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

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Abstract

The integration of ecological and genetic approaches is a particularly powerful strategy to identify natural population structure and to obtain information on foraging habitat and habits and social structure, over different timescales. In order to investigate the potential occurrence of population segregation 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 areas within the North Atlantic: Northwestern Iberian Peninsula, United Kingdom, Faroe Islands and United Sates of America. The genetic data revealed strong oceanic and regional levels of divergence, although AMOVA revealed no differentiation between East and West Atlantic. Results from biogeochemical tracers supported previous dietary studies, revealing geographic and ontogenetic dietary variation in *G. melas*. These results suggest that both ecological and genetic factors may drive the levels of pilot whale differentiation in the North Atlantic. The ecological segregation observed may be related with the exploitation of different foraging niches (e.g. oceanic *vs*. coastal), which can be highly influenced by prey distributions and/or oceanographic phenomena. Genetic differentiation may result from limited dispersal mediated through the social structure presented by this species. These results from multiple approaches provide preliminary information about pilot whale stocks in the North

Atlantic, based on both evolutionary and finer ecological timescales, essential baseline information for conservation and management plans.

Keywords: stable isotopes; fatty acids; mitochondrial DNA; pilot whales; population 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 become a challenge to understand the genetic and/or ecological processes that drive population differentiation, especially in complex environments, such as the marine habitats, since several factors may be important either independently or in combination.

Wildlife populations are, usually, identified based on their evolutionary traits, with genetic stocks representing reproductively isolated units, genetically different from each other (Coyle 1998). Genetic population differentiation is driven by microevolutionary forces such as gene flow, drift and/or selection (Ballard & Whitlock 2004, Selkoe et al. 2006, Xu et al. 2010). Neutral molecular markers such as mitochondrial DNA (mtDNA) and microsatellite have been extensively applied in population genetic studies to detect the effect of neutral microevolutionary forces on the genetic diversity, structure and demography of wild populations, over evolutionary time-scales (depending of the marker used and its evolutionary rate; Frankham et al. 2002, Ballard & Whitlock 2004, Selkoe et al. 2006).

Additionally, the adaptation to environmental and/or ecological variables may also lead to discontinuous ecological or phenotypic stocks (isolated units adapted to specific environmental and/or ecological factors, even in the absence of genetic differentiation; Coyle 1998). 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), isolation by distance or geographic barrier (Fontaine et al. 2007), social organization (Lyrholm et al. 1999) and dietary specializations (e.g. Foote et al. 2009).

For apex marine predators, such as cetaceans, it becomes crucial to complement genetic knowledge with an understanding of their role in marine food webs. 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). Several studies have also used stable isotopes and/or fatty acid analyses to reveal population/ecotype segregation 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 absence of any restriction of gene flow (e.g. Foote et al. 2009, Quérouil et al. 2013). Depending on the tissues 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 (e.g. fatty acids in blubber and stable isotopes in liver and muscle) to years (e.g. when stable isotopes in hard tissues such as teeth are analyzed) (e.g. Hobson & Clark 1992; Nordstrom et al. 2008), highlighting 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) so there is a need to distinguish individual-level and stock-level variability.

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 (e.g. in relation to localized anthropogenic threats) than is the evolutionary time-scale (Evans & Teilmann 2009). Additionally, ecological stocks could be viewed as units likely to become reproductively isolated in the future (Funk et al. 2006), or units whose unique characteristics and/or distribution justify their separate conservation, while bearing in mind that demographic responses to external stressors can be meaningfully interpreted only at the population level (Hoelzel 1998, ICES 2009, 2013). Therefore, the combination of biogeochemical markers with genetic information (diverse integration time-scales) can provide more complete and reliable information about distribution, feeding ecology and social structure while clarifying possible genetic and ecological structure within a population (Frankham et al 2002, Evans & Teilmann 2009).

The conservation status of 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 in three animals, Abend & Smith 1995), parasites (IWC 1990) and morphometric differences (Bloch & Lastein 1993) suggested the segregation of pilot whale in the North Atlantic, particularly between the West and East Atlantic. Additionally, previous stomach contents analysis in the Northeast Atlantic reported the occurrence of dietary variation in pilot whales, based on geographical location, sex and length of the animal (Santos et al. 2014). Considering the contrasting results previously found about the population structure of pilot whales, based on biogeochemical and genetic markers, the main objective of the present study is to combine ecological and genetic approaches to provide new insights regarding potential pilot whale stocks, in this oceanic basin. In this context, mitochondrial DNA and biogeochemical markers (fatty acids and stable isotopes) are analyzed in order 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 animals 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 from the tissue bank of the Museum of Natural History of the Faroe Islands (FI), which had been collected from animals killed by drive fisheries in 2010 (Table 1). Strandings were attended by experienced personnel belonging to the different stranding networks in

each region. Detailed necropsies were performed, if the condition of the animal permitted. Otherwise, basic measurements/information (i.e., length, sex, decomposition state) and samples were collected. Only fresh and moderately decomposed animals (decomposition state ≤ 3; Kuiken & Hartmann 1991) were used for stable isotope and fatty acid analyses, to prevent sampling biases associated to tissue decomposition. The gender 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). A 400bp fragment of the mtDNA control region was sequenced in a total of 102 samples, from the North Atlantic (Fig. 1), using the 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 10µl final volume reaction containing 1x PCR Buffer, 2 mM $MgCl_2$, 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 30s at 92ºC, 30s at 60-50ºC (decreasing 0.5ºC per cycle) and 45s at 72ºC, 19 cycles of 30s at 92ºC, 30s at 50ºC and 45s 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 undertaken 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 direct 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

After thawing the blubber samples, the inner blubber was collected for fatty acid analysis, by selecting the portion of blubber situated approximately 0 - 1cm above the muscle (Samuel & Worthy 2004). Lipids were extracted from the inner blubber of 56 cetaceans, using a modified Folch method (Folch et al. 1957). Briefly, homogenized inner blubber samples (approximately 1g) were left for approximately 24h at 4ºC, in a 2:1 (v/v) solution of chloroform:methanol, containing butylated-hydroxytoluene (BHT) as an antioxidant. A 0.88% (w/v) KCl in water solution was then added to achieve a final ratio of 8:4:3 of chloroform/methanol/water. The final biphasic system was centrifuged at 1800rpm for 20 min and the entire lower phase was transferred to a pre-weighed glass flask and evaporated in a rotary evaporator set at 35ºC, until all detectable traces of solvent were gone. To

remove final traces of solvent, a high vacuum pump was used and the flask was placed on a desiccator with silica gel, overnight. Lipid content was then determined gravimetrically.

