MORGANE DROMBY

Assessment of the ecophysiological conditions and trophic ecology of two cetacean species (*Tursiops truncatus* and *Globicephala macrorhynchus*) in Madeira Archipelago, using RNA/DNA ratios and Carbon/Nitrogen stable isotopes

UAlg FCT
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MASTER THESIS IN MARINE BIOLOGY

Assessment of the ecophysiological conditions and trophic ecology of two cetacean species (*Tursiops truncatus* and *Globicephala macrorhynchus*) in Madeira Archipelago, using RNA/DNA ratios and Carbon/Nitrogen stable isotopes

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Morgane Dromby

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Institution supporting biochemical analysis of bottlenose dolphins and short-finned pilot whales.

Observatório Oceânico da Madeira

Institution supporting skin biopsy samples of bottlenose dolphins and short-finned pilot whales and tissue collection of small fishes (e.g mackerels, sardines, bogues), large pelagic fishes (albacore, bigeye and skipjack tunas), zooplankton and POM (Particulate Organic Matter), off the coast of Madeira.

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ABSTRACT

Madeira is home of up to 22 cetacean species, which makes this place an important spot for conservation measures. There is a need to assess the nutritional state of marine mammals in the region, to prevent or monitor detrimental effects from natural and anthropogenic stressors. This study investigates the nutritional state of bottlenose dolphins and short-finned pilot whales in Madeira, via the usage of RNA/DNA ratios and provides an insight into their trophic ecology and habitat residency thanks to carbon and nitrogen stable isotopes analysis. In 2017-2018, RNA/DNA ratios and stable isotopes were determined from skin samples, and stable isotopes were compared with those in samples of their potential preys. SIMMs (Stable Isotope Mixing Models) determined the contribution of each prey species in the diet of the two cetacean species. RNA/DNA ratios of both species indicated that cetaceans were in good nutritional states and that ratios were different across taxa. Ratios were not significantly different across seasons and residency patterns. The $\delta^{13}C$ values supported preferences for offshore waters for short-finned pilot whales and inshore waters for bottlenose dolphins and underlined spatial segregation between the two species. The $\delta^{15}N$ values informed that pilot whales (mean=12.7‰, SD=0.5) and bottlenose dolphins (mean=10.9‰; SD=0.7) occupied high trophic levels. SIMMs did not support that pilot whales adopted a theuthophagus preferential diet, but rather consumed large amounts of mackerels and tuna species. Moreover, there were no significant differences in their diets across seasons but there were differences between residency patterns. SIMMs predicted that bottlenose dolphins fed on demersal and pelagic fishes and it existed intra-season variabilities in isotopic values. The study provides a better understanding of the two species ecology and highlights their organisation in space in Madeira. It suggests that RNA/DNA ratios, could be good proxies for the determination of nutritional states in cetaceans and help for successful conservation.

Keywords: Nucleic acid derived indices, food web dynamics, marine mammals, stable isotope analysis, biopsy sampling

RESUMO

O arquipélago da Madeira possui seis reservas marinhas, que contribuem para a extensa rede de áreas marinhas protegidas na região da Macaronésia. Embora a zona marinha da ilha tenha ambiente oligotrófico, com limitação de nutrientes, alberga numerosas espécies de cetáceos. No entanto, não foi ainda estudado o estado nutricional dos mamíferos marinhos, nem avaliada a ecologia trófica durante a sua residência no arquipélago, o que é determinante para prevenir ou monitorizar efeitos prejudiciais (imediatos ou a longo prazo) das perturbações antrópicas ou naturais (alterações globais) A contribuição das diferentes espécies de presas na dieta da baleia-piloto-tropical e do golfinho-roaz ao longo do ano necessita também de ser descrita na Madeira. Além disso, as análises isotópicas nunca foram associadas a um indicador de estado nutricional e de saúde, como a razão RNA/DNA. Este estudo investiga a ecologia trófica de duas espécies de delfinídeos, em função das estações do ano e padrões de residência (transitórios, residentes, visitantes) no arquipélago da Madeira, utilizando isótopos estáveis de carbono e azoto, e esclarece o seu estado nutricional através da razão RNA/DNA. Em 2017 e 2018, realizaram-se biópsias de baleia-piloto-tropical e de golfinho-roaz na Madeira. A razão RNA/DNA e isótopos estáveis foram determinadas a partir de amostras de pele e os resultados dos isótopos estáveis foram comparados com os de amostras de suas potenciais presas na região da Madeira. Os valores dos isótopos estáveis permitiram a execução de múltiplos modelos de mistura de isótopos bayesianos, a fim de determinar a contribuição de cada espécie de presa na dieta dos dois mamíferos marinhos. A razão de RNA/DNA de ambas as espécies indicou que, em geral, estavam em bom estado nutricional, e a alimentar-se de presas na zona, apesar de existir diferenças nos valores entre os táxons. As proporções não foram significativamente diferentes em entre as estações e os padrões de residência. Embora, os visitantes tendessem a ter uma proporção maior do que outros padrões, o que poderia ser devido a uma capacidade de predação de animais mais diversificados e maiores, pois também registravam os maiores valores de δ^{15}N ou uma variação intraespecífica entre diferentes populações da mesma espécie. Grandes variações intraespecíficas foram observadas no golfinho-roaz, pelo que novos estudos devem investigar os fatores de variações desta relação. Os valores dos isótopos estáveis de carbono confirmaram que a baleia-piloto-tropical depende de águas profundas, enquanto o golfinho-roaz depende de águas costeiras. Os valores de isótopos estáveis de nitrogénio implicaram que a baleia-piloto-tropical (média 12,7 ‰) e o golfinho-roaz (média de 10,9 ‰) ocupam altos níveis tróficos e que a baleia-piloto ocupa uma posição trófica superior. No entanto, os resultados do modelo misto bayesiano não suportaram a hipótese de que as baleias-piloto adotaram uma dieta de teutófagos no arquipélago, como descrito noutros estudos, mas parecem consumir grandes quantidades de cavala e de atuns. A menor área de nicho dietético para as baleias-piloto sugeriu que tinham mais especialização ou tinham menor variação inter-individual do que os roazess. De facto, os roazes apresentaram pequenas variações na sua dieta ao longo das estações, o que poderia ter explicado a área de nicho mais ampla. As baleias-piloto não tiveram diferenças significativas na contribuição de suas presas ao longo das estações, mas assumiram que endossavam diferentes hábitos alimentares em função de sua residência na ilha. Os transientes e visitantes tendiam a se alimentar de uma grande variedade de espécies de presas, enquanto os residentes podiam ser considerados mais especializados com uma dieta focada em espécies intermediárias de nível trófico. O modelo misto bayesiano previu que os roazes se alimentavam de peixes pelágicos, como cavalas e sardinhas, e dependiam de uma proporção relativamente alta de lulas. Foi registada variabilidade intra-estações nos valores isotópicos para os roazes, provavelmente explicados por variações na disponibilidade de presas, o que levou a uma menor competição entre as duas espécies. A dinâmica das presas deve ser investigada na Madeira para determinar os factores de variação dos isótopos estáveis na baleias-piloto-tropical e no golfinho-roaz. Este estudo permite uma melhor compreensão da ecologia destas duas espécies na região da Madeira, e sugere que a razão RNA/DNA um indicador de condição fisiológica, e a análise isotópica em conjunto poderão ser no futuro uma ferramenta a aplicar na determinação do estado ecofisiológico e ecologia alimentar de mamíferos marinhos, contribuindo assim para uma adequada conservação destes grandes vertebrados.

Palavras-chave: Índices derivados de ácidos nucleicos, dinâmica de teias alimentares, mamíferos marinhos, isótopos estáveis, amostragem por biópsia

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ABBREVIATIONS

- **FP:** Fluorescent plate
- **POM:** Particulate organic matters
- **SIA =** Stable Isotope Analysis
- **SIAR:** Stable Isotope Analysis in R
- **SIMMS:** Stable Isotope Mixing Models
- **TEF:** Trophic enrichment factor
- **δ^{15}N:** Nitrogen stable isotopes
- **δ^{13}C:** Carbon stable isotopes
- **μ=** Mean

1. GENERAL INTRODUCTION

The common bottlenose dolphin (*Tursiops truncatus*) and the short-finned pilot whale (*Globicephala macrorhynchus*) belong to the family delphinidae (Editors of Encyclopeadia Britanica2018). They are characterised by their relatively small sizes when compared to other cetaceans' clade. While the biggest of all cetaceans, the blue whale (*Balaenoptera musculus*) can reach up to 33 meters in length (Mac Clain *et al.* 2015), the pilot whale is only limited to 7.2 meters and the bottlenose dolphin to 3 meters (Editors of Encyclopoedia Britannica2018). Both species are cosmopolitan but are known to occupy rather different habitats. The short-finned pilot whale tends to inhabit offshore, tropical/warm temperate waters (Olson2009, Alessandrini2016), whereas the bottlenose dolphin is more acquainted to coastal, warm/temperate waters (Wilson *et al.* 1999, Wells and Scott 2009, Dinis *et al.* 2016). They seasonally follow the movement patterns of their favourite prey sources (Hui 1985, Shane 1995). However, in some population of short-finned pilot whales, notably in Madeira (Alves 2013), California (Shane and McSweeney 1990), Japan (Miyashita *et al.* 1990), Hawaii (Shane and McSweeney 1990) and Canary Islands (Heimlich-Boran 1993) and in some population of bottlenose dolphins in Madeira (Dinis *et al.* 2016), Florida (Scott *et al.* 1990), and Scotland (Wilson *et al.* 1997), individuals can reside in the same area all year long. This phenomenon is observed in warm waters, where prey resources are enough to sustain the populations the entire year. The resident populations often have to coexist with other migrant populations. When travelling, the migrant bottlenose dolphins form dynamic units varying from stable and resident groups to groups continually changing in size and membership. This input and output of individuals occur over time (Ballance 1990). Each unit usually varies from 2 to 15 animals based on sex and age and deep and opened waters tend to favor larger groups. Besides, groups of 3 to 1000 individuals were observed in the south African population of bottlenose dolphins (Saayman *et al.* 1973). Short-finned pilot whales, travel in bigger groups than bottlenose dolphins and form social units composed of 20 to 90 whales (Editors of Encyclopoedia Britannica 2018). A group of short-finned pilot whales is composed of mixed sex and age animals (Reeves *et al.* 2002), generally belonging to the same family and is always led by a female (Mahaffy 2012). They occasionally, but rarely dissociate from each other over time. These two studied species are similar in a number of ways. They are delphinidae, implying the possession of teeth and a streamlined body which helps them to predate on fish and cephalopods (Thomas *et al.* 1993). However, because the bottlenose dolphin is much smaller than the pilot whale, it is expected to have a higher metabolic rate than pilot whales, based on the rule relating the metabolic rate of mammals to their size (Kanwisher and Ridgway 1983). Therefore, it is expected that the two species exhibit different feeding habits found on different foraging strategies and different habitats occupancy. Many studies indicate that the bottlenose dolphin has a diverse range of food items and feed from demersal fish species to schooling pelagic preys, depending on food availability and local conditions (Shane and Wells 1986, Barros *et al.* 1998, Conor *et al.* 2000, Kiszka *et al.* 2014, Dinis *et al.* 2016). The species is very clever and developed various range of behaviours to make its foraging activity as efficient as possible. These strategies are then learnt from generation to generation via social learning (Krutzen *et al.* 2005). Short-fined pilot whales prefer to feed on deep living preys residing in offshore waters, which they catch during deep dive events (Aguilar Soto *et al.* 2008). Their deep dives require high energy but are compensated by the consumption of high calorific preys (Mintzer *et al.* 2008). Studies from California (Seagars and Henderson 1985), Japan (Kubodera and Miyazaki 1993), Caledonia (Bustamante *et al.* 2003) and Canary island (Hernandez-Garcia and Martin 1994) noticed the importance of the class cephalopods (octopus, squids) in short-finned pilot whales' diet, which were the preferred favourite preys.

1.1 Study area

The Madeira archipelago (Deserta islands (15 km^2), Madeira (728 km^2) and Porto Santo (69 km^2) is part of the Macaronesia region, located in the North Atlantic, off the Northeast coast of Africa. The archipelago raised from volcanic activity 18 million years ago and evolved through complex volcanic history. The isolation of these islands, their mid-Atlantic positions and the difference in temperatures between the west part (cooler) of the island and the east part (warmer) define them as marine oasis for many temperate and tropical species (Alves *et al.* 2018). The coasts of the Madeira island plausibly provide unique fish assemblages, different geophysical and oceanographic conditions favourable to resident cetaceans long stay and to seasonal stay of transient and visitor cetaceans. The waters around the archipelago can reach up to 4000 meters below ground (Geldmacher *et al.* 2000) due to its lack of continental shelf (Cetaceos MADEIRA II 2013). This feature provides habitats for deep living and pelagic fish species as well as benefit deep living cetacean species such as sperm whales or pilot whales. Therefore, Madeira is a primordial place to protect and conserve. In the Madeiran region, it exists an uneven distribution of nutrients, because of a submarine oceanic crest that connects the Madeira Island to the Deserta Islands. This crest disturbs the general water circulation (Campuzano *et al.* 2010). Consequently, the area around the crest is richer, thanks to the upwelling of cold waters with high primary production. This phenomenon is accentuated in spring, with higher temperatures and solar radiations, which makes the archipelago of Madeira flourish in marine life (Campuzano *et al.* 2010).

