UNIVERSITAT DE BARCELONA

Use of chemical markers in the study of distribution range and population structure of large cetaceans

Uso de marcadores químicos en el estudio del rango de distribución y de la estructura de poblaciones de grandes cetáceos

Morgana Vighi

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Morgana Vighi

Morgana Vighi – PhD Thesis - 2015

Cetaceans have historically been heavily exploited, and are still currently subject to different threats. For conservation and management purposes, most of the large cetaceans' populations have been categorized in stocks, considered as isolated and demographically independent management units. Many research techniques may contribute in the definition of these stocks, such as morphometric studies, mark recapture studies, genetics, satellite tracking. This thesis focuses on the development and application to cetaceans' populations of chemical markers of geographical origin and spatial movements. The main objective of the thesis is to evaluate the potentialities of chemical markers, such as stable isotopes and element concentrations, to investigate the patterns of distribution, the level of structuring and, when possible, the migration routes, of cetaceans' populations. All these variables are essential to ensure the implementation of proper management measures and to guarantee the conservation of these animals. Three case studies were considered: the Eastern North Atlantic sperm whale (*Physeter macrocephalus*); the South Western Atlantic right whale (*Eubalaena australis*), and the North Atlantic fin whale (*Balaenoptera physalus*). δ^{15}N, δ^{13}C and δ^{18}O values were analyzed in sperm whale teeth, right whale and fin whale bone; concentrations of fluoride and selected metals (Zn, Pb, Ti, Sr, Cu) were analyzed fin whale bone.

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PhD Thesis

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Facultad de Biología - Departamento de Biología Animal

Programa de Doctorado en Biodiversidad

Use of chemical markers in the study of distribution range and population structure of large cetaceans

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Uso de marcadores químicos en el estudio del rango de distribución y de la estructura de poblaciones de grandes cetáceos

Memoria presentada por

Morgana Vighi

Para optar al título de

Doctora por la Universidad de Barcelona

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Director y tutor	Doctoranda	Directora
Dr. Alejandro Aguilar Vila	Morgana Vighi	Dra. Asunción Borrell Thió

Departamento de Biología animal y IRBio, Universidad de Barcelona

“The uncertain, unsettled condition of this science of Cetology is in the very vestibule attested by the fact, that in some quarters it still remains a moot point whether a whale be a fish (…)

Be it known that, waiving all argument, I take the good old fashioned ground that the whale is a fish (…)

To be short, then, a whale is a spouting fish with a horizontal tail. There you have him. However contracted, that definition is the result of expanded meditation. A walrus spouts much like a whale, but the walrus is not a fish, because he is amphibious. But the last term of the definition is still more cogent, as coupled with the first. Almost any one must have noticed that all the fish familiar to landsmen have not a flat, but a vertical, or up-and-down tail. Whereas, among spouting fish the tail, though it may be similarly shaped, invariably assumes a horizontal position. (…)

Hence, all the smaller, spouting and horizontal tailed fish must be included in this ground-plan of cetology. Now, then, come the grand divisions of the entire whale host.”

Herman Melville – Moby Dick

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Contents

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Abstract

Cetaceans have historically been object of heavy exploitation, and are still currently subject to different threats. For conservation and management purposes, most of the large cetacean populations have been categorized in stocks, considered as isolated and demographically independent management units. Many research techniques may contribute in the definition of these stocks, such as morphometric studies, mark recapture studies, genetics, satellite tracking. This thesis focuses on the development and application to cetacean populations of chemical markers of geographical origin and spatial movements. The main objective of the thesis is to evaluate the potentialities of chemical markers, such as stable isotopes and element concentrations, to investigate the patterns of distribution, the level of structuring and, when possible, the migration routes, of cetaceans populations. All these variables are essential to ensure the implementation of proper management measures and to guarantee the conservation of these animals. Three case studies were considered: the Eastern North Atlantic sperm whale (*Physeter macrocephalus*); the South Western Atlantic right whale (*Eubalaena australis*), and the North Atlantic fin whale (*Balaenoptera physalus*). $\delta^{15}N$, $\delta^{13}C$ and $\delta^{18}O$ values were analyzed in the dentinal growth layers of sperm whales from Denmark and NW Spain, in bone of right whales from Southern Brazil and Northern Argentina, and in bone of fin whales from W Iceland and NW Spain; concentrations of fluoride and selected metals (Zn, Pb, Ti, Sr, Cu) were analyzed in bone of fin whales from W Iceland and NW Spain. To establish the validity of each marker, the degree of compliance of each of the following four properties has been investigated: i) the basal levels of the chemical marker in the environment present significant gradients; ii) the chemical marker can be detected and measured in the tissues of whales; iii) the variability of the chemical marker within each population is moderate or, alternatively, it is predictable through measurable parameters, so that its effect can be taken into account; and iv) the values of the chemical marker in the body tissues reflect basal levels in a predictable and consistent manner. Each marker provided, at different levels, valuable information regarding the studied populations. Some difficulties emerged, connected with the complexity of the migratory cycles of whales, the possible intervention of homeostatic regulation mechanisms, and the characteristics of the tissues investigated. Nevertheless, results highlighted the occurrence of structuring within the North Atlantic sperm whale population, as well as within the South Western Atlantic right whale population, which are both currently considered as a single management unit. Moreover, results regarding the North Atlantic fin whale populations contributed to highlight the complexity of migration patterns and of the level of connectivity among the currently accepted stocks, challenging the proper definition of their limits and their degree of isolation within the basin. Some further research is recommended to deepen the knowledge about the North Atlantic fin whale population structure, as well as to investigate the levels of chemical markers in other tissues with different turnover characteristics, such as skin or baleen plates. Overall, the results obtained from the analyses performed in this thesis underline the power of chemical markers as a complementary tool to the traditional techniques used for investigating the structure and demography of cetaceans' populations.

Keywords: Chemical markers, cetaceans, stable isotopes, elements, right whale, fin whale, sperm whale

Resumen

Los cetáceos han estado y están actualmente sujetos a diferentes tipos de amenazas. Para su correcta gestión y conservación, la mayoría de las poblaciones de grandes cetáceos han sido categorizadas en “stocks”, considerados como unidades de gestión aisladas y demográficamente independientes. Muchas técnicas de investigación pueden contribuir en la definición de estas unidades, como los estudios morfométricos, los estudios de marcaje y recaptura, la genética y el seguimiento por satélite. El objetivo principal de esta tesis es evaluar las potencialidades de los marcadores químicos, tales como los isótopos estables y las concentraciones de algunos elementos, como herramientas para la investigación de los patrones de distribución y del nivel de estructuración de las poblaciones de cetáceos. Para ello, se han considerado tres casos modelo: el cachalote del Atlántico Nororiental, la ballena franca del Atlántico Suroccidental, y el rorcual común del Atlántico Norte. Se analizaron las proporciones isotópicas de $\delta^{15}N$, $\delta^{13}C$ y $\delta^{18}O$ en muestras de dientes de cachalote y de hueso de ballena franca y rorcual común; y las concentraciones de flúor y de metales pesados (Zn, Pb, Ti, Sr, Cu) en muestras de hueso de rorcual común. Cada marcador proporcionó, a diferentes niveles, información valiosa sobre las poblaciones estudiadas. Al interpretar los resultados, surgieron algunas dificultades relacionadas con la complejidad de los ciclos migratorios de los cetáceos, la posible intervención de mecanismos de regulación homeostática, y las características de los tejidos investigados. Sin embargo, los resultados destacaron cierto nivel de estructuración dentro de las poblaciones de cachalotes del Atlántico Norte y de ballenas francas del Atlántico Suroccidental, que actualmente están ambas consideradas como unidades de gestión únicas. Por otra parte, los resultados obtenidos en las poblaciones de rorcual común del Atlántico Norte contribuyeron a evidenciar la complejidad de los patrones migratorios y del nivel de conectividad entre sus poblaciones, desafiando la adecuada definición de sus límites y de su grado de aislamiento. En general, los resultados de la tesis destacan las potencialidades de los marcadores químicos como herramienta complementaria a las técnicas utilizadas tradicionalmente para la investigación de la estructura y demografía de las poblaciones de cetáceos.

Palabras clave: Marcadores químicos, cetáceos, isótopos estables, elementos, ballena franca, rorcual común, cachalote

Advisors' report

Dr. Alex Aguilar and Dr. Asunción Borrell, co-advisors of the PhD thesis entitled "Use of chemical markers in the study of distribution range and population structure of large cetaceans" certify that the thesis presented here has been carried out in its totality by Morgana Vighi. The candidate has participated actively in the planning and preparation of each of the articles presented in this thesis. The article included in chapter 1.1 was planned and mainly prepared by Dr. Borrell, and the candidate performed the data analysis and partially contributed to the manuscript preparation. The contribution of the candidate to each of the remaining articles included: planning of the objectives; laboratory analyses of the samples, results and data analysis, manuscript writing and revision. None of the information here presented have been used for other PhD theses.

Articles included in the thesis with relative publication status and impact factor of the journals:

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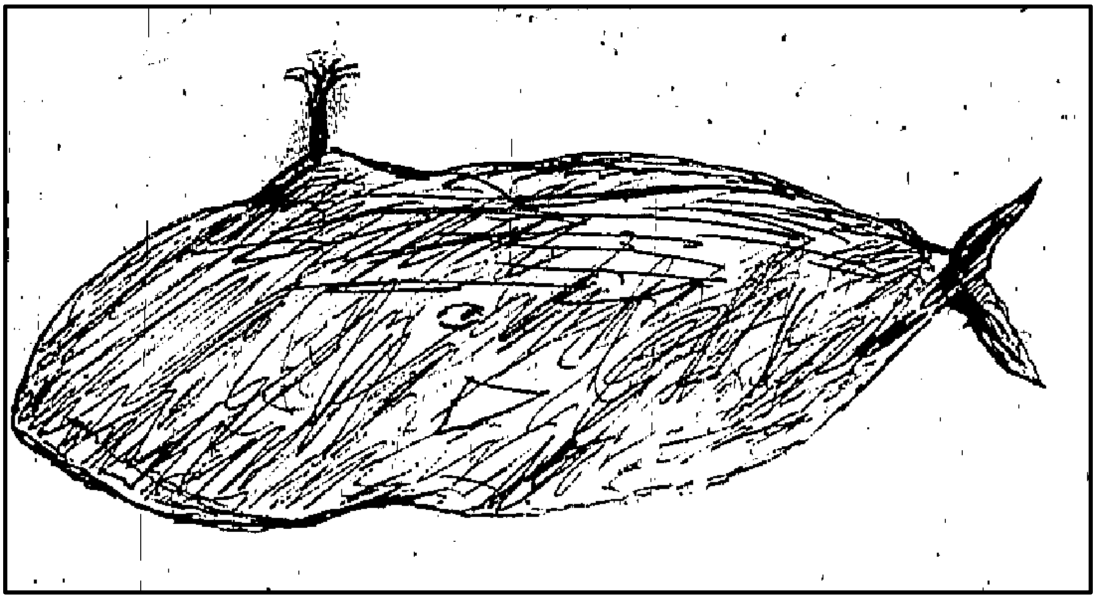
Barcelona, octubre 2015,

Alejandro Aguilar Vila Asunción Borrell Thió

General Introduction



1. The current state of cetaceans' populations

Cetaceans are a wide group of marine mammals which include almost 90 species, encompassing a broad range of sizes, shapes, distributions, biological and ecological characteristics. According to their feeding structure, cetaceans have been traditionally classified in two main subgroups, or suborders: odontocetes (from the ancient Greek *odoús*, "tooth", including toothed cetaceans); and mysticetes (from the ancient Greek *mustikētos*, or perhaps *mystakókētos*, literally "whale-mouse" or "mustache-whale", including baleen whales). Apart from eating through teeth or baleen plates, the two groups differentiate under many crucial aspects.

Odontocetes, with a few exceptions, are in general smaller; they actively feed on fish and cephalopods and are structured in sub-populations distributed over smaller geographical areas, with limited migration flaws. This group include, among others, dolphins, porpoises, beaked whales and sperm whales (Hooker, 2009). The sperm whale (*Physeter macrocephalus*) is the largest odontocete species and, similarly to large mysticete cetaceans, presents a complex population structure and wide range movements (Whitehead, 2009).

Mysticetes are generally larger than toothed whales, including in this group the largest known animal species: the blue and the fin whale. Although with some differences related to each species, all mysticetes are active filter feeders; they use baleen plates to filter water and feed on small organisms including fish, krill and other small invertebrates. Due to their large size, feeding behavior and life history characteristics, mysticetes populations generally extend over broader geographical areas and perform long-range movements. Many species, like rorquals, are highly migratory and follow a typical annual cycle in which they alternate summer high latitude feeding grounds and winter low latitude breeding grounds (Bannister, 2009).

To different extents, all cetaceans' populations have been and are still currently subject to a variety of threats connected, directly or indirectly, with human impacts. If nowadays cetaceans suffer for the direct effects of pollution, anthropogenic noise and disturbance, and generally for the global effects of overfishing and habitat loss, what dramatically affected some populations and species in the past was direct hunting (Reeves, 2009).

Large cetaceans, and in particular mysticetes, are among the marine resources which most severely suffered from human exploitation. Most mysticetes species, as well as sperm whales, were heavily exploited in the past, and many populations are still currently caught (Tønnessen & Johnsen, 1982). Because of their large size, whales were traditionally considered as an invaluable source of fat and protein, thus explaining the huge biomass extraction that took place from the oceans, and that during the 20th century reached values of almost 120 million of tons (Clapham & Baker, 2002).

Due to the low demographic productivity of cetaceans, the consequences of this exploitation were extreme and led most of species and populations to a critical situation, if not to extinction. For this reason, in the second half of the 20th century, the International Whaling Commission (IWC) was established, with the aim to manage worldwide the whales stocks. Since then, the IWC started a policy of progressive protection of the most endangered species, and in 1985 proclaimed a moratorium on commercial whaling (Gambell, 1999).

After decades of protection, many populations started to recover, although following different trajectories. While some populations were almost extinguished, others started a gradual demographic increase, and some of them managed to reach levels similar to those previous to exploitation. The reason of this variability in resilience is unclear but is most probably connected with how whaling impacted on the different subpopulations. Despite species occupying large geographic areas, like whales, are generally more resilient (Pinsky, 2010), populations subject to intense whaling often showed fragmented recovery patterns when compared to the original distribution (Clapham *et al*., 2008; Valenzuela *et al*., 2009). Such a fragmented distribution pattern may be explained by the extirpation through whaling of segments of populations which were ‘faithful’ to certain habitats, thus causing these sites to remain empty (Hoelzel, 1998), as well as by the intervention of other human impacts such as fishing, climate change and alterations of the pristine habitats (Leaper *et al*., 2006).

2. How to define adequate management units?

The IWC is the intergovernmental body charged with the conservation of whales and the management of whaling. It bases its procedures of management on units known as “stocks”, defined as population genetically or biologically isolated which are demographically independent and occupy extended geographical areas (Palsbøll *et al*., 2007). The underlying assumption on the definition of these managements units is that individuals of each stock move homogeneously within its limits (Clapham *et al*., 2008). Such a definition represents an extreme simplification of the complexity of distribution and recovery patterns observed in many cetacean populations after the ban of commercial whaling, which showed that many stocks are not homogeneous, but instead structured in subgroups occupying definite habitats. When exploitation affected severely some of these habitats, recovery of empty habitats could occur only from proximate areas, something impossible if the cultural ‘fidelity’ to these areas was lost. Thus, the use of too wide management units or their incorrect definition led sometimes to the extinction of whole collectives that occupied habitats of reduced size and were fundamental for the survival of the overall meta-population (Taylor, 2005; Clapham *et al*., 2008).

Such management mistakes confirmed that large genetic meta-populations of cetaceans are indeed strongly structured in subunits with independent demographic dynamics. Based on these evidences, many researchers started questioning the currently accepted stock subdivision and proposed a reconsideration of the management and conservation procedures of cetacean populations (Donovan, 1991; Freeman, 2008; Valenzuela *et al*., 2009; Carroll *et al*., 2011). According to them, in order to guarantee demographic conservation, management units should be substantially smaller and their limits should be set in order to prevent local extirpation and assure that each population maintains healthy demographic size.

Despite this, none of the organizations in charge of management and conservation of whales (IWC, International Union for Conservation of Nature, North Atlantic Marine Mammal COmmission, etc.) could yet integrate properly these principles in their management policies, due to the overall lack of knowledge about the mechanisms driving cetacean distribution and the difficulties in identifying the discrete subunits of cetaceans populations (Taylor 2005; Clapham *et al*., 2008).

In this scenario, it is fundamental to increase the general knowledge about population structure, distribution ranges of the potential subunits and migratory routes of cetaceans in order to understand the demographic dynamics of these animals and guarantee adequate measures of management and conservation.

The importance of considering the range and patterns of distribution of populations as essential indicators for species monitoring was also underlined by international conventions and European conservation strategies. Among them, the Habitat Directive of the European Union considers that range and distribution of populations are essential tools to evaluate a ''good conservation status'' of the environment. The OSPAR agreement (Convention for the Protection of the Marine Environment of the North-East Atlantic) and the Ecosystem Approach process of the Barcelona Convention also consider that distribution ranges are priority indicators. Finally, the Horizon 2020 program of the European Union highlights the importance of defining suitable tools and techniques that would support the management of the marine ecosystem in the North Atlantic guaranteeing a sustainable exploitation of its life resources.

As previously stated, the correct definition of management units is extremely important for cetaceans' populations, whose conservation status is globally deficient due to the past exploitation and present exposure to a large variety of anthropogenic threats. Many tools can be used to obtain information about patterns of distribution and structure of cetaceans' populations, each of them exposing strengths and weaknesses according to the case studied. The use of a good combination of these techniques should guarantee a good foundation for the understanding of aspects that are still unknown for many cetacean populations.

3. Techniques for studying cetaceans' populations

The definition of distribution ranges and migration routes of cetaceans is an extremely complex task because these animals are highly mobile, their populations occupy large geographic areas and in many cases they perform long range movements. Thus, cetaceans' populations have been traditionally studied through a wide variety of techniques, ranging from field observations, to the use of natural and artificial marking of individuals, or laboratory techniques such as genetics.

3.1 In the field

At the base of field techniques for studying cetacean's populations lay visual surveys, which can be performed using dedicated platforms -such as research vessels-, platforms of opportunity -such as ferries or recreational boats- or, in case of aerial surveys, small aircrafts (Forney, 2009). Visual surveys provide, through the technique of distance sampling or direct counting, information about the number and location of the individuals, essential for animal censuses. Data obtained from visual surveys can be complemented by data obtained from acoustic surveys, which also give information about the position and density of animals, as well as information about their behavior and social activities. Through repeated surveys over the same area, it is therefore possible to estimate trends in the abundance and distribution of the animals, and also to obtain information about their habitat use and population dynamics (Forney, 2009).

A traditional technique associated with field observations and commonly used for estimating the abundance of a species or population is mark-recapture. Being not feasible to actually perform serial captures, markings, releases and recaptures of cetaceans, this technique was firstly applied during the modern whaling era through the so-called 'discovery marks', which were deployed during dedicated campaigns and recovered when the whales were caught (Buckland & Duff, 1989;

Mizroch *et al*., 2013). This technique was gradually substituted by the totally non-invasive technique of photo-identification, which uses marks naturally present on the body of the animal as distinguishing features (Hammond *et al*., 1990). The underlying principle of both techniques is the same, thus allowing through the percentage of re-captured individuals to estimate the size of a population, and, over the time, to evaluate possible trends in abundance and distribution.

In the recent years, the application of a particular kind of artificial “marks”, in the form of telemetry tags, has proved very useful in the study of cetaceans’ dynamics. Satellite tags, especially, which are small devices transmitting directly to the satellites, have been increasingly used in this field, as they can provide data regarding long range movements of the animals (Mate & Urban-Ramirez, 2003; Zerbini *et al*., 2006; Panigada, 2013). After they are implanted in the blubber of the whale, they allow in fact to follow its movements during weeks, and, with a little luck, months, until they are expelled from the body of the animal or their batteries are used up. The results obtained with this technique would be strikingly useful for studying whale migrations and movements, but this technique is still highly expensive and the number of individuals that can be tagged during a campaign is still too low to draw unambiguous conclusions.

3.2 In the lab

As a complementary tool to field studies, laboratory techniques have traditionally contributed to the study of population structure of cetaceans, especially through genetics, and its wide range of applications, such as a molecular mark-recapture tool, or to estimate the level of isolation between populations (Palsbøll, 1999; Garrigue *et al*., 2004; Gaspari *et al*., 2013). Despite genetics can be very useful to investigate the levels of isolation between populations, the results obtained with this technique are sometimes of difficult interpretation with regards to animals’ movements and migrations (Taylor & Dizon, 1999).

In the past two decades, advances in analytical chemistry and instrumentation made it possible to incorporate chemical markers to the traditional repertoire of techniques. The underlying principle behind the use of these tools is that the composition of the tissues of an organism reflects, either directly or through predictable relations, the baseline values of the environment in which this organism lives or feeds. To be useful, these markers must display a substantial spatial variation and be detected into the tissues of the studied species, allowing the assignment of individuals to certain geographical areas. Also, the markers variability within a population should be moderate or alternatively predictable through parameters that are measurable, so that their effect may be taken into account. If these assumptions are satisfied, chemical markers offer further highly diagnostic information with an excellent cost / benefit ratio (Bianchi & Canuel, 2011).

Chemical marker techniques are commonly used in cetaceans’ research, because conventional techniques are sometimes impractical or extremely expensive due to the elusiveness of the individuals and the difficulty or inability to capture and handle them. Since several years, some markers, such as organochlorine compounds, other xenobiotics, or trace elements have been used with good results in species at the top of food webs, because some of them show high levels due to biomagnification (Aguilar, 1987; Aguilar *et al*., 1999; Born *et al*., 2003). More recently, stable isotopes of certain elements have also been successfully applied for discrimination of populations (Das *et al*., 2003; Newsome *et al*. 2010; Borrell *et al*., 2012).

3.2.1 Stable isotopes

Stable isotope analysis is a widespread tool for ecological analysis and has been applied to the study of patterns of movement and composition of the diet of a variety of animals, from insects to mammals (Rubenstein & Hobson, 2004).

Carbon (δ^{13}C) and nitrogen (δ^{15}N) isotopes have been successfully used for studying various cetacean species (Borrell *et al*., 2006; Bentaleb *et al*., 2011; Giménez *et al*., 2013). While the ^{15}N proportion (δ^{15}N) is strongly influenced by diet and reflects, above all, the trophic level occupied by the target organism (Newsome *et al*., 2010), ^{13}C proportion (δ^{13}C) is influenced by the different contribution of primary producers to the environmental organic carbon and, to a lesser extent, by ecosystem productivity, thus reflecting both the type of prey consumed (benthic, pelagic) and their distribution in relation to the coast (Newsome *et al*., 2010).

Stable isotopes of oxygen (δ^{18}O) have been widely used in geochemical studies, but their application to cetaceans has been limited even though, unlike terrestrial animals, the values present in the tissues of marine animals are potentially good tracers of geographical origin (Clementz & Koch, 2001; Zenteno *et al*., 2013). In addition to varying with latitude, δ^{18}O is also a good proxy for salinity and water temperature (Newsome *et al*., 2010), and it has been widely investigated in paleoclimate studies. Due to these properties, it is a potentially good tracer for species living in the polar region or which are anticipated to be particularly sensitive to climate change, such as cetaceans.

Stable isotopes of carbon, nitrogen and oxygen have well known gradients in the oceans, which have been delineated based on a meta-analysis of baseline isotopic values and are represented in the form of isoscapes (Bowen, 2010, McMahon *et al*., 2013).

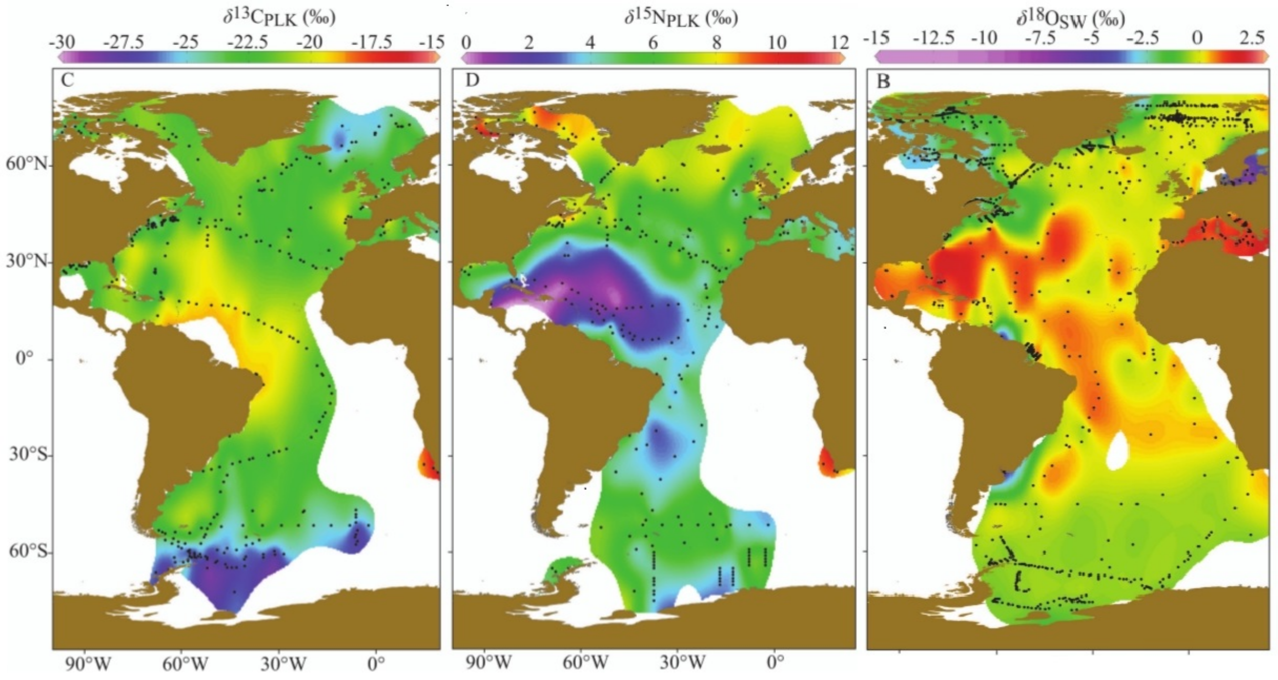


Figure 1. Atlantic Ocean isoscapes of δ^{13}C, δ^{18}O and δ^{18}O. (Reproduced from McMahon *et al.*, 2013.)

3.2.2 Concentrations of elements

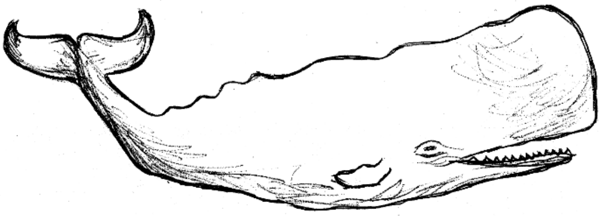
The concentrations of elements can also be used as promising markers for cetaceans' population studies. Trace elements are present in the environment naturally at varying concentrations according to the local geochemistry, although human activities strongly increased the availability of many of them. The combination of natural and anthropogenic sources offers a wide range of gradients, which makes elements' concentrations particularly useful tracers of geographic origin. Among the natural occurring elements, fluoride and metals concentrations present some of the characteristics for being potential markers of cetacean populations.

Fluoride. The main sources of fluoride are anthropogenic pollution, mainly derived from industrial activities, and natural emissions from sediments and volcanic activity (de Angelis & Legrand, 1994). Exposure to high levels of fluoride can be toxic to vertebrates, which incorporate this element into the bone and developing teeth. In the marine environment, fluoride is concentrated by some organisms, most notably the euphausiid crustaceans (krill), which are the main food of the baleen whales (Sands *et al*., 1998). Being accumulated through the food web in the bone of baleen whales (Landy *et al*., 1991), fluoride may therefore represent a potential marker for these animals' populations.

Metals. In animals exposed to sources of pollution, or to naturally high environmental concentrations, metals tend to accumulate in internal, protein rich organs, such as liver and kidney. Concentrations in these tissues of metals such as mercury, cadmium, zinc and copper, have been used as chemical markers for discriminating cetacean populations (Sanpera *et al.*, 1993; 1996). Despite showing lower concentrations, also other tissues, such as skin, have been considered for measuring the concentrations of some metals, because of their easier recollection (Born *et al*., 2003). In general, there is less experience in the investigation of metals or tissues that present lower concentrations and therefore are more complex to detect and quantify, however any element proving to be measurable offers the potential for being used as a marker of populations.

Different markers for the study of cetacean populations may have various levels of utility according to the species, the geographical area, and the tissues studied. Some markers may not be applicable because their geographical gradients in the study area are not well-defined; or because their concentrations in the tissues of the selected species are too low to be detected or not reflect adequately the environmental values because of homeostatic regulation. For this reason a validation process of each marker, which allows to identify the suitable conditions for its application, would provide a significant reference point to be used in future studies. A good selection of chemical markers for the determination of the distribution ranges of cetaceans would represent an essential complementary tool to the data obtained from traditional techniques, allowing a substantial increase of the overall knowledge about population structure and distribution ranges of many species of cetaceans.

Case study 1: The North Atlantic sperm whale



Sperm whale (*Physeter microcephalus*) is the largest odontocete species. It is a large predator situated at the top of the food web that inhabit mesopelagic marine ecosystems and is globally distributed in all the oceanic basins of the world. Its social structure is complex, characterized by the presence of matrilineal primary social groups formed by adult females with their offspring and juveniles, which occupy tropical and temperate waters; bachelor schools, formed by young males not yet socially mature; and solitary mature males, which tend to move in higher latitudes and return to lower latitudes only to join the primary social groups for reproduction (Best, 1979; Best *et al*., 1984; Christal *et al*., 1998; Lettevall *et al*., 2002; Whitehead, 2003).

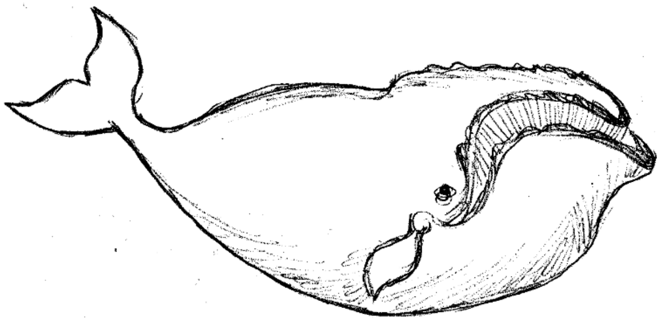
The sperm whale owes its name to the spermaceti, a waxy organ contained in the whale's head and originally mistaken for its semen. Unluckily for this whale, the oil derived from this organ, together with its blubber and teeth, had an extensive range of commercial applications, making the sperm whale a prime target of whaling since the early 18th century (Whitehead, 2009).

In the North Atlantic, during the second half of the 18th century and throughout all 19th century, the species was widely hunted by American, British and French whalers (Davis, 1997). The introduction of modern whaling technology and the beginning of the exploitation of rorquals did not smoothen the pressure on the species, but rather the contrary: between the 1950s and 1980s, the species was caught by Spain, the Azores, Madeira and Iceland, for a total of half a thousand whales killed every year (IWC, 1981). This extreme whaling pressure led in the 60s to a decrease of almost a meter of the average size of captured males in Spain and Iceland (Aguilar *et al*., 2014), thus indicating that older whales were being eliminated at rates impossible to be replenished by the populations (Martin, 1980; 1981; Aguilar & Sanpera, 1982), and inducing the IWC to declare the protection of the species in the North Atlantic in 1981.

It is unknown how many sperm whales survived in the North Atlantic after two and a half centuries of exploitation, but the current population size is estimated to be several tens of thousands of individuals, a figure probably between one half and one third of its original size (Whitehead, 2003).

Based on analyses of commercial whaling data, sightings distribution, evidences of long-range movements from mark-recaptures, similarities in mortality rates and trends in body lengths, the IWC considered the North Atlantic sperm whale as one single stock (IWC, 1981; Dufault *et al*., 1999; Mitchell, 1975). The existence of a single ocean-wide population is supported by studies showing absence of subpopulation structure in other large oceans (Mizroch *et al*., 2013) and by worldwide genetic studies that failed to distinguish subpopulations at less than an ocean basin level (Lyrholm *et al*., 1999; Lyrholm & Gyllensten 1998). However, data from historical exploitation in the North Atlantic showed clearly delineated whaling grounds suggestive of a patchy distribution (Townsend, 1935; Aguilar & Borrell, 2007), and genetic studies revealed differences between sperm whales from the Mediterranean Sea and the eastern North Atlantic, indicating that subpopulation structuring of the stock may indeed occur (Drouot *et al*., 2004).

Case study 2: The Southwest Atlantic right whale

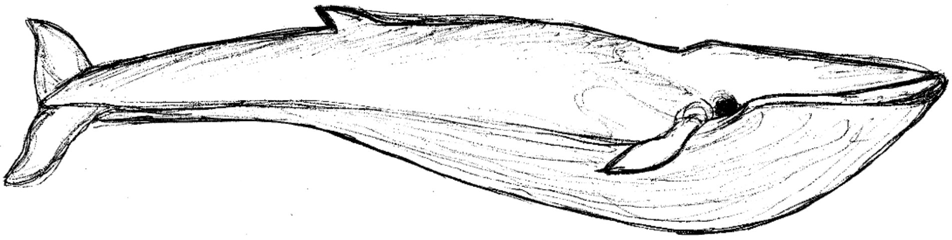


Right whale (*Eubalaena* spp.) owes most probably its name to the early whalers, who looked upon this species as their 'right' target, due to its docile nature, slow surface swimming, high floatability once dead, and the extremely high quantity -and quality- of its blubber. For these reasons the whaling pressure on these species was intense, and led one of them, the North Atlantic *Eubalaena glacialis*, almost to its extinction (Kenney, 2009).

In the South Western Atlantic, Spanish whalers started to catch the southern right whale (*Eubalaena australis*) since the end of the 18th century in the southern Patagonia. Their whaling activity expanded north to include the Brazilian coast (Santa Catarina, Bahía, Río de Janeiro), and was later substituted by local Argentinian and Brazilian whalers. Catches from this early period were probably limited, with reduced consequences on the population demography. Nevertheless, during the 19th century and the beginning of the 20th the species was massively hunted by American and British whalers (Best, 1987). Most of the catches took place between 1820 and 1900 along a continuous band extending over 2,200 km from Portoalegre, in Rio Grande do Sul, Brazil, until Puerto Deseado, in Southern Argentina (Townsend, 1935).

Whales were also killed in the Strait of Magellan and the Falkland Islands and, after the opening of the Antarctic fishery, in the waters near the South Georgia Islands, one of their known summer feeding grounds. Most catches ended in 1935, when the protection of the species was decreed, but Russian whalers ignored the ban and between 1948 and 1973 killed around 3,200 whales, including, in 1962, about 1,300 along the Argentinian coast (Berzin, 2008). Since the early seventies the protection of the species has been effective and the population has recovered to a rate of 7% per annum (IWC, 2001). In recent years, the pup mortality in one of the now more robust cores, breeding on Peninsula Valdés, has substantially increased. This event has been linked to a poor nutritional status of breeding females due to limitations in the availability of food, probably as a consequence of the fact that the population has reached the carrying capacity of the ecosystem. This hypothesis would be surprising, because, although the species has increased in Peninsula Valdés and in Brazil, the two ends of the original distribution, it has not repopulated the intermediate region (Uruguay, Mar del Plata) where, according to the catches of the nineteenth century, it was once abundant (Townsend, 1935). Genetic studies in Peninsula Valdés have shown that individuals of a certain maternal lineage tend to consume the same food and to follow the migratory routes that have learned from their mothers (Valenzuela *et al.*, 2009). According to this hypothesis, the central area of the distribution would not have been repopulated because the segment of the population preserving such cultural memory was lost (Clapham *et al.*, 2008).