Before esterification, lipid classes were measured in blubber samples to test for indications of decomposition (presence of high level of free fatty acids). For this purpose, Thin-Layer Chromatography (TLC) of approximately 5mg lipid samples, on Silica Gel TLC plates, 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 therefore were excluded from further analysis (the excluded samples are not listed in Table 1).

The extracted lipid was then prepared for the esterification procedure. Approximately 10mg of lipid was dissolved in distilled toluene and 1% (v/v) sulphuric acid in methanol and left in a Block Thermostat, at 50ºC for a minimum of 12h. After being washed with 5% (w/v) sodium chloride in water, fatty acid methyl esters (FAMEs) were extracted in hexane and washed again with 2% (w/v) potassium bicarbonate in water. The FAME extract was then dried over anhydrous sodium sulphate.

The 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 0.25mm internal diameter, J & W Scientific Inc. California, USA). Nitrogen was used as the carrier gas and the temperature of the oven was programmed to start at 60°C, then to increase to 150°C at 25°C min^{-1}, followed by an increase to 200°C at 1.0°C min^{-1} (hold for 10min) and a final increase to 230°C at 5°C min^{-1} (hold for 5min). Quality assurance procedures at 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 individual 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. 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 48h and ground to powder. Afterwards, a lipid extraction was performed and consisted of agitating approximately 100mg of powder with 4 ml of cyclohexane, for 1h, followed by a centrifugation at 4000g for 5 min and discard of the supernatant. Samples were then dried in an oven at 45ºC for 48h, and subsamples of 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 $\delta^{13}C$ and atmospheric N_2 for $\delta^{15}N$, in parts per thousand (‰). Replicate measurements of internal laboratory standards (acetanilide) indicated that measurement errors were ± 0.15 and ± 0.2 ‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

Data treatment

Genetic analysis

Sequence variation and alignment was performed using Clustal W (Thompson et al. 1997) and MEGA 4.0 (Tamura et al. 2007). 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 347bp. All the variable sites detected within the 400 base pair amplicon were also within the shorter fragment. Nucleotide (π) and haplotypic (h) diversities (Nei 1987) were estimated for each sampling region and for the entire set of samples of the North Atlantic, using ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010).

The potential occurrence of genetic structure in the North Atlantic was tested through pairwise comparisons and an Analysis of Molecular Variance (AMOVA, Excoffier et al. 1992), in the software ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010). In pairwise analysis, both F_{ST} (based on haplotype frequency data alone, Weir & Cockerham 1984) and its analog Φ_{ST} (which takes into account both haplotype frequencies and genetic distances, Excoffier et al. 1992) were estimated, to assess the divergence between the sequences. For Φ_{ST} estimates, the Tamura-Nei model (Tamura & Nei 1993) was used. Statistical significances of F_{ST} and Φ_{ST} estimates were calculated using 20000 permutations of haplotypes among sampling regions (Fisher's exact test). For the Analysis of Molecular Variance (AMOVA), a hierarchical assessment of structure was examined, partitioning variance between Western and Eastern sides of the Atlantic, between regions within each side of the Atlantic (Western: USA; Eastern: Faroe Islands, UK, NWIP) and among individuals within regions.

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 (Table 2). 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 fatty acids found at low or trace levels (which may be incorrectly identified and separated from nearby peaks belonging to abundant FAs, Iverson et al., 2004) and 2) if the data on 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 to 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 was used to visualize any relationships between the set of response variables (FAs) and geographical location (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-value > 0.1; Legendre & Legendre 2012) and homogeneous covariance matrices between groups (F-test = 0.58, p-value > 0.1 for area; 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 Development Core Team 2014).

Stable isotopes

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

To determine which explanatory variables influence $\delta^{13}C$ and $\delta^{15}N$ 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 geographical location (categorical). If the final model included categorical variables, contrasts were constructed to perform pairwise tests, using the package lsmeans (Lenth 2013). The model fitted for $\delta^{13}C$ included a variance structure related to geographical location (VarIdent) in the error term, to account for the heteroscedasticity observed in the residuals in relation to this variable, while the model fitted for $\delta^{15}N$ included variance structures for both geographical location (VarIdent) and length (VarComb). No random factor was defined, since the variable geographical location 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).

Results

Genetic analysis

A total of five polymorphic sites (2 deletions and 3 transitions) defined six haplotypes across the different geographic 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 (A, B and C correspond to GenBank GMU20926, GMU20928 and GMU20927, respectively; Siemann 1994; 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 2). Within the North Atlantic, the UK presented the highest nucleotide diversity (π = 0.16% ± 0.15%), followed by NWIP and the Faroe Islands, 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 high levels of differentiation between regions in the North Atlantic (F_{ST} = 0.63, p< 0.001; Φ_{ST} = 0.68, p< 0.001). The AMOVA showed no differentiation, either at haplotype or nucleotide levels, between the East and West Atlantic (F_{ST} = 0.16, p> 0.05), highlighting that most of the genetic variance occurred among regions within East and West Atlantic (45.3%; Faroe Islands, UK, NWIP) rather than between oceanic basins (16%), suggesting populations more closely related between than within Western and Eastern Atlantic. This result is in agreement with pairwise regional comparisons, which show high levels of differentiation among areas, except between the United Kingdom and the United States of America (F_{ST} = 0.09, p< 0.05; Φ_{ST} = 0.01, p> 0.05, Table 4).

The network of mtDNA supports the results from AMOVA, since no clear separation seems to occur between the West and East Atlantic. Haplotype A (n = 118) is the most common in the North Atlantic, being shared by all regions (Fig. 1) which together with the star-like pattern exhibited in the network, may suggest that it corresponds to the ancestral haplotype. A phylogeographic pattern seems to occur within the North Atlantic, since several haplotypes are almost unique to one region (B, C, E and G) (Fig. 1).

