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Population Ecology of Long-finned Pilot Whale (*Globicephala melas*) off the Western Coast of the Iberian Peninsula

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Ao meu pai…

To my father…

“The dolphins were having a great relaxed time and there were no major answers they wished to know the question to…”

Douglas Adams

“You're off to Great Places!
Today is your day!
Your mountain is waiting,
So... get on your way!”

Dr. Seuss

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ABSTRACT

This study focused mainly on providing information about the ecological and genetic characteristics of long-finned pilot whales (*Globicephala melas*) from the Western coast of the Iberian Peninsula, as well as the determining the habitat preferences of this species, in that region. Additionally, the inclusion of samples from other regions led to the investigation of the occurrence of population structure within the North Atlantic.

Firstly, stomach contents and fatty acid analyses were used, to assess the dietary preferences and understand the influence of geographical and biological factors in the dietary ecology of *G. melas*. Stomach contents results confirmed pilot whales as mainly teuthophagous species and showed that Iberian whales had a more diverse diet, dominated by Octopodidae species, in comparison to the predominance of Ommastrephids in Scotland. The analysis of prey fatty acids, in the present study, also indicated that, although not conclusive, there is some evidence that Iberian whales are feeding on octopods.

Both stomach contents and fatty acid analyses revealed the occurrence of significant geographical differences between animals from different regions of the North Atlantic (Iberia, Scotland and USA). These results may be a consequence of the ingestion of different types of prey based on prey preference/availability or due to the exploitation of different feeding niches/habitats in the study areas, which suggest the possibility of the occurrence of different ecological groups with specific foraging habits in the North Atlantic. There were also biological influences on the dietary ecology of *G. melas*, particularly evident in the stomach contents analysis, where significant differences in the main prey consumed were associated with the length and sex of the animal. However, no significant differences occurred in the fatty acid profiles of female/male or mature/immature pilot whales.

Secondly, the genetic population diversity and divergence of *G. melas* from six regions in the North Atlantic and adjacent waters were investigated, based on mitochondrial DNA (mtDNA) and MHC DRA and DQB loci. Both mtDNA and MHC diversities were comparable to other abundant widespread cetaceans. Pairwise estimates of genetic differentiation (F_{ST}) indicated the occurrence of genetic structure at both regional and oceanic scales at mtDNA, while MHC suggested that Iberian whales represent a genetically differentiated group.

Population structuring revealed by mtDNA could be related to the social structure presented by this species, associated with high levels of female phylopatry. For the MHC loci, although the occurrence of historical balancing selection appeared to have an important role in shaping population diversity, the spatial patterns of extant diversity across the North Atlantic could be attributable to local selection pressures for specific pathogens/parasites or patterns of gene flow and/or drift.

Therefore, the combination of the results from ecological tracers (i.e. fatty acids, stomach contents) and genetic markers into a multi-tracer approach revealed the occurrence of segregation of long-finned pilot whales from the different regions of the North Atlantic analysed. Furthermore, the results obtained in this thesis consistently show *G. melas* from the Western Iberian Peninsula as a potential different group within the North Atlantic, based on genetic (mtDNA and MHC) and trophic (stomach contents and fatty acids) analyses.

Finally, since the identification of habitat preferences and suitable habitats within a species range has been defined as a priority for effective conservation and management, habitat modeling techniques (presence-only models, i.e. PCA and Maxent) were used to determine pilot whales habitat preferences and suitability in Atlantic Iberia, based on six ecogeographic variables. Both methodologies identified depth and SST gradient as the most important variables for the ecological niche of pilot whales. SST was also an important variable defined by PCA, although Maxent model included it as a variable of minor importance. Higher habitat suitability occurred in locations with shallower waters, higher values of SST gradient (although PCA, based on a shorter temporal scale, showed the opposite result for SST gradient) and SST values between 15 and 17^{o}C. These results may indicate that pilot whales undertake incursions into coastal waters which may be related with a high concentration of Octopodidae spawners in these areas, in the upwelling season. However, it also highlights the importance of thinking carefully about the meaning of findings at different temporal scales, as well as demonstrating the importance of using a fine temporal scale, in marine environments.

The main results of this study contribute to the basic knowledge of this cetacean species, necessary for the determination of its conservation status and the identification of potential conservation concerns. In this context one of the key findings is the good evidence for existence of a separate Iberian population, which might be considered as a management unit for conservation purposes.

RESUMO

Este estudo focou-se na obtenção de informação sobre as características ecológicas e genéticas de Baleia-piloto (*Globicephala melas)*, bem como na determinação das preferências de habitat desta espécie, na Costa Oeste da Península Ibérica. Adicionalmente, foi também investigada a ocorrência de estrutura populacional no Atlântico Norte.

Inicialmente, foram analisados conteúdos estomacais e ácidos gordos para determinar as preferências dietéticas e investigar a influência de factores geográficos e biológicos na ecologia trófica de *G. melas*. A análise de conteúdos estomacais confirma esta espécie como maioritariamente teutófaga, com as baleias Ibéricas a apresentarem uma dieta mais variada e dominada por Octopodidae, comparativamente com a predominância de Omastrephidae, na Escócia. A análise dos ácidos gordos de presas, no presente estudo, também sugere a possível ingestão de polvos por parte das baleias que ocorrem na Península Ibérica.

Os resultados dos conteúdos estomacais e dos perfis de ácidos gordos sugerem a ocorrência de diferenças geográficas significativas entre animais de diferentes regiões do Atlântico Norte (Península Ibérica, Escócia e EUA). Estas diferenças poderão resultar da ingestão de diferentes espécies-presa, consoante a preferência ou disponibilidade de presas ou da exploração de diferentes nichos/habitats na área de estudo, o que sugere a ocorrência de diferentes grupos ecológicos, com hábitos alimentares específicos no Atlântico Norte. Foram também observadas influências biológicas na ecologia alimentar de *G. melas*, principalmente ao nível dos conteúdos estomacais, onde a abundância das principais presas é significativamente influenciada pelo tamanho e sexo do predador. O mesmo não se verificou ao nível dos ácidos gordos, onde não ocorreram diferenças significativas entre machos/fêmeas ou entre animais imaturos/maturos.

Em seguida, foram investigadas a diversidade genética e a estruturação populacional de *G. melas* de seis regiões do Atlântico Norte e águas adjacentes, baseados em ADN mitocondrial (mtADN) e marcadores de MHC. A diversidade genética ao nível do mtADN e do MHC apresentou valores comparáveis com outras espécies de cetáceos. As estimativas de diferenciação genética (F_{ST}) indicam a ocorrência de estrutura populacional a escalas regionais e oceânicas para o mtADN, enquanto o MHC sugere as baleias da Península Ibérica

como uma população geneticamente distinta. A estrutura populacional revelada pelo mtADN poderá estar relacionada com a estrutura social apresentada por esta espécie, com elevados níveis de filopatria feminina. Relativamente ao MHC, apesar de historicamente a selecção aparentar ser determinante para a diversidade genética, a estruturação espacial dessa mesma diversidade poderá ser atribuida a pressões selectivas locais por agentes patogénicos/parasitas específicos ou devido a padrões de fluxo e/ou deriva genética.

Assim, a combinação de marcadores ecológicos e genéticos revelou a ocorrência de segregação de Baleias-piloto de diferentes regiões do Atlântico Norte. Adicionalmente, os resultados desta tese consistentemente sugeriram que as *G. melas* da costa Oeste da P. Ibérica representam um grupo distinto no Atlântico Norte, baseado tanto em análises genéticas como tróficas.

Por último, técnicas de modelação de habitat foram utilizadas (métodos de presença, PCA e Maxent) para determinar as características ambientais e os habitats favoráveis à ocorrência de Baleias-piloto na Costa Atlântica da Península Ibérica, tendo por base seis variáveis ambientais. Ambas as metodologias identificaram profundidade e gradiente de temperatura superficial da água (GrSST) como as variáveis que mais influenciaram a distribuição das baleias. A temperatura superficial da água (SST) foi também considerada uma variável importante pelo PCA, no entanto no modelo do Maxent foi incluída como uma variável de menor importância. As condições de habitat mais favoráveis para as baleias ocorreram em locais com águas menos profundas, valores elevados de GrSST (apesar de a utilização de uma escala temporal mais fina no PCA mostrar um resultado oposto para esta variável) e valores de SST entre 15 e 17^{o}C. Estes resultados sugerem que as Baleias-piloto poderão realizar migrações para águas costeiras devido, provavelmente, à elevada concentração de indivíduos reprodutores de Octopodidae nessas águas, na época de afloramento. No entanto, também evidenciam a importância de uma cautelosa interpretação de resultados provenientes de diferentes escalas temporais e da utilização de escalas temporais finas, em ambientes marinhos.

Os resultados do presente estudo contribuem para o conhecimento desta espécie, necessário para a determinação do seu estado de conservação e identificação de potenciais problemas de conservação. Dentro deste contexto, um dos principais resultados obtidos é a evidência da ocorrência de uma população distinta de *G. melas* na Península Ibérica, o que poderá constituir uma unidade de gestão independente, para fins de conservação.

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Chapter I

Introduction

The importance of studying marine mammal ecology

Marine mammals are, generally, apex predators, given that they represent major consumers of the top trophic levels and have few or no natural predators. The top of the food web position occupied by these animals represents an important ecological role, as top-down regulators of ecosystem functioning (Estes *et al.*, 1998, 2004; Williams *et al.,* 2004; Morissette *et al.,* 2012).

Several studies exemplified the influence of marine apex predators as ecosystem top-down regulators and the consequences resulting from the removal of those species in the environment (Heithaus *et al.,* 2008; Baum *et al*., 2009; Ferretti *et al.,* 2010). A common example refers to the increased sea otter mortality in Alaska, due to a change in predation by transient killer whales, reversing the classic sea otter – sea urchin – kelp trophic cascade (Estes *et al.,* 1998, 2004; Williams *et al.,* 2004), although some controversy still exists around this theory (Wade *et al*., 2009). Likewise, a study in the Bering Sea found results consistent with the hypothesis of top-down control by foraging grey whales, as one of the main causes for ampesliscid populations declines (Coyle *et al.,* 2007). The impact of the decrease of cetacean species resulting mainly from harvesting and incidental captures, in the marine ecosystems, was also reported in studies developed in the Bering Sea (Merrick *et al*., 1997), the north Pacific Ocean (Croll *et al.*, 2006; Essington, 2007) and in the Baltic Sea (Osterblom *et al*., 2007).

One other type of ecosystem regulation is the bottom-up control that consists of a regulation of the food web dominated by the lower trophic levels (primary producers), which is itself strongly influenced by environmental conditions (Aebischer *et al*., 1990; Field *et al*., 2006; Frederiksen *et al*., 2006). There are several examples of the influence of bottom-up regulation on apex predators in the marine environment, namely with seabirds (Kitaysky *et al*., 2000; Cury & Shannon, 2004; Frederiksen *et al*., 2006; Suryan *et al*., 2006) and marine mammals (Cury & Shannon, 2004; Trites *et al*., 2006; Benoit-Bird *et al*., 2012). As an example, the collapse of small pelagic fish populations in the northern Benguela had profound effects on top predators such as marine bird and mammals (Cury & Shannon, 2004).

Top-down and bottom-up processes are not mutually exclusive within ecosystems. In fact, both forms of ecosystem control may act in concert and their relative strength can vary in response to ecosystem alterations (Casini *et al*., 2009).

Changes in apex predator abundance and/or distribution may influence other species and several ecosystems processes, as described above. Many of the factors that can result in changes in the abundance or distribution of marine mammals have anthropogenic origins, namely: 1) incidental capture in fishing gear; 2) decrease in prey availability due to overfishing and habitat degradation 3) pollution and 4) other environmental changes, such as increased water temperatures (Lewison *et al.,* 2004; Bearzi *et al.* 2006; Lambert *et al.,* 2011; Lassalle *et al.,* 2012; Mannocci *et al.,* 2012) or ocean acidification. However, other factors may also be considered, such as ship strikes, hunting and underwater noise.

Cetacean by-catch is one of the major threats to cetacean species worldwide and is due to the competition between cetaceans and fisheries for the same resources, resulting in both economic and conservation consequences (Perrin *et al*., 1994; as reviewed by Read *et al.,* 2006). However, fisheries may also represent an indirect threat to the sustainability of cetacean populations. As top predators in the food web, cetaceans will integrate all the changes that occur in the ecosystem, namely at lower trophic levels, especially in bottom-up regulation situations (Lassalle *et al.,* 2011). Hence, the overexploitation of prey by fisheries (that represents an anthropogenic form of top-down regulation) may result in unpredictable consequences for ecosystem dynamics (Baum & Worm, 2009; Navia *et al*., 2012). The same type of effect can also occur with the decrease in prey availability due to habitat degradation, pollution and climatic change (which can affect productivity worldwide, Aebischer *et al.,* 1990; Field *et al.,* 2006; Frederiksen *et al.,* 2006).

Although the importance of marine mammals in ecosystems is recognized, as exemplified by their inclusion in ecosystem models (e.g. Ecopath) as upper-level trophic species (Libralato *et al.,* 2005; Essington *et al.,* 2007; Lassale *et al.,* 2011, 2012; Morisette *et al.,* 2012), the understanding of how cetacean can either influence or be influenced by lower levels of the trophic chain or by changes in the ecosystem is still poor.

Of the 87 cetacean species evaluated for the IUCN Red List, 45 still remain categorized as “data deficient” (IUCN, 2013). In addition, implementation of the Ecosystem Approach to Fisheries Management (EAFM, FAO, 2003; Morishita, 2008), which in Europe forms part of the reform of the Common Fisheries Policy (COM(2011) 417 and COM(2011) 425) and the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC), require collection of

data on the status of all ecosystem components, including top predators. This highlights the need to obtain solid scientific knowledge about these species.

In a preliminary approach, fundamental information is needed about genetic and ecological characteristics of a species, together with its abundance, distribution and movements, as well as the identification of major anthropogenic threats. This information will then provide foundations for the definition of the ecological role of the species in the ecosystem, the analysis of the influence of ecosystem regulation and structuring processes on animal movements, and the investigation of habitat selection and behaviours.

In relation to management (e.g. to meet conservation objectives), such information can help determine population status and trends, as well as assist with establishment of indicators and reference points to evaluate this status, and to inform management measures including possible mitigation of anthropogenic pressures, among others. At this level of knowledge, marine mammals could then be used as indicator species of lower trophic levels distribution and ecosystem processes (Hooker & Gerber, 2004).

Furthermore, the acquired information can also be applied to support the communication between science and the community and to inform development of policy, e.g. to help define legal limits of habitat exploitation (normally via managers and politicians) and if possible achieve sustainable co-existence between human economic activities (such as, for example, fisheries) and marine mammal populations. Marine mammals represent "flagship or umbrella species" (Hooker & Gerber, 2004). Therefore, conservation strategies applied to these species may also preserve the ecosystem. Hence, it is important that the development of informed conservation strategies in the current context of global change includes the dynamics between apex predators and other elements of the ecosystem.

Background and Conservation status of long-finned pilot whale (*G. melas*)

Phylogeny

Long-finned pilot whale (*Globicephala melas*) is one of the largest odontocetes. The *Globicephala* genus (Delphinidae Family) is only shared with one other species, the short-finned pilot whale (*Globicephala macrorhynchus*). These two species are difficult to distinguish at sea, given that their main differences are the length of pectoral flippers (Bloch et *al.*, 1993), the skull shape and the number of teeth (Olson, 2009) (Figure 1.1).

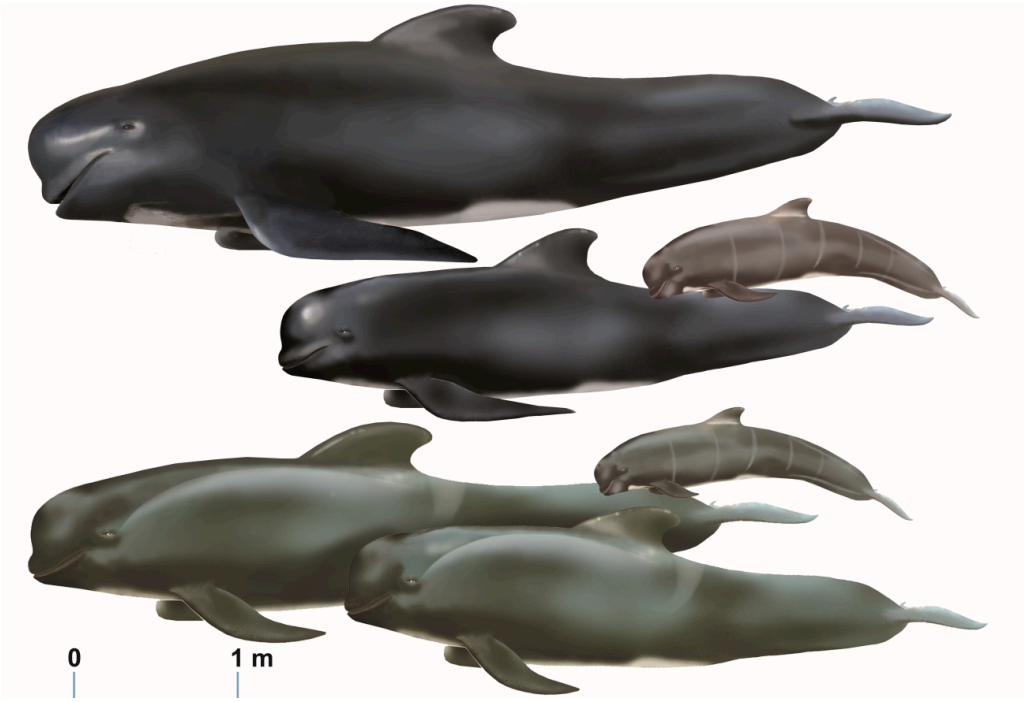


Figure 1.1. Long-finned (above) and short-finned (below) pilot whale (drawing by Tokio).

Distribution

Globicephala species exhibit parapatric distributions. *G. melas* is widely distributed in subarctic and temperate waters of the North Atlantic and Southern Hemisphere, being absent from tropical waters (Figure 1.2; Reid *et al.,* 2003). Populations from the two hemispheres are separated and some authors defend the occurrence of two subspecies (*melas* in Northern Atlantic and *edwardii* in Southern Hemisphere, Rice, 1998). In contrast, G.*macrorhynchus* is present in tropical, subtropical and warm temperate waters around the world, with some overlap existing in range between the two species (Olson, 2009).

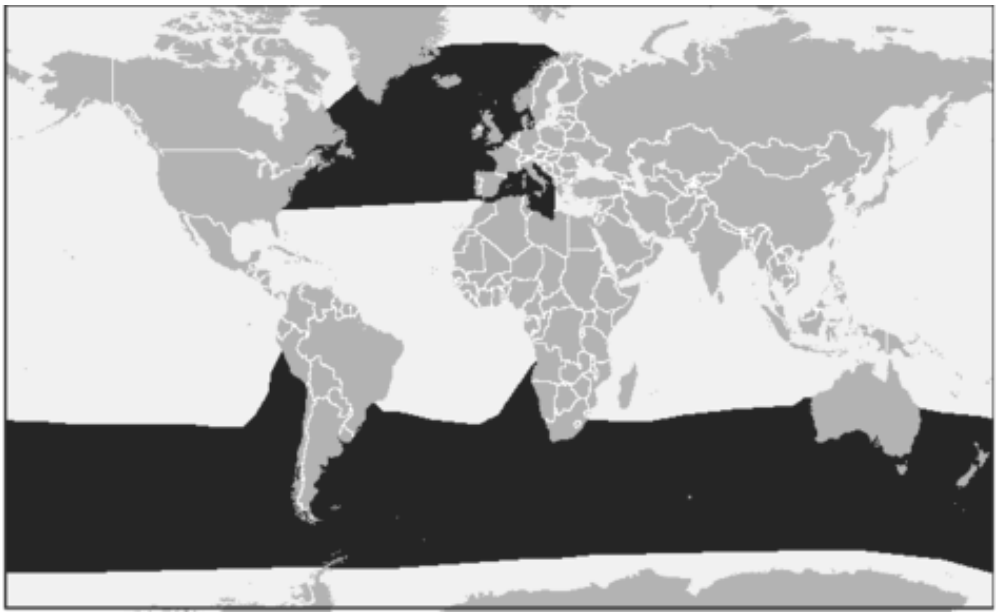


Figure 1.2. Distribution of long-finned pilot whale (from Taylor *et al.* 2008).

During the last two decades, three large-scale sighting surveys studied the distribution and abundance of cetaceans in Europe (SCANS I in 1994 (Hammond *et al.,* 2002), SCANS II in 2005 (Hammond, 2006; Hammond et al., 2013) and CODA in 2007 (CODA, 2009)). Only the survey that intended to evaluate the distribution and abundance of cetaceans in offshore European Atlantic waters (CODA) was able to calculate long-finned pilot whale abundances, estimating the occurrence of 25338 animals, in European waters (CODA, 2009).

Long-finned pilot whale is described as an oceanic species (Reyes, 1991), however incursions to coastal waters have already been registered e.g. during land-based sightings surveys (Pierce *et al.,* 2010a). This species seems to be more frequent in deep waters (100-3000m), near the continental shelf or over deep submarine canyons, with some level of preference for slope regions (Payne & Heineman, 1993; Carwardine, 1995; Cañadas *et al.,* 2002, 2005; Hamazaki, 2002; Reid *et al.,* 2003; Macleod *et al.,* 2007; Kiszka *et al.,* 2007; De Stephanis *et al.,* 2008a; Spyrakos *et al.,* 2011; Fernández et al., 2013; Silva *et al.*, 2013). Oceanographic dynamic variables (such as sea surface temperature (SST) or chlorophyll *a* (Chl *a*)) also seem to influence the distribution of pilot whales. In the North Atlantic and Mediterranean, the presence of *G. melas* seems to be related with areas with high levels of Chl *a* concentrations, and especially low sea temperatures (Fullard *et al.,* 2000; Hamazaki,

2002; Macleod *et al.,* 2007; Doksæter *et al.,* 2008; Praca & Gannier, 2008; Fernández *et al.,* 2013).

Diet

Several previous studies have analyzed the stomach contents obtained from pilot whales stranded in different parts of the world (Desportes & Mouritsen, 1993; González *et al*., 1994; Gannon *et al*., 1997; Santos & Haimovici, 2001; Pierrepont *et al*., 2005; Beatson *et al*., 2007; Beatson & O'Shea, 2009; Spitz *et al*., 2011). In general, these studies described long-finned pilot whales as a mainly teuthophagous species, although consumption of fish species has also been recorded (Overholtz & Waring 1991, Spitz *et al.* 2011). Some authors have described a positive relationship between prey distribution and pilot whale distribution and movements (Mercer, 1975; Desportes & Mouritsen, 1993; Payne & Heinemann, 1993; Zachariassen, 1993; Jákupsstovu, 2002).

Social behaviour

Pilot whales are highly social animals, aggregating in large pods, with close matrilineal associations. Results from studies carried out on samples from the North Atlantic (Faroe Islands) drive fisheries, which analysed polymorphic proteins (Andersen, 1993), microsatellites (Amos *et al.,* 1993; Fullard *et al.,* 2000), organochlorine concentrations (Aguilar *et al.,* 1993), intestinal parasites (Balbuena & Raga 1994) and heavy metals (Caurant *et al.* 1993), along with behavioural (Ottensmeyer & Whitehead, 2003; De Stephanis *et al.,* 2008b) and photo-identification studies (Alves *et al.*, 2013), agree that this species seems to show a similar social structure to that present in killer whales: natal group philopatry, where neither females nor males disperse from their natal groups. However, males do not father offspring from the same pod, apparently 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).

Population structure

Several genetic and ecological studies on pilot whale have suggested the occurrence of different populations within the North Atlantic (Perrin *et al*., 1990; Bloch & Lastein, 1993; Abend & Smith, 1995; Fullard *et al.,* 2000; Oremus *et al.,* 2009; Santos *et al.,* 2013).

Comparing the Atlantic Ocean with other oceanic basins, based on mitochondrial DNA, Oremus *et al.* (2009) found pairwise differences between Atlantic, New Zealand and Australian whales. Another study based on neutral markers (microsatellites), in North Atlantic pilot whales (Fullard *et al*., 2000), revealed differentiation between West Greenland and other regions (Cape Cod, Faroe Islands and UK), potentially associated with sea surface temperature. Also, the analysis of stable isotopes in animals from the Faroe Islands, the mid-Atlantic Bight and Cape Cod areas, suggested the occurrence of dietary segregation of whales from the West and East Atlantic, when fast and medium turnover rate tissues were considered (Abend & Smith, 1995). In the same way, differences in parasite composition between animals from the western Mediterranean, France, Faroe Islands and Newfoundland suggest that individual whales may not routinely move between any of these regions (Perrin *et al*., 1990). Another study described morphometric differences between whales from Faroe Islands and Newfoundland, suggesting the occurrence of two separated populations in these regions (Bloch & Lastein, 1993). Only one genetic analysis, based on the mitochondrial control region of 70 pilot whales from the North Atlantic (USA, Nova Scotia, Newfoundland and United Kingdom) found no evidence of population structure (Siemann, 1994).

Threats and Conservation status

Long-finned pilot whales are strictly protected under Annex IV of the Habitat Directive (92/43/EEC) in the European territory. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and the Bern Convention on the Conservation of European Wildlife and Natural Habitats categorized this species as "Vulnerable" and a "Strictly protected species", respectively. In the IUCN Red List, *Globicephala melas* is one of the cetacean species still categorized as "Data Deficient" (Taylor *et al.,* 2008).

The main anthropogenic threats to this species are the direct exploitation, by-catch in fisheries and exposure to chemical contaminants (Olson, 2009). Historically, directed fisheries for pilot whale occurred in several locations across the North Atlantic. However, nowadays, in the Atlantic Ocean, only in Faroe Islands are drive fisheries for pilot whales still occurring. Although Faroe Islands fisheries are considered sustainable (Bloch *et al.,* 2003), more information is needed to account for the impact of these captures on pilot whales populations, since it is suspected that the limit to be considered sustainable (defined as an anthropogenic removal of 1.7% , ASCOBANS, 2012) is exceeded.

Another human-induced threat to cetaceans, worldwide, is incidental capture in fishing gears. By-catch of long-finned pilot whales seems to occur mainly in longlines, trawl and gillnets across the Atlantic, although captures in purse seine have also been reported (López *et al.,* 2003; Read *et al*., 2006; Rogan *et al.,* 2007; Waring *et al.,* 2007; Leeney *et al.,* 2008; Vingada *et al*., 2011). Most by-catch probably goes unreported since this information is not recorded in many countries (Olson, 2009). Hence, the effect of the mortality rates caused by by-catch is unknown for pilot whales.

Long-finned pilot whales of the North Atlantic seem to show high levels of organochlorine contaminants (such as DDT and PCB, Aguilar *et al.,* 1993; Simmonds *et al*., 1994; Lindstrom *et al*., 1999; Tilbury *et al.*, 1999; Dam & Bloch, 2000; Weisbrod *et al*., 2000, 2001), as well as high heavy metal concentrations (cadmium and mercury, Caurant *et al.,* 1993; Dam & Bloch, 2000; Frodello *et al*., 2000). Because these contaminants accumulate in tissues over time, older animals tend to have higher concentrations (Caurant *et al,.* 1993). The broad distribution range, top trophic position and the existence of detailed data on population parameters from pilot whales captured in Newfoundland and Faroe Islands fisheries (Sergeant *et al*, 1962; Donovan *et al*., 1993), turned long-finned pilot whale into a model for contaminant study for Northwest Atlantic cetaceans (Weisbrod *et al*., 2000). However, the lack of information about other pilot whale populations and about the impact of anthropogenic activities on pilot whale populations worldwide highlight the need to increase scientific knowledge about this species. That information would help to support conservation strategies and possibly define this as an indicator species for the contamination status of cetacean of the Atlantic in order to help achieve a Good Environmental Status (GES) of European marine waters, as required by the Marine Strategy Framework Directive (Directive 2008/56/EC).

Iberian Pilot whales

In the Iberian Peninsula, several national laws demand species conservation, by forbidding the killing, harm or possession of wild animals (Portugal: law 263/81; Habitats Directive: law 140/99. Spain: law 4/1989; Habitats Directive, law 9/2001). However, long-finned pilot whales are categorized in the Portuguese Redbook of the Vertebrates as “Data Deficient” in Portugal (Cabral *et al.,* 2006) and in the “Catálogo Español de Especies Amenazadas” (law 139/2011) as “Vulnerable” (but referring only to the Mediterranean

population), in Spain. In the remaining conservation laws either this species was included in general categories of animals in need of strict protection (Ley del Patrimonio Natural y de la Biodiversidad, law 42/2007, law 1727/2007) or was not included. The exclusion of this species from some conservation legislation and its inclusion in general categories of animals in other laws are both consequences of the poor knowledge about *G.melas* along the Atlantic coast of Iberia. In this region, most studies are based on animals from the Galician coast, with basically no information about pilot whales in Portugal.

In Galician waters, a large-scale sightings survey undertaken in 2007 (Cetacean Offshore Distribution and Abundance in European Atlantic Waters*,* CODA) estimated an abundance of 194 long-finned pilot whales (*Globicepahala melas*) and 238 pilot whales (*Globicephala* sp*.,* that may also include *G.melas*) (CODA, 2009). However, this specifically refers to offshores waters rather than the Continental shelf, and the relatively high numbers of sightings and strandings suggest that this species is common in this area (López *et al*. 2002, 2004; Pierce *et al.,* 2010a; Spyrakos *et al.,* 2011; Vingada *et al.*, 2011; Santos *et al.,* 2012).

In relation to the distribution and habitat use of this species in Iberia (including Mediterranean), recent studies support previous investigations that suggest that pilot whale are mostly present in deep waters or at the limit of the continental shelf (Cañadas *et al.,* 2002, 2005; López *et al.,* 2004; Kiszka *et al.,* 2007; Praca & Gannier, 2008; De Stephanis *et al.,* 2008a; Spyrakos *et al.,* 2011; Santos *et al.,* 2012; Fernández *et al.,* 2013), although incursions into coastal waters were also observed in Galicia (Pierce *et al.,* 2010a; Spyrakos *et al.,* 2011; Vingada et al., 2011; Santos *et al.,* 2012). The only study in Iberia that also included the influence of dynamic variables on the ecological niche of *G. melas* found that along the Galician coast this species seems to prefer areas with lower sea surface temperature and higher values of chlorophyll *a* (Fernández *et al*., 2013).

Several studies in other parts of the Atlantic already related the habitat preferences and movements exhibited by this species with prey distribution (Mercer, 1975; Desportes & Mouritsen, 1993; Payne & Heinemann, 1993; Zachariassen, 1993; Jákupsstovu, 2002). In the Iberian Peninsula, trophic studies seem to also support this hypothesis. The only previously published stomach contents analysis for this region, based on three animals stranded on the Galician coast, found mostly remains of Octopodidae family (*Octopus vulgaris* and *Eledone cirrhosa*), showing that *G. melas* seems to feed on benthic and neritic species (González *et*

al., 1994, the re-analysis of this data was performed in chapter II). This result is supported by a stable isotope analysis on animals stranded in the northwest Iberian coast (Galicia and North of Portugal) that suggests that either a coastal distribution or a preference for benthic resources may be the reasons for the isotopic values of $\delta^{13}C$ exhibited by this species (Méndez-Fernandez *et al*., 2012).

Considering the major threats to long-finned pilot wale, the levels of contaminants (Méndez-Fernandez, 2012) and incidental captures in fisheries (López *et al.,* 2002, 2003) were already investigated for this species, along the Atlantic coast of Iberia. Although *G. melas* from Galicia showed lower PCB, hepatic mercury and renal cadmium concentrations than those reported in individuals from other locations in the Atlantic, some individuals of this species showed concentrations of cadmium in liver and kidney as well as hepatic Hg above the threshold level for toxic effects in mammals (20-200 µg/g w.wt, 50-400 µg/g w.wt and 60 µg/g w.wt, respectively. Law, 1996; Ma, 1996; AMAP, 1998; Méndez-Fernandez, 2012). However, caution is needed when interpreting these results due to low sample size. In relation to fisheries impact on this species, by-catch estimates based on strandings, carcass recovery and interviews with fishermen suggested that around 16 % of the stranded pilot whales along the Galician coast presented signs of by-catch and that around 100 long-finned pilot whales may have been incidentally captured in Galicia (per year), both in gillnets and trawls (López *et al.,* 2003).

In relation to the social structure exhibited by long-finned pilot whales, a photo-identification study analysed the inter-individual association patterns in pilot whales of the Strait of Gibraltar and showed evidence for the occurrence of a social system similar to killer whale matrilineal units (De Stephanis *et al*., 2008b). These results are supported by a stable isotope analysis that suggests that closely associated animals were more likely to share a similar $\delta^{13}C$ signature, compared to individuals belonging to the same clan, but which associate less often (De Stephanis *et al.* 2008b). Furthermore, photo-identification studies (De Stephanis *et al.,* 2008b; Verborgh *et al.,* 2009), together with the stable isotope analysis suggest that pilot whales are resident year round in the Strait of Gibraltar (De Stephanis *et al.,* 2008c), with a mean abundance of 213 individuals (2003-2005, Verborgh *et al.,* 2009).

The population of *G. melas* of the Strait of Gibraltar has been extensively studied, in relation to diet (De Stephanis *et al*., 2008c), social structure (De Stephanis *et al.,* 2008b), abundance, survival rates and residency (Verborgh *et al.,* 2009) and distribution (De

Stephanis *et al.,* 2008a), providing valuable information to understand the biology of this species. However, information about most of the parameters already described for the Strait of Gibraltar population is still missing for the Atlantic coast of Iberia, along with many others, such as abundance, reproductive and genetic information on pilot whales in this area or a more detailed analysis of the threats induced by human activities.

The Western Iberian Peninsula

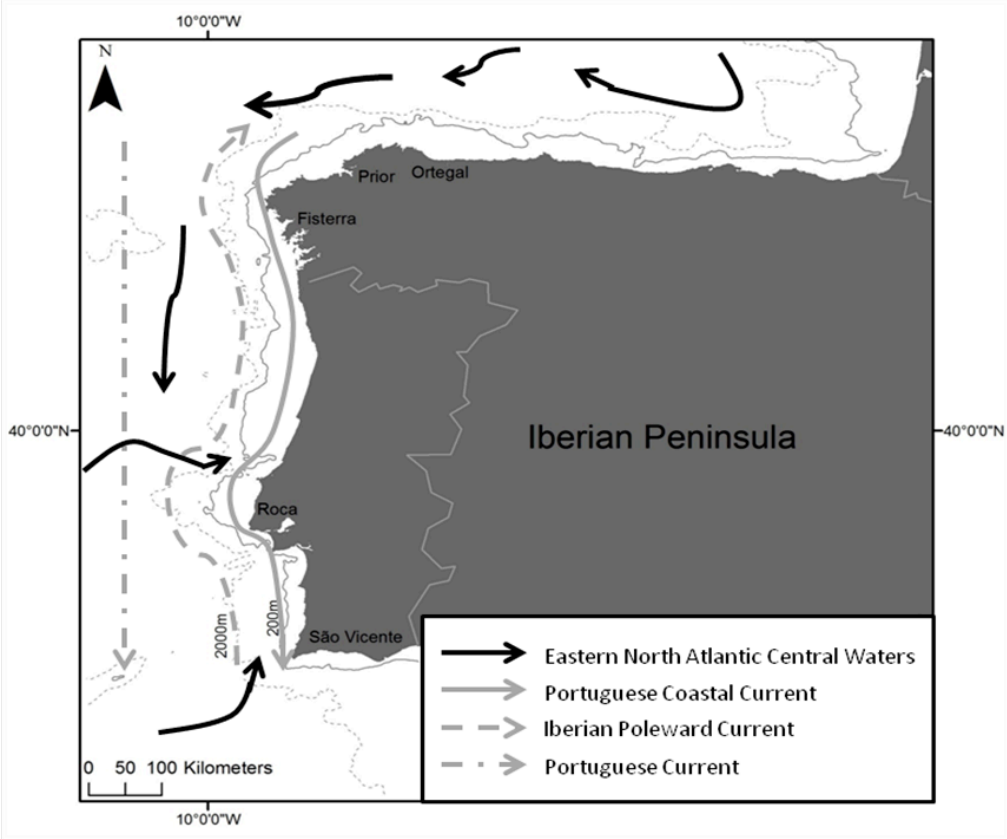


Figure 1.3. Map of the Western Iberian Peninsula. The isobaths of 200m and 2000m, as well as the main capes and main oceanic currents present in the study area are shown (adapted from Hernández-Molina *et al*., 2011).

The Western Iberian Peninsula (WIP) coast, comprising the coast of Portugal and Galicia (northwest Spain), is characterized topographically by a narrow shelf that is, on average, 45km wide and 100-200 m deep (Figure 1.3).

In relation to the oceanographic dynamics, Iberia is situated on the northern limit of the NW Africa upwelling system, a region where the interaction of along-shore winds with

the coastal topography produces an upwelling-downwelling seasonal system (Figueiras *et al*., 2002). During summer, prevalent northerly winds favour the transport of Eastern North Atlantic Central Waters of subpolar origin (ENACW) close to the Iberian coast (probably with the help of the southward flow of the Portuguese Current (PC, Prieto *et al*., 2013)), where upwelling events cause them to reach the surface (Alvarez *et al.,* 2012). These upwelled waters are generally cold and characterized by high concentrations of nutrients that enhance primary production and consequently increase the concentration of chlorophyll *a* (Chl *a*) and the levels of biodiversity in the area (Figueiras *et al.,* 2002; Moreno *et al.*, 2009; Alvarez *et al.,* 2012; Picado *et al.*, 2013).

The upwelling-downwelling pattern shows inter-annual and spatial variations across the Iberian Peninsula. Upwelling phenomena seem to occur mainly from April to September (Figueiras *et al.,* 2002; Moreno *et al.,* 2009; Alvarez *et al.,* 2012), although a shorter period seems to occur on the North coast of Iberia (June to August; Alvarez *et al.,* 2010). In general, there seems to be an influence of coastline orientation on upwelling events, since the WIP shows a higher probability of occurrence of upwelling processes than the North or South coasts of Atlantic Iberia (Relvas & Barton, 2002; Moreno *et al.,* 2009; Alvarez *et al.,* 2010, 2012: Prego *et al.*, 2012). Additionally, upwelling phenomena are generally stronger and more persistent in the former region than in the latter two areas of the Iberia (Relvas & Barton, 2002; Moreno *et al.,* 2009; Alvarez *et al.,* 2010, 2012: Prego *et al.*, 2012).

Having in mind that the presence of capes makes an upwelling event stronger and more persistent (Prego *et al.,* 2012), it is not surprising that WIP upwelling cores are located especially around Cape Fisterra (Alvarez *et al.,* 2008, 2010, 2012: Prego *et al*., 2012), Cape Roca and Cape São Vicente (Alvarez *et al.,* 2008). On the North coast of Iberia, an upwelling core is located between Cape Prior and Ortegal, (with lower values of productivity than the ones described for the west coast) (Alvarez *et al.,* 2010, 2012; Prego *et al.,* 2012). A decrease of Chl *a* concentration is observed eastward of Cape Ortegal (Alvarez *et al.,* 2010, 2012; Prego *et al.,* 2012), where occasional upwelling events were found associated with capes, such as Cape Peñas (Llope *et al.,* 2006; Prego *et al.,* 2012).

In contrast to the upwelling phenomena, between September-October until February-April the surface circulation reverses, resulting in a downwelling process (Figueiras et al., 2002). In this season, the Iberian Poleward Current (IPC) is intensified, driven by prevalent southerly winds that bring ENACW of tropical origin from 39°N to 47°N (Figueiras *et al.,*

2002). However, there is clearly some variability in this pattern as winter upwelling events have already been described (Santos et al., 2001).

The main effect of the upwelling phenomenon is the increase in primary production, which results in high levels of biodiversity in this area, since almost 400 species of fish (Bañon *et al*., 2010), 75 species of cephalopods (Guerra, 1992) and at least 20 marine mammal species (16 cetaceans and 4 pinnipeds) have been reported to inhabit the Western Iberia (Penas-Patiño & Piñeiro-Seage, 1989; Cigoña, 1990; López *et al.,* 2002, 2004; Cabral *et al.,* 2006; Pierce *et al.,* 2010a; Vingada *et al.,* 2011; Santos *et al.,* 2012). In this area, the most frequently recorded cetacean species, both from sightings and strandings are bottlenose dolphin (*Tursiops truncatus*), common dolphin (*Delphinus delphis*), harbour porpoise (*Phocoena phocoena*), minke whale (*Balaenoptera acutorostrata*), long-finned pilot whale (*Globicephala melas*), striped dolphin (*Stenella coeruleoalba*) and Risso's dolphin (*Grampus griseus*) (López *et al.* 2002, 2004, Pierce *et al.* 2010a; Vingada *et al*., 2011; Santos *et al*., 2012).

Multi-approach analyses for the definition of marine mammal population structure

Recently, the Marine Strategy Framework Directive (Directive 2008/56/EC) defined the legislative basis for an ecosystem approach to the management of human activities, in order to minimize their impacts on the marine ecosystems. Eleven descriptors were outlined to ensure the Good Environmental Status (GES) of European marine waters and the first descriptor concerns biodiversity, thus requiring the analysis of the biological diversity of the ecosystem. This framework implies that conservation efforts have to be directed not only for the maintenance of marine species viability, but also for the preservation of the behavioural, ecological and genetic diversity within species, as defended in many studies (Dizon *et al*., 1997; Coyle, 1998; Reeves *et al.,* 2003).