Case study 3: The North Atlantic fin whale



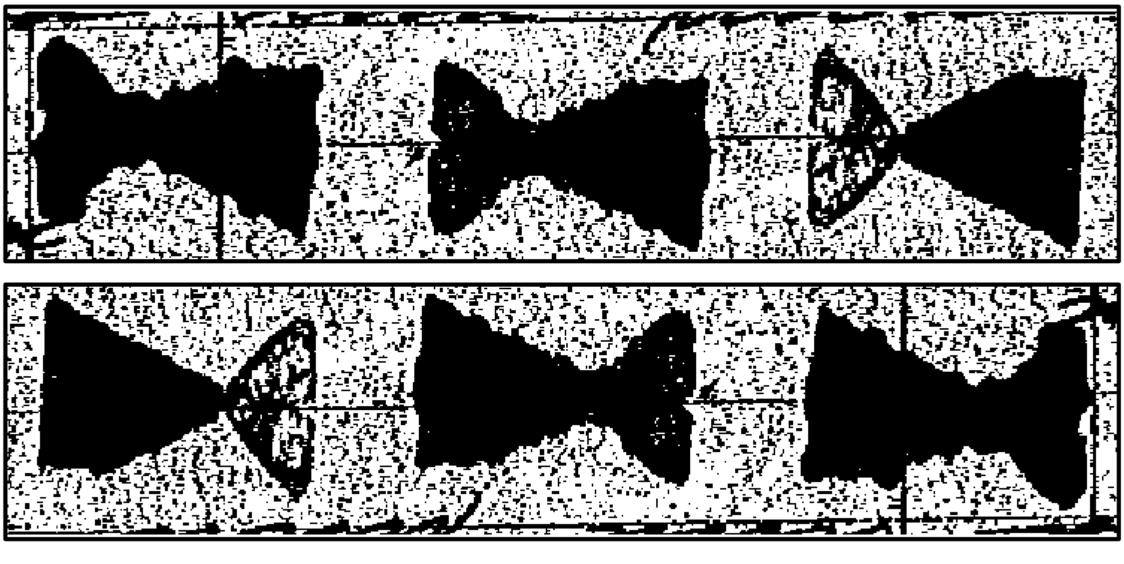
Fin whale (*Balaenoptera physalus*), is one of the most abundant mysticetes in the North Atlantic (Víkingsson *et al.*, 2009), where its original population probably exceeded 70,000 individuals (Christensen, 2006). Like most mysticetes, fin whale performs annual migrations between highly productive summer feeding grounds at high latitudes and low latitude winter breeding grounds. As a consequence, feeding is mainly a seasonal activity which peaks in summer (Lockyer, 1981). Nevertheless, in the North Atlantic, latitudinal migrations seem smaller than in other oceans due to the influence of the Gulf Stream, which creates suitable wintering grounds at relatively high latitudes (Aguilar, 2009).

Since the beginning of modern whaling operations in the Atlantic, the fin whale has been a target species due to its big size (21-22 m and 80 tons) and high yield. The main whaling operations concentrated in Norway, Iceland, Faroe Islands, Ireland, Scotland, Terranova and Iberian Peninsula, where the species was exploited by whaling factories in Gibraltar strait, Portuguese coast and Galicia. Since the 60s catches were limited to Spain, Iceland and Greenland (Jonsgård, 1966). After the IWC moratorium over commercial whaling, the last Spanish factory closed in 1985, but the fin whale is still caught in Iceland and Greenland. Despite some of the populations partially recovered, the IUCN still considers the species as endangered (IUCN, 2015). According to census and recent models, the current size of North Atlantic population is estimated to be over 17,000 individuals, probably averaging the 70% of its original size (Buckland *et al.*, 1992; IWC, 2008). Surprisingly, while at the beginning of exploitation the highest density of fin whales was around Gibraltar strait, this area was never recolonized by fin whales, indicating the total extinction of this segment of the population. On the contrary, in NW Spain, where the species was hunted with equal intensity, the density recovered in the 70s (Aguilar, 1985; 2006).

Based on differences observed in the response to exploitation, marking studies, morphologic differences, genetics and levels of contaminants (Jover, 1992; Víkingsson, 1992; Sanpera *et al.*, 1996; Víkingsson & Gunnlaugsson, 2005; Pampoulie & Danielsdóttir, 2013), the North Atlantic fin whale population has been classified in 7 stocks (IWC, 2009). In recent years, this demographic structure and the proposed limits have been challenged by findings suggesting a smaller scale structuring of the population (Bérubé *et al.*, 1998), erratic movements of the individuals and migratory routes different from those traditionally suggested (Silva *et al.*, 2013; Giménez *et al.*, 2013). At the light of these findings, since 2011 the IWC is carrying on an Implementation Review of North Atlantic fin whale, to reconsider the traditionally accepted demographic structure and pattern of distribution of the different subunits (IWC, 2009).

Among the 7 North Atlantic stocks, the ones summering in the waters off NW Spain and W Iceland have been studied extensively (Aguilar & Lockyer, 1987; Sigurjónsson & Víkingsson, 1997; de Stephanis, 2008; IWC, 2009). Although these studies support the isolation of the two populations at their feeding grounds, the location of the wintering grounds and the patterns of the migratory routes of these populations are still unknown.

Objectives



This thesis focuses on the study of chemical markers of geographic origin and spatial movements and aims to identify those which, with a better cost / benefit ratio, are more useful for investigating the distribution range and population structure of large cetaceans. These markers can become a complementary tool or even an alternative to field surveys and census campaigns, which, at extremely high cost, are now one of the few sources of this information. The expected results obtained through the use of these markers would be relevant for a better understanding of the dynamics of populations of large cetaceans and would therefore be of great interest for the management and conservation of these animals, collectively the most threatened group of vertebrates. The data obtained through the use of chemical markers would in fact help to determine the adequate size and characteristics of the management units that international agencies should apply to make safer management procedures and to avoid the removal of any subpopulation.

Thus, the **main objective** of this thesis is to evaluate the suitability and validity of the use of chemical markers of geographical origin in the study of cetaceans' populations, pointing out strengths and weaknesses of the application of each potential marker in the investigation of cetaceans' population structure and long-term trends.

To achieve this general objective, according to each case study, some **specific objectives** have been pursued:

- When possible, to compare the values of chemical markers with available environmental baselines, either determined through the analysis of organisms at the base of the trophic web, or available through literature;
- To identify levels of variability of each marker within and between the studied populations, and their possible relations with homeostatic mechanisms;
- To evaluate the potential influence of the available biological variables, such as sex, age, reproductive status, on the chemical markers;
- Finally, for each of the studied populations, to test if the markers provide adequate information about population structuring, distribution and migration routes.

The objectives of the thesis were achieved through the analysis of two main groups of chemical markers: stable isotopes and element concentrations, over the populations of three species of cetaceans, including one odontocete (the sperm whale) and two mysticetes (the right whale and the fin whale), sampled in different areas of the Atlantic Ocean.

The thesis is structured in two main sections, referred to the two main groups of chemical markers investigated, and each section is organized in chapters according to the population studied and/or to the specific objectives pursued:

1. **Stable isotopes.**
    - **North Atlantic sperm whale.** To investigate the structure of the population of North Atlantic sperm whales through the analysis of stable isotopes of oxygen, carbon and nitrogen in the dentinal growth layer of teeth (Chapter 1.1).
    - **Southwest Atlantic right whale.** To analyze the carbon, nitrogen and oxygen isotopic values in bone of South Atlantic right whale, to test the existence of segregation between the breeding grounds of Patagonia and southern Brazil. To verify if the results obtained confirm the hypothesis that whaling prevented the

repopulation of the central habitats, once valuable for the population, through the extermination of essential segments of the population, which kept ‘cultural memory’ of the use of these habitats (Chapter 1.2).

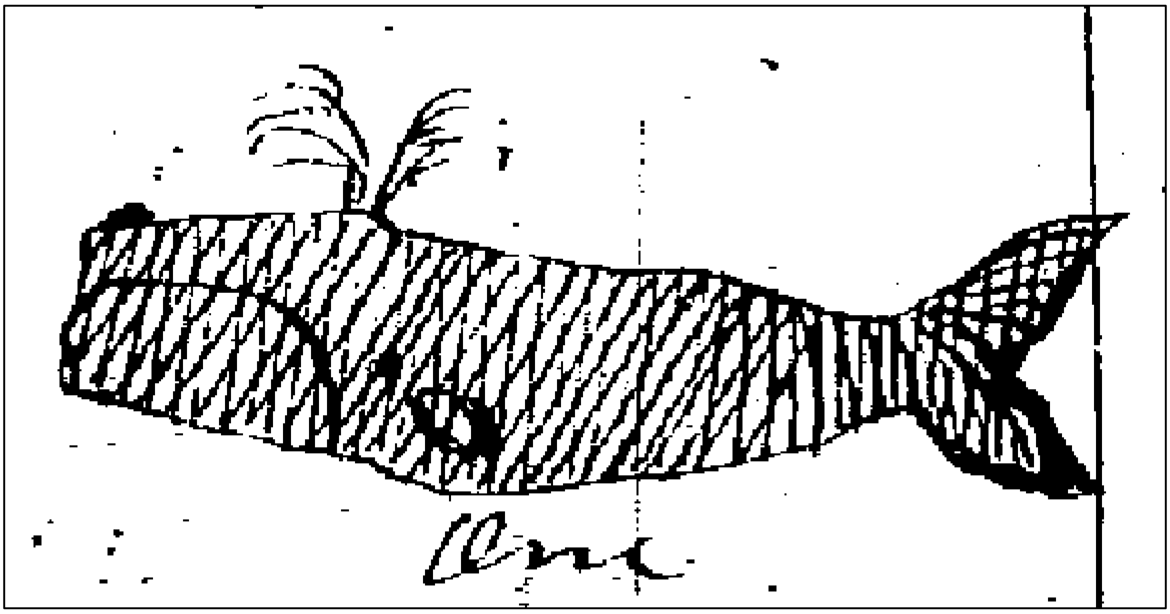
- **North Atlantic fin whale.** To determine stable isotope values of oxygen, carbon and nitrogen in the bone of fin whales sampled in two feeding grounds traditionally classified as isolated: the one located off W Iceland and the one off NW Spain. To estimate the consistency of the results, combined with the carbon and nitrogen stable isotope values of krill sampled from the whales’ stomach, with the available isoscapes of the North Atlantic. To check for differences between subareas in the isotopic values of fin whales’ bones. To contribute with the obtained results to the ongoing discussion about levels of connectivity and migratory movements of the Atlantic fin whale subpopulations (Chapter 1.3).

2. **Elements concentrations.**

   - **Fluoride concentrations in North Atlantic fin whale.** To measure levels of fluoride in krill and bone of fin whales, sampled from the two above mentioned feeding grounds of NW Spain and W Iceland. To test the trends of fluoride concentrations with age and sex of individuals, and to highlight the strengths and weaknesses of the use of fluoride as potential marker for differentiating populations (Chapter 2.1).

   - **Metals concentrations in North Atlantic fin whale.** To analyze concentrations of selected metals (Cu, Pb, Ti, Sr and Zn) in bone of fin whales sampled from the feeding grounds of NW Spain and W Iceland. To describe the trends of metal accumulation with age and sex and to define which, among the metals analyzed, could be good markers for distinguishing subpopulations of fin whales (Chapter 2.2).

1. Stable isotopes



1.1 Stable isotopes provide insight into population structure and segregation in Eastern North Atlantic sperm whales

Authors: Asunción Borrell, Adriana Velásquez Vacca, Ana M. Pinela, Carl Kinze, Christina H. Lockyer, Morgana Vighi, Alex Aguilar

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Título: Los isótopos estables como fuente de información de la estructuración poblacional y la segregación en los cachalotes del Atlántico Nororiental

Resumen:

En las especies pelágicas que habitan los grandes océanos, la diferenciación genética tiende a ser leve y las poblaciones carentes de estructura. Sin embargo, los grandes cetáceos han proporcionado muchos ejemplos de estructuración. En el presente estudio investigamos si el cachalote, una especie pelágica con poblaciones de gran tamaño y supuestamente provista de gran movilidad, muestra indicios de estructuración en el Atlántico Norte oriental, una cuenca oceánica en la que se cree que exista una única población. Para hacer eso, examinamos los valores de isótopos estables en grupos de capas de crecimiento secuencial de los dientes de individuos muestreados en Dinamarca y en el noroeste de España. En cada capa se midió la proporción isotópica de oxígeno ($\delta^{18}O$) en el componente inorgánico (hidroxi-apatita), y las proporciones de isotopos de nitrógeno y de carbono ($\delta^{15}N$; $\delta^{13}C$) en el componente orgánico (principalmente colágena). Se encontraron diferencias significativas entre Dinamarca y NW España en: los valores de $\delta^{15}N$ y $\delta^{18}O$ en la capa depositada a los 3 años (considerada como la mejor representación de la línea de base del área de reproducción); los valores de $\delta^{15}N$, $\delta^{13}C$ y $\delta^{18}O$ en el período de hasta 20 años de edad; la variación ontogenética de los valores de $\delta^{15}N$ y $\delta^{18}O$. Estas diferencias evidencian que la composición de la dieta, el uso de hábitat y / o los destinos migratorios son diferentes entre los cachalotes de la dos regiones y sugieren que la población de cachalotes del Atlántico Norte es más estructurada de lo que se ha aceptado tradicionalmente.

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Stable Isotopes Provide Insight into Population Structure and Segregation in Eastern North Atlantic Sperm Whales

Asunción Borrell[1*], Adriana Velásquez Vacca[1], Ana M. Pinela[1], Carl Kinze[2], Christina H. Lockyer[3], Morgana Vighi[1], Alex Aguilar[1]

1 Department of Animal Biology, Faculty of Biology and Biodiversity Research Institute (IRBio), University of Barcelona, Barcelona, Spain, **2** Zoological Museum, University of Copenhagen, Copenhagen, Denmark, **3** Age Dynamics, Konhgens Lyngby, Denmark

Abstract

In pelagic species inhabiting large oceans, genetic differentiation tends to be mild and populations devoid of structure. However, large cetaceans have provided many examples of structuring. Here we investigate whether the sperm whale, a pelagic species with large population sizes and reputedly highly mobile, shows indication of structuring in the eastern North Atlantic, an ocean basin in which a single population is believed to occur. To do so, we examined stable isotope values in sequential growth layer groups of teeth from individuals sampled in Denmark and NW Spain. In each layer we measured oxygen- isotope ratios ($\delta^{18}O$) in the inorganic component (hydroxyapatite), and nitrogen and carbon isotope ratios ($\delta^{15}N$: $\delta^{13}C$) in the organic component (primarily collagenous). We found significant differences between Denmark and NW Spain in $\delta^{15}N$ and $\delta^{18}O$ values in the layer deposited at age 3, considered to be the one best representing the baseline of the breeding ground, in $\delta^{15}N$, $\delta^{13}C$ and $\delta^{18}O$ values in the period up to age 20, and in the ontogenetic variation of $\delta^{15}N$ and $\delta^{18}O$ values. These differences evidence that diet composition, use of habitat and/or migratory destinations are dissimilar between whales from the two regions and suggest that the North Atlantic population of sperm whales is more structured than traditionally accepted.

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* E-mail: xonborrell@ub.edu

Introduction

In the main oceans of the world, the absence of geographical barriers permits pelagic species to occupy vast geographic ranges inside which they often engage in long migratory movements. This result in high population sizes which, together with the high mobility of many of the species, favours mild genetic differentiation and consequent absence of structuring [1,2]. However, among large cetaceans there have been recurrent examples of structuring and segregation within otherwise large and apparently homogeneous areas of distribution, probably as a consequence of environmental patchiness, the evolutionary history of populations, behavioural traits such as resource partitioning and social affiliations, and cultural or maternally transmitted fidelity of migratory destinations [3-6].

Sperm whales are migratory odontocetes situated at the top of the food web that inhabit mesopelagic marine ecosystems. They are slow-growing animals that live as long as 70 years [7] and their diet varies with sex, age and geographical region [8-10]. Individuals from different segments of the population establish long-lasting bonds and group into social units which often show allopatric geographic distributions. Thus, adult females with their offspring and juveniles form the matrilineally based social units, the so-called primary social groups, which occupy tropical and temperate waters of all large oceans. When reaching an age between 4 and 21 yr males leave the breeding units to join the bachelor schools, structures which are thus formed by males in their teens and twenties that are not considered to be socially mature. From their mid-twenties, males may start migrating from low to high latitudes. Males in their forties and older are usually solitary and tend to move to higher latitudes where they remain until returning on an unknown schedule to lower latitudes to meet the primary social groups and reproduce [11-15]. However, the frequency and duration of the large-scale latitudinal migrations a well as the extent of the geographical segregation are still poorly understood.

Sperm whales have a global distribution and sustain significant populations in all large oceans of the world. In the North Atlantic, since long ago the International Whaling Commission (IWC) has recognized the existence of only one single stock of sperm whales based on analyses of commercial whaling data, distribution of sightings, evidences of long-range movements from mark-recaptures within the North Atlantic and similarities between different locations in mortality rates and trends in body lengths [16-18]. For instance, harpoons or harpoon fragments from the Azores were found, years after their deployment, in the bodies of whales killed off Iceland and NW Spain, indicating long-range movements across the eastern North Atlantic [19-21]. Moreover, the case for the existence of a single ocean-wide population received later support by studies showing absence of subpopulation structure in other large oceans [22] and by worldwide genetic studies that were unable to find clearly distinct subpopulations at less than an ocean basin level [23,24]. However, some doubts have persisted not only because data from historical pelagic exploitation in the North Atlantic showed clearly delineated whaling grounds suggestive of significant patchiness in the species distribution [25,26] but also because genetic studies have revealed differences between sperm whales from the Mediterranean Sea and their neighbouring eastern North Atlantic counterparts, thus indicating that subpopulation structuring may indeed occur [27].

Some light into this subject can be shed by the use of stable isotopes, chemical markers that in the last decades have become a technique of choice to track movement and to define geographic occupancy in many species [28-32], including sperm whales [33,34]. Due to differences in the isotope elemental mass, the isotope composition of animal tissues reflect a combination of the parameters of the environment in which they live, such as temperature, salinity and productivity, in addition to biological parameters characteristic to the individuals involved, such as dietary preferences, trophic relationships, physiology and behaviour [35]. Among the various body tissues, tooth dentine, which is made of proteins and hydroxyapatite, is particularly useful because it is deposited in the crown and root of the teeth during the entire life of the animal without existence of turnover [36]. As a consequence, the growth layers that form each year provide material for the reconstruction of ontogenetic time series of isotopic values [37]. The stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) are the most commonly used in this type of studies, but the stable isotopes of other elements, such as oxygen ($\delta^{18}O$) are also seldom used to provide further resolution [38]. In marine mammals, these three elements are incorporated mostly through dietary protein and ingested water, and their isotopes are subject to different and predictable changes when being transferred through the ecosystem, thus allowing tracing of animal's movements and foraging behaviour [30,37,39-42].

In this study, stable isotope signatures of carbon, nitrogen and oxygen were studied in the dentinal growth layer groups (annual groups of laminae) of nine sperm whales sampled at two different geographical regions in the North Atlantic (NW Spain and Denmark) to investigate potential differences in the deposited isotopic signatures, particularly in the first years of life, which can be indicative of different reproductive regions (breeding units).

Materials and Methods

Ethics Statement

The sperm whale teeth used for this study were obtained from the biological tissue bank of the University of Barcelona (BMA Tissue Bank) and originated either from commercial fisheries (samples from NW Spain) or from naturally stranded individuals (samples from Denmark). No specific approval is required in Spain to undertake research on samples supplied by official channels and coming from commercial fisheries or stranded individuals.

Study site and sampling

Five individuals were caught off northwestern Spain (Galicia, thereafter NW Spain) and flensed at the whaling factories of the company Industria Ballenera S. A. during the summer whaling seasons of 1978 and 1980 under catch quotes issued by the International Whaling Commission. Four individuals were sampled from two mass strandings that took place on the northern shores of the island of Rømø in the southwestern Danish North Sea coast [43] (Figure 1). In both regions the whales were measured and sexed (Table 1), teeth were extracted and preserved at the biological tissue bank of the University of Barcelona (BMA Tissue Bank).

Tooth sampling

Teeth were cut in half, with one half being used for age determination and the other one to obtain the dentine samples for isotope analysis. For the latter purpose, circa 20 mg of dentine powder from each growth layer group (GLG) was drilled off using a Dremel™ driller. The powder was divided into two subsamples, one for carbon and nitrogen determination and one for oxygen determination.

Age estimation

The age of individuals was estimated by counting dentinal GLGs. Independent age estimates were obtained by two different readers (AA and CL) to account for subjectivity. GLGs were interpreted as those identified in the report of the Workshop on Age Determination of Odontocete Cetaceans and Sirenians [44] as "a repeating or semi-repeating pattern of adjacent groups of incremental growth layers within the dentine which is defined as a countable unit involving a change from a ridge to groove". The neonatal line was not included in the total number of GLGs. The final age estimate for each individual was determined as the mean of all counts (pooling the counts of the two readers).

To compare the isotopic signal while individuals were still at the breeding grounds we compared values in GLGs formed during the third year of life. This age was chosen to prevent isotope signature variations caused by: i) lactation, because $\delta^{15}N$ in mammals enriches during maternal nourishment [45-49] and sperm whale calves suckle for approximately 14-16

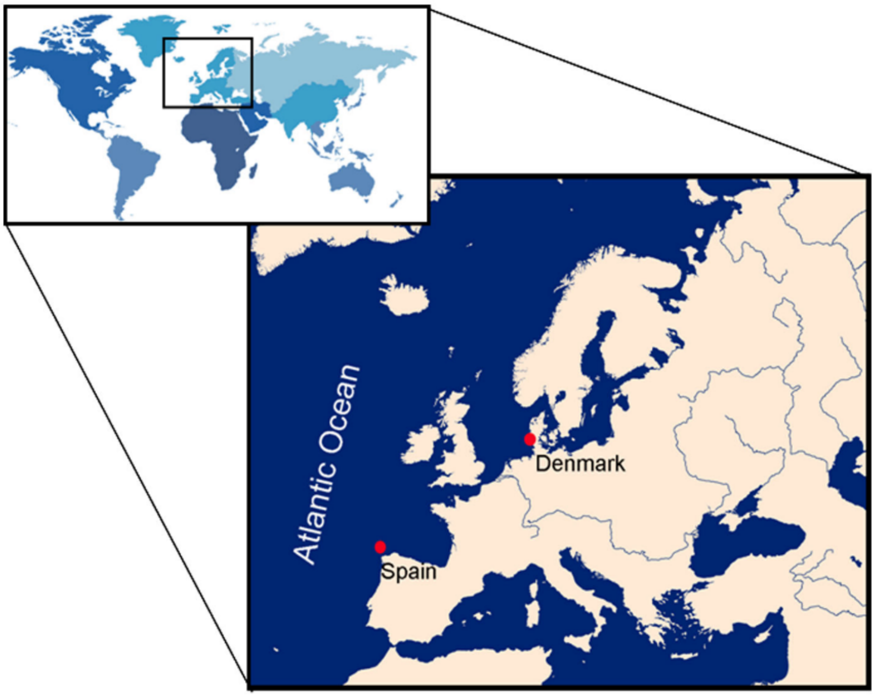

Figure 1. Location of sampling sites.

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months [8]; and ii) migration, because juveniles leave their breeding units at an age of 4-21 years [12,50] so they are expected to start changing their tissue isotopic values thereafter. However, in individual I from Denmark the distal tip of the tooth was worn and the GLG corresponding to the fifth year was the first that could be properly sampled and, because age 5 is still at the bottom of the range of first migration, the isotope values obtained from that GLG were used to characterise the breeding ground signature of that particular individual.

Stable isotopes

Carbon and nitrogen. When analysing teeth, some authors carry out a preventive demineralisation to eliminate the inorganic carbon by treating the teeth with either a 0.5 M or a 1 M hydrochloric acid (HCl) solution [33,34,37,51]. However, concern has been expressed that such treatment could adversely affect the nitrogen isotopic signature [52]. Taking this into account, we conducted a test on a subset of the samples (n = 20) and found that neither $\delta^{13}C$ nor $\delta^{15}N$ values differed between demineralised and untreated samples, so subsequent analyses were carried out without demineralisation.

Table 1. Information on the sperm whales used in the study.

ID	Sex	Body length (m)	Region	Source	Year of sampling	Estimated age (years)	Number of samples analysed
A	Male	12.2	NW Spain	Captured	1980	16	9
B	Male	11.6	NW Spain	Captured	1980	18	10
C	Female	10.9	NW Spain	Captured	1978	18	9
D	Female	9.5	NW Spain	Captured	1978	20	9
E	Female	10.1	NW Spain	Captured	1978	13	6
F	Male	12.6	Denmark	Stranded	1996	24	7
G	Male	13.2	Denmark	Stranded	1996	27	8
H	Male	12.9	Denmark	Stranded	1996	22	7
I	Male	14.0	Denmark	Stranded	1997	55	12

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Approximately 1 mg of the powdered sample was weighed in tin capsules, automatically-loaded, and combusted at 1000 °C to be analysed in a continuous flow isotope ratio mass spectrometer (Flash 1112 IRMS Delta C Series EA Thermo Finnigan). Standards for ^{13}C and ^{15}N were the Vienna Pee Dee Belemnite limestone (V-PDB) standard and atmospheric nitrogen (air), respectively. International isotope secondary standards of known $^{13}C/^{12}C$ ratios in relation to V-PDB, namely: polyethylene (IAEA CH_7; $\delta^{13}C$ = -31.8‰), graphite (USGS24; $\delta^{13}C$ = -16.1‰) and sucrose (IAEA-CH_6; $\delta^{13}C$ = -10.4‰), were used for calibration of $\delta^{13}C$ at a precision of 0.2‰. For nitrogen, international isotope secondary standards of known $^{15}N/^{14}N$ ratios in relation to air, namely: ammonium sulphate (IAEA N1; $\delta^{15}N$ = +0.4‰ and IAEA N_2; $\delta^{15}N$ = +20.3‰) and potassium nitrate (IAEA NO_3; $\delta^{15}N$ = +4.7‰) were used for calibration of $\delta^{15}N$ to a precision of 0.3‰. Atropine (70.56%C, 4.84%N) was used as a standard for elemental composition of C and N. The experimental precision based on the standard deviation of replicates of an atropine standard was 0.3% for both carbon and nitrogen. The reference materials used for the analysis are distributed by the International Atomic Energy Agency (IAEA).

Oxygen. Dentine powder was pre-treated with 30% hydrogen peroxide for 24 hours to remove organics, then rinsed carefully with milli-Q water and treated with 1M calcium acetate /acetic acid buffer for another 24 hours to remove any diagenetic carbonate. Then it was rinsed again following the same procedure and dried for 24 hours. Stable isotopic determinations were carried out in 10 mg of dentine using a Finnigan-MAT 252 mass spectrometer, fitted with a Kiel Carbonate Device III (Thermo Electron - Dual Inlet) where samples were dissolved in 100% phosphoric acid at 70°C with concurrent cryogenic trapping of CO_2 and H_2O. The CO_2 was then admitted to the mass spectrometer for analysis. NBS-19 international standard was used, with $\delta^{18}O$ (in relation to V-PDB) = −2.20‰ values, certified by the IAEA. Analytical reproducibility (1σ parameter) obtained from replicate analyses of the powder fraction of reference material NBS-19 was better than ±0.08 for $\delta^{18}O$. Analyses were carried out in the laboratories of the University of Barcelona (Centres Científics i Teconlògics, CCiT-UB).

The natural abundances of ^{13}C, ^{15}N, and ^{18}O are expressed as permil (‰) deviation from the standards as defined by the following equation [53]:

$$\delta^i E = \left[R(iE/jE)_s - R(iE/jE)_{std}\right] / R(iE/jE)_{std}$$

where R is the ratio of the heavy isotope (^iE) to the light isotope (^jE) of element E, in the sample (s) and in the standard (std).

Because $\delta^{18}O$ values in zoology are more commonly presented relative to standard mean ocean water (SMOW) and, to allow comparison with published data, $\delta^{18}O$ values were converted from V-PDB to SMOW using the following equation [54]:

$$\delta^{18}O_{SMOW} = 1.03086 \times \delta^{18}O_{V-PDB} + 30.86$$

Statistical analysis

Data were tested for normality with a Kolmogorov-Smirnov test of goodness of fit and homogeneity of variances with the Levene's test. Because all δ values from both the NW Spain and the Denmark groups of samples showed a normal distribution ($Z<0.84$ and $p>0.05$), subsequent analyses were conducted with parametric tests. T-student test was used to compare means of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ between regions in the 3rd year dentinal GLG.

To investigate the influence of age and sex in the distinction between the two regions, and the interactions among these factors, an ANCOVA with age as covariate and region and sex as fixed factors was performed. Moreover, as the age span of individuals varied markedly and the individuals from NW Spain were noticeably younger than those from Denmark, the analysis was restricted to the age-segment corresponding to the first 20 years of life.

Age was observed to have a significant influence on the stable isotope values, so the stable isotope age-related trends in each individual were investigated through the application of both a general linear model (GLM) and a non-linear general additive model (GAM) with varying number of knots depending on the number of observations. The fit of the two models for each individual was evaluated through the comparison of their relative AIC (Akaike's Information Criterion) values.

Table 2. Statistical results of Independent samples test between stable isotope values of the 3rd year dentinal growth layer group (GLG) in sperm whales from NW Spain and Denmark.

	Levene's test for equality of variances		t-test for equality of means				
	F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std error difference
$\delta^{13}C$	0.17	0.69	0.26	7	0.80	0.11	0.42
$\delta^{15}N$	0.10	0.76	-3.33	7	0.01	-2.79	0.84
$\delta^{18}O$	0.67	0.44	2.68	7	0.03	0.46	0.17

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Statistical calculations were carried out using the statistical package SPSS15 (SPSS Inc., Chicago, IL, USA), and the mgcv package in the R-3.0.2 software

Results

Table S1 details the stable isotope values and the biological information available from each individual analysed. The C/N ratio of the tooth dentine ranged 2.8-3.4 (mean: 3; sd:± 0.11), values which are well within the range of unaltered collagen, thus indicating absence of contamination by lipids or other materials [55].

Nitrogen isotopes

Specimens from NW Spain showed $\delta^{15}N$ values ranging from 13.5‰ to 17.9‰ (mean:15.4‰; sd:±1.1) and those from Denmark ranging from 11.8‰ to 16.2‰ (mean:13.9‰; sd: ±1.0). The $\delta^{15}N$ in dentine deposited at 3 years of age was significantly lower in sperm whales from Denmark (mean: 13.0‰; sd:±1.0) than in those from NW Spain (mean:15.8‰; sd:±1.4) (Table 2, Figure 2). To exclude the effect of potential variation in isotopic baselines with time, we examined the relationship between $\delta^{15}N$ values and the year of formation of the 3rd year dentinal GLG in sperm whales from both regions and found absence of correlation (Figure 3a, Denmark: Pearson's r=-0.27, p=0.73; Spain: Pearson's r=-0.50, p=0.39).

Results from the ANCOVA indicated that region (F(1, 62)=18.63, $p<0.001$) and age (F(1, 62)=6.72, $p<0.05$) showed strong interaction with $\delta^{15}N$, while sex (F(1, 62)=3.80, p=0.056) was close to show interaction (Table S2).

Trends of $\delta^{15}N$ with age for each individual are depicted in Figures 4a and 4b, while AIC and p values resulting from the application of the GLM and GAM models to each individual set of data are shown in Table 3. Based on the results obtained from the application of the two models and the comparison of

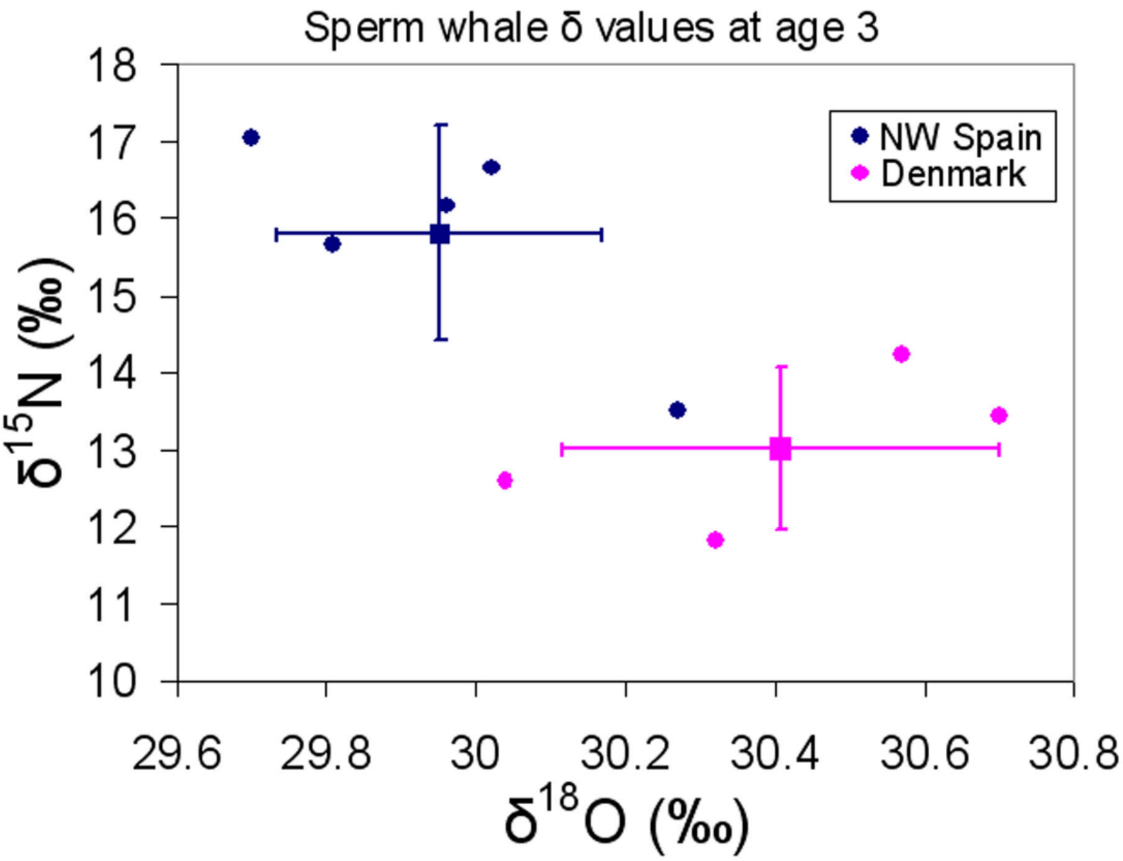

Figure 2. Comparison of $\delta^{15}N$ and $\delta^{18}O$ values (mean±sd) of the 3rd year dentinal growth layer group (GLG) in sperm whales sampled in Denmark (*n*=4) and NW Spain (*n*=5) ($p<0.05$ for $\delta^{15}N$; and $p<0.01$ for $\delta^{18}O$).
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the relative AIC values, $\delta^{15}N$ showed a significant positive linear correlation with age in two (F and G) out of the four males sampled in Denmark, while one of the other males from Denmark (I) showed a non-linear relationship and the other (H) showed a non-significant trend (Figure 4b). Regarding the five individuals from NW Spain, $\delta^{15}N$ significantly decreased linearly with age in the three females (C, D and E) and in one male (A), while another male (B) showed a non-significant trend (Figure 4a). No substantial differences in the AIC values of the two models were found in any of the NW Spain individuals (Table 3).