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 5). The predominant FAs were 18:1 (28.68% ± 5.79%), 16:0 (12.7% ± 2.08%), 22:6(n-3) (9.22% ± 4.11%), 22:1 (8.99% ± 6.39%) and 20:1 (9.03% ± 3.23%), with clear variation between the different geographical locations, notably for 20:1 and 22:1, which had low values in NWIP compared to other areas, and for 20:4(n-3) which showed high values in NWIP. In addition, several FAs showed high variability within areas, e.g. 16:1 (n-7), 18:1 and 22:1 (Table 5).

Overall, 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 32.5% and 7.9% of the variation, respectively. Although some caution is needed, since the first two RDA axes only explain 40.4% 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). Additionally, the three geographical locations analysed in this study are well separated in the RDA, suggesting differences in FA profiles in these regions, especially related with the higher values of 16:1(n-7) presented by animals from USA (Fig. 2). In relation to the length of the animal, it seems to be negatively correlated with 16:1 (n-7) (Fig. 2). Significance tests confirm effects of geographic location ($F = 13$, $p < 0.001$) and length of the animal ($F = 7.2$, $p < 0.001$) but no influence of gender on fatty acid signatures.

Linear Discriminant Analysis (LDA) was used to determine which fatty acids best identified each location (i.e. which FAs were more important in separating animals from different areas). The 2-dimensional model, which best separated the three locations, is shown in Figure 3. There was a clear separation of the locations using 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 $p < 0.001$). LDA indicated that the 1st discriminant function mostly separated NWIP profiles from those in other locations, 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 6). A slight overlap occurred between individuals from the UK and the USA.

The ability of the model to predict location based on these eight FAs was tested using cross-validation, which achieved a correct assignment of 96.5% of blubber samples to their respective locations (jackknife approach with a leave-one-out cross validation). Results indicated 100%, 92.3% and 100% correct assignment for NWIP, UK and USA samples, respectively. The low misclassification rate (two UK samples incorrectly classified as USA), demonstrates that (at least in our small sample) pilot whale location can be determined with acceptable reliability from fatty acid analysis of inner blubber.

Stable isotopes

Overall, mean values of $\delta^{13}C$ and $\delta^{15}N$ 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 $\delta^{13}C$ (-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.

Regarding the distribution of pilot whales along a coastal-oceanic axis, the best performing GLS model for $\delta^{13}C$ revealed a significant effect of geographical location, when the heterogeneity of this explanatory variable was taken into account ($F = 11.30$, $p < 0.001$), since adding the variance structure related to geographical location to this model yielded significant improvement (likelihood ratio $p = 0.001$). A significant effect of geographical location on $\delta^{13}C$ supports the occurrence of differences between the studied areas of the North Atlantic. Pairwise analyses showed significant differences among all the different areas, except between IB and FI (Tukey test = 5.30, $p = 0.08$) and between UK and FI (Tukey test = -0.90, $p = 0.81$). There were no significant effects of either sex or length of pilot whales on $\delta^{13}C$ values. The inclusion of these variables decreased the AIC and the significance of the likelihood ratio; therefore they were excluded from the final model (model 3, Table 7).

Considering the trophic level occupied by pilot whales, high levels of intra-specific $\delta^{15}N$ 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 $\delta^{15}N$ revealed significant effects of geographical location ($F = 70.53$, $p < 0.001$) 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. In fact, adding the variance structure related to geographical location and length to this model yielded a significant improvement of the model fit (likelihood ratio $p = 0.001$). Most of the animals 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 geographical location on $\delta^{15}N$, the model suggests the occurrence of differences between the areas of the Atlantic. Pairwise analyses showed significant differences among all the different areas, except between the UK and FI (Tukey test = -1.74, $p = 0.31$). There was no significant effect of sex of pilot whales on $\delta^{15}N$ values and this variable was, therefore, excluded from the final model (model 2, Table 7).

The three approaches used here give different views of the degree of segregation between pilot whales from different sample locations. All three methods distinguished UK and NWIP samples; the two shorter-term markers (FAs, SIs) also differentiated USA from the other two areas whereas, mtDNA data suggested links between UK and USA samples. Regarding FI samples, which were available for only two of the three sub-studies, mtDNA showed segregation between FI and remaining areas analysed, whereas the short-term marker (SI) showed a strong similarity between the UK and FI samples (although some similarity also occurred between FI and NWIP considering $\delta^{13}C$) (see Fig.5 in discussion).

Discussion

Understanding the occurrence of population structure within wild species is crucial to identify their behavioural, ecological and genetic diversity (Coyle 1998) and support informed conservation and management strategies. The results of this study showed that the combination of different methodologies supported the occurrence of genetically and ecologically segregated units of pilot whales in the North Atlantic.

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). Levels of mitochondrial diversity reported in this study are comparable to those reported previously for this species and in other cetaceans believed to have similar social systems (long- and short-finned pilot whale (*Globicephala melas* and *G. macrorhynchus*, respectively) Oremus et al. 2009; killer whales (*Orcinus orca*), Hoelzel et al. 2002; sperm whale (*Physeter macrocephalus*), Lyrholm et al. 1996).

Although the AMOVA showed no differentiation between Western and Eastern sides of the Atlantic, there were significant oceanic and regional differences in the North Atlantic, with high levels of

genetic variation in mtDNA, revealed by pairwise analysis. There is compelling evidence for the occurrence of natal group phylopatry in pilot whales, where neither males nor females disperse from their natal group (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 (Andersen & Siegismund 1994, Amos et al. 1993), resulting in groups of "multiple matrilines" (e.g De Stephanis et al. 2008b, Oremus et al. 2013). Although the influence of microevolutionary forces such genetic drift cannot be discounted, the occurrence of sex-biased dispersal with high levels of female phylopatry could help to explain the elevated values of genetic divergence exhibited by the maternally inherited haploid marker.

The social structure and resource specialization may play key roles in the levels of population structure presented by cetaceans. A social structure with low rate of dispersal from a natal group could be explained by foraging specialization, with significant investment in learning strategies associated with the exploitation of local resources (Hoelzel et al. 2007, Pilot et al. 2010). As an example, specialized techniques for the exploration of different preys (e.g. benthic preys in NWIP vs. pelagic prey in FI or UK) or different foraging habitats may involve some social learning (e.g. Krutzen et al. 2005), leading to a potential reduction in an individual's fitness, if it disperses from a natal habitat. As a consequence, a reduction in the dispersal rate and gene flow can then be imprinted in pilot whales mitochondrial genetic diversity, especially considering their social structure system.