The geographic distribution of a species is frequently large relative to the dispersion ability of the individuals, resulting in structuring in most natural populations. These local populations can either be defined based on evolutionary traits, behaving as reproductively isolated units with low or no genetic flow between them (genetic stock) or based on ecological characteristics, where geographically isolated units adapted separately to the

different habitats, even if no genetic differentiation has occurred (i.e. these are ecological or phenotypic stocks) (Coyle, 1998; Waples & Gaggiotti, 2006).

Logically, management efforts should be scaled according to population structure. Measures designed to protect or enhance the status of animals in an area may not have the expected consequences if those animals are part of a larger population or indeed if they belong to several different populations (Evans & Teilmann, 2009; ICES 2009). Predictable effects on population trends are likely to occur only when a management unit corresponds to a "real" biological population.

Several studies have used genetic markers to provide information about wild population divergence and to support the definition of management units (Wang *et al.,* 1996; Rosel *et al*., 1999; Mendez *et al.,* 2007), because it was evident that reproductively isolated units should normally be recognized as separate management units (Palumbi & Cipriano, 1998; Moritz, 2002; Palsbøll *et al*., 2007; ICES 2009), since their responses to perturbations would be distinct. Recently, there has been an increasing use of ecological markers to study the definition of "ecological populations" (Caurant *et al*., 2009; Evans & Teilmann, 2009; ICES *et al*., 2009, 2013). The justification for separation of management units based on ecological populations is less clear, however it has been suggested that some species, such as for example *Delphinus delphis*, should be managed using an ecological time-scale, since it represents a finer scale that may be more relevant to management issues than the evolutionary time-scale (Evan & Teilmann, 2009). Additionally, ecological populations could be viewed as units likely to become reproductively isolated in the future, or units whose unique characteristics and/or distribution justify their separate conservation (Hoelzel, 1998; Schluter, 2001; Caurant *et al*., 2009; ICES, 2009, 2013).

For conservation and management purposes, it is therefore essential to identify the occurrence of population structure or segregation within a species, in order to preserve genetic and ecological diversity. Several studies have applied multi-approach analyses (i.e. analyses of several genetic and/or ecological parameters in the same individuals) to quantify marine mammal stock structuring or ecological segregation between species (Herman *et al*., 2005; Borrell *et al.,* 2006; Born *et al*., 2007; Krahn *et al.,* 2007; Caurant *et al.,* 2009; Foote *et al.,* 2009; Méndez-Fernandez *et al*., 2013; Quérouil *et al.,* 2013). As an example, Born *et al*. (2007) used a combination of heavy metals, organochlorines and fatty acid analyses to

identify subpopulations of minke whales (*Balaenoptera acutorostrata*) of the North Atlantic, being able to discriminate four subpopulations across this area.

Another example consists of a long-term investigation of killer whales (*Orcinus orca*) in order to confirm the existence of a third ecotype of this species, besides the previous described "resident" and "transient" ecotypes, through genetic (Hoelzel *et al*., 1998), acoustic (Barret-Lennard *et al.,* 1996), morphological (Ford *et al*., 2000) and feeding (Ford *et al*., 1998) ecological analyses. The third ecotype ("offshore") was firstly suggested by the analysis of killer whales' offshore distribution between California and Alaska (Krahn *et al.,* 2007). It was then confirmed by the analysis of stable isotope ratios of carbon and nitrogen, persistent organic pollutants and fatty acids in biopsy samples (Herman *et al.* 2005; Krahn *et al.,* 2007), which shown results consistent with genetic differences (mtDNA) in relation to resident and transient whales (Barrett-Lennard, 2000).

In the past decades, the use of genetic approaches to answer ecological questions and support conservation and management strategies has become more efficient, powerful and flexible (Selkoe *et al.,* 2006; Palsbøll *et al.,* 2007). One of the advantages leading to the widespread use of molecular biology by ecologists relates to the fact that genetic markers can provide long-term information about wildlife population parameters, such as genetic diversity, population structure, migration rates, population size, demographic processes (bottlenecks or expansion), kinship and even conservation-related issues, such as detection the genetic impact of human-induces threats (by-catch, Baker *et al.,* 2006; Méndez *et al.,* 2007) or identification of illegally traded cetacean species at whale meat markets (Baker *et al.*, 2006). Furthermore, the constant technical and statistical advancements in molecular biology made it easier and less expensive to investigate the genetic diversity and divergence of wild species (Selkoe *et al.,* 2006). Several examples of the application of genetic procedures to study the parameters described above, but especially for population and ecotypes structure analyses, exist for marine mammals (Bérubé *et al.,* 1998; Hoelzel *et al.,* 1998; Fullard *et al.,* 2000; Born *et al.,* 2003; Natoli *et al.,* 2004, 2006, 2008a, 2008b; Fontaine et al., 2007; Méndez *et al.,* 2007; Mirimin *et al.,* 2009; Oremus *et al.,* 2009; Tezanos-Pinto *et al.,* 2009; Anderwald *et al.,* 2011).

As apex predators, it is important to complement the genetic information on cetaceans with an understanding of their role in marine food webs. The food web is the main route by

which marine mammals incorporate bioavailable environmental elements and compounds into their tissues, materials which were ultimately generated either by biochemical (fatty acids, Carbon and Nitrogen stable isotopes), geochemical (trace elements) or anthropogenic (organic contaminants) processes (Caurant *et al.,* 2009). Consequently, these materials may be considered as ecological tracers of food resources and/or habitats exploited by individuals, and, by extension, indicators of population structuring.

The differences in tracer signatures between groups of animals potentially reveal ecological differentiation between those groups, on an integration time-scale that is linked to the half-life of the tracer and the turnover rate (i.e. the time taken for the replacement of old tissue signatures with new dietary signatures during tissue repair, Sweeting et al., 2005) of the tissue analysed (Caurant *et al.,* 2009). Therefore, depending of the type of tracer used and the relative turnover rate of the tissue analysed, tracers are able to provide information referring to time periods ranging from some days of integration (stomach content analysis), days to life times (fatty acids, stable isotopes, trace elements, vital rates) or even generations or evolutionary time-scales (genetic markers or morphometrics) (Hobson & Clark, 1992; Wagemann et al., 1990 in Das et al., 2000; Hobson & Sease, 1998; Kirsch *et al*., 2000; Nordstrom *et al.,* 2008; Caurant *et al*., 2009 and references therein).

The difficulty of directly observing the foraging behaviour of cetaceans in their natural habitat has led to the development of several techniques to obtain information about these species' feeding ecology, habitat use and trophic position, namely analysis of hard parts in stomach contents and scats and, more recently, stable isotope and fatty acid analyses of predator tissues, and molecular analysis of prey tissues remaining in stomachs and scats (Tollit *et al.,* 2009). All the analytical methods mentioned present advantages and disadvantages (reviewed in Tollit *et al*., 2010). While stomach contents and scat analyses provide only a snapshot of the diet of an individual, with potential biases associated with differential rates of digestion of prey hard parts, they still provide useful direct information about the diet composition. Ecological tracers, such as fatty acids and stable isotopes, are able to supply less (or at least differently) biased and longer-term information about top predator feeding habits (Budge *et al*., 2006; Caurant *et al.,* 2009; Tollit *et al*., 2010; Hobson, 1999; Chouvelon *et al*., 2012; Kelly & Scheibling, 2012). However, as implied by the term "tracer" they represent indirect methods for studying dietary intake, constituting a proxy of diet composition, trophic level or habitat occupied by the marine mammals (Hebert *et al*.,

2006; Caurant *et al.,* 2009; Méndez-Fernandez, 2012). Additionally, while all the ecological tracers mentioned may provide information about the habitat and the feeding ecology of the predator, their concentrations are highly influenced by other factors such as their bioavailability, spatio-temporal variations in the food webs or intrinsic biological factors such as sex, age, growth, metabolism and physiology of the species and individuals, all of which factors must be carefully considered (Honda & Tatsukawa, 1983; Hobson & Welch, 1992; Wagemann *et al*., 1995; Bustamante *et al.,* 1998; Koopman, 2001; Das *et al*., 2003; Vanderklift & Ponsard, 2003; Iverson *et al*., 2004; Budge *et al.,* 2006; Koopman, 2007; Caurant *et al.,*2009; Newland et al., 2009; Méndez-Fernandez, 2012).

Due to the advantages and disadvantages of the different types of tracers (as described above), there is a consensus that the combination of complementary ecological tracers with diverse integration time-scales (e.g. stomach contents, fatty acids, stable isotopes, trace elements and contaminants) along with genetic information can provide more complete and reliable information about habitat use, distribution, feeding ecology and social structure and at the same time allow the understanding of possible genetic and ecological structure within a population (Caurant *et al.,* 2009; Evans & Teilmann, 2009; ICES, 2012).

Habitat preferences and suitability as a complement for population structure studies for management purposes

Changes in the distribution patterns are probably a more rapid response to the variability in marine ecosystems, exhibited by wide-ranging cetacean species, than changes in population parameters, such as survival or reproductive rates (Forney, 2000; Redfern *et al.,* 2006). Consequently, besides understanding levels of genetic and ecological population structure in order to preserve the diversity within a species, the identification of suitable habitats within a species range, along with the understanding of the links between cetacean distribution, environmental variables and local variation in prey choice, have been defined as a priority for effective conservation and management (Torres *et al.,* 2003; Cañadas *et al.,* 2005). In addition, changes in both range and abundance of various marine species are used as indicators for the biodiversity descriptor of the MSFD.

As a first approach it is fundamental to determine which habitats are used with higher frequency by marine mammals (Cañadas *et al.,* 2005), especially when little is known about

the ecology of a species (Redfern *et al.,* 2006). Therefore, it is important to perform a regular and comprehensive monitoring of wild populations, as described by numerous investigations (Payne & Heinemann, 1993; Gannier *et al.,* 2002; Moore *et al.,* 2002; Hammond *et al.,* 2002, 2006, 2013; Macleod *et al*., 2003; López *et al.,* 2004; Wall *et al.,* 2006; Kiszka *et al.,* 2007; Shirakihara *et al.,* 2007; Weir *et al.,* 2007; Nichols *et al.,* 2008).

The following steps consist of determining which ecogeographical variables (biotic and abiotic) influence the distribution of the species and the identification of suitable areas. Several cetacean distribution studies aimed at identifying suitable habitats, in order to understand the ecology of these animals and establish management strategies to protect critical habitat and maintain a favourable conservation status (Cañadas *et al.,* 2005). The definition of those suitable habitats were usually influenced by biological (Baumgartner *et al.,* 2003; Tynan *et al*., 2005; Torres *et al*., 2008; Doniol-Valcroze *et al.,* 2012; Goetz *et al.,* 2012; Moura *et al.,* 2012; Pendleton *et al.,* 2012; Pirotta *et al.,* 2013) or anthropogenic features of the inhabited area (Kaschner, 2004; ASCOBANS, 2005; Gregr *et al*., 2011; Lambert *et al.,* 2011; Goetz *et al.,* 2012). When substantial *a priori* knowledge exists about cetacean-habitat relationships, models can be used to test specific hypotheses about the ecological processes determining cetacean distributions (Redfern *et al*., 2006).

The application of species distribution modelling (SDM) techniques, namely presence-absence (GLM,GAM, zero inflated models) or presence-only methods (PCA, ENFA, GARP, MAXENT) attempts to understand the spatial distribution and abundance of cetacean species, based on the preference for habitats defined by the combination of environmental (topographic, climatic, prey availability) or anthropogenic characteristics (Cañadas *et al*., 2005; Elith *et al.,* 2006; Redfern *et al*., 2006; Macleod *et al.,* 2008; Praca *et al.,* 2009). This is the reason why habitat modelling has recently become extensively used among ecologists.

Presence-absence (PA) methods require data on the distribution of survey effort so that, even if absence was not explicitly recorded, it can be inferred, while presence–only (PO) models only require the occurrence data (Macleod et al., 2008). Both type of techniques present advantages and disadvantages. Several authors argue that presence-absence (PA) methods should be preferred over presence-only (PO) techniques to predict species distribution, when absence data are available (Brotons *et al.,* 2004; Macleod *et al.,* 2008). However, another consideration is that it is not always possible to have accurate

absence data for cetaceans, due to either logistic and/or ecological constraints (Macleod *et al.,* 2008), which may hamper the detection of these species at sea. Failure to detect animals that are present on a survey route ("false absence") can present as a problem for PA methods, since it can lead to potentially biased model predictions (Hirzel *et al.,* 2001; Pearson *et al.,* 2007). However, if the probability of false absences is constant, relative occurrence or abundance estimates instead of absolute abundance or occurrence probabilities can be analysed (Mackenzie, 2005). Furthermore, if the heterogeneity in the probability of detection is evaluated on the survey stage and associated with abundance or occupancy modeling approaches that account for imperfect detection (Buckland et al., 2001; Mackenzie et al., 2002, 2005; Cañadas et al., 2004; Martin *et al.,* 2005 and references therein; Rota et al., 2011; Zuur et al., 2012), then there is less chance to result in spatially biased predictions (Mackenzie et al., 2002, 2005; Rota et al., 2011). However, there is still some controversy around this subject (Manel et al., 2001; McPherson et al., 2007; Santika et al., 2011; Welsh et al., 2013).

Presence-only modelling can be an alternative technique to PA methods, since it does not require the use of absence data. Furthermore, PO analysis allows the use of sightings data that were collected opportunistically from a wide range of sources (i.e. data *not* collected from dedicated effort-based surveys at sea). Finally, several studies of marine organisms have shown that PO techniques can produce models of habitat suitability significantly better than random and which exhibit comparable performances to PA approaches (Macleod *et al.,* 2008; Tittensor *et al.,* 2009). However, PO models also present disadvantages. As presence-only methods do not take into account the survey effort, such models may be affected by sample selection bias (whereby some areas in the "landscape" (of available habitats) are sampled more intensively than others) (Macleod *et al.,* 2008; Phillips et al., 2009; Elith et al., 2011). Moreover, absolute prevalence (proportion of sampled sites where a species is present, Santika, 2011) is not identifiable from presence-only data (Ward et al., 2009). However, as indicated in the previous paragraph, absence data also have several problems associated with detection probability (Manel *et al*., 2001; McPherson et al., 2007; Santika et al., 2011), so that even presence-absence data may not yield a good estimate of prevalence (Elith et al., 2011).

The development of habitat modelling approaches represents a great improvement over using simple measures of occurrence, such as distribution maps or encounter rates (Cañadas *et al.,* 2005). Furthermore, habitat suitability studies can sometimes predict cetacean distribution and animal movements in areas not surveyed due to logistic or inaccessibility limitations, being a good approach for the exploration of potentially new habitats (Torres *et al.,* 2008).

Thesis Objectives

Given the limited available information about the *Globicephala melas* on the Atlantic coast of the Iberian Peninsula and given that the determination of population structure and habitat preferences and suitability are among the main research needs for adequate management and conservation, this thesis will address the objectives described below. The study focuses on the Iberian Peninsula, making use of samples and data from strandings and data from sightings surveys. However, it was also possible to obtain samples from several other parts of the North Atlantic, thus permitting an investigation of large-scale stock structuring.

Objective 1. To describe the feeding habits of long-finned pilot whales in the northeast Atlantic, with a particular focus on Iberian Peninsula but making use of other available data, based on the analysis of stomach contents from animals stranded in Portugal, Northwest Spain (Galicia) and Scotland. Dietary variability of *G. melas* was related to geographical (area), seasonal (year, season) and biological (length and sex) variables and the existence of different diet preferences between putative populations was explored.

Objective 2. To determine if there are long-term dietary differences between long-finned pilot whale of Atlantic Iberian Peninsula (Portugal and Galicia), Scotland and USA (Cape Cod), blubber fatty acids were analysed. Fatty acids were used as ecological tracers of habitat segregation, and inferred dietary variability of *G. melas* was related to geographical (area) and biological (sex and maturity) variables. The existence of different diet preferences between putative populations was explored and the outcome was compared to stomach contents results.

Objective 3. To determine the genetic population diversity and divergence in long-finned pilot whales in the North Atlantic and adjacent waters. The spatial distribution of mitochondrial DNA and Major Histocompatibility Complex variation was used to characterize levels of population genetic diversity and structure among putative populations from Atlantic Iberian Peninsula (Portugal and Galicia), Scotland, Faroe Islands and USA (Cape Cod).

Objective 4. To determine habitat preferences and identify suitable habitats of long-finned pilot whales from Atlantic and Cantabrian coasts of Iberia, using presence–only methods (PCA and MAXENT). This analysis was based on the relationship between pilot whale occurrences and six ecogeographic variables known to be important in determining the distribution of cetacean species. Results were compared to stomach contents and fatty acids results, to understand if habitat use may be related with prey distribution and movements.

Additional objectives include:

Objective 5. To combine the results of the genetic (objective 3) and ecological tracers analyses (objective 1 and 2) in order to synthesize the occurrence of potential population differentiation of long-finned pilot whales from the North Atlantic, based on a multi-tracer approach.

Objective 6. To obtain valuable information of genetic and ecological characteristics of G.melas in Western Iberia, in order to help define their conservation status.

Thesis outline

The thesis consists of six chapters of which four are presented as scientific articles at different stages of the publication process. Authorship of chapters for publication is shared with other researchers who have made significant contributions to the work. All co-authors are listed at the beginning of the chapters concerned.

Chapter 1 (the present chapter) provides a general introduction to the importance of studying cetacean ecology, the background and conservation status of long-finned pilot whale (*Globicephala melas*), the characterization of the study area and the application of multi-approach analyses for the definition of marine mammals population structure, as well as the application of habitat preferences and suitability as a complement for population structure studies for management purposes.

Chapter 2 investigates variation in *G. melas* diet in Portugal, Northwest Spain (Galicia) and Scotland, through stomach contents analyses. The existence of different diet preferences between putative genetic populations was explored. In addition, diet variation in relation to other variables (e.g. year, season, sex and length) was examined (Objective 1).

Chapter 3 examines potential population structure between putative genetic populations (Iberia, Scotland and United States of America), using blubber fatty acid analysis. Variation in fatty acid signatures was examined in relation to other aspects of long-finned pilot whale biology (e.g. sex,) and comparisons with results obtained from stomach content analyses were undertaken (Objective 2).

Chapter 4 examines the potential population structure of long-finned pilot whales off North Atlantic waters (Iberia, Scotland, Faroe Islands and USA), with the use of genetic markers (mitochondrial DNA and Major Histocompatibility Complex) (Objective 3).

Chapter 5 includes the analysis of habitat preferences of long-finned pilot whales from Atlantic and Cantabrian coasts of Iberia and identification of suitable habitats for pilot whales along this area, based on six ecogeographic (Objective 4).

A general discussion is provided in **Chapter 6** (where objectives 5 and 6 will be discussed), which concludes and offers some broader perspectives on this work.

Chapter II

Patterns and trends in the diet of long-finned pilot whales (*Globicephala melas*) in the Northeast Atlantic.

Santos, M. B., S. S. Monteiro, J. V. Vingada, M. Ferreira, A. López, J. Martinez-cedeira, R. J. Reid, A. Brownlow and G. Pierce. 2013. Patterns and trends in the diet of long-finned pilot whales (*Globicephala melas*) in the northeast Atlantic. *Marine Mammal Science* doi: 10.1111/mms.12015.

Abstract

There is little previous information on feeding habits of long-finned pilot whales (*Globicephala melas*) in the Northeast Atlantic. The present study analysed stomach contents of pilot whales stranded in Portugal (*n*=6), Galicia (Northwest Spain) (*n*=32), and Scotland (UK) (*n*=10), from 1990 to 2011. These animals ranged from 213 to 555 cm in length (24 females, 19 males and 5 of unknown sex). The main prey identified were cephalopods of the families Octopodidae and Ommastrephidae, the former being numerically more important in Iberia (Portugal and Galicia) and the latter more important in Scotland, with Iberian whales also showing a more diverse diet. Multivariate analysis revealed evidence of geographical and seasonal variation in diet. Generalized Additive Modelling results indicated that more octopus *Eledone cirrhosa* were eaten in Iberia than in Scotland, more in the first half of the year, and more in larger whales. Numbers of ommastrephid squids in the stomach decreased over the study period and varied with season and whale length. This study confirms cephalopods as the main prey of pilot whales, as previously reported, although our results also suggest that, in the northeast Atlantic, ommastrephid squid are largely replaced as the main prey by octopods at lower latitudes.

Introduction

The long-finned pilot whale (*Globicephala melas*), herein after referred to as pilot whale, is one of the largest odontocetes, with maximum length recorded as 625 cm (Bloch *et al*., 1993). The species is distributed throughout temperate and subarctic regions of the northern and southern hemisphere, being absent from tropical waters (Reid *et al*., 2003). Although occupying mainly oceanic habitats (Bloch *et al*., 2003; Macleod *et al*., 2007; Azzellino *et al*., 2008; De Stephanis *et al*., 2008*a*), with most sightings recorded in waters over 2,000 m (Baird *et al*., 2002), pilot whales can range over the continental shelf and, in Galicia, the species has occasionally been observed during land-based sightings surveys (Pierce *et al*., 2010a).

Several studies have analysed the stomach contents obtained from pilot whales stranded in different parts of the world (*e.g.*, Desportes & Mouritsen, 1993; Gannon *et al*., 1997; Santos & Haimovici 2001; Pierrepont *et al*., 2005; Beatson *et al*., 2007; Beatson & O'Shea 2009; Spitz *et al*., 2011). In general, these studies have found cephalopods to be the main component of pilot whale diet, although fish may also be important (Overholtz &

Waring, 1991; Spitz *et al.,* 2011). The only previous study for the NW Iberian Peninsula was by González *et al*. (1994), who described cephalopod remains in stomach contents of three individuals stranded in Galicia: material from these three samples has been included in the present analysis. There are no previous studies of the diet of this species in UK waters.

Due to the difficulty of carrying out direct observations in their natural habitat, obtaining information on the feeding ecology of cetaceans has traditionally involved the examination of stomach contents of dead animals (either from stranded or directly caught individuals). Although several indirect methods to obtain information on the feeding habits of marine mammals have been developed over the last 2-3 decades and include the use of fatty acid and stable isotope profiles of predator tissues, DNA analysis of prey remains in feces, *etc*. (for a recent review see Tollit *et al.,* 2010), such techniques are most useful once some information on diet is already available, since they rely on the existence of a library of prey "signatures". Because of these limitations, examination of stomach contents remains the most widely used method to study cetacean diet.

Provided that possible biases in the samples available are kept in mind, *i.e*., that the sample could show an overrepresentation of sick animals not able to feed properly, that prey hard structures are subject to differential digestion, *etc*. (see Pierce *et al*., 2004; Tollit *et al.,* 2010 for discussions on the topic), strandings monitoring programs afford an excellent opportunity to study feeding habits and factors affecting cetacean diet. Stomach contents can often be extracted even from partially decomposed carcasses and important ancillary data such as location, date, sex, and body size can also be obtained together with cause of death in some cases. These data can be used then to investigate differences in diet between different population components. In addition, the use of all hard remains has been shown to increase the rate of prey detection, especially for those species which have small and/or fragile otoliths (for example, Brown & Pierce, 1998).

As top predators, cetaceans play an important role in marine food webs and improved knowledge of their diet and the factors that can affect it (*e.g.*, season, year, ontogeny, *etc*.) are of considerable importance to help us determine their ecological role, to quantify the predator-prey relationships, and to evaluate the possible threats these predators could be facing (*e.g.*, prey depletion due to overfishing, changes in prey distribution, and availability due to other anthropogenic pressures such as climate change, Pierce *et al*., 2004). In the case of pilot whales their oceanic habitat and deep diving capabilities make direct

observations of whale feeding a challenge, and as with many other odontocete species, information on diet and on basic life history has been obtained by the study of stranded individuals and those obtained by direct hunt, which is still carried out in the Faroe Islands (*e.g*,. Desportes & Mouritsen, 1993).

The main goals of the present study are therefore: i) to describe the feeding habits of pilot whales in the Northeast Atlantic based on the analysis of the stomach contents obtained from animals stranded in three different geographical locations (Portugal, Scotland, and Northwest Spain) and ii) to analyze the dietary variability in relation to area, year, season, length, and sex of the whales.

Methodology

Sample collection

In our study area, three stranding monitoring programs are responsible for the examination of marine mammal carcasses and the collection of samples. Strandings are attended in all cases by experienced personnel, from the Sociedade Portuguesa de Vida Selvagem (SPVS) in northern Portugal, from the Coordinadora para o Estudio dos Mamíferos Mariños (CEMMA) in Galicia (NW Spain) and from the Scottish Agriculture College Veterinary Science Division (SAC) in Scotland. In all cases, when the condition of the animal permitted it, detailed necropsies were performed. Otherwise, basic measurements/information (*i.e.*, length, sex, decomposition state) and samples were collected (*i.e.*, teeth, blubber, and, when possible, stomach contents). Since not all animals were assessed for maturity status, we summarized the likely distribution of maturity stages based on body length, following Bloch *et al*. (1993).

Monitoring of strandings along the Galician coast started in 1990. A mean of 183 animals stranded per year between 1990 and 2010. Of 232 long-finned pilot whales recorded over this period, detailed necropsies were carried out on 56 whales and stomach contents were obtained from 32 of them. In Scotland, the strandings monitoring network started in 1992 and registered a mean of 152 cetacean strandings per year, with a total of 149 pilot whales strandings up until June 2011. Of these, only the animals in a fresh state were sent for detailed necropsies (*n*=24) and of the 24, stomach contents were recovered from 10 animals. A detailed monitoring program in the centre and north of Portugal (with active search and detailed necropsies on stranded animals carried out whenever possible)

began in 2000, registering *ca.* 160 strandings per year. A total of 17 pilot whales was recorded stranded in this area up to 2011, with stomach contents being recovered from 7 out of the 8 animals which were fully necropsied. One of these 7 animals with non-empty stomachs had only milk in its stomach and further analysis therefore refers to 6 whales from Portugal. Thus, from 1990 to June 2011, a total of 48 nonempty stomachs were collected and analysed (Figure 2.1 and Table 2.1).

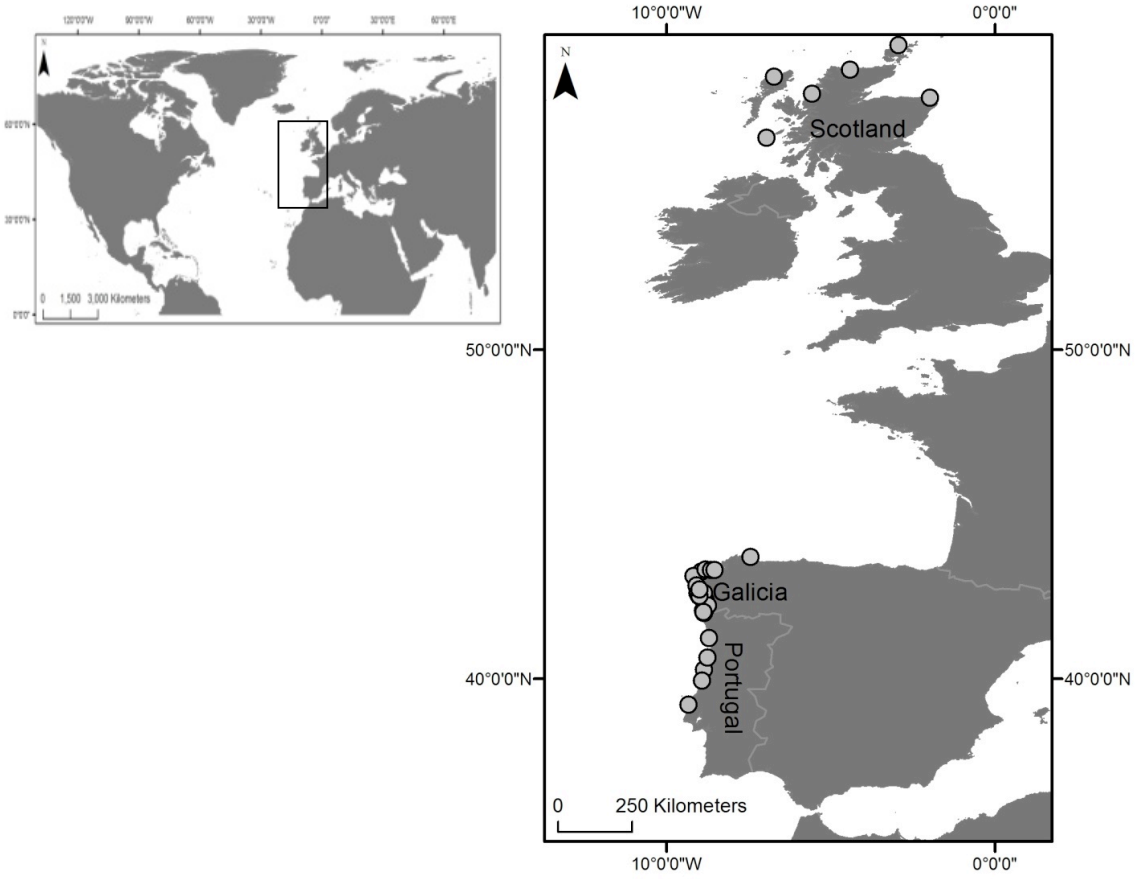


Figure 2.1. Map showing the locations of the strandings of pilot whales analysed in this study (n =48)

Table 2.1. Summary of composition of sampled pilot whales. Data are described in each year period, by (a) season (quarter), (b) sex, (c) area and (d) maturity.

Year		Quarter				Sex			Area			Maturity		
		Q1	Q2	Q3	Q4	F	M	U	PT	GAL	SCO	I	M	U
1990-1995	13	3	8	1	1	6	6	1	0	11	2	11	2	0
1996-2000	12	5	5	0	1	6	6	0	0	10	2	5	7	0
2001-2005	7	3	3	2	0	3	3	1	2	2	3	5	1	1
2006-2011	16	10	10	2	0	9	4	3	4	9	3	6	8	2
Total	48	15	26	5	2	24	19	5	6	32	10	27	18	3

Location		Sex			Maturity		
		F	M	U	I	M	U
PT	6	2	2	2	1	3	2
GAL	32	17	12	3	19	10	1
SCO	10	5	5	0	5	5	0
Total	48	24	19	5	25	18	3

Year periods: 1990-1995; 1996-2000; 2001-2005; 2006-2011. Season: Q1, Jan- March; Q2, Apr-Jun; Q3, Jul-Sept; Q4, Oct-Dec. Sex: F, female; M, male; U, unknown. Location: PT, Portugal; GAL, Galicia; SCO, Scotland; Maturity: I, immature; M, mature; U, unknown.

All nonempty stomachs were either taken to the laboratory whole or dissected on the beach. Stomachs contents were preserved frozen or in 70% ethanol prior to further analysis. Prey remains consisted almost exclusively of cephalopod mandibles (beaks), which were preserved in 70% ethanol, as were crustacean and other mollusc remains. Some fish otoliths, bones, and eye lenses were also found and these remains were stored dry.

Sample analysis

Analysis of diet composition

The cephalopod beaks, and fish otoliths and bones were identified using published guides (Clarke, 1986; Härkönen, 1986; Watt *et al*., 1997; Tuset *et al*., 2008) and reference collections of cephalopod beaks (provided by Malcolm Clarke from his extensive collection identified from the stomach of predators) and of fish otolith and bones from the Northeast Atlantic held at the University of Aberdeen. In practice, very few fish otoliths were recovered

and other fish remains (*e.g.*, vertebrae, other bones, and eye lenses) were therefore also used to identify the prey taken, when possible, and to quantify the number of fish taken. Not all remains could be identified to species. Thus, the highest number of otoliths (18) was recovered from a whale stranded in Scotland but these otoliths could not be identified since they did not correspond to any of the many species available in the reference collection or in the published guides for the Northeast Atlantic.

The minimum number of individual cephalopods of a taxon present in each stomach was estimated from the numbers of upper or lower beaks, whichever was higher. Likewise, the minimum number of fish of each taxon present in each stomach was estimated by counting sagittal otoliths and three of the jaw bones (premaxilla, dentary, maxilla), and using the most numerous. Each otolith, premaxilla, dentary, or maxilla was assumed to represent 0.5 fishes, while each upper or lower beak represented 1 cephalopod. Crustacean and other mollusc remains were identified to the lowest possible taxon, although identification was usually difficult due to the poor state of preservation in which they were found.

Prey length and weight were estimated from beak and otolith dimensions using a compilation of published regressions (see Appendix 1, in Supplementary Material). For cephalopods, since complete pairs of beaks were rarely present, weight and length were estimated using, in most cases, the lower beak measurements (rostral length for squid and hood length for octopus and sepiolids; Clarke, 1986). For stomachs in which a cephalopod species was represented by more than 30 beaks, we measured a random sample of around 10% of the total number of beaks of that species (not less than 30 beaks). In fishes, size estimates were mainly based on otolith length (Härkönen, 1986) or width for any otolith broken lengthways. All measurements were taken with a binocular microscope, fitted with an eyepiece graticule, or with calipers. When identification to species level was not possible and remains were assigned to a group of species (*e.g.*, family or genus), the regression used to estimate fish size was based on a combination of data from all (relevant and available) species of that grouping (see Appendix 1, in Supplementary Material). No correction was applied to the estimates of fish size obtained from otoliths to take account of potential gastric erosion. The measurement of only uneroded otoliths, which has been suggested as a possible solution to this problem, was not possible in our case since all fish material was found in a digested state with no flesh remaining.

Although all identifiable hard remains were used to estimate the numerical proportion of each prey taxa, only measurements of cephalopod beaks and fish otoliths were used to calculate original prey size. Therefore, because prey (generally fish) were sometimes represented only by other remains, *e.g*., bones or eye-lenses, the proportion of fish (by weight) in the diet could be underestimated.

Analysis of dietary variation

Overall diet of pilot whales in each area was quantified using three standard indices (Hyslop, 1980): (1) frequency of occurrence of each prey type (calculated as the number of stomachs where prey *i* was found divided by the total number of non-empty stomachs examined), (2) numerical proportion of each prey type *i* in relation to the total number of individual prey (calculated by adding all individuals of prey type *i* identified in all stomachs and dividing this total by the summed number of all individuals of all prey in all the stomachs) and (3) proportion of the total reconstructed prey weight represented by each prey type (calculated similarly to (2)). For the latter two indices, the totals are those for all stomachs combined. This approach implies that no explicit weighting is applied to each sample (stomach) when estimating overall diet, so that animals with larger amounts of food in the stomach contribute relatively more to the estimated overall diet. Alternative weightings, for example equal weighting, are possible but this latter approach would assume that all whales, regardless of their size or the amount of food in their stomachs, contribute equally to the overall amount of food removed. For a discussion of the issue and the consequences of applying different weightings see Pierce *et al*. (2007) and Tollit *et al*. (2010).

To determine which explanatory variables may influence the stomach contents of pilot whales, the numerical importance of the main prey types in the diet was analysed using a combination of multivariate exploration based on Redundancy Analysis (RDA) and univariate modeling using Generalized Additive Models (GAM), as implemented in Brodgar 2.7.2 (www.brodgar.com). The response variables were numbers of each type of prey present in individual stomach samples rather than estimated total weights since the latter are subject to additional errors. Specifically, not all individual prey were identified from cephalopod beaks or fish otoliths but only beaks and otoliths were measured to obtain prey sizes and weights, it was not possible to account for digestive size reduction of measured hard parts,

and, finally, some weights were estimated using regression equations constructed using combined data from several prey species.

All data series were explored for outliers, collinearity, heterogeneity of variance and interactions between variables, and to visualize the relationships between response and explanatory variables, following the protocol proposed by Zuur *et al*. (2010). RDA was then used to visualize any patterns in the set of response variables (prey numbers) as well as any relationships between the set of response variables and the various explanatory variables. To avoid the results being unduly influenced by rare prey types, to deal with prey groups such as the genus *Histioteuthis* for which a substantial proportion of individuals could not be identified to species, and to use as much of the available stomach contents information as possible, prey categories were amalgamated, leaving the following groups: *Eledone cirrhosa*, *Octopus vulgaris*, *Chiroteuthis* spp., *Histioteuthis* spp., *Illex*/*Todaropsis*, *Todarodes sagitattus*, *Sepia* spp., *Teuthowenia megalops*, *Gonatus* spp., Sepiolidae and fish. RDA employs permutation-based tests to identify statistically significant effects of explanatory variables. Here we used 9,999 permutations of the data (see Zuur *et al*., 2007). The explanatory variables considered were year, month, area of stranding (Portugal, Galicia or Scotland, using Galicia as the reference value), sex (females used as the reference), and length. Because RDA assumes approximately linear relationships between response variables and explanatory variables, scores on axes 1 and 2 were plotted against continuous explanatory variables to check for evidence of serious nonlinearity.

Secondly, we used GAMs to analyze the effect of the explanatory variables on the numerical importance of the two most abundant prey categories (*Eledone cirrhosa* and *Illex/Todaropsis*). In addition, since exploratory analysis suggested a strong pattern in fish occurrence we also analysed numerical importance of fish. Since the response variables were based on abundance (count data), a discrete probability distribution was applied. For the cephalopods we used a negative binomial error distribution with log link to account for overdispersion. Fish numbers adequately fitted a Poisson distribution. The explanatory variables were the same used for the RDA. We treated length, year, and month as continuous variables and their effects were thus included as smoothers. Although year and month are strictly speaking discrete variables, this approach has the advantage of providing a visualization of trends and the possibility of reducing degrees of freedom. For length and month, the complexity of smoothers was constrained by setting a maximum number of

"knots" (*k*=4). Since there is no reason to expect a simple relationship with year, no constraint was set for the year effect. Backwards selection was applied to identify the best models, with the optimum model being the one that presented the lowest Akaike Information Criterion (AIC, Akaike, 1974) value, together with no obvious patterns in the residuals or highly influential data points ("hat" values) (see Zuur *et al*., 2007). If "final" models contained nonsignificant terms, the consequence of removing these was tested using an *F*-test; they were retained if they significantly improved the model fit.

Results

Composition of the sample of pilot whales

Of the 48 pilot whales for which stomach contents were obtained, 6 had stranded along the coast of northern Portugal, 32 in Galicia (Northwest Spain), and 10 in Scotland (Table 2.1). The final set of samples comprised stomach contents from 24 females, 19 males, and 5 individuals for which sex could not be determined due to the poor state of preservation of the carcasses. Most of the whales in the sample had stranded in the first half of the year (1st and 2nd quarters). The length of the animals ranged between 213 and 555 cm (Figure 2). Following the length-based criteria of Bloch *et al*. (1993) most of the sample set comprised immature individuals (Table 2.1).

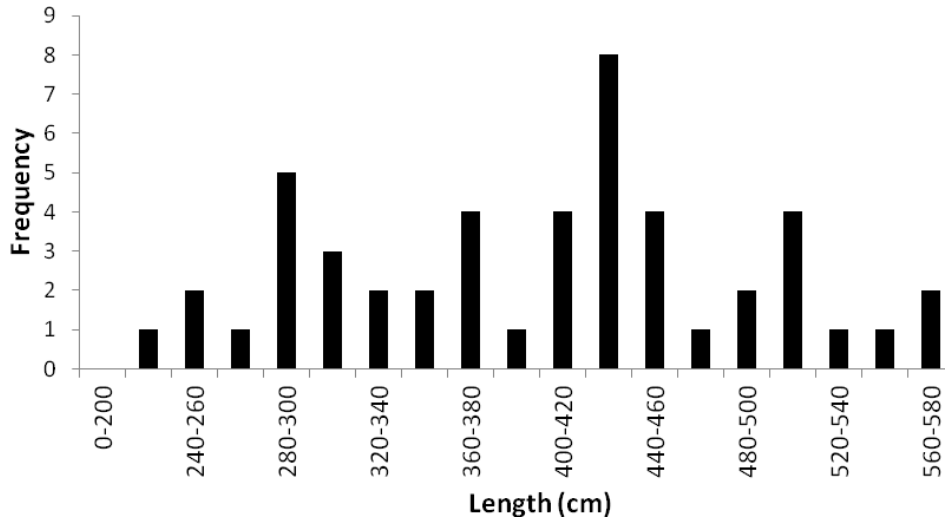


Figure 2.2. Size distribution of pilot whales analysed in this study.

Diet composition

Remains of 2,347 individual prey items were recovered from the stomachs. Pilot whale diet consisted mainly of cephalopods (98.9% by number), but also included fish, crustaceans, and other molluscs (0.9%, 0.1%, and <0.1% by number, respectively) (Table 2.2).

Overall, remains of 2,322 individual cephalopods belonging to at least 18 species of 12 families were found, corresponding to a total reconstituted mass of *ca.* 694 kg. In terms of numerical importance, Octopodidae were the most abundant group in Iberian samples (58.2% in Portugal and 72.3% in Galicia), with *Eledone cirrhosa* being the most abundant species (Table 2.2 and Figure 2.3).

