Fatty acid methyl ester (FAME) profiles of inner blubber of pilot whales from NWIP, UK and USA. Values are presented as means ±SD of fatty acids normalized areas (NA %) for each FA plus summed values for MUFA, PUFA and SFA categories. For the monounsaturated acids 18:1, 20:1 and 22:1, the chromatographic area used includes two structural isomers. Predominant sources of fatty acids in predator (in this case, pilot whale) adipose tissue: B: relatively large contributions from both biosynthesis and diet; B?: not fully understood but believed to be relatively large contributions from both biosynthesis and diet; D: all or primarily from direct dietary intake; NFU: not fully understood (Iverson et al. 2004).# FAs used in RDA and LDA analysis; \* FAs selected by LDA forward stepwise method, as the most important to separate animals from different areas. Abbreviations are described in Table 1.

**Table S2.** Standardized and structured coefficients for Linear Discriminant Analysis (LDA) after forward selection (α = 0.05) for the inclusion of FA in the model.