Biogeochemical markers

The segregation of pilot whale in the North Atlantic revealed by the mtDNA was mirrored in the results of biogeochemical markers, at fine timescale. Both stable isotope and fatty acid signatures showed the occurrence of geographical differences in pilot whales from the North Atlantic, highlighting the role of trophic ecology when investigating the ecological stocks of a species.

Previous dietary (stomach contents and stable isotopes) and habitat preference studies provided insights regarding the preferences of pilot whales in the different regions analyzed. In the NWIP, pilot whales seem to be present in neritic habitats and exhibit a preference for benthic prey species (such as curled octopus, *Eledone cirrhosa*) (e.g. Pierce et al. 2010, Méndez-Fernandez et al. 2012, Santos et al. 2014). In the USA, pilot whales seem to perform seasonal inshore-offshore movements (e.g. Payne & Heinemann 1993) and prefer the demersal long-finned squid *(Loligo pealei)* and the epipelagic Atlantic mackerel (*Scomber scombrus*) (Gannon et al. 1997). Off the UK and Faroe Islands, pilot whales exhibit an oceanic preference in terms of both habitat and prey species, since they mostly occur in areas off the continental shelf in both locations (Bloch et al. 2003, MacLeod et al. 2007), although sightings also occur from land in FI (Zachariassen et al. 1993), and European flying squid (*Todarodes sagitattus)* and *Gonatus sp.* squids seem to be the most important prey species (Desportes & Mouritsen 1993, Santos et al. 2014).

The geographical differences in both type of prey consumed and habitat use, revealed by both biogeochemical markers in the present study, seem to be in agreement with the trophic and habitat preferences previously described for this species.

Most of the FAs responsible for separation of the different regions of the North Atlantic were of dietary origin (Iverson et al. 2004), although some could also be biosynthesized by the predator (Iverson et al. 2004). The separation between NWIP and remaining regions, based on a higher proportion of arachidonic acid (AA, 20:4n-6) in Iberian animals, may provide useful information regarding the foraging ecology of pilot whales in these regions. Since AA is proposed to be a marker of benthic and coastal feeding (Piché et al. 2010) or an inherent characteristic in octopuses (e.g. Navarro & Villanueva 2000), results of the present study suggest that pilot whales in Iberia consume octopuses, while they may be feeding on other prey species in the UK and the USA, as verified in previous stomach contents analysis (Gannon et al. 1997, Santos et al. 2014).

Stable isotope analysis indicates that pilot whales exhibit some degree of dietary plasticity, both in terms of foraging areas and prey consumed, evident in the variability shown by $\delta^{13}C$ and $\delta^{15}N$. The only exception seems to be the animals from the Faroe Islands, who probably belonged to the same pod. Therefore, to obtain a range of diversity representative of this region, samples from different pods should be analyzed. Nevertheless, in general, stable isotope results seem to be in agreement with the habitat and dietary preferences of pilot whales described above. Regarding $\delta^{13}C$, significant differences existed between most of the regions analysed, with a ^{13}C-depletion observed in animals from the UK relative to most of the remaining regions, which may result from the exploitation of oceanic habitats and/or ingestion of relatively ^{13}C-depleted resources, while Iberian pilot whales seem to be feeding on more coastal habitats and/or benthic prey. The $\delta^{15}N$ values indicate that different trophic levels of prey are being targeted in the most study regions, similarly to results of previous studies, with the only exception occurring between FI and UK, which is not surprising considering the similar diet reported in whales from both locations (Desportes & Mouritsen 1993; Santos et al. 2014).

The differences detected between regions, in terms of prey consumed and foraging habitats reflect trophic regime changes along the geographic range analyzed and some level of feeding niche segregation, potentially associated with coastal vs. oceanic feeding habits, where either prey movement and/or oceanographic features (gyres, upwellings, topography) may play an important role. Although the lack of local abundance data about most of pilot whales' prey prevents their categorization as generalist (which would imply that dietary differences are related to prey availability) or specialist consumers, links between prey and pilot whale abundance and/or movements have been reported in the USA and FI (e.g. Desportes & Mouritsen 1993, Payne & Heinemann 1993, Jákupsstovu 2002). Furthermore, some studies showed the influence of oceanographic phenomena, such as gyres and upwelling processes on pilot whale distribution, which may be mediated by environmental effects on the abundance of target prey species. As an example, there seems to be a link between the abundance of pilot whales in the Faroe Islands, their main prey and the marine climate in the northeastern Atlantic (subpolar and subtropical gyres) in a bottom-up process (Hatún et al. 2009). In the NWIP, a recent analysis of pilot whale (*Globicephala* sp.) distribution in summer months (Monteiro 2014), suggests that these species may indirectly be affected by seasonal upwelling due to its effect on the spawning and recruitment of octopus (e.g. Otero et al 2008), one of the main prey targeted by pilot whales in that region. Oceanographic phenomena, such as the upwelling occurring in NWIP, may also influence the isotopic baseline, with an increase of the phytoplankton growth rates (and higher $\delta^{13}C$ values) (Pancost et al.

1997). It is important to be aware that 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). This variation may present a challenge when stable isotope studies aim at discerning ecological segregation of highly mobile animals that explore different marine regions, since it can be difficult to tease apart differences in consumer stable isotope values 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 $\delta^{15}N$ and $\delta^{13}C$ baseline geographic gradients observed in the 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 segregation. However, it would not explain some level of similarity between FI and NWIP isotopic niches, especially when the zooplankton organic $\delta^{13}C$ seem to vary around 2‰ between both 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 segregation 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 the signatures of the biogeochemical tracers analysed. As a size-dimorphic species (Bloch et al. 1993), differences in diet could be expected in pilot whale, in order to fulfill the higher energy requirements of the larger sex. However, in the present study, no evidence of differences in the foraging habits of female and male pilot whales was found, in agreement with stomach contents analysis from the Northeast Atlantic (Santos et al. 2014) and with results of stable isotope analysis for the Mediterranean (De Stephanis et al. 2008), suggesting that gender is not an important factor at defining feeding niches in this species.