In terms of biomass, Octopodidae were by far the most important prey group for the whales stranded in Galicia (representing more than 78% of the reconstructed weight of all prey), with *E. cirrhosa* again being the most important prey species (58.6% by weight) (Table 2.2). The family Ommastrephidae was the most abundant prey group taken by the pilot whales stranded in Scotland (36.6% by number), contributing more than 80% to the reconstructed prey weight. It was also the most important group by weight in the diet of whales stranded in Portugal, although not the most numerous. The ommastrephid squid *Todarodes sagitattus* was the main prey species by weight in both Scotland and Portugal (80.6% and 53% by weight, respectively) although it only represented 1/3 of the prey numbers in Scotland and half that amount in Portugal, reflecting the relative large size of the individual squid (*e.g.*, those in samples from Scotland ranged from 21 to 54 cm dorsal mantle length) (Table 2.2). Fish remains appeared in a total of 12 stomachs across the three areas, almost always representing very small numbers of fish (1 or 2), the exception being a Scottish sample that contained 18 otoliths. Although identification of the eroded fish remains was difficult, fish belonging to the family Gadidae were identified in Scotland and fish of the Gadidae, Merluccidae, and Carangidae in Galicia. Crustacean remains were found in 3 stomachs, generally in a poor state of preservation, and only remains of the swimming crab *Polybius hemslowii* could be identified to species level in the stomach of one of the Galician whales.

Table 2.2. Prey species identified from the stomach contents of *Globicephala melas*.

	PT			GAL			SCOT			TOTAL		
	%F	% N	%W	%F	% N	%W	%F	% N	%W	%F	% N	%W
CEPHALOPODS	**100.0**	**99.6**	**100.0**	**100.0**	**99.5**	**99.9**	**100.0**	**78.9**	**99.5**	**100.0**	**98.9**	**99.9**
Sepiidae	33.3	2.9	0.4	28.1	2.45	0.2	0.0	0.0	0.0	22.9	2.4	0.2
Sepia sp.	33.4	2.9	0.4	28.1	2.45	0.2	0.0	0.0	0.0	22.9	2.4	0.2
Sepiolidae	0.0	0.0	0.0	9.4	0.4	0.0	0.0	0.0	0.0	6.3	0.3	0.0
Rossia sp.	0.0	0.0	0.0	3.1	0.1	0.0	0.0	0.0	0.0	2.1	0.1	0.0
Sepiola atlanticus	0.0	0.0	0.0	3.1	0.1	0.0	0.0	0.0	0.0	2.1	0.1	0.0
Gonatidae	16.7	1.8	0.1	9.4	0.5	0.2	40.0	15.5	3.3	16.7	1.1	0.3
Gonatus sp.	16.8	1.8	0.1	9.4	0.5	0.2	40.0	15.5	3.3	16.7	1.1	0.3
Lepidoteuthidae	0.0	0.0	0.0	3.1	0.1	0.4	0.0	0.0	0.0	2.1	0.0	0.3
Lepidoteuthis grimaldii	0.0	0.0	0.0	3.1	0.1	0.4	0.0	0.0	0.0	2.1	0.0	0.3
Histioteuthidae	33.3	4.3	0.6	9.4	1.2	0.3	20.0	14.1	6.9	14.6	1.9	0.7
Histioteuthis reversa	16.7	3.6	0.3	3.1	1.0	0.2	10.0	1.4	0.1	6.3	1.3	0.2
Histioteuthis Type A	33.3	0.7	0.3	6.3	1.0	0.1	10.0	12.7	6.8	10.4	0.6	0.4
Brachioteuthidae	0.0	0.0	0.0	3.1	0.1	0.0	10.0	1.4	0.0	4.2	0.1	0.0
Brachioteuthis riisei	0.0	0.0	0.0	3.1	0.1	0.0	10.0	1.4	0.0	4.2	0.1	0.0
Ommastrephidae	50.0	24.3	57.6	84.4	18.3	19.8	60.0	36.6	83.5	75.0	19.6	28.9
Illex /Todaropsis	50.0	8.6	4.6	65.6	16.5	14.4	0.0	0.0	0.0	50.0	15.1	12.1
Todarodes sagitattus	33.3	15.7	53.0	25.5	1.2	5.3	40.0	32.4	80.6	29.2	3.9	16.5
Chiroteuthidae	50	7.1	1.4	18.8	0.7	0.1	0.0	0.0	0.0	18.8	1.5	0.3
Chiroteuthis veranii	33.3	3.2	0.3	12.5	0.3	0.1	0.0	0.0	0.0	12.5	0.6	0.1
Chiroteuthis Type II	33.3	3.9	1.1	3.1	0.1	0.0	0.0	0.0	0.0	6.3	0.6	0.2
Mastigoteuthidae	0.0	0.0	0.0	3.1	0.1	0.0	0.0	0.0	0.0	2.1	0.0	0.0
Mastigoteuthis schmidti	0.0	0.0	0.0	3.1	0.1	0.0	0.0	0.0	0.0	2.1	0.0	0.0

Table 2.2. (Continued)

	PT			GAL			SCOT			TOTAL		
	%F	% N	%W	%F	% N	%W	%F	% N	%W	%F	% N	%W
Cranchiidae	33.3	0.7	0.1	15.6	3.3	0.3	10.0	1.4	0.5	16.7	2.9	0.3
Taonius pavo	0.0	0.0	0.0	3.1	0.1	0.0	0.0	0.0	0.0	2.1	0.0	0.0
Teuthoweniia megalops	33.3	0.7	0.1	15.6	3.2	0.3	10.0	1.4	0.5	16.7	2.9	0.3
Alloposidae	0.0	0.0	0.0	3.1	0.1	0.2	0.0	0.0	0.0	2.1	0.0	0.2
Haliphron atlanticus	0.0	0.0	0.0	3.1	0.1	0.2	0.0	0.0	0.0	2.1	0.0	0.2
Octopodidae	66.7	58.2	40.0	81.3	72.3	78.3	20.0	7.0	5.3	66.7	68.7	68.7
Eledone cirrhosa	66.7	51.4	38.6	78.1	64.3	58.6	20.0	7.0	5.3	64.6	61.1	52.8
Octopus vulgaris	50.0	6.8	1.4	37.5	8.0	19.7	0.0	0.0	0.0	31.3	7.6	15.9
Unidentified Cephalopoda	16.7	0.4	0.0	9.4	0.3	0.0	20.0	2.8	0	12.5	0.3	0.0
FISH	16.7	0.4	0.0	21.9	0.4	0.1	40.0	18.3	0.5	25.0	0.9	0.1
Gadidae	0.0	0.0	0.0	3.1	0.1	0.0	10.0	2.8	0.5	4.2	0.1	0.0
Micromesistius	0.0	0.0	0.0	3.1	0.1	0.0	0.0	0.0	0.0	2.1	0.0	0.0
Merlucciidae	0.0	0.0	0.0	3.1	0.1	0.1	0.0	0.0	0.0	2.1	0.0	0.1
Merlucius merlucius	0.0	0.0	0.0	3.1	0.1	0.1	0.0	0.0	0.0	2.1	0.0	0.1
Carangidae	0.0	0.0	0.0	3.1	0.1	0.0	0.0	0.0	0.0	2.1	0.0	0.0
Trachurus sp.	0.0	0.0	0.0	3.1	0.1	0.0	0.0	0.0	0.0	2.1	0.0	0.0
Unidentified Fish*	16.7	0.4	0.0	12.5	0.2	0.0	30.0	15.5	0.0	16.7	0.7	0.0
CRUSTACEA	0.0	0.0	0.0	6.3	0.1	0.0	10.0	1.4	0.0	6.3	0.1	0.0
Polybius hemslowii	0.0	0.0	0.0	3.1	0.1	0.0	0.0	0.0	0.0	2.1	0.0	0.0
Unidentified Crustacea	0.0	0.0	0.0	3.1	0.1	0.0	10.0	1.4	0.0	2.1	0.1	0.0
MOLLUSC	0.0	0.0	0.0	0.0	0.0	0.0	10.0	1.4	0.0	2.1	0.0	0.0

PT: Portugal; GAL: Galicia; SCO: Scotland. Dietary importance is shown as % frequency of occurrence (%F), % importance by number (%N) and % importance by weight (%W).

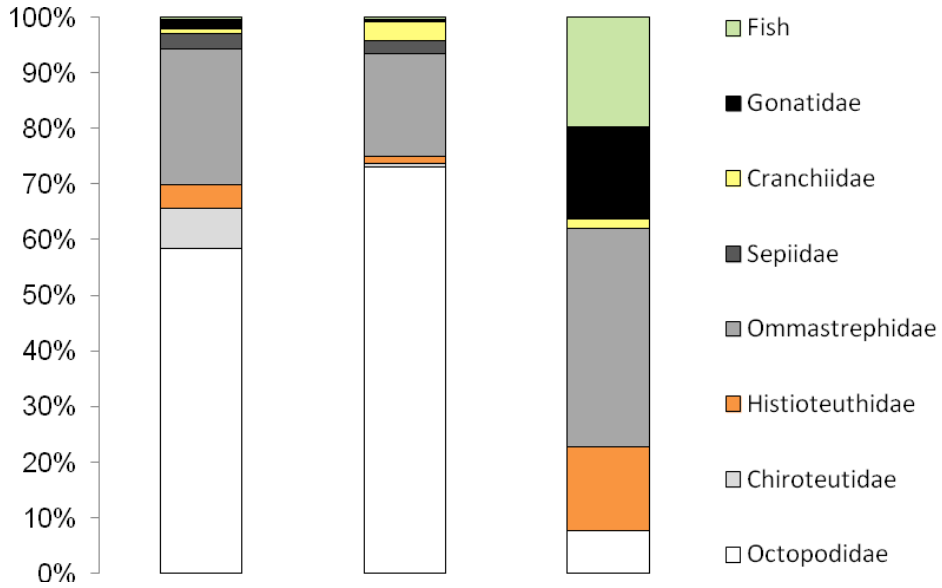


Figure 2.3. Numerical importance of the main prey families identified from the stomachs of the pilot whales. The bars represent pilot whales from Portugal (left), Galicia (middle) and Scotland (right).

Dietary variation

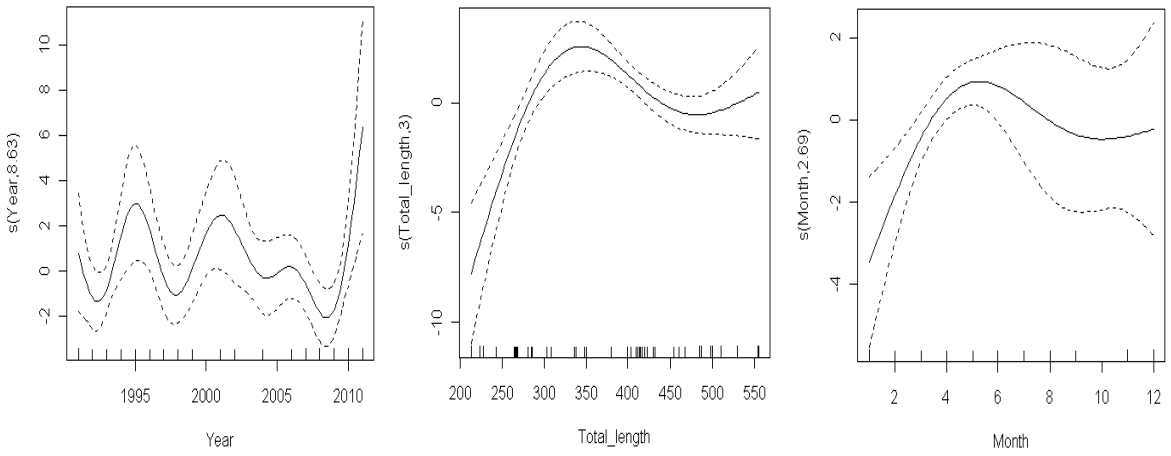
RDA on the 11 response variables indicated that overall 17% of dietary variation was captured in the RDA axes, with axes 1 and 2 explaining 6.0% and 4.7% of variance respectively. The first RDA axis was most strongly related to numbers of *Octopus vulgaris* while axis 2 was related to the occurrence of fish, sepiolids, *Chiroteuthis* spp. and *Teuthowenia megalops*. Numbers of fish were negatively related to numbers of most cephalopod groups except *O. vulgaris* and *Gonatus* spp. Statistical tests for conditional effects indicated effects of region (Scotland differed from Galicia) and year (P=0.037 in both cases). Examination of biplots also suggested a possible relationship between numbers of fish and body length. Retrospective exploration of relationships between RDA axis scores and continuous explanatory variables suggested possible non-linear relationships between the axis 1 score and both month and length. The existence of non-linear relationships between response and explanatory variables would violate the assumptions of RDA and may have prevented detection of effects of month and length. Since the multivariate dietary patterns were weak, no further analysis was carried out using RDA.

Results from the GAMs indicated that the numbers of *Eledone cirrhosa* (N_E) in pilot whale stomachs were significantly related to area (P<0.0001), whale length (P<0.0001), and month of stranding (P=0.0078), and year (P=0.0443). The model explained 71.4% of deviance. There was a wide range of hat values with four values exceeding 0.8 although none exceeded 1.0. Smoothers illustrated in Figure 2.4a suggest that the numerical importance of *E. cirrhosa* in the diet increased with whale length (reaching an asymptote around 350 cm) and increased during the first half of the year (although wide confidence limits, especially in the second half of the year obscure any further trend). There was also a significant effect of region, with fewer *E. cirrhosa* in the stomachs of the pilot whales stranded in Scotland than in whales stranded in Spain or Portugal (P<0.0001 in both cases). Numbers of *E. cirrhosa* found were highest in 1995, 2001, and 2011.

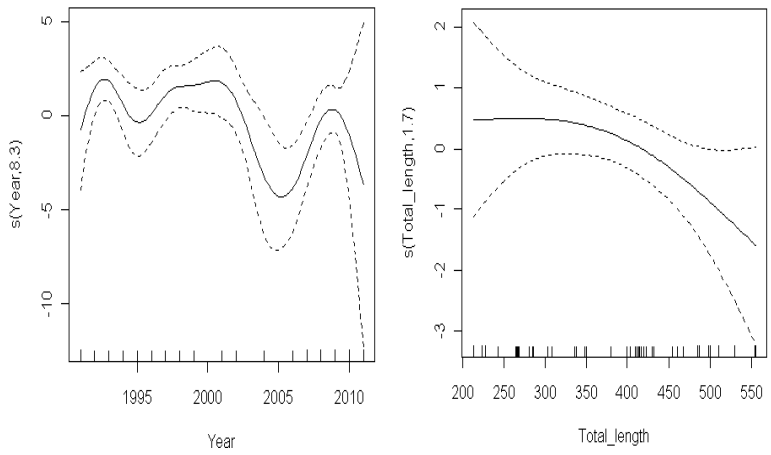
The final model for the numerical abundance of the ommastrephid group *Illex/Todaropsis* in pilot whale stomach contents (chosen on the basis of lowest AIC and absence of patterns in residuals or influential data points) explained 50.7% of deviance and included a significant effect of year (P=0.0065) and a non-significant effect of pilot whale length (P=0.0611), which, nevertheless, significantly improved overall goodness of fit (F test, P<0.05). Smoothers illustrated in Figure 2.4b suggest that the numerical importance of these ommastrephids in the diet decreased with increasing pilot whale length. Numbers eaten were lowest in 2005.

The final (Poisson) model for numerical importance of fish (selected using the same criteria mentioned in the previous paragraph) included effects of sex (females ate more than males, P=0.0057), year (most fish taken around 1996, P=0.0138), and length (increased predation on fish in larger individuals, P<0.0001) (Figure 2.4c).

a)


s(Year,8.63)
Year
s(Total_length,3)
Total_length
s(Month,2.69)
Month

b)


s(Year,8.3)
Year
s(Total_length,1.7)
Total_length

c)

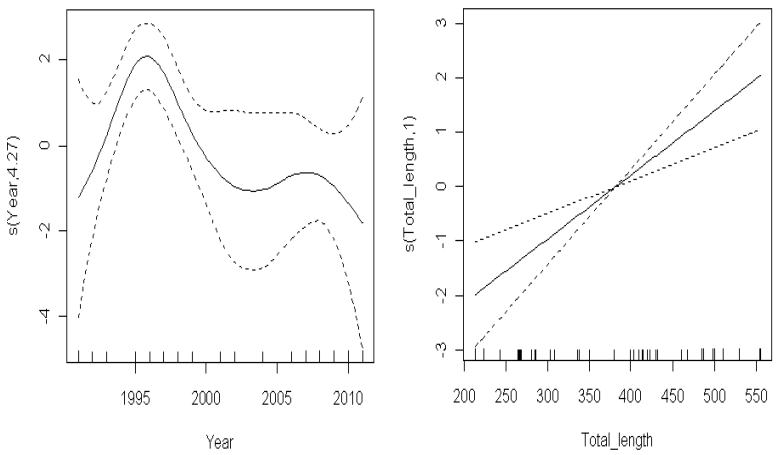

s(Year,4.27)
Year
s(Total_length,1)
Total_length

Figure 2.4. GAM results for the numerical importance of *Eledone, Illex/Todaropsis* and fish in the stomachs of pilot whales. (a) Smoothers for the effect of year, pilot whale length (cm), and month on *Eledone* numerical importance. (b) Smoothers for the effect of year and pilot whale length on *Illex/Todaropsis* numerical importance. (c) Smoothers for the effect of year and pilot whale length on numerical importance of fish. Dotted lines are 95% confidence intervals.

Discussion

Diet composition

Remains of at least 22 prey species belonging to 16 families were identified from the stomach contents in our study and, with the exception of 3 species of fish and one crustacean, all remaining prey types were cephalopods. This apparent preference for cephalopods as prey is consistent with most previous studies of the diet of pilot whales carried out in other areas, which described the diet of this species as consisting mainly of squid. In the Faroe Islands, analysis of stomach contents from 391 animals killed for human consumption showed the main prey species to be the oceanic squids *Todarodes sagittatus* and *Gonatus* sp. (Desportes & Mouritsen, 1993). In the Western Atlantic, the main prey of 30 whales accidentally captured off the Northeastern United States consisted of the neritic squid *Loligo pealei*, followed by oceanic squids of the families Ommastrephidae and Histioteuthidae (Gannon *et al*., 1997). In Brazil, the stomachs of fives whales stranded from 1985 to 1998 contained remains of squid of the oceanic families Lycoteuthidae, Histioteuthidae, and Cranchiidae (Santos & Haimovici, 2001). Cephalopods were also found as the main prey category in pilot whales stranded in France (Pierrepont *et al*., 2005), New Zealand (Beatson *et al*., 2007, Beatson & O'Shea, 2009), and the Bay of Biscay (Spitz *et al.,* 2011).

The number of cephalopod species (18) identified from Galicia (our biggest sample set with 32 stomachs analysed) is quite high, particularly when compared with the numbers identified from other studies with bigger sample sizes, although our samples were collected over an extended time period (almost 20 years). Desportes and Mouritsen (1993) identified 13 cephalopod taxa in 391 stomachs contents obtained from the carcasses of pilot whales landed in the Faroe Islands as part of their annual hunt.

Diet variability

We found evidence of geographical, seasonal, and ontogenetic variation in the diet of the pilot whales examined. Scottish whales had consumed a higher number of squids (oceanic species in all cases) when compared with the Iberian whales (northern Portugal and Galicia), for which the lesser octopus (*Eledone cirrhosa*), constituted the most numerous prey in the diet. *E. cirrhosa* is a benthic species found over a wide range of water depths. Although mainly recorded between 50 and 300 m (Belcari *et al*., 2002; Hastie *et al*., 2009), it has also been found in waters up to 800 m depth (Belcari *et al*., 2002; Pierce *et al*., 2010b and references therein). Other prey found in the stomachs included the common octopus, *Octopus vulgaris*, another benthic species but with a more restricted depth distribution, having been recorded from the coast to 200 m depth (Hastie *et al*., 2009; Pierce *et al*., 2010*b* and references therein). It is worth noting that in Northwest Iberia, long-finned pilot whales are occasionally sighted from the coast (in Galicia they are the 5th most frequently sighted species from land-based surveys; Pierce *et al*., 2010a), although most sightings in the area have taken place in waters off the shelf or on the shelf break.

The prevalence of octopus in the diet of long-finned pilot whales is also reported in a recent study based on analysis of 11 stomachs of pilot whales stranded in the Bay of Biscay (Spitz *et al.*, 2011). The authors found benthic octopods to be the main prey in the stomachs analysed (21.1% of prey biomass), followed by oceanic squids, such as *Todarodes sagitattus* and *Histioteuthis reversa* (17.2% and 10.7% of prey biomass, respectively). Cuttlefish (*Sepia sp*.) have also been recorded in the diet of long-finned pilot whales, being the most numerous prey in stomachs of two pilot whales that stranded on the French Atlantic coast, with *E. cirrhosa* representing only 14.3 % of the total number of prey (Pierrepont *et al*., 2005). The second most important prey family identified in our study is the squid family Ommastrephidae. Of the species present in the diet, *Todarodes sagitattus* has an oceanic distribution, while *Illex coindettii* and *Todaropsis eblanae* are also recorded in shelf waters (Guerra, 1992).

Long-finned pilot whales are widely distributed in the cold temperate waters of the Northeast Atlantic but little is known on its population structure and movements in the area. Fullard *et al.* (2000) analysed microsatellite DNA of whales from the East coast of the USA, West Greenland, the Faroe Islands and the UK and the authors reported that their results did not support a simple isolation-by-distance model of population differentiation. The authors

explained the pattern found in their samples as possible if population differentiation occurs in areas of different sea surface temperature. Smaller-scale studies based on genetic and stable isotope results, together with photoidentification studies carried out in the Strait of Gibraltar, suggest that at least some pilot whales are resident all year round and show a complex social structure constituted by several clans containing several pods each (De Stephanis *et al.,* 2008b). No information exists for other areas of the Northeast Atlantic. Desportes and Mouritsen (1993) noted that all prey species found in the stomach contents of pilot whales killed off the Faroes were common species in the area, but the authors also suggested that pilot whales showed a preference for the oceanic ommastrephid squid *Todarodes sagitattus* when this species was available in high numbers, information that these authors obtained from fishery data since this cephalopod species is also exploited commercially. As a mainly teuthophagous species, long-finned pilot whale is clearly in some respects a specialist feeder. However, the wide range of prey species recorded in the diet by several authors and the geographical differences in the main prey taken by the pilot whales would suggest a more generalist feeding behaviour, with whales feeding on the most abundant cephalopod species in each area with several authors suggesting that it is the abundance and movements of prey that drives pilot whale abundance and movements. In addition to this suggestion being made for pilot whales and *T. sagittatus* off the Faroe Islands (Desportes & Mouritsen, 1993; Zachariassen, 1993; Jákupsstovu, 2002), pilot whales have also been reported to be associated with *Illex illecebrosus* off Newfoundland (Mercer, 1975) and *Loligo pealei* and *Scomber scombrus* off the United States (Payne & Heinemann, 1993).

The three main prey categories for pilot whales identified in our study are also among the most important cephalopod species marketed in Spain and Portugal, with mean annual landings in Galicia alone of 1423 tons and 2,800 tons, for *Eledone cirrhosa* and *Octopus vulgaris* respectively and 3154 tons of ommastrephids, between 1997 and 2010 (http://www.pescadegalicia.com). Little is known on the abundance of non-commercial cephalopods since many of these species live in oceanic open waters and therefore they are rarely found in research surveys which tend to cover mainly fish resources in shelf waters. Because of this lack of data, the assumption that pilot whales feed on the most abundant prey species, so that diet differences would be due to the local availability of potential prey,

is difficult to prove since there is no contemporary information on the local abundance of many of the prey species (and sizes) identified in the diet.

Besides the variation in pilot whale feeding habits in relation to geographical area, evidence of ontogenetic changes in diet was detected in our samples. Larger whales ingested a higher number of *E. cirrhosa*, this relationship reaching an asymptote at around 350cm whale length, *i.e*., before the animals normally reach sexual maturity (Bloch *et al*., 1993), and also more fish. There was also a nonsignificant tendency for larger whales to eat fewer ommastrephid squids of the genera *Illex/Todaropsis*. Smaller whales, in contrast, showed a more varied diet. Juvenile whales could be limited in their ability to capture prey, either due to inexperience or physiological limitations. Thus they may not be able to swim as fast as adults, perhaps an issue for the capture of fast swimming prey species or may lack the capacity to carry out deep and/or long dives needed to reach and search the seafloor for benthic octopus, at least in deeper waters. Variation in the diet of individuals of different reproductive status, length and age has been previously described for this species (Desportes & Mouritsen, 1993), as well as for other odontocetes such as bottlenose dolphin (Blanco *et al*., 2001; Santos *et al.,* 2007), common dolphin, *Delphinus delphis* (Silva *et al*., 1999), and *harbor porpoise*, *Phocoena phocoena* (Santos *et al*., 2004). Desportes and Mouritsen (1993) found that although cephalopods represented the main prey of Faroese pilot whales, calves measuring less than 300 cm ate smaller cephalopods and that the consumption of shrimp and fish also varied between groups of whales of different length and reproductive status.

Our results suggest that the consumption of several prey categories fluctuates significantly year to year. Few data are available to indicate abundance of the main prey categories, although fishery statistical data from ICES sub-area IX (west of the Iberian Peninsula) suggest that ommastrephid (virtually all of which will be *Illex coindetii* and *Todaropsis eblanae*, Pierce *et al.,* 2010b) abundance has fluctuated widely. Landings in the early 1990s were low, as little as 250 tons in 1993, before rising to a peak of almost 3,000 tons in 1997 before declining again reach slightly over 300 tons in 2007. A similar trend was seen in Bay of Biscay waters (ICES 2000, 2011). Our dietary data are clearly inadequate to test whether diet has tracked prey abundance, but there was evidence of a decline in the numerical importance of *Illex* and *Todaropsis* in pilot whale diet during approximately 2000 to 2005.

The higher importance of octopus in the diet of pilot whales found in the present study (and by Spitz *et al.,* 2011) compared to most previous studies probably reflects a latitudinal trend, with squids (mainly ommastrephids) dominating the diet at higher latitudes while octopods are more important at lower latitudes. These differences could relate to differences in prey availability, but there are no relevant abundance estimates for these cephalopod groups and this hypothesis is not presently testable.

Improving our knowledge of the factors affecting the diet of deep divers such as pilot whales could help us to understand the trophic links within these systems and also the relationships between oceanic and shelf waters that this predator seems to be able to exploit simultaneously. It would be interesting to understand why the whales appear to take mostly prey species of relatively low energy density. Few data exist on the calorific values of oceanic cephalopods although some figures are available for neritic species. For example, Spitz *et al*. (2011) gave values of 4.7 kJ g^{-1} for *E. cirrhosa* and 4.4 kJ g^{-1} for squid of the family Ommastrephidae (only *Illex coindetti* and *Todaropsis eblanae* were analysed). These values are similar to those for fish of the family Gadidae but are quite low when compared with the energetic content of some other fish such as clupeids and some myctophids. In principle, diet selection is expected to reflect a trade-off between calorific content of the prey and the energetic cost of capturing them, suggesting that prey species such as *Eledone cirrhosa* may be particularly abundant and/or easy to capture. However, it is also true that not all biases can be accounted for when inferring the diet of a species by the analysis of the stomach contents of stranded individuals *e.g*., complete digestion of certain prey, lack of information from animals with empty stomachs, and, ultimately, the combination of the information obtained from several methods (stomach contents analysis, stable isotopes, fatty acids, *etc*.) probably represents the best approach to improve our knowledge on the feeding ecology of these species.

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through the EEA Financial Mechanism) and by the Project MarPro - Life09 NAT/PT/000038 (funded by the European Union - Program Life+). The collection of samples in Galicia is supported by the programs of the Dirección Xeral de Conservación da Natureza of the Xunta de Galicia. Strandings work carried out by SAC are funded by Defra and Marine Scotland.

Chapter III

Fatty acid signatures reveal geographical variation in feeding ecology of long-finned pilot whale (*Globicephala melas*) in Atlantic waters.

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Abstract

The feeding ecology of top predators can be influenced by both extrinsic and intrinsic factors, such as geographical location and/or gender, length, age, body condition or reproductive status of the animal. In addition to complementing stomach contents results, fatty acid (FA) analysis can help identify potential sources of variation for long-term dietary preferences of wild animals and provide insights into stock structure**.** We examine the extent to which the fatty acid composition of pilot whale depot fats can be used to investigate spatial, sex-related and ontogenetic differences in the diet of this species in three regions of the North Atlantic, by analyzing samples of blubber collected from pilot whales stranded off the Northern Iberia (n=18), Scotland (n=26) and the Northeast coast of the USA (n=12). Additionally, prey muscle samples were analysed in order to investigate if fatty acid profiles of prey species help explain the results obtained in pilot whale stomach contents. Multivariate analysis showed no significant variation between male/female and immature/mature pilot whale FA profiles, but there seem to be significant pairwise differences among all three different areas analysed over the North Atlantic. Both the geographical variation in FA signatures, based mostly on so-called "dietary FAs" and the evidence (although not conclusive) that Iberian whales ingest octopods, is consistent with previous results from stomach contents analysis (available for two of the three areas) and suggests that the dietary differences across these three regions of the North Atlantic may reflect the occurrence of different feeding niches. These results reveal the possibility of the occurrence of different ecological groups with specific foraging habits in the North Atlantic.

Introduction

The difficulty of directly observing the foraging behaviour of cetaceans in their natural habitat has led to the development of several techniques to obtain information about these species' feeding ecology, namely analysis of hard parts in stomach contents and scats and, more recently, stable isotope and fatty acid analyses of predator tissues, and molecular analysis of prey tissues remaining in stomachs and scats (reviewed in Tollit *et al.,* 2010). The food web is the main route by which marine mammals incorporate bioavailable environmental elements and compounds into their tissues, materials which were ultimately generated either by biochemical (fatty acids, Carbon and Nitrogen stable isotopes),

geochemical (trace elements) or anthropogenic (contaminants) processes (Caurant *et al.,* 2009). Consequently, these compounds may be considered as ecological tracers of food resources and/or habitats exploited by individuals, with differences in tracer signatures between groups of animals potentially revealing ecological differentiation between those groups, on a time-scale that is linked to the half-life of the tracer and the turnover rate of the tissue (i.e. the time taken for the replacement of old tissue signatures with new dietary signatures during tissue repair, Sweeting et al., 2005) in which the analysis are made (Caurant *et al.,* 2009).

All the analytical methods mentioned above present advantages and disadvantages (reviewed in Tollit *et al*., 2010). While stomach contents and scat analyses provide only a snapshot of the diet of an individual, ecological tracers such as fatty acids and stable istopes are able to supply less (or at least differently) biased and longer-term information about top predator feeding habits (Iverson *et al.,* 2004; Budge *et al*., 2006; Tollit *et al*., 2010; Kelly & Scheibling, 2012). However, as implied by the term "tracer" they represent indirect methods of determining dietary intake constituting a proxy of diet composition, trophic level or habitat occupied by the marine mammals (Hebert et al., 2006; Caurant et al., 2009). Therefore, there is a consensus that the combination of complementary ecological tracers with diverse integration time-scales (e.g. stomach contents, fatty acids, stable isotopes along with trace elements and contaminants) can provide more complete and reliable information on habitat use and distribution, feeding ecology and social structure and, at the same time, allow the understanding of possible ecological structuring within a population (Caurant et al., 2009; Evans & Teilmann, 2009).

Fatty acids (FA) are the predominant constituent of most lipids (Tollit *et al.,* 2010). Marine ecosystems contain a wide range of FA, which can be transferred through the food web, from prey to predators (Iverson*,* 2009; Budge *et al.,* 2006; Tollit *et al.,* 2010). FA signatures in predator tissues may thus function as dietary indicators and provide information on diet integrated over a long-term period (weeks to months, Iverson et al., 2004; Nordstrom et al., 2008), due to several characteristics: 1) the storage of fat in predator reservoir tissues, such as blubber, allows access to FA accumulated over time, representing an integration of days to months, depending on the species, energy intake and storage rates (reviewed in Budge *et al.,* 2006; Learmonth, 2006; Iverson *et al.,* 2004; Iverson, 2009; Tollit *et al.,* 2010); 2) the limited ability of mammalian predators to synthesize FA suggests that

most of the lipidic components incorporated into the consumer´s adipose tissue comes from dietary sources (Nakamura & Nara, 2003; Iverson et al., 2004) and 3) some metabolism of fatty acids occurs within the predator such that the composition of predator tissue will not exactly match that of its prey (slight modification within the carbon chain of FA may occur between ingestion and deposition, as well as differential deposition and mobilization of ingested fatty acids (Koopman *et al.,* 1996; Budge *et al.,* 2006)). However, most fatty acids are deposited in adipose tissue with little modification and where this is not the case, it is thought that there is a predictable relationship between dietary intake and the modified FA , which permits the estimation of ingested FA, from the FA in fat depots of marine predators.

Fatty acid signature analysis, alone or in combination with other methods, has been used extensively in several studies, with two main purposes. Mainly it has been used to obtain qualitative and semi-quantitative information about predator diet, including determining spatial and/or temporal dietary variation within populations and ecotypes, and investigating sex- and/or age-related dietary differences (Iverson *et al.,* 1997; Lea *et al.,* 2002; Walton *et al.,* 2000, 2003, 2008; Møller *et al.,* 2003; Beck *et al*., 2005, 2007; Herman *et al.,* 2005; Born *et al.,* 2007; Budge *et al.,* 2008; Tucker *et al*, 2009a; Quérouil *et al.,* 2013). Increasingly, as mentioned above, FA profiles are also viewed as a possible ecological marker to quantify stock structuring, especially when considering a multi-tracer approach, based on the idea that consistent differences in trophic ecology may be considered sufficient to delimit so-called “ecological stocks” (Walton *et al.,* 2000; Herman *et al*., 2005; Born *et al*., 2007; Krahn *et al*., 2007; Caurant *et al.,* 2009; ICES, 2009).

Secondly, and rather less frequently, fully quantitative estimates of diet have been obtained from the combined analysis of predator and prey FA signatures, through the QFASA method described by Iverson *et al.* (2004). Several studies used this model to predict diet in marine mammals and seabirds and describe potential spatial, temporal and gender dietary variations (Iverson *et al.*, 2007; Williams *et al*., 2008; Tucker *et al.,* 2009b; Wang *et al.,* 2009). However, this model has three key requirements: 1) appropriate sampling of predator tissue; 2) appropriate sampling and analysis of prey and determination of reliable differentiation between them; 3) accounting for predator metabolism (since there is some transformation and differential deposition of ingested fatty acids), by using calibration coefficients (CCs) (Iverson *et al.*, 2004, 2009; Budge *et al.,* 2006). The main limitation to apply QFASA to cetacean studies, at the moment, is the lack of calibration coefficients

specifically developed for these species, although they are available for pinnipeds, mink and seabirds (Iverson *et al.,* 2009). A recent study questioned the validity of the use of CCs derived from alternative predators and suggested effects of both predator phylogeny and prey type on calibration coefficients (Rosen & Tollet, 2012), highlighting the need to perform further investigations in this area. Hence, the present study will focus primarily on qualitative interpretation of fatty acids in pilot whale samples.

In cetaceans, several studies correlated the location of the blubber used in FA analysis with the dietary information that it can provide. It has been shown that outer blubber (i.e. near the skin) is more structural (playing a relatively minor role in lipid metabolism, Aguilar & Borrell, 1990) and inner blubber (i.e. near to the muscle) is more active metabolically (in terms of lipid deposition and mobilization, being subjected to continuous turnover) and more appropriate for diet analysis, since it is where dietary fatty acids (i.e. FA that arise from dietary origin, Iverson *et al.*, 2004) are preferentially deposited (Koopman *et al.,* 1996, 2002, 2007; Koopman, 2001; Iverson *et al.,* 2004). While biopsies of subcutaneous blubber samples from live cetaceans are widely used for FA analysis, this technique is more suitable to obtain samples of outer blubber. A possible solution is the use of samples from dead stranded animals, although is important to keep in mind possible biases associated with this type of sample, such as possible degradation and oxidation of (especially) polyunsaturated FA resulting from air exposure and accelerated by light and temperature (Pond, 1998), or the overrepresentation of sick animals that are not able to feed properly or that are fasting (Iverson *et al.,* 2004; Tollit *et al.,* 2010). Several studies compared lipid content and classes, as well as FA signatures between stranded and biopsied animals (Krahn *et al.,* 2001, 2004), concluding that similar type of results can be obtained from the two sampling methods. Also, a study that compared FA signatures in stranded harbour porpoises, with different levels of decomposition state (from extremely fresh (2a) to moderately decomposed (3)) found only slight differences and concluded that it is admissible to use samples from stranded animals showing signs of moderate decomposition (3) (Learmonth, 2006).

The long-finned pilot whale (*Globicephala melas*), hereafter referred to as pilot whale, is one of the largest odontocetes. Several studies have suggested the occurrence of different populations of this species across the North Atlantic, based on the application of genetic and ecological markers (Fullard *et al.*, 2000; Bloch & Lastein, 1993; Abend & Smith, 1995; Perrin *et al*., 1990). An analysis of neutral markers (microsatellites) within North Atlantic pilot

whales (Fullard *et al.,* 2000) revealed differentiation between West Greenland and remaining regions (USA East Coast, UK and Faroe Islands). In addition, the analysis of stable isotopes in animals from the Faroe Islands, the mid-Atlantic Bight and Cape Cod areas, suggested the occurrence of dietary segregation of animals from the West and East Atlantic, when fast and medium turnover rate tissues were considered (Abend & Smith, 1995), while differences in parasite composition between animals from the western Mediterranean, France, Faroe Islands and Newfoundland suggest that individual whales may not routinely move between any of these regions (Perrin *et al*., 1990). Another study showed the occurrence of morphometric differences between whales from Faroe Islands and Newfoundland, suggesting segregation of long-finned pilot whales between East and West Atlantic (Bloch & Lastein, 1993). A recent study, described in chapter II, analysed stomach contents of pilot whales from Portugal, Northwest Spain and Scotland (with 13 samples in common with the present study from Iberia (n=7) and Scotland (n=6)) and the authors found evidence of geographical, seasonal and ontogenetic (size of the predator) differences in the diet of this species (Santos *et al.,* 2013; chapter II). Geographical differences in diet consisted mainly of a higher importance of squid (mainly ommastrephids) at higher latitudes and a high importance of octopods in Iberian Peninsula (Santos *et al.,* 2013).

It is important to underline that "intrinsic" factors may also be responsible for intra-specific differences in diet. Thus, inter-individual differences in FA profiles may be influenced by factors such as animal length, age, sex, reproductive status, and body condition (Samuel & Worthy, 2004; Beck *et al.,* 2005, 2007; Smith & Worthy, 2006; Budge et al., 2008; Newland et al., 2009; Tucker *et al.,* 2009a; Quérouil et al., 2013).

In the present study, FA signatures of pilot whale from three regions of the North Atlantic were examined to investigate sources of geographical, sex-related and ontogenetic variation in the foraging behaviour of this species, based on the assumption that FA profiles of predator tissues reflect diet composition. Also, in order to detect if fatty acid profiles of prey species help explain the results obtained in pilot whale stomach contents and investigate the Iberia-Scotland difference in the consumption of Octopodidae and Ommastrephidae (Santos *et al*., 2013; chapter II), samples of prey species from Scottish and Iberian waters were also included in this study. The main goals of the present study are 1) to investigate if there are sex-related and ontogenetic differences in foraging behaviour, 2) to analyze if the occurrence of geographical variation in the fatty acid signatures of pilot whales

is in accordance with stomach contents analysis results, reflecting dietary variation, 3) to investigate if fatty acid profiles of prey species help explain the results obtained in pilot whale stomach contents, and 4) to evaluate evidence for stock structuring based on FA analysis.

Methodology

Sample collection

Full-depth blubber samples were collected from a total of 56 pilot whales (*Globicephala melas*) stranded along Northwest Iberia (n=18), Scotland (n=26) and Northeast United States of America (Cape Cod, n=12) (Figure 3.1 and Table 3.1).

Four stranding monitoring programs were responsible for the examination of marine mammal carcasses and the collection of samples. Strandings were attended in all cases by experienced personnel, from the Sociedade Portuguesa de Vida Selvagem (SPVS) in northern Portugal, the Coordinadora para o Estudio dos Mamíferos Mariños (CEMMA) in Galicia (NW Spain), the Scottish Agriculture College Veterinary Science Division (SAC) in Scotland and the International Fund for Animal Welfare (IFAW) Marine Mammal Rescue & Research Program in Northeast United States of America (USA).

In all cases, when the condition of the animal permitted, detailed necropsies had been performed. Otherwise, basic measurements/information (i.e., length, sex, decomposition state) and samples were collected. The gender of the animals was assessed either during the necropsy procedure or through genetic analysis. Full-depth blubber samples were collected from mid-region of the body, wrapped in aluminium foil and frozen (-20ºC) until analysis.

To prevent sampling biases associated to FA oxidation, only animals recently dead (decomposition state ≤ 3, moderate decomposition) were used in this analysis and those of poor quality (obvious rendering, decomposition, dehydration or other post-mortem/storage effects) were excluded.

Table 3.1. Summary of the composition of sampled pilot whales. Data are described for each location, by sex and maturity of the animals. This table excludes samples rejected due to advanced decomposition.

Location		Sex		Maturity		
		F	M	I	M	U
NW IBERIA	18	12	6	8	10	0
SCOTLAND	26	13	13	9	16	1
USA	12	7	5	6	6	0
TOTAL	56	32	24	23	32	1

F: female; M: male. I: immature; M: mature; U: unknown.