Carbon isotopes

$\delta^{13}C$ values in whales from NW Spain ranged from -13.6‰ to -11.6‰ (mean:-12.4‰; sd:±0.5), whereas in those from Denmark ranged from -14.3‰ to -11.7‰ (mean:-12.9‰; sd:± 0.7). No significant difference was found in dentine deposited at 3 years of age between sperm whales from Denmark (mean:-12.4‰; sd:±0.6) and NW Spain (mean:-12.6‰; sd:± 0.3) (Table 2). No correlation was found between $\delta^{13}C$ values and the year of formation of the 3rd year dentinal GLG in the sperm whales from any of the regions (Figure 3b. Denmark: Pearson's r=0.78, p=0.22; Spain: Pearson's r=-0.14, p=0.82).

Results from the ANCOVA indicated that region (F(1, 62)=6.01, $p<0.05$) showed strong interaction with $\delta^{13}C$, while sex (F(1, 62)=1.40, p=0.241) and age (F(1, 62)=0.29, p=0.593) did not show interaction (Table S2).

Trends of $\delta^{13}C$ with age for each individual are depicted in Figures 4c and 4d, while AIC and p values resulting from the application of the GLM and GAM models to each individual set of data are shown in Table 3. $\delta^{13}C$ showed a significant negative linear correlation with age in two (H and I) out of the four males from Denmark, while none of the other males from Denmark showed any significant trend (F and G) (Figure 4d). Regarding the five individuals from NW Spain, $\delta^{13}C$ showed a significant negative linear correlation with age in one male (A) and a significant non-linear correlation in a female (C). In the other three individuals (B, D and E), no significant $\delta^{13}C$ trends were detected (Figure 4c). In these three individuals, the AIC values resulting from the application of the GAM model were lower than those resulting from the GLM model (Table 3).

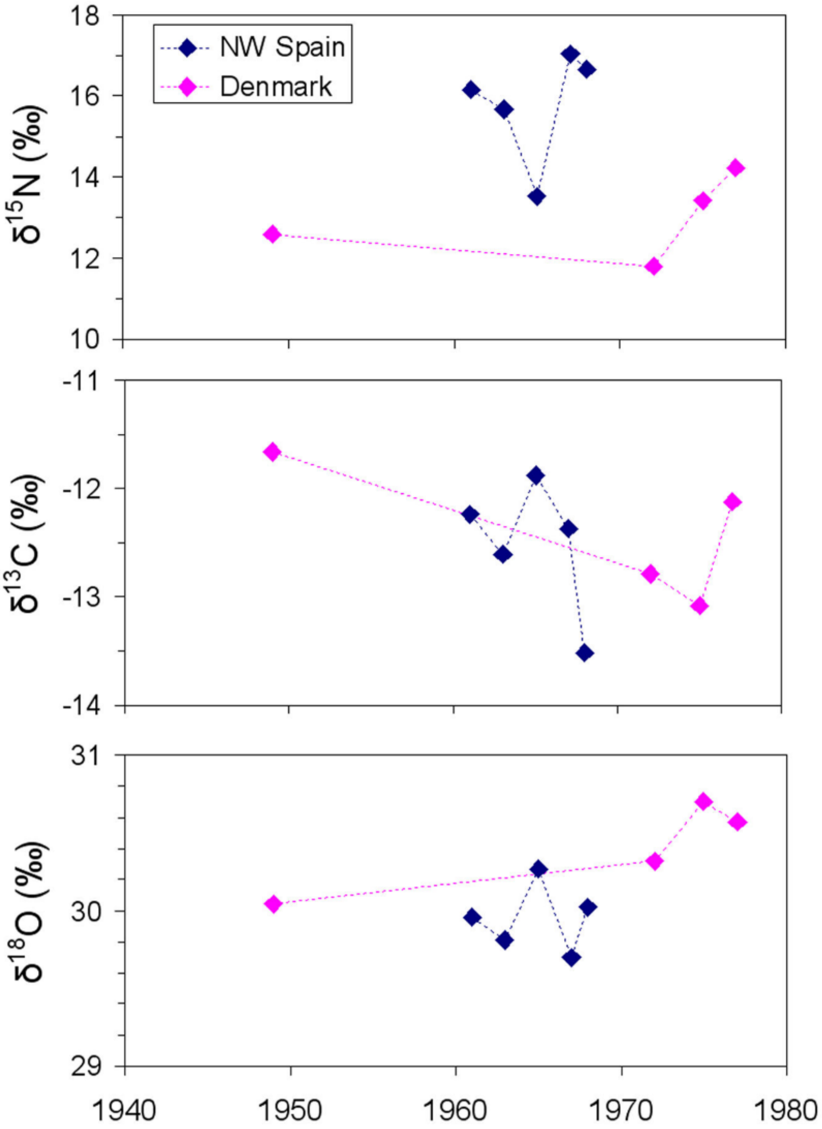

Figure 3. Relationship between δ^{15}N, δ^{13}C and δ^{18}O values and the year of formation of the 3rd year dentinal growth layer group (GLG) in sperm whales from NW Spain and Denmark.

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Oxygen isotopes

δ^{18}O values in whales from NW Spain ranged from 27.9‰ to 30.5‰ (mean:29.8‰; sd:±0.5), whereas in whales from Denmark ranged from 28.2‰ to 30.7‰ (mean:29.9‰; sd:±0.6). A significant difference was found in the dentine deposited at 3 years of age between sperm whales from Denmark (mean: 30.4‰; sd:±0.3) and NW Spain (mean:30.0‰; sd:± 0.2) (Table 2, Figure 2). No correlation was found between δ^{18}O values and the year of formation of the 3rd year dentinal GLG in the sperm whales from any of the regions (Figure 3c, Denmark: Pearson's r=-0.84, p=0.16; Spain: Pearson's r=0.65, p=0.23).

Results from the ANCOVA indicated that region (F(1, 58)=6.16, $p<0.05$) and age (F(1, 58)=16.23, $p<0.001$) showed strong interaction with δ^{18}O, while sex (F(1, 58)=0.7, p=0.405) did not show interaction (Table S2).

Trends of δ^{18}O variations with age for each individual are depicted in Figures 4e and 4f, while AIC and p values resulting from the application of the GLM and GAM models to each individual set of data are showed in Table 3. δ^{18}O significantly linearly decreased in two (G and H) out of the four males sampled in Denmark, while it did not show any trend in the remaining two (Figure 4f) individuals from Denmark, nor in the five individuals from NW Spain (Figure 4e). The AIC values calculated in the two models were nearly similar for all individuals (Table 3).

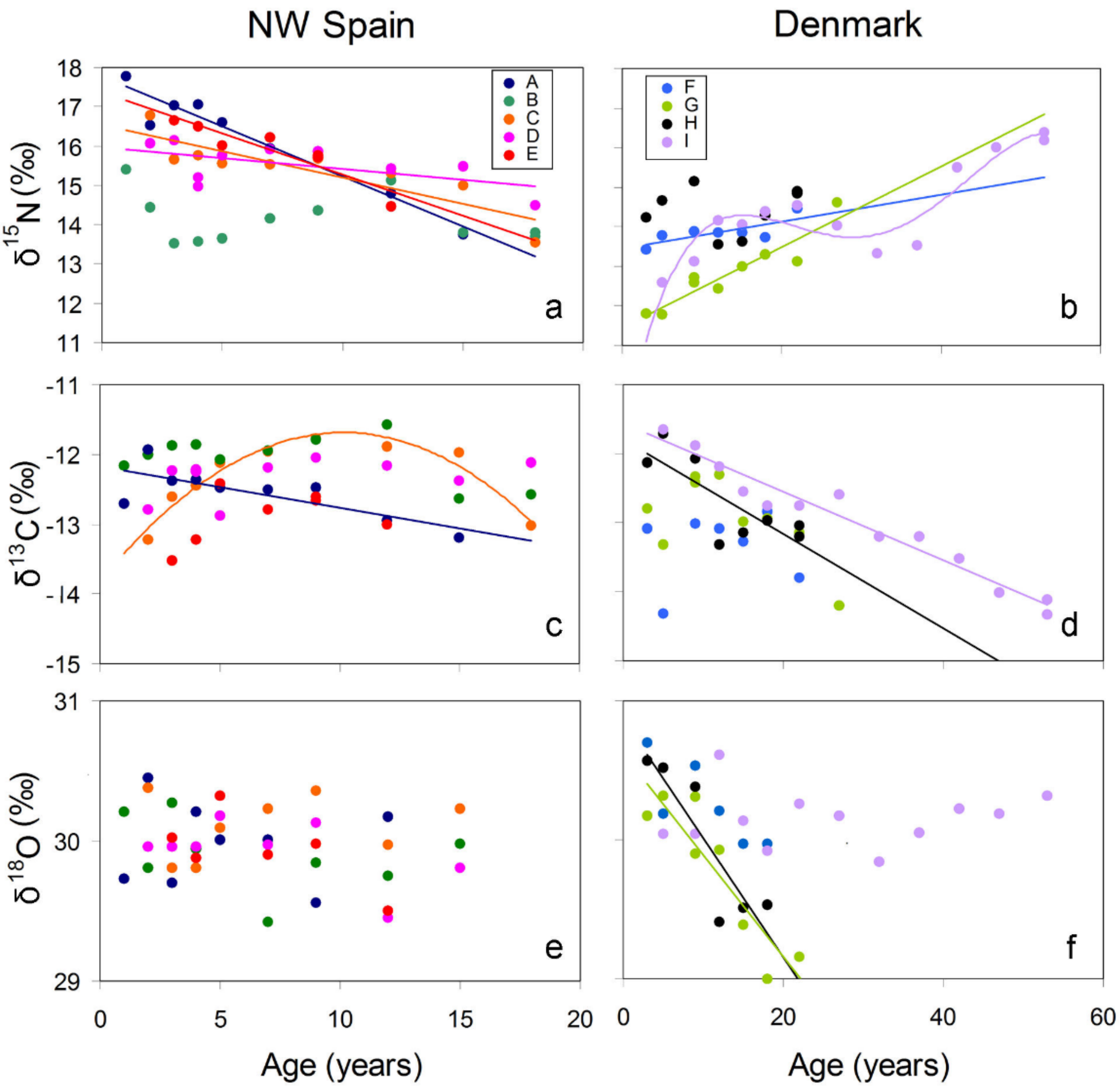

Figure 4. Relationship between stable isotope values ($\delta^{15}N$: a, b; $\delta^{13}C$: c, d and $\delta^{18}O$: e, f) and age in sperm whales sampled in Denmark and NW Spain. Each individual, identified as A-I according to details shown in Table 1, is represented by a different colour.

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Discussion

The goal of the present study was to explore the utility of stable isotopes to investigate the occurrence of structure in the eastern North Atlantic population of sperm whales. More specifically, through the examination of individual age-related variation of stable isotope values in the GLGs in teeth we sought to determine potential segregation in breeding areas and/or dissimilarities in long-range movements throughout life-span.

The departing hypothesis was that, if the studied individuals belonged to the same population, females and young individuals of both sexes would stay at the same breeding grounds, while adult males would migrate towards northern latitudes during at least part of their cycle. Because the isotopic signal of the environment is permanently recorded in the teeth dentine deposited each season [33-35], if the hypothesis were true we anticipated that the isotopic values of the GLGs corresponding to the first years of life would be similar in males and females, while those deposited at older ages would differ. We considered the GLG formed at age 3 as the best indication

Table 3. AIC and p values resulting from the application of the linear GLM and non-linear GAM models to each individual set of data to investigate the correlation between stable isotope values and age.

		GLM		GAM	
	ID	**AIC**	**p**	**AIC**	**p**
$\delta^{15}N$	A	11.47	<0.001	11.47	<0.001
	B	24.46	0.61	24.46	0.61
	C	15.28	<0.01	15.28	<0.01
	D	12.02	<0.05	12.02	<0.05
	E	7.01	<0.001	6.86●	<0.001
	F	4.39	<0.05	4.39	<0.05
	G	9.40	<0.001	9.40	<0.001
	H	17.19	0.90	17.19	0.90
	I	31.66	<0.001	18.11●	<0.001
$\delta^{13}C$	A	4.71	<0.05	4.71	<0.05
	B	8.26	0.17	3.47●	0.07
	C	17.88	0.64	-0.83●	<0.001
	D	6.97	0.24	6.97	0.24
	E	9.91	0.53	9.21●	0.69
	F	15.70	0.82	15.70	0.82
	G	15.90	0.13	10.83●	0.62
	H	10.87	<0.05	10.86●	<0.05
	I	-0.09	<0.001	-0.09	<0.001
$\delta^{18}O$	A	8.84	0.98	8.84	0.98
	B	5.92	0.42	5.49●	0.60
	C	4.19	0.64	4.19	0.64
	D	-0.92	0.10	-0.92	0.10
	E	2.09	0.14	2.09	0.14
	F	1.47	0.09	1.47	0.09
	G	5.78	<0.05	5.78	<0.05
	H	5.53	<0.05	5.53	<0.05
	I	1.45	0.69	1.45	0.69

● indicates a lower AIC value resulting from the application of the GAM model than that of GLM.

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of the isotopic signal of the breeding grounds because at that age sperm whales have already been usually weaned but have not yet abandoned the breeding school [8,12,50]. The investigation had necessarily to focus on a limited number of individuals because in a long-lived species like the sperm whale the detailed study of age-related trends requires extensive sub-sampling of GLGs. Indeed, the sample size used here is well within the range of previous similar studies conducted on this and other comparable long-lived species [33,34,56].

Individuals from Denmark were all males, as expected given that only sperm whales of this gender occupy waters situated north of 40-45°N, while individuals from NW Spain belonged to both sexes (3 females and 2 males), again as expected because this region is within the latitudinal range of occurrence of the breeding schools [7,14]. Irrespectively of their gender, at age 3 individuals from Denmark showed lower $\delta^{15}N$, similar $\delta^{13}C$ and higher $\delta^{18}O$ than those from NW Spain (Table 2, Figure 2). The high variability among individuals from the same region (see Figure 2) could be due to shifts in maternal foraging areas by year or time period [22]. Because individuals had been sampled in different years and their total age was very variable, the year corresponding to age 3 differed greatly. To exclude the effect of potential variation in isotopic baselines with time, we examined the relationship between $\delta^{15}N$, $\delta^{13}C$ and $\delta^{18}O$ values and the year of formation of the 3rd year dentinal GLG in sperm whales from both regions and found absence of trend (Figure 3). Environmental factors such as temperature, light intensity and nutrient concentration are known to affect the $\delta^{18}O$, $\delta^{13}C$ and $\delta^{15}N$ values of primary producers [57] and therefore the dissimilarities in these profiles indicate that the geographical areas where the individuals had been born presented substantially different environmental characteristics and, very likely, were distant.

However, the difference between sperm whales sampled in Denmark and in NW Spain was not apparently restricted to the early period of life. Thus, the ANCOVA test also demonstrated significant differences during the first 20 years, the segment of lifespan that was represented in the two regions. Most males from Denmark showed a significant increase, either linear or quadratic, in $\delta^{15}N$ values with age (Figure 4b). Conversely, in NW Spain one male and all females showed a significant decreasing trend (Figure 4a). Previous studies [33,34] have shown considerable variation in both values and trends between individuals. However, $\delta^{15}N$ in males usually tended to increase with age, as is the case in individuals from Iceland and Scotland [33,34]. This trend is attributed to the increasing strength and diving capacity that individuals acquire when growing old, characteristics that allow them to progressively feed on bigger prey, mostly giant squids, situated at higher trophic levels [8,58-60]. Why that trend was observed in most males from Denmark but the two males from NW Spain showed either no trend or a decreasing trend is unclear but again the difference is suggestive of dissimilarities between individuals from the two regions, either on their foraging behaviour or in their patterns of movements and, consequently, on the $\delta^{15}N$ baselines of the waters they inhabit. Indeed, other males previously analysed from the Mediterranean and from India also showed age-related decreasing trends in their $\delta^{15}N$ values [33]. On the other hand, the decreasing trend in $\delta^{15}N$ values found in the females from NW Spain is probably explained by the fact that after the onset of reproductive activity they are likely to reduce the depth of their dives both during pregnancy and lactation and therefore to shift to shallower prey [9,10]. This is confirmed by previous analyses on another female from NW Spain which did not show any age-related $\delta^{15}N$ trend and on two other females, one from the Azores and another from the Mediterranean Sea, that did show a decreasing trend [33].

$\delta^{13}C$ values were also highly variable between individuals but again showed significant differences between regions (Figures 4c and 4d). In males, values decreased in about half of the sample both in Denmark and in NW Spain, as has been previously found to occur in individuals from Scotland [34], while the other half showed no definite trend as seen in whales from other regions [33]. In the females from NW Spain, values of one individual showed a non-linear correlation with age, while those of the other two individuals did not show any trend, again as previously observed elsewhere [33]. The high variability observed in all sample groups probably reflected periodic or seasonal changes in diet composition or in movements, like shifting from inshore to offshore waters. In particular, published carbon isoscapes suggest that the age-related decrease in $\delta^{13}C$ values found in Denmark males (Figure 4d) could reflect a tendency to move to higher latitudes when they grow older [30,34,61].

As expected in a marine species [39], variability of $\delta^{18}O$ values was small but still displayed substantial inter-annual fluctuation, the pattern of which differed between individuals. Two males from Denmark displayed a decreasing trend of $\delta^{18}O$ with age (Figure 4f) but neither the other males from the same region nor any individual from NW Spain, irrespective of its gender, showed any trend (Figure 4e). Because $\delta^{18}O$ tends to be depleted in higher latitudes and in colder water [41,62], the decrease found in the former group of males is likely to reflect migration to higher latitude or an increasing tendency to consume deeper, colder water prey as the individual grows old.

Overall, the differences observed in C, N and O stable isotopes and trends point towards the existence of heterogeneities in the habitat used by sperm whales from Denmark and NW Spain, particularly in the location of their breeding grounds. This suggests the occurrence of structure in the eastern North Atlantic population of the species.

Given that currently only one single management unit of the species is recognized for the whole North Atlantic [16,17], further research is needed to deepen into such potential structuring and, if occurring, to define the actual borders of any subpopulation.

Supporting Information

Table S1. Individual isotope values and biological information.
(XLS)

Table S2. Statistical results of One-way Analysis of Covariance (ANCOVA) with isotope values as dependent variable and age as covariate and region and sex as fixed factors.
(DOC)

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Author Contributions

Conceived and designed the experiments: AB AA CHL CK. Performed the experiments: AB AVV AMP AA. Analyzed the data: AB AVV MV. Contributed reagents/materials/analysis tools: AB AA AVV AMP CHL CK MV. Wrote the manuscript: AB AA.

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1.2 Stable isotopes indicate population structuring in the Southwest Atlantic population of right whales (*Eubalaena australis*)

Authors: Morgana Vighi, Asunción Borrell, Enrique A. Crespo, Larissa R. Oliveira, Paulo C. Simões-Lopes, Paulo A. C. Flores, Néstor A. García, Alex Aguilar

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Título: Los isótopos estables indican estructuración poblacional en la población de ballena franca (*Eubalaena australis*) del Atlántico suroeste

Resumen:

Desde principios del siglo XVII hasta la década de 1970 la ballena franca austral, *Eubalaena australis*, fue objeto de una intensa explotación a lo largo de la costa atlántica de América del Sur. Las capturas registradas por los balleneros a lo largo de esta costa formaban originalmente un continuo desde Brasil hasta Tierra del Fuego. Sin embargo, la recuperación de la población se ha producido aparentemente de manera fragmentaria, y con dos principales áreas de concentración, una frente al sur de Brasil (Santa Catarina) y otra frente Argentina central (Península Valdés). Este patrón sugiere un cierto nivel de heterogeneidad de la población, lo que entraría aparentemente en contradicción con los registros históricos, que indicaban que los individuos se desplazaban a lo largo de toda la extensión geográfica cubierta por la especie en el suroeste Atlántico. Para probar la hipótesis de la existencia de subpoblaciones discretas explotando hábitats específicos hemos investigado los valores isotópicos de N, C y O en 125 muestras óseas obtenidas de factorías balleneras que operaban en la década de 1970 en el sur de Brasil (n = 72) y de varamientos producidos en época reciente el centro de Argentina (n = 53). Los resultados indicaron diferencias significativas entre las dos zonas de muestreo, siendo los valores de $\delta^{13}C$ y $\delta^{18}O$ significativamente más altos en las muestras del sur de Brasil que en las del centro de Argentina. Esta variación era consistente con las bases de referencia isotópicas de las dos áreas, lo que indica la existencia de un cierto nivel de estructura en la población de ballena franca del Atlántico sur y al mismo tiempo que probablemente las ballenas se alimentan en zonas que comúnmente se consideraban ser exclusivamente de reproducción. Los resultados apuntan a reconsiderar las unidades que actualmente se utilizan en la gestión de la ballena franca austral en el Océano Atlántico Sudoccidental. En el contexto de la actual mortalidad que afecta la especie en la Península Valdés, estos resultados también evidencian la necesidad de comprender mejor los movimientos de los individuos y de identificar precisamente sus áreas de alimentación.

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Stable Isotopes Indicate Population Structuring in the Southwest Atlantic Population of Right Whales (*Eubalaena australis*)

Morgana Vighi[1]*, Asunción Borrell[1], Enrique A. Crespo[2], Larissa R. Oliveira[3,6], Paulo C. Simões-Lopes[4], Paulo A. C. Flores[5], Néstor A. García[2], Alejandro Aguilar[1]

1 Department of Animal Biology and IRBio, Faculty of Biology, University of Barcelona, Barcelona, Spain, **2** Laboratory of Marine Mammals, Centro Nacional Patagónico (CENPAT-CONICET), National University of Patagonia, Puerto Madryn, Argentina, **3** University of Vale do Rio dos Sinos (UNISINOS), São Leopoldo, Brazil, **4** Department of Ecology and Zoology, Center of Biological Sciences, Federal University of Santa Catarina,Trindade, 88010-970 - Florianopolis, Santa Catarina, - Brazil, **5** Centro Mamíferos Aquáticos - CMA (Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos), ICMBio, MMA,, Jurerê Florianópolis, Santa Catarina, 88053-700, Brazil, **6** Study Group of Aquatic Mammals of Rio Grande do Sul (GEMARS), Imbé, Brazil

Abstract

From the early 17th century to the 1970s southern right whales, *Eubalaena australis*, were subject to intense exploitation along the Atlantic coast of South America. Catches along this coast recorded by whalers originally formed a continuum from Brazil to Tierra del Fuego. Nevertheless, the recovery of the population has apparently occurred fragmentarily, and with two main areas of concentration, one off southern Brazil (Santa Catarina) and another off central Argentina (Peninsula Valdés). This pattern suggests some level of heterogeneity amongst the population, which is apparently contradicted by records that traced individuals moving throughout the whole geographical extension covered by the species in the Southwest Atlantic. To test the hypothesis of the potential occurrence of discrete subpopulations exploiting specific habitats, we investigated N, C and O isotopic values in 125 bone samples obtained from whaling factories operating in the early 1970s in southern Brazil (n = 72) and from contemporary and more recent strandings occurring in central Argentina (n = 53). Results indicated significant differences between the two sampling areas, being δ^{13}C and δ^{18}O values significantly higher in samples from southern Brazil than in those from central Argentina. This variation was consistent with isotopic baselines from the two areas, indicating the occurrence of some level of structure in the Southwest Atlantic right whale population and equally that whales more likely feed in areas commonly thought to exclusively serve as nursing grounds. Results aim at reconsidering of the units currently used in the management of the southern right whale in the Southwest Atlantic Ocean. In the context of the current die-off affecting the species in Peninsula Valdés, these results also highlight the necessity to better understand movements of individuals and precisely identify their feeding areas.

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* E-mail: morgana.vighi@gmail.com

Introduction

Cetacean populations have been historically subject to different threats. Whaling, in particular, depleted many species which, due to their long life cycle and low demographic productivity, showed limited resilience. Consequently, many populations and several species of whales are currently catalogued by the International Union for the Conservation of Nature (IUCN) as endangered or critically endangered [1]. Since the International Whaling Commission put in place a protection policy and cut off commercial whaling, some populations have recovered although the pace of demographic increase has markedly varied with no evident reason for such heterogeneity.

An example of a strongly depleted population that is now recovering is the Southwest Atlantic population of the southern right whale (*Eubalaena australis*). Although the intensity of exploitation changed with time, the population was exploited almost continuously from 1602 to the mid1970s. Thus, the early 17th century fishery conducted by Spanish and Portuguese coastal whalers [2] was substituted in the 18th and 19th centuries by a pelagic fishery dominated by open-boat British and American whalers [3] turning into a modern multinational operation [4,5]. Exploitation of the species during the second half of the 19th century and along the 20th century was fragmentary and characterized by low levels of catches, undoubtedly due to the meagre state of the population. Thus, in the 20th century about 800 whales were caught mostly in South Georgia between 1904 and 1931, a figure that represents only a small portion of the more than 200,000 whales caught during that period in these grounds [6]. Later on, between 1950 and 1973 about 350 whales were removed by coastal operations in Brazil [5] and about 1,300 were illegally caught by Soviet whalers in the late 1960s [7,8]. From the late 1970s the protection of the species has been effective and the population has been recovering at an annual rate of about 7% [9,10].

However, in the last years the population has experienced an enhanced mortality of calves that has raised concern about a potential curving of the recovery process [11]. The observed association between increased mortality and sea surface temperature oscillations [12] as well as the fact that the Peninsula Valdés right whales have fewer calves than expected in years of low krill abundance [11], may be suggestive that the population is approaching the carrying capacity of the ecosystem. However, this hypothesis would be apparently contradicted by the fact that, despite recorded catches by 19th century whalers originally formed a continuum along the Southern American Atlantic coastline [3], the repopulation process has yet to complete filling empty spaces in the original distribution range. Thus, evidences for population increase only exist for two main areas of concentration, one off southern Brazil (around Santa Catarina) [13] and the other off central Argentina (Peninsula Valdés) [9], but not for the intermediate area (south of Uruguay and Mar del Plata), where the species appears to have been originally very abundant [14,15,16].

Such a fragmented pattern of recovery, which has also been observed elsewhere in other already well-recovered southern right whale populations [15], suggests some level of heterogeneity in the population which is apparently contradicted by movements of individuals recorded throughout the geographical range occupied by the species in the South Atlantic [17]. This apparent contradiction is not novel, since many examples are known among mysticetes of subpopulation units that never recovered after being extirpated by whaling [18]. Although mysticetes are highly mobile and migratory animals, individuals tend to return to particular feeding and breeding sites [19,20] purportedly as a consequence of behavioural learning from their mothers during the first migration [21]. This matrilineal fidelity to specific breeding and foraging areas has been proposed to be acting as a limiting factor in the re-colonisation and population growth of the con-specific New Zealand population of southern right whales [22] and, indeed, genetic studies made in Peninsula Valdés showed that southern right whales concur in this behaviour and that they use non-homogeneous food sources [23]. In this context, and having whaling strongly depleted the Southwest Atlantic population of this species, it may be hypothesized that groups relying on different breeding or feeding grounds could have been kept isolated, and the lack of recovery in the central area of South America could be due to a loss of cultural memory, as proposed by Clapham *et al.* [18].

A sound understanding of the actual distribution and structure of a given population is essential for its management and conservation. For the Southwest Atlantic population of right whales this is particularly relevant because current management recognizes this to be a single stock, and therefore promotes integrated conservation actions along the Atlantic coast of South America [24]. When genetics do not delineate subpopulations, chemical markers and other tools, such as stable isotopes, may assist in achieving this goal [25,26]. Stable isotope values in body tissues strongly correlate to the characteristics of the water masses in which feeding takes place and this property has made these markers a suitable tool to investigate diet, trophic ecology and migration in a variety of species, including marine mammals [27,28,29,30].

Stable isotope values can be investigated in any body tissue, but bone has a slow isotopic turnover rate and therefore integrates a much wider temporal span than other more metabolically active tissues. This makes bone a tissue of choice for investigating long-term processes [31]. Bone is made up of two matrices, both of which are useful to investigate habitat use, pattern of migrations and dietary history of organisms. The organic matrix, mostly composed of collagen, is a complex structural protein that has been used to study nitrogen and carbon stable isotopes, the values of which are mainly related to trophic position and diet. The inorganic matrix, largely composed of carbonated hydroxyapatite, has been used to investigate variations in the oxygen stable isotopic value. This latter value correlates with characteristics of the water such as temperature and salinity and, as a consequence, is a good indicator of habitat use and migration patterns [29].

In this study we look into the nitrogen, carbon and oxygen stable isotopic values in bone samples of southern right whales from two separate locations on the southern coasts of South America, southern Brazil and central Argentina, to investigate potential structuring and isolation of subpopulations exploiting specific habitats.

Materials and Methods

Ethics Statement

All necessary permits were obtained to collect the samples for the described study, which complied with all relevant regulations. Samples from protected areas were collected with permits SISBIO 24429, 27927 and 17890 issued by the 'Instituto Chico Mendes de Conservaçao da Biodiversidade' in Brazil and with written provincial permits issued by the 'Dirección de Fauna y Flora Silvestre, Subsecretaria de Recursos Naturales, Ministerio de Industria, Agricultura y Ganaderia'; and by the 'Subsecretaría de Conservación y Áreas Protegidas, Secretaría de Turismo', in Península Valdés, Argentina. No samples were donated or purchased, and no whales were killed for the purpose of this study. All the samples used for this research derived from animals stranded and naturally dead or from animals that were legally caught during the period of commercial exploitation.

Sample collection and preparation

Bone samples were obtained either from individuals stranded or from disposal sites at the Southern Brazil whaling factories. In total, 125 samples were collected: 72 from the area off Santa Catarina, Brazil, and 53 from central Argentina, mainly from Peninsula Valdés, in the Chubut area (Figure 1). Samples from disposal sites were collected from sites located far apart in order to avoid the replicated sampling of a given individual; however, the occurrence of duplicates in the sample set cannot be totally excluded. All samples were modern (less than 50 years old), but information about the precise date of stranding or capture, as well as the sex or the age of the individuals involved, was not provided in most cases. Samples included different skeleton parts but in most cases they were parts of ribs, cranial bones, jaw bones and vertebrae.

The samples were preserved dry. For the analysis, a small subsample was ground to fine powder with mortar and pestle. Half of the powder was used for the analysis of carbon and nitrogen stable isotopes in the organic matrix, and half for that of oxygen stable isotopes in the inorganic matrix.

Carbon and nitrogen stable isotope analysis

Because the depletion in ^{13}C that occurs in lipids as compared with other constituents may affect the analytical results by decreasing the overall $\delta^{13}C$ value in a sample [32,33], prior to the analysis of carbon and nitrogen isotopes the bone powder was treated with a chloroform-methanol (2:1) solution to extract the

Figure 1. Sampling areas. Map of the Eastern coast of South America showing the two sampling areas: Peninsula Valdés, in central Argentina; and the area of Santa Catarina, in southern Brazil.
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lipophilic fraction [34]. Also, as the analysis of carbon and nitrogen stable isotopes was performed in the organic matrix of bone samples, and inorganic carbon is liable to affect the carbon isotopic value [35,36], samples were decalcified with a 1 M solution of HCl.

After these treatments and subsequent drying, approximately 0.7 mg of each powdered sample was weighted in tin capsules, loaded, and combusted at 1000°C and analysed in a continuous flow stable isotope ratio mass spectrometer (Flash 1112 IRMS Delta C Series EA Thermo Finnigan) coupled with an elemental analyser.

Standards for ^{13}C and ^{15}N were the Vienna Pee Dee Belemnite (V-PDB) standard and atmospheric nitrogen, respectively. International isotope secondary standards of known $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios, namely: polyethylene (IAEA CH7; $\delta^{13}C = -31.8‰$), sucrose (IAEA CH6; $\delta^{13}C = -10.4‰$), ammonium sulphate (IAEA N1; $\delta^{15}N = +0.4‰$ and IAEA N2; $\delta^{15}N = +20.3‰$), potassium nitrate (USGS 34; $\delta^{15}N = -1,7‰$), L-glutamic acid (USGS 40; $\delta^{15}N = -4,6$ ‰; $\delta^{13}C = -26,2‰$), and caffeine (IAEA 600; $\delta^{15}N = 1,0‰$; $\delta^{13}C = -27,7‰$) were used for calibration of $\delta^{13}C$ and $\delta^{15}N$. The reference materials used for the analysis were selected based on previous calibration experiments performed on the same type of samples and in order to ensure an optimum range of reference values. All the reference materials used are distributed by the International Atomic Energy Agency (IAEA).

Results were expressed following the delta (δ) notation, in which the relative variations of stable isotope ratios are calculated as:

$$\delta^{13}C \; or \; \delta^{15}N(‰) = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] * 10^3$$

Where R is the heavy-to-light isotope ratio ($^{15}N/^{14}N$; $^{13}C/^{12}C$) in the sample and in the reference standards certified by the International Atomic Energy Agency (IAEA, Vienna).

Oxygen stable isotope analysis

Because the analysis of oxygen stable isotopes was performed in the inorganic matrix of the samples, the bone powder was treated with 30% hydrogen peroxide for 24 hours to remove the organic compounds. Samples were then rinsed carefully with milli-Q water and treated with 1 M calcium acetate/acetic acid buffer for another 24 hours to remove any diagenetic carbonate. Finally, samples were rinsed again repeating the same procedure and afterwards dried for 24 hours [28].

An average of 4 mg of each powdered sample was analysed using a Carbonate Kiel Device III carbonate preparation system (Thermo Electron-Dual Inlet) linked to a gas source mass spectrometer. Samples were dissolved in 100% phosphoric acid at 70°C with concurrent cryogenic trapping of CO_2 and H_2O. The CO_2 was then admitted to the mass spectrometer for analysis. An internal standard calibrated with international standard NBS-19 was used. Precision was $1\sigma = \pm 0.05‰$ for $\delta^{18}O$.

Values, reported in parts per thousand (‰), were calculated using the formula:

$$\delta^{18}O(‰) = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] * 10^3$$

Where R is the heavy-to-light isotope ratio ($^{18}O/^{16}O$) and the standard is V PDB. Because $\delta^{18}O$ values in animal studies are more commonly presented relative to SMOW, to allow comparison with published data $\delta^{18}O$ values were converted from V-PDB to SMOW using the following formula [28]:

$$\delta^{18}O(SMOW) = [\delta^{18}O(PDB) * 1.03086] + 30.86$$

All the above analyses were conducted at the *Centres Científics i Tecnològics* (CCiT-UB) of the University of Barcelona.

Statistical analysis

Results were tested for normality of distribution with a Kolmogorov-Smirnov test of goodness of fit and for homoscedasticity with a Levene test. The variability of the results and the presence of outliers were tested graphically through boxplots.