In contrast, there was a significant effect of the length of pilot whales on fatty acid profiles and $\delta^{15}N$ values. Individuals smaller than 239cm (weaning stage, Sergeant 1962) showed the expected $\delta^{15}N$ isotopic values of un-weaned individuals (i.e. higher, on average, than in juveniles or adults, Hobson et al. 1997). However, the separation of the un-weaned and weaned pilot whales was not so evident in results from fatty acid analysis. The length of pilot whales seems to be negatively correlated with the relative abundance of 16:1(n-7) (FA originated either from diet or byosinthesis, Iverson et al. 2004) and 20:5(n-3) (dietary FA, Iverson et al. 2004) in the inner blubber, which may suggest an effect of animal size on fatty acid biosynthesis and/or that diet may vary with the length of the whale, a result also described in previous stomach contents analysis (Desportes & Mouritsen 1993, Santos et al. 2014).

Conclusion and Management implications

The integration of ecological and genetic methodologies allowed the evaluation of pilot whale segregation in the North Atlantic. Biogeochemical markers were able to evidence higher levels of segregation between the Western and Eastern basin of the North Atlantic, when compared to genetic analysis, revealing an ecological basis for the segregation of USA relatively to remaining regions. This difference between genetic and biogeochemical markers highlights the ability of the latter to detect differentiation between regions at a finer time scale than genetic markers and the importance of using complementary tools to detect putative ecological segregation in a genetically uniform population (Coyle 1998). Within the Northeast Atlantic basin, both genetic and biogeochemical markers suggest a long

and strong segregation between UK and NWIP, evidencing the occurrence of more than one management unit (Fig.5). Biogeochemical markers (SI) revealed a high similarity between FI and UK feeding niche, than between NWIP and FI pilot whales, which may indicate the occurrence of higher ecological segregation between NWIP and IF than between UK and FI. However, it would be useful to investigate more biogeochemical (e.g. POPs) and genetic markers, more samples from different pods from Faroe Islands, as well to study intermediate geographical locations (e.g. Bay of Biscay, English channel, Greenland, Azores) in order to be able to detect potential migratory routes and define stock boundaries.

Stock identification is a key prerequisite for successful conservation management. The relative value of defining genetic and ecological stocks has been the subject of much debate and, indeed, management units do not always correspond to separate stocks (ICES 2014). However, while threats may be localized, their impact 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). Thus knowledge of both ecological and genetic stocks can be useful to define management units. This knowledge may be useful in order to design monitoring programmes and management measure to meet requirements of policy drivers such as the Habitats Directive and the Marine Strategy Framework Directive (2008/56/EC).

This study demonstrates the ability of biogeochemical markers to distinguish between different stocks, highlighting their usefulness, if evidence of subpopulations divergence is otherwise absent.

Acknowledgments

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Tables and Figures

Table 1. Number of pilot whale samples analyzed in each methodology, along with the length range (in cm) of the animals analyzed using the biogeochemical markers. NWIP: Northwestern Iberian Peninsula; UK: United Kingdom; FI: Faroe Islands; USA: United States of America.

	mtDNA	SI	FA	Length (cm)
NWIP	34	22	18	190 - 532
UK	38	45	26	264 - 576
FI	23	19	-	194 - 573
USA	73	28	12	275 - 508

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.

		Nucleotide position					East Atlantic			West Atlantic
		1	**1**	**1**	**2**	**2**				
Hap	Freq	**0**	**0**	**5**	**2**	**2**	**NWIP**	**UK**	**FI**	**USA**
		3	**4**	**6**	**6**	**8**				
A	118	**-**	**-**	**T**	**C**	**A**	8	25(7)	5(1)	24(48)
B	1	.	.	.	T	G	0	0	0	1
C	18	.	.	.	T	.	0	1	7(10)	0
E	4	T	A	.	.	.	1	3	0	0
F	26	.	.	C	.	.	25	1	0	0
G	1	T	A	C	.	.	0	1	0	0

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.

		NWIP	UK	FI	USA	Overall
	n	34	38	23	73	168
	haplotypes	3	5	2	2	6
mtDNA	**h**	0.42 ± 0.08	0.29 ± 0.10	0.40 ± 0.09	0.03 ± 0.03	0.47 ± 0.04
	Π (%)	0.15 ± 0.14	0.16 ± 0.15	0.12 ± 0.12	0.02 ± 0.04	0.17 ± 0.15
	s	3	4	1	2	5

Table 4. Pairwise regional comparisons based on the mtDNA of pilot whale in the North Atlantic. Below diagonal: F_{ST}; Above diagonal: ΦST; Bold: p-value < 0.05. Abbreviations are described in Table 1.

	NWIP	UK	FI	USA
NWIP	-	**0.62**	**0.72**	**0.76**
UK	**0.55**	-	**0.67**	0.01
FI	**0.56**	**0.55**	-	**0.79**
USA	**0.78**	**0.09**	**0.82**	-

Table 5. 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. Predominant sources of fatty acids in predator 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.