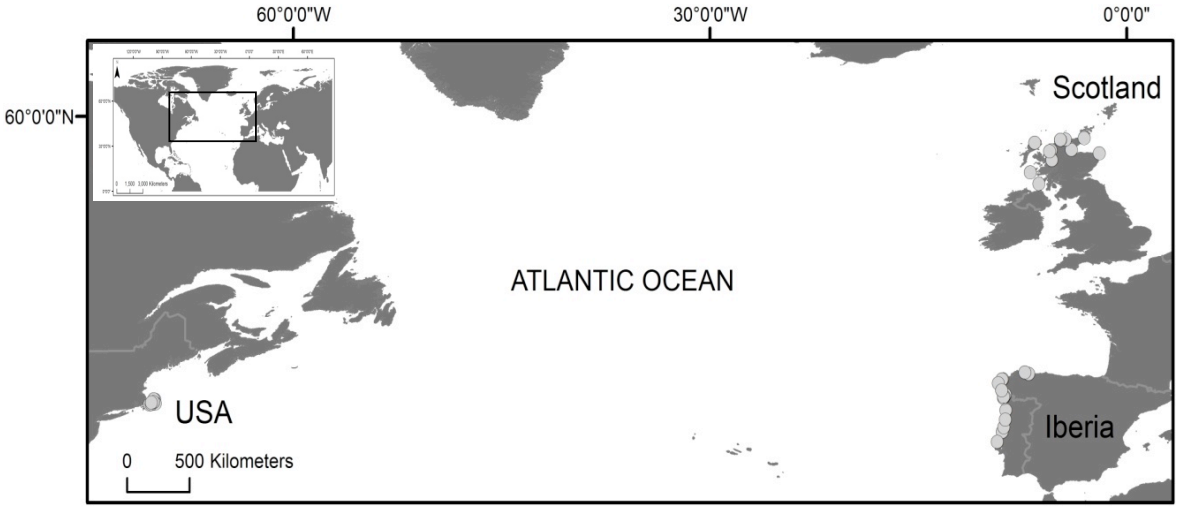

Figure 3.1. Map showing the locations of the strandings of pilot whales analysed in this study (n =56).

Prey species belonging to Octopodidae and Ommastrephidae families (as described in previous stomach contents studies, chapter II and Santos *et al.,* 2013), from Iberian and Scottish waters were also included in this study (Table 3.2). Prey samples were mostly collected during oceanographic surveys undertaken by Instituto Português do Mar e da Atmosfera in Portugal (IPMA), Instituto Español de Oceanografía, in Galicia (NW Spain) (IEO) and Marine Scotland Laboratory, in Scotland. However, some samples were also obtained from commercial fishing vessels landings, in Portugal and Spain. Mantle muscle samples were wrapped in aluminium foil and frozen (-20ºC) until analysis. Additionally, data from

prey samples previously collected in these areas and analysed by Stowasser (2004) were also included in the analysis.

Table 3.2. Summary of the sampled cephalopods in each location.

Location	Species	N	Family
NW IBERIA	*Eledone* sp.	10	Octopodidae
	Octopus vulgaris	15	
	Illex coindetii	115	Ommastrephidae
	Todaropsis eblanae	8	
	Todarodes sagittatus	6	
SCOTLAND	*Todarodes sagitattus*	34	Ommastrephidae

Fatty acid analysis

Lipid was extracted from inner blubber of cetaceans (i.e. the blubber closest to the muscle as described in Samuel & Worthy, 2004) and the mantle muscle from prey, using a modified Folch method (Folch *et al.,* 1997). Briefly, homogenized inner blubber and mantle muscle samples (approximately 1g) were left for approximately 24h at 4^{o}C, in a 2:1 (v/v) solution of chloroform:methanol, containing butylated-hydroxytoluene (BHT) as 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 35^{o}C, 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.

Before esterification, lipid classes were measured in blubber samples to test for indications of decomposition (presence of high level of free fatty acids), with all the samples with signs of decomposition being excluded from further analysis. For this purpose, Thin-Layer Chromatography (TLC) of approximately 5mg of 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 were excluded from the analysis (the excluded samples are not listed in Table 3.1).

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 50^{o}C for a minimum of 12h. After being washed with 5% (w/v) sodium chloride in water, fatty acid methyl esters (FAME) were then extracted in hexane and washed again with 2% (w/v) potassium bicarbonate in water. The FAME extract was then dried over anhydrous sodium sulphate. To ensure that methylation took place and no contamination occurred, standard laboratory reference materials (LRM145 and LRM 144) and a blank sample were methylated with each batch of samples.

The FAME were analysed 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 calibration standard EO23 was run at the beginning of each batch and after every 10th sample, to validate instrument performance, verify normalized area percentage of components and check for component retention time drift. The FAMEs were identified by comparison with individual Standard Reference materials LRM 144 and LRM 145, and the normalized area percentage (NA%) was calculated for each fatty acid as a percentage of the total area values for all identified fatty acids. A solvent blank was run at the beginning of each run. 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.

Statistical analysis

Pilot whales

To determine which explanatory variables could have an influence on the FA profiles of pilot whales, the effects of geographic location, sex and sexual maturity of the animals, were

investigated using Multivariate Analysis of Variance (MANOVA) and Linear Discriminant Analysis (LDA), using R v.2.9.1 (R Development Core Team, 2011) (Table 3.1). Since not all animals were assessed for maturity status, we inferred maturity from body length, according to Bloch et al. (1993) (Table 3.1).

A total of 24 FAs was routinely identified in all pilot whales (Table 3.3). However, the number of FAs identified exceeded the number of individuals present in the smallest group used in the analysis (having grouped the Iberian samples, n values ranged from 12 to 26). Therefore, two criteria were used in order to reduce the number of FAs to be used in the multivariate analysis: 1) only FAs with proportions >0.4% to avoid fatty acids found at low or trace levels (and which thus may not be correctly identified and separated from abundant nearby peaks, Iverson et al., 2004), were selected and 2) if the normalized areas of two FAs were highly correlated (Pearson's r >0.8), one of them was discarded. The 12 FAs finally 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 FA comprised 82.7% of the normalized area of the total FAs.

Assumptions of MANOVA and LDA were tested using the package *vegan* in R (Oksanen *et al.,* 2011): 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; Anderson, 2006). Results indicated there was no need to transform the variables.

To test for geographical, sex and sexual maturity differences in FA profiles, we first ran an overall three-way MANOVA (using type III sum of squares), where no interaction of explanatory variables was tested, due to small sample size within categories (Table 3.1). If this overall model was significant, contrasts were constructed to perform pairwise tests and a Bonferroni correction was applied as an adjustment of critical p-values, due to multiple comparisons. Wilk's Lambda was the test used to assess the significance of the influence of independent variables (location, sex and sexual maturity) in the dependent variables (FAs) (Johnson & Field, 1993).

For the independent variables presenting significant values in the MANOVA, a LDA was performed using a forward-stepwise method in order to assess which FA subset optimally separated the pilot whales by group. 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).

Prey samples

To investigate whether it is possible to distinguish the main prey families (Octopodidae and Ommastrephidae) of long-finned pilot whale and detect which fatty acids could be responsible for that separation, multivariate (Principal Component Analysis, PCA) and univariate (Mann-Whitney test) analyses were applied, since even with variable transformation, the assumptions of multivariate normality and homogeneity were not accomplished.

As occurred with pilot whale data, a total of 24 FAs was routinely identified in all cephalopods analysed in the present study (identified in Table 3.3, for pilot whales). However, to be able to match this dataset with the fatty acid data analysed by Stowasser (2004), the fatty acids 18:3(n-3), 20:0, 22:0 and 21:5(n-3) were excluded from this analysis. Hence, the dataset used in PCA and Mann-Whitney test included 20 fatty acids, as described in Tables 3.5 and 3.6. It is important to mention that to account for potential geographical variation in the Ommastrephidae, this group was divided into two groups in these analyses, namely Ommastrephidae from Iberia and Ommastrephidae from Scotland.

PCA summarises all explanatory variables into a few orthogonal principal components (PC). Each PC has an associated eigenvalue that represents the amount of variation explained by that axis (Zuur *et al.,* 2007). For the present study, the selection of the most important PCs to be presented was based on the "Kaiser-Guttman criterion", whereby PCs whose eigenvalues are larger than the mean of all eigenvalues are analysed (Legendre & Legendre, 2012). Fatty acids of the cephalopods were analysed in a correlation biplot of Principal Component Analysis. All calculations were performed using the package *vegan* (Oksanen *et al.,* 2011) implemented in R v.2.9.1 (R Development Core Team, 2011).

In the univariate analysis, individual fatty acids were compared between Octopodidae and Ommastrephidae and between Ommastrephidae from Iberia and Ommastrephidae from Scotland groups using Mann-Whitney test, as implemented in R v.2.9.1 (R Development Core Team, 2011). A Bonferroni correction was applied as an adjustment of critical p-values, due to multiple comparisons.

Results

Pilot whales

Overall, the FA profiles of the pilot whales were generally high in MUFAs (55.72% ± 7.29%), with SFAs and PUFAs showing similar but lower contributions (22.92% ± 2.59% and 20.78% ± 7.04%, respectively) (Table 3.3). 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 (Table 3.3). Iberian samples showed the highest values of 18:1, 16:0 and 22:6(n-3) and the lowest values of 20:1 and 22:1. These last two FAs showed the highest values in Scottish animals.

There was no significant difference in FA profiles of male and female pilot whales (MANOVA: Wilk's λ $F_{[12,39]}$=1.6, p>0.05), nor between immature and mature pilot whales (MANOVA: Wilk's λ $F_{[12,39]}$=1.8, p>0.05). However, FA profiles of pilot whales differed significantly with location (MANOVA: Wilk's λ $F_{[24,78]}$=19, p<0.001). A pairwise analysis showed significant differences among all the different areas (MANOVA: Wilk's λ $F_{[12,31]}$=20.0 (Iberia vs. Scotland), Wilk's λ $F_{[12,17]}$=37.9 (Iberia vs. USA), Wilk's λ $F_{[12,25]}$=13.0 (Scotland vs. USA), p<0.001).

LDA was used to determine which fatty acids best identified each location (i.e. were more important in separating animals from different areas and determined how well groups were classified). The 2-dimensional model which best optimized the separation of the three locations is shown in Figure 3.2. 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-value < 0.001). LDA indicated that the 1st discriminant function mostly separated Iberian profiles from those in other locations, mainly because of higher proportion of 20:4(n-6) in Iberian samples and 18:4(n-3) in the Scotland/USA group, while the 2nd discriminant function separated Scotland and USA, based on the proportions of 16:2(n-6) (higher in Scottish samples) and 16:1(n-7) and 20:5(n-3) (higher proportion in whales from the USA) (Table 3.4). A slight overlap occurred between individuals from Scotland and the USA.

Table 3.3. Fatty acid methyl ester (FAME) profiles of inner blubber of pilot whales from different locations. Values are presented as means ±SD (NA %).

	NW IBERIA	SCOTLAND	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	?
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	

*: FAs selected by LDA forward stepwise method, as the most important at separating animals from different areas. Predominant sources of fatty acids in predator adipose tissue: B: all or primarily from biosynthesis; b: relatively large contributions from both biosynthesis

and diet; D: all or primarily from direct dietary intake; ?: not fully understood (Iverson *et al*., 2004).

Table 3.4. Standardized and structured coefficients of the LDA. The FA were included in the model, after a forward selection (α = 0.05).

	Standardized		Structured	
	LDA1	**LDA2**	**LDA1**	**LDA2**
16:0	-0.873	0.514	-0.493	0.020
16:1(n-7)	-0.471	0.302	-0.191	0.505
16:2(n-6)	0.151	-0.841	-0.046	-0.853
18:1	-0.459	-0.667	-0.514	-0.281
18:2(n-6)	-0.127	0.731	0.123	0.266
18:4(n-3)	-0.470	-1.074	0.623	0.039
20:4(n-6)	-2.114	-0.671	-0.801	0.189
20:5(n-3)	1.399	0.767	-0.232	0.595

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

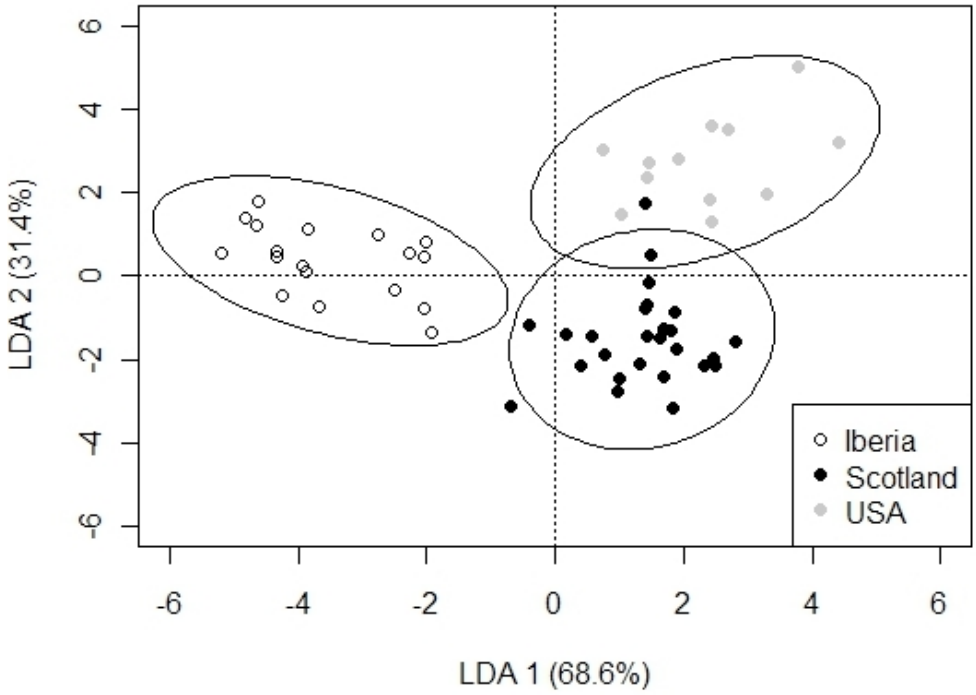

Figure 3.2. LDA results for pilot whales from different locations of the North Atlantic. Ellipses represent 95% data point clouds.

Prey samples

The first three PC were the most important at explaining the variation of the pilot whale prey dataset. The three first axes of PCA explained 94.3% of total variation, with PC1 and PC2 accounting for 71.8% and 13.3%, respectively (Table 3.5).

Table 3.5. PCA results for long-finned pilot whale prey. Eigenvalues and coefficients for each fatty acid are described for the first three principal components.

	PC1	PC2	PC3
14:0	-0.121	0.463	-0.191
15:0	0.015	0.105	0.026
16:0	3.112	2.840	1.417
16:1(n-7)	-0.358	0.246	-0.229
16:2(n-6)	-0.042	-0.061	0.010
16:3(n-6)	-0.037	-0.012	-0.007
16:4(n-3)	0.109	0.199	0.049
18:0	-1.558	-0.594	0.437
18:1	-1.450	0.579	-1.077
18:2(n-6)	-0.163	0.014	-0.096
18:3(n-6)	0.005	0.027	0.003
18:4(n-3)	-0.069	0.029	-0.074
20:1	-0.765	-0.437	-0.902
20:4(n-6)	-2.052	-0.367	0.583
20:4(n-3)	-0.076	0.009	-0.100
20:5(n-3)	-1.693	-1.606	2.107
22:1	-0.935	0.314	-1.044
22:5(n-3)	-0.685	-0.115	-0.080
22:6(n-3)	7.522	-1.647	-0.319
24:1(n-9)	-0.055	0.038	-0.167
Eigenvalue	45.479	8.420	5.850
Proportion of variation explained	0.718	0.133	0.092
Accumulated variation explained	0.718	0.851	0.943

Although some overlap occurs, a separation between Octopodidae and Ommastrephidae individuals seems to occur, as evidenced by the correlation biplot presented in figure 3.3. PCA indicated that PC1 mostly separated Octopodidae from Ommastrephidae, based on the proportions of 20:4 (n-6), which showed higher proportions in Octopodidae (5.97±2.62%), than in Ommastrephidae (1.19±0.51%), both from Iberia (0.54±0.26%) and Scotland (1.35±1.18%). PC1 also separated Octopodidae and Ommastrephidae based on the proportions of 22:6 (n-3), which showed higher proportions in Ommastrephidae (42.62±3.91%), both from Iberia (46.20±1.73%) and Scotland

(41.74±3.80%), than in Octopodidae (29.74±3.98%)**.** Variation in PC2 was mostly related with the proportions of 22:6(n-3) and 16:0, while PC3 was mostly related with the proportions of 20:5(n-3) and 16:0.

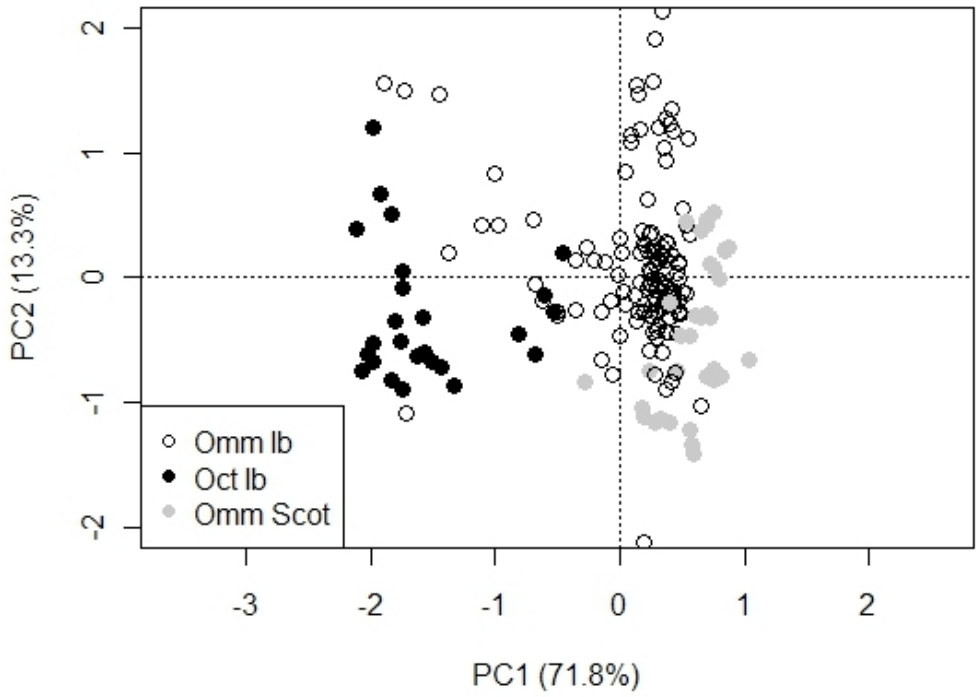

Figure 3.3. PCA results for Octopodidae and Ommastrephidae prey. Omm: Ommastrephidae; Oct: Octopodidae; Ib: Iberia; Scot: Scotland.

There were significant differences, in several individual fatty acids, between Octopodidade and Ommastrephidae (Table 3.6). Within the fatty acids considered primarily of dietary origin in predators (Table 3.3, Iverson et al., 2004) 16:2(n-6), 16:3(n-6), 20:4(n-6), 20:5(n-3), 22:1, 22:5(n-3), 22:6(n-3) were significantly different between the two cephalopod families. When comparing Ommastrephidae individuals from different geographical locations there were significant differences between Ommastrephidae from Iberia and Scotland, in most of the fatty acids (table 3.6).

Table 3.6. Mann-Whitney results for the comparison between Octopodidae and Ommastrephidae prey.

	Oct (n=25) vs. Omm (n=163)		Omm Ib (n=138) vs. Omm Scot (n=34)	
	Mann-Whitney U	p-value	Mann-Whitney U	p-value
14:0	3132	***	4090	***
15:0	3257	***	4589	***
16:0	4067	***	2643	ns
16:1(n-7)	437	***	1700	*
16:2(n-6)	1099	***	2885	*
16:3(n-6)	1002	***	1843	*
16:4(n-3)	2544	ns	1367	***
18:0	4	***	2998	*
18:1	392.5	***	3821	***
18:2(n-6)	556	***	1591	***
18:3(n-6)	2535	ns	2391	ns
18:4(n-3)	2124	ns	3239	***
20:1	1983	ns	2398	ns
20:4(n-6)	4	***	4445	***
20:4(n-3)	1848	ns	2751	ns
20:5(n-3)	557	***	3101	***
22:1	408	***	1831	*
22:5(n-3)	127	***	3376	***
22:6(n-3)	4189	***	297	***
24:1(n-9)	2305	ns	2771	ns

ns: non-significant; *:p-value <0.05; **:p-value<0.01; ***:p-value<0.001; Omm: Ommastrephidae; Oct: Octopodidae.

Discussion

In the present study, the influence of geographical location, gender and sexual maturation in the FA signatures of pilot whale across three regions of the North Atlantic were investigated. The results revealed the occurrence of geographical differences in fatty acid profiles in the North Atlantic, which in combination with previous stomach contents results may suggest the existence of different ecological groups in this region. Furthermore, it was investigated whether a comparison of the fatty acid profiles of the whales and the two

main prey families of pilot whales from Iberia and Scotland would provide evidence of a preference for octopus in the Western coast of Iberia, which would support previous stomach contents results. Although not conclusive, the analysis of the prey FA signatures suggest that Iberian pilot whales may be feeding on octopods, as indicated by the importance of amounts of 20:4(n-6) in discriminating octopus from ommastrephids, and Iberian whales from other whales, which coincides with the findings in the stomach contents of whales from that region.

Sex-related and ontogenetic variation in the foraging behaviour of pilot whales

Differences in sex-specific costs of reproduction could contribute to variation in male and female FA profiles, due to FA mobilization to accommodate the physiological requirements of pregnancy and lactation, as described in pinnipeds (Iverson et al., 1995, 1997; Wheatley *et al.,* 2008). Differences between sex and/or reproductive states were also analysed in cetacean (Koopman, 2001; Samuel & Worthy, 2004; Smith & Worthy, 2006; Budge et al., 2008; Quérouil et al., 2013). As an example, female and male, as well as lactating and non-lactating females of bottlenose dolphins from the Eastern coast of the USA showed significant differences in FA signatures (Samuel & Worthy, 2004). In contrast, no FA signature differences were observed between male and female of bowhead whales (Budge et al., 2008). Similarly, in harbour porpoises, the overall fatty acid composition of the blubber of lactating females was not distinguishable from that of non-lactating females, being suggested that age had a greater influence than reproductive class on the FA of this species (Koopman, 2001).

In the present study, no evidence of differences in the foraging habits of female and male pilot whales was found. This result is consistent with stomach contents analysis that showed no sex differences in the consumption of the main cephalopod prey of pilot whales from the Northeast Atlantic (Santos *et al.,* 2013) and stable isotope analysis of Mediterranean pilot whales that showed no sex-related differences in either $\delta^{15}N$ or $\delta^{13}C$ (De Stephanis *et al.,* 2008c), suggesting that gender is not an important factor at defining feeding niches in this species. However, it is important to mention that although 59% of the sampling group is composed by mature individuals, no information exists about the reproduction state (pregnancy or lactation) of the females, which could mask the possible effect of reproduction costs.

As a size-dimorphic species (Bloch *et al.,* 1993), a difference in diet could be expected between sexes of *G. melas*, in order to fulfill the higher energy requirements of the larger sex. Several studies have already analysed the influence of the length or age of cetaceans in the composition of blubber fatty acids (Koopman et al., 1996; Koopman, 2001; Learmonth, 2006; Budge et al., 2008). In harbour porpoise, the influence of body length in FA profiles was mainly related with the presence of neonates or pre-weaned propoises in the sampling group (Koopman et al., 1996; Learmonth, 2006), although some differences were also found between animals with different ages (Koopman, 2001). Body length of individuals was found to have a significant effect on bowhead blubber FA composition, being suggested that the diet of smaller animals (pre-weaned or animals feeding of higher proportions of euphasiids or fish) was different from the one of larger whales (Budge et al., 2008).

In the present study, there were no significant differences between FA profiles in immature and mature animals. If these categories were considered as a proxy for length (Bloch et al., 1993), this result would contrast with the one obtained in the stomach contents analysis that shown that length of pilot whales significantly influenced the proportions of the main prey species consumed (Santos et al., 2013). However, an effect of the predator length in the FA signatures would only be expected to occur if the change in the diet resulted in ingestion of different proportions of prey with different FA profiles. Additionally, having in mind that the length of the pilot whales at the weaning stage was defined as 239cm (Sergeant, 1962) most of the animals analysed in the present study were post-weaned individuals. Neverthless, it may be that the FA signal of ontogenetic dietary changes is too weak to be detected or simply that samples size in the FA analyses was too small to get a good handle on ontogenetic dietary changes.

Geographical variation in the foraging behaviour of pilot whales

Although there was no evident variation in the foraging behaviour between females and males or between immature and mature animals, there were significant differences between the FA signatures of animals from different locations. Several studies have examined potential population structure of marine mammals, by using fatty acids (Iverson *et al.,* 1997; Walton *et al.,* 2000, 2008; Møller *et al.,* 2003; Herman *et al.,* 2005; Born *et al.,* 2007; Tucker *et al,* 2009; Quérouil *et al*., 2013) and all of them attributed the occurrence of geographical differences in FA profiles to possible geographical differences in dietary habits.

In the present study, among the eight FA selected as being the most important for separating Iberia, Scotland and Northeast USA, five were dietary FA (i.e. FA that arise from dietary origin, rather than being (bio)synthesised by the predator; Iverson *et al*., 2004) and three were major FA that can have a dietary origin, but are also biosynthesized by the predator, according to Iverson *et al.* (2004). Hence, the results found in this study seem to suggest that pilot whales across the North Atlantic have different dietary characteristics, reflecting the potential existence of different ecological groups.

It is theoretically possible, if unlikely, that individuals from the three areas differ in how they deposit, metabolize and integrate FA in the blubber, due to different intrinsic characteristics (Newland *et al.,* 2009). Furthermore, it is possible that pilot whales show identical diets in the different locations, but fatty acid profiles of the prey species are different, due to geographic, seasonal or intrinsic factors (Budge *et al.,* 2002). However, the broad geographical range covered by this study suggests that it is more plausible that pilot whales from different locations consume different prey types (likely to represent different species, but possibly different age classes and/or different proportions of the same prey species).

A study based on the analysis of stomach contents of pilot whales from Portugal, Galicia and Scotland (with 13 samples in common with the present study from Iberia (n=7) and Scotland (n=6)), found evidence of geographical variation in diet across these areas (chapter II). Iberian whales showed a more diverse diet, with a prevalence of octopus that contrasted with the mainly Ommastrephidae-dominated diet of Scottish animals (Santos *et al.,* 2013). The analysis of prey fatty acids, in the present study, revealed some evidence that Iberian whales are feeding on octopods, since the same dietary fatty acid (20:4(n-6)) that seems to be responsible for the separation between whales from Iberia and whales from Scotland/USA, was also one of the fatty acids that showed significant differences between Octopodidae and Ommastrephidae, and more importantly was the FA identified by LDA as the most important for discrimination. However this evidence is not conclusive since, first of all, it is very risky making assumptions about diet composition based on one or two fatty acids (Budge *et al.,* 2006). Additionally, in the present study, other dietary fatty acids showed significant differences between Octopodidade and Ommastrephidae, but not between Iberian and Scottish whales and vice-versa (as it happens with the higher proportions of 18:4(n-3) in Scottish/USA whales compared to Iberian whales, which were not evidenced in

the PCA). Furthermore, significant differences between Ommastrephidae of Iberia and Scotland were observed, suggesting that the differences found between fatty acids of cephalopod families may also be related with other sources of variation. It is important to mention that the metabolism of pilot whales was not considered in this analysis. Several studies have already suggested that predator and prey fatty acid profiles will not match exactly, since some level of metabolism of fatty acids occurs within the predator (Koopman *et al.*, 1996; Budge *et al.,* 2006). This highlights the need to investigate how dietary fatty acids incorporate into cetacean blubber, through diet experiments that allow the determination of calibration coefficients for individual fatty acids, as already implemented in pinnipeds (Iverson *et al.,* 2004).

Similar to the stomach contents study performed in chapter II, the prevalence of benthic octopus (21.1% of prey biomass), followed by oceanic squids was also found in a study of stomach contents of pilot whales stranded in the Bay of Biscay (Spitz *et al.,* 2011), while Pierrepont *et al.* (2005) found *Sepia* sp. to be the most numerous prey found in pilot whales stranded along the French Coast, with curled octopus (*E. cirrhosa*) only representing 14.3% of the total number of prey. At higher latitudes, in the Eastern Atlantic, Desportes & Mouritsen (1993) found *Todarodes sagitattus* and *Gonatus* sp. to be the main prey present in pilot whale stomach contents from schools around Faroe Islands. In the Western Atlantic, stomach contents studies described a diet consisting largely of the neritic squid *Loligo pealei*, but also including oceanic squids of the families Ommastrephidae and Histioteuthidae (Gannon *et al.,* 1997). Although the wide range of prey species described by the previous stomach contents studies provides only snapshots of the dietary intake of pilot whales across the different areas, it is useful to help interpret results observed in the present study, since it indicates geographical shifts in the prey species consumed.

The geographical shifts in the feeding habits can either be due to the preference for different prey species or to different prey availability in the studied areas. The lack of contemporary data related to the local abundance of many of the prey species eaten by pilot whales, makes it difficult to determine if pilot whales are generalist consumers (feed on the most abundant prey species, hence diet differences are related with prey availability) or show some type of specialist behaviour towards particular cephalopod species (since being mainly teuthophagous already reflects some level of specialist behaviour).

Additionally, the geographical differences in FA profiles of pilot whales may also be due to exploitation of different feeding niches. A study on nitrogen isotopes in different tissues (representing different turnover rates) of pilot whales showed significant differences between the West and East Atlantic, suggesting that pilot whales are feeding at different trophic levels in those locations (Abend & Smith, 1995). Moreover, a recent study on stable isotopes of different odontocete species occurring in Northwestern Iberia found that carbon isotopes signatures suggested that pilot whales of this region may occur in coastal habitats (a result supported by habitat distribution analysis and the occurrence of coastal sightings of this species, Pierce *et al.,* 2010a; Spyrakos *et al.,* 11; Santos *et al.,* 2012) and/or that this species was mainly foraging on neritic and/or benthic prey species (Méndez-Fernandez *et al*., 2012). These results, together with stomach content results for Iberian Peninsula, that showed a preference for *Eledone cirrhosa* (Santos *et al.,* 2013), a eubarythic species, the main distribution of which is situated at depths of less than 300m (Boyle, 1983) contrast with the oceanic preferences shown by this species in other locations, both in terms of habitat (Macleod *et al.,* 2003, 2007; Kiszka *et al.*, 2007; De Stephanis et al., 2008a; Praca & Gannier, 2008) and oceanic prey species consumed (Desportes & Mouritsen, 1993; Santos *et al.*, 2013). Hence, Iberian pilot whales may be occupying a different feeding niche, when compared to other locations analysed.

Although a quantitative analysis was not performed in the present study, the results related with the geographical variation in FA signatures of pilot whales, based mostly on dietary FA suggest that dietary differences occur across these three regions of the North Atlantic, which may reflect the occurrence of different ecological stocks, with specific foraging habits. These results, together with previous stomach content and stable isotope analysis, and habitat distribution studies suggest that Iberian whales may occupy a different (more coastal) feeding niche compared to animals from other locations. Therefore, this study highlights the usefulness of the combination of fatty acid analysis that can provide a longer term history of dietary habits of a species, with more conventional approaches such as stomach contents analysis that supplies snapshots of detailed diet composition. The integration of these two approaches, together with other ecological tracers, in the study of natural populations is a particularly powerful strategy for obtaining information on feeding ecology over different time-scales.

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Chapter IV

Sequence polymorphism and geographical variation at mitochondrial and MHC loci in Long-finned pilot whale (*Globicephala melas*) from the North Atlantic.

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Abstract

Comparison of how genetic diversity is apportioned among populations at neutral and adaptive markers can provide insights about the influence of selection versus other evolutionary forces in shaping population genetic structure, as well as important foundations for potential management and conservation strategies. Sequence polymorphism and geographical variation at a putatively neutral locus (mitochondrial control region, mtDNA) and two adaptive loci (MHC DRA and DQB) were investigated in long-finned pilot whales (*Globicephala melas*), from six regions in the North Atlantic and adjacent waters. For the mtDNA locus, haplotype (0.56±0.04) and nucleotide (0.22%±0.18%) diversities were comparable to other abundant widespread cetaceans. There were high and significant levels of mtDNA differentiation between most regional groups from the North Atlantic, indicative of genetic structure at both regional and oceanic scales. MHC analyses revealed three alleles at each locus, with a nucleotide diversity of 0.56±0.42 and 4.63±2.40 for DRA and DQB loci, respectively. Patterns of population divergence from the MHC are consistent with the occurrence of genetic structure among populations in the North Atlantic, with Iberian whales representing a significantly genetically differentiated group ($0.07<F_{ST}<0.18$, $p<0.05$, between Iberia and other regions, across both loci). Population structuring within mtDNA could be related to the social structure presented by this species, associated with high levels of female phylopatry and short-term movements of males to reproduce. For the MHC loci, the occurrence of historical balancing selection was evident (especially in the DQB locus), as shown by the trans-specific allele sharing and the d_N/d_S ratio. However, although historically it seems that balancing selection had an important role in shaping population diversity, the spatial patterns of extant diversity across the North Atlantic could be attributable to local selection pressures for specific pathogens/ parasites or patterns of gene flow and/or drift.

Introduction

Determining the spatial distribution of genetic diversity for a species is important for understanding both long term adaptive potential and for identifying demographically and evolutionarily independent populations that require specific management or conservation action (Alcaide *et al.,* 2008; Valenzuela et al., 2009; Witteveen *et al.,* 2011). Generally, markers such as mitochondrial DNA (mtDNA) and microsatellite polymorphisms have been

extensively applied in population genetic studies, due to features like high levels of polymorphism and expected non-deviation from neutral models of evolution (Ballard & Whitlock, 2004). These have contributed significantly to our understanding of how drift and migration operate across populations to shape patterns of extant diversity. However, much of the genetic diversity occurring within individuals is adaptive, being also influenced by selection, which may act in favour or against stochastic microevolutionary forces to provide a different pattern of population genetic structure (Meyer & Thomson, 2001; Piertney & Oliver, 2006; Bos *et al.,* 2008; Spurgin & Richardson, 2010). As such, there is increasing focus on mapping how adaptive genetic variation of key genes of ecological or adaptive importance changes over both space and time. Interactions between demographic processes (i.e. population bottlenecks, subdivision, genetic drift and gene flow) and selection, may hamper the understanding of which micro-evolutionary forces are responsible for the maintenance of neutral and adaptive diversity in wild populations (Nielsen *et al.*, 2005; Bos *et al*., 2008; Radwan *et al.,* 2010). However, considering that demography is expected to affect all loci, while different types and strength of selection target specific genes and will not influence the allele frequency distribution of neutral markers (Hedrick, 2001; Piertney & Oliver, 2006; Spurgin & Richardson, 2010), the combination of analysis based on both adaptive and neutral markers could be a good approach to discern which evolutionary forces underpin population genetic structure (Nielsen *et al.,* 2001, 2005).

A common non-neutral marker that is widely used is the Major Histocompatibility Complex (MHC). The main function of the MHC is to trigger adaptive immune responses, since it is responsible for encoding proteins that recognize and present foreign antigens to the vertebrate immune system (Piertney & Oliver, 2006). Several studies describe pathogen-mediated selection as one of the main ecological mechanisms believed to underpin MHC diversity, based on three (not exclusive) hypotheses: heterozygote advantage, frequency-dependent selection and fluctuating selection (Wegner *et al.,* 2003; Piertney & Oliver, 2006; Oliver *et al*., 2009; Spurgin & Richardson, 2010). These processes may explain potential rapid and episodic changes in patterns of genetic diversity in populations, based on spatial (Landry & Bernatchez, 2001; Miller *et al.,* 2001; Charbonnel & Pemberton, 2005; Oliver *et al.,* 2009) and temporal (Charbonnel & Pemberton, 2005; Oliver *et al.,* 2009) variation of selection effects, thus making selection a possible strong but frequently temporary force (Elena *et al.,* 1996). Moreover, mating behaviour correlated with social structure has also been suggested,

as another ecological process through which selection could operate on MHC loci in natural populations (Wenink *et al.,* 1998; Sommer *et al.,* 2003; Kundu & Faulkes, 2004; Cutrera & Lacey, 2006).

The long-term effects of balancing selection can be detected by phylogenetic patterns (like trans-species polymorphism where allelic lineages are shared among species and persist over long evolutionary time (Piertney & Oliver, 2006)) or the d_N and d_S ratio (ratio between nonsynonymous and synonymous substitutions). The effects of balancing selection on patterns of contemporary population genetic structure are less clear (reviewed by Radwan *et al.,* 2010). On the one hand, a corollary of trans-species polymorphism, that retains allelic lineages across species, would be that alleles are retained in populations. This would reduce the levels of genetic divergence relative to a neutral marker (Piertney & Oliver, 2006). However, it may be that the broad-scale signature of balancing selection is a consequence of local selection favouring specific alleles in specific regions (Piertney & Oliver, 2006). This would generate a pattern similar to neutral markers when dispersal is limited, with localized genetic divergence. Teasing apart the effects of selection and reduced gene flow would be difficult, since different localised selection pressures may not mirror patterns of gene flow between adjacent populations (Landry & Bernatchez, 2001; Miller & Lambert, 2004; Campos *et al.,* 2006; Alcaide *et al.,* 2008; Babik *et al.,* 2008; Peters & Turner, 2008; Miller *et al.,* 2010). From a management and conservation perspective however, this distinction may not be relevant as it is the consequence of microevolution that is important for defining a set of demographically or evolutionarily independent units, rather than whether this is driven by stochastic or deterministic processes

The long-finned pilot whale (*Globicephala melas*), hereafter referred to as pilot whale, is one of the largest odontocete cetaceans. The species is distributed throughout temperate and subarctic regions of the north and southern hemisphere, being absent from tropical waters (Reid *et al*. 2003). Several genetic and ecological studies on pilot whale have suggested the occurrence of different populations in the North Atlantic (Perrin *et al.,* 1990; Bloch & Lastein, 1993; Abend & Smith, 1995; Siemann *et al.,* 1994; Fullard *et al.,* 2000; Oremus *et al.,* 2009; Santos *et al.,* 2013). Comparing genetic divergence between the Atlantic Ocean with other oceanic basins, from mitochondrial DNA, Oremus *et al.* (2009) found pairwise differences between Atlantic, New Zealand and Australian whales. Another study using neutral microsatellite markers within North Atlantic pilot whales (Fullard *et al*.,

2000), revealed differentiation between West Greenland and other regions (Cape Cod, Faroe Islands and UK), potentially associated with sea surface temperature. Also, the analysis of stable isotopes in animals from the Faroe Islands, the mid-Atlantic Bight and Cape Cod areas suggested the occurrence of dietary segregation of animals from the West and East Atlantic (Abend & Smith, 1995). Likewise, a stomach contents study with animals from Iberia and Scotland found dietary differences between both areas, which could also suggest the occurrence of different dietary niches (Santos *et al.,* 2013). In the same way, differences in parasite composition between animals from the western Mediterranean, France, Faroe Islands and Newfoundland suggest that population structure is prevalent (Perrin *et al.,* 1990). A study showed the occurrence of morphometric differences between whales from Faroe Islands and Newfoundland, suggesting the occurrence of two separated populations in these regions (Bloch & Lastein, 1993). Only one genetic analysis, based on the mitochondrial control region of 70 pilot whales from the North Atlantic (USA, Nova Scotia, Newfoundland and United Kingdom), found no evidence of population structure (Siemann, 1994).

Two studies have characterized adaptive genetic diversity within the *Globicephala* genus. The first characterized the MHC DQB locus in several cetacean species and revealed three alleles in two short-finned pilot whales (*G. macrorhynchus*) from Japan (Hayashi *et al*., 2003) while the second analysed the DQA and DQB loci and revealed the occurrence of 8 alleles at each locus, in a total of 237 mass stranded long-finned pilot whales (G. *melas*) from New Zealand (Heimeier, 2009).

Main goals of the present study are: 1) Characterise genetic diversity at the mitochondrial control region and MHC DRA and DQB loci in six putative populations of pilot whale from the North Atlantic and adjacent waters and compare them with populations of the Pacific Ocean; 2) Examine how neutral and adaptive diversity is structured across the North Atlantic and 3) Determine which evolutionary forces are responsible for maintaining variability in populations, by assessing whether patterns of contemporary population genetic structure inferred from neutral and adaptive markers are concordant with the influence of balancing selection or in accordance with the effects of demographic processes, such as migration or genetic drift.