1.2 The bottlenose dolphins and short-finned pilot whales in Madeira

The abundance and frequent occurrence of bottlenose dolphins and short-finned pilot whales in Madeira (Freitas *et al.* 2004, Freitas *et al.* 2006) suggest that they are important predators in the region and that the coastal waters of Madeira have an essential role in their sustainment. Off the island, both species were observed using the area on regular basis for diverse activities such as feeding, socializing, and calving (Dinis *et al.* 2016, Alves *et al.* 2018). Besides, for both species, the percentage of calves in the area is not negligible, more than 40 % for short-finned pilot whales and up to 24 % for bottlenose dolphins (Alves *et al.* 2018). Thus, Madeira is a relevant place to be monitored and protected as its conditions allow the development of future generations of bottlenose dolphins and short-fined pilot whales (Reggente *et al.* 2016). Photo-ID studies of the two species in Madeira, described an island-association pattern for some individuals, others being regular visitors, and transient animals belonging to a bigger Atlantic pelagic population (Alves *et al.* 2013, Dinis *et al.* 2016). Up to 25 % of bottlenose dolphins photographed were considered associated with the island (cetaceo MADEIRA II 2013). Regarding pilot whales, they form a single population in Madeira, divided in clans (Alves *et al.* 2013). The clans are composed of matrilineal pods of 15 individuals average with long lasting relationship. They are suggested to exhibit natal group philopatry (Alves *et al.* 2013). Resident individuals very likely reproduce with transients and visitors, which share the same space as proven by photo-ID (Alves *et al.* 2013). Although, no studies demonstrated a variation in morphotype between migrants and residents, it is tenable that there is in fact one. Transient and visitor individuals are hypothesised to be bigger, as they need stronger muscles for travelling long distances (Vermeulen and Cammareri 2009). On overall, the two species and especially short-finned pilot whales prefer the warmer waters of the southeast of the island (Alves 2013), which provide good conditions for small calves and cows. Likewise, the southeast waters are richer in nutrients as a result of the upwelling of cold waters with high primary production (Campuzano *et al.* 2010). However, it is also in this region of the island that human activity at sea increases. It is manifested by maritime traffic of recreational crafts, touristic vessels and fishing activity (Cunha 2013), which can compromise the Cetacea wellbeing. Bottlenose dolphins and pilot whales coexist in Madeira, but they do not share the same space. Short-finned pilot whales stick to oceanic waters, whereas bottlenose dolphins live on the neritic zone (Alves *et al.* 2013, Dinis *et al.* 2016).

1.3 Conservation status

The bottlenose dolphin and the short-finned pilot whale are described as least concerned in Madeira and are protected in the Exclusive Economic Zone (EEZ) since 1986 (Cabral *et al.* 2005). However, the short-finned pilot whale is regarded as Data Deficient on the global scale, and the population trend stays unknown (IUCN 2012), which is the result of hunting activities off Japan, Indonesia, Sri Lanka and the eastern Caribbean (Reeves *et al.* 2002) along with bycatch off California, Hawaii, and the Northeast US waters. Short-finned pilot whale is listed in the Annex II of Bern Convention, Annex IV of Habitats Directive and Appendix II of CITES. The bottlenose dolphin in Madeira is principally distributed around the coasts (Dinis *et al.* 2016), which directly expose it to human activities. The species is listed in the Annex II of Habitats Directive (Natura 2000 network), Appendix II of CITES, and special areas of conservation (SACs) are established for it (Wilson *et al.* 1997, Cañadas 2006). In general, cetaceans are increasingly threatened nowadays. Indeed, they are apex predators and long-life spanned (Seixas *et al.* 2009); thus, they are more inclined to experience the most serious anthropogenic impacts in the marine milieu (Bowen 1997, Bearzi *et al.* 2003, MacLeod *et al.* 2005).

Fisheries, the whale watching industry, pollutants, and finally climate change could have detrimental effects on cetaceans´ survival, and for that reason more ecological studies are needed. Moreover, the geographical location and the insular propriety of Madeira makes its marine ecosystem vulnerable to long term impacts. Several threatened species of cetaceans have been recorded in Madeira, such as the North Atlantic right whale, the blue whale, the fin whale and the sei whale (IUCN 2007 2008). An evaluation of the entire region healthiness and stability via ecophysiological studies on two of the most frequent cetacean species in Madeira (bottlenose dolphin and short-finned pilot whales) may help in the protection of other cetacean species, and in the protection of the Macaronesia ecosystem in general.

1.4 Objectives

Bottlenose dolphins and short-finned pilot whales are very common in Madeira Archipelago, but it is only this last decade that scientists started to study their ecology. The study of the ecology of marine mammals is difficult to inspect as it is expensive, manipulative experiments rarely possible and the interaction between systems occur at different spatial and temporal scales. Therefore, for many cetaceans two main questions are unanswered 1) When and where do cetacean forage? 2) What do they eat? In this context, the aims of this study were 1) To check if the biochemical indice of ecophysiological conditions (i.e. RNA/DNA ratio) could be efficiently used on cetacean’s species and provide information on their nutritional state; thus, on the environmental conditions of Madeira. 2) To determine the trophic ecology of the two cetaceans in function of residency patterns, based on the analyses of stable isotopes (carbon and nitrogen). 3) Draw a potential link between the diet aspect of cetaceans and their nutritional state. The validation of RNA/DNA ratio as a relevant tool for the determination of the physiological state of Cetaceans related to their environments is important for the identification of crucial habitats and the threats they face because of human activities. With this study, I expect by combination of physiological and trophic information to inform on the ecological status of cetaceans in the Madeira region and help on their successful conservation.

This study will verify if as expected bottlenose dolphins are generalist and feed in pelagic waters in Madeira. It will also confirm if short-finned pilot whales feed predominantly on squids, living in deep waters around the island. We expect spatial niche segregation between the two species in Madeira, to mitigate competition for resources and habitats. The study will enlighten this point.

2. RNA/DNA RATIO, AN INDICATOR OF NUTRITIONAL STATE OF BOTTLENOSE DOLPHINS AND SHORT-FINNED PILOT WHALES IN MADEIRA

2.1 Introduction

Knowledge of the physiology of any species in function of their environments and the factors contributing to its variability is essential in a changing world (Dahlhoff 2004). Most of health indices in cetaceans derive from photogrammetry of body condition and stress hormones (Hunt *et al.* 2013). In this study, the RNA/DNA ratio was used as a new health assessment tool, and as a stress biomarker of environmental (e.g. upwelling, season) or human (e.g. fisheries, pollution, whale watching) disturbances in cetacean species. The ratio, known as 'standardized RNA/DNA ratio'- sRD, provides a measure of cellular protein synthesis capacity and relies on the fact that the concentration of cellular DNA is relatively constant in the somatic cells regardless of any changes in the organism's environment, while the RNA content of a cell increases as the cellular demand for protein synthesis and growth increases (Chicharo and Chicharo 2008). Thus, the amount of RNA can be age, life-stage, organism size, disease-state and environmental condition dependant (Bullow *et al.* 1970, Dahlhoff 2004, Buckley *et al.* 2008, Li *et al.* 2010, Kobari *et al.* 2012, Vieira *et al.* 2014). The RNA/DNA is the most widely used biochemical indicator of physiological (growth, reproduction, secretion) and nutritional states of a wide range of marine organisms since its first apparition, 48 years ago (Burow *et al.* 1970). It is an innovation in the context of the assessment of physiological conditions in marine vertebrate species like cetaceans. In addition, there is no potential to create handling artefacts, like it can be observed during blood sampling methods to determine stress hormones (Pedemera-Romano *et al.* 2010, Champagne *et al.* 2012, Keogh *et al.* 2013, Fair *et al.* 2014). Although, the RNA/DNA ratio is a good indicator of organisms health, it can lack precisions on the reasons for its variation as many biotic and abiotic factors can be interacting at the same time. This method will be completed with carbon and nitrogen stable isotopes in chapter 3, in order to verify a link between aspects of diet and physiological responses. The specific aims of this chapter is to analyse the nutritional condition and potential growth of sampled cetaceans.

2.2 Sampling and data collection

2.2.a *Study site*

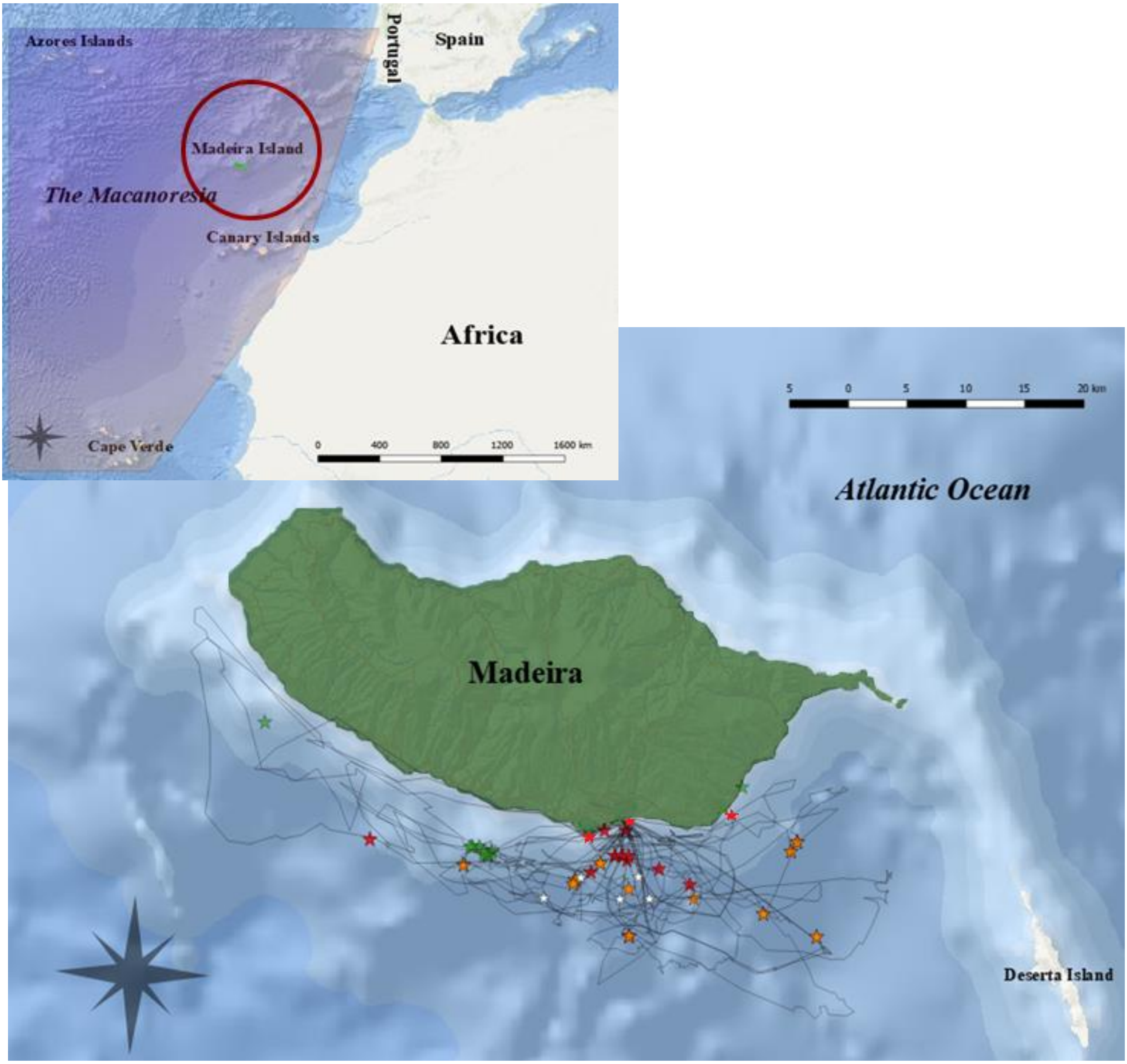

Fig 2.1 *Map of the Macaronesia region and the study site, Madeira circled in red (top map). RNA/DNA and stable isotope sample collection locations in Madeira, Portugal and track lines (black lines) (bottom map). Bottlenose dolphin sampling locations are indicated by the red stars. Short-finned pilot whales sampling locations are indicated by the orange stars. Small fishes sampling location are indicated by the green stars and POM and Zooplankton sampling locations are indicated by the white stars.*