	NWIP	UK	USA	OVERALL	SOURCE
14:0#	5.26 ± 0.87	5.97 ± 1.13	6.65 ± 1.17	5.89 ± 1.16	B
15:0	0.79 ± 0.13	0.58 ± 0.10	0.56 ± 0.06	0.64 ± 0.15	B
16:0#*	14.07 ± 2.18	12.15 ± 1.87	11.86 ± 1.35	12.7 ± 2.08	B
16:1(n-7)#*	9.93 ± 3.76	6.48 ± 3.25	10.74 ± 4.37	8.50 ± 4.08	B
16:2(n-6)#*	0.59 ± 0.14	0.74 ± 0.16	0.36 ± 0.06	0.61 ± 0.20	D
16:3 (n-6)	1.09 ± 0.18	0.62 ± 0.16	0.49 ± 0.13	0.75 ± 0.29	D
16:4 (n-3)	0.05 ± 0.02	0.09 ± 0.04	0.18 ± 0.08	0.09 ± 0.07	D
18:0#	4.30 ± 1.05	3.14 ± 0.63	2.25 ± 0.50	3.32 ± 1.07	B
18:1#*	32.31 ± 4.58	28.44 ± 5.39	23.77 ± 4.57	28.68 ± 5.79	B
18:2(n-6)#*	1.34 ± 0.16	1.33 ± 0.28	1.48 ± 0.20	1.36 ± 0.24	D
18:3(n-6)	0.09 ± 0.04	0.18 ± 0.06	0.13 ± 0.04	0.14 ± 0.06	D
18:3(n-3)	0.46 ± 0.10	0.58 ± 0.27	0.70 ± 0.11	0.56 ± 0.22	D
18:4(n-3)#*	0.21 ± 0.08	0.57 ± 0.37	0.68 ± 0.18	0.48 ± 0.33	D
20:0	0.40 ± 0.13	0.28 ± 0.08	0.19 ± 0.08	0.30 ± 0.13	B
20:1	5.09 ± 1.07	11.00 ± 1.81	10.68 ± 2.31	9.03 ± 3.23	D
20:4(n-6)#*	1.46 ± 0.54	0.63 ± 0.13	0.69 ± 0.18	0.91 ± 0.50	D
20:4(n-3)	0.43 ± 0.11	0.60 ± 0.22	0.63 ± 0.20	0.55 ± 0.20	D
20:5(n-3)#*	2.59 ± 1.50	1.26 ± 0.65	2.88 ± 1.28	2.03 ± 1.33	D
22:0	0.10 ± 0.05	0.08 ± 0.04	0.03 ± 0.03	0.07 ± 0.05	B?
22:1#	2.30 ± 0.84	12.70 ± 4.92	10.99 ± 6.17	8.99 ± 6.39	D
21:5(n-3)	0.21 ± 0.07	0.27 ± 0.11	0.31 ± 0.10	0.26 ± 0.10	B

22:5(n-3)	3.91 ± 1.82	2.60 ± 0.90	3.06 ± 0.97	3.12 ± 1.38	D
22:6(n-3)[#]	10.91 ± 4.90	8.08 ± 3.30	9.17 ± 3.85	9.22 ± 4.11	D
24:1(n-9)	0.41 ± 0.21	0.63 ± 0.23	0.37 ± 0.18	0.51 ± 0.24	NFU
SFA	24.91 ± 2.90	22.18 ± 1.90	21.56 ± 1.57	22.92 ± 2.59	
MUFA	50.03 ± 7.69	59.26 ± 5.01	56.57 ± 5.85	55.72 ± 7.29	
PUFA	24.49 ± 8.61	18.03 ± 4.88	21.17 ± 6.24	20.78 ± 7.04	

Table 6. Standardized and structured coefficients for Linear Discriminant Analysis (LDA) after forward selection (α = 0.05) for the inclusion of FA in the model.

	Standardized		Structured	
	LDA1	**LDA2**	**LDA1**	**LDA2**
16:0	-0.87	0.51	-0.49	0.02
16:1(n-7)	-0.47	0.30	-0.19	0.50
16:2(n-6)	0.15	-0.84	-0.05	-0.85
18:1	-0.46	-0.67	-0.51	-0.28
18:2(n-6)	-0.13	0.73	0.12	0.27
18:4(n-3)	-0.47	-1.07	0.62	0.04
20:4(n-6)	-2.11	-0.67	-0.80	0.19
20:5(n-3)	1.40	0.77	-0.23	0.59

Table 7. Comparison of the generalized least squares (GLS) models fitted to $\delta^{13}C$ and $\delta^{15}N$ values of pilot whales. AIC: Akaike Information Criterion; LR: Likelihood ratio. Bold: Final model

Model	AIC	Test	LR	P
$\delta^{13}C$				
1.Sex+Area+Length	227.1			
2.Area+Sex	225.5	1 vs. 2	0.50	0.48
3.Area	**224.6**	**2 vs. 3**	**1.10**	**0.29**
$\delta^{15}N$				
1.Sex+Area+Length+$(Length)^2$	190.0			
2.Area+Length+$(Length)^2$	**188.0**	**1 vs. 2**	**0.006**	**0.94**

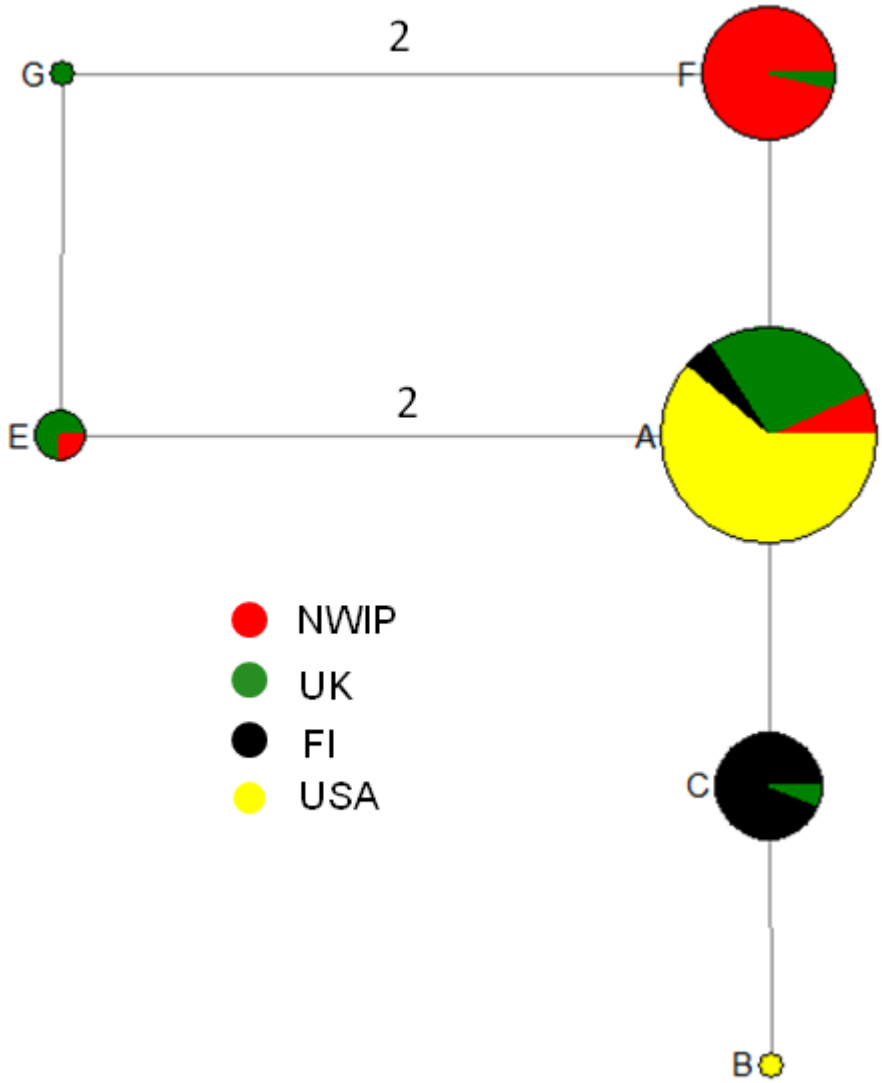

Figure 1. Median Joining network of the haplotypes of North Atlantic pilot whales, with different weights of transitions, transversions and insertions/deletions. Nodes are proportional to haplotype frequencies. All branches between haplotypes represent a single mutational step, unless stated otherwise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