No analysis of the effect of age, sex or date of death in determining the isotopic values of the samples could be made because of lack of appropriate information.

The variances of the isotopic values from the two sampling areas did not satisfy the assumption of homoscedasticity, so the non-parametric test U of Mann-Whitney was used to detect differences between them. Also, a linear Discriminant Function Analysis with origin as grouping variable and $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ as independent variables was used to establish the best combination of variables that distinguished the samples from the two areas and to determine the percentage of correct assignments to each of them.

A graphical comparison between the isotopic values of the two sampling areas was carried out with standard ellipses analysis (SEAc) of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ values performed using SIBER (Stable Isotope Bayesian Ellipses in R, [37]).

SPSS 20 and R-2.15.2 statistical software were used for all the above analysis.

Results

Out of the 125 samples available, successful analyses could be conducted in 118 samples for nitrogen stable isotopes, 120 for carbon stable isotopes and 122 for oxygen stable isotopes.

The analysis of the data distribution indicated an extremely high variability for the three elements (figure 2). $\delta^{13}C$ ranged from −27.83‰ to −13.63‰ (mean±SD = −20.39±3.08‰); $\delta^{15}N$ from 4.00‰ to 14.95‰ (mean±SD = 9.31±2.3‰) and $\delta^{18}O$ from 22.97‰ to 31.59‰ (mean±SD = 29.54±1.17‰). Only one outlier was detected as being higher to 3*IQR ($\delta^{18}O$ = 22.97‰). In order to check for potential errors in the processing of this sample, three aliquots from it were subsequently subject to independent analytical runs under the same laboratory conditions. The $\delta^{18}O$ values obtained in the resulting four analytical runs were extremely variable, undoubtedly indicating the existence of heterogeneity within the sample. Taking this result into account, this outlier was eliminated from subsequent analysis. Excluding it, data obtained from the two sampling areas resulted normally distributed (Kolmogorov-Smirnov test) and with significantly different variances (Levene test, $p<0.05$).

Being the assumption of homoscedasticity unsatisfied, the comparison of mean $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ between the two sampling areas was carried out through the non-parametric U of Mann-Whitney test. The results indicated that values for both elements were significantly lower ($p<0.05$) in the individuals from central Argentina (mean $\delta^{13}C$±SD = −20.39±3.08‰; mean $\delta^{18}O$±SD = 29.22±1.28‰) than in those from Brazil (mean $\delta^{13}C$±SD = −16.75±2.13‰; mean $\delta^{18}O$±SD = 29.87±0.62‰). No significant differences were found in $\delta^{15}N$ between the two sampling areas.

The linear Discriminant Function Analysis correctly classified 77.6% of samples as Argentinian and 81.8% as Brazilian. Globally, the distinction between the two sampling areas based on their isotopic values was supported by the 80% of samples. The standard coefficients calculated for the discriminant function were 0.238 for $\delta^{18}O$; −0.7 for $\delta^{15}N$ and 1.139 for $\delta^{13}C$. The statistics produced by this function for the Argentinian group of samples were: centroid (mean): −1; standard deviation: 1.112; and for the Brazilian group of samples: centroid (mean): 0.74; standard deviation: 0.909. The eigenvalue resulting from this function was 0.757, with an associated λ (Lambda of Wilks) value of 0.569, which was highly significant ($p>0.001$), indicating that the two sampling areas were statistically different.

Standard Ellipses Analysis showed that the two sampling areas graphically differentiated mostly for $\delta^{13}C$ and to a smaller extent for $\delta^{18}O$ (figure 3). The overlapping area calculated from the plot of $\delta^{13}C$ and $\delta^{15}N$ values resulted to be the 6.08% of the area of the ellipse representing Argentinian samples and 9.95% of the area of the ellipse representing Brazilian samples. On the other hand, the overlapping area calculated from the $\delta^{18}O$-$\delta^{13}C$ plot resulted to be the 10.37% of the area of the ellipse representing Argentinian samples, and the 40.72% of the ellipse representing Brazilian samples. Finally, as already announced by the U of Mann-Whitney test, $\delta^{15}N$ values did not contribute to differentiating the samples coming from the two areas; the $\delta^{15}N$-$\delta^{18}O$ plot shows how the ellipse obtained from the Brazilian samples is almost completely encompassed by the ellipse representing the Argentinian samples.

All results of the stable isotopes analyses described above are publicly available in the Supporting Information (Table S1).

Discussion

Coincidentally with previous studies conducted in central Argentina [23], results obtained here for the three stable isotopes were highly variable in the two sampling areas. This large variability might be partially due to heterogeneities in the age, sex and death date of the individuals sampled and, in the particular case of our study, as well as to the type of bone used for the analysis. Unluckily, the actual influence on the isotopic values of these variables could not be established because adequate information from most individuals was not available. However, being whales highly mobile and thus able to cross long distances across the region, the high isotopic variability observed may be very likely reflecting the heterogeneous isoscapes of the Atlantic waters off South America, especially with regards to carbon and oxygen [38,39,40].

The various statistical tests highlighted a significant difference in mean $\delta^{13}C$ and $\delta^{18}O$ but not in $\delta^{15}N$ between the two sampling areas. Being trophic position the main driver of $\delta^{15}N$ variation [41,42], the absence of significant distinction between areas is likely to indicate that all animals were feeding at similar trophic level. The diet of the southern right whale is mainly based on calanoid copepods and krill, particularly on species belonging to the genus *Euphasia* and *Munida*, which are generally situated at comparable trophic levels [43,44,45]. $\delta^{13}C$ values, which in marine mammal tissues reflect those of the prey, vary with latitude, and show a strong correlation with the conditions of productivity prevailing in the area, appeared to be significantly higher in whales from Brazil than in those from central Argentina. This result is consistent with the baseline values available for the study areas as shown by the isoscapes delineated by McMahon *et al.* [40] for the Atlantic Ocean based on a meta-analysis of published plankton $\delta^{13}C$ values. Thus, according to the isoscape, zooplankton $\delta^{13}C$ baseline values in the Brazilian area appear slightly higher (around −20‰) than those in the Argentinian area (around −22‰). The difference between these values and our results is also consistent with the estimated diet-tissue discrimination factor available for bone of baleen wales, which has been estimated to be around +3‰ [46].

Similarly, the $\delta^{18}O$ values showed a significant enrichment in the whales from southern Brazil as compared to those from central Argentina. Stable oxygen isotopes have rarely been used to investigate the migrations of marine vertebrates [47,48] but $\delta^{18}O$, being strictly correlated with water salinity and temperature, can be a useful tracer for studying the use of habitat made by marine mammals in areas with defined environmental gradients. Again, comparing on a qualitative level the results here obtained with the available baselines for the sampling areas [39,40], we found that the observed difference is consistent with proposed isoscapes for the South American Atlantic coast, which indicate slightly higher $\delta^{18}O$ values in the Brazilian area than in the Argentinian one.

The standard ellipses analysis proved to be useful in graphically depicting the degree of isolation and overlap between the two sampling areas. Running the analysis with the pairwise combinations of the three isotopes was a good method for highlighting the contribution of every isotope in distinguishing the two sampling areas, and the calculation of the overlapping area numerically strengthened the results obtained.

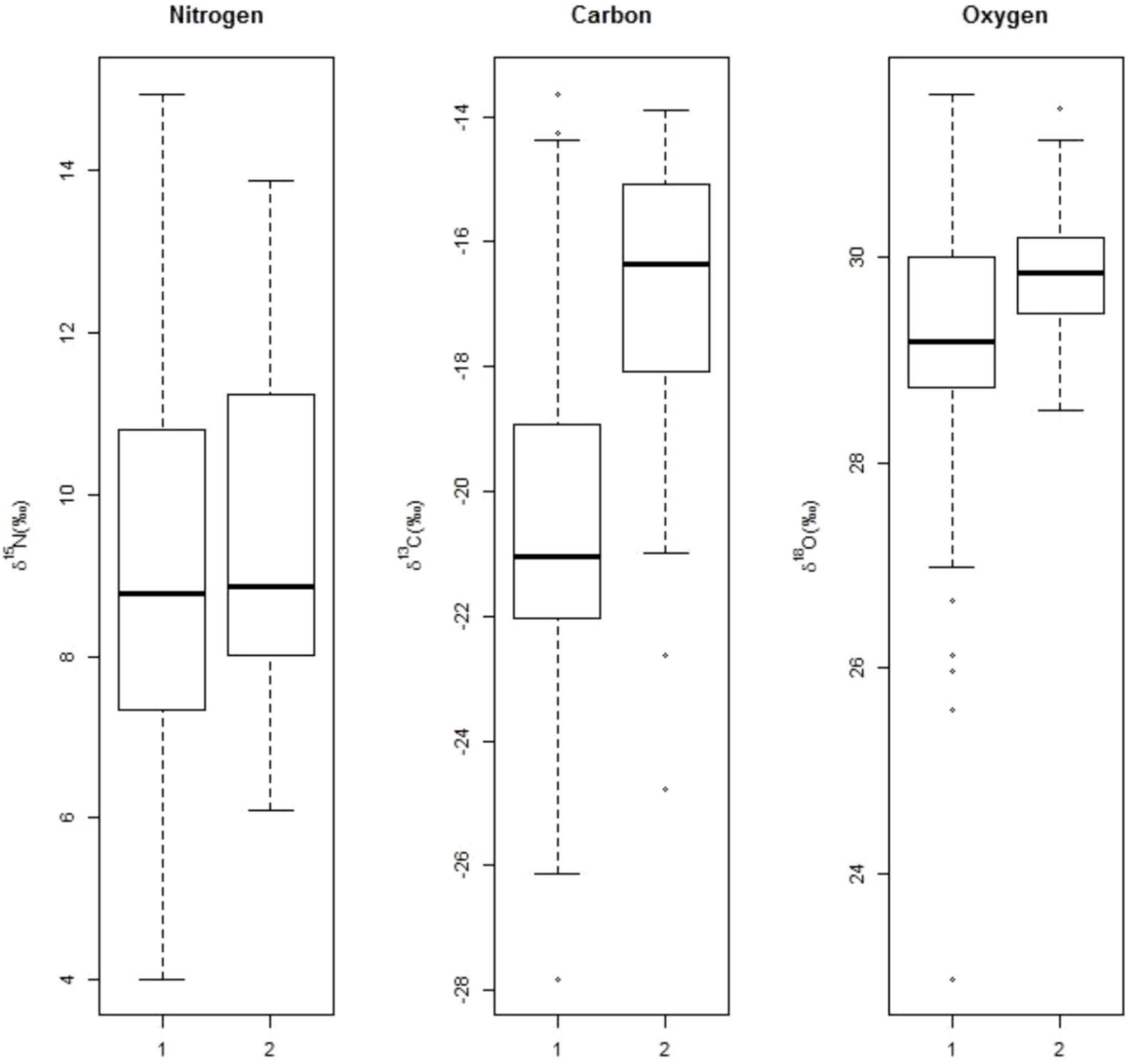

Figure 2. $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ values distribution. Boxplots depicting the distribution of values obtained from the isotope analysis in the samples from the two sampling areas (1: central Argentina; 2: southern Brazil).
doi:10.1371/journal.pone.0090489.g002

Overall, individuals from southern Brazil were isotopically distinct from those from central Argentina. Being the carbon and oxygen isotopic composition of animal tissues mainly deriving from the food [49], a straightforward explanation for this difference is that individuals from the two areas exploit different feeding grounds. All previous evidences pointed to the Antarctic area as the main feeding destination for southwest Atlantic right whales as some individuals photo-identified in Peninsula Valdés had been re-sighted in South Georgia [50], a well-known feeding ground for the species [44], but the consistency of our results with the isotopic values around the sampling areas suggests that whales substantially feed also in their breeding grounds. Indeed, despite it is generally accepted that southern right whales primarily fast while they occupy lower latitudes, individuals have also been repeatedly observed feeding on plankton blooms, particularly at the end of the season [43,45]. Feeding at lower latitudes has also been reported in other populations of southern right whales [51] as well in the northern right whale, *Eubalaena glacialis* [52] and would be further supported by frequent records in logbooks from open-boat whaling expeditions to the eastern coast of South America confirming the presence of "whale food" in the whaling grounds (unpublished data, extracted from 19th century whalers logbooks), and the finding of floating faeces in Peninsula Valdés waters [11].

The isotopic difference between the individuals from the two sampling areas also might indicate that intermingling between the two areas is limited or non-existing, supporting the hypothesis of isolation between the right whales off central Argentina and southern Brazil. However, despite finding some level of genetic structuring, genetic results presented by Ott *et al.* [53] supported the hypothesis that whales from southern Brazil and central Argentina belong to the same population although recognizing that these individuals could mix on the feeding grounds with

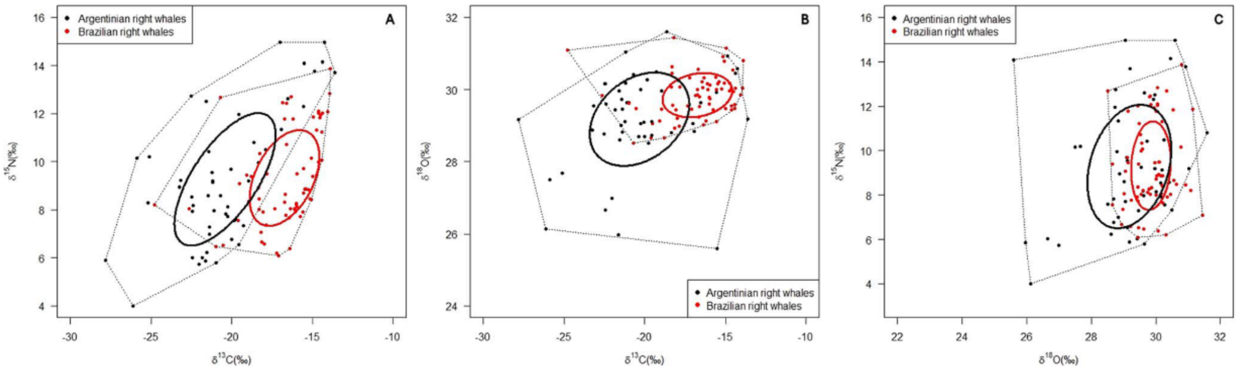

Figure 3. Standard ellipses analysis. Output graphs resulting from the standard ellipses analysis (SEAc) performed using SIBER (Stable Isotope Bayesian Ellipses in R, Jackson *et al*., 2011). Results from the analysis performed with $\delta^{13}C$ and $\delta^{15}N$ values (A), $\delta^{13}C$ and $\delta^{18}O$ values (B); and $\delta^{18}O$ and $\delta^{15}N$ values (C) are shown.
doi:10.1371/journal.pone.0090489.g003

whales from other genetically distinct calving grounds (*e.g.* South Africa, [54]). Moreover, the isotopic result also appears to be contradicted by the re-sighting off Laguna (Brazil) of three mother-calf pairs that had previously been photo-identified in Peninsula Valdés [50]. Both the areas of Laguna (Brazil) and Peninsula Valdés (Argentina) are considered to be nursery grounds, even if calves appear more frequently in Peninsula Valdés [55]. To explain these occasional re-sightings it has been hypothesised that some females may not display a strict site fidelity and use therefore different nursery areas in different years or, else, that some females may move between the two areas in the year in which their calves are born [50]. However, records of long range movements of both the southern and northern right whales implying migrations over 4,000 km distances [56] confirm the high mobility of the species.

The absence of re-colonization of areas where the Southwest Atlantic right whales had been previously recorded and which now only appear to be or marginal use, strengthened by the isotopic differences found in the present study, seem to support the hypothesis that the recorded re-sightings are to be interpreted as random movements of the individuals. This hypothesis is also consistent with the recognized fidelity of females to the two current nursery grounds and the almost non-existing intermingling between the two areas. Another factor further supporting the differentiation between the two subpopulations of Southwest Atlantic right whales would be the occurrence of lesions caused by the attack of kelp gulls (*Larus dominicanus*), so far massively recorded in whales from central Argentina [57] and beginning only recently to be reported in whales from Brazil [58].

At the light of the above findings, the use of a single management unit for the conservation plans being developed for the Southwest Atlantic right whale and the application of an integrated approach for the species along the whole Atlantic coast of South America [24] should be reconsidered, as the population is likely to be structured into at least two units that exploit different feeding and nursery grounds. This distinction is particularly relevant not only from the demographic perspective, but also for the implementation of corrective measures since conservation threats are dissimilar in the two areas.

Thus, the main threats for the species in Southern Brazil are considered to be entanglements in gillnets, collisions due to the intense marine traffic and the whale-watching industry [11,59]. Despite the number of right whales using the Brazilian nursery ground has been increasing since their exploitation ended in 1973 [60], the number of strandings in the last 20 years has been increasing [61,62]. The higher rate of strandings is located in the Rio Grande do Sul area, which now appears to be only a migratory passage towards the nursery grounds situated further north, off Santa Catarina [63]. It is unclear whether the waters of Rio Grande do Sul have always been only a transit area or whether it ceased to be used as a nursery ground due to anthropogenic influence. If this latter were the case, regulation of shipping and fishing activities would be instrumental for promoting re-colonization and strengthening the conservation of this putative management unit [11].

On the other hand, the right whales calving in Central Argentina have recently experienced persistent high mortality events which affected 90% of the calves in their first year of life [11]. Causes adduced to explain the mortality include, among other potential factors, reduced food availability for adult females, bio-toxins and infectious diseases, as well as anthropogenic disturbance caused by whale watching, marine traffic and fisheries. However, harassment by kelp gulls appears to be central in the process, since in 2008 77% whales in the area presented wounds caused by those birds [64]. It has been suggested that kelp gull harassment is likely to affect behaviour and compromise calf survivorship [57] and therefore take a most relevant role in the whale mortality [65].

As a consequence of such dissimilar conservation scenarios, the two putative management units of Southwest Atlantic right whales would require independently focused conservation regulations and their biological parameters and demographic trends would have to be examined separately. Also, to deepen into the origin of the apparent demographic structuring of the population inhabiting the Atlantic waters of South America, the stable isotope analysis should be complemented with research based on other chemical and genetic markers as well as with historical reconstruction of past distribution, migration routes and localization of feeding grounds.

Supporting Information

Table S1 Stable isotope analysis results. Table showing the list of samples with their corresponding sampling area and $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values.
(XLS)

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Author Contributions

Conceived and designed the experiments: MV AA AB EAC. Performed the experiments: MV AA AB. Analyzed the data: MV AA AB. Contributed reagents/materials/analysis tools: MV EAC LRO PCSL PACF NAG. Wrote the paper: MV AA.

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1.3 Stable isotope analysis and fin whale subpopulation structure in the eastern North Atlantic

Authors: Morgana Vighi, Asunción Borrell, Alex Aguilar

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Resumen:

El rorcual común del Atlántico Norte fue objeto de una fuerte explotación en el pasado y, a pesar de haberse recuperado parcialmente, todavía se considera en peligro de extinción. Investigaciones recientes han cuestionado la estructura sub-poblacional y los movimientos migratorios actualmente aceptados para esta población, desafiando los esfuerzos de manejo y conservación actuales. En el presente estudio contribuimos a este debate presentando los resultados del análisis de isótopos estables en muestras de hueso de rorcual común y de krill recogido en el estómago de los rorcuales procedentes de dos áreas explotadas, una situada al oeste de Islandia y la otra al noroeste de España, y comparando estos resultados con los isoscapes (mapas de los valores isotópicos basales) del Atlántico Norte. Los valores de $\delta^{15}N$ en krill eran muy variables y no se evidenciaron diferencias significativas en $\delta^{15}N$ y $\delta^{13}C$ entre las dos zonas. Los valores de $\delta^{15}N$ y $\delta^{13}C$ en hueso de rorcual eran mayores que en el krill, debido al enriquecimiento trófico, y no eran significativamente diferentes entre las zonas. Los resultados de krill y de hueso estaban ligeramente en contraste con los valores basales locales, tal vez debido a las variaciones estacionales en la productividad local y en la dieta del krill y, en el caso de los huesos, a su capacidad para integrar los valores isotópicos de largo plazo, derivados de alimentos consumidos en áreas lejanas. En cambio, los valores de $\delta^{18}O$, que reflejan la señal basal del agua de mar, eran congruentes con los valores basales y significativamente más altos en las ballenas de España noroccidental, lo que posiblemente indica migraciones a zonas de reproducción situadas en latitudes más bajas.

Stable isotope analysis and fin whale subpopulation structure in the eastern North Atlantic

Morgana Vighi*, Asunción Borrell, Alex Aguilar

Department of Animal Biology and Institute of Biodiversity Research (IRBio), Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain.

Abstract

The North Atlantic fin whale was subject to heavy exploitation in the past and, despite partial recovery, it is still considered endangered. Recent research has questioned its currently accepted subpopulation structure and migratory movements, challenging management and conservation efforts. Here we contribute to this discussion by presenting results of stable isotope analysis of fin whale bones and krill samples collected from fin whale stomachs from two exploited areas, W Iceland and NW Spain, and comparing these results with North Atlantic isoscapes. In krill, $\delta^{15}N$ values were highly variable and no significant differences in $\delta^{15}N$ and $\delta^{13}C$ between areas emerged. $\delta^{15}N$ and $\delta^{13}C$ values in bone were higher than in krill, due to trophic enrichment, and were not significantly different between areas. Both krill and bone results were slightly inconsistent with the local isoscapes, maybe due to seasonal variations in local productivity and in krill diet and, in the case of bone, to its capability to integrate long-term isotopic values, derived from food consumed in distant areas. Conversely, $\delta^{18}O$ values, which reflect the basal signal of sea water, were consistent with isoscapes and significantly higher in whales from NW Spain, possibly indicating migration to breeding grounds located at lower latitudes.

Introduction

Wild animal populations are managed through a hierarchy of structural units that range from species to subspecies, distinct population segments and stocks (Taylor 2005, Palsbøll *et al.* 2006). The first three categories entail morphological and genetic differences and their preservation is therefore critical to ensure the species ability to respond to environmental change. On the contrary, stocks, which are defined biologically as groups of animals with independent demography and generally occupying a defined geographic area (Taylor 2005), are often the structural unit on which management focuses. This is because the responses of a stock to environmental impacts are self-regulating and independent of those of other stocks. Therefore, strong pressure from exploitation on a particular stock may result in its extirpation and, as a consequence, in gaps in the distribution range of the whole species, as repeatedly observed along the history of whaling (Clapham *et al.* 2008). Consequently, the aim of the study of demography and population structure to define biological stocks is essential to the creation of management units for these mammals. Biological stocks, unlike other structural units, may not be distinguished solely by genetic isolation. Other aspects may also play an important role in their identification, such as potential differences in the response to exploitation, or dissimilarities in ecological or behavioral traits like migratory routes, acoustic parameters, use of habitat, or diet (Donovan 1991).

The fin whale (*Balaenoptera physalus*) is one of the most abundant baleen whales in the North Atlantic (Víkingsson *et al.* 2009) where its original population ranged at least over 70,000 individuals (Christensen 2006). Being a large and fast whale that sinks once dead, the species was not a target during the early whaling operations and its large-scale exploitation started with the introduction of steam ships and the use of explosive harpoons at the end of the 19th century. The main operations concentrated around Norway, Iceland, the Faroe Islands, Ireland, Scotland, Newfoundland, and the Iberian Peninsula (Jonsgård 1966, International Whaling Commission 1992). During the first decades of whaling the intensity of the exploitation was extreme and the size of the subpopulations drastically reduced in very short periods of time (Jonsgård 1966, Tønnessen and Johnsen 1982). Whaling pressure declined in the 1940s but increased again in Spain and Iceland during the late 1970s, until the moratorium on commercial whaling was established by the International Whaling Commission (IWC) in 1985. After 1985, only a reduced number of individuals have been caught off Iceland and Greenland (Sanpera and Aguilar 1992, IWC 2009).

The demographic recovery that has followed the decrease in the harvesting pressure has been slow and the species is still classified by the International Union for the Conservation of Nature (IUCN) as endangered (IUCN 2015). The current estimate of the total population for the eastern North Atlantic is about 35,000 individuals, a figure considered to represent about 70% of the original population size (Buckland *et al.* 1992*a*, *b*; North Atlantic Marine Mammal Commission 2005; IWC 2009). Nevertheless, recovery appears to have been spatially heterogeneous and the current distribution differs from that occurring at the beginning of the whaling operations: areas where the fin whale was originally very abundant, like the Gibraltar strait (Tønnessen and Johnssen 1982, Aguilar 2013) or the Faroe Islands (North Atlantic Marine Mammal Commission 2000), are now apparently devoid of this species (North Atlantic Marine Mammal Commission 2004, Clapham *et al.* 2008, Gutiérrez-Expósito *et al.* 2012), while in other areas where whales were caught in comparable numbers, abundance has recovered rapidly (Sanpera and Jover 1989, Buckland *et al.* 1992*b*). Such heterogeneous demographic trajectories suggest a complex structure in subpopulations.

In the North Atlantic, the fin whale distributes all over the basin from at least 80ºN to the Equator. This range includes the Mediterranean and the Baltic seas, but not the Black sea (Aguilar 2009, Øien 2009). Since its early years of activity, the IWC has been working for defining appropriate management units for this species. Despite the absence of ubiquitous physical barriers, a number of discrete units or stocks have been recognized within the basin. Based on dissimilarities in the response to exploitation between different localities, Jonsgård (1966) and Sergeant (1977) drew some preliminary stock boundaries. These were later redefined according to results obtained from studies on tagging (Gunnlaugsson and Sigurjonnson 1989), genetics (as reviewed by Pampoulie and Daníelsdóttir 2013), internal and external morphology (Lockyer 1982, Jover 1992, Víkingsson 1992), pollutant levels (Sanpera *et al.* 1993, 1996), and other sources of data (Víkingsson and Gunnlaugsson 2005), to end up with the recognition of 7 North Atlantic stocks (IWC 2009) (Fig. 1). Parallel to this redefinition of boundaries, fin whales from the Mediterranean Sea were found to have limited gene flow with their North Atlantic counterparts (Bérubé *et al*. 1998, Palsbøll *et al.* 2004), so fin whales from this region were incorporated into the IWC management scheme as a further, independent unit (IUCN 2012).

However, a number of elements challenges this demographic structure and the proposed boundaries between stocks: the scarce knowledge about the location of the breeding grounds in the North Atlantic, the likely structuring into subunits over relatively short distances (Bérubé *et al.* 1998), and the recent finding that movements and migration of individuals are less well defined than expected, straddling accepted boundaries (Silva *et al.* 2013, Giménez *et al.* 2013). Moreover, the stocks were largely delimited according to information or tissue material obtained during the early phases of exploitation. Habitat may vary with population abundance and ranges may expand or contract according to changes in density or the exploitation pressure experienced by discrete segments of the population (Clapham *et al.* 2008). Thus, in whale populations subject to severe exploitation recovery of numbers does not always bring complete repopulation of the original range (Clapham *et al.* 2008). In addition movement of individuals and distribution patterns are not static and may be liable to change according with variations of the environmental conditions. A remarkable example of this is the recent crossing of North Pacific gray whales (*Eschrichtius robustus*) into the North and South Atlantic and the Mediterranean Sea as a likely consequence of the progressive melting of the Arctic Sea ice caused by climate change (Scheinin *et al.* 2011, Elwen and Gridley 2013).

Stable isotope analysis traces the features of the environment in which animals live and feed (Newsome *et al.* 2010), and in the last decade has proved useful to investigate demographic structure and migration in both terrestrial (Hobson 1999, Kelly 1999, Crawford *et al*. 2008) and marine mammals (Mendes *et al.* 2007; Witteveen *et al.* 2009*a*, *b*; Borrell *et al.* 2013; Giménez *et al.* 2013; Vighi *et al*. 2014). Among other animal tissues, bone, due to its slow isotopic turnover rate, integrates large time scales and has proven to be a tissue of choice for investigating long-term processes (Riofrío-Lazo and Aurioles-Gamboa 2013). Both the organic and inorganic matrices of bone have been used: collagen, the organic matrix, has been analyzed to study nitrogen and carbon stable isotope values, while the inorganic matrix, mostly composed of carbonated hydroxyapatite, has been analyzed to study the values of stable isotopes of oxygen. Isotopes of nitrogen and carbon are the most widely used in ecological studies, being both related to the diet. While nitrogen isotopes mainly reflect the trophic position, carbon isotopes reflect the prevailing condition of primary productivity of the foraging area, and are therefore commonly used to distinguish pelagic from coastal areas (Newsome *et al*. 2010). Stable isotopes of oxygen are less commonly used in the study of marine vertebrates, although they may provide useful information about the movements and migrations of these animals. Being strictly correlated with water temperature and salinity, oxygen stable isotopes show defined latitudinal gradients and can therefore provide further resolution of habitat use and migration patterns of highly migratory animals such as marine mammals (Bowen 2010, Zenteno *et al.* 2013).

In this study we analyzed N, C and O stable isotopes in fin whale bone samples from W Iceland and NW Spain, two areas traditionally considered to pertain to different stocks, with the objective of further elucidating the structure and movements of the fin whale in the North Atlantic. To assist in establishing the environmental isotopic baselines from the two sampling areas, the C and N stable isotopes were also determined in food (krill) found in the stomach of fin whales from each area.

Materials and methods

Sample collection and composition

Bone samples (central section of a thoracic rib) were collected from fin whales caught off NW Spain (Galicia Bank) during the 1983 and 1984 summer whaling seasons and from fin whales caught off W Iceland (Denmark Strait) during the 1986 summer season (Fig. 1). 24 samples (9 males and 15 females) were analyzed from each area, with ages ranging from 7 to 27 yr in the sample from W Iceland, and from 6 to 33 yr in the sample from NW Spain. Results of carbon and nitrogen stable isotope analysis of 9 more samples from NW Spain (3 females and 6 males) obtained during the 1983-1985 whaling campaigns and analyzed in the same laboratories were available from the study of Borrell *et al.* 2012. Because the preparation protocol used in that study did not include sample decalcification, a treatment liable of affecting the overall carbon signature, only values of nitrogen were incorporated into the statistical analysis. Data on body length, sex, and age were collected from most individuals.

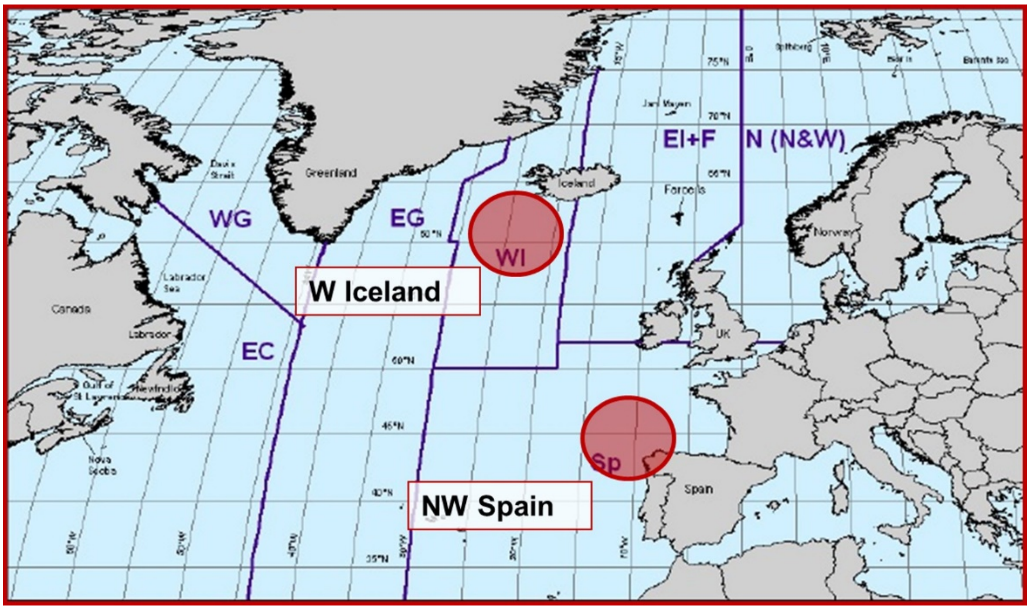

Figure 1. Sampling areas and proposed fin whale stock structure. Map of the North Atlantic Ocean, representing the stock structuring of the North Atlantic fin whale population proposed by the IWC (IWC, 2009). Sampling areas are marked with circles.

Samples of the whales' food, consisting entirely of krill, or planktonic euphausiids, were collected from the fore-stomach (first chamber) of fin whales caught off NW Spain during the 1983 and 1985 seasons, and of fin whales caught off W Iceland during the 1986 season. In total, 9 krill samples from W Iceland were analyzed, all taken from the same whales whose bone samples were analyzed in the present study, while 15 samples from NW Spain were analyzed, 6 of them collected from whales analyzed in the present study, and 9 collected from other whales. Identification of the krill species composition was impossible because, despite the fore-stomach being nongladular, the exoskeleton of the euphausiids was partially digested by mechanical maceration and indigenous bacteria, a process that had been possibly enhanced by postmortem fermentation. However, the body size of the euphausiids and the overall aspect of the remains suggested that the dominant species was *Meganyctiphanes norvegica*, the prey most commonly consumed by fin whales off both NW Spain and W Iceland (Aguilar 1985, Víkingsson 1997).

All tissue samples were kept frozen until analysis.

Age determination

Age was determined by counting the growth layers through a longitudinal section of the fin whale ear plug core, following the procedures described by Lockyer (1984). Age determination of each sample was performed by more than one reader, and in about 70% of samples it was repeated twice by the same reader. If the ages determined for the same sample differed more than 10% of the lowest age estimated, then the age determination of that sample was not considered valid; otherwise the mean of all readings was considered to be the age of the sample.

Carbon and nitrogen stable isotope analysis

Prior to stable isotope analysis, samples were dried at 60 ºC for 48 h in a stove and subsequently ground to a fine powder by mortar and pestle. In the case of bone, half of the powder was used for the analysis of carbon and nitrogen stable isotopes, and half for the analysis of oxygen stable isotopes. In the case of krill, the whole sample was used for the analysis of carbon and nitrogen stable isotopes.

Because lipids are depleted in ^{13}C compared with proteins (De Niro and Epstein 1977, Tieszen *et al*. 1983), prior to the analysis of carbon and nitrogen isotopes all powdered samples were treated with a chloroform-methanol (2:1) solution to extract the lipophilic fraction (Bligh and Dyer 1959). Also, as the analysis of carbon and nitrogen stable isotopes was performed in the organic matrix of bone samples, all bone samples were decalcified with a 1 M solution of HCl (Fry 1988, Jacob *et al*. 2005) to prevent inorganic carbon from affecting the carbon isotopic value.

After these treatments and subsequent drying, approximately 0.7 mg of each powdered sample was weighted in tin capsules, loaded, combusted at 1,000 ºC and analyzed in a continuous flow stable isotope ratio mass spectrometer (Flash 1112 IRMS Delta C Series EA Thermo Finnigan) coupled with an elemental analyzer.