Methodology

Sample collection

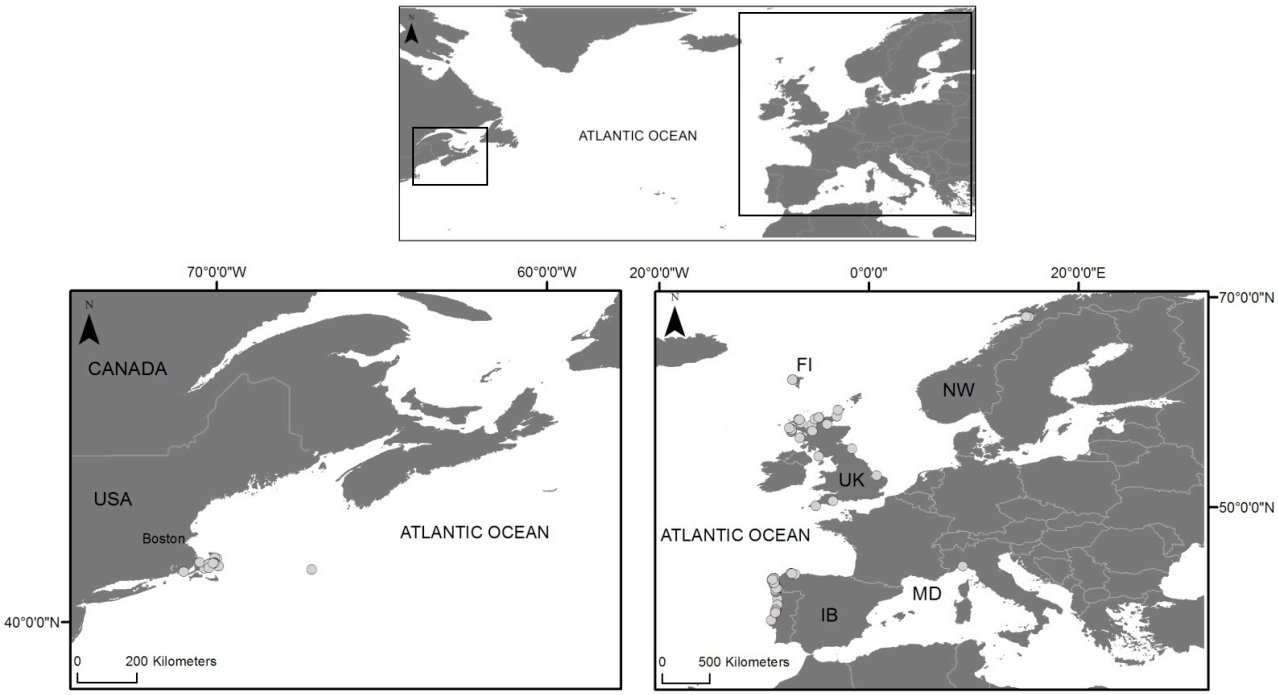


Figure 4.1. Map showing the location of the strandings of pilot whale analysed in this study (n = 123). IB: Northwest Iberia; MD: Mediterranean; UK: United Kingdom; FI: Faroe Islands; NW: Norway; USA: United States of America.

A total of 123 pilot whale samples were collected mostly from stranded animals, in several areas of the North Atlantic and Mediterranean (Northwest Iberia (n=34), Mediterranean (n=1), United Kingdom (n=34), Faroe Islands (n=25), Norway (n=3) and United States of America (n=27), Figure 4.1). Samples collected in Norway were taken from biopsied free-ranging animals and samples from Faroe Islands are from animals caught in drive fisheries. All tissue samples were either frozen or preserved in 70% ethanol.

DNA extraction, amplification and genotyping

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). After quantification in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific), samples were diluted to 30µg/µl.

For the mitochondrial analysis, a 400bp fragment of the mtDNA control region was sequenced in each of a total of 106 samples, from the North Atlantic and Mediterranean (Figure and Table 4.1), 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 95° C, 20 cycles of 30s at 92°C, 30s at 60-50°C (decreasing 0.5°C per cycle) and 45s at 72°C, 19 cycles of 30s at 92°C, 30s at 50°C and 45s at 72°C, followed by a final extension at 72°C 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 a direct and reverse direction.

Data obtained from this study were augmented with previously published mtDNA control region haplotypes representing a total of 643 whales from the Atlantic (n=70, U20926-U20928, Siemann (1994)) and Pacific (Australia (n=215) and New Zealand (n=358), FJ513342-FJ513354, Oremus *et al.* (2009)).

For the MHC DRA locus, a 188bp fragment of the exon 2 region was amplified in each of a total of 115 samples from the North Atlantic (Figure 4.1 and Table 4.2), using the primers DRAf (5'- AAT CAT GTG ATC ATC CAA GCT GAG TTC-3') and DRAr (5'- TGT TTG GGG TGT TGT TGG AGC G -3') (Xu *et al.,* 2007). For the MHC DQB locus, a 171bp fragment of the exon 2 region was amplified in a total of 100 samples from the North Atlantic (Figure 4.1 and Table 4.2), using the primers DQB1 (5 -CTGGTAGTTGTGTCTGCACAC-3') and DQB2 5 -CATGTGCTACTTCACCAACGG-3 (Murray *et al.,* 1995). To allow analysis using Denaturing Gradient Gel Electrophoresis (DGGE), an additional 40bp GC-rich sequence (GC clamp) was added to the 5' end of the primers DRAf and DQB1. The optimal GC clamp sequence was ascertained using WinMelt software (Bio-Rad). For both MHC loci, PCR reactions were carried out in a 10µl final volume reaction containing 1x PCR Buffer, 1,5 mM $Mgcl_2$, 0.2 mM DNTPs, 0.5 units of BIOTAQ DNA Polymerase (Bioline) and 0.4 µM of each primer. Cycling conditions for the DRA locus were: 2 min at 94°C, 19 cycles of 30s at 91°C, 30s at 60-50°C

(decreasing 0.5°C per cycle), 19 cycles of 30s at 91°C, 30s at 50°C followed by a final extension at 72°C for 1 min, while for the DQB locus conditions were: 3 min at 94°C, 30 cycles of 30s at 94°C, 30s at 58°C and 30s at 72°C followed by a final extension at 72°C for 5 min. PCR products were first visualized on a 1.5% agarose gel.

DGGE was performed in a Bio-Rad DCode System. One microlitre of each PCR product was applied directly onto 1 mm thick 10% polyacrylamide gels (acrylamide:bis-acrylamide at 37:5:1) in 1x TAE (40 mM Tris-acetate and 1 mM EDTA, pH 8.3), mixed with varying concentrations of denaturing agents (according to the desired denaturing gradient) and polymerized by the addition of 0.1% TEMED and 0.1% ammonium persulfate. Denaturing gradients consisted of increasing concentrations of urea and formamide in the polyacrylamide solutions (0.07 M urea and 0.4% of formamide per % denaturant) and were formed using the Gradient Delivery System (Bio-Rad). Electrophoresis conditions were optimized for maximum band separation and resolution to 14 h at a constant voltage (50 V) and temperature (60°C), in a linear 40% to 50% denaturing agent gradient for the DRA locus and 50% to 70% for the DQB locus. After electrophoresis, the gels were stained using silver staining, consisting of a 30 min immersion of the gel in a fixative solution (10 % absolute ethanol/0.5 % acetic acid), followed by a 20 min immersion in 0.1 % silver nitrate solution and a final immersion in a developing solution (3% sodium hydroxide and 1.5% of 37% formaldehyde solution), until total development of the bands. Gels were photographed with a CANON 550D and allele comparison with the allele standard was performed with ImageJ (Schneider *et. al,* 2012). DGGE bands were sequenced after excision from the gel and re-amplification. Briefly, bands were excised, resuspended in 25 ml of sterilized water, and stored at 4°C for 12h. An aliquot (2µl) of supernatant was used for PCR re-amplification with the original primer set of each locus, as described above, except that for DQB locus the PCR comprised 28 cycles and the annealing temperature was increased to 60°C. PCR products were purified using QIAquick PCR purification columns (Qiagen), according to the manufacturer's protocol, and sequenced as described previously. A sequence variant was identified as a new allele only when it was in accordance with criteria laid out in Kennedy *et al.* (2002), namely that when using DNA cloning and sequencing there have to be at least three identical clones, identified in either two separate PCRs from the same individual, or from PCRs from at least two different individuals. Therefore, to validate each allelic sequence, at least 12 replicates of each putative allelic band (when possible), taken from

different individuals across random gels, were sequenced using forward primer and every allele was re-run with the reverse primer at least three times. To ensure consistency in scoring between runs, alleles found in previously cloned pilot whales, according to the standard pGEM (Promega Ltd protocol), were used as a standard and added to each gel.

Both DRA and DQB exon 2 alleles of pilot whale were designated according with the nomenclature described by Klein *et al*. (1990) for MHC in non-human species. The alleles resulting from the analyses of DRA and DQB loci were phylogenetically compared with previously published sequences of the DRA (31 sequences) and DQB (21 sequences) exon 2 regions of different cetacean species (see Figure 4.3 for Genbank accession numbers).

Statistical analysis

For both mitochondrial DNA and MHC loci, sequence variation, alignment and translation into amino acid sequences were performed using Clustal W (Thompson *et al.,* 1997) and MEGA 4.0 (Tamura *et al.*, 2007). Sequences were confirmed as mitochondrial control region and MHC DRA and DQB sequences by National Center for Biotechnology Information (NCBI) BLAST comparison.

Genetic diversity

For the mitochondrial DNA, in order to allow direct comparison with sequences available in GenBank, the size of the sequences obtained for the North Atlantic and Mediterranean samples analysed in this study 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 and Mediterranean, using ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010). Two tests on substitution and deletion weights were performed, with deletion, transition and transversion weights being either equal or different, as described by Hoelzel *et al.* (1991). These provided similar results and therefore only un-weighted results are presented.

For the MHC, allelic richness and nucleotide diversity were calculated using ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010) and FSTAT 2.9 (Goudet, 1995). The average pairwise nucleotide distances (Tamura-Nei model), average pairwise amino acid distances (JTT model,

with alpha=1 for the DQB locus, Jones *et al.,* 1992) and the relative rate of nonsynonymous (d_N) to synonymous (d_S) mutation, applying the method of Nei & Gojobori (1986), with Jukes–Cantor correction for multiple mutations at single sites, were calculated using MEGA 4.0 (Tamura *et al.,* 2007). The probability that d_N=d_S was determined using a Z-test (Nei & Kumar 2000). Standard errors for these estimates were estimated through 100000 bootstrap replicates. Nucleotides within the Protein Binding Region (PBR) were determined as predicted by Brown et al., (1993).

Population differentiation and Phylogenetic analysis

For mitochondrial DNA, the potential occurrence of genetic structure in North Atlantic or worldwide 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) (the Mediterranean and Norway were not included in pairwise comparisons due to low sample size). 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). To quantify the genetic divergence between samples an Analysis of Molecular Variance (AMOVA) was undertaken using ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010). A hierarquical assessment of structure was examined, partioning variance between Western and Eastern sides of the Atlantic; between regions within each side of the Atlantic (Western: USA; Eastern: Faroe Islands, UK, Norway, Northwest Iberia, Mediterranean) and among individuals within regions. For a global analysis, the hierarquical assessment of structure was examined, partioning variance between Atlantic and Pacific Oceans; between regions within each ocean (Atlantic: USA, Newfoundland, Nova Scotia, Faroe Islands, UK, Norway, Northwest Iberia, Mediterranean; Pacific: New Zealand, Australia) and among individuals within regions.

As for the analysis of genetic diversity, two tests were performed with deletion, transition and transversion weights, with equal or different weights among substitutions and deletions, as described by Hoelzel *et al.* (1991). These provided similar results and therefore only un-weighted results are presented.

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 (weighted genetic distance to the known sequences in the dataset, within which potential median vectors may be constructed) was set to 10.

For the MHC, the potential occurrence of genetic structure in North Atlantic was tested through pairwise comparisons and an Analysis of Molecular Variance (AMOVA, Excoffier *et al*., 1992), available in the software ARLEQUIN 3.5.1.2. F_{ST} based on allele frequency was used to test population differentiation (Weir & Cockerham, 1984). Statistical significance of the estimates F_{ST} was calculated using 20000 permutations of alleles among sampling regions (Fisher's exact test). As for mtDNA, to quantify the genetic divergence between samples an Analysis of Molecular Variance (AMOVA) was undertaken using ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010). A hierarquical assessment of structure was examined, partioning variance between Western and Eastern sides of the Atlantic; between regions within each side of the Atlantic (Western: USA; Eastern: Faroe Islands, UK, Northwest Iberia) and among individuals within regions.

Phylogenetic relationships were assessed using Maximum Likelihood as implemented in PAUP 4.0 (Swofford, 2003) and Bayesian analysis as implemented in MrBayes 3.2 (Ronquist *et al.,* 2012). Likelihood analysis was performed using 1000 bootstrap replicates with tree-bisection-reconnection branch swapping. For Bayesian phylogeny estimation, two independent runs of four Metropolis-coupled MCMC chains (temperature=0.2) were run for 1000000 generations (every 1000^{th} tree was sampled). The first 25% of trees were discarded as burn-in, resulting in 750 trees from which parameter values and trees were then summarized and a consensus tree was drawn using the program TREEVIEW 1.6 (Page, 1996). The model of sequence evolution recommended by JModeltest 2.1 (Darriba *et. al*, 2012), for the MHC data, is Kimura 2 Parameter (Kimura, 1980), with gamma-distributed rate variation across sites for DRA locus (gamma distribution =0.295) and HKY+G (A=0.2234, C=0.2542, G=0.3835, T=0.1389 and gamma distribution =0.1540) for DQB locus. *Homo sapiens* (Genbank accession number: AM259941) was used as an outgroup for the DQB locus.

The effects of historical selection on MHC loci, were determined using a d_N/d_S ratio >1 in the Peptide Binding Region (PBR), together with the retention of allelic lineages across

speciation events (trans-species polymorphism), while the occurrence of contemporary selection was examined from the levels of population differentiation in comparison to a neutral locus (population differentiation F_{ST}).

Results

Genetic Diversity

Mitochondrial DNA

A total of six polymorphic sites (2 deletions, 3 transitions and 1 transversion) defined seven haplotypes along the different geographic regions in the North Atlantic and Mediterranean (Table 4.1, Figure 4.2). The haplotypes D, E and G had previously not been described in pilot whales (Genbank accession numbers: KC934932-34), but A, B, C, 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 4.1).

Overall, haplotype and nucleotide diversity were 0.56 ± 0.04 and π=0.22% ± 0.18%, respectively (Table 4.2). Within the North Atlantic, the United Kingdom presented the highest nucleotide diversity (π=0.18% ± 0.16%), followed by Northwest Iberia and the Faroe Islands, while the highest haplotype diversity was seen in the Faroe Islands (h=0.53 ± 0.08) (Table 4.2). The USA showed the lowest values for both nucleotide and haplotype diversities (h= 0.08 ± 0.07; π=0.04±0.07) (Table 4.2).

Table 4.1. Variable nucleotide positions in worldwide pilot whale mitochondrial control region sequence. Haplotype frequencies within different regions of North Atlantic, Mediterranean and Pacific are also shown (347bp).

																		East Atlantic				MD	West Atlantic			Pacific	
Hap	Freq	3 9	4 5	1 0 0	1 0 1	1 0 1 3	1 0 4	1 1 9	1 1 2	1 1 4	1 2 6	2 2 1	2 2 6	2 2 8	2 5 7	3 4 4	IB	UK	FI	NW	MD	USA	NF	NS	NZ	AU
A	124	T	A	A	T	*	*	C	A	T	C	C	C	A	T	C	8(8)	33(26)	6(5)	1(1)	0	73(25)	0	0	3	0
B	4	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	0	0	0	0	0	1(1)	0	3	0	0
C	366	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	0	1(1)	17(7)	0	0	0	1	0	316	31
D	1	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	0	0	0	0	1(1)	0	0	0	0	0
E	4	.	.	.	.	T	A	.	.	.	.	.	.	.	.	.	1(1)	3(3)	0	0	0	0	0	0	0	0
F	80	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	25(25)	1(1)	0	0	0	0	0	0	3	51
G	1	.	.	.	.	T	A	.	.	C	.	.	.	.	.	.	0	1	0	0	0	0	0	0	0	0
O	14	.	.	*	*	.	.	.	.	.	.	T	.	.	.	.	0	0	0	0	0	0	0	0	14	0
Q	72	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	0	0	0	0	0	0	0	0	3	69
T	1	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	0	0	0	0	0	0	0	0	1	0
U	18	.	.	.	.	.	.	.	.	C	.	T	.	.	.	.	0	0	0	0	0	0	0	0	17	1
V	1	.	G	.	.	.	.	.	.	C	.	T	.	.	.	.	0	0	0	0	0	0	0	0	1	0
W	15	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	0	0	0	0	0	0	0	0	0	15
Y	3	.	.	.	.	.	.	.	.	C	.	T	.	.	.	T	0	0	0	0	0	0	0	0	0	3
Z	31	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0	31
O2	14	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	0	0	0	0	0	0	0	0	0	14

Hap: Haplotype; Freq: frequency summed across all samples; Dots represents nucleotide identity with haplotype A; *: nucleotide insertion/deletion; within brackets are haplotype frequencies found in the present study. Abbreviations are described in figure 4.1, except NF: Newfoundland; NS: Nova Scotia; NZ: New Zealand; AU: Australia.

Table 4.2. Summary of genetic diversity statistics for the mtDNA and MHC DRA and DQB loci of long-finned pilot whale. Mean values ± Standard Deviation are shown.

		IB	UK	FI	NW	USA	MD	Overall
MtDNA	**n**	34	32	12	1	26	1	106
	haplotypes	3	5	2	1	2	1	7
	h	0.42±0.08	0.34±0.10	0.53±0.08	-	0.08±0.07	-	0.56±0.04
	Π (%)	0.15±0.14	0.18±0.16	0.15±0.15	-	0.04±0.07	-	0.22±0.18
	s	3	4	1	-	2	-	6
DRA	**n**	26	33	24	3	27	-	115
	alleles	3	3	2	2	2	-	3
	Allelic richness	2.15	1.88	1.72	1.95	2.00	-	2.00
	Π (%)	0.61±0.45	0.84±0.57	0.22±0.23	4.66±3.71	0.27±0.26	-	0.56±0.42
DQB	**n**	18	29	26	2	22	-	100
	alleles	3	3	3	3	3	-	3
	Allelic richness	1.85	2.00	1.83	3.00	1.85	-	3.00
	Π (%)	4.53 ± 2.4	4.48±2.37	4.21±2.24	6.53±4.50	4.36±2.34	-	4.63±2.40

n: sample size; h: haplotype diversity; π: nucleotide diversity; s: number of polymorphisms. Abbreviations are described in figure 4.1.

MHC

No more than two sequences were resolved for any individual at either the DRA or DQB loci, suggesting that one single locus was amplified in both case.

The DRA exon 2 region (186 bp) was sequenced from 115 pilot whales, across five different geographic locations in the North Atlantic. A total of 13 variable sites (6.9%) in the nucleotide sequence defined three unique DRA allelic sequences: Glme-DRA*01, Glme-DRA*02 and Glme-DRA*03 which were previously described by Xu *et al*. (2009) in other cetacean species (Table 4.3a). Nucleotide distances between the three DRA exon 2 alleles ranged from 0.5-7.4% with an average nucleotide divergence of 4.9%. The three nucleotide alleles translated to two different amino acid sequences (of 62 amino acids in length), with eight variable sites (Table 4.3a). When translated to amino acids, the distance between the alleles ranged between 0-13.8%, with an average amino acid divergence of 9.5%. For DRA exon 2 region, the proportion of nonsynonymous substitutions (d_N=4.7%) was not significantly different from synonymous substitutions (d_S= 5.5%) (d_N/d_S=0.8545,p>0.05), which provided no evidence for selection acting on this locus. None of the amino acid substitutions is located in a site that is considered to be involved in peptide binding, as described by Brown *et al.* (1993) (Table 4.3a). DRA exon 2 diversity is given for each Atlantic region in Table 4.2. Overall, there was an average nucleotide diversity of 0.56±0.42%, ranging between 0.22±0.23% (Faroe Islands) and 4.6±3.7% (Norway) (Table 4.2 and Figure 4.2). Allelic richness was highest in Northwest Iberia and lowest in the Faroe Islands (Table 4.2 and Figure 4.2).

The DQB exon 2 region (171 bp) was sequenced from 100 pilot whales, from five different geographic locations in the North Atlantic. A total of 17 variable sites (9.9%) in the nucleotide sequence defined three unique DQB allelic sequences: the new allele Glme*DQB*01; Glme*DQB*02, which was previously described in *Tursiops truncatus* by Kita *et al*. (2007); and the new allele Glme*DQB*03 (Table 4.3b). Nucleotide distances between the three DQB exon 2 alleles ranged from 3-10.1% with an average nucleotide divergence of 7.5%. Each of the three nucleotide alleles translated to a different amino acid sequence (57 amino acids), with 10 variable sites (Table 4.3b). When translated to amino acids, the distance between the alleles ranged between 7.7-25.3%, with an average amino acid divergence of 17.6%. A significantly higher value for the proportion of nonsynonymous substitutions (d_N= 9.7± 3.3%) was shown when compared to the proportion of synonymous

substitutions (d_S=0.8± 0.8%) over all sites (d_N/d_S=2.81,Z test p-value<0.01), suggesting selection acting in this locus. For the 14 amino acids corresponding to the PBR, variability was detected at two sites (14.3%), while for the remaining 43 amino acids variability was detected at 8 sites (18.6%) (Table 4.4)**.** For codons within both the PBR and non-PBR, the rate of nonsynonymous substitutions (d_N=8.9±6.7% and 9.5±3.9%, respectively) exceeded that of synonymous substitutions (d_S=0% and 1.1± 1.1, respectively) (Z test p-value>0.05 for PBR and p<0.05 for non-PBR), providing evidence that positive selection was acting only in non-PBR. Table 4.2 summarizes the data on indicators of DQB exon 2 diversity in each sampling group. Overall, there was an average nucleotide diversity of 4.63±2.39%, ranging between 4.21 ± 2.24% (Faroe Islands) and 6.53 ± 4.50% (Norway) (Table 4.2 and Figure 4.2). Allelic richness was higher in Norway and lower in the Faroe Islands (Table 4.2).

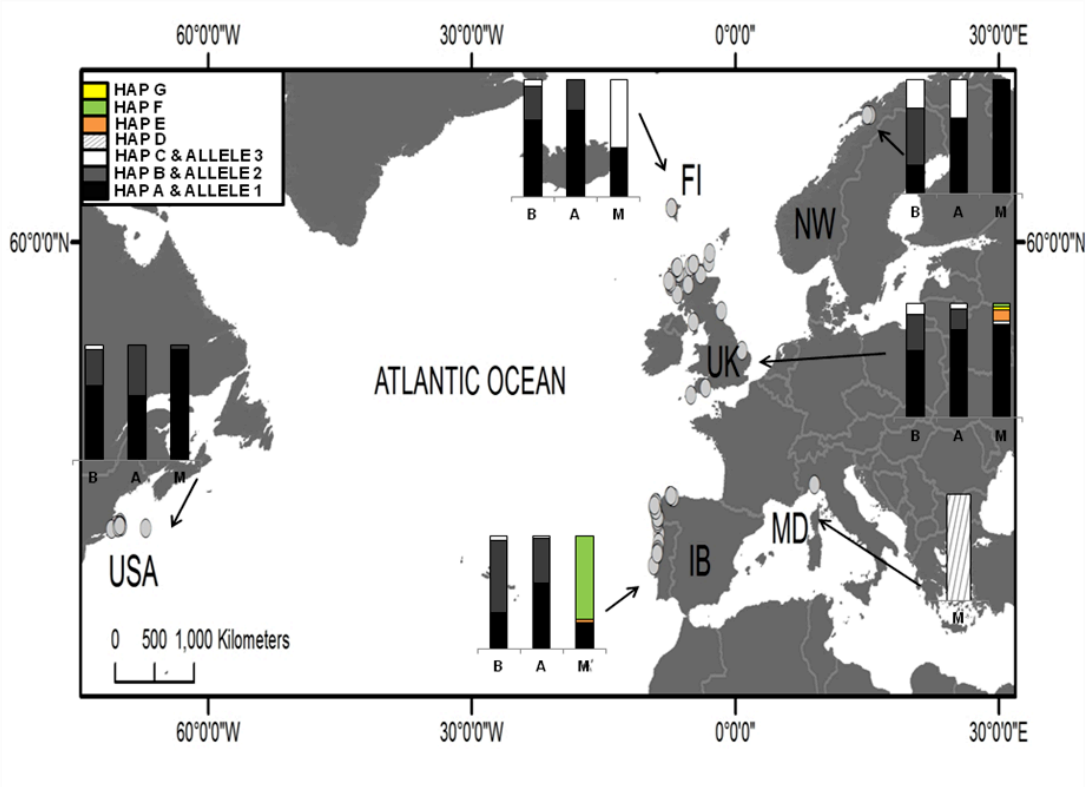


Figure 4.2. Map showing the distribution of DRA (A) and DQB (B) MHC class II alleles and mtDNA (M) haplotypes in the study area. Bars represent the absolute frequency of the different alleles and each allele/haplotype is represented by a different shade of grey. MtDNA haplotypes are represented as described in table 4.1. Abbreviations are described in figure 4.1.

Table 4.3 Nucleotide and predicted amino acid sequences for: a) MHC class II DRA exon 2 alleles and b) MHC class II DQB exon 2 alleles in *G.melas*. A dot indicates identity with the top sequence. Asterisks and numbers in bold identify sites in the putative peptide-binding region as predicted by Brown et al (1993).

a) DRA

Glme*DRA*01	TCTCTGTCCCCTTATCAATCAAACGAGTTTATGTTTGACTTTGATGGTGATGAGATTTTCCA
Glme*DRA*02	....
Glme*DRA*03	......GT....G.C..G...GG.......................................

Position										22		24		26					31	32	
PBR										*		*		*					*	*	
Glme*DRA*01	S	L	S	P	Y	Q	S	N	E	F	M	F	D	F	D	G	D	E	I	F	H
Glme*DRA*02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Glme*DRA*03	.	.	V	.	D	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.

Glme*DRA*01	CGTGGATATGGAAAAGAAGGAGACAGTGTGGCGGCTTAAAGAATTTGGAAATTTTGCCAGTT
Glme*DRA*02	....C.
Glme*DRA*03	.......T......G...C.

Position										43								51		53	**54**
PBR										*								*		*	*
Glme*DRA*01	V	D	M	E	K	K	E	T	V	W	R	L	K	E	F	G	N	F	A	S	F
Glme*DRA*02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Glme*DRA*03	.	.	L	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Glme*DRA*01	TTCAGGCTCAGGGTGCATTGGCCAATATGGCTGTGGGAAGAGCCAACCTGGACATCTTGATA
Glme*DRA*02	....
Glme*DRA*03	............................T..........A................A.....

Position	55			58							65			68	69			72		
PBR	*			*							*			*	*			*		
Glme*DRA*01	Q	A	Q	G	A	L	A	N	M	A	V	G	R	A	N	L	D	I	L	I
Glme*DRA*02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Glme*DRA*03	.	.	.	.	.	.	.	.	I	.	.	.	K	.	.	.	.	.	M	.

a) DQB

```
Glme*DQB*01   A C G G A G C G G G T G C G G G T C A T G A A C A G A T A C A T C T A T A A C C G G G A G G A G T T C G T G C G C T T C G A
Glme*DQB*02   . . . . . . . . . . . . . . . C A . G . . . G . . . . . T G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Glme*DQB*03   . . . . . . . . . . . . . . . T C . G . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Position                                                            2           2           2                             3     3
                                                                    2           4           6                             1     2
PBR                                                                 *           *           *                             *     *
Glme*DQB*01   T     E     R     V     R     V     M     N     R     Y     I     Y     N     R     E     E     F     V     R     F     D
Glme*DQB*02   .     .     .     .     .     H     V     S     .     L     .     .     .     .     .     .     .     .     .     .     .
Glme*DQB*03   .     .     .     .     .     S     V     D     .     .     .     .     .     .     .     .     .     .     .     .     .

Glme*DQB*01   C A G C G A C G T G G G C G A G T A C C G G G C G G T G A C C G A G C T G G G C C G G C C G G A C G C C G A G T A C T G G A
Glme*DQB*02   . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T C .
Glme*DQB*03   . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Position                                                              4                                               5           5     5
                                                                      3                                               1           3     4
PBR                                                                   *                                               *           *     *
Glme*DQB*01     S     D     V     G     E     Y     R     A     V     T     E     L     G     R     P     D     A     E     Y     W     N
Glme*DQB*02     .     .     .     .     .     F     .     .     .     .     .     .     .     .     .     .     .     .     .     F     .
Glme*DQB*03     .     .     .     .     .     F     .     .     .     .     .     .     .     .     .     .     .     .     .     .     .

Glme*DQB*01   A C A G C C A G G A G G A C A T C C T G G A G G A G G A A C G G G C C G C G C T G G A C A C G
Glme*DQB*02   . . . . . . . . A . . . . . . . . . . . . . . C G . . . . . . . . . . C A C G . . . . . . . .
Glme*DQB*03   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Position          5                 5                                         6                 6     6
                  5                 8                                         5                 8     9
PBR               *                 *                                         *                 *     *
Glme*DQB*01       S     Q     E     D     I     L     E     E     E     R     A     A     L     D     T
Glme*DQB*02       .     .     K     .     .     .     .     R     .     .     .     H     V     .     .
Glme*DQB*03       .     .     .     .     .     .     .     .     .     .     .     .     .     .     .
```

Table 4.4. The relative rate of d_N and d_S substitutions among alleles for codons in the PBR and non-PBR of DQB exon 2, of North Atlantic pilot whale.

	N	d_N	d_S	d_N/d_S	*P*
Overall	57	0.097±0.033	0.008±0.008	12.12	0.006
PBR	14	0.089±0.067	0	infinite	0.185
Non-PBR	43	0.095±0.039	0.011±0.011	8.63	0.018

d_N: Nonsynonymous substitutions; d_S: synonymous substitutions; PBR: peptide binding region; P: probability of the null hypothesis of neutrality ($d_N=d_S$) (Nei & Kumar, 2000); N: Number of codons used for the test. Mean value ± standard error based on 100000 replicates.

Population differentiation and Phylogenetic analysis

Mitochondrial DNA

From mitochondrial DNA there is evidence of high levels of differentiation between regions in the North Atlantic (F_{ST}= 0.49568, p<0.001; Φ_{ST}= 0.57207, p<0.001). This result is in agreement with pairwise regional comparisons which show high levels of differentiation among areas except between United Kingdom and United States of America (F_{ST}= 0.043, p>0.05; Φ_{ST}=-0.03, p>0.05, Table 4.5). The AMOVA (that included both Mediterranean and Norwegian samples) showed no differentiation, either at haplotype or nucleotide levels, between the groups East and West Atlantic (F_{ST}=-0.165, p>0.05; Φ_{ST}=-0.428, p>0.05), highlighting that most of the genetic variance occurred among regions within East and West Atlantic (Faroe Islands, UK, Norway, Northwest Iberia and Mediterranean, F_{ST}= 0.465, p<0.001; Φ_{ST}=-0.491, p<0.001) rather than among oceanic basins, suggesting populations more closely related between rather than within Western and Eastern Atlantic. In a global analysis, there is evidence of high levels of differentiation between regions worldwide (F_{ST}= 0.407, p<0.001; Φ_{ST}= 0.323, p<0.001). An AMOVA showed no differentiation, either at haplotype or nucleotide level, between North Atlantic and Pacific (F_{ST}= 0.224, p>0.05; Φ_{ST}=0.156, p>0.05), revealing that most of the genetic variance occurred among regions within oceanic basins (F_{ST}= 0.606, p<0.001; Φ_{ST}=0.55, p<0.001) and within sampling regions (F_{ST}= 0.491, p<0.001; Φ_{ST}=0.472, p<0.001), rather than among the North Atlantic and the Pacific basins.

In the mitochondrial median joining network, the two most common haplotypes were A (n=124) and C (n=366) (Figure 4.3b). The frequencies of these two haplotypes showed strong phylogeographic patterns, with A being the most common haplotype in North Atlantic, shared by all North Atlantic's regions, D being present only in the Mediterranean and C the most common haplotype in the Pacific. Haplotype F (n=80) was the haplotype most shared between Atlantic and Pacific whales. Considering the regions within North Atlantic and Mediterranean, a phylogeographic pattern seems to occur, since several haplotypes are almost unique to one region (B, D, E and G) (Figure 4.2 and 4.3b). It is apparent that there are still some haplotypes mainly occurring in Atlantic and Mediterranean samples (A,B,D,E and G) and others mainly present in Pacific whales (O,T,U,V,W,Y,Z,O_2 and Q).

Table 4.5. Pairwise regional comparisons based on the mtDNA (below diagonal) and MHC locus (above diagonal) of long- finned pilot whale in the North Atlantic.

	NI	**UK**	**FI**	**USA**
NI	-	**0.10**	**0.18**	**0.07**
UK	**0.52 (0.60)**	-	0.01	**0.02**
FI	**0.49 (0.66)**	**0.35 (0.52)**	-	0.05
USA	**0.66 (0.64)**	0.04 (-0.003)	**0.51 (0.59)**	-

Below diagonal: F_{ST} (Φ_{ST}); Bold: Fisher's exact test of differentiation p-value < 0.05. Abbreviations are described in figure 4.1.

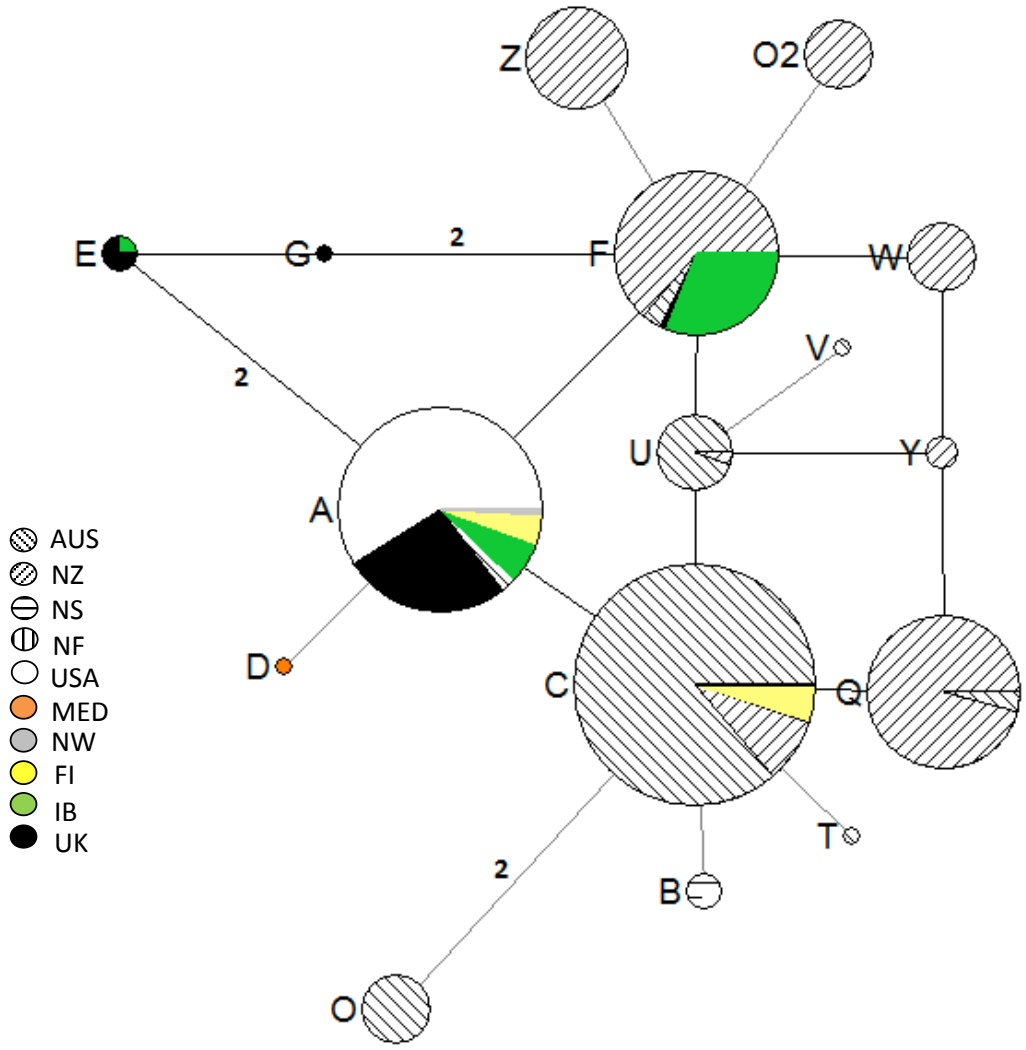


Figure 4.3. Median Joining network of the haplotypes of worldwide long finned pilot whales. Nodes are proportional to haplotype frequencies. All branches between haplotypes represent a single mutational step, unless stated differently (numbers). Haplotypes refer to the ones described in table 4.1. Abbreviations are described in figure 4.1, except NF: Newfoundland; NS: Nova Scotia; NZ: New Zealand; AU: Australia.

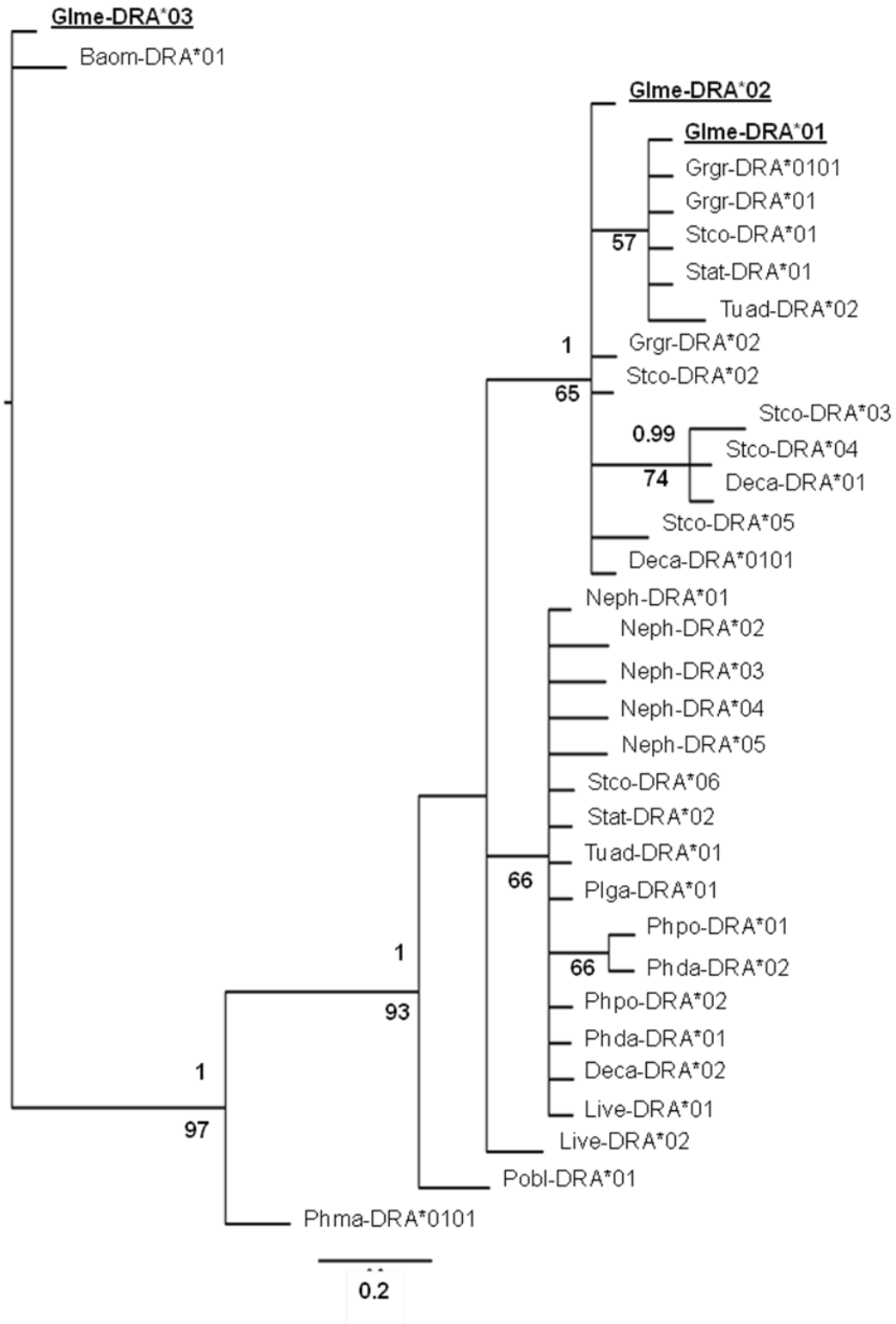
MHC

In MHC, there were significant moderate levels of genetic differentiation (F_{ST}=0.07, p<0.001). In general, F_{ST} values across both MHC loci were much lower compared to the mitochondrial data (overall F_{ST}= 0.49568, p<0.001). Northern Iberia was the only region to show significant moderate levels of differentiation from the other sampling groups

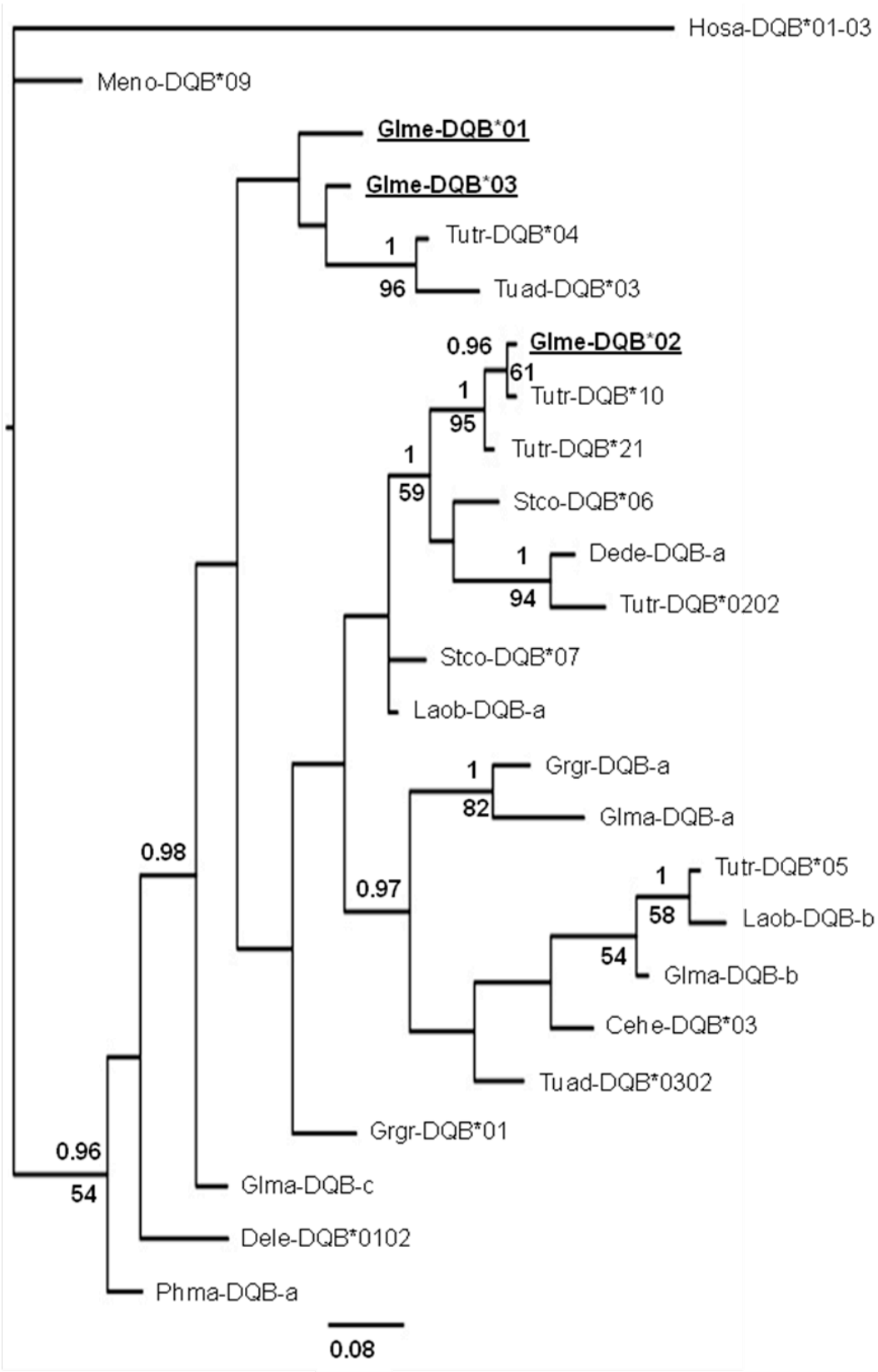
((0.07<F_{ST}<0.18, p<0.05); Table 4.5). An AMOVA showed no differentiation between East and West Atlantic (F_{ST}= -0.05, p>0.05), revealing that most of the genetic variance occurred within sampling regions (F_{ST}= 0.09, p<0.001), rather than among East and West Atlantic or among sampling regions, within oceanic basins. There was no clear separation on the MHC alleles among the different Atlantic regions, resulting in a homogeneous distribution of the alleles, with the probable exception being Glme-DQB*02 in Iberia (Figure 4.1). For DRA, both Glme-DRA*01 (the most common allele) and Glme-DRA*02 were present in all sampling groups. In contrast, Glme-DRA*03, was the only allele to be shared only by samples from the East Atlantic (Northern Iberia and United Kingdom) (Figure 4.2). Similar results were obtained with DQB sequences, which did not show any phylogeographic pattern, since all populations shared the different alleles (Figure 4.2).