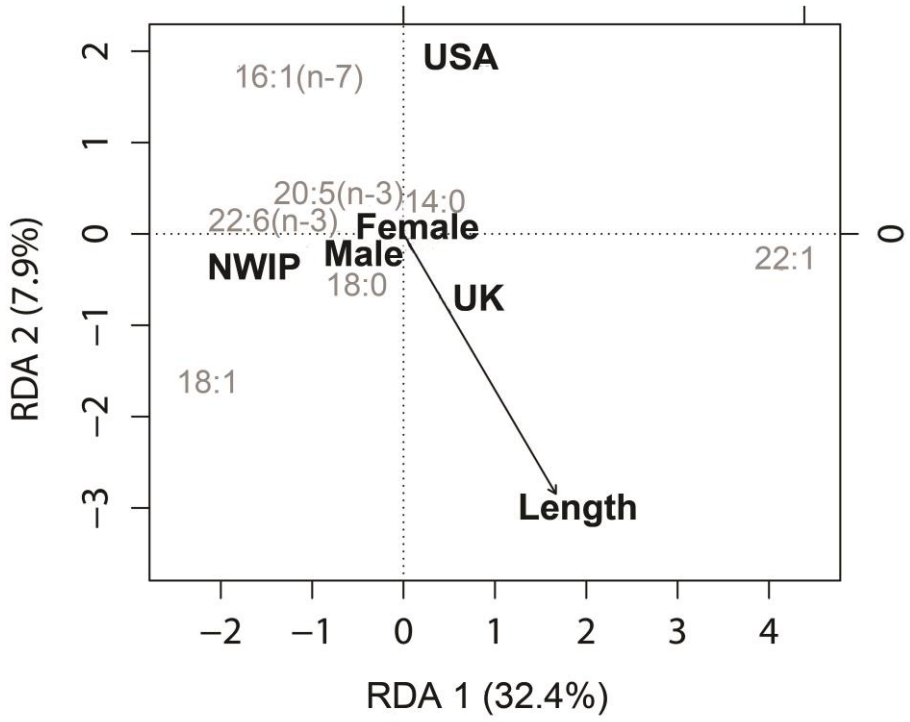

Figure 2. Redundancy analysis results for variables affecting the fatty acid signatures of pilot whales. Explanatory variables (black and bold) and the response variables (grey) are presented. Remaining FA included in the analysis (Table 1) were located under “Female” and “Male” and excluded of the plot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

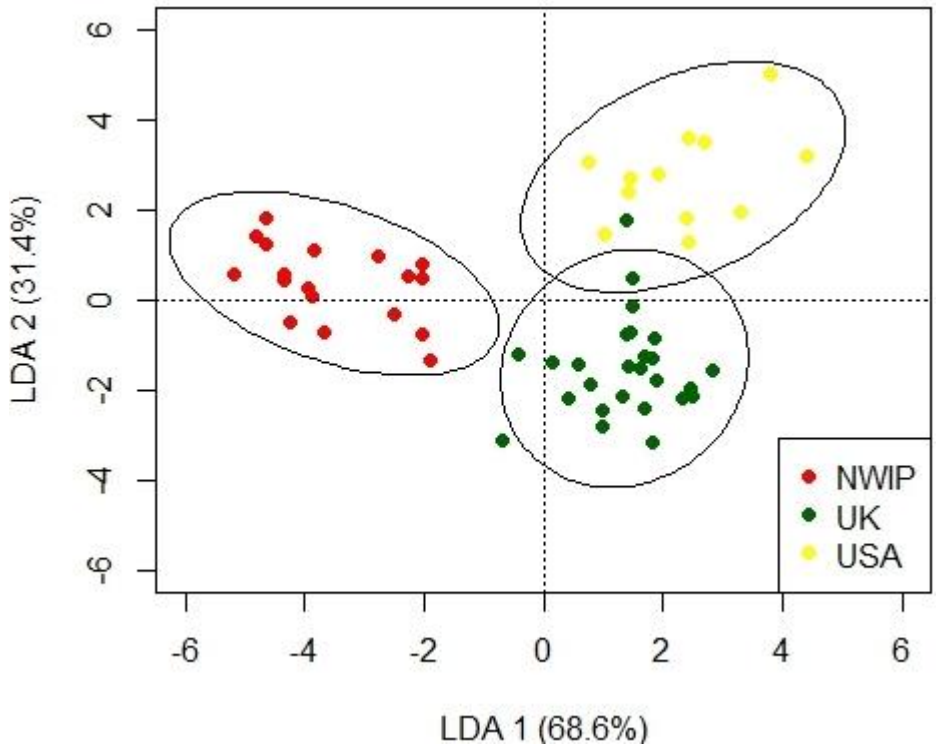

Figure 3. Geographical differences in the fatty acid (FA) profiles from pilot whales from the North Atlantic, based on linear discriminant analysis (LDA). Each dot represents a pilot whale and ellipses represent 95% data point clouds. Abbreviations are described in Table 1.