2.2.b *In the field*

Thirty-three dermis samples (skin and blubber) of bottlenose dolphins *(Tursiops truncatus)* and 23 dermis samples of short-finned pilot whales *(Globicephala macrorhyncus)* were collected in Madeira (**Fig 2.1**). The biopsies were obtained from dedicated surveys over two times period from the 3rd of November until the 20th of November 2017 and from the 16th of March until the 18th of April 2018. Look-out posts were usually used to indicate the location of the two species. Although, random search was sometimes undertaken when cetaceans could not be localised. Once individuals were spotted, they were approached by boat and sampled via the use of a crossbow and biopsy darts that prevailed from 1.5 to 2 cm of dermis **(Fig 2.2** and **Fig 2.3).** The sampling targeted the side, posterior part of the cetacean, so that the spine, the head and dorsal fins were not injured and underwent complications during the healing. Once taken, biopsies were kept in a liquid nitrogen container in the boat before being frozen at -80 °C at the University of Madeira until laboratory analysis. Freezing is considerate a relatively safe way to preserve samples for the analysis of RNA/DNA ratios (Amaral *et al.* 2008, Barrow *et al.* 2008). The date, GPS location, number of individuals in the group, number of species, number of calves and reaction to the biopsies were registered for all samples. Sampling areas were always in the range of five to six nautical miles from the coast. During the sampling, an attempt to digitally photograph sampled individual was made. The photographs, together with field observations allowed to categorise all sampled cetaceans as adults or subadults and also differentiate transient, resident and regular visitor individuals thanks to their comparisons with the photo-id catalogues of the Oceanic Observatory of Madeira, which comprise data from 2004 until 2017 for bottlenose dolphin and from 2003 until 2017 for short-finned pilot whale. Pilot whales were considerate resident to the island when they were captured more than 5 times in at least 3 years and 3 seasons since 2003. They were considered transient when they were captured only once since 2003 and regular visitors when they were seen more than once since 2003 but had not been recognised as a resident (based on Alves *et al.* 2013). Bottlenose dolphins were considerate resident to the island when they were seen more than two consecutive years and during 3 seasons around the island since 2004 (Pradel *et al.* 1997, Dinis *et al.* 2016). They were considered transient when they were seen only once in Madeira since 2004 and regular visitors when they were seen several times but in non-consecutive years since 2004 (Kendall *et al.* 1997, Dinis *et al.* 2016). Samples were only taken from adults and subadults cetaceans to minimise the stress impact on the individuals and because the physiological conditions of calves and mothers might be different from standard individuals.

Fig 2.2 *Biopsy-dart with a crossbow used on a short-finned pilot whale by the coast of Madeira.*

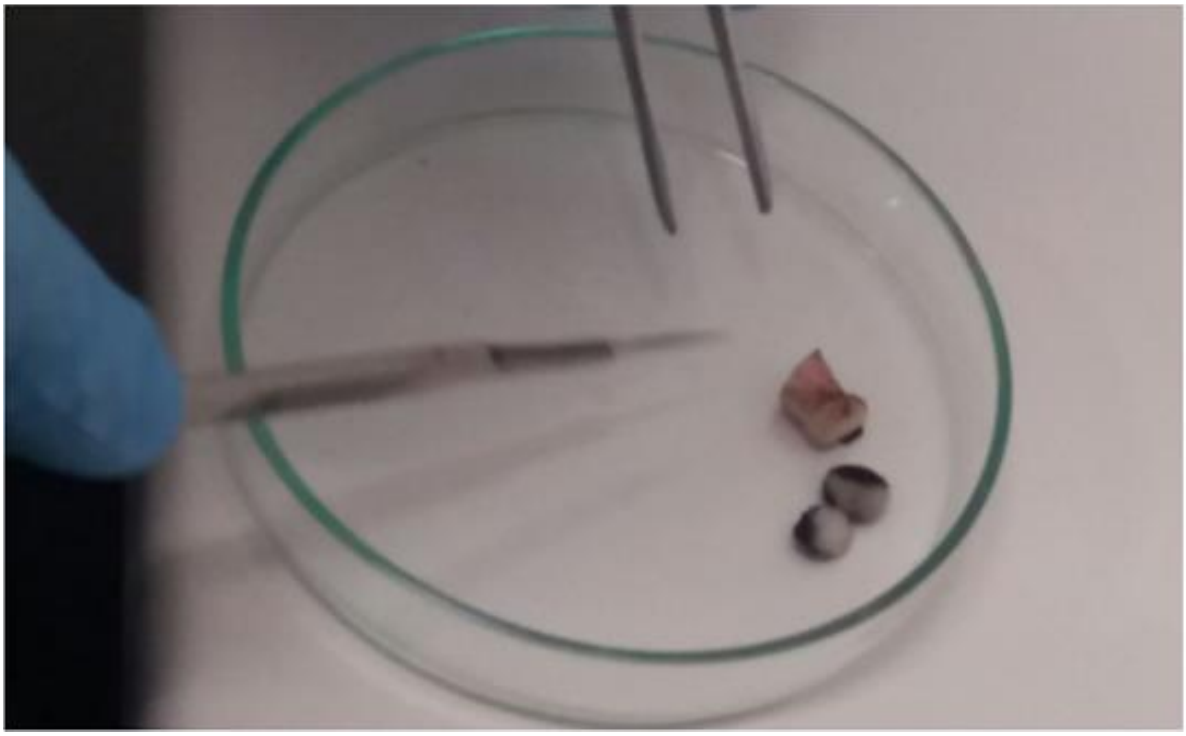

Fig 2.3 *Example of a biopsy-dart sample obtained with a crossbow, showing epidermis (black tissues) and blubber (pinkish tissue) of a short finned-pilot whale.*

2.2.c *In the lab*

Biopsy-dart samples were kept in azote liquid at – 80 °C. Before the analyses, the skin of each samples was separated from the blubber and were then divided in four little pieces for various analysis including carbon and nitrogen stable isotopes (chapter 3) and the RNA/DNA ratio. Nucleic acid analysis was carried out on 33 adult bottlenose dolphins and 23 adult short-finned pilot whales. Only 31 samples for bottlenose dolphins and 22 samples for short finned pilot whales were usable for statistical analysis. The procedures used to quantify nucleic acids in individuals are outlined in Esteves *et al.* (2000) and Caldarone *et al.* (2001)

The skin tissues of each individual were put in 600 µl of Sarcosine (0.5%) and sonicated 3 times for 10 seconds at 70 A, in order to separate cells tissues from the genetic material. Samples were then vortexed for 30 minutes and centrifuged for 15 minutes at 12 000 r P. M at 0 to 4 °C so that dense particles were separated from the lighter particles, and the supernatant containing the genetic material was able to be analysed further. In order to determine the DNA and RNA concentrations of the samples via the reading of absorbances, standard curves needed to be established. In this study, DNAstock and RNAstock produced from bovine material were used. In the wells of a fluorescent plate, 50 µl of Aliquot of supernatant of each sample was pipeted. Then, 120 µl of Tris buffer was added in each well in order to maintain a stable PH as DNA is PH sensitive. Various concentrations of RNAstock and DNAstocks were pipeted into different lines of the fluorescent plate. Those lines were completed with various concentrations of Sarcosine (0.05 %). Thereafter, 30 µl of RNAase was pipeted into the DNAstock wells and in all samples. The enzyme RNAase allowed later, after warming up at 37 °C to remove the RNA from the DNA. Then, 30 µl of GelRED, was added into each wells of the Fluorescent Plates. It allowed to make nucleic acids fluorescent. The plates were read in a multiple reader for absorbance measures at 360 nm and 590 nm to determine the total amount of DNA and RNA in each sample. After reading, RNAase was activated by incubating the Fluorescent Plates at 37 °C for 30 minutes. Samples were read again at 360 nm and 590 nm. It allowed the determination of DNA concentrations within samples and DNAstock. Finally, RNA concentrations were calculated via the equation: [RNA] = Total fluorescence – fluorescence after RNAase activation (**Annexes**).

2.3 Statistical treatment

All graphics and statistical analyses were performed using the open source software R version 3.5.1 (R Development Core Team; www R.-project O R.g). Data were analysed after removal of statistical outliers and testing for normality (Shapiro-Wilkoxon normality test) and homoscedasticity (Barlett´s test). When the assumptions were not met, the Kruskal wallis test was used. For all tests, significance was assigned at p,0.05. The influence of independent factors (resident/visitor/offshore; spring/autumn; bottlenose dolphin/short-finned pilot whale) on dependent variables (ecophysiological condition of two species of cetaceans based on RNA/DNA ratio) were analysed. Two regression analyses for both species were performed to investigate the relationship among RNA concentrations or DNA concentrations and RNA/DNA values. A Two-samples t-test was used to compare the RNA/DNA of the two cetaceans. Then, two other two-samples t-test were used to compare first the RNA concentration of the two species and second the DNA concentration of the two species. RNA/DNA values of collected data of regular visitor, resident and transient individuals were compared by a Kruskal Wallis test, in the case that sample sizes were too small and parametric tests are very sensitive to sample sizes. Two samples t-tests were used to determine the influence of sampling season on RNA/DNA values for each species, and a two ways ANOVA test with Posthoc Bonferroni test tested a difference between season within short-finned pilot whales residency pattern. One-sided t-tests were applied to detect a difference in RNA/DNA values between individuals present in a group with calves and individuals present in a group without calves. Correlation tests were effectuated to check a relationship between size of the group in which the individual lived and the RNA/DNA values. Another t-test was utilised to compare coefficient of variations between the two species.

In the statistical treatment of the data, the individuals Gma 24 and Tt 5 were excluded of the analyses since their coefficient of variation were very high (Gma 24= 76%; Tt 5= 100%). The values of the data for these two individuals were not uniform to the arithmetic means of the data.

2.4 Results

The average values, standard deviation (SD) and coefficient of variation of the RNA/DNA ratios are presented in (**Annexe; Table 2.3)** for bottlenose dolphins and presented in (**Annexe, Table 2.4)** for short-finned pilot whales.

Bottlenose dolphins

The average value of RNA/DNA for bottlenose dolphin was 0.492, SD= 0.074. The ratio varied from 0.264 to 0.690 and the greatest value was held by the individual Tt 9 (0.690), followed by Tt 16 (0.684) and Tt 29 (0.684) **(Annexe, Table 2.3** and **Fig 2.4).** One third of the individuals sampled (n=10) had a ratio comprised between 0.35 and 0.44 and individuals ranging from 0.62 and more were also greatly represented, with a contribution close to one third in the sample (n=7). Individuals present in groups accompanied with calves (n= 10) did not exhibit a significant (One sided t-test: t, -0.907; p, 0.188) lower RNA/DNA ratio (0.438) than individuals present in groups with no calves (n= 10) (0.531) **(Table 2.5)**. Although, the mean value was higher for the group with no calve. There were not significant correlations (Pearson´s correlation, t, -0.035; p, 0.972; cor, -0.007) between the size of the pod in which the individuals swam and their RNA/DNA ratio **(Table 2.5)**.

Season

The analysis of the RNA/DNA ratio for the two distinct groups autumn (n=13) and spring (n= 17) affirmed non-significant (Two-samples t-test: t, 0.867; p, 0.394) differences for the biochemical index **(Table 2.5** and **Fig 2.9).** Ecophysiological conditions of bottlenose dolphin, were not significantly different in spring (0.509, SD= 0.08) compared to autumn (0.470, SD= 0.07). Bottlenose dolphins sampled in spring, did not exhibit significantly higher RNA or DNA concentrations than bottlenose dolphins sampled in autumn (Two-samples t-test: t, 0.925, p, 0.363; t, 0.6 19, p, 0.541, respectively). Bottlenose dolphins sampled in spring ([RNA] =5.49, SD= 0.77, [DNA] =5.37, SD= 0.1) had roughly the same RNA and DNA concentrations than bottlenose dolphins sampled in autumn ([RNA] = 4.68, SD= 0.68, [DNA] =4.95, SD= 0.16)

Table 2.5 *Mean RNA/DNA ratio with standard deviation for bottlenose dolphin sampled in Madeira during November 2017 and March-April 2018 and results of statistical analyses at a level of 95 % confidence interval.*

Season	**BOTTLENOSE DOLPHINS**	
	Mean	SD
Autumn (n= 13)	0.470	0.066
Spring (n= 17)	0.509	0.080
Total (n= 30)	0.490	0.074
Comparison		
Autumn vs Spring	Two-samples t-test: t, 0.87; p, 0.39	
Group size	Pearson´s correlation, t, -0.035; p, 0.972; cor, -0.007	
Presence of calve		
With calves (n= 10)	0.438	0.053
No calves (n= 10)	0.531	0.082
One sided t-test: t, -0.907; p, 0.188		