Phylogenetic relationships of DRA exon 2 were based on sequences described in this study for pilot whale and sequences from different species described in previous studies (Xu *et al.,* 2007, 2008, 2009; Ballingall, 2010) (Figure 4.4a). In general, no species-specific or even suborder-specific clades were observed, since the three alleles described in this study for pilot whale were more closely related with alleles from other species or suborders than with intraspecific alleles, revealing a trans-specific sharing of alleles. Glme-DRA*01 grouped together with Risso's dolphin, Striped dolphin, Pantropical spotted dolphin and Indo-Pacific bottlenose dolphin, with posterior probability almost significant (0.90, not shown in the tree). In this group, all alleles were identical except Tuad-DRA*01. Glme-DRA*02 grouped with Risso's dolphin, striped dolphin and long-beaked common dolphin, although this cluster was not supported by a significant posterior probability (Figure 4.4a). In this group, all alleles were identical except Stco-DRA*05 and Deca-DRA*0101. The cluster containing both Glme-DRA*01 and 02 formed a separated group supported by a posterior probability of 0.99. Glme-DRA*03 showed a strong relationship with Omura's whale, supported by posterior probability of 1. Hence, there was not a clear separation between Mysticeti and Odontoceti suborders, since both Sperm and Pilot whale (Glme-DRA*03) alleles grouped together with Omura's whale, the only representantive of Mysticeti in this study (Figure 4.4a).

a)


Glme-DRA*03
Baom-DRA*01
Glme-DRA*02
Glme-DRA*01
Grgr-DRA*0101
Grgr-DRA*01
Stco-DRA*01
Stat-DRA*01
Tuad-DRA*02
Grgr-DRA*02
Stco-DRA*02
Stco-DRA*03
Stco-DRA*04
Deca-DRA*01
Stco-DRA*05
Deca-DRA*0101
Neph-DRA*01
Neph-DRA*02
Neph-DRA*03
Neph-DRA*04
Neph-DRA*05
Stco-DRA*06
Stat-DRA*02
Tuad-DRA*01
Plga-DRA*01
Phpo-DRA*01
Phda-DRA*02
Phpo-DRA*02
Phda-DRA*01
Deca-DRA*02
Live-DRA*01
Live-DRA*02
Pobl-DRA*01
Phma-DRA*0101
1
57
1
65
0.99
74
66
1
66
1
93
97
0.2

b)


Hosa-DQB*01-03
Meno-DQB*09
Glme-DQB*01
Glme-DQB*03
1
Tutr-DQB*04
96
Tuad-DQB*03
0.96
Glme-DQB*02
61
1
Tutr-DQB*10
95
Tutr-DQB*21
1
59
Stco-DQB*06
1
Dede-DQB-a
94
Tutr-DQB*0202
Stco-DQB*07
Laob-DQB-a
1
Grgr-DQB-a
82
Glma-DQB-a
0.98
1
Tutr-DQB*05
0.97
58
Laob-DQB-b
54
Glma-DQB-b
Cehe-DQB*03
Tuad-DQB*0302
Grgr-DQB*01
0.96
54
Glma-DQB-c
Dele-DQB*0102
Phma-DQB-a
0.08

2; b) MHC DQB exon 2. Numbers above branches indicate posterior probability support values (only probabilities above 0.95 are shown), whereas Maximum Parsimony or Likelihood bootstrap values above 50% are shown below branches. For the DQB locus, *Homo sapiens* (AM259941) was used as outgroup. Dede-*Delphinus delphis* (AB164220), Stco-*Stenella coeruleoalba* (EU698969-70), Tutr-*Tursiops truncatus* (AB302053, AB302064, AB302047, EF507877, EF690297), Tuad-*Tursiops aduncus* (EF507876, EU698975), Glma-*Globicephala macrorhynchus* (AB164226-28), Grgr-*Grampus griseus* (EU698953, AB164222), Phma-*Physeter macrocephalus* (AB164208), Laob-*Lagenorhynchus obliquidens* (AB164224-25), Meno-*Megaptera novaeangliae* (DQ354650), *Dele-Delphinapterus leucas* (U16987), Cehe- *Cephalorhynchus hectori* (EU024809). For the DRA locus, Deca-*Delphinus capensis* (EF375603-04, FM986350), Phph-*Phocoena phocoena* (EF375597-98), Stco-*Stenella coeruleoalba* (EF375585-90), Stat-*Stenella attenuata* (EF375591-92), Tuad-*Tursiops aduncus* (EF375593-94), Pobl-*Pontoporia blainvillei* (EF375595), Plga-*Platanista gangetica* (EF375596), Phda-*Phocoenoides dalli* (EF375599-600), Neph-*Neophocaena phocaenoides* (DQ843609-13), Live- *Lipotes vexillifer* (DQ851844-45) Baom-*Balaenoptera omurai* (EF375605), Grgr-*Grampus griseus* (EF375601-02, FM986351), Phma-*Physeter macrocephalus* (FM986352).

Phylogenetic relationships for DQB exon 2 were based on the sequences described in this study for pilot whale and sequences from different species described in previous studies (Figure 4.4b). There was a separation of most cetacean species relative to the outgroup (*Homo sapiens*), with high bootstrap and posterior probability values. No species-specific clade was observed, since trans-specific sharing of alleles was evident. The Glme-DQB*02 allele grouped with Tutr-DQB*10 (identical alleles) described by Kita *et al*. (2007), with high bootstrap (61%) and posterior probabilities (0.96) values.

Discussion

In the present study considerable genetic diversity was resolved for both mitochondrial DNA and MHC loci, for long-finned pilot whales. Both type of markers revealed the occurrence of genetic structure among regional groups of *G. melas*, across the North Atlantic, with consistent divergence patterns describing Atlantic Iberia as a separate group, when compared with remain regions.

The detection of three new 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, Oremus *et al.,* 2009; killer whales, Hoelzel *et al*., 2002; sperm whale, Lyrholm *et al.,* 1996). Likewise, at MHC loci, the variation presented by the DRA and DQB loci in the present study is in accordance with the results of previous studies with several cetacean species (Hayashi *et al.,* 2006; Xu *et al.,* 2007, 2008, 2009; Heimeier *et al.,* 2009). However, the extent to which diversity at adaptive loci can be compared across species with different ecological and life histories is still debated.

This study showed the occurrence of oceanic and regional differences in pilot whales from different regions in the North Atlantic, with significant high and moderate levels of genetic variation at mtDNA control region and MHC loci, respectively. The only area to show similar structural patterns in both types of locus was Northwest Iberia, which shown significant high and moderate levels of differentiation from other regions, at the mtDNA and MHC loci, respectively. Mitochondrial and MHC AMOVAs showed no differentiation between Western and Eastern sides of the Atlantic, revealing that most of the genetic variation occurred among or within the sampling regions analysed, rather than between oceanic basins.

The patterns of structure shown by mitochondrial DNA may be due to several factors, namely, limited gene flow (due for example to limited dispersal between sampling regions), genetic drift or social organization of pilot whales. Although this possibility cannot be discarded, the high mobility presented by this species (200km per day; Bloch et al., 2003), suggests that isolation by distance does not seem to be the explanation for the levels of mitochondrial structure found across the Atlantic, a finding also described by an analysis based on eight microsatellites on North Atlantic pilot whales populations (Fullard et al., 2000). Pilot whale social structure is still not completely understood, however results from studies carried out on samples from the North Atlantic (Faroe Islands) drive fisheries, which analysed polymorphic proteins (Andersen, 1993), microsatellites (Amos *et al.,* 1993; Fullard *et al.* 2000), organochlorine concentrations (Aguilar *et al.,* 1993), intestinal parasites (Balbuena & Raga, 1994) and heavy metals (Caurant *et al.,* 1993), along with behavioural (Ottensmeyer & Whitehead, 2003; De Stephanis *et al.,* 2008b) and photo-identification

studies (Alves *et al.*, 2013), agree that this species seems to show a similar social structure to that present in killer whales: natal group philopatry, where neither females nor males disperse from their natal groups. However, males do not father offspring from the same pod, being only able to mate when two pods meet or when males perform short-term dispersal in order to reproduce (Andersen & Siegismund, 1994; Amos *et al.,* 1993). Hence, the movements performed by the males for reproduction associated with high levels of female philopatry could explain the high values of genetic divergence exhibited by the maternally inherited haploid marker relatively to the MHC loci, similar to results found in terrestrial mammals with a similar social organization (Sommer *et al*., 2003, Wenink *et al.,* 1998). Also, it is important to consider the potential bias associated with samples originating from strandings, given there is some uncertainty about the individual's origin. However, a recent study that analysed the drift of cetacean carcasses in the sea in the Bay of Byscay showed that 57% and 85% of the stranded animals were bycaught in waters of 100 and 500m depths, at 20 to 78 km from the coast, respectively (Peltier *et al*., 2012), which considering the broad geographical range of the study shows that samples from stranded animals do not bias patterns of dispersal and gene flow. Moreover, a genetic diversity and divergence comparison between stranded and biopsy sampled free-ranging dolphins reports that population studies based solely on animal carcasses may underestimate the levels of genetic differentiation (Bilgmann *et al.,* 2011).

The MHC showed evidence of historical balancing selection, which was evaluated upon the occurrence of phylogeographic patterns related with the trans-species sharing of polymorphisms and d_N/d_S ratio value in the Peptide Binding Region (PBR). Alleles of both DQB and DRA loci grouped together with other cetacean species, showing the occurrence of common MHC polymorphisms among these species. Regarding the d_N/d_S ratio value, for DQB locus, overall nonsynonymous (d_N) substitutions were significantly more frequent than synonymous (d_S), being a similar result obtained for Non-PBR and, although not significant, for PBR. These results are in accordance with previous cetacean studies which usually found values of d_N/d_S greater than 1 for PBR, representing balancing selection (beluga, Murray *et al,* 1995; minke whale, Hayashi et al., 2003; finless porpoises, Du *et al*., 2010; Hector's dolphin, Heimeier *et al.,* 2009). For the DRA locus, a non-significant $d_N<d_S$ suggests no effects of selection at this locus. Similar results were obtained by Xu *et al.* (2009), where the analysis of several cetacean species revealed purifying historical selection on this locus. This may be

due either to loss of variation via random drift or it may represent a nonclassical MHC locus, where low variation is due to a functional constraint (Babik *et al*., 2008).

Although there is evidence of occurrence of historical effects of balancing selection in the North Atlantic pilot whale samples analysed, it is yet not clear how these historical signatures reflect contemporary selection in those sampling groups. MHC loci analyses revealed the occurrence of population structure across the North Atlantic, with Iberian whales representing a separate group from remain regions. The structure levels evidenced by adaptive loci may reflect the occurrence of local selection pressures acting in antagonistic coevolution with pathogens/parasites or may be due to other forces such as migration or genetic drift. Several studies used pathogen-mediated selection theory to explain the differentiation of populations, based on the MHC locus (reviewed by Spurgin & Richardson, 2010; Murray *et al.*, 1995; Xu *et al*., 2010).

Pathogen-mediated selection can be an ecological factor responsible for spatial variation in selective forces and may potentially explain the separation of Northern Iberian whales from the remaining sampled regions, at MHC level, due to proximity to the Mediterranean Sea. A lethal morbillivirus infection of long-finned pilot whales, thought to be caused by a different morbillivirus strain from the one previously described as causing pilot whale morbillivirus (Duignan *et al,* 1992; Taubenberger *et al.,* 2000) occurred in the Mediterranean. It may be the case that the different possible strains of morbillivirus found in pilot whales worldwide may be acting as a trigger for selection to improve individual fitness and confer stronger pathogen resistance. Nevertheless, further studies based on the analysis of this disease in other populations should be performed in order to provide more robust insights about this theory. It is important to note that, although the occurrence of local selection pressures may be an explanation for the structural patterns presented by MHC, it still has to be considered that the structural patterns observed at adaptive loci in pilot whale populations may be due to the same forces that drive neutral markers variation, as migration or genetic drift may have an important role in shaping contemporary population genetic structure and diversity or may be masking contemporary signatures of selection at North Atlantic pilot whale population diversity and structure.

Previous evidence either for or against genetic population substructure in the North Atlantic is scarce. A study based on the mitochondrial control region of 70 pilot whales from Northeast USA, Nova Scotia, Newfoundland and United Kingdom found no evidence of

population structure between the Western and Eastern basins of the North Atlantic, it being suggested that the low genetic variability could be due to a recent origin of the North Atlantic population or to the strong matrilineal structure (Siemann, 1994). Neverthless, a nuclear marker study, based on eight highly polymorphic microsatellite loci, which analysed samples from the East Coast of USA (Cape Cod), West Greenland, the Faroe Islands and the UK, indicated the occurrence of substructure, particularly pronounced between West Greenland and other sites. However, the magnitudes of the various pairwise comparisons did not support a simple isolation-by-distance model, it being suggested instead, that population isolation occurs between areas of the ocean which differ in sea surface temperature (Fullard *et al*., 2000).

In agreement with the findings of Siemann (1994), in the present study, UK and Northeast USA did not seem to show the occurrence of population differentiation, at mitochondrial DNA. Additionally, the AMOVA showed that most of the variance occurred within rather than between oceanic basins. The signs of population differentiation found at mitochondrial DNA may be due to the inclusion of previously unstudied areas. Several studies have already mentioned that population structure may sometimes remain undetected due to sampling limitations associated with opportunistic schemes used for collection of cetacean samples (such as strandings), that may prevent the analysis of individuals from genetically distinct populations (Evans & Teilmann, 2009; Mirimin *et al*., 2009). Regarding the microsatellite analysis performed by Fullard *et al*. (2000), the differences between those results and the ones found in the neutral marker (mitochondrial DNA) analysed in the present study may either be due to the different levels of mutation rates exhibited by both type of markers (Selkoe *et al.,* 2006) and/or due to the four-fold smaller efective population size of mitochondrial DNA comparatively with nuclear autosomal genes with biparental transmission (Moore, 1995), which makes it a more sensitive detector of population subdivision, by random genetic drift (Wilson *et al.,* 1985; Balloux *et al*., 2000). Furthermore, the social structure presented by pilot whales may also result in higher levels of divergence at this maternally inherited marker. Therefore, the present study provided new insights about previously unsampled regions of the North Atlantic and about adaptive diversity that was not previously studied in this region.

In conclusion, both mitochondrial and MHC analyses indicate the occurrence of genetic substructure in pilot whales in the North Atlantic. Mitochondrial DNA shows the occurrence

of regional and oceanic differences among populations, while MHC analysis shows Iberian whales as a separate group when compared with other sampled regions. The most likely explanation for the levels of genetic diversity and differentiation shown by mitochondrial DNA seems to be associated to the social organization of this species, with high levels of female phylopatry and short-term movements of the males for reproduction. For the MHC loci, the occurrence of historical balancing selection was evident (especially in the DQB locus), as showed by the trans-specific allele sharing and the d_N/d_S ratio. However, although historically it seems that balancing selection had an important role in shaping population diversity, the spatial patterns of contemporary diversity across the North Atlantic could be attributable to local selection pressures for specific pathogens/parasites or evolutionary forces such as gene flow and/or drift.

Acknowledgements

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Chapter V

Assessing pilot whale (*Globicephala* sp.) distribution around Atlantic and Cantabric coasts of Iberia, using presence-only methods.

Silvia Monteiro; Pedro Rodrigues; Alfredo López; Graham J. Pierce; Marisa Ferreira; Jorge Bastos; Helder Araújo; Angela Llavona; Begoña Santos; Joana Andrade & José V. Vingada

Abstract

The geographical and ecological distribution range and the availability of suitable habitats are important points to be considered for conservation and management strategies of wildlife species. The main objectives of the present study were to identify ecogeographical variables (EGVs) that may influence pilot whale (*Globicephala* sp.) distribution and at the same time discover suitable areas for this species along the Atlantic and Cantabrian coast of Iberia. Sightings of pilot whales were recorded from April to September, from 2007 to 2012, using different types of platforms. These records were then analysed together with information relative to six EGVs, namely depth, slope, sea surface temperature (SST), chlorophyll a (Chl *a*) and gradients of SST and Chl *a*, using two presence-only analyses: Principal Component Analysis (PCA) and Maximum entropy model (Maxent). Both methodologies identified depth and SST gradient as the most important variables for the ecological niche of pilot whales. SST was also an important variable defined by PCA, although Maxent model included it as a variable of minor importance. Higher habitat suitability occurred in locations with shallower waters, with higher values of SST gradient (although PCA showed the opposite result) and SST values between 15 and 17°C. These results may indicate that pilot whales undertake incursions into neritic foraging waters that may be related with a high concentration of Octopodidae spawners in these areas, due to the link between the reproductive season of these prey species and coastal upwelling during summer months (April-September) in Atlantic Iberia. However, the present study also highlights the importance of thinking carefully about the meaning of findings at different temporal scales, as well as evidencing the importance of using fine temporal scale, in marine environments.

Introduction

Along with the understanding of the biological characteristics of wild species, is also important to consider the geographical and ecological distribution range and the availability of suitable areas for those species, in conservation and management strategies. Species distribution modelling (SDM) has been widely used as an effective tool for spatial and conservation ecology, in order to understand the links between species movements or abundance patterns and environmental (topographic, climatic) or anthropogenic characteristics (Macleod *et al.,* 2008).

Several SDM techniques have been developed to infer species' environmental requirements, from conditions at locations of known occurrences, enabling distribution predictions in areas where no biological information is currently available. Distribution modelling has been extensively used in marine habitats, namely with fishes (Chatfield *et al.,* 2010; Monk *et al.,* 2010, 2012; Huff *et al.,* 2012; Mckinney *et al.,* 2012; Sequeira *et al.,* 2012), corals (Tittensor *et al.*, 2009; Williams *et al.,* 2010), crustaceans (Compton *et al.,* 2010), and cetaceans (Cañadas *et al.,* 2005; Macleod *et al.,* 2008; Praca & Gannier, 2008; De Stephanis *et al.,*2008a; Praca *et al.,* 2009; Pierce *et al.,* 2010a; Whitehead *et al.,* 2010; Gill *et al.,* 2011; Gregr, 2011; Best *et al.,* 2012; Moura *et al.,* 2012).

Species distribution modelling is generally based on two types of methodologies: presence-absence (PA, such as GAM, GLM, zero-inflated models) or presence-only (PO, such as PCA, ENFA, GARP, MAXENT) models. The former requires data on the distribution of survey effort so that, even if absence was not explicitly recorded, it can be inferred, while the latter only requires the occurrence data (Macleod *et al.*, 2008).

Several authors argue that presence-absence methods should be preferred over presence-only techniques to predict species distribution, when absence data are available (Brotons *et al.,* 2004; Macleod *et al.,* 2008; Elith *et al.,* 2011). However, another consideration is that it is not always possible to have accurate absence data for cetaceans, due to either logistic and/or ecological constraints (Macleod *et al.,* 2008). First of all, dedicated cetacean surveys are usually time-consuming and require significant financial and logistic resources, which may limit their spatial and temporal extent (Evans & Hammond, 2004; Macleod *et al.,* 2008), and can lead to the frequent use of opportunistic observations for which effort data are unavailable or for which effort is difficult to measure. Furthermore, the high mobility associated with conspicuous or deep-diving behaviours presented by many cetaceans (Baird *et al.,* 2002) may hamper the detection of these species at sea, implying a potential "false absence" if a PA approach is used, one of the major assumptions when applying PA methods to distribution studies.

Some potential solutions for the "false absence" limitation are available: firstly, if the heterogeneity in the probability of detection is evaluated on the survey stage and associated with abundance or occupancy modeling approaches that account for imperfect detection (Buckland *et al.*, 2001; Mackenzie *et al.,* 2002; Cañadas *et al*., 2004; Mackenzie, 2005; Martin *et al.,* 2005 and references therein; Rota *et al.,* 2011; Zuur *et al.,* 2012), then there is less

chance to result in spatially biased predictions (Mackenzie *et al*., 2002; Mackenzie, 2005; Rota *et al.,* 2011). However, there is still some controversy around this subject (Manel *et al*., 2001; McPherson *et al*., 2007; Santika*,* 2011; Welsh *et al.,* 2013). Secondly, if relative abundance is modeled instead of absolute abundance or occurrence, then a non-zero probability of false absences is not an issue, although it can only be applied if detectability is always constant (Mackenzie, 2005). An additional alternative is to use presence only methods, especially if no effort data are available (Brotons *et al.*, 2004).

Presence-only modelling appears to be an alternative technique to address some constraints related to the application of PA methods to marine environments, namely the uncertainty in determining absences that can lead to potentially biased model predictions (Hirzel *et al.,* 2001; Pearson *et al.,* 2007). Furthermore, PO analysis allows the use of sightings data that were opportunistically collected from a wide range of sources (i.e. data *not* collected from dedicated effort-based surveys at sea). Finally, several studies of marine organisms have shown that PO techniques can produce models of habitat suitability significantly better than random and which can exhibit comparable performances to PA approaches (Macleod *et al.,* 2008; Tittensor *et al.,* 2009). However, presence-only methods also present disadvantages. Firstly, the proportion of sampled sites where a species is present (prevalence, Santika, 2011) is not identifiable from presence-only data (Ward *et al*., 2009). However, as mentioned above, absence data may also present several problems related with the detection probability (Manel *et al.,* 2001; McPherson *et al.,* 2007; Santika, 2011), so that even presence-absence data may not yield a good estimate of prevalence (Elith *et al.*, 2011). Secondly, another major implication of PO models is related with the potential influence of sample selection bias (whereby some areas in the landscape are sampled more intensively than others), resulting from non-random distribution of survey effort (Macleod *et al*., 2008; Phillips *et al.,* 2009; Elith *et al.,* 2011). Sample selection bias has a much stronger effect on PO models than on PA models (Phillips *et al*., 2009; Elith *et al*., 2011).

Pilot whales (*Globicephala* sp.) are one of the largest odontocetes inhabiting the Atlantic Ocean. This genus includes two species: long-finned pilot whales (*Globicephala melas*) and short-finned pilot whales (*Globicephala macrorhyncus*). These have parapatric distribution ranges, with *G. melas* occurring in cold-temperate and subpolar waters and G. *macrorhyncus* inhabiting tropical warm-temperature waters (Reid *et al*., 2003). Animals from

this genus seem to prefer deep waters, near the continental shelf or over deep submarine canyons (Carwardine, 1995), with depths ranging between 100-3000m, with some level of preference for slope regions (Payne & Heineman, 1993; Cañadas *et al.,* 2002, 2005; Hamazaki*,* 2002; Reid *et al,* 2003; Macleod *et al.,* 2007; Kiszka *et al.,* 2007; De Stephanis *et al.,* 2008a; Silva *et al*., 2013). Previous studies along the Galician coast showed that pilot whales were observed in deep waters (170m to 900m, López *et al.,* 2004; Spyrakos *et al.,* 2011; Fernández *et al.,* 2013), although dietary evidence suggests that this species sometimes forages in coastal waters, taking more coastal cephalopod species (Santos *et al.,* 2013). In addition, observations of pilot whales from coastal observation points are documented (Pierce *et al.,* 2010a). Apart from the link with topographical variables, oceanographic dynamic variables such as sea surface temperature (SST) or chlorophyll *a* (Chl *a*) also seem to be related with the distribution of these cetacean species. Pilot whale presence in the North Atlantic seems to be associated with areas with higher concentrations of Chl *a* and, especially, low sea temperatures (Fullard *et al.,* 2000; Macleod *et al.,* 2007; Doksæter *et al.,* 2008; Fernández *et al.,* 2013). Several studies have proposed that the habitat preferences and movements exhibited by this species are dependent or related to prey distribution (Payne & Heinemann 1993; Jákupsstovu, 2002; Doksæter *et al.,* 2008; De Stephanis *et al.,* 2008a; Santos *et al.,* 2013).

In the present study, to model pilot whale occurrence along the Iberian coast six ecogeographic variables (EGV), either known or suspected to be correlated with pilot whale distribution will be used (López *et al.,* 2004; Macleod *et al.,* 2007; Spyrakos *et al.,* 2011; Fernandez *et al.,* 2013), namely mean depth (m), seabed slope (degrees) and mean values and spatial gradients of Sea Surface Temperature (SST, °C) and Chlorophyll *a* (Chl *a*, mg/m^3). Some distribution studies of marine mammals used SST and Chl *a* as a proxy for primary production or prey distribution (Canãdas *et al.,* 2005; Macleod *et al.,* 2007; Panigada *et al.,* 2008; Torres *et al.,* 2008; Gilles *et al*., 2011). It is still unclear how do dynamic variables, such as SST and Chl *a*, directly or indirectly affect cetaceans (Polovina et al., 2001; Whitehead et al., 2010; Thompson et al., 2012), however a study that related bottlenose dolphin distribution with the actual prey distribution or with ecogeographic variables that are normally used as proxy for prey distribution (including SST and Chl *a*), indicated that due to high habitat heterogeneity, the spatial variability of prey patches and the difficulty of having data on prey distribution, fine-scale models of dolphin habitat selection will be more

successful if environmental variables are used as predictor variables of predator distributions rather than relying on prey data as explanatory variable (Torres *et al.,* 2008). Furthermore, both SST and Chl *a* were included in the present study, along with the respective gradients, because they can capture general phenomena which are indicative of the productivity in the study area, such as upwelling (Figueiras *et al.,* 2002; Cañadas et al., 2005; Moreno *et al.,* 2009; Alvarez *et al.,* 2012; Picado *et al.*, 2013).

Several studies have investigated the occurrence of oceanic fronts in upwelling systems (Castelao *et al*., 2006; Kahru *et al*., 2012). Oceanic fronts are regions of sharp spatial gradients of ocean properties between adjacent waters and occur at a wide range of spatial and temporal scales (Kahru *et al.*, 2012). Fronts are indicators of many oceanographic processes and often mark dynamically active regions in the ocean, either horizontally (due to ocean currents and wind forcings) and/or vertically (Park *et al*., 1999). They are often associated with biologically active regions that affect all oceanic life forms from microbes to seabirds and marine mammals (Polovina *et al*., 2001; Tynan *et al.,* 2005; Etnoyer *et al.,* 2006; Doniol-Valcroze *et al.,* 2007; Gannier *et al*., 2006; Bost *et al*., 2009; Dragon *et al*., 2010; Scott *et al.,* 2010; Louzao *et al*., 2011). Several studies already analysed the influence of oceanic fronts in the distribution of several cetacean species of the mid-western North Atlantic (Hamazaki*,* 2002) and Pacific oceans (Tynan *et al.,* 2005), blue whales off the Baja California Peninsula (Mexico, Etnoyer *et al*., 2006), rorqual species in Canadian waters (Doniol-Valcroze *et al*., 2007) and several cetacean species in the North Sea (Scott *et al.,* 2010).

Little information is available about the distribution of pilot whales along the Atlantic and Cantabrian coasts of Iberian Peninsula (to which we refer herein as Atlantic Iberia), especially along the Portuguese coast. Most available data do not arise from dedicated surveys. Hence, in the present study, presence-only methods were applied (Principal Component Analysis (PCA) and the Maximum Entropy approach) in order to: a) identify which environmental variables influence pilot whales' distribution and define the ecological niche of these species, in this area and b) identify suitable geographical areas that satisfy the environmental demands of pilot whales along the Atlantic coast of the Iberian Peninsula.

Methodology

Study Area

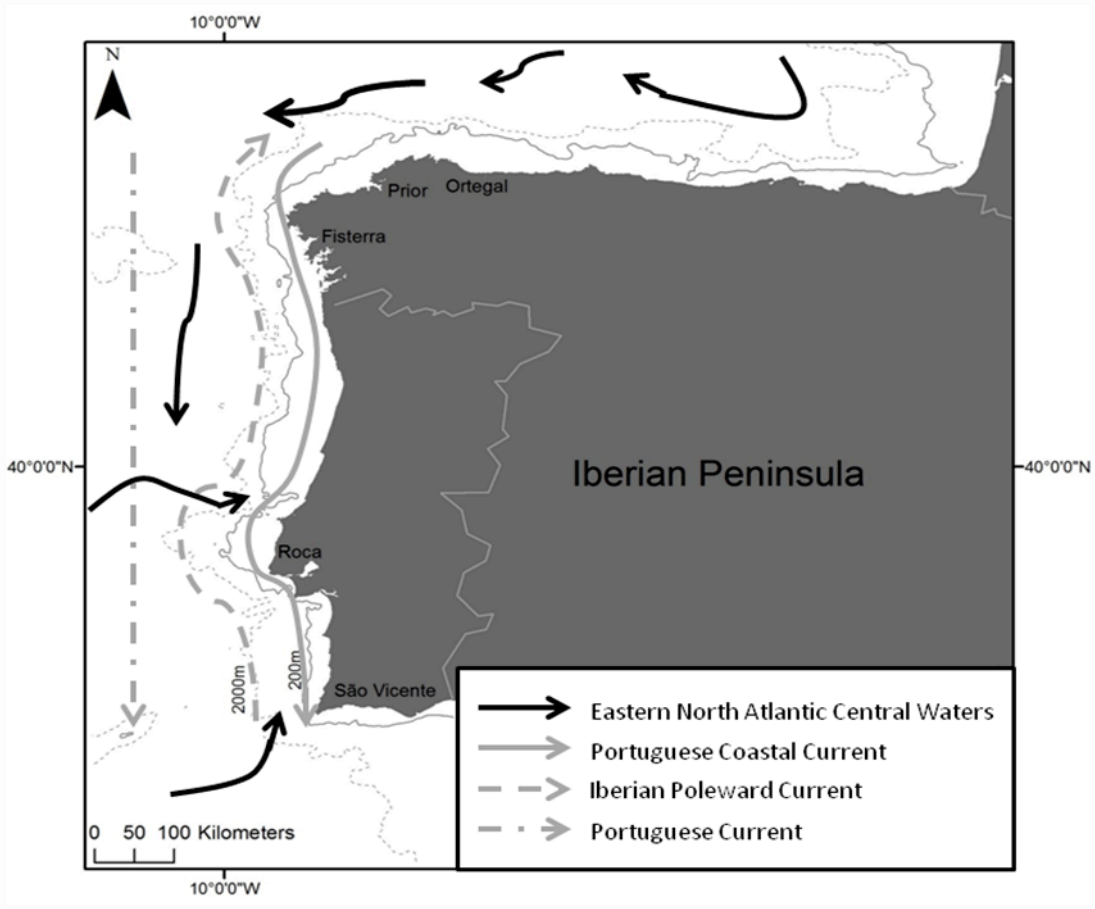


Figure 5.1. Map of study area. The isobaths of 200m and 2000m, as well as main capes and main ocean currents present in the study area are shown (adapted from Hernández-Molina *et al*., 2011).

The study area comprises three regions of the Iberian coast: the North Iberian Peninsula (NIP), the Western Iberian Peninsula (WIP) and the South Atlantic coast of the Iberian Peninsula (SIP), corresponding to the south coast of Portugal (see Figure 5.1).

The entire extent of the study area is characterized topographically by a narrow shelf that is, on average, 45km wide and 100-200 m deep. In relation to oceanographic dynamics, Iberia is situated on the northern limit of the NW Africa upwelling system where the interaction of along-shore winds with the coastal topography produces an upwelling-downwelling seasonal system (Figueiras *et al*., 2002). During summer, prevalent northerly winds favour the transport of Eastern North Atlantic Central Waters (ENACW) of subpolar origin, close to the Iberian coast (probably with the help of the southward flow of the

Portuguese Current (PC, Prieto *et al*., 2013)), where upwelling events cause them to reach the surface (Alvarez *et al.,* 2012). These upwelled waters are generally cold and characterized by high concentrations of nutrients that enhance primary production and consequently increase the concentration of chlorophyll *a* (Chl *a*) and the levels of biodiversity in the area (Figueiras *et al.,* 2002; Moreno *et al.,* 2009; Alvarez *et al.,* 2012; Picado *et al.*, 2013). The upwelling-downwelling pattern shows inter-annual and spatial variations across the Iberian Peninsula. Upwelling phenomena seem to occur mainly from April to September (Figueiras *et al.,* 2002; Moreno *et al.,* 2009; Alvarez *et al.*, 2012), although a shorter period seems to occur on the NIP, when compared to other regions (June to August; Alvarez *et al.*, 2010). In general, there seems to be an influence of coastline orientation on upwelling events, since the WIP shows a higher probability of occurrence of upwelling processes than the NIP and the SIP (Relvas & Barton, 2002; Moreno *et al.,* 2009; Alvarez *et al.,* 2010, 2012: Prego *et al.*, 2012). Additionally, upwelling phenomena are generally stronger and more persistent in the former region than in the other two areas of the Iberia (Relvas & Barton, 2002; Moreno *et al.,* 2009; Alvarez *et al.,* 2010, 2012; Prego *et al.*, 2012).

In contrast to the upwelling phenomena, from September-October until February-April the surface circulation reverses, with the Iberian Poleward Current (IPC) being intensified, driven by prevalent southerly winds that bring ENACW of tropical origin from 39°N to 47°N, resulting in a downwelling process (Figueiras *et al.,* 2002).

Occurrence data

In the present study, due to the difficulty of distinguishing these species at the sea, along with a possible distribution range overlap over the study area (confirmed by strandings of both species in Portugal and Galicia; López *et al*., 2002, 2004; pers.com.: Marisa Ferreira) sightings are assumed to be from pilot whales (*Globicephala* sp.).

Analyses were performed using sightings recorded in the study area, from April to September (hereafter referred to as summer months) from 2007 to 2012. Pilot whale sightings were collected from different types of platforms: opportunistic data collection onboard fishing and oceanographic vessels, and dedicated cetacean surveys on boats and plane and from coastal observation points. It is important to mention that the dataset used

here partially overlaps with that used by Fernández *et al.* (2013) but included a wider range of data sources.

Opportunistic surveys onboard fishing and oceanographic vessels

Trained marine mammal observers of CEMMA (Coordinadora para o Estudio dos Mamíferos Mariños) and SPVS (Sociedade Portuguesa de Vida Selvagem) accompanied commercial fishing vessels in Galicia (2007-2009, n=122 survey days) and along the North/Centre Portuguese Coast (2007-2012, n=874 survey days). Survey routes were determined by the primary activities of the boats. One observer was onboard during each sampled trip, recording data on the presence of cetaceans and on environmental conditions. Marine mammal observations were carried out continuously except when catch compositions were being recorded or weather conditions were unsuitable. For further details on the protocol followed see López *et al.* (2004).

Fish surveys carrying marine mammal observers were conducted by the Instituto Español de Oceanografía, in Spain (March-April, 2007-2012, n=111 survey days) and by Instituto Português do Mar e da Atmosfera, in Portugal (March-April, 2008-2012, n=75 survey days), as part of the annual survey series to monitor and study the distribution and abundance of small pelagic fish resources of the study area, using acoustic methods. A series of transects perpendicular to the coast and spaced approximately 8 nm apart were followed, covering the continental shelf from South of Portugal to the Cantabrian Sea (North of Spain). Exceptionally, areas of the continental slope were also surveyed. In Spain, data were collected by two observes while in Portugal there were one observer and one data recorder. For further details on the protocol followed, see Riveiro Alarcón & Vazquez (2011).

Dedicated cetacean surveys

Boat-based surveys have been performed periodically over the Galician shelf by CEMMA since 2003. The present study includes data recorded from April to September, between 2007 and 2010 (n=65 survey days). Additionally, two surveys were performed in offshore waters: one in Galicia (minimum distance from shore: 100nm, September 2007, CEMMA) and another along the Portuguese coast (between 50 and 250nm from the coast, August 2011, SPVS). Sightings recorded within the study area (maximum 60 nm from the coast), were included in this analysis. At least two experienced observers were working at all

times, recording data on cetacean presence and environmental conditions. For further details on the Portuguese survey, see Santos *et al*. (2012).

Systematic monthly coastal surveys for cetaceans were carried out in Galicia (CEMMA, 2003-2012) and in the North/Centre region of Portugal (2008-2012, SPVS). In each area, a series of 30 sites spread approximately evenly along the two regions was used as observation stations. Detailed methodology is described in Pierce *et al.* (2010a).

From 2010 to 2012, dedicated annual aerial surveys were performed by the University of Minho/SPVS, in September, along the Portuguese Continental Coast (from Caminha to Vila Real de Santo António), to Fisterra in Galicia. The surveys were conducted using the line transect sampling method under a systematic sampling scheme, with perpendicular 50 nm transects and with 10 nm spacing between transect lines. The survey team included 2 cetacean observers and 2 data recorders. For further details see Santos *et al*. (2012).

In all cases, sightings were recorded by experienced observers. The basic methodology used in the different types of surveys described above usually consisted of two dedicated observers working simultaneously, scanning the horizon in search of cetacean, together covering a field of view of 180° centered on the direction of travel (each observer covers 90°, except in cases were only one observer was present, such as the opportunistic surveys onboard commercial fishing vessels). The information collected for each cetacean sighting included: species, GPS location of the boat (proxy for location of the animal), distance and horizontal or vertical angle to the animal, number of individuals, number of calves and behaviour (Evans & Hammond, 2004). Generally, observers also recorded information on environmental conditions. Cetacean sighting records during all the above mentioned surveys were accessible for analysis, although information on effort was only available for all the surveys, except the opportunistic surveys onboard commercial fishing vessels (which included, approximately, 50% of the sightings analysed).