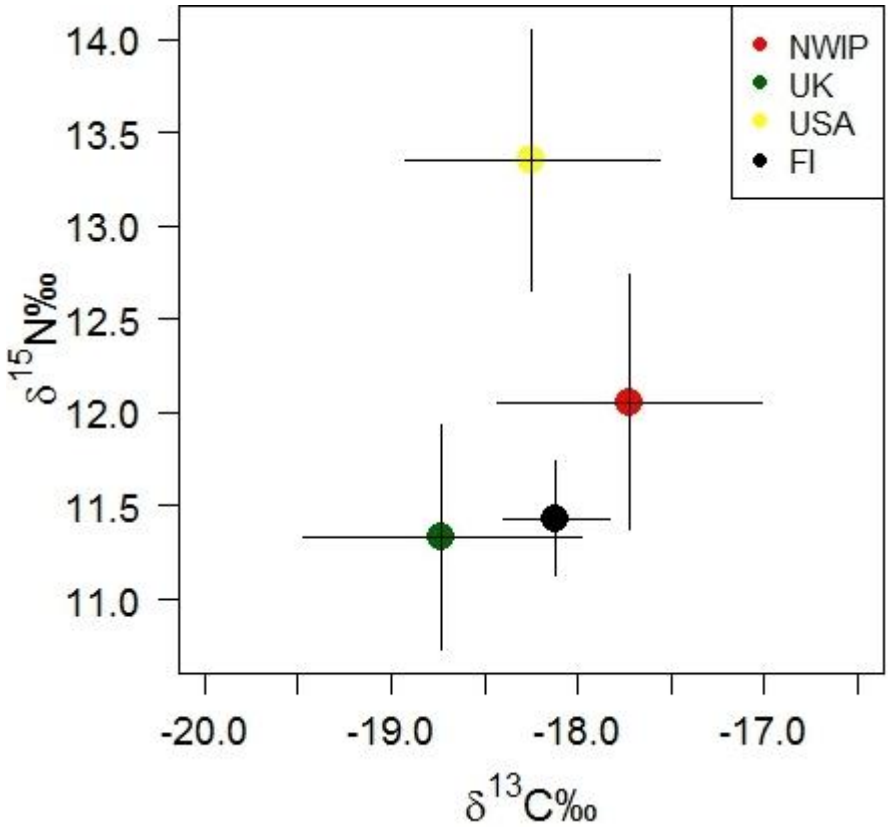

Figure 4. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values (mean ± SD and ranges, ‰) in pilot whales from different regions of the North Atlantic. Abbreviations are described in Table 1.

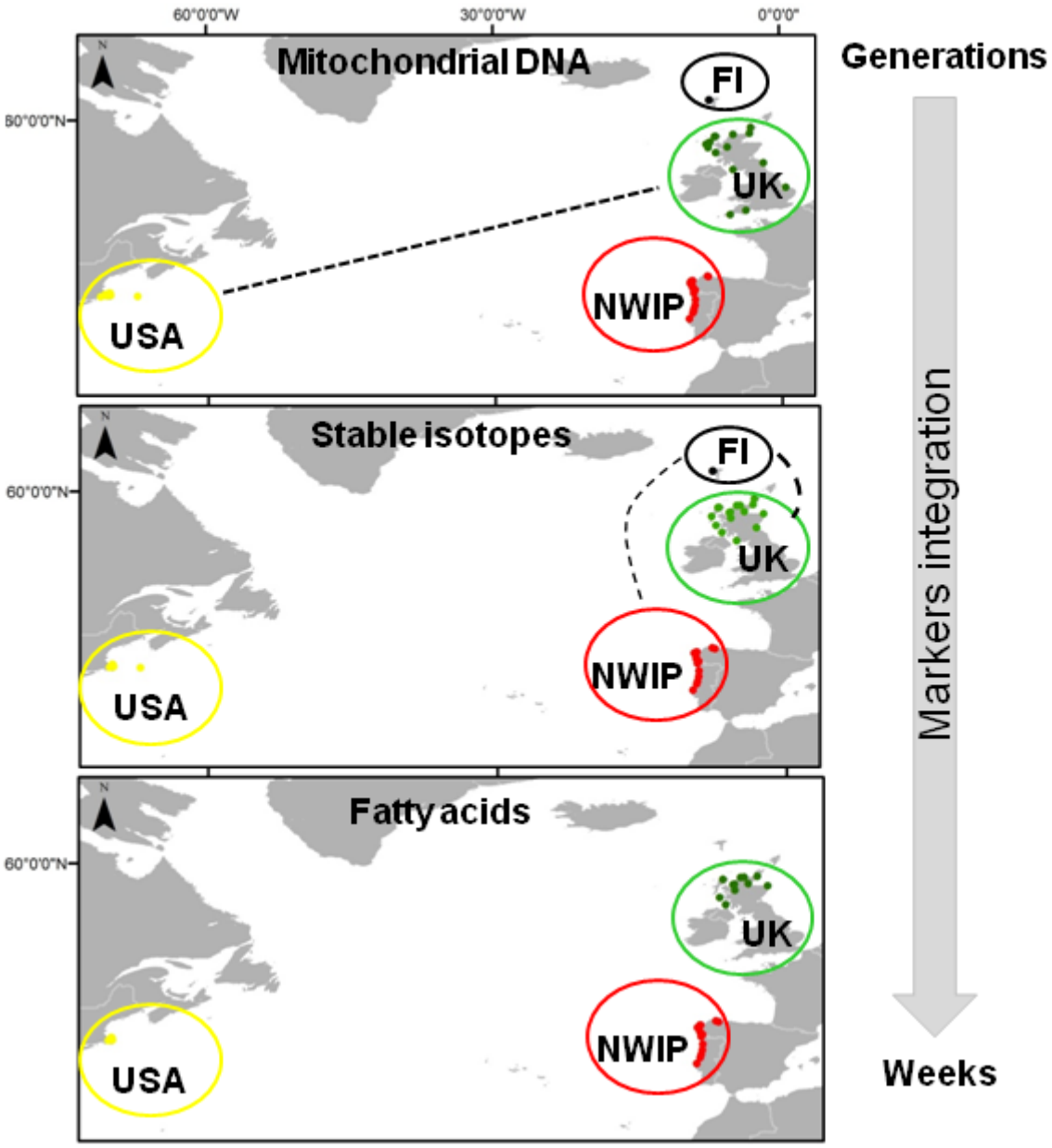

Figure 5. Conceptual scheme of the information given by biogeochemical and genetic markers, with different levels of integration periods, regarding pilot whales' segregation in the North Atlantic. The size of the circles has no specific meaning. Dashed lines represent potential links between regions. Thicker lines represent stronger evidence towards the links between regions. Dots represent the geographical locations of pilot whale samples. Abbreviations are described in Table 1.

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