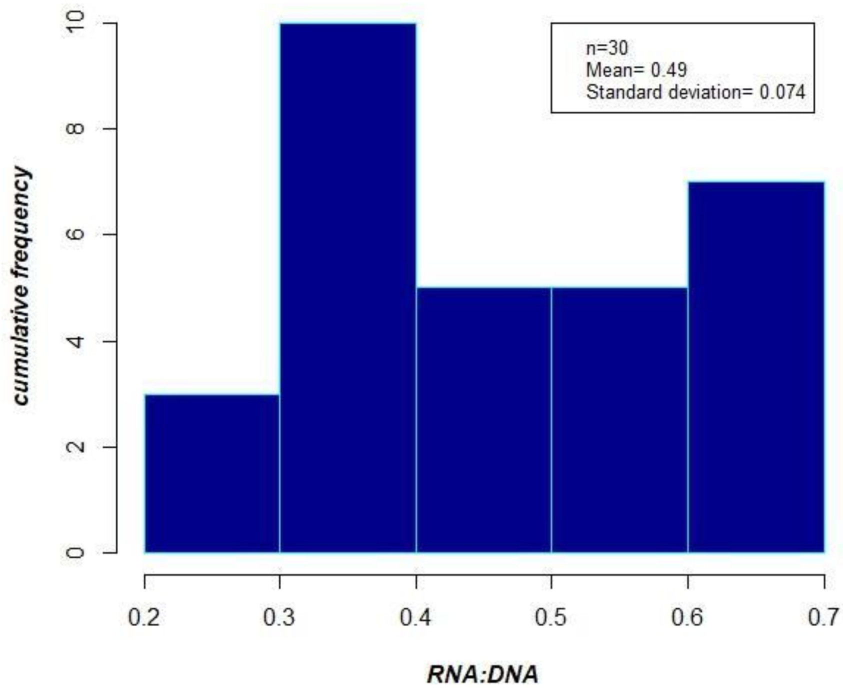

Fig 2.4 *Frequency distributions of RNA/DNA ratios of bottlenose dolphin from samples prevailed during November 2017 and March-April 2018.*

Short-finned pilot whales

Residency pattern

The three residency patterns (resident, visitor and transient), in which RNA/DNAs varied from 0.205 to 0.488 **(Table 2.4)**, were not significantly different (Kruskal-Wallis test, p, 0.432). The average value of the RNA/DNA in short-finned pilot whales off the coast of Madeira was 0.309, SD= 0.07. The greatest value was held by the individual Gma 8, a regular visitor in Madeira, while the smallest value was held by the individuals Gma 7 a transient animal in Madeira **(Table 2.4)**. Although, the three different residency patterns did not significantly differ, visitor and transient animals seemed to be the most likely to differ (Kruskal Wallis test and Dunn test: p, 0.21, $\mu_{transient}$ = 0.286, SD= 0.07; $\mu_{visitor}$= 0.337, SD= 0.08). Transient and resident animals seemed to be the least likely to differ (Kruskal Wallis test: p, 0.43, $\mu_{transient}$= 0.286, SD= 0.07, $\mu_{resident}$= 0.99, SD= 0.08). (**Table 2.6** and **Fig 2.6**). Visitor, transient, and resident individuals did not significantly differ by their DNA (Kruskal-Wallis and Dunn test: p, 0. 19; p, 0.26 and p, 0.08, respectively; $\mu DNA_{visitor}$= 3.89, $\mu DNA_{transient}$= 4.46; $\mu DNA_{resident}$ = 3.32) neither did they significantly differ from their RNA (Kruskal Wallis and Posthoc dunn test: p, 0.47; p,0.28; p, 0.33; respectively $\mu RNA_{visitor}$= 3.87, $\mu RNA_{transient}$= 3.88; $\mu RNA_{resident}$= 2.86) **(Table 2.6)**. Nonetheless, the sample sizes for each group were very small ($n_{transient}$= 6, $n_{visitor}$= 9, $n_{resident}$ =6); thus, the precision, certainty and confidence in our estimates are low. There were no significant differences (Wilcoxon rank sum test: W, 53; p, 0.569, $\mu_{transient}$ = 0.286, $\mu_{resident+visitor}$= 0.318) between transient and the two other groups combined. There were very little variations in the RNA/DNA ratios of resident individuals (range: 0.253-0.342) while the variations within transients and visitors seemed to fluctuate further (range: 0.214-0.378 and 0.205-0.488, respectively) **(Table 2.4** and **Fig 2.6)**.

Among the 21 short-finned pilot whales, without taking into consideration the residency patterns, half of the whales had RNA/DNAs comprised between 0.27 and 0.33, less than one third of them had RNA/DNA comprised between 0.21 and 0.27 and the rest had a ratio higher than 0.33 **(Table 2.6** and **Fig 2.5)**. Residents, transients and visitors were all encompassed between 0.25 and 0.35. Individuals present in groups accompanied with more than two calves (n= 7) did not exhibit a significant (One sided t-test: t, -0.626, p, 0.731) lower RNA/DNA ratio (0.324) than individuals present in groups with 0-2 calves (n= 14) (0.301) **(Table 2.6)**. There was not a significant relationship (Pearson´s correlation: t, 0.185, p, 0.855, cor, 0.04) between the size of the pod in which the individuals were sampled and their RNA/DNA ratios **(Table 2.6)**.

Season

The analysis of the RNA/DNA ratio for the two distinct groups autumn (n=14) and spring (n= 7) showed non-significant differences (Two-samples t-test: t, -0.28492; p, 0.779) for the biochemical index for the population as a whole and within the three residency patterns (2-ways ANOVA, and post-hoc Bonferroni test: p, 0.989) **(Table 2.6** and **Fig 2.9)**. The ecophysiological conditions of short-finned pilot whales were not significantly different in spring (0.303, SD= 0.08) compared to autumn (0.312, SD= 0.07). Short-finned pilot whales in spring did not exhibit significant (Two samples t-test: RNA: t, 0.233, p, 0.409; DNA: t, 0.350, p, 0.365, respectively) higher RNA or DNA concentrations than short-finned pilot whales sampled in autumn, so that short finned pilot whales sampled in spring ([RNA] =3.69, SD= 0.9; [DNA]= 4.03, SD= 0.14) had roughly the same RNA and DNA concentration than short-finned pilot whales sampled in autumn ([RNA] = 3.53, SD= 0.7; [DNA] = 3.82, SD= 0.13).

Table 2.6 *Mean RNA/DNA ratio with standard deviation for short-finned pilot whales sampled in Madeira during November 2017 and March-April 2018 and results of statistical analyses at a level of 95 % confidence interval.*

Residency pattern	**Short-finned pilot whales**	
	Mean	SD
Resident (n= 6)	0.291	0.080
Visitor (n= 9)	0.337	0.075
Transient (n= 6)	0.286	0.067
Total (n=21)	0.309	0.08
Comparison		
Resident vs Visitor	Kruskal Wallis test: p, 0.27 > 0.05	
Resident vs Transient	Kruskal Wallis test: p, 0.43 > 0.05	
Visitor vs Transient	Kruskal Wallis test: p, 0.21 > 0.05	
Season	Mean	SD
Autumn (n= 14)	0.312	0.070
Spring (n= 7)	0.303	0.080
Total (n= 21)	0.309	0.074
Comparison		
Autumn vs Spring	Two-samples t-test t, -0.28; p, 0.78	
Group size	Pearson´s correlation test: t, 0.185; p, 0.855; cor, 0.04	
Presence of calve		
With 0 to 2 calves	0.30	0.07
With 2 or more calves	0.32	0.07
One-sided t-test: t, -0.626, p, 0.731		

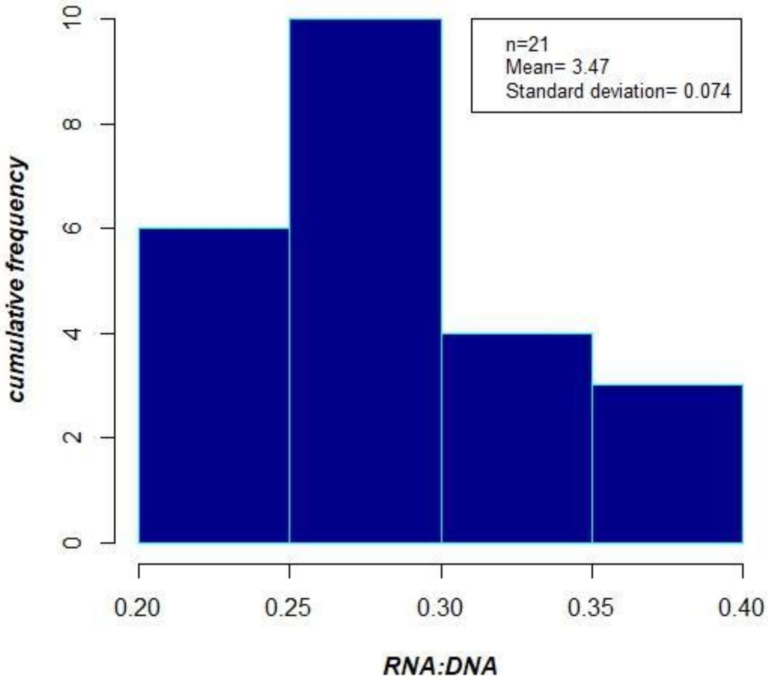

Fig 2.5 *Frequency distributions of RNA/DNA ratios of short-finned pilot whale from samples prevailed* during *November 2017 and March-April 2018.*

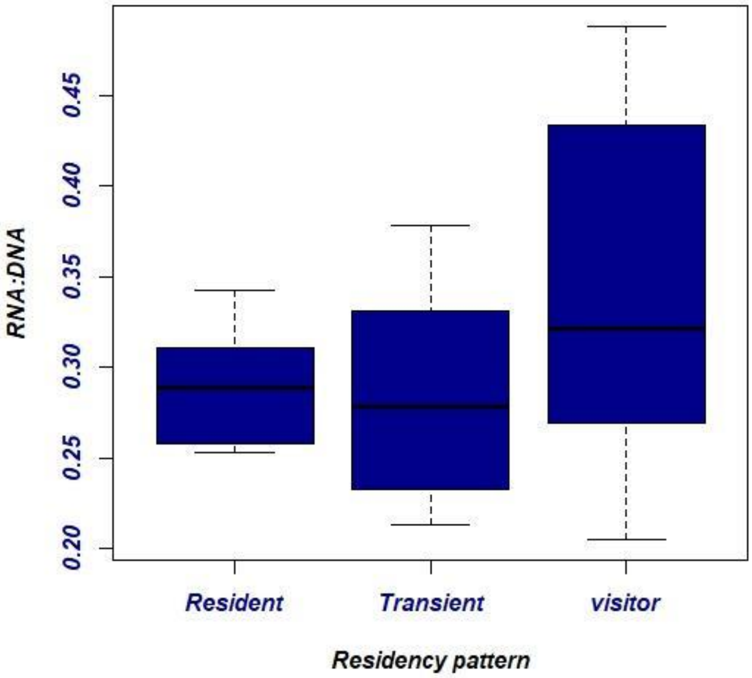

Fig 2.6 *Biochemical condition of the three-residency pattern of short-finned pilot whale. RNA/DNA for resident (n= 6), transient (n=6) and visitors (n=9) sampled in Madeira during November 2017 and March -April 2018.*

For the two species, there were strong positive correlations between the amount of RNA and the RNA/DNA (Linear regression: Tt: F, 37.59, p < 0.05, and Gma: F, 12.73, p, 0.002, respectively). These relationships accounted for Tt: 57 % and Gma: 40 % of the variances of RNA/DNA ratios. However, there were no significant correlations between the amount of DNA and the RNA/DNA ratio (Pearson´s correlation test: p, 0.13, cor = 0.28 and p, 0.48, cor= 0.16). Relationships between the amount of RNA and DNA in function of their RNA/DNA ratio are presented in **Fig 2.7.** These outcomes confirm that only RNA was responsible for fluctuation in the ratios, and that the DNA amount is stable at the individual scale, while the RNA amount fluctuates in function of environmental or biological parameters.