Standards for ^{13}C and ^{15}N were the Vienna Pee Dee Belemnite (V-PDB) standard and atmospheric nitrogen, respectively. International isotope secondary standards of known $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios, namely: polyethylene (IAEA CH7; $\delta^{13}C$ = -31.8‰), sucrose (IAEA CH6; $\delta^{13}C$ = -10.4‰), ammonium sulphate (IAEA N1; $\delta^{15}N$ = +0.4‰ and IAEA N2; $\delta^{15}N$ = +20.3‰), potassium nitrate (USGS 34; $\delta^{15}N$ = -1,7‰), L-glutamic acid (USGS 40; $\delta^{15}N$ = -4,6‰; $\delta^{13}C$ = -26,2‰), and caffeine (IAEA 600; $\delta^{15}N$ = 1,0‰; $\delta^{13}C$ = -27,7‰) were used for calibration of $\delta^{13}C$ and $\delta^{15}N$. Reference materials used for the analysis were selected based on previous calibration experiments performed on the same type of samples and in order to ensure an optimum range of reference values. All reference materials used are distributed by the International Atomic Energy Agency (IAEA).

Results were expressed following the delta (δ) notation, in which the relative variations of stable isotope ratios are calculated as:

$$\delta^{13}C \text{ or } \delta^{15}N\ (‰) = [(R_{sample}/R_{standard})-1] \times 10^3$$

Where R is the heavy-to-light isotope ratio ($^{15}N/^{14}N$; $^{13}C/^{12}C$) in the sample and in the reference standards.

Oxygen stable isotope analysis

Oxygen stable isotope analysis was performed in the inorganic matrix and therefore could only be carried out in the bone samples. Powdered samples were treated with 30% hydrogen peroxide for 24 h to remove any organic compound. Samples were then rinsed carefully with milli-Q water and then treated with 1 M calcium acetate/acetic acid buffer for another 24 hours to remove any diagenetic carbonate. Finally, samples were rinsed again following the same procedure and then dried for 24 h, Koch *et al*. 1997.

An average of 4.5 mg of each powdered sample so treated was analyzed using a Carbonate Kiel Device III carbonate preparation system (Thermo Electron - Dual Inlet) linked to a gas source mass spectrometer. Samples were dissolved in 100% phosphoric acid at 70 °C with concurrent cryogenic trapping of CO_2 and H_2O. The CO_2 was then admitted to the mass spectrometer for analysis. An internal standard calibrated with international standard NBS-19 was used. Precision was $1\sigma = \pm 0.05‰$ for $\delta^{18}O$.

Values, reported in parts per thousand (‰), were calculated using the formula:

$$\delta^{18}O\ (‰) = [(R_{sample}/R_{standard})-1] \times 10^3$$

Where R is the heavy-to-light isotope ratio ($^{18}O/^{16}O$) and the standard is V–PDB. Because $\delta^{18}O$ values in animal studies are more commonly presented relative to the standard mean oceanic water index (SMOW), to allow comparison with published data $\delta^{18}O$ values were converted from V-PDB to SMOW using the following formula, Koch *et al*. 1997:

$$\delta^{18}O\ (SMOW) = [\delta^{18}O\ (V\text{-}PDB) \times 1.03086] + 30.86.$$

All the above analyses were conducted at the *Centres Científics i Tecnològics* (CCiT-UB) of the University of Barcelona.

Statistical analysis

Results were tested for normality of distribution with a Kolmogorov-Smirnov test of goodness of fit and for homoscedasticity with a Levene test. The presence of outliers was tested graphically through boxplots. As stable isotope results from krill were normally distributed and with homogeneous variances, a Student's *t-test* was used to investigate differences between the two sampling areas. As results from bone were normally distributed and with homogeneous variances, differences between the sampling areas and potential interactions among variables and factors where investigated through a General Linear Model (GLM) applied considering respectively $\delta^{15}N$, $\delta^{18}O$ and $\delta^{13}C$ as dependent variables, origin and sex as fixed factors and alternatively length and age as covariates.

A graphical comparison between the isotopic values of the two sampling areas was carried out with standard ellipses analysis performed using SIBER (Stable Isotope Bayesian Ellipses in R, Jackson *et al*. 2011) on $\delta^{13}C$ and $\delta^{15}N$ from krill samples and on $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ from bone samples. Both the convex hull, representing the smallest area encompassing all the observations, and the standard ellipses, representing a better estimate of the isotopic niche width when sample size is small, were represented. The percentage of overlapping between sampling areas was calculated from the overlapping area of the standard ellipses for each combination of stable isotopes.

SPSS 20 and R-3.1.2 statistical software were used for all analyses.

Results

Krill stable isotope analysis

All krill samples were successfully processed and analyzed. Boxplots (Fig. 2) indicated a moderate variability of δ^{13}C values, which ranged from -20.41‰ to -18.56‰ (mean ± SD = -19.67 ± 0.49‰), and a higher variability of δ^{15}N values, which ranged from 5.12‰ to 9.06‰ (mean ± SD = 7.37 ± 1.07‰). 3 outliers were detected but they were not excluded from the analysis because they did not exceed the 3*IQR limit. A Student's *t-test* did not indicate significant differences in δ^{13}C and δ^{15}N values between the two areas, and the graphs obtained from the Standard Ellipses Analysis (Fig. 3) highlighted an almost complete overlap between the standard ellipses calculated from these values. Thus, the overlapping area between the ellipses calculated from δ^{13}C - δ^{15}N values of the two areas represented 75% of the area of the ellipse representing NW Spain and 60% of the ellipse representing W Iceland.

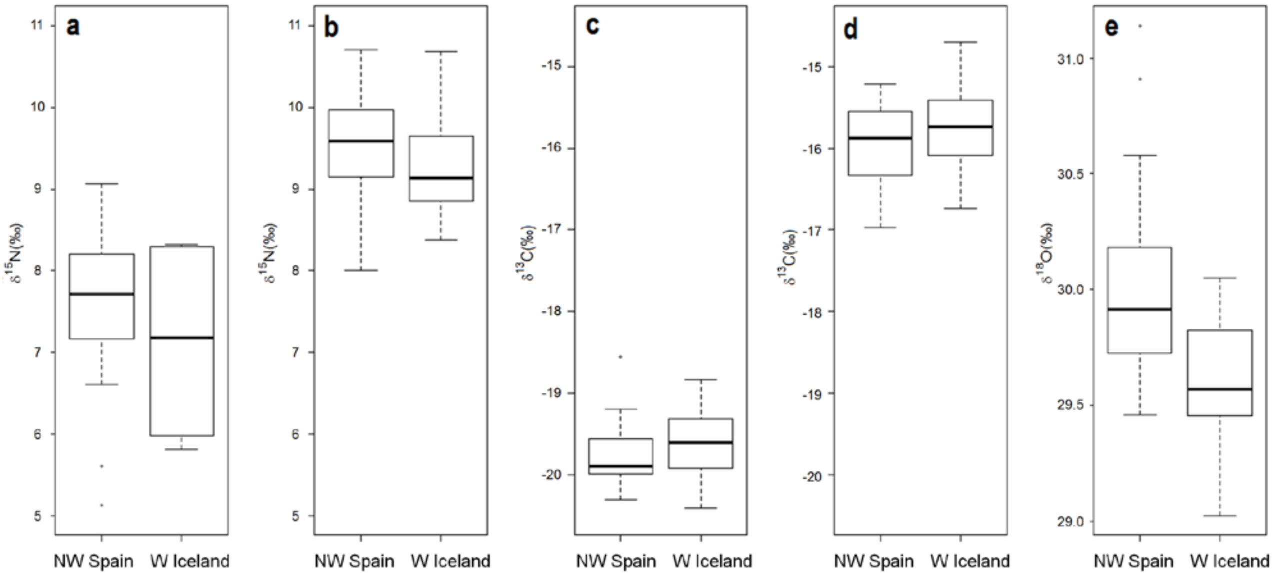

Figure 2. Boxplots. Distribution of δ^{15}N isotopic values (expressed in ‰) in krill (a) and fin whale bone (b); of δ^{13}C in krill (c) and fin whale bone (d); and of δ^{18}O in fin whale bone (e) sorted by geographic origin. Each box represents the median, first and third quartiles, and the whiskers represent the 95% confidence interval of the median.

Bone stable isotope analysis

Successful analyses could be conducted in all samples except one in which carbon and nitrogen stable isotopes signatures could not be measured. Boxplots indicated relatively low variability, with δ^{13}C values ranging from -16.97‰ to -14.7‰ (mean ± SD = -15.85 ± 0.5‰), δ^{15}N values from 8‰ to 10.70‰ (mean ± SD = 9.43 ± 0.64‰), and δ^{18}O values from 29.03‰ to 31.14‰ (mean ± SD = 29.8 ± 0.41‰) (Fig. 2). Two outliers were detected but they were not excluded from the analysis because they did not exceed the 3*IQR limit. A significant correlation was found between the length and both the age and sex of the individuals, therefore the General Linear Models were built excluding this variable. Only the model built for nitrogen values included the variable length instead of age because for samples included from the study of Borrell *et al.* (2012) age data were not available. However, none of these variables was found to correlate with $\delta^{13}C$, $\delta^{15}N$ or $\delta^{18}O$ values, or to change significantly across the sampling areas. While no significant differences were found in mean $\delta^{15}N$ and $\delta^{13}C$ between the two sampling areas, the model showed significant correlations between the origin and $\delta^{18}O$ values (Table 1). Thus, mean $\delta^{18}O$ was significantly ($P < 0.01$) lower in the samples from W Iceland (mean $\delta^{18}O$ ± SD = 29.59 ± 0.27‰) than in those from NW Spain (mean $\delta^{18}O$ ± SD = 30.01 ± 0.42‰).

	$\delta^{15}N$ ~ Origin + Sex + Length		$\delta^{13}C$ ~ Origin + Sex + Age		$\delta^{18}O$ ~ Origin + Sex + Age	
	Est. ± SE (*t*)	*P* (>|*t*|)	Est. ± SE (*t*)	*P* (>|*t*|)	Est. ± SE (*t*)	*P* (>|*t*|)
(Intercept)	8.52±1.7 (5.01)	$6.71e^{-06}$***	-16.01±0.34 (-47.63)	$<2e^{-16}$***	29.11±0.24 (119.04)	$<2e^{-16}$***
Origin	0.28±0.18 (0.16)	0.12	-0.19±0.15 (-1.27)	0.21	0.44±0.11 (4.13)	0.000***
Sex	0.03±0.2 (0.18)	0.859	0.18±0.15 (1.15)	0.258	0.11±0.11 (0.98)	0.333
Length	0.02±0.08 (0.26)	0.796	-	-	-	-
Age	-	-	0.01±0.01 (1.36)	0.18	-0.01±0.01 (-1.02)	0.312

Table 1. Main GLM outputs. Summary of the results obtained from the three complete GLM models built for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ as response variables and using origin and sex as fixed factors and alternatively length and age as covariates. For each of the three models, the estimated values of intercept, factors and covariates are shown ± the associated standard error, as well as the *t* value (in brackets) and probability (*** indicate significant probability at level P<0.001).

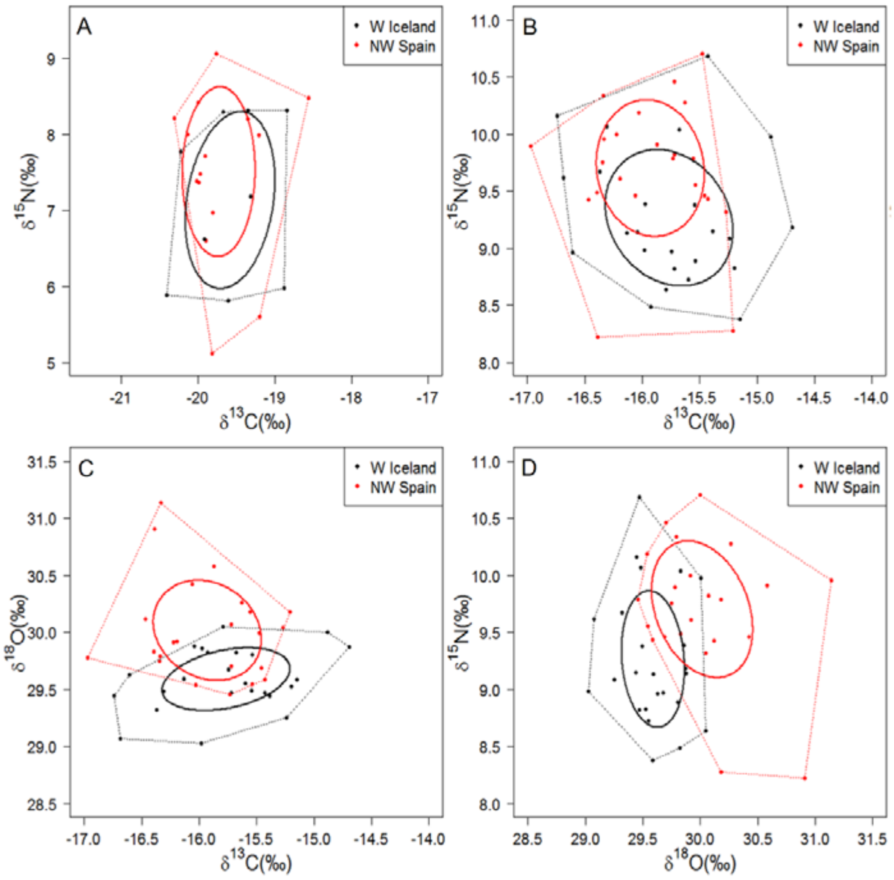

Figure 3. Standard ellipses. Plots resulting from the standard ellipses analyses performed using SIBER (Stable Isotope Bayesan Ellipses in R, Jackson *et al.* 2011). The convex hull (dotted lines) and the standard ellipse (solid lines) are represented. (A): $\delta^{13}C$ and $\delta^{15}N$ values in krill; (B): $\delta^{13}C$ and $\delta^{15}N$ in bone; (C): $\delta^{13}C$ and $\delta^{18}O$ in bone; and (D): $\delta^{18}O$ and $\delta^{15}N$ in bone.

The graphs obtained from the Standard Ellipses Analysis performed with values of all three stable isotopes showed that the two sampling areas could be mostly differentiated by their $\delta^{18}O$ values and, to a smaller extent, by their $\delta^{15}N$ values (Fig. 3). The overlapping area calculated from the $\delta^{18}O$ - $\delta^{15}N$ graph was 9.76% of the area of the standard ellipse representing samples from NW Spain and 15.17% of the area of the ellipse representing samples from W Iceland.

Discussion

The results of the stable isotope analysis and the comparison between the two geographical areas can be summarized in three main findings: i) $\delta^{15}N$ values were highly variable in krill and to a smaller extent in fin whale bone, but did not significantly differ between areas ii) $\delta^{13}C$ values in krill and in fin whale bone did not significantly differ between areas; and iii) $\delta^{18}O$ values in bone were higher in whales from NW Spain than in whales from W Iceland. These results and the observed differences can be examined with respect to the baseline data of the North Atlantic Ocean. These are available for the stable isotopes of carbon, nitrogen and oxygen in the form of isoscapes, result of a comprehensive meta-analysis of $\delta^{13}C$ and $\delta^{15}N$ at the base of the food web, and of $\delta^{13}C$ and $\delta^{18}O$ in seawater (McMahon *et al.* 2013).

Values resulting from the analysis of $\delta^{15}N$ in krill were variable in both areas, but no significant difference was found between areas, in contrast with the baseline values showed in the North Atlantic isoscapes, which are slightly higher in the waters off W Iceland (McMahon *et al.* 2013). This apparent contradiction may be due to two reasons: dissimilarities in the diet of krill and/or heterogeneity in krill species composition. Thus, *M. norvegica* is known to vary its diet from exclusively phytoplanktonic to carnivorous (Schmidt 2010). As the trophic level is the main driver of $\delta^{15}N$ variation (Minagawa and Wada, 1984, Post 2002), dissimilarities in the krill diet may explain both the apparent inconsistency of $\delta^{15}N$ values in krill with the regional baselines and the large variability in the values observed. Also, despite previous studies having shown that during the summer fin whales in the two areas base their diet mostly on *M. norvegica*, (Aguilar 1985, Víkingsson 1997), in our study the identification of krill at a species level was not feasible. For this reason the presence of other species displaying different $\delta^{15}N$ values cannot be ruled out. In particular, fin whales have been reported to occasionally consume Euphasiids of the genus *Thysanoessa* (Jonsgård 1966, Quiroga *et al*. 1983), whose $\delta^{15}N$ values are slightly lower (Ólafsdóttir *et al.* 2013), and which in Iceland constitute a significant component of the diet of other mysticetes like the minke whale, *Balaenoptera acutorostrata* (Sigurjónsson *et al.* 2000).

Mean values of $\delta^{15}N$ in fin whale bone samples were slightly variable, and about 2 per-mil points higher than values in krill samples, consistently with the trophic enrichment expected to occur between these species (Borrell *et al.* 2012). Values of $\delta^{15}N$ in fin whales showed no differences between the two areas, most probably reflecting similarities in their diet. Nevertheless, variability of $\delta^{15}N$ values in fin whales was high compared with that of the other isotopes, probably reflecting some degree of heterogeneity in prey items between individuals. Thus, the species is known to rely on krill when this food source is readily available (Sigurjónsson and Víkingsson 1997), but when this does not happen it behaves as an opportunistic feeder and relies on a variety of small schooling fishes (Ingebrigsten 1929, Nemoto 1957, Nemoto and Kasuya 1965, Jonsgård 1966). Both Jonsgård (1966) and Sergeant (1977) proposed krill to be the prevalent food when this is available, while schooling fish would be an alternative resource when krill is scarce and the schooling fish aggregates for spawning, typically in spring or autumn (*e.g.* Reid *et al.* 1999, Stratoudakis *et al*. 2007). Such an opportunistic behavior would explain not only the range of $\delta^{15}N$ values found in our study but also the comparatively wide trophic niche exhibited by North Atlantic fin whales as compared to other balaenopterid species (Mitchell 1975, Ryan *et al*. 2013).

Variability of $\delta^{13}C$ values in both krill and fin whale bone was smaller than that of $\delta^{15}N$ values in the same tissues, and consistent with previously studies from the same geographical areas (Borrell *et al.* 2012, Ólafsdóttir *et al*. 2013). Similarly, mean $\delta^{13}C$ values in fin whale bone samples were almost 4 per-mil points higher than in krill samples, a result consistent with the trophic enrichment factor between krill and fin whale bone estimated by Borrell *et al.* (2012). However, neither the $\delta^{13}C$ values in krill nor in bone showed any significant difference between areas, apparently in contrast with available baselines showing higher values in the waters around NW Spain than in those off W Iceland (McMahon *et al.* 2013). The reasons for this apparent contradiction may be different for the two tissues. Values of $\delta^{13}C$ in in the marine environment are influenced by the productivity conditions prevailing in the area, which in turn depend from highly seasonal variables such as the photoperiod, water temperature and salinity (McMahon *et al.* 2013). In the case of krill, sampling was restricted to the summer, and therefore it may not have been fully representative of the year-round conditions shown by the isoscapes. In the case of bone, the absence of geographical differences may be explained by the capability of this tissue to integrate long-term isotopic fluctuations (Newsome *et al.* 2010), thus blurring the locally-acquired signature with the isotopic signatures acquired from other areas where the whales could have been moving and feeding.

What clearly differentiated fin whales from the two sampling areas was $\delta^{18}O$ values. In the marine environment, $\delta^{18}O$ strongly correlates with water salinity and temperature, and it is therefore a good tracer of migrations and habitat use of marine mammals, especially in areas with well-defined environmental gradients (Yoshida and Myazaki 1991, Clementz and Koch 2001). $\delta^{18}O$ values in bone were significantly higher in whales from NW Spain than in whales from W Iceland, thus matching the regional variation of the $\delta^{18}O$ isoscapes proposed for the North Atlantic Ocean (Bowen 2010, McMahon *et al*. 2013) and substantiating that the two groups of whales exploit distinct feeding areas.

The large difference between areas found in $\delta^{18}O$ bone values and the slight inconsistencies between the $\delta^{15}N$ and $\delta^{13}C$ values found in bone and in the sampling location baselines deserve attention. Fin whales, like most mysticetes, undertake annual migrations that involve seasonal movements between productive high-latitude feeding grounds, which they occupy in the summer, and low-latitude breeding grounds, which they occupy in the winter. As a consequence of this geographical shift, feeding is generally considered to be a highly seasonal activity that strongly peaks during the summer. However, in the North Atlantic the latitudinal amplitude of the migration has been suggested to be smaller than in other oceans because of the influence of the Gulf Stream, which provides adequate wintering grounds for breeding at relatively high latitudes (Aguilar 2009). Consistently with this, several studies have shown that some individuals remain in their summer grounds even during winter (Ingebrigtsen 1929, Jonsgård 1966, Evans 1992, Sanpera and Aguilar 1992, Gunnlaugsson and Víkingsson 2014), a fact that suggests year-round feeding. Fin whales have also been observed feeding during migration (Silva *et al.* 2013) and recent research on stable isotope values in baleen plates has indicated that food intake during periods other than the summer may be enough to influence the isotopic composition of the body tissues (Aguilar *et al.* 2014). According to this, as already suggested, we can conclude that the isotopic values observed in bone represent the integration of those typical of the food ingested during the feeding season, with those characteristic of food consumed in distant areas during periods other than the summer.

Unfortunately, while much is known about the location of North Atlantic fin whale summer breeding grounds, questions are still open on where the winter breeding grounds of the species may be located, and which may be the migration routes from the summer grounds to such breeding grounds. Different hypotheses have been brought forward about the possible relationships between the putative stocks and the degree of mixing in the different feeding and breeding grounds (Mizroch *et al.* 1984, Daníelsdóttir *et al.* 2006, IWC 2009). Stable isotopes may shed some light to this discussion. According to our analysis, we can confirm the currently accepted distinction of the Icelandic stock from that off NW Spain. Indeed, the statistical analysis and the graphical interpretation made through the standard ellipses analysis highlighted that $\delta^{18}O$ values clearly differentiate the two subpopulations, thus confirming that they exploit separated feeding grounds and move between areas isotopically well differentiated. However, the migratory patterns of the two stocks and their distribution ranges, particularly in the winter, are not easy to establish based on the results of the stable isotope analysis and their comparison with the available isoscapes (Graham *et al.* 2009, Bowen 2010, McMahon *et al*. 2013).

Most hypotheses about stock subdivisions (IWC 2009) concur that the fin whale stock feeding off NW Spain is composed of individuals belonging to a unique stock, whose breeding grounds location remains to be identified. The high $\delta^{18}O$ values here found firmly indicate that these grounds should be located in an oceanic area with higher baseline values of oxygen. According to the North Atlantic isoscapes (Graham *et al.* 2009, Bowen 2010, McMahon *et al*. 2013), such an area may be located at lower latitudes, on a wide band extending off the eastern coast of Africa.

Regarding the Icelandic stock, many hypotheses concur that it is composed of a mixture of animals breeding in the central Atlantic with animals breeding in the Western Atlantic (IWC 2009). This hypothesis cannot be discarded, because our results represent an integration of the isotopic values typical of the sampling area with others characteristic of other areas. The co-existence of groups breeding in other grounds would be also supported by the recent finding through satellite tracking of a central fin whale migratory corridor that runs over 2,700-3,000 km along the Gulf Stream and links the Azores with the summer feeding grounds located between E Greenland and W Iceland (Silva *et al.* 2013).

Thus, the results of the stable isotope analyses point to an intricate structure of fin whale subpopulations exploiting different habitats and with latitudinal migrations that are still far to be completely understood. The lack of complete knowledge about the North Atlantic fin whale population structure and movements highlights the need for a careful revision of some aspects of the subdivision into stocks currently used in the management of the North Atlantic fin whale (Fig. 1). Further studies with satellite tracking and genetics would undoubtedly contribute to clarifying these questions, particularly the actual structure of stocks. Also, the analysis of chemical markers in other tissues like the baleen plates may shed further light on fin whale migration patterns (Best and Schell 1996, Lee *et al.* 2005, Mitani *et al.* 2006, Bentaleb *et al.* 2011, Giménez *et al.* 2013). These tissues, which provide permanent records of at least the last two migration cycles of the whales, together with the analysis of tissues such as skin, which reflect the short-term diet composition, may prove extremely effective tools to clarify such complex picture.

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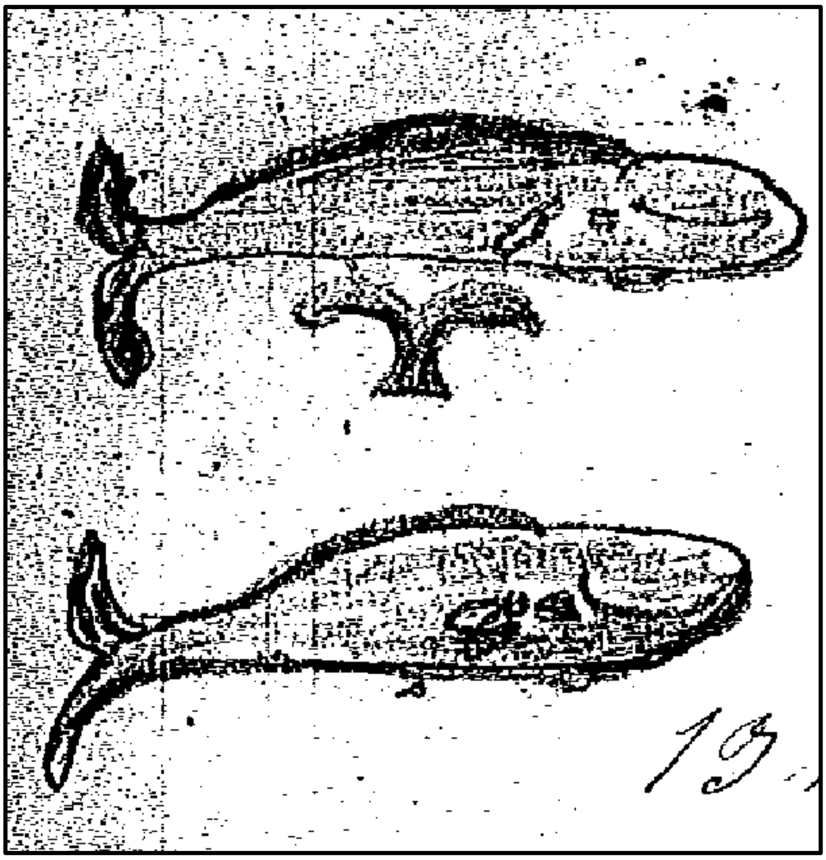
2. Elements concentrations



2.1 ***The fin whale, a marine top consumer, exposes strengths and weaknesses of the use of fluoride as ecological*** *tracer*

Authors: Morgana Vighi, Iván García-Nisa, Asunción Borrell, Alex Aguilar

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Título: El rorcual común, un consumidor apical en el ambiente marino, expone fortalezas y debilidades en la utilización del flúor como trazador ecológico

Resumen:

El flúor es retenido en los tejidos óseos de los animales y su disponibilidad en el medio ambiente varía entre regiones en función de las fuentes naturales y antropogénicas. Estas propiedades sugieren que este elemento pueda ser un trazador adecuado del origen, la distribución y los movimientos de los animales. En ambientes marinos, el krill acumula concentraciones de flúor que se transfieren a sus depredadores. En este estudio examinamos el potencial de las concentraciones de flúor en el hueso para discriminar dos poblaciones separadas de un consumidor de krill, el rorcual común. Los niveles basales de las áreas de muestreo (Islandia occidental y noroeste de España) fueron determinados a través del análisis de muestras de krill. Como se esperaba, debido a la alta carga de flúor de origen volcánica en las aguas de Islandia, el krill de W Islandia mostró concentraciones de flúor mucho más altas que el de NW España. Las concentraciones en el hueso ballenas estaban correlacionadas con el sexo y la edad, aumentando linealmente con la edad en las hembras y mostrando valores significativamente más bajos y con un diferente patrón de acumulación con la edad en los machos. Las concentraciones de flúor en los huesos de las ballenas eran mucho más altas que en el krill, lo que indicaría acumulación del elemento, pero, inesperadamente, la zona de origen no tuvo influencia en las concentraciones. Esta aparente contradicción puede explicarse bien por la integración en el hueso de los alimentos consumidos en otras áreas, o por la activación de respuestas homeostáticas a muy altos niveles de exposición al flúor. Se concluye que el flúor puede ser un trazador útil sólo si se integran en el análisis datos de edad y sexo de los individuos, si hay disponible información sobre su dieta durante todo el año y / o si la población investigada está expuesta a niveles leves de este elemento.

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The fin whale, a marine top consumer, exposes strengths and weaknesses of the use of fluoride as ecological tracer

Morgana Vighi *, I. García-Nisa, A. Borrell, A. Aguilar

Department of Animal Biology and Institute of Biodiversity Research (IRBio), Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain

HIGHLIGHTS

- We analyzed fluoride levels in krill and fin whale bone from two separate areas.
- Krill levels of fluoride were significantly higher in W Iceland than in NW Spain.
- Fin whale bone accumulated significantly higher fluoride concentrations than krill.
- Fluoride levels in fin whales were influenced by sex and age, not by sampling area.
- Fluoride accumulation in females was higher than in males and increased with age.

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ABSTRACT

Fluoride is retained in bone tissues of animals and its availability in the environment varies between regions according to natural and anthropogenic sources. These properties suggest this element as a suitable tracer of origin, distribution or movements of animals. In marine environments, krill builds-up fluoride concentrations that are transferred to its predators. In this study we examine the ability of bone fluoride concentrations to discriminate two separate populations of a krill consumer, the fin whale. Background levels of the sampling areas (Western Iceland and North-Western Spain) were determined through the analysis of krill samples. As expected, due to the high load of volcanic-derived fluoride in Icelandic waters, krill from W Iceland showed much higher fluoride concentrations than that from NW Spain. Concentrations in whales' bone were correlated with sex and age, increasing linearly with age in females and showing significantly lower values and a different age-related pattern of accumulation in males. Fluoride concentrations in whales' bone were much higher than in krill, indicating accumulation of the element but, rather unexpectedly, the area of origin had no influence on concentrations. This apparent contradiction may be explained either by the integration in bone of food consumed in other areas, or by the activation of homeostatic responses at very high levels of fluoride exposure. It is concluded that fluoride can be a useful tracer only if age and sex data are integrated into the analysis, year-round information on diet is available and/or the investigated population is exposed to mild levels of this element.

1. Introduction

Elucidation of distribution range, structure of populations, migratory routes and origin of individuals are central elements for understanding the population dynamics of animals and to inform their management. These variables can be investigated through a number of techniques that range from examination of population trends to morphological studies, natural and artificial tagging, genetics or photo-identification, but the development in the last decades of analytical techniques at reasonable cost has led to the emergence of a variety of chemical tracers able to further contribute to this end (Bianchi and Canuel, 2011; Ramos and González-Solís, 2012). The underlying principle behind the use of these tools is that the composition of the tissues of a given organism directly reflects, or is related through predictable processes, to the background levels of the environment in which it lives or feeds. To be useful, these tracers should moreover display substantial spatial variation to allow traceability. In marine mammals, the use of chemical tracers has nowadays become a standard tool in

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* Corresponding author. Tel.: +34 633656763.
E-mail address: morgana.vighi@gmail.com (M. Vighi).

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population and ecological studies, with particular emphasis on the use of xenobiotic organic pollutants, stable isotopes or trace elements (Aguilar, 1985; Hobson, 1999; Kunito et al., 2002; Das et al., 2003; Borrell et al., 2006; Krahn et al., 2007; Newsome et al., 2010; Méndez Fernandez, 2012; Vighi et al., 2014).

Fluoride is an element naturally present in the environment which at high levels of exposure can become toxic, particularly to vertebrates. In these animals, ingested fluoride is mainly absorbed and incorporated into developing teeth and bone, where it can replace the hydroxyl group of the main component of the inorganic matrix, hydroxyl apatite. The resulting compound, called fluorapatite, is potentially harmful to the skeletal integrity and at high levels it produces osteofluorosis, which in its turn results in exostoses, an increase of bone mass and density accompanied by related symptoms. Dental lesions have also been observed in a variety of mammals, affecting the enamel and causing an excessive decay of the teeth. Other symptoms caused by fluoride intoxication can be emaciation, thickening of the joints, decreased milk yield, and digestive, reproductive, renal, neurological and endocrine alterations (Walton, 1988; Ozsvath, 2009; Kierdorf et al., 2012). This toxicity has elicited numerous ecophysiological studies on populations of domestic and wild terrestrial mammals exposed to high background levels of this element which contributed to improve the knowledge on its dynamics in ecosystems (Walton, 1988; Camargo, 2003; Ozsvath, 2009).

In seawater, fluoride originates both from natural and anthropogenic sources. Releases from sediments, precipitation of blown dust from the atmosphere and volcanic activity, particularly the deposition of volcanic ash, are the main natural sources (de Angelis and Legrand, 1994). Apart from natural fluoride releases, significant anthropogenic emissions are produced by a variety of industrial activities that range from aluminum smelters to paper mills, coal-fired power stations, coking plants and oil refineries, as well as the generalized use of fluoride-rich fertilizers, refrigerants, aerosols, lubricants and plastics (Walton, 1988; Paranjpe et al., 1994; Kierdorf and Kierdorf, 2001). Such diversity of sources makes background levels of this element extremely variable between locations. Once in the seawater, fluoride is dissolved and ingested by marine organisms. In their digestive system, the low pH of the gastric fluids allows the formation of the fluorhydric acid, which is absorbed from the stomach and small intestine by simple diffusion (Ozsvath, 2009) and is deposited in the target tissues, such as bone in vertebrates and the chitinous exoskeleton in invertebrates (Camargo, 2003). The ample variation in fluorine background levels, coupled with its deposition in structural tissues, provide this element with the necessary basis for being a useful indicative tracer of the water masses in which organisms live.

Some species of crustaceans, particularly the Euphasiids or krill, show a remarkable capacity to build-up extremely high fluoride concentrations in their tissues. Soevik and Braekkan (1979) found levels of fluoride in the range 1330–2400 mg kg^{-1} fat free dry weight in whole body of North Atlantic *Meganyctiphanes norvegica* and Antarctic *Euphausia superba*, and Sands et al. (1998) reported levels of up to 5977 mg kg^{-1} dry weight in the exoskeleton of Antarctic *Euphausia crystallorophias* and of 12800 mg kg^{-1} dry weight in the mouth parts of Antarctic *E. superba*. Consistently with these high levels in krill, most vertebrates feeding on these krill, such as Adélie penguins (*Pygoscelis adeliae*), crabeater seals (*Lobodon carcinophagus*), minke whales (*Balaenoptera acutorostrata*) and fin whales (*Balaenoptera physalus*), also show high fluoride content in their bones and other skeletal structures (Schneppenheim, 1980; Adelung et al., 1985; Walton, 1988; Landy et al., 1991; Alne, 1995). However, similarly as marine fishes (Julshamn et al., 2004), these species appear to tolerate well high levels of exposure to fluoride. Indeed, despite accumulating elevated concentrations of the element in their bones, these species do not suffer rates of skeletal lesions or other clinical signs higher than other comparably exposed species (Landy et al., 1991; Alne, 1995; Mikaelian et al., 1999). Besides the interest raised about the potential impact of fluoride as a pollutant, the concentrations above described were high enough to produce concern toward the potential consumption of such organisms, whether by humans or by wildlife (Grave, 1981). Indeed, the interest in the use of krill or krill derived products, both for human and for aquaculture, has dramatically increased (Nicol and Foster, 2003), and the potential of fluoride to accumulate along the food web raises concern. However, the actual risks from the perspective of food security are usually considered low because the fluoride content of meat and other edible tissues of vertebrates is very low even in the highly polluted species (Wright and Davison, 1975; Adelung et al., 1985).