Ecogeographic Variables

To model pilot whale occurrence along the Iberian coast, in the summer, from 2007 to 2012, six ecogeographic variables (EGV), either known or suspected to be correlated with pilot whale distribution were used (López *et al.,* 2004; Macleod *et al.,* 2007; Spyrakos *et al.,* 2011; Fernández *et al.,* 2013). Topographical variables were mean depth (m) and seabed

slope (degrees), while dynamic oceanographic variables were mean values and spatial gradients of Sea Surface Temperature (SST, °C) and Chlorophyll *a* (Chl *a*, mg/m^3). All these variables were analysed with a spatial resolution of 9 Km, based on the available resolution of dynamic variables and because it is believed that it will incorporate adequate information for the highly mobile pilot whale.

Initially, bathymetry data was extracted at approximately 1.8 Km resolution using the One Arc-*Minute Global Relief Model* of ETOPO1 (Amante & Eakins, 2009). Mean depth and slope were then calculated for each 9 Km grid cell. Slope was calculated using a Custom Transverse Mercator coordinate system, centered for the study area.

SST and Chl *a* data were extracted with a 9 km resolution, as monthly daytime averages from Aqua-MODIS (http://oceancolor.gsfc.nasa.gov/cgi/l3), from 2007 to 2012. Dynamic variables spatial gradients indicate the presence of frontal systems and were calculated, over a 3x3 grid cell moving window, as described in Louzao *et al.*, (2011):

$$\text{Spatial Gradient} = \frac{(\text{maximum value} - \text{minimum value}) \text{ x } 100}{\text{maximum value}}$$

It is important to mention that the analysis of the gradients of SST and Chl *a* were chosen over the analysis of SST and Chl *a* standard deviation due to the high collinearity presented between mean and standard deviation of both SST and Chl *a* (Pearson's r>0.7).

Data analysis

A suitable habitat for a species may result from the interaction of several ecogeographic variables, meaning that a species may only occur at a determined location when the combination of individual EGVs occurs (Fernández *et al.,* 2013). Initially it is important to know which variables influence the definition of the ecological niche of pilot whales. Several studies used Principal Component Analysis (PCA) to achieve that purpose (Macleod *et al.,* 2008; Praca *et al.,* 2009; Weir *et al.,* 2012; Férnandez *et al.,* 2013). Secondly, it would be interesting to identify suitable habitats for *Globicephala* sp., based on the ecological requisites of these species. Several modelling methods are capable of identifying suitable habitats for wild species. The maximum entropy approach as implemented in Maxent (Phillips *et al.,* 2006; Phillips & Dudik, 2008), has recently been extensively used, since it has been shown to provide comparable results to presence-absence and outperform

other presence-only techniques (Brotons *et al.,* 2004; Elith *et al.,* 2006, 2011; Phillips *et al.,* 2006; Wisz *et al.,* 2008).

The use of a multi-year average situation, instead of a smaller temporal scale, was needed in Maxent, because a compilation of pilot whale presence data across all years was required to have sufficient data. Since other PO methods would also require an across-year compilation of the EGVs, in order to detect potential seasonal variations, the Principal Component Analysis was used. Therefore, data were analysed at two levels of temporal resolution, namely: a) the whole period study (2007-2012), as implemented in Maxent, which provides a coarse-scale view of distribution, without the possibility to examine temporal trends; b) monthly averages, as implemented in Principal Component Analysis, in order to identify the environmental variables that seem to influence pilot whale distribution, using a fine time-scale. While the multi-year composite will not provide fine-scale temporal information about habitat use (such as the information provided by PCA analysis), it will provide information on pilot whale distribution in relation to longer-term local oceanographic processes, which may also affect habitat preferences.

Principal Component Analysis (PCA)

PCA summarises all explanatory EGVs into a few orthogonal principal components (PC). Each PC has an associated eigenvalue that represents the amount of variation explained by that axis (Zuur *et al.,* 2007). For the present study, the selection of the most important PCs to be presented was based on the "Kaiser-Guttman criterion", where PCs whose eigenvalues that are larger than the mean of eigenvalues are analysed (Legendre & Legendre, 2012). Limitations of PCA are mainly related with the fact that PCA measures linear relationships between variables for each PC (Zuur *et al.,* 2007), while most relationships in ecological research are non-linear. However, this limitation may be overcome by the use of several PCs.

When applied to spatial distribution, this method requires only presence data and continuos environmental variables (Brocard et al., 2011). For this analysis, EGV values were extracted for each pilot whale sightings considering the smallest temporal scale for dynamic variables (corresponding month of the observation). Then, both the sightings data and the environmental variables were analysed in a correlation biplot of Principal Component Analysis. All EGVs were standardized to mean = 0 and SD =1 to avoid scale effects. All

calculations were performed using the package *vegan* (Oksanen *et al.,* 2011) implemented in R v.2.9.1 (R Development Core Team, 2011).

Maximum Entropy (MAXENT)

MAXENT is a maximum entropy-based machine learning software that attempts to find the most uniform distribution of habitat suitability (i.e. maximum entropy) subject to a set of constraints that represent our incomplete information about the target species distribution, over the entire study area. Those constraints are a complex suite of transformations on the environmental variables believed to be important to the target species (Phillips *et al.,* 2006). This method requires only presence data and environmental predictor variables (continuous or categorical) (Phillips *et al.,* 2006). All calculations were performed in MAXENT 3.3.1 (http://www.cs.princeton.edu/~schapire/maxent/; Phillips *et al.,* 2006).

Model building

First, tests were performed to select the best regularization parameter (β or LASSO penalty) in MAXENT. This parameter constrains modelled distributions to lie within a certain interval around the empirical data mean, in order to avoid overfitting the input data (Elith *et al.,* 2011; Warren & Siefert, 2011; Merow *et al.,* 2013). Different values of β were tested (β = 1(default), 1.5, 2 and 5), with the same settings described below. The best model was selected based on the analysis of model fit metrics described below.

Settings used for model training were: logistic output, duplicates removal, convergence threshold set to 0.00001, 500 maximum iterations, hinge features, regularization multiplier set to 1.5 and 10000 background points. The logistic output (logistic model) is a transformation of the raw output (exponential model) of Maxent that makes certain assumptions about prevalence and sampling effort (Phillips & Dudik, 2008; Elith *et al.,* 2011). These two output types (raw and logistic) are monotonically related, so if the purpose of a study is to rank sites according to suitability, both will yield identical ranking and hence identical rank-based measures (e.g., AUC values) (Elith *et al*., 2011). However, it is not intuitive to work with raw values (Phillips & Dudik, 2008), hence using logistic output make it easier to conceptualize Maxent results. The features represent a set of transformations of the original predictors used in Maxent. A model using hinge features fits

a piecewise linear response and is recommended when the number of samples exceed 15 occurrences, in order to avoid overly-complex model fits (Elith *et al*., 2010; 2011).

Model predictor importances (normalized to percentage) were assessed, indicating the influence of each covariate on the final model. A jackknife procedure systematically removes each variable, creating a model with the remaining variables, in addition to a model with each variable in isolation, measuring the decrease in AUC (defined below) and regularized gain along the process. Regularized gain is similar to deviance in GLM, a statistic that measures how well a variable distinguishes occurrences from the total area under study (Monk *et al*., 2010). Variables with importance values lower than 1% that, at the same time, caused a low decrease of the regularized gain when omitted, were removed from the model.

To assess how the variation of the predictors influenced habitat suitability, response curves were used. These curves show how the probability of habitat being suitable changes as each environmental predictor is varied, while keeping remaining predictors at their average value (marginal response curves). Additionally, curves are also produced for models containing each variable in isolation, which are easier to interpret when strong correlation between variables occurs. All the variables were tested for collinearity, prior to MAXENT modelling, by examining correlations (Pearson correlation coefficient, r), using ENMTools 1.3 (Warren *et al.,* 2010) and by analyzing the PCA correlation biplot. Due to high levels of collinearity with GrSST (Pearson's r≥0.70), Grchla gradient was excluded from the posterior analysis.

Testing or validation is required to assess the predictive performance of spatial distribution models (SDM) (Pearson *et al.,* 2007). The validation technique used was to subsample 25% of the dataset, over 10 replicates, to be used as test data for model validation, with the remaining 75% used as training data.

Model evaluation

Several lines of evidence were considered in evaluating the best model (Pearson *et al.,* 2007; Baldwin, 2009; Elith & Graham, 2009; Elith *et al.,* 2011; Merow *et al.,* 2013): 1) the threshold independent metric: area under the Receiver Operating Characteristic (ROC) curve (AUC) and 2) the threshold-dependent metrics like omission rates (false absences, i.e. predicted as absent when in reality there was a recorded sighting).

The AUC provides the probability that the model correctly ranks a set of random presence site vs. a random background (Phillips *et al.,* 2009). It ranges between 0.5 (randomness) and 1 (perfect discrimination). Omission rate values (models with lowest values of omission rate are selected) along with significant results from a binomial test that determines whether a model predicts a test locality better than random (Phillips *et al.,* 2006) can be also used as model performance measure.

For validation of SDMs and to obtain the omission rates, reclassification of habitat suitability maps into binary maps (suitable/unsuitable) by setting a decision threshold is a widely used method in wildlife applications (Lobo *et al.,* 2008). As highlighted by Phillips *et al.* (2006), determining the optimal threshold still remains little explored, when using Maxent. Hence, two alternative thresholds were applied in the present study: the "minimum training presence" (MTP) and "10 percentile training presence" (P10) thresholds. The former can be explained ecologically as identifying grid cells predicted as being at least as suitable as those where pilot whale presence occurred (conservative approach). The latter assumes that 10% of the occurrence data may suffer from errors or lack of resolution (liberal approach), being appropriate for datasets collected over long time periods (Rebelo & Jones, 2010).

Results

A total of 59 pilot whale sightings was registered in Atlantic Iberia, in summer months from 2007 to 2012. Around 46% and 20% of the sightings occurred in April and September, respectively. Pilot whales occurred at locations where the average depth was 846m, where the seabed slope was shallow (approximately 2°). Dynamic variables at pilot whale locations presented mean values of 16.38°C and 1mg/m^3 for SST and Chl *a*, respectively, with 54% and 60.9% of the sightings occurring at lower than 17°C (SST) and 1mg/m^3 (Chl *a*). However, these average characteristics do not tell the whole story: 52% of the sightings took place over the continental shelf (i.e. in waters of less than 200m depth) and occurrence was recorded over wide ranges of depth, slope, SST and Chl *a* (table 5.1).

Table 5.1. Mean and range values of EGV at pilot whales sightings, in summer months from 2007 to 2012 (n=59).

Depth (m)		Slope (°)		SST (°C)		Chl *a* (mg/m^3)	
mean	range	mean	range	mean	range	mean	range
-846.10	-9 - -4739	2.13	0.13 – 7.63	16.38	12.50 – 23.09	1.00	0.07 – 7.54

PCA

The EGVs used in the PCA were depth, slope, mean SST, mean Chl *a,* SST gradient (GrSST) and Chl *a* gradient (GrChl *a*). The two first axes of the PCA explained 64.5% of total variation, with PC1 and PC2 accounting for 42.3% and 22.3%, respectively.

Table 5.2. PCA results for pilot whales sightings. Coefficients for each EGV and eigenvalues are presented for the first two principal components.

	PC1	PC2
Depth	-1.458	0.422
Slope	1.104	-0.720
SST	1.173	-0.704
GrSST	-0.548	-1.446
Chl *a*	-1.193	-0.334
GrChl *a*	-1.202	-0.869
Eigenvalue	2.538	1.335
Proportion of variation explained	0.423	0.222
Accumulated variation explained	0.423	0.645

The correlation biplot presented in figure 5.2 shows that variation in PC1 is related to water depth and SST (table 5.2), with most animals showing a preference for shallower waters (or lower concentrations of Chl *a*, since some level of correlation seems to occur), reflecting the occurrence of a more coastal habitat preference. There was also a preference for colder waters. However, the reverse is also found for some animals (as may be expected since the arithmetic mean depth of waters where sightings occurred was 846m). PC2 is mainly related with GrSST (table 5.2) with most animals showing a preference for locations with a shallower gradient of SST (or Grchla, since some level of correlation seems to occur). However, the contrary situation was also found for some individuals.

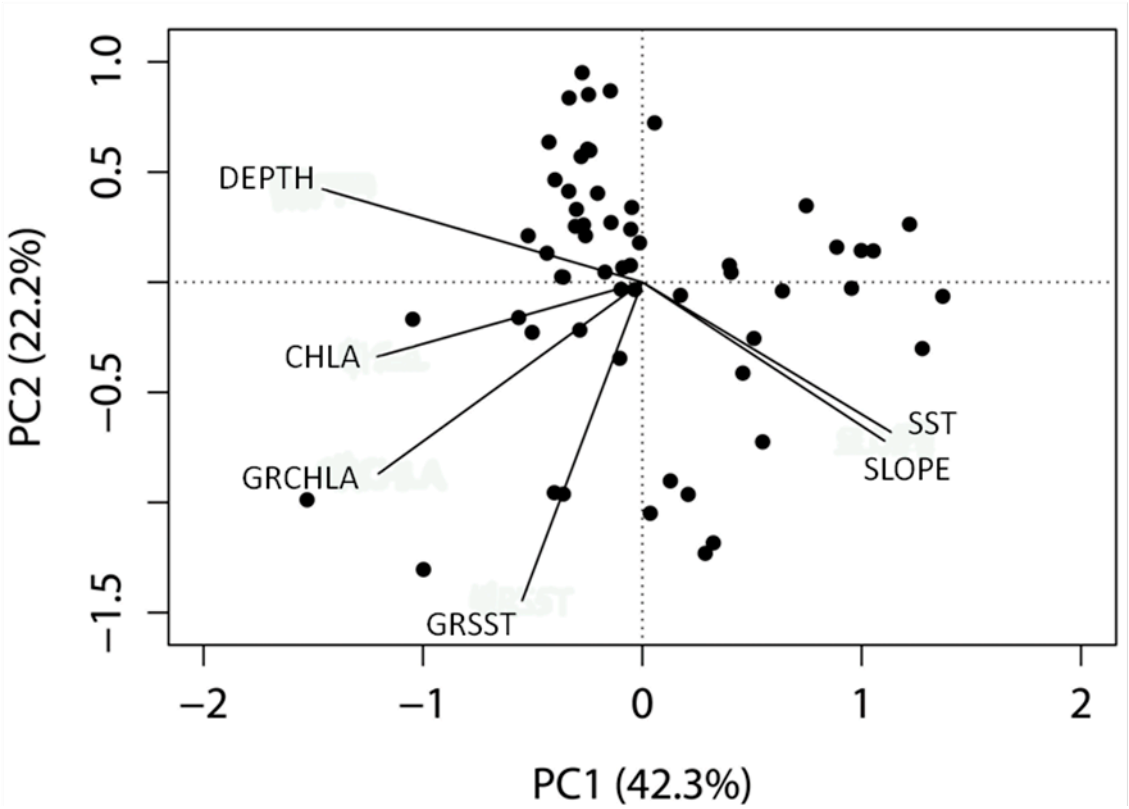


Figure 5.2. PCA correlation biplot for pilot whale sightings. Black dots represent pilot whale observations. Note: Depth is expressed as negative values (zero equals the surface of the water).

MAXENT

Model evaluation

The Maxent model developed to analyze the distribution of pilot whales in Iberia during summer months, considering a 6-year period (2007-2012), discriminated suitable habitats for this species satisfactorily. Average area under the curve (AUC) values for all replicates of both training and test data were above 0.8, considered to be a good model performance (Table 5.3). Omission rates (false absences) for test data ranged between 2% and 6%. A binomial test of omission for the null hypothesis that test points are predicted no better than random produced one-sided p-values <0.05, providing further validation of model reliability.

Table 5.3. Model evaluation metrics.

Training AUC	0.8568
Test AUC	0.8679
Regularized gain	0.6743
Omission rates MTP	0.0214 (0.002)
Omission rates P10	0.0571 (0.000)

MTP: minimum training presence threshold; P10: 10 percentile training presence threshold; p-values are described within brackets.

Environmental predictor importances

Depth was the most important predictor influencing pilot whale summer distribution in the Atlantic coast of Iberia, when considering the total range of years analysed (2007-2012: 49.2%), while the second most important EGV was GrSST (45.8%) (Figure 5.3). The remaining variables – slope and SST - showed less than 5% importance (Figure 5.3).Depth was one of the most important predictors among the variables analysed and the depth curve showed that habitat suitability for pilot whales was superior to 50% for water depths inferior to 3000m and decreased at deeper waters (Figure 5.4). Higher probability of habitat being suitable for pilot whales is found at locations with GrSSt values superior to 2.44 (Figure 5.4). The remaining variables - slope and SST- showed little importance, but suggest that higher probability of habitat suitability occurred at lower values of SST (Figure 5.4). It is important to mention that response curves built with each variable alone (allow the interpretation of the effect of each predictor on the distribution, without potential interaction with other variables, which can reveal collinearity) show that habitat suitability is less than 50% at locations with SST values higher than 17°C.

The gradient of SST (GrSST) was both responsible for the highest decrease of the regularized gain, when omitted, and the highest gain when used in isolation, showing that this variable has a strong influence on this species distribution model (Figure 5.3).

Summarizing, the Maxent model suggests that in summer months, in Atlantic Iberia, the suitable areas for pilot whales occur in shallower waters, with levels of SST variation (GrSST) ranging between 2.45 and 11.4, and SST values mainly concentrated between 15°C and 17°C.

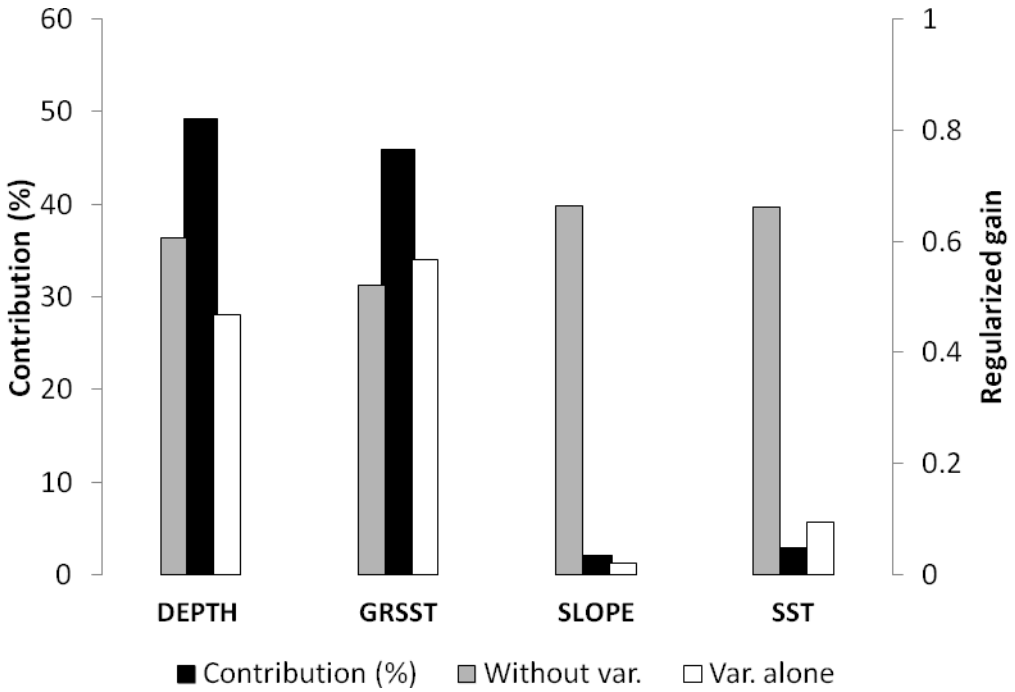
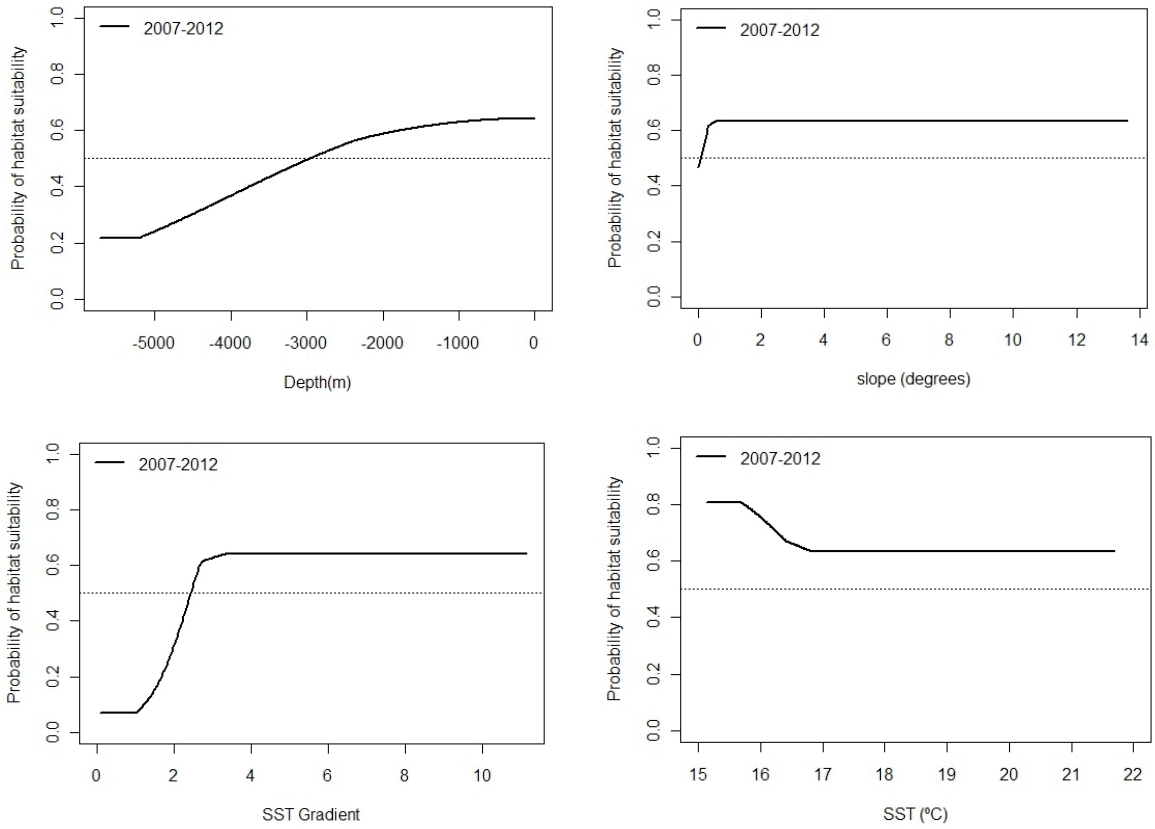


Figure 5.3. Representation of the importance of each variable for the Maxent models. The contribution of each variable (%) to the model is represented by the black bar. Remaining bars represent the jackknife results for the regularized gain in a model with only one variable (Var. alone) and for a model with all the variables, except the one analysed (Without var.). Values for the regularized gain are represented on the right axis.

a)


2007-2012
Probability of habitat suitability
Depth(m)
slope (degrees)
SST Gradient
SST (ºC)

b)

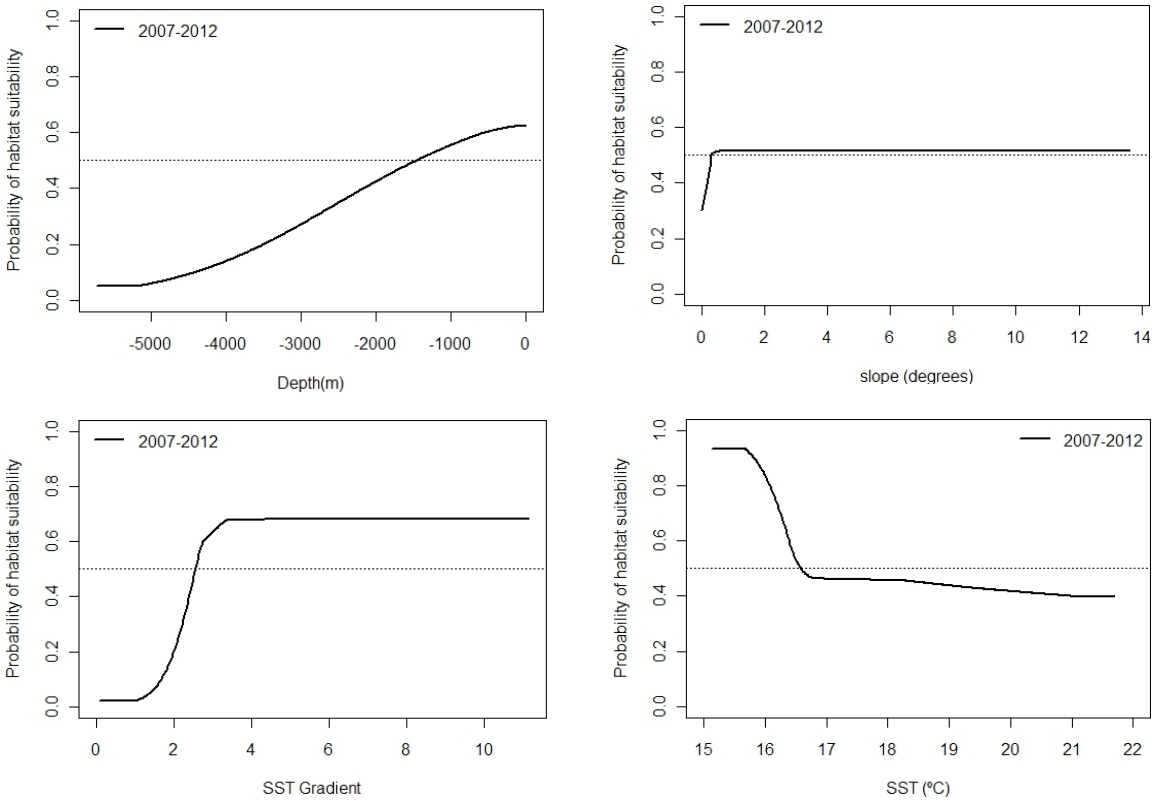

2007-2012
Probability of habitat suitability
Depth(m)
slope (degrees)
SST Gradient
SST (ºC)

Figure 5.4. Response curves of the environmental predictors of pilot whale habitat suitability. a) Marginal response curves; b) Response curves (considering the predictor in isolation). Note: Depth is expressed as negative values (zero equals the surface of the water).

Predicted distribution

Maxent model indicated that the most appropriate habitat for pilot whales along the Atlantic coast of the Iberian Peninsula in summer months seems to be located around Fisterra and Prior Capes, mostly over water depths of <200 m (i.e. on the continental shelf). A high probability of suitable habitat was also observed in Capes Roca and Cape São Vicente in WIP and Cape Ortegal in NIP (Figure 5.5).

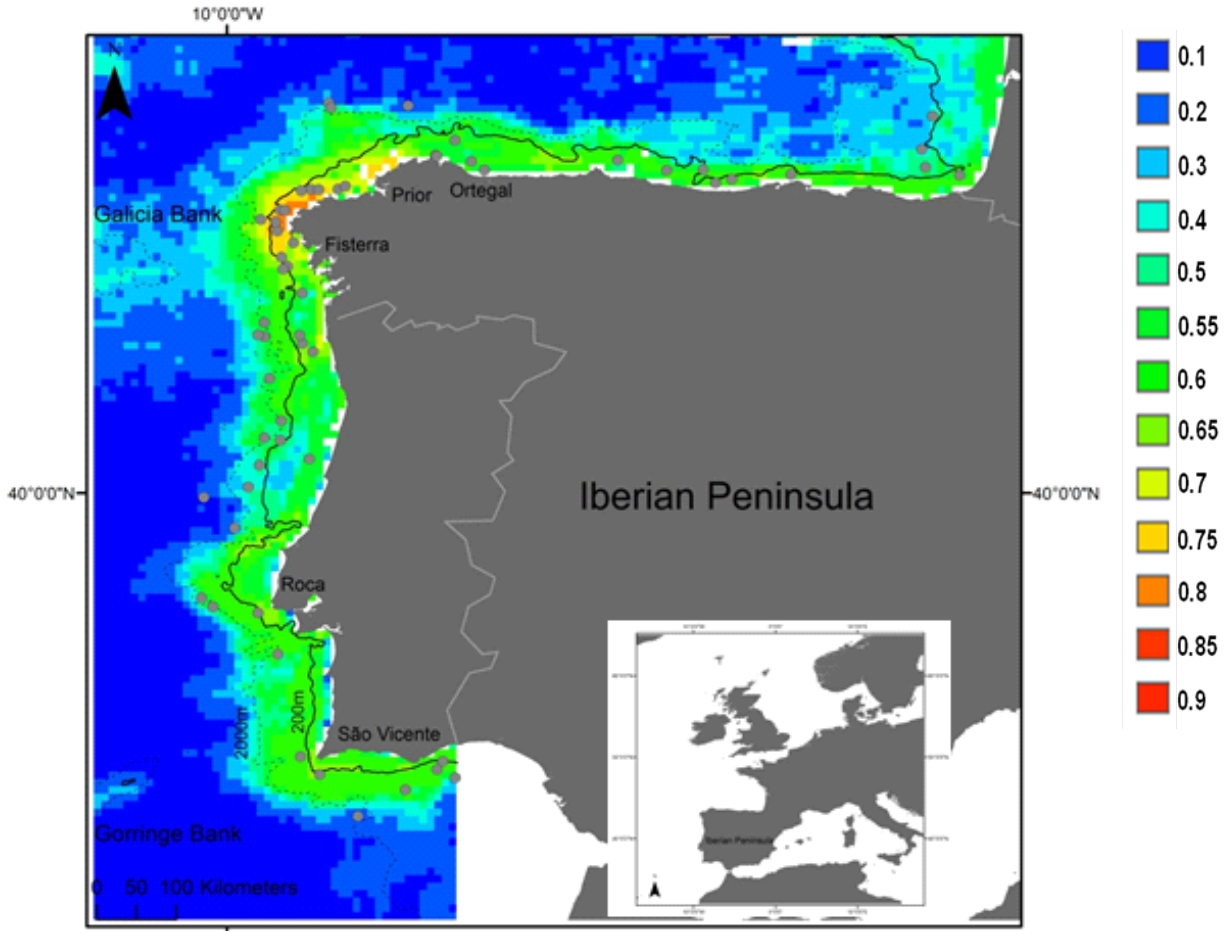


Figure 5.5. Predicted habitat suitability of pilot whales in Iberia (summer months, 2007 to 2012). Grey dots represent pilot whale occurrence data. Warmer colors (red) and colder colors (blue) indicate high and low habitat suitability, respectively. White pixels represent No Data.

Discussion

Pilot whales (*Globicephala* sp.) have been described as oceanic species, occurring at a wide range of depths and seabed slopes (Payne & Heinemann, 1993; Cañadas *et al.,* 2002, 2005; Macleod *et al*., 2007; Kiszka *et al.,* 2007; Certain *et al.;* 2008; Doksæter *et al.,* 2008; De Stephanis *et al.,* 2008a; Spyrakos *et al.,* 2011; Fernández *et al.,* 2013), although some sightings have already been described over the continental shelf (Kiska *et al.,* 2007; Certain *et al.,* 2008; Pierce et al., 2010a; Spyrakos *et al.,* 2011; Fernández *et al.,* 2013). A few studies also analysed the influence of dynamic variables such as sea surface temperature and chlorophyll *a* on the distribution of these species (Hamazaki, 2002; Macleod *et al.,* 2007; Doksæter *et al.,* 2008; Fernández *et al.,* 2013). In Scotland, the most important variable influencing the occurrence of pilot whales was water depth, followed by Chl *a* and then SST values (Macleod *et al.,* 2007). A study that analysed the distribution of several cetacean species along the mid-Atlantic ridge showed that SST and salinity were the most important variables determining the habitat preferences of pilot whales (i.e., they occurred in colder and less-saline waters), while depth seemed to be less important (Doksæter *et al.,* 2008).

Within the study area, in Galicia, pilot whales have previously been observed in deep waters but also occurring on the continental shelf (170m to 900m, López *et al.,* 2004; Spyrakos *et al.,* 2011; Santos *et al.,* 2012; Fernández *et al.,* 2013) and being occasionally sighted close to the coast (Pierce *et al.,* 2010a). Also, a recent study has shown that in this area, the ecological niche of *G. melas* seems to be related especially with depth, slope and SST (Fernández *et al.,* 2013), with pilot whales in NW Iberia (Galicia) being found in deeper (average depths of -275m) and cooler waters (average SST values of 16°C), with stronger and more variable slopes (average values of 4.27°), than other cetacean species recorded in the area. Furthermore, trophic studies suggest that *G. melas* may be feeding in coastal areas (Méndez-Fernandez *et al.,* 2012; Santos *et al.,* 2013). A stomach contents study revealed that the diet of *G. melas* is mainly composed of benthic and neritic cephalopod species in the Iberian Peninsula, although oceanic squids were also eaten (chapter II, Santos *et al.,* 2013), while a stable isotope study suggested that this species may occur in coastal habitats and/or may be mainly foraging on neritic and/or benthic prey species (Méndez-Fernandez *et al*., 2012).

In the present study, pilot whales were found at average depths of -846m, however more than 50% of the sightings occurred over the continental shelf (<200m depth),

supporting the idea that in Atlantic Iberia, pilot whales seem to exhibit a more coastal habitat, than in other parts of the Atlantic and Mediterranean (Cañadas *et al.,* 2002; 2005; Macleod *et al*., 2003, 2007; Kiska *et al.,* 2007; Praca & Gannier, 2008; De Stephanis *et al.*, 2008a), or at least make more regular incursions into coastal waters. These results are supported by both PCA and Maxent analysis that show depth to be one of the most important variables influencing pilot whale distribution in the study area.

Previous SDM studies performed on pilot whales showed that along with depth, sea surface temperature plays an important role in the identification of suitable habitats for these species (Hamazaki, 2002; Macleod *et al.,* 2007; Doksæter *et al.,* 2008; Fernández *et al.,* 2013). Although Maxent results suggest that SST is a variable of minor importance for pilot whales, still it shows that there is a higher probability of occurrence between 15 and 17°C, and PCA results support evidence for the occurrence of pilot whale's ecological niche in colder waters. Is important to mention that the range of water temperature preferred by pilot whales, in the present study, seems to suggest that most of the individuals analysed are *Globicephala melas* (Reid *et al*., 2003; Macleod *et al*., 2007; Doksaeter *et al*., 2008; Fernández *et al*., 2013).

The second most important variable described by both methodologies is SST gradient, which is intended to capture the effect of SST variation and the occurrence of thermal fronts, on pilot whale distribution. It has been suggested that sometimes habitat selection cannot be explained solely by the absolute values of the EGVs at single locations. In particular, some processing of these variables is needed to reveal mesoscale oceanographic features such as fronts, which show great influence on the distribution of some marine mammals species (Hamazaki, 2002; Tynan *et al.,* 2005; Etnoyer *et al.,* 2006; Doniol-Valcroze *et al.,* 2007; Scott *et a*l., 2010). Oceanic fronts are often associated with biologically active regions and several studies have already investigated the occurrence of oceanic fronts in upwelling systems (Castelao *et al.,* 2006; Kahru *et al.,* 2012).

Although higher habitat suitability occurred at higher levels of GrSST in the Maxent model, the PCA indicated the opposite situation. The temporal resolution used by the different methodologies could be a potential explanation of the results obtained. Although the use of a coarse temporal scale can provide information of the influence of macro-scale conditions and events on the distribution of the marine species, it prevents the analysis of the influence of intra- and inter-annual fluctuations of the oceanic physical and biological

features. It is particularly relevant to account for those fluctuations in the context of highly variable oceanographic conditions, such as upwelling systems. Several studies suggest the occurrence of intra- and inter-annual variation in upwelling events in the Western Iberian coast (Alvarez *et al*., 2008, 2010; Soares, 2009). As an example, a study analysed the upwelling event off Aveiro (West coast of Portugal) in 2007 and indicated the occurrence of alternation between upwelling and relaxation periods, within the upwelling season (Soares, 2009). Additionally, some studies imply an upwelling weakening in recent years, in most months of the year (except February, June and July, Alvarez *et al.,* 2008) or a decrease in the upwelling favourable days in Western Galicia (Alvarez *et al*., 2010). However, other studies indicate contradictory trends, possibly due to analyzing data over different time periods (Solow *et al.,* 2002, Alvarez *et al.,* 2010): Santos *et al*. (2005) found an intensification of coastal upwelling off the Western Iberia since 1992, while Miranda *et al*. (2013) suggested that upwelling intensity is likely to increase with global warming, in this region.

A possible explanation for the contradictory effects of GRSST, suggested by the two modelling approaches in the present study, is that pilot whales tend to prefer areas with more variable SST, associated with the presence of fronts (as suggested by MAXENT), but within these areas are not specifically occurring at the fronts (as shown by PCA). Some studies have found that the presence of fronts influenced other cetacean species, but in some cases, animals were not directly on top of the fronts and the authors hypothesized that a spatial or temporal lag could either occur because fronts are not necessarily straight lines under the surface, or because it takes time for prey to aggregate at the fronts (Gannier *et al*., 2006; Doniol-Valcroze *et al*., 2007).

Primary production hotspots, normally exhibited in upwelling areas (such as the upwelling events off Iberian Peninsula), may be either directly or indirectly related with the occurrence or abundance of cetaceans, mainly due to the attraction of potential prey to those areas (Thompson *et al.,* 2012). Given the association of fronts with high prey abundance the explanation above may sound counter-intuitive. However, in this region, a recent stomach contents analysis of *Globicephala melas* showed a dietary preference mainly for Octopodidae sp. (58.2% and 72.3% in prey number, in Portugal and Galicia, respectively), although some oceanic species were also consumed (chapter II, Santos *et al.,* 2013). Both *Eledone cirrhosa* and *Octopus vulgaris* spawning season seems to occur in the spring (May-June) (Silva *et al.,* 2004; Otero *et al.,* 2007; Moreno *et al.,* 2009; Regueira *et al.,* 2013) and

seems to be linked with the seasonal upwelling process, so that hatchlings benefit from the greatest abundance of zooplankton (Moreno *et al.,* 2009). Hence, although it may seem that pilot whales may not specifically target frontal areas, since their prey in this region appears to be mainly benthic and neritic species, nevertheless, the high productivity associated with upwelling likely extends to the whole shelf area and may contribute to high abundance of benthic species such as octopuses, as indicated by the strong association of octopus spawning season and the fishery landings in Galicia, with annual upwelling conditions (Otero *et al.* 2008; Moreno *et al.,* 2009). These results suggest that SST gradients may indirectly affect the pilot whale distribution, in Iberia.

This could also explain the suitable habitat in coastal areas and the hotspots around the main capes of Atlantic Iberia, especially Fisterra and Prior capes described by Maxent, since the presence of capes makes the upwelling event stronger and more persistent (Prego *et al.,* 2012) and the upwelling cores in Atlantic Iberia are located in Fisterra, Prior, Ortegal, Roca and São Vicente capes (Relvas *et al*. 2007; Alvarez *et al.,* 2008, 2010, 2012; Prego *et al.,* 2012).

However, it is also important to bear in mind the limitations of the dataset analysed, which could have contributed to discrepancies between the results from the two modeling approaches. The occurrence of temporal sampling bias, as a consequence of imperfect survey designs, with non-random distribution of survey effort (one of the major limitations of presence-only methods, Elith *et al.,* 2011) resulted in around 46% of the samples being collected in April. This month is coincident with the beginning of the upwelling season in Atlantic Iberia. Therefore, higher GRSST values will probably be obtained if a temporal scale of the total months of the upwelling season is considered (as in Maxent). This may also be the reason for chl *a* not being an important variable in both distribution methodologies used, contrary to results obtained in previous studies (Macleod *et al.,* 2007; Fernández *et al.,* 2013). In the beginning of the upwelling season, recently upwelled phytoplankton cells are still adapting to light conditions in order to start photosynthesis. Besides, top predators do not consume phytoplankton, resulting in a significant time or spatial lags between primary production, the presence of phytoplankton consumers and latter the presence of cetaceans (Grémillet *et al.,* 2008; Otero *et al.,* 2008; Thompson *et al*., 2012).

Although the analysis of the influence of upwelling on pilot whales distribution, at a macro temporal scale, may still provide useful information to elucidate the distribution of

the pilot whales in relation to macro-scale phenomena such as the upwelling season, the difference in effects of GrSST obtained between methods highlights the importance of thinking carefully about the meaning of findings at different temporal scales. Furthermore, it evidences the importance of using fine temporal scale, in marine environments, when dynamic variables are included in the analysis, in order to be able to detect seasonal variations in species distribution. Therefore, a further improvement to the present study would be to increase the sample size and sample homogeneity across the upwelling season in order to be able to reduce the temporal scale used and, at the same time, allow investigation of potential seasonal variations in the distribution of pilot whale.

Acknowledgements

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Chapter VI

General Discussion

The present study aimed at providing an understanding of the status and ecology of long-finned pilot whale (*Globicephala melas*) in the Western Iberian Peninsula, based on the investigation of trophic ecology, genetic diversity and habitat use. A multi-tracer approach was also used to detect the occurrence of population structure in *G. melas* in the North Atlantic. The main results of this study contribute to the basic knowledge of this cetacean species, necessary for the determination of its conservation status and identification of potential conservation and management concerns.