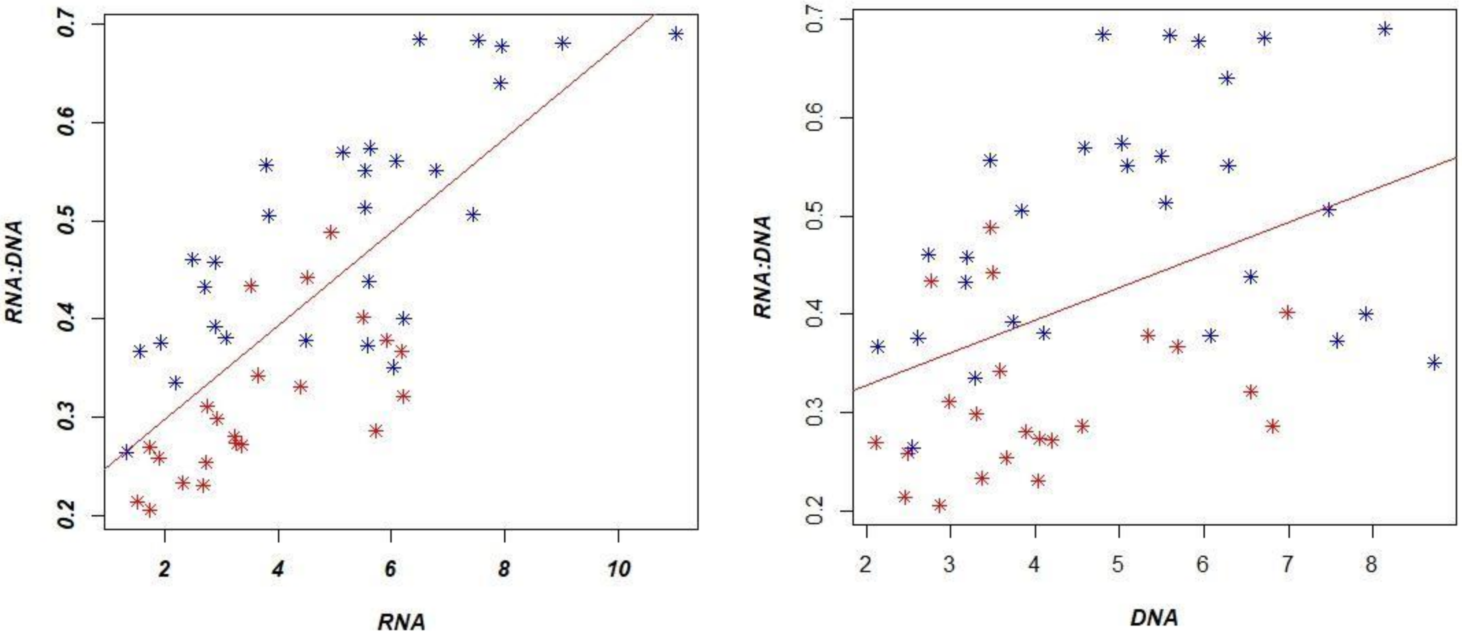

Fig 2.7: *RNA/DNA as a function of RNA (on the left) and DNA (on the right) concentration in bottlenose dolphin (blue dots) and short finned pilot whales (red dots), sampled in Madeira during November 2017 and March-April 2018.*

RNA/DNAs between species were significantly different (Two sample t-test: t, -6.049, P < 0.05). The highest average RNA/DNA ratio (0,49) was obtained for bottlenose dolphin whereas, the average RNA/DNA of short-finned pilot whale was of 0,30. The two species differed significantly by both their average DNA values (Two sample t-test: t2.95, p, 0.005. Tt: DNA = 5. 19; SD= 0.13 and Gma: DNA= 3.90; SD= 0.13) and their average RNA values (Two sample t-test: p, 0.023. Tt: RNA= 4.98, SD= 0.73 and Gma: RNA= 3.47, SD= 0.80)

(Fig 2.8). On overall, short-finned pilot whales seem to have less variations within their RNA/DNAs (range: 0.205-0.378), while bottlenose dolphins have a wider range of RNA/DNA ratios (range: 0.335-0.69) **(Fig 2.8)**.

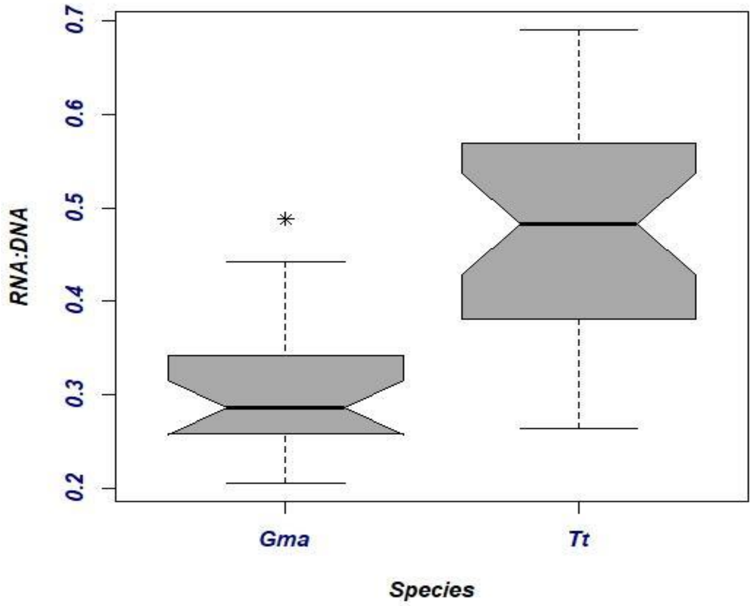

Fig 2.8 *Biochemical condition of bottlenose dolphin and short-finned pilot whale. RNA/DNA for bottlenose dolphin (n= 30) and short-finned pilot whale (n= 21) sampled in Madeira during November 2017 and March -April 2018.*

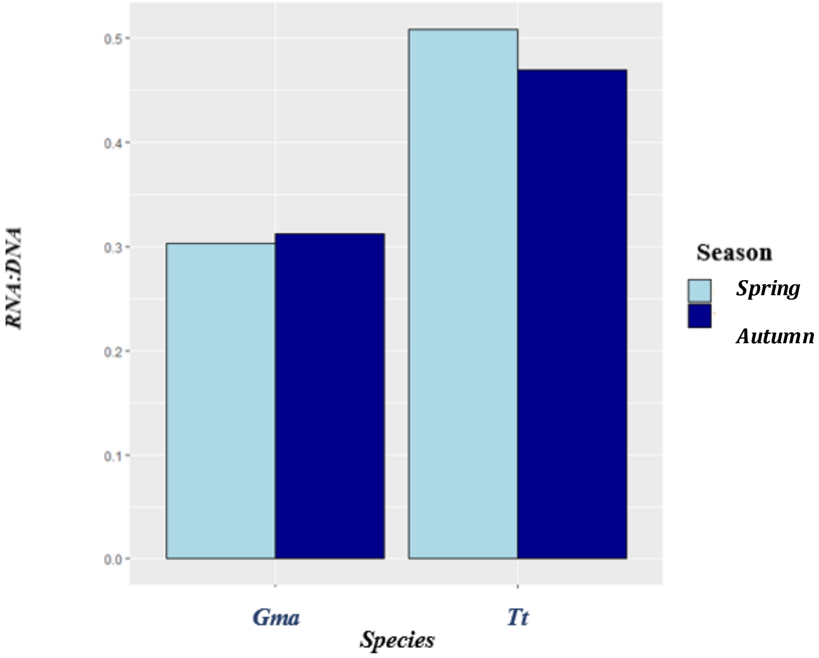

Fig 2.9 *Biochemical condition of bottlenose dolphin and short-finned pilot whale. RNA/DNA for bottlenose dolphin sampled in autumn (n =13) and spring (n =17) and short-finned pilot whale sampled in autumn (n =14) and spring (n =7) in Madeira in November 2017 and March -April 2018.*

2.5 Discussion

The main goal of this chapter was to demonstrate that the RNA/DNA could be used as an indicator of nutritional conditions for marine mammals, in their natural environments. The over year sampling of the two cetaceans and the distinction of different residency patterns within species, allowed a spatial and temporal comparison of within species physiological conditions. The method used can be rather expensive and required manipulation of the individuals, but it evidenced positive results. In addition of reflecting the growth and physiological conditions of a broad array of larval fishes and other marine invertebrates (Buckley 1979, Rooker and Holt 1996, Pierce *et al.* 1999, Chicharo *et al.* 2001, Amaral *et al.* 2008, Frommel and Clemmesen 2009, Li *et al.* 2010, Vieira *et al.* 2014), the RNA/DNA can henceforth be considerate another tool for the health assessment of marine mammals. Previous studies effectuated on ectotherm marine animals, strongly supported the evidence that the RNA/DNA was temperature dependant (Buckley *et al.* 2008). In the present study, it was impossible to evaluate this parameter, as Madeira tends to have relatively stable sea surface temperatures through the year, reaching its coolest temperatures in March-April (19 °C) and its warmest temperatures in September-October (25 °C) (sea temperature O R.g). Moreover, the temperatures in Madeira fluctuated only of 3,5 °C between the two sampling periods. Therefore, any variations in the RNA/DNAs between autumn and spring is not likely explained by a thermal factor but would rather be explained by resource availability. In the study, bottlenose dolphins and pilot whales sampled in spring did not exhibit a significant higher ratio; thus, better physiological conditions than individuals sampled in autumn (**Tables 2.5 and 2.6, Fig 2.9**). However, the mean concentration of the ratios in spring for both species were slightly higher than in autumn, which hypothesises that seasons and biological variations related to it could potentially affect the species physiological conditions. Indeed, Madeira is characterised by a pronounced spring phytoplankton bloom pattern, related to higher sunlight and coastal upwellings (Martins *et al.* 2007), which could be responsible for both the accumulation of the megafauna such as cetaceans and their preys during the spring season (Gleiss *et al.* 2016) and could explain a slightly higher RNA/DNA ratio due to better nutritional states.

In Madeira, both concerned species exhibit different residency patterns, with specimens living off the coast the whole year long and others only passing by for short period of times. Among pilot whales, the ratio was on average higher for visitors than for their other counterparts (resident and transient); although, the differences were not statistically different

(**Table 2.6 and Fig 2.6**). This latter result is plausibly due to the fact that the sample sizes were very small in this study, which increased the risk of type I error. In addition, there were greater variations in the range of RNA/DNA values among visitor and transient individuals than among residents (**Fig 2.6**). Visitors likely migrate to exploit seasonal peaks of resources in various highly nutritive areas, while escaping the seasonal resource depletion in Madeira (Alerstam *et al.* 2003). This behaviour would contribute to their slightly higher nucleic acid concentration; thus, their overall better physiological conditions. However, the migrative behaviour of visitor and transient pilot whales, could compromise their overall physiological states in function of their hunting success during their travels, which would explain great variations in their RNA/DNA values. The dependency of resident individuals on Madeira, might be genetically predominated and related to the good climatic conditions and the demography of the island, which allows the subsistence of permanent prey populations. As a consequence, resident individuals do not need to adapt their feeding behaviours like migrants could do through their travelling, and may feed on the same preferential prey resources, which contribute to their small variations in RNA/DNAs. The reason for visitor and transient individuals to move away from the island, might have occurred through a density-dependant selection, where migrants had been outcompeted due to lack of habitats and resources (Alerstam and Enckell 1979). These results underline the importance to monitor fisheries on a global scale, as migrant individuals seem to be the most affected by resource availability. Indeed, they require to be well fed, so that they can survive the cost of the journey. Visitor individuals recorded the highest RNA/DNAs. In general, long distance swimmers are favoured by increased body sizes (Alerstam *et al.* 2003). Although, short-finned pilot whales are naturally bulky and design to support high activity events during their hunts (Velten *et al.* 2013, Aoki *et al.* 2017), a larger muscular body would significantly favour the animal in its chances of hunt success and explain higher RNA/DNAs in visitor pilot whales.

Residency patterns were unable to be determined for bottlenose dolphins due to the poor quality of the pictures taken during the sampling. However, two trends in the RNA/DNAs were determined. Individuals comprised between 0.35 and 0.44 were the most represented in the bottlenose dolphins sampling, but still a big majority of individuals had a ratio higher than 0.62 (**Fig 2.4**). Yet, the RNA/DNA has been proven to fluctuate in function of genders and in function of the life stage of the entities in marine invertebrates (Chicharo *et al.* 2007, Buckley *et al.* 2008, Frommel *et al.* 2009). This is partly due to different feeding habits depending on physiological demand in relation to the individual´s state (life stage, gender, gestation, lactation) (Ensminger *et al.* 2014). Gates and Edmunds (1999), also found that it could exist intraspecific variations in the RNA/DNA Further studies need to investigate the ratio in function of the residency patterns of bottlenose dolphins, genders and life stages, in order to optimise the use of the indice on marine mammals and avoid mistakes on the interpretation of the impacts of environmental conditions on physiological states. The present study could not provide a direct interspecies comparison; thus, only differences in the range of RNA/DNA ratio between the two species were able to be exploited. Indeed, this biochemical tool has numerous sources of variations, and generalisation across taxa is impossible, as the ratio varies taxonomically and ontogenetically (Foley *et al.* 2016). Nonetheless, it was possible to depict a difference in the range of RNADNAs between bottlenose dolphins and short-finned pilot whales, with bottlenose dolphins having greater variations in the ratios (**Fig 2.8**) The wide range observed in bottlenose dolphins can´t yet be explained. Residency patterns, sex, and age might be the causes for such differences. However, a wider range for the ratio in the species when compared to pilot whales, could highlight that bottlenose dolphins are more sensitive to variations in resource availabilities than pilot whales and might be further disturbed by environmental or anthropogenic impacts. In addition, it was previously proven that boats could have an impact on bottlenose dolphins´ inter-breath interval, inter-animal distance, direction and swimming speed when at proximity of vessels (Nowacek *et al.* 2006). These responses evidence the stress induced by such activities and hypothesize negative side effects on their chance of hunting success. Pollution could also induce a physiological stress to resident individuals, as there is a high human occupancy in Madeira, which involves higher pollution rate off the coasts. Li (2010), noticed that pollution could decrease the RNA/DNA ratio of invertebrate species. This result should be investigated for vertebrate marine species.