Independently of the toxicological implications, the high tissue levels of fluoride present in these marine organisms facilitate the use of this element as tracer of their distribution and movements. The present study examines the ability of fluoride concentrations in bone to discriminate two separate populations of a marine consumer subject to different background levels of this element. The only previous investigation examining variation of fluoride levels between populations of marine mammals has been conducted on beluga whales (*Delphinapterus leucas*) from Canada. A significant difference was found between individuals from St. Lawrence Estuary (Quebec) and Hudson Bay (North Western Territories) but, because the study had been designed with an ecotoxicological focus, fluoride background levels in each area were not investigated and the cause for the difference not identified (Mikaelian et al., 1999).

The species selected for the present study was the fin whale, a krill-eating species already known to accumulate high concentrations of fluoride in its tissues (Landy et al., 1991). In the North Atlantic the species structures in a number of independent stocks or demes that purportedly occupy segregated geographical ranges (International Whaling Commission, 2009, Fig. 1). Taking advantage of material collected during the period of commercial whaling in the 1980s and stored at the tissue bank of the University of Barcelona, we analyzed samples of bone (rib) from individuals caught off North Western (NW) Spain ($n = 54$) and Western (W) Iceland ($n = 24$), as well as krill obtained directly from the stomach of the sampled whales. These locations correspond to the feeding grounds of two distant fin whale stocks whose isolation is supported by investigations carried out using a wide range of techniques, from morphological studies to tagging, genetics and chemical tracers (Gunnlaugsson and Sigurjónsson, 1989; Jover, 1992; Lockyer, 1981, 1982; Sanpera et al., 1996; Bérubé et al., 1998; Víkingsson and Gunnlaugsson, 2005). The distance between the two whaling grounds is about 2.700 km and the Icelandic whaling grounds are moreover subject to chronic pollution by fluoride as a consequence of the volcanic eruptions that periodically afflict the region (Stefánsson and Sigurjónsson, 1957; Thorarinsson, 1979; Witham et al., 2005), for which reasons it was anticipated that the sampling locations would provide the variation in fluoride background levels required for the test.

2. Materials and methods

2.1. Ethics statement

Samples used for this study were obtained from the biological tissue bank of the University of Barcelona (BMA Tissue Bank), which preserves samples from whales that were legally caught during the period of commercial exploitation in the 1980s for purposes other than research. No specific approval is required in Spain to undertake research on samples supplied by official channels and coming from commercial fisheries.

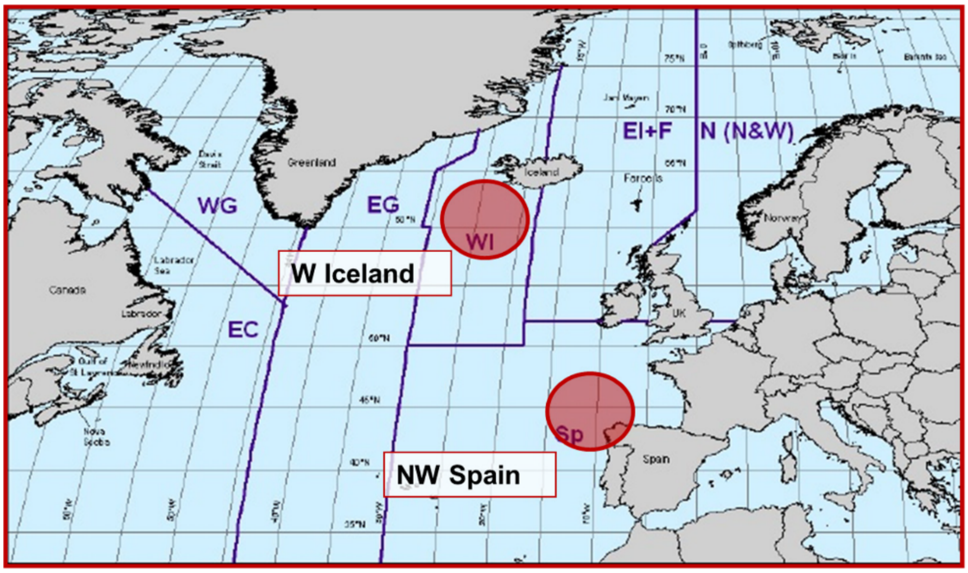

Fig. 1. Fin whale stock structure. Map of the North Atlantic Ocean, representing the stock structuring of the North Atlantic fin whale population made by the International Whaling Commission (IWC, 2009). Sampling areas are marked with circles.

2.2. Sample collection and preparation

A total of 78 bone samples were obtained from sections of the ribs of fin whales: 24 from fin whales caught in 1986 off W Iceland (Denmark Strait), and 54 from fin whales caught in 1983 and 1984 off NW Spain (Galicia) (Fig. 1). Data about sex, age and length were available from all individuals except one. The sample from W Iceland was composed of specimens aged 7–27 years (9 males and 15 females) while the sample from NW Spain was composed of specimens aged 4–84 years (39 males and 15 females). Age and sex distribution in the two sampling areas are resumed in Table 1.

Krill samples were obtained from the stomach content of 5 fin whales caught off NW Spain in 1983 and 1985 (2 replicates from each were analyzed); and of 2 fin whales caught off Iceland in 1986 (3 replicates from each were analyzed). Identification of the krill species composition was impossible because samples were partially digested, but, being parts of the exoskeletons and other anatomical structures still intact, all samples were identified as euphasiids, belonging to the order *Euphasiacea*.

After collection, all samples were frozen in plastic bags at −20 °C and stored until analysis.

2.3. Fluoride analysis

Before the analysis, all samples were thawed at room temperature and dried in a stove at 60 °C for 24 h. After drying, a sub-sample of each bone, 2 sub-samples of each krill sample from NW Spain and 3 sub-samples of each krill sample from W Iceland were finely ground to powder with mortar and pestle.

As some of the samples from NW Spain were already treated for lipid extraction during previous analyses, to avoid possible biases in the comparison among samples, and to prevent possible interferences caused by lipids with the detection of fluoride, all powdered samples were treated with a chloroform–methanol (2:1) solution to extract their lipophilic fraction. Trials were made with random samples from NW Spain and W Iceland in order to verify if the treatment had an effect on the detection of fluoride; results of a Student's *t*-test ($p > 0.05$) performed to compare fluoride concentration between treated and non-treated samples did not highlight

Table 1
Age and sex distribution; mean length and fluoride concentrations of samples. Distribution of age and sex of samples from the two sampling areas; mean and standard deviations (SD) of length and fluoride concentrations (mg kg^{-1} ash) in rib bone of males (M) and females (F) fin whales from W Iceland and NW Spain.

	W Iceland ($N = 24$)		NW Spain ($N = 54$)	
Age (y)	M ($N = 9$)	F ($N = 15$)	M ($N = 39$)	F ($N = 15$)
4			2	
5			2	
6			4	1
7		2	3	1
8	2		2	1
9		2	5	2
10	1	2		3
11		1	2	
12	1		2	1
13			1	
14	1	1	4	
15		2	1	1
16	1		1	
17		2	1	
18			1	1
19				
20	1			
21		1	1	
22				
23				1
24				
25			1	1
26	1	1		1
27	1			
28			2	
33				1
35			1	
36			1	
68				1
82			2	
84			1	
NA		1		
Length (m, mean ± SD)	19 ± 1.01	19.1 ± 0.96	18.4 ± 0.84	19.9 ± 0.95
Fluoride (mg kg^{-1} ash, mean ± SD)	8718 ± 2652	7623 ± 1955	7671 ± 1395	10118 ± 2562

any significant effect of the treatment. To eliminate the organic component of the samples, sub-samples of 100–200 mg were burned to ash in porcelain containers in a muffle furnace at 500 °C for 24 h. Ashes were cooled in a desiccator to prevent the absorption of moisture. The average percentage of organic component and lipid content in bone samples were calculated by subtracting the ash weight from the initial dry weight of treated and non-treated samples, and comparing the results. The total organic component resulted to be 54.6% of the original dry weight, of which the 17.4% was constituted by lipids. Three aliquots of 8 mg of each bone sample were dissolved in 4 mL of 0.1 M perchloric acid; volumes were brought to 10 mL with distilled water and the solution was buffered with 10 mL of a total ionic strength adjustment buffer (TISAB II, Orion Research, Beverly, MA, USA). 1–2 aliquots of 8 mg of each krill sample were dissolved in 2 mL of 0.1 M perchloric acid; volumes were brought to 5 mL with distilled water and the solution was buffered with 5 mL of a total ionic strength adjustment buffer (TISAB II, Orion Research, Beverly, MA, USA).

Fluoride determination was performed with a fluoride ion-specific combination electrode (Orion Research 96-09). A certified Orion reference solution containing 1000 mg NaF L^{-1} was used to prepare standards for electrode calibration. Standards of 2, 5, 10 and 20 mg NaF L^{-1} were also measured between each set of three bone aliquots and at the beginning and the end of the analysis to build calibration curves and adjust all determinations. For fluoride determination in krill, the set of standard concentrations used for electrode calibration were 0.2, 0.5, 1 and 2 mg NaF L^{-1}.

Fluoride concentrations were calculated as mg of fluoride contained in 1 kg of bone or krill ash, and expressed as mg kg^{-1} ash.

2.4. Statistical analysis

All results were tested for normality of distribution with a Kolmogorov–Smirnov test of goodness of fit and for homogeneity of variances with a Levene test.

Krill fluoride concentrations were found to be normally distributed and with homogeneous variances. As a consequence, the distribution of results in the two sampling areas was verified through boxplots and differences between krill samples from the two sampling areas were tested with a Student's *t*-test.

Bone fluoride concentrations were found to be not normally distributed and with homogeneous variances. The distribution of results between the two sampling areas and between sexes was verified through boxplots.

Also, differences of fluoride concentrations between krill and whale bone were investigated through a non-parametric U of Mann–Witney test applied to the whole data set.

Relations among variables were first investigated through a partial correlation analysis, performed using the Pearson's coefficient to estimate pairwise correlations between sex, length, age and origin of the fin whale samples.

Basing on the result of the preliminary data exploration, variables were selected, and a series of general lineal models (GLMs) and general additive models (GAMs) were applied to verify trends in fluoride concentration and whether these were dependent to any of the selected variables. Bone fluoride concentration was considered as dependent variable; and the effect of combinations of the remaining variables were estimated. Comparison between the resulting GAMs and GLMs was performed based on the AIC (Akaike's Information Criterion) values; the best fitting model was selected according to its lowest AIC and the highest R^2 and percentage of explained deviance.

As the distribution of age values in the samples was not homogeneous, causing an increased standard deviation in the oldest samples, the same series of models was applied to a reduced set of data, excluding from the analysis all samples obtained from individuals aged over 36 years. Also in this case, the process of model selection was conducted according to the AIC values, R^2, and the percentage of explained deviance.

Finally, because a significant difference was highlighted in fluoride concentration and trends between males and females, two different reduced models, considering only the effect of origin and age, were performed for the two sexes both in the whole and in the reduced data set.

SPSS 20 and R-2.15.2 statistical software were used for all above analyses.

3. Results

3.1. Krill samples

Krill fluoride concentrations ranged 148–1994 (mean ± SD = 1000 ± 621) mg kg^{-1} ash in the samples from NW Spain and 4242–5675 (mean ± SD = 4819 ± 525) mg kg^{-1} ash in the samples from W Iceland. The *t*-test performed with fluoride concentration as dependent variable and sampling area as fixed factor revealed a significant difference between sampling areas ($p = 0.016$), being mean fluoride concentration in W Iceland samples almost five times higher than in those from NW Spain (Fig. 2a).

3.2. Fin whale bone samples

Bone fluoride concentrations ranged 5051–14790 (mean ± SD = 8307 ± 2429) mg kg^{-1} ash in samples from W Iceland, and 5390–14913 (mean ± SD = 8370 ± 2103) mg kg^{-1} ash in samples from NW Spain (Table 1).

Results were not normally distributed (Kolmogorov–Smirnov test, $p < 0.05$), with homogeneous variances (Levene test, $p > 0.05$), and showed a limited variability in the two sampling areas (Fig. 2b).

The U of Mann–Witney test performed on the whole data set considering the results of the two sampling areas together revealed a significantly ($p < 0.001$) higher fluoride concentration in bone samples (mean ± SD = 8350 ± 2192 mg kg^{-1} ash) than in krill samples (mean ± SD = 2432 ± 1992 mg kg^{-1} ash) (Fig. 2).

The partial correlation analysis highlighted some level of correlation between the variable "fluoride concentration" and the variables "sex" (Pearson's $\rho = 0.43$) and "length" ($\rho = 0.41$), between the variable "length" and the variables "sex" ($\rho = 0.51$) and "age" ($\rho = 0.39$), and at a smaller extent of the variable "origin" with the variable "sex" ($\rho = 0.31$).

Because the variable "length" was correlated with almost each variable, and in particular it showed a significant linear correlation with the variable "age", it was decided to exclude it from subsequent analysis, being that it could mask the effect of the variable "age", more interesting under a biological point of view. With the remaining variables, a series of GLM and GAM models was applied with fluoride concentration as dependent variable and combinations of the other variables as covariates, starting considering pairs of them, and then considering the combination of the three variables "age", "sex", and "origin" together. None of the models applied highlighted a significant difference between fluoride concentrations in the two sampling areas, while all the models highlighted a significant effect of the variable "sex", and many indicated also a significant effect of the variable "age". From the process of model selection, the model which best explained the data resulted to be a GAM with sex and origin as fixed factors, and a smoothing structure of correlation with age (Fluoride ~ Origin + Sex + s(Age)) (Table 2). Even if the variable "origin" was not significant in any of the tested models, it was kept in the final

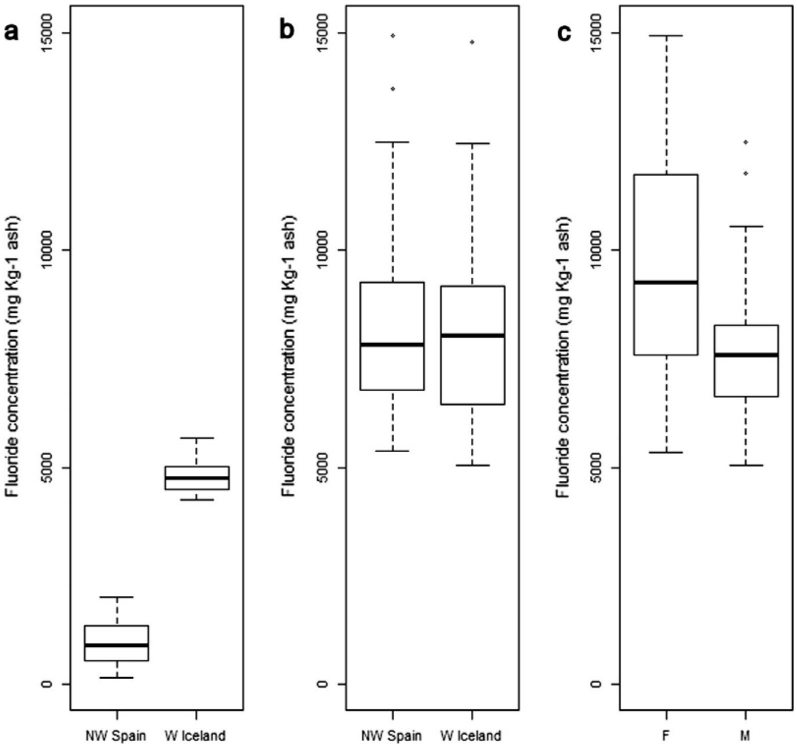

Fig. 2. Boxplots. Fluoride concentration (mg kg^{-1} ash) distribution in krill samples sorted by sampling origin (a), and in fin whale bone samples sorted by sampling origin (b), and sex (F = females, M = males), (c). Each box represent median, first and third quartile, whiskers represent 95% confidence interval of median.

Table 2
GLMs and GAMs. Series of GLMs and GAMs applied to the whole data set and to the reduced data set including only samples aged up to 36 years. For every model AIC values, % of deviance explained, R^2, and the level of significance of the variables ($*** = p < 0.001$, $* = p < 0.05$, ns = $p > 0.05$) are indicated. The best fitting model is underlined.

Model	Variables	All samples				Samples up to 36 y of age			
		Significance	AIC	% Dev. Exp.	R^2	Significance	AIC	% Dev. Exp.	R^2
GLM	Origin + Sex	Sex***	1412	18.4	0.162	Sex***	1321	17.8	0.154
GLM	Sex + Age	Sex***	1392	19.8	0.176	Sex*** Age*	1317	22.5	0.203
GAM	Sex + s(Age)	Sex*** Age*	1386	32	0.271	Sex*** Age*	1314	28.5	0.248
GLM	Origin + Age	ns	1409	1.1	0	Age*	1330	7.2	0.045
GAM	Origin + s(Age)	Age*	1400	18	0.125	Age*	1327	14.3	0.101
GLM	Origin + Sex + Age	Sex***	1393	21.2	0.18	Sex***	1317	24.5	0.212
GAM	Origin + Sex + s(Age)	Sex*** Age*	1385	33.4	0.282	Sex*** Age*	1314	31.1	0.265

model because its inclusion increased both the R^2 and the percentage of deviance explained by the model.

According to this model, fluoride concentration was significantly lower in males (mean ± SD = 7661 ± 1491 mg kg^{-1} ash) than in females (mean ± SD = 9452 ± 2688 mg kg^{-1} ash), ($p < 0.001$), (Fig. 2c), and a significant correlation of fluoride concentration with age emerged (Fig. 3a). Trends in males and in females were investigated separately, and resulting models and graphs indicated the absence of any significant trend of fluoride concentration with age in males, and a significant linear increase of fluoride concentration with age in females ($p < 0.05$; Fig. 3b).

As in any of the model applied the amplitude of standard deviation of the correlation function between age and fluoride concentration was dramatically increasing in samples aged over 36 years, due to the scarcity of elder samples, the same series of models was applied to a reduced set of data, excluding from the analysis all samples obtained from individuals aged over 36 years. In total, 4 samples were excluded from the analysis. The resulted AIC values obtained with the reduced set of data were considerably lower than those obtained from the equivalent models based on the full set of data (Table 2). Also in this case the best fitting model resulted to be the GAM (Fluoride ~ Origin + Sex + s(Age)), which led us to the same conclusion of a significant difference in fluoride concentration between males and females and a significant trend of fluoride concentration with age, modeled by the smoothing function in Fig. 3c. Investigating trends in males and females separately, concentrations of fluoride resulted to be modeled by a smoothing function with age at a significance of $p = 0.05$, while they were still significantly linearly increasing with age in females ($p < 0.05$; Fig. 3d).

4. Discussion

To be a good tracer, a chemical would ideally meet at least four properties: (i) its background levels in the environment should present significant variation; (ii) the tracer should be incorporated

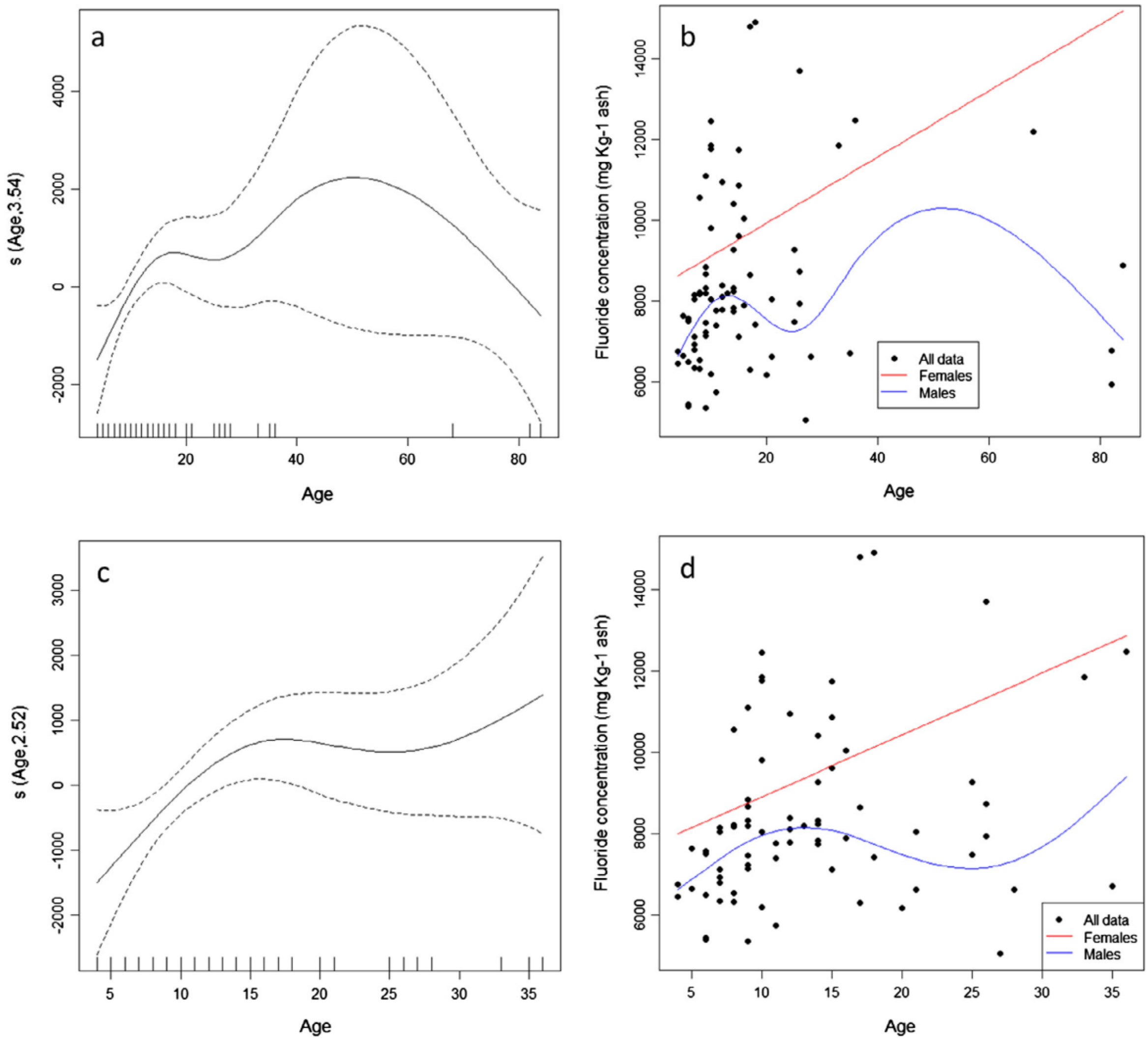

Fig. 3. Trends of fluoride concentration with age. Plots representing trends of fluoride concentration with age, (a, b) in the whole data set, and (c, d) in the reduced set of data limiting the age to individuals aged up to 36 years old. (a, c) Smoothing function of the selected GAM (dotted lines represent standard deviation intervals). (b, d) Distribution of samples (black points) and predicted trends of males and females plotted separately.

into the tissues of the studied species in measurable concentrations; (iii) the variability of the tracer within a population should be moderate or predictable through measurable variables to allow their inclusion in the statistical model; and (iv) tissue concentrations of the tracer should reflect background levels in a consistent and predictable manner.

Krill, which was assumed to reflect fluoride background levels, presented high variability of fluoride concentrations within each location. In Euphausiids, fluoride is mostly deposited in the exoskeleton, where it might play a role as a hardener (Soevik and Braekkan, 1979; Adelung et al., 1987; Sands et al., 1998). However, the exoskeleton is discarded in each molt and fluoride is therefore lost to be re-accumulated from the environment when the new exoskeleton is built. Although in our sample the molting phase of krill could not be determined, the observed variation in fluoride levels is probably not unrelated to the molt cycle, which in Euphausiids like *M. norvegica* occurs every 9–13 d (Keck, 1984). This makes krill a complex proxy for assessing fluoride background levels and, when differences in concentration between areas are moderate, a determination of the physiological phase at which krill is sampled may be required. In our study the krill collected from the stomach of the whales was partially digested, so neither the molting state nor the species composition could be established. However, previous studies have shown that the diet of North Atlantic fin whales is mainly composed of *M. norvegica* in both areas (Soevik and Braekkan, 1979; Aguilar 1985; Sigurjónsson and Víkingsson, 1997), and the difference between areas was found to be so large that within-areas variation was considered not able to obscure it. Thus, concentrations of fluoride were significantly higher in the krill from W Iceland than in that from NW Spain. Being that krill from the two areas was demonstrated to belong to two genetically distinct populations, isolated by patterns of ocean circulation (Papetti et al., 2005), this difference in fluoride levels undoubtedly reflects the fact that the Icelandic whaling grounds, which are located about 250 km offshore (Víkingsson, 1997), are enriched by fluoride originated from volcanic eruptions, which produce fluoride-rich ash that disperses to distances of several hundred kilometers (de Angelis and Legrand, 1994). Indeed, fluoride is the principal toxic element adsorbed on volcanic ash (Cronin et al., 2003; Witham et al., 2005), and intermittent or semi-continuous volcanic activity has been documented to result in widespread contamination of neighboring water (Stefánsson and Sigurjónsson, 1957) and mortality by fluorosis of animals (Sigurðsson and Pálsson 1957; Thorarinsson, 1979; Stewart et al., 2006). It is unclear what is the contribution of volcanic activity to the difference in the fluoride background levels of the areas here studied, but it is clear that the water masses in which the whales here studied live and feed can be characterized using this element.

The fulfillment of the second property required to the tracer, implying that it should be incorporated into the tissues of the studied species in measurable concentrations, had already been documented beforehand (Landy et al., 1991). When compared with krill, fluoride concentrations in the bone of the studied fin whales were more than three times higher than those in krill, indicating some level of bio-accumulation of this element through the food web. Thus, bone concentrations ranged 5051–14913 mg kg^{-1} ash, values similar to those obtained by Landy et al. (1991) in a previous study on W Icelandic fin whales, and of the same order of magnitude of those found in bone of other marine mammals, like beluga whales from St. Lawrence Estuary (873–7336 mg kg^{-1} ash) and Hudson Bay (7186–17686 mg kg^{-1} ash) (Mikaelian et al., 1999), Antarctic crabeater seals (1337–6380 mg kg^{-1} dry weight), and Antarctic Weddell seals, *Leptonychotes weddellii* (878–2200 mg kg^{-1} dry weight) (Adelung et al., 1985).

Checking for the third property, implying that the variability of the tracer within the study populations is moderate or predictable through measurable variables, was not as straightforward. Consistently with other studies, variability in bone fluoride concentrations was not negligible: the coefficient of variation was 0.29 for the sample of W Iceland and 0.25 for that of NW Spain. Previous studies had already highlighted that fluoride concentrations in mammals were related to the sex and age of the individual (Landy et al., 1991; Alne, 1995; Mikaelian et al., 1999); this effect was also found in our study, where all models indicated that concentrations were higher in females than in males and correlated with age. In a similar study on minke whales, Alne (1995) suggested that females would incur in a higher mobilization of calcium during pregnancy and breastfeeding periods and that, therefore, the degree of calcium mobilization in certain periods of their life cycle might lead to a higher rate of incorporation of fluoride in bone. The observed increase of fluoride concentration with age, which was also found by Landy et al. (1991) in Icelandic fin whales and by Mikaelian et al. (1999) in beluga whales from the St. Lawrence Estuary, can only be explained by a rate of intake higher than that of excretion which inevitably leads to the accumulation of the element in bone tissues. However, as observed in Fig. 3, fluoride concentrations appeared to increase dramatically in all samples in the first years of age, at least until 10–15 years of age. After this age, that is when fin whales are generally considered to be sexually mature (Aguilar and Lockyer, 1987), the global trend (Fig. 3a and c) seems to reach a sort of plateau, with no further increase in fluoride concentration. This would probably indicate the intervention of some mechanisms of regulation or homeostasis when concentrations go beyond certain thresholds. When looked in detail, considering males and females separately, this trend resulted more complex, indicating that after maturity, levels of fluoride in females continue to increase linearly, while in males they seem to follow an unclear trend of small fluctuations around 8000 mg kg^{-1} ash (Fig. 3d). These differences might be explained by the fact that when physical growth stops, accumulation of fluoride in males reaches a sort of threshold and fluoride levels get more stable. On the contrary, in females no threshold is reached, and fluoride continues to accumulate, due to the more dynamic structure of female bone connected to cycles of pregnancy and lactation, as already hypothesized by Alne (1995). Thus, Mikaelian et al. (1999) observed that belugas from the Hudson Bay, which carried in their tissues higher fluoride loads than those from the St. Lawrence Estuary, did not show correlation of concentrations with age. In this case, absence of correlation was tentatively explained by the small age range of that sample, which did not allow testing satisfactorily the relationship between bone fluoride concentration and age, but could also reflect the fact that this highly polluted population had already reached a threshold eliciting homeostasis. According with these findings, concentrations of fluoride in cetaceans bone cannot be precisely predicted through measurable variables, but whatever the case, sex and age are variables that must be considered when investigating levels of fluoride in cetacean populations, and must be incorporated into any statistical model allowing the accounting for their effect.

The fourth property of the tracer, that tissue concentrations reflect background levels in a consistent and predictable manner, was apparently not fulfilled. Thus, despite krill, and by extension the background levels, were higher off W Iceland than off NW Spain, none of the models showed a significant difference between fluoride concentrations in the bone of whales from the two areas. This unexpected result may be explained by at least two reasons. The first one is the potential incidence in the bone concentrations of the fluoride contents of other prey items. We departed from the assumption that the whales would be reflecting the food consumed in their feeding grounds, where they had been sampled. As other baleen whales, the fin whale undergoes seasonal migrations between high latitude summer grounds, where it feeds intensively, and lower latitude winter grounds, where it breeds (Aguilar, 2009). As a consequence, feeding is a highly seasonal activity that strongly peaks in the summer, a period when in both areas the species is known to rely almost only on krill (Aguilar, 1985; Sigurjónsson and Víkingsson, 1997; Víkingsson, 1997). However, it is also known that during seasons when krill is scarce schooling fish may become an alternative resource, particularly when the fish aggregates for spawning (Jonsgård, 1966; Sergeant, 1977), something which occurs in spring or autumn (Reid et al., 1999; Stratoudakis et al., 2007). In this scenario, fin whales from W Iceland could be displaying lowered fluoride concentrations in their tissues because they are integrating non-krill food sources poor in fluoride, or even krill from other locations which present lower levels of fluoride. This could be corroborated by the fact that the sympatric common minke whale, whose diet is composed of 59% fish and 41% crustaceans mainly belonging to the gender *Thysanoessa* sp. (Sigurjónsson and Víkingsson, 1997; Skaug et al., 1997), which contains lower concentrations of fluoride than *M. norvegica* (Moren et al., 2007), accumulates in its bone significantly lower fluoride concentrations (6510 ± 2969 mg kg^{-1} ash, Alne, 1995) than the fin whale (8369 ± 2103 mg kg^{-1} ash).

However, it cannot be discarded that, as suggested above, the whales may activate some homeostatic mechanism that regulate fluoride absorption. If this were true, fluoride concentrations in fin whale bone would not merely be a reflection of those in the diet, but the result of a dynamic regulation involving enhanced excretion by the renal system (Ozsvath, 2009). This possibility had been anticipated by other authors (Landy et al., 1991) and may be supported by the highly dynamic bone structure characteristic of the animals that undergo frequent dives in deep waters. Because high pressure tends to result in lowered bone density (Taylor, 2000; Reidenberg, 2007; Gray et al., 2007), and fluoride is mainly accumulated in bone, it may be suggested that in baleen whales this element is more mobile than in terrestrial mammals and is therefore subject to some specific regulation mechanisms.

Whatever the reason, the fact that in our study fluoride concentrations in each area were significantly distinct in the krill purportedly reflecting background levels but indistinguishable in the whales consuming that krill, questions the utility of fluoride as a tracer of distribution, origin or movements unless the apparent discrepancy is clarified. Further investigations are required to deepen in this subject and determine whether our inability to discriminate the fin whale populations was caused by incomplete information on feeding biology or by the very high levels of exposure of the species, which elicited homeostatic responses. However, a different mechanism of accumulation between males and females was highlighted possibly reflecting differences in physiology after sexual maturity is reached. According to these conclusions, fluoride could still be a useful tracer for cetacean species only as long as the targeted species is exposed through food to low or moderate levels of this element, and data about sex and age of sampled individuals are available and integrated into the analysis.

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2.2 Metals concentration and accumulation trends in the bone of two populations of fin whale, Balaenoptera physalus

Authors: Morgana Vighi, Asunción Borrell, Alex Aguilar

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Resumen:

Los metales se depositan de forma masiva, a través de emisiones directas o deposiciones atmosféricas secas y húmedas, en el medio marino. La contaminación por metales en los organismos marinos se ha investigado cada vez más, pero la mayor parte de la investigación se centra en unos pocos tejidos y elementos, y algunas especies, como los cetáceos misticetos, han sido poco estudiadas. Aquí contribuimos a esta base de datos examinando las concentraciones de zinc, cobre, plomo, titanio y estroncio en los huesos del rorcual común (*Balaenoptera physalus*) del noroeste de España y oeste de Islandia, evaluando las posibles tendencias con la edad y el sexo, y averiguando la ocurrencia de pasaje trans-placentario mediante el análisis de muestras de fetos. Los resultados destacaron altas concentraciones de Sr y tendencias significativas de acumulación de Pb con la edad en las muestras de España noroccidental. A excepción del Sr, todos los metales son transferidos a través de la barrera placentaria, a diferentes niveles, y Cu, Pb y Zn mostraron concentraciones más altas en los fetos que en los adultos. Después del nacimiento, las concentraciones de Zn y Pb mostraron un incremento significativo con la edad.

Metals concentration and accumulation trends in the bone of two populations of fin whale, *Balaenoptera physalus*

Morgana Vighi*, Asunción Borrell, Alex Aguilar

Department of Animal Biology and Institute of Biodiversity Research (IRBio), Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain.

Abstract

Metals are deposited massively, through direct emissions or atmospheric dry and wet depositions, in the marine environment. Metal contamination in the marine organisms has been increasingly investigated, but most research focuses on a few tissues and elements, and some species, like mysticete cetaceans, have been scarcely studied. Here we contribute to this fragmented data examining zinc, copper, lead, titanium and strontium concentrations in bone of fin whales (*Balaenoptera physalus*) from NW Spain and W Iceland; evaluating possible trends with age and sex; and testing the occurrence of placental transfer through the analysis of fetal samples. Results highlighted higher Sr concentrations and significant Pb accumulation trends with age in NW Spain samples. Except from Sr, all metals were transferred, at different levels, through the placental barrier, and Cu, Pb and Zn showed higher concentrations in fetuses than in adults. After birth, Zn and Pb concentrations showed a significant increase with age.

Keywords: Cetaceans; Cu; Zn; Ti; Sr; Pb.

Introduction

Metals reach the environment both through natural processes, such as volcanic activity or geological weathering, and human activities, including waste disposal, industrial emissions, mining operations or the use of fossil fuels (Bowles, 1999). The majority of metals are toxic, at different levels, for living organisms. Some, essential at low concentrations due to their role in metabolism, become toxic at high concentrations; others, with no recorded functions in metabolism, can be toxic also at very low concentrations (Law, 1996).

The rapid and almost uncontrolled development of industrial activities in the last century caused a dramatic increase of emissions of heavy metals in the environment (Nriagu, 1990), raising concern about the potential threats of metal pollution to living organisms, human health, and, in general, ecosystems.