Trophic ecology of long-finned pilot whales from the North Atlantic

Due to the advantages and disadvantages of both traditional and recent dietary methods (see chapter I, also reviewed in Tollit *et al.,* 2010), in order to obtain a more complete knowledge about the feeding habits of *G. melas* in the Northeast Atlantic and potential sources of variation in dietary preferences, the results of trophic ecology obtained through the analysis of stomach contents of long-finned pilot whales from East Atlantic (chapter III), were complemented with the analysis of blubber fatty acids of animals stranded in the East Atlantic (Scotland and Atlantic Iberian Peninsula). The latter work also extended to the West Atlantic (Northeast USA), thus potentially providing larger-scale information on population structure (chapter IV).

The stomach contents analysis revealed a wide range of prey species ingested by pilot whales and confirmed them as mainly teuthophagous animals, although fish species were also present, as described in previous studies (Overholtz & Waring, 1991; Spitz *et al*., 2011). Animals from Northern latitudes (Scotland) consumed more oceanic squid (namely *Todarodes sagitattus*), while in the Iberian Peninsula (Portugal and Galicia) animals showed a more diverse diet, dominated by Octopodidae species. This result was also evidenced in the analysis of potential sources of variation in pilot whale diet, since some level of geographic variation was revealed, mainly related with a higher abundance of lesser octopus (*Eledone cirrhosa*) consumed by animals off the Iberian Peninsula.

The findings of the present study, along with previous results from stomach contents studies, that show the occurrence of geographical shifts in prey species consumed by pilot whales (Desportes & Mouritsen, 1993; Gannon *et al.*, 1997; Pierrepont *et al.,* 2005; Spitz et *al.*, 2011), support the significant differences in fatty acid signatures of animals from different locations (Atlantic Iberia, Scotland and Northeast USA), based on five dietary FAs

(i.e. FAs that arise from dietary origin, rather than being (bio)synthesised by the predator. Iverson *et al*., 2004), plus three FA that can have a dietary origin, but are also biosynthesized by the predator, according to Iverson *et al.* (2004).

The geographical dietary differences, between regions in the North Atlantic, revealed in stomach contents analysis and fatty acid signatures, may be a consequence of the ingestion of different types of prey based on prey preference/availability or due to the exploitation of different feeding niches/habitats in the study areas (Budge *et al.,* 2002). In reality, relating dietary ecology of long-finned pilot whales with either predator dietary preferences or prey availability is difficult to prove. Although some level of specialist behaviour is reflected by the mainly teuthophagous diet, pilot whales may also be viewed as generalist consumers, due to the wide range of prey species detected in their stomach contents. Related to this, many authors have referred to the relationship of prey abundance and movements with the presence and movements of *G. melas* (Mercer, 1975; Desportes & Mouritsen, 1993; Payne & Heinemann, 1993; Zachariassen, 1993; Jákupsstovu, 2002). However, the lack of contemporary data related to the local abundance of many of the prey species eaten by this cetacean species, makes it difficult to test assumptions about the predatory behaviour exhibited by pilot whales and prevents the discrimination between the ingestion of preferred prey (specialist behaviour) versus feeding on abundant prey (generalist behaviour) species.

As mentioned above, the geographical differences in both stomach contents and blubber fatty acid profiles of pilot whales may also be due to exploitation of different feeding niches. A study on nitrogen isotopes in different tissues (representing different turnover rates) of pilot whales showed significant differences between West and East Atlantic, suggesting that pilot whales are feeding at different trophic levels in those locations (Abend & Smith, 1995). Furthermore, recent studies base on stable isotopes of different odontocete species occurring in Northwestern Iberia suggested that pilot whales of this region may occur in coastal habitats (a result supported by habitat distribution analysis and the occurrence of coastal sightings of this species, Pierce *et al.,* 2010a; Spyrakos *et al.,* 2011; Santos *et al.,* 2012 and chapter V of the present thesis) and/or that this species may mainly be foraging on neritic and/or benthic prey species (Méndez-Fernandez *et al*., 2012, 2013). The stomach contents results for the Iberian Peninsula (chapter II), suggest a preference for *Eledone cirrhosa* (chapter III), a eubarythic species, the main distribution of which is situated

at depths of less than 300m (Boyle, 1983). Additionally, the analysis of prey fatty acids, in the present study (chapter III), revealed that there is some evidence, although not conclusive, that Iberian whales are feeding on octopods, in particular the importance of high levels of FA 20:4(n-6) in discriminating both octopods from ommatrephids and Iberian pilot whales from pilot whales elsewhere. The dietary results related with Iberian pilot whales (stable isotope and stomach contents) confirm the presence of this cetacean species in coastal areas, in this region, which contrasts with the oceanic preferences reported for this species in other locations, both in terms of habitat use (Hamazaki, 2002; Macleod *et al.,* 2003, 2007; Kiszka *et al.*, 2007; Praca & Gannier, 2008; Silva *et al*., 2013) and oceanic prey species consumed (Desportes & Mouritsen, 1993).

Regarding sex-related and ontogenetic dietary variations, previous studies have already shown differences in diet related with reproductive status, length and age, for this species (Desportes & Mouritsen, 1993), as well as for other odontocetes (Silva, 1999; Blanco *et al*., 2001; Koopman, 2001; Samuel & Worthy, 2004; Santos *et al.,* 2004; Learmonth, 2006; Smith & Worthy, 2006; Santos *et al*., 2007; Budge *et al.,* 2008; Quérouil *et al*., 2013). The abundance of *Eledone cirrhosa* and fish in the stomach contents of pilot whales of the present study seemed to be correlated with the length of the predator. These results may be due to limitations in the ability of juveniles to capture prey, either due to inexperience or physiological characteristics. A study with Faroese pilot whales that found that calves measuring less than 300 cm in length ate smaller cephalopods and that the consumption of shrimp and fish also varied between groups of whales of different length and reproductive status (Desportes & Mouritsen, 1993). However, in the present study, there was no significant influence of sexual maturity (a proxy for length as well as reproductive status of the animal) on the fatty acid profiles of pilot whales. The different results obtained between stomach contents and fatty acid analyses relative to the influence of length in the dietary habits may be due to the fact that an effect of the predator length on the FA signatures would only be expected to occur if the change in the diet resulted in ingestion of prey with different FA profiles, independently of the abundance of prey individuals consumed.

In both stomach contents and fatty acids analyses, no evidence of differences in the foraging habits of female and male pilot whales was found. The only exception was related with the occurrence of higher abundance of fish in female stomachs. These findings are in

agreement with a stable isotope analysis of Mediterranean pilot whales, which showed no sex-related differences in either $\delta^{15}N$ or $\delta^{13}C$ (De Stephanis *et al.,* 2008b).

Genetic diversity and divergence of long-finned pilot whales from the North Atlantic

In order to understand the genetic diversity and population structure in long-finned pilot whales from the North Atlantic, the spatial distribution of mitochondrial DNA and Major Histocompatibility Complex variation was used to characterize levels of genetic structure among putative populations from Western Iberian Peninsula (Portugal and Galicia), Scotland, Faroe Islands and the United States of America (Cape Cod) (chapter II).

Levels of mitochondrial diversity reported in this study are comparable to those reported previously for this species and in other cetaceans (long- and short- finned pilot whale, Oremus *et al.,* 2009; killer whales, Hoelzel *et al*., 2002; sperm whale, Lyrholm *et al.*, 1996). Likewise, at the MHC loci, the variation presented by the DRA and DQB loci in the present study is in accordance with the results of previous studies with several cetacean species (Hayashi *et al.,* 2006; Xu *et al.,* 2007, 2008, 2009; Heimeier *et al.,* 2009). However, the extent to which diversity at adaptive loci can be meaningfully compared across species with different ecological and life histories is still debated.

Previous evidence either for or against genetic population substructure in the North Atlantic is scarce. A study based on the mitochondrial control region of 70 pilot whales from Northeast USA, Nova Scotia, Newfoundland and United Kingdom found no evidence of population structure between the Western and Eastern basins of the North Atlantic, it being suggested that the low genetic variability could be due to a recent origin of the North Atlantic population or to the strong matrilineal structure (Siemann, 1994). Neverthless, a nuclear marker study, based on eight highly polymorphic microsatellite loci, which analysed samples from the East Coast of USA (Cape Cod), West Greenland, the Faroe Islands and the UK, indicated the occurrence of substructure, particularly pronounced between West Greenland and other sites. However, the magnitudes of the various pairwise comparisons did not support a simple isolation-by-distance model, it being suggested instead, that population isolation occurs between areas of the ocean which differ in sea surface temperature (Fullard *et al*., 2000).

In the present study, both mitochondrial DNA and MHC class II DQB and DRA loci analyses suggest that in the North Atlantic pilot whales do not comprise a single population. Mitochondrial DNA showed high and significant levels of differentiation between most regional groups from the North Atlantic, indicative of genetic structure at both regional and oceanic scales. Regarding adaptative loci, patterns of population divergence determined from the MHC revealed Iberia to be a separate group. Mitochondrial and MHC AMOVAs showed no differentiation between Western and Eastern sides of the Atlantic, revealing that most of the genetic variation occurred at a smaller spatial scale (i.e. among or within the sampling regions analysed), rather than between oceanic basins.

In agreement with the findings of Siemann (1994), in the present study, pilot whales from UK and Northeast USA seem to belong to the same population, based on mitochondrial DNA. The signs of population differentiation found in the present study, using mitochondrial DNA may be due to the inclusion of previously un-studied areas. Several studies have already mentioned that population structuring may sometimes remain undetected due to sampling limitations associated with opportunistic schemes used for collection of cetacean samples (such as strandings), that may prevent the analysis of individuals from genetically distinct populations (Evans & Teilmann, 2009; Mirimin *et al*., 2009). The differences between the levels of population divergence found between the microsatellite analysis performed by Fullard *et al*. (2000) and the neutral marker (mitochondrial DNA) analysed in the present study may either be due to the different levels of mutation rates exhibited by both type of markers (Selkoe *et al.,* 2006) and/or to the four-fold smaller efective population size of mitochondrial DNA, as compared to nuclear autosomal genes with biparental transmission (Moore, 1995), which makes the latter a more sensitive detector of population subdivision, by random genetic drift (Wilson *et al.,* 1985; Balloux *et al*., 2000). Furthermore, the social structure presented by pilot whales may also result in higher levels of divergence at this maternally inherited marker.

Genetic drift and gene flow may explain the levels of mitochondrial diversity and differentiation found in the present study. However, the social organization of pilot whales, specifically the occurrence of natal group phylopatry (Aguilar *et al.,* 1993; Amos *et al.,* 1993; Andersen, 1993; Balbuena & Raga 1994; Caurant *et al.,* 1993; Fullard *et al.,* 2000; Ottensmeyer & Whitehead, 2003; De Stephanis *et al.,* 2008c; Alves *et al.*, 2013), may also be one of the explanations for the diversity structure of this maternally inherited marker.

Concerning the MHC results, although historical balancing selection had an important role in shaping population diversity, the spatial patterns of extant diversity across the North Atlantic could either result from local selection pressures for specific pathogens/parasites or evolutionary forces such as gene flow or drift, since teasing apart the effects of selection and reduced gene flow would be difficult (Landry & Bernatchez, 2001; Miller & Lambert, 2004; Campos *et al.,* 2006; Alcaide *et al.,* 2008; Babik *et al.,* 2008; Peters & Turner, 2008; Miller *et al.,* 2010).

Population differentiation of long-finned pilot whales from the North Atlantic based on a multi-tracer approach

In order to ensure a Good Environmental Status (GES) of European marine waters, the Marine Strategy Framework Directive (Directive 2008/56/EC) defined 11 descriptors of GES to be analysed, which included the maintenance of biological diversity (descriptor 1) and normal trophic functioning (descriptor 4). As is also the case for the Habitats Directive, a key issue is the identification or definition of the population units for which status should be measured and indicators developed. Therefore, understanding the occurrence of population structure within marine species is crucial for conservation, including for the implementation of this legal conservation framework (ICES, 2013), in order to guarantee the long-term survival in a changing environment and at the same time preserve behavioural, ecological and genetic diversity within a species (Dizon *et al.*, 1997; Coyle, 1998; Reeves *et al.,* 2003, ICES 2009, 2013).

The heterogeneity and dynamics of marine ecosystems can define boundaries for marine mammal populations (Fullard *et al*., 2000; Fontaine *et al*., 2010).The occurrence of population substructure may be based on evolutionary traits (genetic stock) or on ecological characteristics, since isolated units can adapt separately to the different habitats, even if no genetic differentiation occurs (environmental or phenotypic stock) (Coyle, 1998; Waples & Gaggiotti, 2006).

Several studies have used genetic markers to provide information about wild population divergence and support the definition of management units (Wang *et al.,* 1996; Rosel *et al.,* 1999; Mendez *et al*., 2007). It is evident that reproductively isolated units should be typically recognized as separated management units (Palumbi & Cipriano, 1998; Moritz, 2002; Palsbøll *et al.,* 2007; ICES 2009), since their responses to perturbations would be

distinct. Recently, there has been an increasing use of ecological markers to define "ecological populations" (Caurant *et al.*, 2009; Evans & Teilmann, 2009; ICES, 2009, 2013). The justification for separation of management units based on ecological populations is less clear, however it has been suggested that some species, such as for example *Delphinus delphis*, should be managed using an ecological time-scale, since it represents a finer scale that may be more relevant to management issues than the evolutionary time-scale (Evans & Teilmann, 2009). Additionally, ecological populations could be viewed as units likely to become reproductively isolated in the future, or units whose unique characteristics and/or distribution justify their separate conservation (Hoelzel, 1998; Schluter, 2001; Caurant *et al*., 2009; ICES, 2009, 2013).

Several studies have examined potential population structure of marine mammals through the application of multi-tracer approaches (Herman *et al.,* 2005; Borrell *et al.,* 2006; Segura *et al.,* 2006; Born *et al*., 2007; Foote *et al*., 2009; Evans & Teilmann, 2009; ICES, 2009, 2013; Queróuil *et al.,* 2013). As example, a combination of fatty acids and stable isotope analyses suggested that individuals of *Stenella frontalis* from Madeira and Azores belonged to different ecological stocks (Queróuil *et al.,* 2013), despite the occurrence of gene flow between archipelagos, as evidenced by genetic analysis (Queróuil *et al*., 2010). Another example, already mentioned in introduction, refers to the long-term investigation of killer whales (*Orcinus orca*) in order to confirm the existence of a third ecotype of this species ("offshore"), besides the previously described "resident" and "transient" ecotypes (Barret-Lennard *et al*., 1996; Hoelzel *et al*., 1998; Ford *et al*., 1998, 2000), using genetic markers, stable isotopes, persistent organic pollutants and fatty acid analyses (Barret-Lennard *et al*., 2000; Herman *et al.,* 2005; Krahn *et al*., 2007).

Multi-approach analysis may present several advantages. First of all, the combination of different independent methodologies to infer substructure patterns within a population may overcome potential limitations of each individual technique, providing more accurate and robust results than those obtained using a single metric. Furthermore, genetic and ecological tracers can cover a wide range of time scales, depending of the type of tracer used (stomach contents, fatty acids, stable isotope, trace elements, pollutants, mtDNA, microsatellites, MHC loci, vital rates, etc), and the type of tissue analysed – since different tissues have different turnover rates (e.g. blood, milk, blubber, muscle, bone, teeth) (Caurant *et al*., 2009). Therefore, they are able to provide information referring to time

periods ranging from some days of integration (stomach contents analysis), days to life times (fatty acids, stable isotopes, trace elements, vital rates) or even generations or evolutionary time-scales (genetic markers or morphometrics) (Hobson & Clark, 1992; Wagemann et al., 1990 in Das et al., 2000; Hobson & Sease, 1998; Kirsch *et al*., 2000; Nordstrom *et al*., 2008; Caurant *et al.,* 2009 and references therein). However, it is important to mention that the nature of the information and the biological processes involved in determining the signals present in the different ecological tracers may be very different, which can make it difficult to interpret the combined results from several tracers (Hobson, 1999; Bustamante *et al*., 2002; Das *et al.,* 2003; Iverson *et al*., 2004; Budge *et al*., 2006). Furthermore, it is important to account for factors that may influence the information provided by the ecological tracers, including both intrinsic (metabolic, ontogenetic, sex-related) and extrinsic factors (geographical location, water temperature, trophic position) (Honda & Tatsukawa, 1983; Hobson & Welch, 1992; Wagemann et al., 1995; Bustamante *et al*., 1998; Koopman, 2001, 2007; Das *et al.*, 2003; Iverson *et al.,* 2004; Newland *et al*., 2009; Caurant *et al*., 2009).

In spite of the difficulties described above, the incorporation of different ecological markers in a multi-tracer approach can provide knowledge on animals' movements and habitat (mainly through trophic characteristics) and information about the potential ecological structure of populations, especially if combined with genetic analysis results. The combination of the results from all tracers analysed in the present study (chapter II, III and IV) indicates the occurrence of segregation of long-finned pilot whales from the different regions of the North Atlantic analysed, both ecologically (based on trophic ecology) and genetically (Figure 6.1). Genetic results suggest that North Atlantic pilot whales do not exist as a single population (chapter II). Consistent with this result, the differences in trophic ecology of this species, observed in stomach contents and fatty acids analyses (chapter III and IV) suggest the occurrence of ecological stocks of *G. melas* from the North Atlantic, with specific foraging habits, as observed in other cetaceans (Walton *et al.,* 2000; Møller *et al.,* 2003; Herman *et al*., 2005; Born *et al*., 2007; Khran *et al*., 2007; Caurant *et al.,* 2009; Tucker *et al,* 2009b; Quérouil *et al*., 2013).

Several studies have suggested the occurrence of different populations of *G. melas* across the North Atlantic, based on the application of genetic and ecological markers (Bloch & Lastein, 1993; Abend & Smith, 1995; Perrin et al., 1990; Fullard *et al.*, 2000). An analysis of neutral markers (microsatellites) in North Atlantic pilot whales (Fullard *et al.,* 2000) revealed

differentiation between West Greenland and remaining regions (USA East Coast, UK and Faroe Islands). Moreover, several non-genetic studies based on morphometrics (Bloch & Lastein, 1993), parasites (Perrin *et al*., 1990) or stable isotopes (Abend & Smith, 1995) generally favoured some level of subdivision of the North Atlantic long-finned pilot whale population. As an example, the analysis of stable isotopes in animals from the Faroe Islands, the mid-Atlantic Bight and Cape Cod areas, suggested the occurrence of dietary segregation of animals from the West and East Atlantic, when fast and medium turnover rate tissues were considered (Abend & Smith, 1995), while differences in parasite composition between animals from the western Mediterranean, France, Faroe Islands and Newfoundland suggest that individual whales may not routinely move between any of these regions (Perrin *et al*., 1990). Additionally, another study showed the occurrence of morphometric differences between whales from Faroe Islands and Newfoundland, suggesting segregation of long-finned pilot whales between East and West Atlantic (Bloch & Lastein, 1993).

Therefore, the results of the multi-tracer approach used in the present study are consistent with previous genetic and ecological studies of population structure of long-finned pilot whales from the North Atlantic, in the sense that they suggest the existence of population differentiation within this region. Additionally, it provides new information about the previously studied regions, due to the incorporation of different ecological tracers (such as fatty acids) and genetic markers (such as MHC loci). Finally, the present study provides new knowledge about animals inhabiting regions previously un-studied, such as Southwestern Europe.

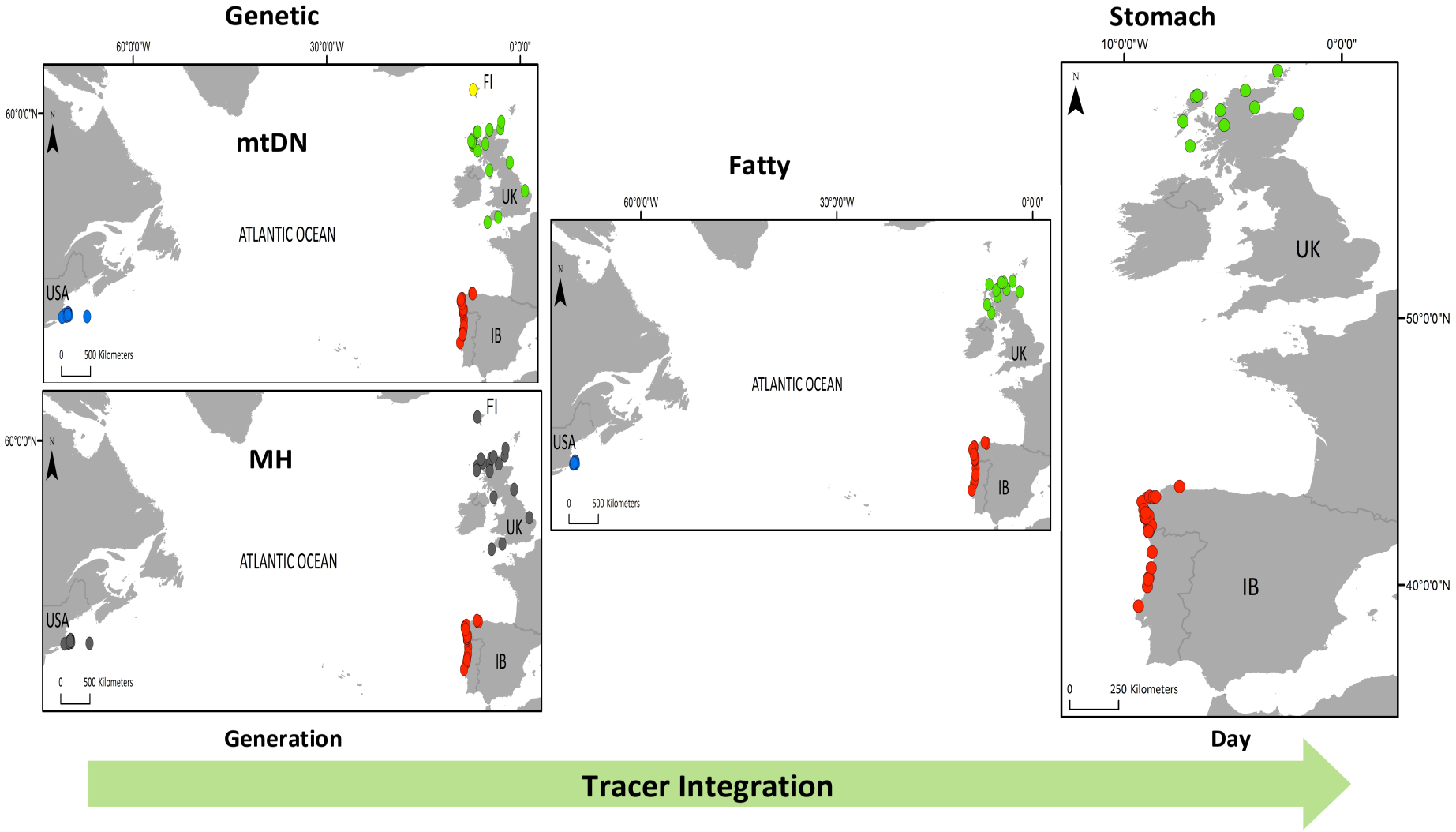

Figure 6.1. Map-based scheme for population structuring in long-finned pilot whale groups from the North Atlantic, as investigated through genetic and ecological tracers. The different colours in the locations represent different groups of *G. melas*, according to the results described for each methodology.

Habitat preferences of pilot whales from the Atlantic and Cantabrian coasts of Iberia

Besides the value of understanding the levels of genetic and ecological population structuring (e.g. in order to define management units and help to preserve species diversity), identifying suitable areas within the distribution range of a species and understanding the relationship between cetacean distribution and environmental variables have been considered as one of the priority research areas for effective conservation and management (Torres *et al.,* 2003; Cañadas *et al.,* 2005; ICES, 2008). Therefore, summer habitat preferences and suitable habitats were determined for pilot whales from Atlantic (Portugal and Galicia) and Biscay (Cantabrian) coasts of Iberia, using presence–only methods (PCA and MAXENT), based on six ecogeographic variables selected for their availability and likely relevance.

Depth and sea surface gradient (that indicates the effect of SST variation on pilot whale distribution) seem to be the most important variables influencing pilot whale distribution off Iberia, as revealed by PCA and MAXENT. Furthermore, although Maxent results suggest that SST is a variable of minor importance for pilot whales, it is apparent that there is a higher probability of occurrence between 15 and 17°C, and PCA results support evidence for the occurrence of pilot whale's ecological niche in colder waters. This latter result is in agreement with previous studies of pilot whales distribution which showed that along with depth, sea surface temperature plays an important role in the identification of suitable habitats for these species (Hamazaki, 2002; Macleod *et al.,* 2007; Doksæter *et al.,* 2008; Fernández *et al.,* 2013).

Although the species is also found in much deeper waters, more than 50% of the sightings occurred over the continental shelf (in waters <200m depth). As mentioned above, this result is consistent with previous distribution (Pierce *et al*., 2010a; Spyrakos *et al*., 2011; Santos *et al*., 2012: Fernández *et al.,* 2013) and trophic studies of Atlantic Iberian whales (stable isotopes, Méndez-Fernandez *et al*., 2012; stomach contents, chapter III) that suggest that, in this region, pilot whales may be occurring regularly in coastal habitats and/or that this species in mainly foraging on neritic and/or benthic prey species. Therefore, these analyses suggest that in the Atlantic Iberian region, pilot whales seem to occur in a more coastal habitat, than in other parts of the Atlantic and Mediterranean (Cañadas et al, 2002; 2005; Hamazaki*,* 2002; Macleod et al., 2003, 2007; Kiska et al., 2007; Praca & Gannier, 2008;

De Stephanis et al., 2008a; Silva *et al*., 2013), or at least make more regular incursions into coastal waters.

The second most important variable described by both methodologies is the sea surface temperature (SST) gradient. However, in this case, there was inconsistency in the results of the two methods, with the Maxent model suggesting higher habitat suitability at locations with higher SST variation and PCA describing the opposite situation. The temporal resolution used by the different methodologies could be one of the potential explanations for the results obtained. Several studies suggest the occurrence of intra- and inter-annual variation in upwelling events in the Western Iberian coast (Santos *et al*., 2005; Alvarez *et al*., 2008, 2010; Soares, 2009; Miranda *et al*., 2013). The use of a coarse temporal scale, as necessary with Maxent due to the relatively small dataset, precludes the analysis of the influence of intra- and inter-annual fluctuations of the oceanic physical and biological features, which are particularly important to account for in highly variable oceanographic conditions, such as upwelling systems. A possible explanation for the contradictory effects of GRSST is that pilot whales tend to prefer areas with more variable SST, associated with the presence of fronts (as suggested by MAXENT), but within these areas they are not specifically occurring at the fronts (as shown by PCA). This type of result was also described in other cetacean species, with the suggestion that a spatial or temporal lag could occur because fronts are not necessarily straight lines under the surface, or because it takes time for prey animals to aggregate at the fronts (Gannier *et al*., 2006; Doniol-Valcroze *et al*., 2007).

The results of the present study seem to indicate that SST gradients may indirectly affect the pilot whale distribution, in Iberia, mainly due to the high abundance of potential prey in highly productive areas, as already suggested by Thompson *et al*. (2012). Although pilot whales may not specifically target frontal areas, since their prey in this region appear to be mainly benthic and neritic species (*Eledone cirrhosa* and *Octopus vulgaris*, chapter II), the high productivity associated with upwelling likely extends to the whole shelf area and may thus contribute to high abundance of benthic species such as octopuses, as indicated by the strong association of octopus spawning season and the fishery landings in Galicia, with annual upwelling conditions (Otero *et al.,* 2008; Moreno *et al.,* 2009). This could also explain the suitable areas described for pilot whales, by Maxent, that seem to be concentrated in coastal areas and around the main capes of Atlantic Iberia, especially Fisterra and Prior

capes, since the presence of capes makes the upwelling event stronger and more persistent (Prego *et al.,* 2012).

However, it is also important to bear in mind the limitations of the dataset analysed, which could have contributed to discrepancies between the results from the two modeling approaches. In particular, the occurrence of temporal sampling bias, as a consequence of imperfect survey designs, with non-random distribution of survey effort (one of the major limitations of presence-only methods, Elith *et al.,* 2011) could have resulted in those differences.

Although the analysis of the influence of upwelling on pilot whales distribution, at a large temporal scale, may still provide useful information to elucidate the distribution of the pilot whales in relation to macro-scale phenomena such as the upwelling season, this study highlights the importance of thinking carefully about the meaning of findings at different temporal scales. Additionally, it shows the importance of considering a fine temporal scale, in marine environments, when dynamic variables are included in the analysis, in order to be able to detect seasonal variations in species distribution. Therefore, a further improvement to the present study would be to increase the sample size and sample homogeneity across the upwelling season in order to be able to reduce the temporal scale used and, at the same time, allow investigation of potential seasonal variations in the distribution of pilot whale. Ideally, availability of more sightings data with associated information on sampling effort could permit the use of presence-absence methods (such as GAM), which can take the distribution of sampling effort into account.

Long-finned pilot whales in Western Iberia and management implications

Management strategies are frequently established based solely on genetic results which reject the occurrence of panmixia between geographic locations. However, recently, there has been a move to complement this information with demographic and ecological data (Palsbøll *et al*., 2007; Caurant *et al*., 2009). The data gathered in this thesis may be a starting point to improve the knowledge about long-finned pilot whales in Western Iberia (IB) and in particular to determine whether they should be considered as a separate management unit. Results obtained in this thesis consistently show IB long-finned pilot whales as a potential different group in the North Atlantic, based on genetic (mtDNA and MHC) and trophic (stomach contents and fatty acids) analyses (Figure 6.1). In Western Iberia,

the diet of *G. melas* seems to be dominated by benthic and mostly neritic cephalopod species (chapter III) which, together with the distribution analysis (chapter IV) and previous stable isotope and distribution studies in this region, shows that this species may perform incursions to coastal habitats, especially in the summer (Pierce *et al*., 2010a; Spyrakos *et al*., 2011; Méndez-Fernández *et al*., 2012; Santos *et al*., 2012; Fernández *et al.,* 2013). These results suggest that off the Western Iberia pilot whales may be exploiting foraging niches in shallower waters, when compared with other regions of the Atlantic and Mediterranean (Macleod *et al.,* 2003, 2007; Kiszka *et al.*, 2007; Praca & Gannier, 2008; De Stephanis et al., 2008a).

This behaviour may increase the potential for harmful interaction of this species with human activities, namely incidental captures or exposure to persistent organic contaminants and other pollution sources. Along the Atlantic coast of Iberia, some studies have already investigated the levels of contaminants (Méndez-Fernandez, 2012; Méndez-Fernandez *et al*., 2013) and the occurrence of incidental captures in fisheries (López *et al.,* 2002, 2003). In fact, long-finned pilot whales from Galicia showed lower PCB, hepatic mercury and renal cadmium concentrations than those reported in individuals from other locations in the Atlantic (Méndez-Fernandez, 2012; Méndez-Fernandez *et al*., 2013). However, the same study described high concentrations of cadmium in liver and kidney, as well as hepatic Hg, above the threshold levels for toxic effects in mammals, in some individuals of *G.melas* (20-200 µg/g w.wt, 50-400 µg/g w.wt and 60 µg/g w.wt, respectively; Law, 1996; Ma, 1996; AMAP, 1998; Méndez-Fernandez, 2012). However, caution is needed in the interpretation of these data, due to low sample size.

In relation to the impact of fisheries on this species, by-catch estimates based on strandings, carcass recovery and interviews with fishermen suggested that around 16 % of the stranded pilot whales along the Galician coast presented signs of by-catch (López *et al.,* 2003). In addition to by-catch, competition for resources between cetaceans and fisheries may also adversely affect the cetaceans due to resource depletion. The three main prey categories ingested by Iberian pilot whales (chapter III) are also among the most important cephalopod species marketed in Spain and Portugal. Mean annual landings in Galicia (1997-2010) were 1423 tons and 2800 tons, for *Eledone cirrhosa* and *Octopus vulgaris* respectively and 3154 tons of ommastrephids (http://www.pescadegalicia.com). However, due to the

lack of information about the abundance of these main prey species no assumption can be made about this indirect impact of fisheries on pilot whales from Western Iberia.

At present, there is still a lack of information for the Western Iberia population of *G. melas*. Real estimates of by-catch in fishing gear and the investigation of the impact of pollutants in this species are critical to assess the degree to which the pilot whales are threatened in Western Iberia. Furthermore, research focusing on pilot whale demography, namely reproduction and survival rates, as well as continuous monitoring of the population to determine seasonal movements and abundance estimates, is fundamental to understand the resilience of this species against the impact of anthropogenic activities. These investigations will enrich the basic scientific knowledge about long-finned pilot whales in this region of the Atlantic, allowing the definition of its conservation status, as well as helping to ensure the achievement of Good Environmental Status (GES) of European marine waters, as required by the Marine Strategy Framework Directive (Directive 2008/56/EC).

Study Limitations

One of the main limitations of the present study is related with the number and coverage of samples used in each analysis. There is little control over sampling opportunities and conditions when acquiring non-invasive samples of oceanic species of cetaceans. Relying on opportunistic sampling, such as the use of strandings and/or fishery by-catches may lead to sampling bias. However, the stomach contents and even the inner blubber of cetaceans used in the trophic analyses of the present study could not realistically be obtained with another sampling technique.

Biopsy sampling to obtain blubber samples of this species in all the areas studied, aside from raising ethical issues and the need for relevant permits would require a high investment of time (not least to find the animals and get close enough to collect samples), funding and equipment, unlikely to be feasible in the context of a PhD project, if at all. While it is theoretically possible to obtain stomach contents from a live cetacean (and this can be achieved by trained veterinary personnel in captive animals), it would be logistically enormously difficult and stressful to do this by capturing free ranging individuals. The alternative of “research whaling” is not generally considered ethically acceptable in Europe or the United States, except for a limited amount of aboriginal whaling of certain species in certain areas.

A limitation in the analysis of the dietary ecology of *G. melas* is related to the impossibility of using the QFASA model to obtain reliable quantitative estimates of diet from the combined analysis of predator and prey FA signatures, due to the non-existence of calibration coefficients (CCs) for cetacean species. There is a need to perform studies to determine the extent to which specific fatty acids undergo selective deposition, metabolism or synthesis in cetaceans, enabling the development of calibration coefficients to "weight" the proportions of individual fatty acids found in the blubber, so as to accurately reconstruct their relative importance in ingested food. A good approach would be to develop CCs for different species of cetaceans, considering different experimental diets since both predator phylogeny and prey type seem to influence calibration coefficients (Rosen & Tollit, 2012). However, as suggested in the later study, it is also possible that no universal set of CCs exists for any predator species, and if CCs vary significantly with diet, the prospect of reconstructing the true diet is remote indeed. Even if adequate CCs can be obtained, variability in FA profiles of individual prey species also presents an important challenge.

Finally, the limited number of sightings of pilot whales precluded the analysis of seasonal variations in this species' habitat use and led to the use of a coarse temporal scale for the dynamic variables used in Maxent. It is important to underline the fact that both the samples and the distribution data could not be obtained without the support of regional stranding networks, highlighting the need for long-term and continuous monitoring for sample and data collection.

Conclusion

The present study provides some useful data on the feeding habits, genetic characteristics and suitable habitat areas of long-finned pilot whales inhabiting the North Atlantic, with a special focus on Western Iberian whales. Results provide evidence for the occurrence of genetic and ecological structure of *G. melas* in the North Atlantic. Consistent results between genetic (mtDNA and MHC) and trophic tracers (stomach content and fatty acids) suggest that Western Iberian animals can be considered as a separate group when compared with other areas in the Atlantic, at least so far as investigated here. The distribution data along with a diet dominated by mostly coastal cephalopod species in this region, suggest that *G. melas* perform coastal incursions, especially in the summer months. This behaviour may increase the exposure of this species to human activities, notably

incidental captures in fishing gear. However, further studies are necessary in the Iberian Peninsula to enrich the knowledge about this species and to support its conservation.

Future Research

To enrich our knowledge of long-finned pilot whales in the North Atlantic, further several lines of further study may be suggested:

- The application of the QFASA model to quantitatively determine the diet long-finned pilot whale based on fatty acids, once calibration coefficients are developed for cetaceans.
- The analysis of more ecological tracers, with different turnover periods, such as stable isotopes, POPs and trace elements, would enhance the information about the feeding ecology and trophic position of long-finned pilot whales, as well as support the investigation of the suggested ecological segregation in the North Atlantic.
- An increase in the numbers of samples for ecological markers would help generate more robust results about temporal trends (seasonal or annual) in feeding habits of *G. melas*, especially for longer-term ecological tracers. This would allow the identification of potential shifts in prey consumption, providing basic knowledge for the understanding of the feeding behaviour of this species, although information about prey abundances and availability would also be necessary.
- The inclusion of higher number of samples per location and analysis of other genetic markers such as for example SNPs or microsatellite would complement and help resolve genetic divergence between different regions of the North Atlantic.
- The analysis of more functional markers in order to detect genetic structure due to potential adaptations associated with reproduction, thermoregulation, diving ability, among others. This investigation would help to establish a bridge between genetic and ecological groups of long-finned pilot whales.
- To obtain more useful results on stock structuring it would be useful to obtain samples from different areas, for analysis of both genetic and ecological markers.
- A continuous monitoring programme would increase distribution and abundance information and allow a better understanding of the relationship of the distribution of *G. melas* with environmental variables and, if such data are available, local variation in prey abundance. An increase in the number of sightings would allow the investigation of potential

seasonal variations in habitat use. The use of alternative modelling techniques would also complement the analysis of the present study. Availability of effort data would permit use of more robust techniques which can account for variation in search effort.

- Additionally, in order to provide further information for the identification of potential conservation issues, demographic studies on survival and reproductive rates, as well as studies about the human impact on Atlantic Iberian population, namely fishery by-catch and contaminants would allow a better understanding of the pressures suffered by this species and the biological ability to overcome these threats.

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Supplementary Material

Appendix 1

Regression equations used to estimate fish and cephalopod sizes.

	Estimated prey length (mm)	Source	Estimated prey weigth (g)	Source
CEPHALOPODS				
Sepia sp.	L=-2.14+21.89xLHL	Cl	$w= 0.12xLHL^{4.06}$	Cl
Sepiolidae sp.	L=18.54+1.65xLHL	Cl	$w= 2.65xLHL^{0.54}$	Cl
Rossia sp.	L=17.81+10.09xLHL	Cl	$w= 8.84xLHL^{1.65}$	Cl
Sepiola atlantica	L=15.02+0.75xLHL	Cl	$w= 1.49xLHL^{0.35}$	Cl
Gonatus sp.	L=-43.4+42.87xLRL	Cl	$w= 0.52xLRL3^{.33}$	Cl
Lepidoteuthis grimaldii			$w= 1.54xLRL^{2.8}$	Sa
Histioteuthissp.	L=-13.60+22.21xLRL	Cl	$w= 4.92xLRL^{2.31}$	Cl
Brachioteuthis riisei	L=16.31+20.18xLRL	Cl	$w= 1.73xLRL^{1.41}$	Cl
Ommastrephidae sp.			$w= 2.34xLRL^{2.82}$	Cl*
			$w= 1.07xURL^{3.15}$	GP
llex/Todaropsis			$w= 2.42xLRL^{2.82}$	Cl*
Illex coindetti			$w= 2.87xLRL^{3.17}$	AG
Todaropsis eblanae	L=-10.32+35.04xLRL	Cl	$w= 1.80xLRL^{3.17}$	Cl
Todarodes sagittatus	L=-11.3+41.36xLRL	Cl	$w= 2.19xLRL^{2.83}$	Cl
Chiroteuthis sp.	L=11.40+24.46xLRL	Cl	$w= 0.79xLRL^{2.7}$	Cl
Mastigoteuthis schmidti	L=-1.8+29-08xLRL	Cl	$w= 1.2xLRL^{2.88}$	Cl
Taonius pavo	L=-12.3+61.43xLRL	Cl	$w= 2.16xLRL^{2.19}$	Cl
Teuthowenia megalops	L=12.2+40.78xLRL	Cl	$w= 2.07xLRL^{2.34}$	Cl
Haliphron atlanticus			$w= 1.7xLHL^{3.2}$	Sa
Eledone cirrhosa	L=3.38+26.57xLHL	Cl	$w= 5.37xLHL^{2.85}$	Cl
			$w= 8.25xUHL^{2.34}$	GP
Octopus vulgaris			$w= 6.17xLHL^{3.03}$	Cl
			$w= 0.17xUHL^{4.52}$	Cl
FISH				
Gadidae sp.	L=-61.59+33.30xOL	Ha	$w= 0.02x(L/10)^{2.87}$	Ha
Micromesistius	L=-2.14+22.09xOL	Ha	$w= 0.007xOL^{3.89}$	Ha
poutassou	L=-17.8+70.77xOW	Ha	$w= 0.002x(L/10)^{3.34}$	Ha
Merlucius merlucius	L=-0.63+23.88xOL	Ha	$w= 0.01xOL^{2.91}$	Ha
Trachurus trachurus	L= -27.02+34.94xOL	S	$w= 0.003x(L/10)^{3.29}$	Co

L, total length (mm) for fish and dorsal mantle length (mm) for cephalopods; W, total weight (g); OL, otolith length (mm); OW, otolith width (m); LHL, lower hood length; LRL, lower rostral length (mm); UHL, upper hood length; URL, upper rostral length. Sources are as follows: Cl, Clarke (1986); Co, Coull et al. (1989); GP, Graham Pierce (unpublished data); Ha, Harkönen (1986); Sa, Santos et al. (2002); AG, Angel González (unpublished data); S, Santos et al. (2007); *, combined data from more than one species.