2.6 Conclusion

In cetaceans, the RNA/DNA ratio can be utilised to determine the influence of environmental conditions on their physiology. It is especially relevant the case for the determination of their nutritional status which may also support further use of other indicators of feeding ecology, as the stable isotopes and diet studies. Organisms active and feeding, hardly will show very low, close to zero RNA/DNA ratios, and then SIA results when compare to local preys will be able to give us some interpretations of the type prey that are contributing more to their growth. inter-species studies using the biochemical indice are not relevant, because of great fluctuations between taxa. Calibration studies for different species studied, are indispensable for accurate results. The study hypothesizes that visitor and transient pilot whales had greater variations in their RNA/DNA values than residents. It involves that they are more prompt to undergo the effect of natural and anthropogenic impacts, and further studies should ensure of their overall states. Bottlenose dolphins recorded greater variations in their RNA/DNA ratios than pilot whales. Bottlenose dolphins should be further investigated in order to determine the factors of variations in the ratio, and make sure that the populations are not in danger. In the future, more data need to be analysed in order to increase the sample sizes, which would represent more accurately the populations. Moreover, the relationship between the biochemical indice and the size, gender, development stage and protein content of entities needs to be investigated, to understand its application and limits. The residency patterns of bottlenose dolphins should be determined and related with the RNA/DNA Whale watching, pollution, climate change and fisheries could impact the broad-scale distribution and composition of preys in the area. They are considerable threats for both short-finned pilot whales and bottlenose dolphins in Madeira. Physiological changes in the two cetaceans could monitor the occurrence, extent and severity of these threats, and prevent future disequilibrium in the Madeiran ecosystem.

3. TROPHIC ECOLOGY OF BOTTLENOSE DOLPHINS AND SHORT-FINNED PILOT WHALES IN MADEIRA ARCHIPELAGO AS INTERPRETED FROM CARBON AND NITROGEN- ISOTOPE RATIOS

3.1 Introduction

The analysis of stable isotopes such as carbon and nitrogen in organisms' tissues, has become a popular and powerful tool in marine trophic ecology studies (Arthur *et al.* 2008). Indeed, trophic ecology tends to be a scarce and unreliable science regarding marine organisms as it is rare to assist to predation events or get information from stomach contents. Therefore, isotope analyses turn out to be fair complementor to these technics. These analyses allow to trace source of feeding, establish trophic models and delineates spatial information on where animals feed (Barnes and Hughes 2009). The method rests on the fact that stable isotopes are differentially distributed in nature within inorganic substrates, as a result of different biogeographic reactions. These isotopes are then differentially incorporated by primary producers and transferred to higher trophic levels over time, via their consumptions (Bisi 2013). The rate of change prey-predator can be predicted (DeNiro & Epstein 1978, 1981, Tieszen *et al.* 1983, Peterson & Fry 1987, Arthur *et al.* 2008). For instance, stable carbon isotopes ($\delta^{13}C$) values are relatively constant through a given food web in a given environment. It is only enriched by ~1‰ from prey to predator (DeNiro & Epstein 1978, Rau *et al.* 1983). Consequently, it is possible to determine the primary producer source (seagrass, phytoplankton, algae) responsible for the energy flow in an ecosystem, as well as to identify the habitat in which it grows. In fact, the primary source of carbon in coastal areas is different from the one in the open ocean, recording different $\delta^{13}C$ values (Haro *et al.* 2016. On overall, $\delta^{13}C$ values in marine organisms tend to be higher in coastal or benthic environments than in oceanic or pelagic environments (Chicharo and Chicharo 2008). This is probably a consequence of low $\delta^{13}C$ values in primary producers in these areas, because of the depletion of nutrients (France 1995, Laws *et al.* 1995). Regarding stable nitrogen isotope values ($\delta^{15}N$), they increase as an individual ascends in the food chain. It is the result of $\delta^{15}N$ retention in the body during excretion (Newsome *et al.* 2010). The magnitude of the trophic enrichment within a food chain is vary variable in function of the species studied and of the tissue sampled. Generally, this increase is on the range of 2.5 to 5 ‰ from prey to predators

(DeNiro & Epstein 1981, Minagawa & Wada 1984). For marine mammals, the $\delta^{15}N$ trophic enrichment factor can be considered around 3.4 ‰ (Post 2002) and vary in function of the taxa. Therefore, the stable nitrogen isotope ($\delta^{15}N$) is a reliable tool to determine the trophic level position of an individual, if the $\delta^{15}N$ values of the baseline food web is known. In addition, it allows to explore the extend of the food web in which it belongs. For example, since modern plants are between 0 to 5‰, their direct consumers will have an average nitrogen isotopic value of 9‰. The use of stable isotopes in this study will enlighten complex ecological interactions of a wide variety of species in Madeira and investigate how individuals respond to environmental conditions. It will provide us knowledge on the diet habits of the two cetaceans and determine, if there is a detrimental effect of fisheries in the region.

3.2 Sampling and data collection

3.2.a *In the field*

Sampling of cetaceans

Thirty-three dermis samples of bottlenose dolphins and 23 dermis samples of short-finned pilot whales were collected in the waters of Madeira from the 3rd of November until the 20th of November 2017 and from the 16th of March until the 18th of April 2018. In addition, samples of their potential preys large pelagic fishes (tuna fish-like species) and small fishes (e.g. mackerels, sardines, bogues), zooplankton and particulate organic matter (POM) were also collected to analyse their carbon and nitrogen stable isotope contents. Biopsy processes are described in chapter 1. Only adults, not accompanied by calves were sampled to avoid possible nursing effect, because nursing adults can usually present higher $\delta^{15}N$ values) (Newsome *et al.* 2009, Riccialdelli *et al.* 2013).

Sampling of fishes, zooplankton and POM

The cetaceans' potential prey were collected between May 2017 and March 2018 and included: small fishes: (Sardines (*Sardinella aurita*), Atlantic chub mackerel (*Scomber colias*), blue jack mackerel (*Trachurus picturatus*), European pilchard (*Sardina pilchardus*), bogue (*boops boops*), Roudi escolar (*Promethichthys prometheus),* snipefish (*Macroramphosus gracilis*), and large pelagic fishes: skipjack tuna (*Katsuwonus pelamis),* Albacore Tuna (*Thunna alalonga*) and bigeye tuna (*Thunna obesus*)). After collection, samples were immediately frozen and kept at -20°C, until laboratory handling.

In this study, stable isotope values from squids, retrieved from literature values were also used 11.3 ± 0.3 and -18 ± 0.3 (Navaro *et al.* 2013).

Zooplankton and POM samples were collected in order to establish a baseline for the food web. Zooplankton were collected on a monthly basis from December 2017 to April 2018, using dragging net of 325 µm mesh size coupled with a flowmeter, during 10 mins at 1kt between 3 and 5 meters depth. At the end of the sampling, roughly 20 m3 of water were collected. For each sampling, one replicate of zooplankton and three replicates of POM were made.

3.2.b *In the lab*

Cetaceans:

For each biopsy samples, the skin was separated from the blubber and a quarter of the piece of skin was then used for stable isotope analyses. The skin was preferentially used in this study, as it has a slow turnover rate when compared to blood (Boecklen *et al.* 2011). Therefore, it provides long term feeding information (two to three months). Samples were stored in small glass tubes to avoid a risk of carbon contamination with plastic tubes. After separation, skin tissues were dried in an oven at 60 °C for roughly 24 hours, in order to render samples easy to powder afterward. Any warmer temperatures would have denatured protein and make substances volatile. Freezing and freeze drying are methods recognised to leave the carbon and nitrogen isotope values intact during their preservations (Bosley and Wainright *et al.* 1999). After drying, samples were grounded to a fine powder inside the tubes, thanks to a stainless steal spoon.

Fish species:

Muscle tissue from the dorsal area was collected from fish samples. Samples were stored in small glass tubes and then, dried in an oven at 60 °C for roughly 24 hours. Before analyses, samples were grounded to fine powder inside the tubes thanks to a stainless steal spoon.

Zooplankton and POM:

Zooplankton and POM were filtered on a a pre-combusted GF/F glass microfilter (pre calcinated in a Muffles at 500 C° for 12 hours) directly after their samplings. Replicate water samples (10 L each) for POM analyses were prefiltered on a 150 µm filter in order to remove zooplankton from the water. For December and January, an average of 20 m³ of water was filtered through GF/F for zooplankton analyses whereas from January until March, an average of 10 m³ of water was filtered through GF/F for the same purpose. One replicate of zooplankton was obtained each time. Filters for POM analysis were fumigated with concentrated HCl to remove inorganic carbonates, rinsed with deionized water, placed in a sterile Petri dish, and dried at 60 _C for 24 h (Lorrain et al.2003). Zooplankton samples were also dried an oven at 60 °C for roughly 24 hours. The date, geographical location, time and weather conditions were registered for each sampling event.

Stable isotope analysis:

Stable isotopes were determined using aThermo Scientific Delta V Advantage isotope ratio mass spectrometer (IRMS) via Conflo IV interface (MARE, Coimbra).

The analytical error, the mean standard deviation of replicate reference material, was ±0.1‰ for δ13C and δ15N.

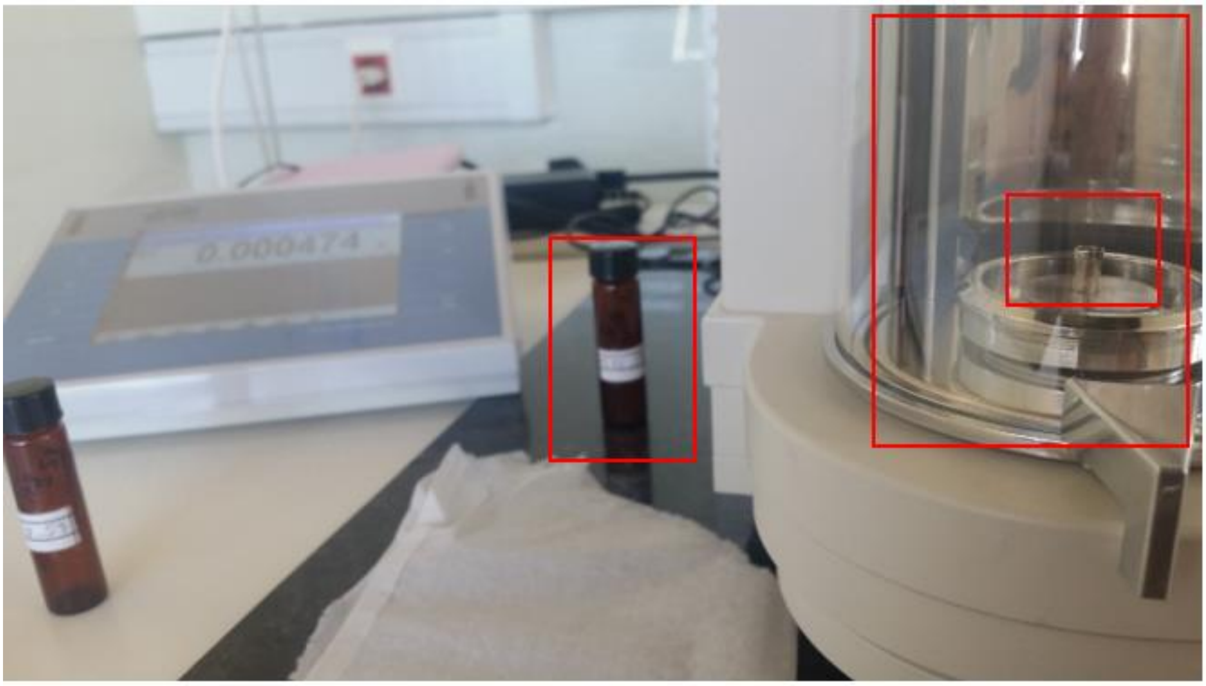

Fig 3.1 *Loading of the powdered samples in aluminium tins and weighting in a precision balance.*

Results were expressed in δ notation relative to PeeDee Belemnite and atmospheric N2 for $\delta^{13}C$ and $\delta^{15}N$, according to the equation below:

$$\delta \mathbf{X} = (\mathrm{Rsample}/\mathrm{Rstandard}-1)1000$$

- **X** is $\delta^{13}C$ or $\delta^{15}N$
- R is the isotope ratio 13C/12C or 15N/14N (Peterson and Fry 1987)
- Measurement errors are ±0.1 in $\delta^{13}C$ and ±0.1 ‰ in $\delta^{15}N$
- % carbon and nitrogen elemental composition of tissues obtained using the elemental analyser and used to calculate the sample C/N ratio

3.3 Statistical treatment

All graphics and statistical analyses were performed using the open source software R version 3.5.1. The influence of sampling season on the isotopic signature of short-finned pilot whales and bottlenose dolphins’ skin were analysed through a two samples t-test. When all required assumptions were met, an ANOVA was used to compare isotopic values of short-finned pilot whales and bottlenose dolphins’ skins with those of other biotas; otherwise, a Kruskal-Wallis test was used, followed by a pair-wise ranking with the Mann- Whitney-Wilcoxon test. A Kruskal Wallis test was carried out to compare the isotopic values of prey species, as function of the three different residency patterns of pilot whales. All univariate comparisons between 2 group means were significance tested using a two-sample t-test. Multigroup comparisons of stable isotopes were effectuated using a Tukey HSD test.

Data were analysed using linear models, after removal of statistical outliers and testing for normality (Shapiro-Wilk normality test) and homoscedasticity (Barlett´s test). For all tests, significance was assigned at p, 0.05.