The marine environment constitutes a sink where metals are deposited massively, through direct emissions or atmospheric dry and wet depositions (Thompson, 1990; Ansari *et al*., 2004). Despite metal contamination in the oceans has been increasingly studied in the last decades (*e.g.* Thomson, 1990; Eisler, 2010), most of the research focuses on a few elements, especially in the most toxic ones, like mercury or cadmium. Data about concentrations and effects of the other metals in marine organisms are scarce or lacking, and information regarding key organisms, such as marine mammals, is still incomplete.

The importance of studying metal concentrations in marine mammals has been stressed since the late ’70, when the FAO working party on marine mammals stated that metals such as mercury, cadmium, lead, arsenic, chrome, copper, nickel, iron and titanium were probably causing serious problems to marine mammals and thus required special consideration (FAO, 1978). The International Whaling Commission (IWC) further highlighted in 1999 the lack of knowledge about these contaminants in marine mammals and warranted attention to metals other than cadmium and mercury and tissues other than kidney and liver (IWC, 1999).

Despite the knowledge about metal contamination in marine mammals increased during the past 15 years, data about certain elements, tissues and species are still lacking. Pinnipeds and odontocetes have been the main groups investigated, while species belonging to the groups of mysticetes and sirenians have been less studied. A number of authors reviewed available data (Wagemann and Muir, 1984; Law, 1996; Aguilar *et al*., 1999, Das *et al*., 2003), which are still incomplete due to the difficulties related with collecting samples and obtain information about sex, age, body size or reproductive state of the individuals, factors which could affect the accumulation of metals. Moreover, the interpretation of the concentrations observed and their possible relation with toxic effects are challenged by the limited knowledge about the mechanisms of physiological regulation in marine mammals (Bowles, 1999).

Moreover, the information regarding metal accumulation in the different tissues of marine mammals is not homogeneous. Protein-rich tissues such as liver, kidney and muscle, due to their capacity to accumulate and store trace metals, have been mostly studied (Aguilar *et al*., 1999), generally in samples collected from stranded, bycaught or directly caught individuals. Skin, despite being a promising tissue because it can be non-invasively sampled from free ranging individuals, has not been frequently studied (O’Hara *et al*., 2008; Aubail *et al*., 2013; Borrell *et al*., 2015), and data about metal concentrations in hard tissues like bone and teeth are scarce.

In the present study we contribute to this scenario investigating concentrations of zinc (Zn), copper (Cu), lead (Pb), titanium (Ti) and strontium (Sr) in bone of fin whales (*Balaenoptera physalus*) from NW Spain and W Iceland. The two areas represent the summer feeding grounds of two groups of whales, which the IWC classified as isolated stocks (IWC, 2009) and whose isolation is confirmed by a number of evidences including genetic, chemical markers, morphologic data, and satellite tracking studies (Lockyer, 1982; Bérubé *et al*., 1998; Víkingsson and Gunnlaugsson, 2005; Vighi *et al*., in press).

Concentrations of mercury (Hg), cadmium (Cd), Cu and Zn have been already determined in liver, kidney and muscle in samples from these fin whale populations (Sanpera *et al*., 1993; 1996). Moreover, arsenic (As), selenium (Se), Hg, Cd, Pb, Cu, Zn, chromium (Cr), nickel (Ni), manganese (Mn) and iron (Fe) concentrations were examined in skin, muscle, kidneys, and livers of minke whales (*Balaenoptera acutorostrata*) from the Icelandic waters (Auðunsson and Víkingsson, 2013). Nevertheless, data about metal concentrations in the bone of these species are absent, and data regarding concentrations of elements such as Ti and Sr are rare in cetaceans, and in general in marine vertebrates (Viale, 1978, Holsbeek *et al*., 1999; Bryan *et al*., 2007; Griesel *et al*., 2008).

Bone was selected as target tissue in the attempt to fill the gaps in the record of tissues investigated, and because, despite being a poorly studied tissue, it can provide useful information about long-term accumulation trends of specific elements.

Being an inert tissue with a long turnover rate, it reflects concentrations of elements over a long time, independently of short-term variations that may be caused by metabolic status or short-term environmental changes.

The availability of data regarding sex, body length, age and reproductive status of all sampled individuals allowed us to investigate the accumulation trends of the metals with age, considering possible variations caused by the other biological variables. Also, through the analysis of fetuses' samples it was possible to verify the placental transfer of metals and their level of accumulation from the mothers during gestation.

Materials and methods

Sample collection and composition

Bone samples, obtained from the biological tissue bank of the University of Barcelona (BMA Tissue Bank), were collected from the central section of a thoracic rib of fin whales caught during the 1983-1984 summer whaling campaigns off NW Spain (Galicia Bank) and the 1986 summer whaling campaign off W Iceland (Denmark Strait) (Fig. 1). Overall, the analyses included 24 samples from W Iceland (9 males and 15 females, with ages ranging from 7 to 27 years), and 25 samples from NW Spain (8 males and 14 females with ages ranging from 6 to 26 years; plus 3 fetuses). Data regarding body length, sex, female reproductive status (lactating, pregnant, or none of the above) and age (determined through the number of growth layers in earplugs as described by Lockyer, 1984), were available from most individuals, and apart from the 3 fetus samples, the sex and age composition of the samples from each area were similar.

All samples were preserved frozen until preparation for the analysis.

Age determination

Age determination was carried out according to the procedures described by Lockyer (1984). Growth layers were counted through a longitudinal section of the fin whales ear plug core. Each sample count was repeated by more than one reader, and for about 70% of samples it was repeated twice by the same reader. Thus, if the difference between the age estimates for the same sample was higher than 10% of the lowest age estimate, the age determination of that sample was not considered valid; otherwise the mean of all readings was considered as a reliable estimate of the age of the sample.

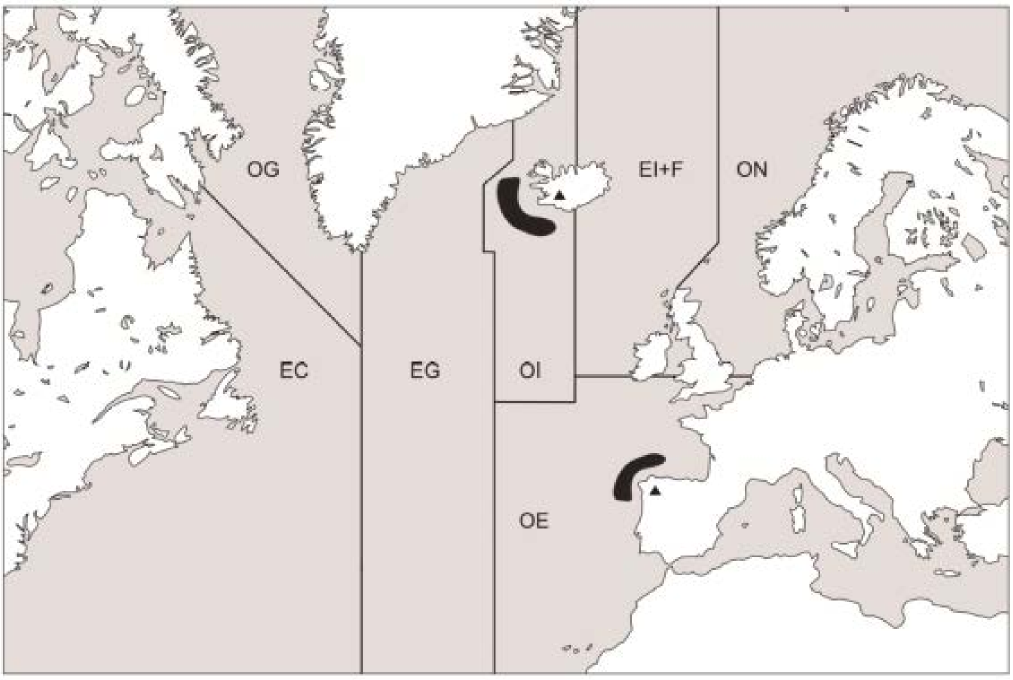

Figure 1. Sampling areas. Map of the North Atlantic Ocean, representing subdivision of the North Atlantic fin whale population proposed by the IWC (IWC, 2009) and the two sampling areas marked in black. The black triangles indicate the localization of the ancient whaling factories where our samples were collected.

Metals analysis

A subsample of each frozen sample was dried at 60 ºC for 48 h in a stove and subsequently ground to a fine powder by mortar and pestle.

A preliminary semi-quantitative analysis was carried out in 2 randomly selected samples to evaluate which elements among the naturally occurring ones were enough concentrated to be quantified in the samples and were therefore suitable for the aims of the present study. Basing on the results obtained, Zn, Cu, Pb, Ti and Sr were selected for quantitative trace element analysis.

Samples were prepared following the same protocol for both for the preliminary test and the quantitative analysis. Approximately 0.1 g of each sample was weighted and transferred to clean Teflon reactors. Each sample was then treated through acid digestion: 3 ml of HNO_3 (70%) and 1 ml of H_2O (30%) were added to each Teflon reactor and the solution was left at 90 ºC during 12 h. The digested samples were then diluted in 30 ml of distilled water, and 10 ml of each diluted sample were transferred to plastic tubes to be analysed (with a Perkin-Elmer Elan 6000) via inductively coupled plasma mass spectrometry (ICP-MS). The entire analytical procedure was validated by analysing one blank (10 ml of H_2O_2), one replicate and one certified reference material (Bovine liver 1577a) every 10 samples. Replicates differed below 10% and the recovery percentage fell between 90 and 100%. Detection limits for each metal were set as 0.5, 0.1, 0.05, 0.2 and 0.1 μg Kg-1 for Zn, Cu, Pb, Ti and Sr respectively. The results, expressed in ng ml^{-1} solution, were converted to ng g^{-1} dry sample (μg Kg^{-1}) through adequate transformations.

All the analyses were conducted at the *Centres Científics i Tecnològics* (CCiT-UB) of the University of Barcelona.

Statistical analysis

Results obtained from each element analysis were tested separately for normality of distribution with a Kolmogorov-Smirnov test of goodness of fit and for homoscedasticity with a Levene test. The distribution of results and the presence of outliers were tested graphically through boxplots, sorted by sampling area and then distinguishing fetuses, males and females, and the different reproductive status of females. Due to the non-normal distribution of most of the results, a non-parametric *U* of Mann Whitney test was used to detect differences between fetuses and the other individuals. Basing on the results of these tests, the results from fetuses were excluded from the data set and the possible relationships among the concentration of each metal, the age of the individuals, and the other variables (length, sex and sampling area) were investigated through a series of GLM models. For each metal the model which better explained the results was selected basing on the value of R^2 and the amount of explained deviance; significant trends were plotted graphically.

R-3.1.2 statistical software was used for all above analyses.

Results

All 49 samples were successfully processed and analyzed. The concentrations of none of the metals (except Zn) were following normal distribution nor had homogeneous variances. Boxplots highlighted the presence of 3 outliers exceeding the 3*IQR limit in the concentrations of Cu (Fig. 2). The highest of these extreme values (19,456 μg Kg^{-1}) derived from a sample whose concentrations of Zn and Pb were also extremely high when compared to the others (31,296 and 499 μg Kg^{-1} respectively). As a consequence, all results derived from this sample were excluded from the subsequent analysis. On the contrary, the other two Cu outliers were not accompanied by values exceeding the concentration ranges of other metals, and therefore only the Cu values of these samples were excluded from subsequent analysis. Excluding the outliers, variability of results was in general high, with concentrations ranging from 39 to 495 μg Kg^{-1} (mean ± SD = 145 ± 94 μg Kg^{-1}) in Pb, from 179 to 636 μg Kg^{-1} in Ti (mean ± SD = 279 ± 110 μg Kg^{-1}), from 45 to 952 μg Kg^{-1} in Cu (mean ± SD = 178 ± 204 μg Kg^{-1}), from 15,889 to 31,951 μg Kg^{-1} in Zn (mean ± SD = 22,295 ± 3,872 μg Kg^{-1}) and from 20,066 to 113,616 μg Kg^{-1} in Sr (mean ± SD = 82,764 ± 20,158 μg Kg^{-1}). Boxplots did not highlight relevant differences in the distribution of concentrations between sampling areas, sexes or female reproductive statuses, but when grouping the fetuses separately from the other individuals, some visible differences emerged (Fig. 2). The U of Mann Whitney test highlighted significant differences between the fetuses and the other individuals in all elements except for Ti, with higher concentrations in fetuses for Pb, Cu and Zn, and lower concentrations in fetuses for Sr (Table 1).

Figure 2 (next page). Boxplots. Distribution of the concentrations (μg Kg^{-1}) of the metals analyzed in fetus samples from NW Spain (NWS F) and in adults samples, sorted by sampling areas (NWS = NW Spain; WI = W Iceland). The y axis representing Cu concentrations is cut to show the three extreme outliers: the sample corresponding to the points marked with a red circle was excluded from the statistical analysis; the values corresponding to the two points marked with a red diamond were excluded only from Cu statistical analysis.

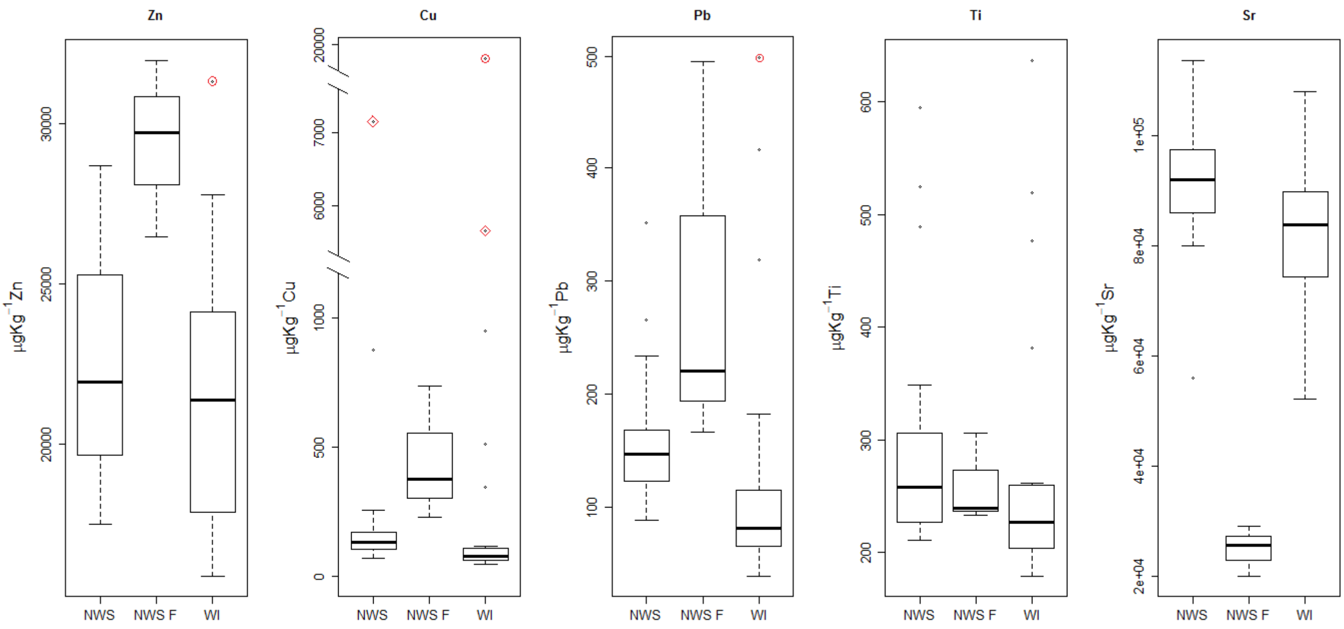

Table 1. Number of samples analyzed, mean concentrations (µg Kg^{-1}) and relative standard deviations of each metal, in fetus and adult samples, also sorted by sampling area (in brackets: NWS = NW Spain; WI = W Iceland). The levels of significance (*P*) of the *U* of Mann Whitney test performed comparing the element concentrations in fetuses and adults are shown (* indicates significant results, at a level of P < 0.05).

Metal	Group	N (NWS;WI)	Mean (NWS;WI)	SD (NWS;WI)	*P*
Zn	Fetuses	3	29,367	2,759	0.002*
	Adults	45 (22;23)	21,824 (22,591;21,090)	3,469 (3,364;3,480)	
Cu	Fetuses	3	446	263	0.011*
	Adults	43 (21;22)	159 (171;148)	190 (170;210)	
Pb	Fetuses	3	294	176	0.017*
	Adults	45 (22;23)	135 (162;109)	80 (60;90)	
Ti	Fetuses	3	260	40	0.599
	Adults	45 (22;23)	280 (294;268)	113 (107;120)	
Sr	Fetuses	3	24,894	4,524	0.000*
	Adults	45 (22;23)	86,622 (91,397;82,056)	13,768 (11,639;14,325)	

Excluding the samples obtained from fetuses, which showed significant differences from the rest of the individuals in most of the metals analyzed, a series of General Linear Models were fitted. Each different metal was analyzed separately; age was considered as independent variable, and length, sex and sampling area, or a subset of them, as covariates/factors. None of the models highlighted a significant contribution of the factor sex. Sampling area had a significant effect only over Sr concentrations, which resulted significantly lower (P < 0.05) in the samples from W Iceland (Table 1); and over Pb concentrations, which showed different accumulation trends in the two sampling areas. Given these results, the relations of Sr and Pb concentrations with age were also tested separately in the two sampling areas. Sr concentrations were not significantly related with age in any of the sampling areas. A significant positive correlation with age emerged instead in Pb concentrations, both when considering all data together ($P < 0.05$), and in samples from NW Spain ($P < 0.01$), while in samples from W Iceland no significant trend with age was detected (Fig. 3). Age had a significant effect also over Zn concentrations, which increased significantly in older samples ($P < 0.001$) (Fig. 4). No significant correlation with age was found for Cu and Ti concentrations.

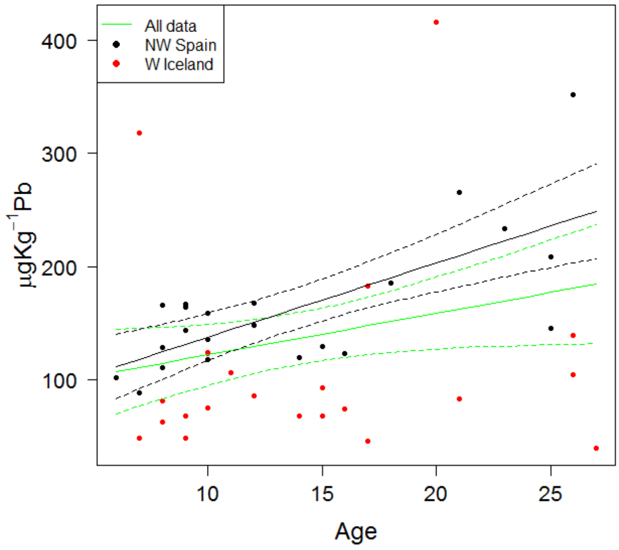

Figure 3. Pb concentrations (µg Kg^{-1}) plotted against age. The significant correlation between Pb concentrations and age is plotted for all data (green line) and for samples from NW Spain (black line) with the relative standard errors (dotted lines, green and black respectively). No significant relation emerged in samples from W Iceland.

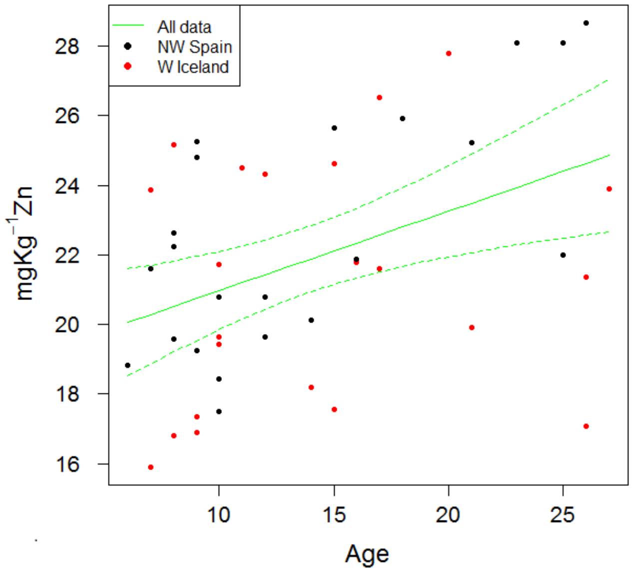

Figure 4. Zn concentrations (mg Kg^{-1}) plotted against age. The significant correlation between Zn concentrations and age is plotted for all data (green line) with the relative standard errors (dotted lines).

Discussion

This study represents the first determination of metals concentration and accumulation trends with age in the bone of fin whales, including samples deriving from fetuses. Most of the research on metal concentrations in cetaceans has been carried out on highly metabolic internal tissues, such as liver or kidney, as well as muscle sampled from stranded animals (*e.g*. Capelli *et al*., 2007; Rosas *et al*., 2012; Borrell *et al*., 2015); while studies on free ranging animals were possible only on skin biopsies (*e.g*. Kinito, 2002; Bryan *et al*., 2007). In both cases, it is complex to have a wide record of animals of different ages from the same study area. In this study, the availability of data related to age, sex and female reproductive status of the individuals allowed us to relate the metal concentrations with these variables, to investigate accumulation trends with age and the possible influence of other biological factors.

Comparison of concentrations with literature data

The comparison of our results with literature was challenged by the scarcity of data regarding metal determination in cetaceans' bone and by a number of variability factors related with the species, the tissue and the element analyzed.

Tissue metals concentrations are affected by a number of factors, including individual biologic variability, the feeding habits and exposure rate of the individuals (Reijnders *et al*., 2009). The pollutant load can be highly variable among cetacean species occupying different trophic niches and displaying distinct ecological traits and distribution ranges. The variability connected with the tissue analyzed is also an important factor to keep in consideration, as the patterns of distribution of the elements within an organism are tissue-specific. Several trace elements (*e.g*. Hg, Se, Cu, Zn, Fe) tend to concentrate in internal tissues, primarily in the liver, followed by kidney and muscle (Das *et al*., 2003; O'Hara *et al.,* 2008; Auðunsson and Víkingsson, 2013; Borrell *et al*., 2015). However, several studies report that the main body-burden of Sr and Pb (> 90%), and also great portion of Zn, are incorporated into the skeleton (Silbergeld *et al*., 1992; Burton and Price, 2002; Rabinowitz, 1991; Murray and Messer, 1981).

Considering all these variability factors, we first compared our results with those obtained from the analysis of the same species and populations, although in different tissues. This was possible only for Zn and Cu, which were analyzed in muscle, kidney and liver of fin whales from NW Spain and W Iceland by Sanpera *et al.* (1996). The comparison of results highlighted that overall Cu mean concentrations in bone (0.16 ± 0.19 mg Kg^{-1}) were more than one order of magnitude lower than those in muscle (2.15 ± 0.57 mg Kg^{-1}), kidney (13.92 ± 2.71 mg Kg^{-1}) and liver (13.44 ± 3.45 mg Kg^{-1}), and Zn concentrations in bone (21.82 ± 3.47 mg Kg^{-1}) were five times lower than those found in muscle (145.1 ± 67.7 mg Kg^{-1}), liver (121.8 ± 29 mg Kg^{-1}) and kidney (116.04 ± 26.5 mg Kg^{-1}) (Sanpera *et al*., 1996). These results agree with the pattern of distribution of Cu and Zn in the body compartments described above, suggesting higher levels in internal tissues than in bone. Both Cu and Zn tend to accumulate at lower concentrations in bone than in other tissues, probably because the percentage of proteins where these metals can adhere is lower in bone. Nevertheless, some authors suggested that Zn can also incorporate in hydroxyapatite and accumulate with calcification (Honda *et al*., 1984), thus explaining the relatively higher concentrations of this metal when compared to the concentrations of the other metals in the fin whale bone.

When data about the same species and populations were not available, we compared our results with data regarding similar species and/or similar sampling areas. As the minke whale (*Balaenoptera acutorostrata*) has been reported to feed upon similar sources as the fin whale in Iceland (Víkingsson, 1997; Sigurjónsson *et al*., 2000), we assumed that metal concentrations would be similar in the two species. Thus, Cu and Zn concentrations in minke whale liver (14.6 ± 3.2 mg Kg^{-1} and 186 ± 49 mg Kg^{-1} respectively, Auðunsson and Víkingsson, 2013) were of the same magnitude to those of Icelandic fin whales (12.30 ± 4.14 mg Kg^{-1} and 191.76 ± 79.48 mg Kg^{-1} respectively, Sanpera *et al*., 1996). However, Cu and Zn concentrations in minke whale skin (2.0 ± 0.3 mg Kg^{-1} and 57 ± 7 mg Kg^{-1} respectively, Auðunsson and Víkingsson

2013) were lower than in internal tissues, but still much higher than concentrations in fin whale bone (0.16 ± 0.19 mg Kg^{-1} and 21.82 ± 3.47 mg Kg^{-1}, respectively).

Pb concentrations in fin whale bone (mean 0.135 ± 0.08 mg Kg^{-1}), on the contrary, were higher than those found in livers of minke whales (mean 0.09 ± 0.05 mg Kg^{-1}) in Iceland (Auðunsson and Víkingsson, 2013), but lower than those in the skin (mean 0.54 ± 0.143 mg Kg^{-1}; Auðunsson and Víkingsson, 2013). Studies on Pb bioaccumulation in bones of cetaceans are rare, but all of them show that Pb concentrations are usually higher in the hard tissues than in the soft ones (Honda *et al*., 1982; Kemper *et al*., 1994; De Luna, 2004; Caurant *et al*., 2006; Lavery *et al*., 2008). Pb intakes derive mainly from the food and, once in the organism, it may behave as a calcium-competitor, tending to accumulate preferentially in the bone (Pain, 1996; Law, 1996).

When data about the same species, similar species and/or similar sampling areas were not available, we compared our data with the only published research available for cetaceans. This was the case of Ti and Sr, which have been scarcely studied in marine mammals.

Ti concentrations in fin whale bone were ranging from 0.179 to 0.636 mg Kg^{-1}, in accordance with the limited data available from literature, reporting overall concentrations below 0.1 mg Kg^{-1} in liver and kidney of sperm whale, and a maximum concentration of 1.3 mg Kg^{-1} in muscle of the same species (Holsbeek *et al*., 1999). Despite Viale *et al*. (1978) stated that the highest concentrations of Ti in marine animals were those from cetaceans, recent data from pinnipeds reported concentrations of Ti ranging from 1.13 to 10.9 mg Kg^{-1} in the blood of harbor seal (Griesel *et al.,* 2008). Nevertheless, any further comparison with other tissues or marine mammal species was impossible due to the lack of investigations on this element.

Concentrations of Sr in bone were ranging from 20.1 to 113.6 mg Kg^{-1}, a value not comparable to any similar species due to the lack of data, but much higher than concentrations found in skin or blood samples of dolphins, ranging from 0.077 to 0.173 mg Kg^{-1} (Bryan *et al*., 2007). These values may be explained by the chemical characteristics of Sr, which is a calcium-like element, and tends to be integrated in the bone constitution when its availability through the diet is high (Pors Nielsen, 2004).

Effects of sex and sampling area

Metals concentrations in fin whale bone did not show significant differences between sexes. In cetaceans, the two sexes generally show limited differences in metal concentrations, although in some species concentrations of specific trace elements (e.g. Hg, Se) were found to differ between sexes (Honda *et al*., 1986; Andre *et al*., 1990; Caurant *et al*., 1994; Aguilar *et al*., 1999; Zhou *et al*., 2001). Caurant *et al*. (1994) associated such results to the different metabolic pathways linked to hormone cycles. However, a clear pattern of different metal accumulation trends between sexes has not been described to date.

Respective to sampling area, the only differences found between the two areas were in Sr concentrations and Pb trends with age. Geographical variations in metal concentrations may be related with natural processes or human activities. Such differences are reflected in cetacean tissues if the dietary intake is high, and the metals are not submitted to physiological regulation. Basing on these assumptions, metal concentrations can represent a chemical marker for differentiating population, as it has been confirmed by several studies (e.g. Sanpera *et al*., 1996; Monaci *et al*., 1998, Kunito *et al*., 2002).

Being Sr a naturally abundant element, higher Sr concentrations in bone of fin whales from NW Spain may be linked to particular geological characteristics of that area. On the contrary, Pb concentrations are highly determined by human activities, and tend to increase in proximity of anthropogenic sources (Law, 1996). The higher accumulation rate of Pb in fin whales from NW Spain might be therefore related to the more industrialized nature of Spain when compared to Iceland, implying a higher contamination of the waters off the Spanish coast. These results suggest that Sr and Pb are the two only metals, among the ones here analyzed in bone, that significantly contribute to differentiate the fin whale populations of NW Spain and W Iceland.

Fetal concentrations and trends with age

While sampling area and sex did not substantially affect the variability of metal concentrations in bone, interesting results emerged from the analysis of concentrations in the fetuses' samples and from the study of metal accumulation trends with age. Although the results deriving from fetuses must be considered with some care, due to the small sampling size, the statistical significance of the observed differences supports some considerations.

Apart from Ti, which showed no clear pattern with age, nor significant different concentrations in fetuses' samples, all other elements showed significantly different concentrations in fetuses, and both Pb and Zn showed significant accumulation trends with age.

The absence of differences in Ti concentrations in fetuses and of trends with age may indicate that some kind of placental transfer of the metal occurs during gestation, as already reported by Viale (1978), but that this metal is not subsequently accumulated in the bone during any of the phases of life.

Sr was the only metal with significantly lower concentrations in fetuses than in the other samples, indicating that it is not transferred through the placental barrier. Also, no significant trend was found in concentrations of this metal with age, in accordance with what was found in the blood and skin of bottlenose dolphins (Bryan *et al*., 2007).

The concentrations of the other metals in fetuses were significantly higher than in the other samples, indicating the occurrence of some level of accumulation from the mothers during gestation, as it has been previously described for many contaminants including metals (Bowles, 1999).

High placental transfer of Cu was already highlighted from previous research, highlighting higher concentrations in fetuses and neonates than in adults (Honda *et al*., 1986; Fujise *et al*., 1988, Law *et al*., 1992, Lahaye *et al*., 2007; Yang *et al*., 2004). After birth, concentrations of Cu decreased and no trends were observed with age. This behavior might be explained, similarly to Sr, by the occurrence of a close regulation of the metal concentrations, which prevents any substantial age-related increase (Thompson, 1990). Results here found for Cu are supported by the studies of Honda *et al*. (1983) and Wagemann *et al*. (1983), which reported a decrease in concentrations from 0 to 1 year old dolphins and no subsequent relations with age, suggesting that Cu metabolic turnover has more importance in determining its concentrations than the exposure time.

Differently from Ti, Sr and Cu; Pb and Zn showed both higher concentrations in fetuses and significant accumulation trends with age. These results are partially in contrast with the scarce studies published, which, to our knowledge, relate bone concentrations in cetaceans with age, including fetuses (Honda *et al*., 1986; Caurant *et al.*, 2006). According to these studies, Zn and Pb concentrations in striped dolphin (*Stenella coeruleoalba*), and Pb concentrations in common dolphins (*Delphinus delphis*) fetal bone were lower than in adults. Nevertheless, in both species levels of Pb were considerably higher in the early fetal period than in the late one, probably as a consequence of placental transfer of Pb in the early embryonic stage and its posterior dilution due to the increase of the weight of the fetal bone (Honda *et al*., 1986; Caurant *et al*., 2006). However, for both metals the transfer across the placenta with subsequent fetal accumulation was observed also in other cetacean species (Law, 1992). It was suggested that higher levels encountered in fetuses or newborns might be connected with specific requirement in newborns or a very low excretion rate of these metals by the fetus; and in some species fetal accumulation was accompanied by a decrease of concentrations in liver of gestating and/or lactating females (Honda *et al*., 1983; 1987). In the present study no variability was found among females at different reproductive statuses, most probably due to the fact that the transfer of the metals from the mother to the fetus implies more metabolically active tissues than bone, not affecting female bone concentrations.

However, the increase of Pb and Zn concentrations with age found in fin whales bone are confirmed by previous literature, highlighting positive accumulation trends of these metals with age, once the neonate is born (Honda *et al*., 1986, Caurant *et al*., 2006). The overall increase found in Pb concentrations with age is consistent with results obtained by Honda *et al.* (1983) in striped dolphins. When analyzing data of the sampling areas separately, we observed a steep increase of Pb concentrations with age in samples from NW Spain, but no significant trends in samples from W Iceland. As bone is the long term target organ for Pb accumulation, these higher levels in bone can represent past contamination (Lavery *et al*., 2008). Our results suggest a higher Pb contamination of Spanish waters: Pb concentrations have been historically higher in the temperate waters of North Atlantic, possibly due to higher leaded petrol emissions in the crowded European countries. Thus, while in samples from W Iceland Pb levels remain nearly constant, fin whales from the more polluted Spanish area continue to integrate Pb in their bone during time.

Also Zn concentrations were increasing with age, in contrast with the common pattern followed by essential metals, which tend to show little variations with age and are more finely regulated, as showed by studies performed on internal soft tissues of cetaceans in which any increase of Zn concentrations with age or length was found (Law, 1996; Aguilar *et al*., 1999; Borrell *et al.*, 2015). On the contrary, our results are consistent with studies on the bone and skin of striped dolphins, highlighting an increase Zn concentrations with age (Honda *et al*., 1986; Borrell *et al*., 2015). We may hypothesize that the accumulation of Zn in bone might be related with the inert nature of this tissue and the possibility that it is used as a storage for elements which, like Zn, are toxic at high concentrations.

Most of the findings emerged from our analyses were in agreement with trends previously described in cetaceans, despite the scarcity of data regarding the species, the tissue, and some of the metals analyzed challenged a proper interpretations of some of our results. The body storage, excretory, metabolic and detoxification mechanisms of metals in cetaceans are in fact still unknown for most metals and species, as well as the actual concentrations causing toxicity and the adverse effects on these organisms (Bowles 1999). An in-depth understanding of the physiological regulation mechanisms in cetaceans will be necessary in order to fully interpret the available data.

Nevertheless, we could detect some relevant trends, and we underlined the potential of bone as a good tissue for investigating long term dynamics, being capable of integrating short-term variations due to transient metabolic or environmental conditions.

Through this study we contributed to increase the record of metals concentration and accumulation trends in cetaceans, providing information about a species, a tissue and some elements which had been scarcely investigated before.

Aknowledgements

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Discussion

The main objective of this thesis was to evaluate the suitability and validity of the use of chemical markers for the investigation of geographical origin, population structure and long-term trends of cetaceans' populations. To do so, three populations of cetaceans were studied through the application of different sets of markers.

In this section, the general findings obtained from the application of each set of markers will be first discussed, pointing out the strength and weaknesses of each of them. Then, the specific results concerning each of the studied population will be considered. Finally, the implication of these results for general management of populations and stock assessment will be pointed out, as well as future perspectives and possible improvements of chemical markers application.

1. Strengths and weaknesses of chemical markers

To be considered a good marker for population structure and origins, a chemical would ideally meet some basic properties:

1.1 "… To be useful, these markers must display a substantial spatial variation…" - a note on BASELINES.

Data about the baseline values of the markers in each of the sampling areas are necessary to verify this property. This information can be obtained either from literature or from the direct analysis of organisms at the base of the food web on which each cetacean species bases its diet.