Diet Through Bayesian Isotopic Mixing Model

The relative contribution of preys to the diet of short finned pilot whales and bottlenose dolphins were calculated using the Bayesian Isotope Mixing Model in the R package “SIAR” (Parnell *et al.* 2010, R Development Core Team 2013), after biplot inspection and identification of the most likely prey. Stable Isotopes Analysis in R (SIAR) uses the isotopic values of consumers and preys, and trophic enrichment factors (TEFs, Δ) to calculate the probability distribution of source contribution as percentages of total diet (Parnell *et al.* 2010). In this study, TEFs $\delta^{13}C$ = 1.01 ±0.3 ‰, and $\delta^{15}N$ = 1.57 ±0.52‰ for bottlenose dolphins (Gimenez *et al.* 2016) and TEFs $\delta^{13}C$ = 0.4 ±1.2‰ and $\delta^{15}N$ = 3.2 ±1‰ for short-finned pilot whales (Vander Zandem and Rasmussen 2001) were used. In the present study, it was not possible to get tissues from squids in the region of Madeira. However, this taxon is considered an essential to the diet of short-finned pilot whales. Consequently, stable isotopes from Atlantic squids previously studied (Navaro *et al.* 2013) were incorporated in the models, so that the proportion of potential preys could be predict as correctly as possible. Bayesian isotope mixing models, were run without lipid normalisation for predator species, as their C:N ratios were around 3.6, which is not extremely different from the 3.5 limit settled by Post (2007). Moreover, Gimenez (2017), affirmed that because lipid extraction had a relatively low effect on isotopes´ values and that normalization equations had a low explanatory power, it was more relevant to use bulk values when C:N ratios were in the range of 3.6 or less. Nonetheless, there was a positive relationship between C/N ratios and δ13C values for the two cetaceans, which indicates that the degree of enrichment in skin δ13C values were correlated with the percent lipids. The C:N ratio of most prey species were higher than 3.5. Therefore, δ13C values were normalized using an arithmetic correction based on C: N for all for deep-sea fish (Hoffman and Sutton2010) (Equation 1).

Equation 1:

$$\delta^{13}C_{bulk} = \delta^{13}C_{protein} \times (C{:}N_{protein}/C{:}N_{bulk}) + \delta^{13}C_{lipid} \times [(C{:}N_{bulk} - C{:}N_{protein})/C{:}N_{bulk}].$$

Ideally, $\delta^{13}C$ and $\delta^{15}N$ would have been analysed in separate sample aliquots, where $\delta^{13}C$ would have been analysed after chemical lipid extraction, as lipid tend to underestimate the $\delta^{13}C$ values in tissues, and $\delta^{15}N$ analysed without chemical lipid extraction. Indeed, lipid extraction is known to decrease $\delta^{15}N$ values in Balaenopteridae skins tissues (Ryan *et al.* 2012) or increase it for other cetaceans (Lesage *et al.* 2010). These procedures would have considerably increased the cost of the analyses but would have also provided the best estimates of prey contribution to predators´ diet. Moreover, in the present study, the samples were prevailed with dart biopsies, thus the amount of tissue was too small to duplicate samples for analysis.

Four mixing models were run in SIAR. One model analysed the contribution of each preys to bottlenose dolphins' diet, the three other models analysed the contribution of potential preys in the diet of visitor, resident and transient short-finned pilot whales. For each species, different TEFs values, based on literature were used. The one showing better fit for the stable isotope ratios determined, and producing the model with the lowest error, was chosen for the following analyses. For bottlenose dolphins the following TEFs were tested:

Models	**$\delta^{15}N$**	**$\delta^{13}C$**
Model 1, from Giménez *et al*. 2016	1.57 ± 0.52‰	1.01 ± 0.3‰
Model 2, from Vander Zandem and Rasmussen 2001	3.2 ± 1.0‰	0.4 ± 1.2‰

After comparison of the results, the model of Gimenez *et al* (2016) was the one producing better results. For short-finned pilot whales, the following TEFs were tested:

Models	**$\delta^{15}N$**	**$\delta^{13}C$**
Model 1, from Giménez *et al*. 2016	1.57 ± 0.52‰	1.01 ± 0.3‰
Model 2, from Vander Zandem and Rasmussen 2001	3.2 ± 1.0‰	0.4 ± 1.2‰
Model 3, Borrell *et al*. 2012	2.8 ± 0.3‰	1.3 ± 0.4‰

Model 2 was the one producing better estimates. Prey species were grouped into four prey categories for bottlenose dolphins (Small fish, Blue Jack mackerel, skipjack tunas and squids) and four for short-finned pilot whales (Small fish, blue jack mackerels, skipjack tunas and tuna). Small fishes were composed of the taxon: Atlantic mackerel, bogue and European pilchard, whereas tunas were composed of the taxon: albacore and bigeye in the respective models, as no significant isotopic differences ($p < 0.05$) were found between the taxon present in each group suggesting feeding preferences. Thus, each group was represented by a single set of isotopic values which corresponded to the means (and SD) of the individual source isotopic values (Philips *et al.* 2014). Albacore and bigeye tunas were not included in the diet of bottlenose dolphins, because they are very rarely consumed by the species, due to their large sizes. In addition, their nitrogen isotope values were higher than bottlenose dolphins, which could suggest that they feed on higher trophic level species than dolphins in

Madeira; or they rely more on benthic prey. Tuna tend to live in deeper waters than bottlenose dolphins and therefore it is expected that they rely more on the benthic food web.

Moreover, studies demonstrated that dolphins often coexist and collaborate with tuna species, as they depredate on same prey sources such as mackerels (Das *et al.* 2000, Scott *et al.* 2012). Skipjack could potentially be a part of bottlenose dolphins´ diet, since they are relatively smaller than the two other tuna species (Atuna *et al.* 2018) and share same ecological characteristics as mackerels. Tuna species were included in the diet of short-finned pilot whales, since they were observed depredating on tunas in longlines tunas' fisheries in the northwest Atlantic (Wapples and Read, Garrison 2007). Besides, there is an important tuna fishery activity in Madeira, which suggests that they could benefit from this activity (Gouveia 2017). The contributions were reported as mode values with 95% of credibility intervals (CI).

Assuming constant isotopic enrichment at a rate of 3.20‰ and 1.57‰ (the TEFs of short finned pilot whales and bottlenose dolphins, respectively) per trophic level, relative trophic levels for biota were calculated (Hobson and Welch 1992, Liu et al 2015): TL = (δ15N consumer - δ15N mean baseline)/ 3.20‰ or 1.57‰ + 2 (Hobson and Welch 1992).

Trophic partitioning among the two cetaceans

Two one-way ANOVA tests were utilised to test first the absolute differences in $\delta^{13}C$ and second the absolute differences in $\delta^{15}N$ among the two species. Then post-hoc Tukey honest significant difference (HSD) analyses were used to identify pairwise differences between species. The SIBER package (Stable Isotopes Bayesian Ellipses in R) was used to construct parameters of ellipses based on sampling points. SIBER allowed to determine potential ecological niches for the species of interest. The standard ellipse area (niche breadth) for each species was estimated based on 10,000 posterior draws and corrected for small sample size.

3.4 Results

The average values, standard deviation, minimum and maximum values of carbon and nitrogen stable isotopes of POM, zooplankton, small pelagic fishes, large pelagic fishes, bottlenose dolphin and pilot whales are presented in **Table 3.1.** C:N values were also reported.

Table 3.1 *$\delta^{13}C$, $\delta^{15}N$ and C:N ratio values (mean, standard deviation, range of values) for POM, zooplankton, small pelagic fish, large pelagic fish and cetaceans (bottlenose dolphin and short-finned pilot whales) sampled in Madeira.*

Species	**δ^{15}N ± SD**	**min/max**	**δ^{13}C ± SD**	**min/max**	**C: N ± SD**	**min/max**
POM	4.58 ± 0.6	3.5/5.4	-2.19 ± 0.8	- 23.2/-20.4	5.69 ± 0.07	4.83/ 7.20
Zooplankton	4.67 ± 0.8	4.1 /6.1	-20.5 ± 1.2	-2.19/ -19.2	5.82 ± 0.96	4.96/ 7.26
Small pelagic fish						
Slender snipefish *Mg*	6.5 ± 0.4	6.0/6.9	-19.5 ± 0.69	-20.3/-18.7	3.47 ± 0.25	3.23/3.82
Round sardinella *Sa*	7.05 ±	NA	-19.3± NA	NA		NA
Madeiran sardinella *Sm*	7.14 ± NA	NA	-19.9 ± NA	NA	3.64 ± NA	NA
European pilchard *Sp*	7.65 ± 0.4	7.0 /8.4	-20.9 ± 1.3	-23.2/-18.8	4.56 ± 1.1	3.0/6.7
Atlantic chub mackerel *Sc*	7.9 ± 0.6	6.9/9.2	-20.3 ± 1.3	-22.5/-18.7	4.1 ± 1.1	2.33/2.86
Bogue *Bb*	8.29 ± 0.6	NA	-19.5 ± 0.8	NA	3.47 ± 0.59	3.06/3.89
Blue jack mackerel *Tp*	9.02 ± 0.3	8.2/9.6	-19.2 ± 0.4	-20.0/-18.7	3.2 ± 0.11	3.1/3.5
Large pelagic fish						
Skipjack tuna *Kp*	9.7 ± 0.3	9.4/10.2	-18.7 ± 0.46	-19.3/-18.1	3.25 ± 0.14	3.23/3.82
Bigeye tuna *To*	11.0 ± 0.3	10.6/11.5	-18.5 ± 1.30	-21.1/-16.9	3.54 ± 0.60	3.15 /4.98
Albacore tuna *Ta*	11.1 ± 0.4	10.5/11.6	-18.8 ± 0.9	-20.7/-18.1	3.42 ± 0.39	3.14/3.49
Cephalopod						
Squids	11.3 ± 0.3		-18 ± 0.3		Based on Navaro *et al.* 2013	
Cetaceans						
Bottlenose dolphin	10.9 ± 0.7	9.2/11.5	-18.6 ± 0.4	-19.5/-17.7	3.6 ± 0.2	3.3 ± 4.1
Short-finned pilot whale	12.7 ± 0.5	1.19/14.0	-17.3 ± 0.6	-18.8/-15.8	3.6 ± 0.2	3.2 ± 4.2

Isotopic Composition in bottlenose dolphin and short-finned pilot whales

The skin samples taken from 33 bottlenose dolphins had mean values (±SD) of -18.6 ± 0.4 ‰ (range = -19.5 to -17.7‰) for δ^{13}C and 10.9 ± 0.7‰ (range = 9.2 to 11.5‰) for δ^{15}N (**Table 3.2**). While The skin samples taken from 23 short-finned pilot whales had mean values (±SD) of -17.3 ± 0.6 ‰ (range = -18.8 to -15.8‰) for δ^{13}C and 12.7 ± 0.5‰ (range = 1.19 to 14.0‰) for δ^{15}N. The season isotopic values showed no significant differences in δ^{15}N (Two samples t-test: t, -1.66; *p*, 0.12) and in δ^{13}C (Two samples t-test: t2.12; *p*, 0.06) between autumn (November sampling) and spring (March and April sampling) for short-finned pilot whales but showed significant differences in δ^{15}N (Two samples t-test: t2.4; p, 0.025) and δ^{13}C (Two samples t-test: t2.14; p, 0.04) for bottlenose dolphins **(Table 3.2** and **Fig 3.2)**

Table 3.2 *δ^{13}C, δ^{15}N and C:N ratio values (mean, standard deviation, range of values) in skin samples of short-finned pilot whales and bottlenose dolphins collected during 2017 and 2018 in Madeira in function of the season of collection and results of statistical analysis.*

Season	BOTTLENOSE DOLPHINS (n= 33)			S-F PILOT WHALES (n= 23)		
	n	**δ^{15}N ± SD**	**δ^{13}C ± SD**	**n**	**δ^{15}N ± SD**	**δ^{13}C ± SD**
Autumn (November)	14	9.8 ± 0.64	-18.4 ± 0.36	14	12.8 ± 0.64	-17.1 ± 0.52
Spring (March -April)	19	12.5 ± 0.63	-18.7 ± 0.39	9	12.5 ± 0.63	-17.7 ± 0.54
Comparison	**δ^{15}N**		**δ^{13}C**	**δ^{15}N**		**δ^{13}C**
Autumn vs Spring	t-test: t 2.4; p, 0.025		t-test: t 2.14; p, 0.04	t-test: t -1.66; p, 0.12		t-test: t 2.12; p, 0.06