Literature provide comprehensive data regarding the stable isotopes baselines of the oceans. They are available in the form of isoscapes: maps representing the baseline isotopic values of N, C and O of the oceans. Information contained in these maps have been delineated based on the results of a meta-analysis of $\delta^{13}C$ and $\delta^{15}N$ in organisms at the base of the food web (zooplankton), and of $\delta^{13}C$ and $\delta^{18}O$ in dissolved carbon and seawater (McMahon *et al.* 2013). Thus, we could confirm that for each of the three species studied, the sampling areas considered have well known isotopic characteristics, and present definite gradients.

Unluckily, such a complete and useful literature is not available for element concentrations. Fluoride or metal concentrations have not been thoroughly measured in the oceans and data regarding the sampling areas off NW Spain or W Iceland were not available. For these markers, we could rely only on some generic information pointing out that higher fluoride contamination is likely to occur close to volcanic areas (Cronin *et al*., 2003; Whitman *et al*., 2005), such as Iceland (Stefánsson & Sigurjónsson, 1957), while depositions of metals derived from human activities, such as Pb, should be more abundant in the waters off more industrialized countries, like Spain.

To complement literature data, we analyzed fluoride concentrations, $\delta^{15}N$, and $\delta^{13}C$ in krill obtained from the stomach of the fin whales sampled from W Iceland and NW Spain, which was assumed to reflect background levels. Some problems emerged from the analysis of krill. First of all, due to partial digestion, we could not identify our krill samples at a species level. Despite this, we assumed that they were composed mainly on *M. norvegica*, which is considered the main food of fin whales in the two areas (Aguilar, 1985; Víkingsson, 1997). This species presents a complex life cycle, as it seasonally varies its diet from exclusively phytoplanktonic to carnivorous (Schmidt, 2010), a phenomena that highly affects $\delta^{15}N$ values. Also, as other Euphasiids, *M. norvegica* discards its exoskeleton in each molt, every 9 to 13 days (Keck, 1984), causing variations in the overall concentrations of fluoride, which mainly accumulates in the exoskeleton (Soevik & Braekkan, 1979; Adelung *et al*., 1987; Sands *et al*., 1998). Due to all these reasons, the three markers analyzed showed high variability in krill samples, and only fluoride concentrations were significant different between the two sampling areas, probably due to the extremely higher load of fluoride of NW Iceland waters. We concluded that krill is not a good proxy for estimate background levels and, when differences between areas are moderate, a precise determination of the species and its physiological phase are required.

Nonetheless, we had sufficient data to conclude that stable isotopes of N, C and O, as well as fluoride concentrations, presented substantial spatial variation among the areas analyzed, while data were not sufficient to draw similar conclusions about metal environmental variations.

1.2 "… be detected in the tissues of the studied species…"

All the markers considered in this thesis were easily measured in the studied populations. The mass spectrometry techniques used for the determination of stable isotope values are well established, and both bone and teeth proved to be good tissues for the measurement of $\delta^{15}N$, $\delta^{13}C$ and $\delta^{18}O$. The techniques used for the measurement of fluoride were on the contrary not completely set up in our laboratories, but after proper instrument calibrations it was possible to easily measure the concentrations of this element in fin whale bone. Finally, metals showed different patterns. Also in this case, the mass spectrometry techniques used for metal determination are commonly used, but only some metals could be detected in fin whale bone. Many metals tend to concentrate in internal tissues, which are rich in proteins (Das *et al*., 2004; Borrell *et al*., 2015), while only a few accumulate in measurable concentrations in bone. The metals which could be successfully measured in fin whale bone and thus considered as possible chemical markers were Pb, Zn, Cu, Ti and Sr.

1.3 "… Also, the markers variability within a population should be moderate or alternatively predictable through parameters that are measurable, so that their effect may be taken into account..." – effect of sex, age, and other biological variables.

Information about sex, age, or length were available for fin whales, as well as for sperm whales, while those data regarding right whales were fragmentary, making impossible to relate the variability found in this species with any individual biological variable.

Globally, each marker presented different levels of variability in the populations studied. Generally, the isotope values showed little or no correlation with sex or age of the individuals, while the element concentrations were more related with these variables.

Variability of $\delta^{15}N$, $\delta^{13}C$ or $\delta^{18}O$ values in the right whale bone was extremely high, but, as previously explained, the possible effect of any biological variable could not be investigated. In the fin whale, the isotopic values of $\delta^{15}N$, $\delta^{13}C$ or $\delta^{18}O$ were more homogeneous and they did not correlate with the sex or age of the individuals. For both mysticete species, we assumed that the isotopic variation was mainly related with environmental variability, animals' movements, and possible variations in their diet.

The results of stable isotope analysis in sperm whale showed, on the contrary, some correlations with the sex of the samples, and different trends with age, although with a high individual variability. These relations were explained at the light of the complex social structure of sperm whales, implying shifts in the movements' patterns and feeding behavior in individuals of different age and sex. Thus, higher strength and diving capacity, which allow feeding on bigger prey at higher trophic levels (Clarke *et al*., 1993; Jennings *et al*., 2002), would cause higher $\delta^{15}N$ values in older males, while the reduction of the dives depth and a shift to shallower prey during pregnancy and lactation (Mizue, 1951; Best, 1999) would imply lower $\delta^{15}N$ values in sexually mature females. Similarly, the tendency to move to higher latitudes of males (Mendes *et al*., 2007; Graham *et al*., 2010), and/or an increasing tendency to consume deeper, colder water prey when they grow old, would result in an age-related decrease in $\delta^{13}C$ and $\delta^{18}O$ values.

These correlations with age and sex are not evident in the fin whale, maybe because in this species both sexes perform similar pattern of migrations during all their lives. Thus, in order to interpret the results of stable isotope analysis, the complexity of the social structure and behavior of every single species must be taken into account.

Contrarily to stable isotope values, variability of fluoride concentrations in fin whale was high, and, consistently with previous studies, it was related with sex and age of the individuals (Landy *et al*., 1991; Alne, 1995; Mikaelian *et al*., 1999). Fluoride concentrations increased in all individuals in their first years of age, at least until sexually maturity. After maturity, they continued to increase linearly in females, while they seemed to fluctuate around a sort of plateau in males. We suggested a possible correlation of these results with the more dynamic structure of female bone, connected to cycles of pregnancy and lactation (Alne, 1995), and we concluded that, even if concentrations of fluoride in cetaceans bone cannot be precisely predicted through measurable variables, sex and age must be considered as sources of variability.

Similarly to fluoride, also concentrations of Pb and Zn increased significantly with age in fin whale bone; but no relations were found for any of the metal analyzed with the sex of individuals. Interesting results emerged from the analysis of metal concentrations in fetal bone, indicating that, apart from Ti, all the other metals which accumulate in bone are subject to placental transfer. Nonetheless, after birth, Pb and Zn were the only metals showing a measurable variation with age, while the variability found in all the other metals could not be related with any predictable parameter.

1.4 "... allowing the assignment of individuals to certain geographical areas." - Do individuals reflect background levels?

Considering the complex migratory patterns, feeding and social behavior of large cetaceans, this property was the most difficult to verify.

Environmental factors, such as temperature, light intensity and nutrient concentration, affect the $\delta^{18}O$, $\delta^{13}C$ and $\delta^{15}N$ values of primary producers (Walker & Mako, 1999), which, in turn, are reflected in the tissues of the consumers. Thus, any dissimilarity in the isotopic values of the individuals may indicate that they consumed resources in geographical areas with substantially different environmental characteristics.

$\delta^{15}N$ values, apart from being related with environmental characteristics, are mainly influenced by trophic position (McMahon *et al.*, 2013; Minagawa & Wada, 1984). $\delta^{15}N$ values were not different between populations of fin or right whales, indicating that the populations studied fed at the same trophic level. On the contrary, some differences were found in the sperm whales from the two sampling areas, indicating both possible switch in the size of the prey and differences in the baselines values of the foraging areas.

$\delta^{13}C$ values vary mostly with latitude, and show a strong correlation with the productivity prevailing in the area, which in turn depends from highly seasonal variables such as the photoperiod, water temperature and salinity (McMahon *et al.,* 2013). The values of $\delta^{13}C$ were significantly different in the right whales from the two sampling areas, and consistent with the geographic baselines; while they were not in the fin whales, apparently in contrast with available baselines of the two sampling areas (McMahon *et al.,* 2013). $\delta^{13}C$ values were also similar in the early years of sperm whales from the two sampling areas, but then different trends emerged, indicating movements to different latitudes.

$\delta^{18}O$, whose values correlate with water salinity and temperature, was the marker providing better information about migrations and habitat use of the species studied, contributing significantly to differentiate both fin whales and right whales groups, and varying consistently with the isoscapes of the relative areas (Bowen 2010, McMahon *et al*., 2013). In sperm whales, differences were more subtle, but some of the trends observed could be related to the migration patterns of the individuals.

The concentration of fluoride did not contribute substantially to differentiate the two groups of fin whales studied, apparently not fulfilling this fourth property. Despite the baseline values obtained from literature and the analyses of krill samples were different in the two areas, the concentrations of this element in the bone of both populations of whales were similar.

Finally, a proper evaluation of this property with regard to metal concentrations was not possible, due to the absence of reliable baseline values. The only metals which differentiated the two groups of fin whales were Sr and Pb: in samples from NW Spain Sr concentrations were significantly higher, and Pb concentrations showed a significantly higher accumulation rate with age than those in Iceland. These differences may be related with the environmental characteristics of the two sampling areas, especially for Pb, which tend to be accumulated in areas with higher pollutant load, but the absence of data about environmental concentrations prevented us to test this hypothesis.

2. Unrestrained difficulties: random migrations; diet shifts; target tissues; homeostatic mechanisms

Each of the selected markers provided different levels of information and presented different levels of difficulties; results from stable isotopes were more easily interpreted thanks to exhaustive literature, while element concentration posed some difficulties due to the lack of baseline data. All the results obtained must be interpreted taking into account the inner complexity of the migratory behavior of whales, and some intrinsic characteristics of the tissues analyzed.

The chemical markers described in this thesis have been analyzed in bone and teeth, which are both tissues with slow metabolic rates and a long turnover. This means that the isotopic signals or the element concentrations measured in bone or in a dentinal GLG are in fact reflecting an integration of the environmental isotopic signals/element concentrations to which the animal was exposed at least in the previous year. Thus, teeth are particularly useful because they provide information on chemical signals that are deposited in the crown and root during the entire life of the animal without existence of turnover (Bada *et al*., 1990). As a consequence, the teeth growth layers allow the reconstruction of ontogenetic time series of isotopic values (Newsome *et al*., 2010). Nonetheless, if the animal moved between areas with different baselines or fed at different trophic levels within one year, all the chemical information of that year will be integrated with the resolution of one GLG, and the 'instantaneous' signal will be lost.

The same happens with bone, which has a slow turnover rate and integrates a much wider temporal span than other more metabolically active tissues. This makes bone a tissue of choice for investigating long-term processes including habitat use and dietary history of organisms (Riofrío-Lazo & Aurioles-Gamboa, 2013). However, as the species investigated are highly migratory, and move between geographic areas with well-defined environmental characteristics, the use of bone may represent a limitation for the fine reconstruction of their migration patterns.

In fact, despite mysticetes are believed to migrate between areas which are used exclusively for foraging, and areas which are used for breeding (Aguilar, 2009), recent literature supported that in some populations feeding is not strictly seasonal but may occur also in breeding areas (Hoffmeyer *et al*., 2010). Thus, the isotopic signal detected in bone may represent the integration of both: the isotopic signature acquired in the feeding grounds and the isotopic signatures acquired from other areas where the whales have been moving and feeding.

This capacity of bone to integrate long-term chemical fluctuations (Newsome *et al.,* 2010) may explain the apparent inconsistencies found between the isotopic values of the samples analyzed and the isotopic baselines of the sampling areas; and also partially justify the contrasts found in fluoride concentrations between fin whales and krill. Thus, fin whales from W Iceland could be displaying lower fluoride concentrations than those expected according baselines because they are integrating non-krill food sources poor in fluoride, or even krill from other locations with low levels of fluoride.

Apart from the confounding effects related with migration patterns and shifts in diet, and the long-term integration effect of bone, the results obtained may also be affected by the intervention of physiological and homeostatic mechanisms. These may play a particular role in element accumulation. Many elements are subject to strict regulation due to their essentiality in body processes. Others, non-essential but highly toxic, are subject to detoxification mechanisms, like mercury, that is sequestered by Se in cetaceans' liver (Wagemann *et al*., 1984; Nigro, 1994). Although bone is poor in proteins and presents a low metabolic rate, some authors suggest that bone structure of animals that undergo frequent dives in deep waters is more mobile, because the high pressure tends to lower bone density (Taylor, 2000; Reidenberg, 2007; Gray *et al*., 2007). So, it may be hypothesized that fluoride and other elements like Pb and Sr, which are mainly accumulated in bone, are subject to some specific mechanisms that regulate their absorption. If this were true, concentrations of these elements in fin whale bone would not merely be a reflection of those in the diet, but the result of a dynamic regulation implying elicitation homeostatic responses (Oszvath, 2009).

Despite the complexities raised and the limitations connected with individual variability, levels of exposure, integration and/or regulation mechanisms, the results obtained from chemical markers allowed us to draw relevant conclusions about the structuring of the selected populations, with possible implications over their management.

3. Outcomes for the studied populations

North Atlantic sperm whale

Based on various evidences, the IWC considers the North Atlantic sperm whale as a single population (IWC, 1981). According to this classification, all the North Atlantic females and young individuals of both sexes would share the same breeding grounds, while adult males would migrate towards northern latitudes during at least part of their adult lives. If this were true, the isotopic values recorded in the teeth dentine deposited in the first years of life the individuals sampled from Denmark and NW Spain would be similar in males and females, while those deposited at older ages would differ. On the contrary, our results highlighted various differences between the individuals from Denmark and NW Spain. $\delta^{15}N$ values were different in the early period of life and showed different trends during the first 20 years of age of the individuals, suggesting dissimilarities either in the foraging behaviour or in the patterns of movements of the individuals from the two regions. $\delta^{13}C$ values were not different in the first years of life of individuals, but were highly variable in all sample groups, and showed decreasing trends with age in some individuals, probably reflecting periodic or seasonal changes in diet composition or in movements, from inshore to offshore waters, or to lower latitudes. Finally, $\delta^{18}O$ values were different in the early period of life and displayed substantial inter-annual fluctuations, following different patterns, and decreasing with age in some individuals, likely reflecting migration to higher latitudes or an increasing consumption of deeper, colder water preys.

These dissimilarities in $\delta^{18}O$, $\delta^{13}C$ and $\delta^{15}N$ values and trends indicate that the individuals were born, and subsequently had moved and fed, across geographical areas which have substantially different environmental characteristics. The results suggest that sperm whales from Denmark and NW Spain use heterogeneous habitats and breeding grounds, and indicate some level of structuring in the eastern North Atlantic population of the species.

Southwest Atlantic right whale

Current management recognizes the Southwest Atlantic population of right whale as a single stock, and promotes integrated conservation actions along the Atlantic coast of South America (IWC, 2013). Nevertheless, the current pattern of distribution of the population appears in contrast with the pattern recorded previously to whaling exploitation, suggesting a fragmented re-population which left empty some valuable habitats.

The results obtained through stable isotope analysis revealed that individuals from the two main areas of concentration along the South American coast are isotopically distinct, confirming the isolation of individuals from southern Brazil and central Argentina. Despite the high variability of results, $\delta^{15}N$ values were similar in the individuals from the two areas, while $\delta^{18}O$ and $\delta^{13}C$ were significantly higher in samples from southern Brazil, in agreement with available baselines. Being the C and O isotopic composition of animal tissues mainly reflecting that of the food (Hobson, 1999), these results suggest that the areas around southern Brazil and central Argentina, traditionally considered to be exclusively nursing grounds, may also be occasional areas of foraging for the whales. Indeed, despite it is generally accepted that southern right whales primarily fast while they occupy lower latitudes, individuals have also been repeatedly observed feeding on plankton blooms, particularly at the end of the season (Matthews, 1938; Hoffmeyer *et al*., 2010). The isotopic difference between the individuals from the two sampling areas indicates also that intermingling between them is limited or non-existing. Although occasionally some females may not display a strict site fidelity or some individuals may randomly move between the two areas (Best *et al.*, 1993), the results obtained support the hypothesis of isolation between the two groups.

These findings suggest that the Southwest Atlantic right whale population is structured into at least two units that exploit different feeding and nursery grounds: such structuring originated probably from whaling exploitation, which caused the loss of the segments of the population occupying the habitats of the central coast of South America.

North Atlantic Fin whale

The IWC has been working during many years for the definition of adequate management units for the North Atlantic fin whale, which is a species still under exploitation and thus requiring special attention. Based on results obtained from a long series of studies performed through different techniques (Jonsgård, 1966; Sergeant, 1977; Lockyer, 1982; Gunnlaugsson & Sigurjonnson, 1989; Jover, 1992; Víkingsson, 1992; Sanpera *et al.,* 1993, 1996; Bérubé *et al.*, 1998; Víkingsson & Gunnlaugsson 2005; Pampoulie & Daníelsdóttir, 2013), the IWC ended up with the recognition of the currently accepted 7 stocks (IWC, 2009). Each of them corresponds to the summer feeding grounds of groups of fin whales which supposedly follow different migratory routes to reach isolated breeding grounds, whose location is currently unknown. The samples analyzed in this thesis proceed from two of these stocks: Sp (Spain) and WI (Western Iceland). Although they are already considered as isolated populations, the pattern of migrations, location of breeding grounds and level of mixing of the individuals belonging to these stocks are still unknown.

The results obtained from the stable isotope analysis were the most useful for speculating about possible movements of the individuals, through a comparison with isotopic baseline values of the Atlantic Ocean. Of the three isotopes, only $\delta^{18}O$ values clearly discriminated the two subpopulations, confirming that they exploit separated feeding grounds and move between areas isotopically well differentiated. $\delta^{15}N$ and $\delta^{13}C$ values, on the other hand, were similar in the two groups, but slightly inconsistent with the sampling location baselines, suggesting that fin whales may feed also during migration and integrate in their tissues the isotopic values typical of the food ingested during the feeding season, with values characteristic of food consumed in distant areas during periods other than the summer. Considering these complexities, the comparison with the available isoscapes (Graham *et al.,* 2009; Bowen, 2010; McMahon *et al*., 2013) does not provide sufficient information to reconstruct the migratory patterns of the two stocks and their winter distribution ranges. From the results obtained, we concurred in defining the fin whale stock feeding off NW Spain as a unique stock, and, according to the baselines values of $\delta^{18}O$ in the North Atlantic (Graham *et al.,* 2009; Bowen, 2010; McMahon *et al*., 2013), we suggested that its breeding grounds should be located in an oceanic area at lower latitudes, on a wide band extending off the eastern coast of Africa. With regard to the Icelandic stock, based on our data and recent studies (Silva *et al.,* 2013), we concurred with the hypothesis indicating that it is composed of a mixture of animals breeding in the central Atlantic and animals breeding in the Western Atlantic (IWC, 2009), which migrate through a corridor along the Gulf Stream. Thus, the results of the stable isotope analyses suggested an intricate structure of the North Atlantic fin whale population, in which subpopulations exploit different habitats and perform latitudinal migrations that are still far to be completely understood.

Analysis of elements did not provide any further insight into the possible migratory patterns and location of breeding grounds. Bone fluoride concentrations were expected to differentiate the two stocks, due to high concentrations of fluoride derived from volcanic depositions in Iceland (Stefánsson & Sigurjónsson, 1957; Thorarinsson, 1979; Witham *et al*., 2005), but no difference emerged. The same happened for the majority of metals: only concentrations of Sr and Pb showed different patterns in the two populations. However, as detailed baseline information of these elements is not available in the North Atlantic Ocean, it was not possible to speculate on the fine scale structuring of the populations.

4. Implications for stock management and future perspectives

The results obtained through the analysis of chemical markers, particularly of stable isotopes, contributed to increase the current knowledge of population structure of all the populations studied and, at different levels, questioned the management units that are currently accepted and implemented.

The North Atlantic sperm whale is currently considered as a whole as a single management unit (IWC, 1981; Dufault *et al*., 1999). Our results emphasized the existence of a higher level of complexity within this stock, suggesting that sperm whales from Denmark and Spain are born in separated breeding grounds and follow distinct migration patterns. Further research will be needed to test the occurrence of potential structuring across all the North Atlantic Ocean, and to define the actual borders of any potential subpopulation, in order to guarantee adequate management and protection of this species, that is classified by the IUCN as vulnerable (IUCN, 2015).

The Southwest Atlantic right whale is also considered as a single management unit, and the currently implemented conservation plans apply an integrated approach for the species along the whole Atlantic coast of South America (IWC, 2013). Our results suggest that the population is structured into at least two units that exploit different feeding and nursery grounds. This highlights the need of reconsideration of the current management procedures and the implementation of corrective measures that take into account the different threats menacing the whales of the two areas. In Southern Brazil entanglements in gillnets, collisions due to the intense marine traffic and the whale-watching industry (IWC, 2011; Pontalti & Danielski, 2011) represent the main menaces: in this area regulation of shipping and fishing activities would promote re-colonization and strengthen the conservation of whales of this putative management unit (IWC, 2011). On the other hand, in Central Argentina, events of high mortality of calves have been related to: reduced food availability for adult females; bio-toxins and infectious diseases; anthropogenic disturbance caused by whale watching, marine traffic and fisheries; and, above all, harassment by kelp gulls (Sironi *et al*., 2009). At the light of such dissimilar conservation scenarios, our results stress the urgency of a revision of the currently accepted stock subdivision and the implementation of independent measures of conservation for the two populations of Southwest Atlantic right whales.

The North Atlantic fin whale population is currently considered to be composed by 7 management stocks. A number of authors already pointed out some weak aspects of the proposed structuring (Bérubé *et al*., 1998; Silva *et al*., 2013, Giménez *et al*., 2013), which takes into account only what is observed in the summer feeding ground, but does not consider the level of connectivity among them at the breeding grounds and during the migrations. The results obtained through the stable isotope analyses contributed to this discussion, pointing to an intricate structure among the subpopulations of fin whale in the North Atlantic, with complex pattern of latitudinal migrations and a level of mixing that is still poorly understood. Thus, the lack of complete knowledge about the North Atlantic fin whale population structure and movements highlights the need of further studies that would make possible a careful revision of some aspects of the currently used management units.

The papers included in this thesis provide an overview of the potentialities of the use of chemical markers in the study of cetaceans' populations. As previously discussed, the results obtained have a wide spectrum of implications, and provide, in some cases, information necessary for a better management and conservation of the populations.

Nevertheless, some limitations were detected on the use of bone as target tissue for our analyses of right whales and fin whales. Bone provides useful information on long-term trends and it is not affected by temporary environmental conditions or metabolic status, but these same characteristics may represent an obstacle when short-term movements and migration patterns are investigated.

This limitation can be overcame through the analysis of more metabolically active tissues, which have a shorter turnover rate, such as skin. Skin reflects the characteristics of the food ingested –and thus of the environment where the animal have fed- in the last few weeks, and may therefore represent a better marker for the study of whale migrations. This tissue presents also the big advantage of being available through non-invasive sampling methods, performed through biopsies on alive and free-ranging animals. The sperm whale teeth are also a good tissue for investigating movement trends in a scale of years, because each GLG represents one year of life of the individual. Baleen plates, in mysticete species, can be used in a similar manner but with a finer resolution. This tissue is not easily collected, because it can only be sampled from dead animals, but the information provided by one single plate can encompass the chemical signals of the last two years of life of the whale, at a resolution of a few weeks, giving detailed information on the chemical characteristics of the food ingested and the environments through which the animal has moved during at least two migration cycles (see box "work in progress"). Examination of the permanent record of stable isotopes, trace elements and other chemical markers left in the baleen plates, in the line of the studies by Best and Schell (1996), Lee *et al.* (2005), Mitani *et al.* (2006), Bentaleb *et al.* (2011), or Giménez *et al.* (2013), as well as the analysis of tissues such as skin, which reflect the short-term diet composition of the whales, may therefore prove extremely effective to clarify such complex population structures and migration patterns like those of the North Atlantic fin whale population.

Finally we must remark that, despite the benefits of chemical markers in the study of cetaceans' populations and the value and potential application of the results obtained, the power of other study techniques such as genetics or satellite tracking must not be underestimated. Thus, to deepen into the populations demographic structuring, the migration routes of individuals, the level of mixing among subpopulations, and to clarify the actual structure of stocks, the analysis of chemical markers' should be complemented with research based on other techniques such as genetic and satellite tracking, as well as with the historical reconstruction of the exploitation and the past distribution of the species.

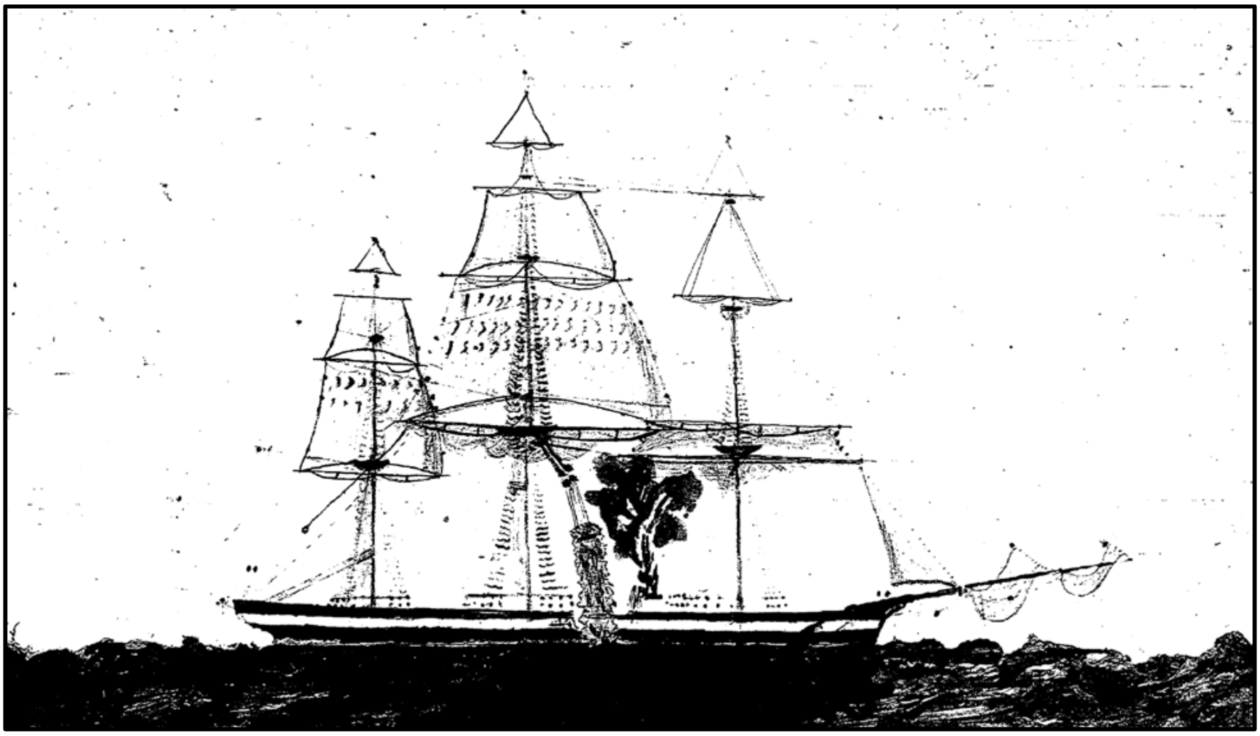
“Work in progress”: Baleen plates as a continuous time-recorder of chemical markers: analysis of stable isotopes and trace elements

(Study partially presented at the 29th Conference of the European Cetacean Society –Malta, 2015; the related article is in preparation)

Morgana Vighi, R. Lloret, A. Borrell, G. Víkingsson, Th. Gunnlaugsson, A. Aguilar

Fin whales, like other mysticetes, are highly migratory organisms that undergo annual migrations between high latitude summer feeding grounds and low latitude winter breeding grounds. In the North Atlantic, after decades of heavy exploitation, the fin whale population was divided for management purposes into 7 stocks occupying separate feeding grounds. However, migration patterns, location of the breeding grounds, and level of mixing between the organisms belonging to these stocks, all aspects which are central for understanding the range and degree of connectivity between populations, still remain controversial. Baleen plates, composed of metabolically inert tissue, grow continuously and keep a permanent record of the variations of the elements present in the environments and which accumulate during the individuals’ growth. Thus, they may represent a useful source of information for investigating migration patterns and movements, providing a continuous time record of the variations in elements concentrations and isotopic signals that occur during the migratory cycle of the whale. In this study we investigated the potential for stable isotopes and trace elements analysed in baleen plates to reflect the movements of fin whales feeding off West Iceland in summer. The nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotopic values, as well as Zn, Cd, Cu, Hg, Pb, Sr and Ti concentrations were analyzed in 30 samples taken along the growth axis of 5 baleen plates sampled from five individuals. The isotopic fluctuation of the stable isotope values of both elements, as well as the patterns found in Hg, Cu, Zn and Sr for some individuals confirmed previous findings that the growth rate of the plate is about 20 cm per year. The $\delta^{15}N$ value presented a regular sine wave pattern similar to that observed in other mysticete populations. The $\delta^{13}C$ also showed a similar pattern, although less regular. Cd concentrations resulted to be below analytical detection limits; Pb and Ti concentrations did not show any pattern with time. Hg concentrations were relatively low (731-1942 μg Kg^{-1}), but 3 individuals showed some fluctuation with time. Analogous results were found in Cu and Zn, but at much higher concentrations (10-18 mg Kg^{-1} and 303-427 mg Kg^{-1}). Finally, Sr concentrations appeared to decrease from distal to proximal layers of the plate. Examination of the pattern of variation of all the chemical markers combined suggests that fin whales individual variability was very large probably due to individual-specific biological traits or dissimilar migratory patterns. These results highlight a complex behavior of each marker in baleen plates. Some of them are possibly reflecting the exposure to changing environments during migration while others may not be good tracers of migrations. Besides this, tissues like baleen plates, which keep a constant record of the environmental signals, proved to be a potentially powerful tool for investigating whale movements over time.

Conclusions



- ✓ Cetaceans' populations have been historically subject to intense whaling activity, which affected dramatically their size and structure, challenging their conservation status. The use of chemical markers, among other techniques, provides information regarding the level of isolation, distribution and migration patterns of the individuals within the accepted stocks, which is fundamental for the conservation of these animals.

- ✓ The analysis of stable isotopes of nitrogen, carbon and oxygen in the dentinal GLG of sperm whales from Denmark and NW Spain highlighted different feeding and migratory behavior of males and females of different ages in this species. Results showed also that diet composition, use of habitat and/or migratory destinations are dissimilar in the two groups, indicating that the North Atlantic population of sperm whales is more structured than traditionally accepted.

- ✓ The analysis of stable isotopes of nitrogen, carbon and oxygen in the bone of right whales from southern Brazil and central Argentina indicated the occurrence of some level of structuring in the Southwest Atlantic right whale population, and that whales are likely to feed also in the two areas, which were commonly thought to serve exclusively as nursing grounds.

- ✓ The analysis of stable isotopes of nitrogen, carbon and oxygen in krill and bone of fin whales from W Iceland and NW Spain indicated the inefficiency of using krill as reliable proxy of isotopic baselines, and some limitations of using bone to define migratory patterns of whales. Overall, these markers showed a clear isolation of the two subpopulations and suggested that North Atlantic fin whale subpopulations have an intricate structure, that they exploit different habitats and perform latitudinal migrations still not completely understood. Thus, the pattern of migrations and level of mixing among the North Atlantic fin whale subpopulations may be more complex than the currently accepted.

- ✓ Fluoride concentration in bone of fin whales from W Iceland and NW Spain did not prove to be a good marker to differentiate the two subpopulations, probably due to homeostatic regulation of this element. However, krill supported different loads, likely reflecting the high prevalence of volcanic effluents in the Icelandic waters.

- ✓ Among the metals analyzed in bone of fin whales from W Iceland and NW Spain, only Sr and Pb contributed to differentiate the two subpopulations; Pb trends of accumulations with age in samples from NW Spain could be related to the higher level of industrialization of continental Europe than that of Iceland. Analyses pointed out the potentiality of most metals to be transferred across the placental barrier, and the tendency of Zn and Pb to accumulate in bone along whales' lives.

- ✓ Each marker presented strong and weak aspects. With regard to baseline data, results from stable isotopes, which showed definite gradients in the study areas, were more easily interpreted thanks to the availability of oceanic isoscapes and exhaustive literature. On the contrary, data regarding baseline element concentrations were scarce.

- ✓ None of the markers presented analytical difficulties: all of them were easily analyzed and detected in the selected tissues.

- ✓ The effect of individual variables such as sex or age was more pronounced over elements concentrations, probably due to physiological mechanisms and homeostatic regulation, than over stable isotopes values, which tend to relate more with feeding ecology and habitat.

- ✓ The use of chemical markers presented some weaknesses, connected with the complexity of migratory patterns, feeding and social behavior of big cetaceans, which determined high individual variability. Also, bone showed some limitations related to its capacity to integrate long-term chemical variations.

- ✓ The application of the selected markers to the three studied populations lead to definite conclusions about their structure, with implications over their management. Both the North Atlantic sperm whale and the South Western Atlantic right whale populations present a level of structuring, and should not be considered as a unique management unit. Further investigation is needed to clarify the intricate structure of North Atlantic fin whale populations.

- ✓ Overall, chemical markers proved to be valuable tools for the study of some aspects of cetaceans' populations, but further investigation of other tissues with different integration characteristics is necessary to improve the current knowledge. Also, a combined use of these techniques with traditional laboratory techniques, satellite telemetry, as well as data provided by historical information, is recommended.

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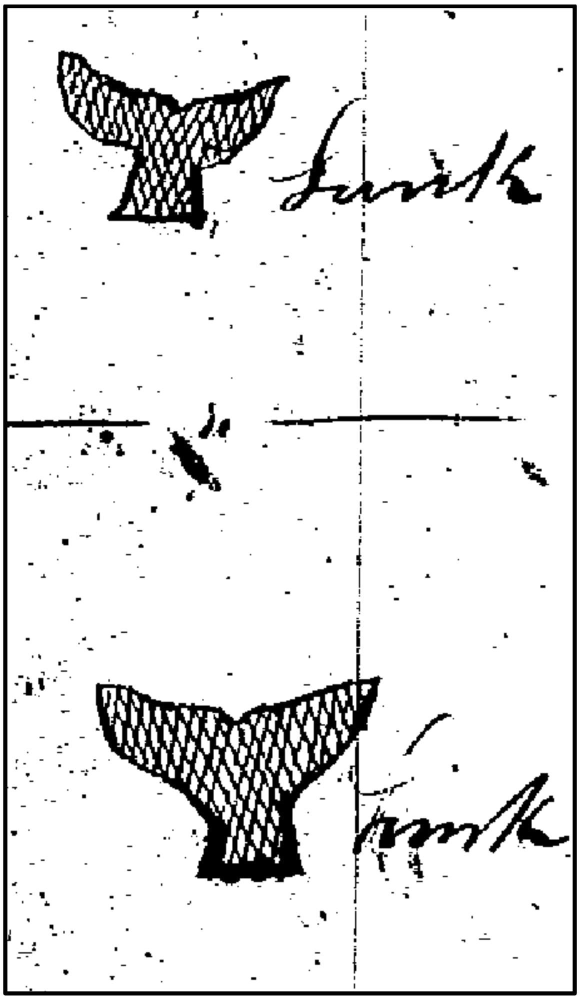

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