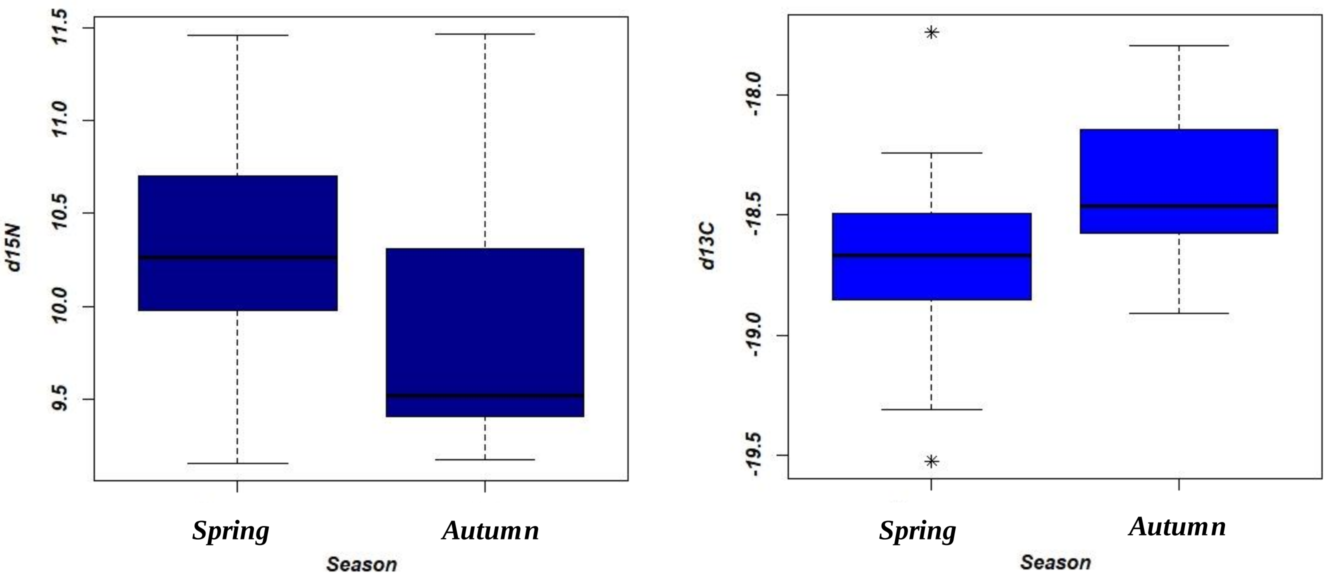

Fig 3.2 *Nitrogen (left side) and carbon (right side) stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$) of bottlenose dolphins in Madeira Archipelago during spring and autumn seasons.*

Visitor short-finned pilot whales had somewhat higher $\delta^{15}N$ than resident and transient (visitor: 12.9, SD= 0.5 ‰ $\delta^{15}N$, *n* = 9; resident: 12.8, SD= 0.27 ‰ $\delta^{15}N$, *n* = 6; transient: 12.4, SD= 0.5 ‰ $\delta^{15}N$, *n* = 6; **Fig 3.3).** However, these differences were not significant (Kruskal-Wallis and posthoc test: p, 0.44 (visitor vs resident); p, 0.06 (visitor vs transient); p, 0.06 (transient vs resident); visitors: 12.9, SD= 0.5 ‰ $\delta^{15}N$, *n* = 9; resident: 12.8, SD= 0.27 ‰ $\delta^{15}N$, *n* = 6; transient: 12.4, SD=0.5 ‰ $\delta^{15}N$, *n* = 6, **Table 3.3; Fig 3.3).** There were no significant differences either for $\delta^{13}C$ values between visitors, residents and transients (Kruskal wallis and posthoc test: p, 0.32; p, 0.12; p, 0.07; visitors: –17.3, SD= 0.4‰ $\delta^{13}C$, *n* = 9; resident: –17.3, SD= 0.38‰ $\delta^{13}C$, *n* = 6; transient: –17.5, SD= 0.9‰ $\delta^{13}C$, *n* = 6. **Table 3.3; Fig 3.3).**

Table 3.3 *δ13C, δ^{15}N and C:N ratio values (mean, standard deviation, coefficient of variation) in skin samples of short-finned pilot whales collected during 2017 and 2018 in Madeira in function of the residency pattern of the whales and results of statistical analysis.*

Residency pattern	SHORT-FINNED PILOT WHALES (n= 23)		
	n	**δ^{15}N ± SD**	**δ^{13}C ± SD**
Visitor	9	12.9 ± 0.5	-17.3 ± 0.4
Resident	6	12.8 ± 0.27	-17.3 ± 0.38
Transient	6	12.4 ± 0.5	-17.5 ± 0.9

Comparison	**δ^{15}N**	**δ^{13}C**
Resident Vs Visitor	KW + Dunn test: p, 0.44	KW + Dunn test: p, 0.32
Visitor Vs Transient	KW + Dunn test: p, 0.06	KW + Dunn test: p, 0.12
Resident Vs Transient	KW + Dunn test: p, 0.06	KW + Dunn test: p, 0.07

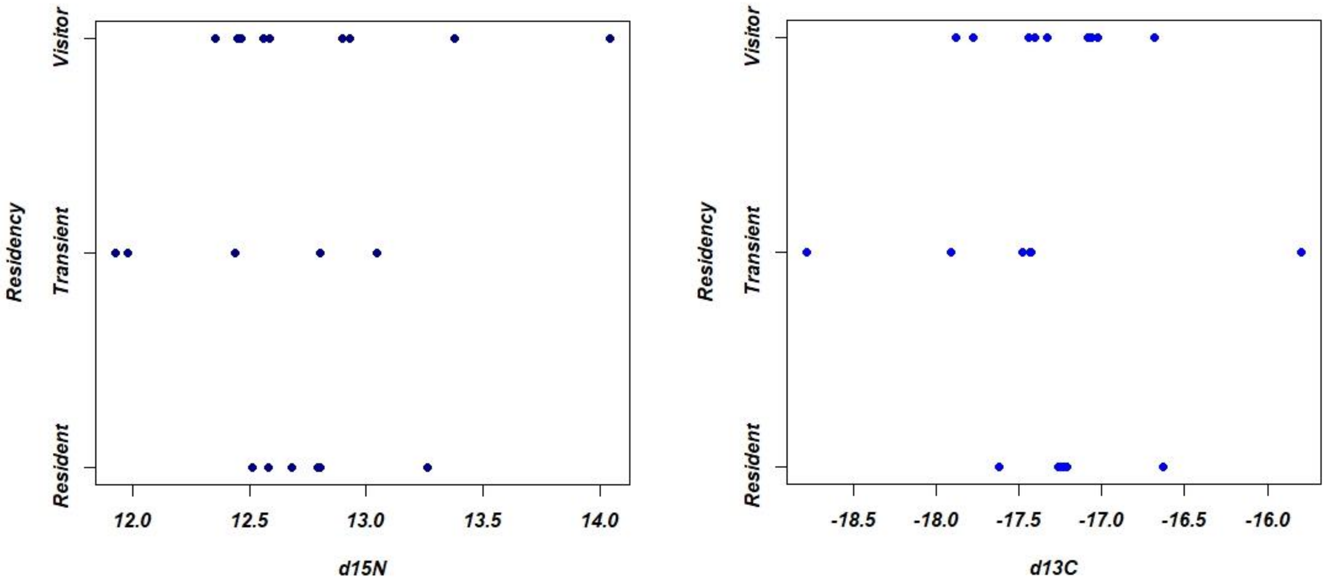

Fig 3.3 *Nitrogen (left side) and carbon (right side) stable isotope values (δ^{15}N and δ^{13}C) of short-finned pilot whales biopsied in Madeira archipelago segregated in function of their residency patterns (resident, transient and visitor).*

Significant differences in δ^{15}N (Wilcoxon test: W, 693; p, 8.3e-10) and δ^{13}C (Two sample t-test: t, 8.6; p, 7.2 e-10) were found between bottlenose dolphins (n=33) and short-finned pilot whales (n= 21) for the three months sampled. Pilot whales had higherδ^{15}Nvalues (µ= 12.7, SD=0.5) and δ^{13}C values (µ= -17.3, SD= 0.6) than bottlenose dolphins (µ= 10.9, SD= 0.7 and µ= -18.6, SD= 0.4; **Fig 3.4)**

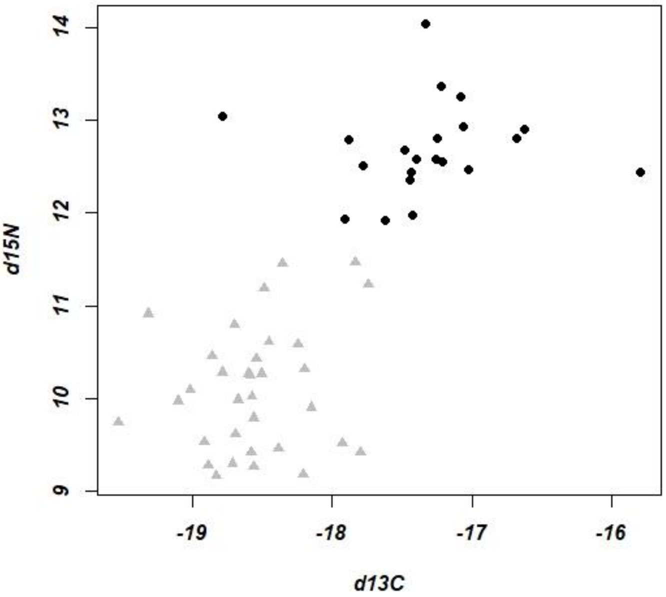

Fig 3.4 *Nitrogen and carbon stable isotope values (δ^{15}N and δ^{13}C) of short-finned pilot whales (black dots) and bottlenose dolphins (grey triangle) biopsied in Madeira archipelago*

There was a significant moderate positive correlation between δ^{15}N and δ^{13}C values for short finned pilot whales (Pearson´s correlation test: t,2.5; p, 0.02; cor= 0.5; **Fig 3.4**), which indicates that when hunting on more oceanic or deep living species, the trophic level of the prey species are higher. This relationship was not significant for bottlenose dolphins (Pearson´s correlation test: t,1.1; p, 0.29; cor= 0. 19; **Fig 3.4**), which indicates that wherever bottlenose dolphins depredate, the cetacean will focus on feeding on similar trophic level prey species.

Isotopic Composition of Preys related to cetaceans

Significant differences were found among prey species for both δ^{15}N and δ^{13}C ($p< 0.05$).

The stable isotope signatures of the potential prey items ranged between −23.2 ‰ and −18.1‰ for δ^{13}C and 3.5 ‰ to 11.6 ‰ for δ^{15}N **(Table 3.1)**. Specifically, big pelagic fishes (bigeye tuna, albacore tuna, skipjack tuna), and small pelagic fishes (bogue and blue mackerel) had significantly (Tukey test: $p < 0.05$; **Table 3.1** and **Fig 3.5)** enriched nitrogen isotopic values in comparison to other small pelagic fishes (European and Atlantic mackerel), while snipefish, Madeiran and round sardinella had even smaller nitrogen isotopic values, significantly (Tukey test: $p < 0.05$; **Table 3.1** and **Fig 3.5)** different from large pelagic fishes and other small pelagic fishes. For δ^{15}N values, albacore tunas had the highest values (11.1 ‰), while POM the lowest (4.58 ‰). Big pelagic fishes had significantly higher δ^{13}C values compared to the other pelagic fishes (Tukey test: $p < 0.05$). For δ^{13}C values, POM followed by zooplanktons had the lowest values (-2.19 and -20.5 ‰, respectively), while albacore tunas the highest (-18.8 ‰) (**Table 3.1** and **Fig 3.5**). The skin of short-finned pilot whales (n = 21) had significantly (Kruskal–Wallis and Dunn tests: $P < 0.001$) higher δ^{15}N values than muscle tissues of potential prey species except for albacore and bigeye tuna, which did not evidence a significant (Kruskal–Wallis and Dunn tests: p, 0.11; p, 0.11) difference. The δ^{13}C values of short-finned pilot whales were all significantly higher than the δ^{13}C values of potential prey species (Kruskal–Wallis and Dunn tests: $P < 0.05$). The skins of bottlenose dolphins (n= 33) had significantly (Kruskal–Wallis and Dunn tests: $P < 0.001$) higher δ^{15}N values than muscle tissues of potential prey species except for albacore, bigeye and skipjack tunas as well as bogue which did not express significant (Kruskal–Wallis tests and Dunn tests: p, 0.1; p, 0.12; p, 0.34; p, 0.06) differences. Strikingly, bottlenose dolphins had an average δ^{15}N value inferior to bigeye and albacore Tuna (10.9; 11.0 and 11.1, respectively; **Table 3.1** and **Fig 3.5**). When comparing the δ^{13}C values of bottlenose dolphins to their potential preys, bottlenose dolphins had significantly (Kruskal–Wallis and Dunn tests: $p <0.05$) higher values than snipefish, European pilchard, Atlantic and blue mackerels. Albacore tunas, bigeye tunas, skipjack tunas, bogue, Madeiran and round sardinella did not have significant (Kruskal–Wallis tests Dunn tests: p, 0.38; p, 0.38; p, 0.33; p, 0.09; p, 0.28; p, 0.17, respectively) differences in their δ^{13}C values. In the present study, POM, Atlantic and blue mackerels were tested on inter-season variations. They were the three only species which had enough data for both seasons to be analysed. There were no significant (Two samples t-test: t, 19; p, 0.09; t, 0.64; p, 0.53; t, 1.8, p, 0.13, respectively) inter-season variations in δ^{15}N and no significant (Two samples t-test: t, 0.55, p, 0.60; Wilcoxon rank sum test: W, 53, p, 0.27, two sample t-test: t, 0.14, p, 0.90) inter-season variations in δ^{13}C within species

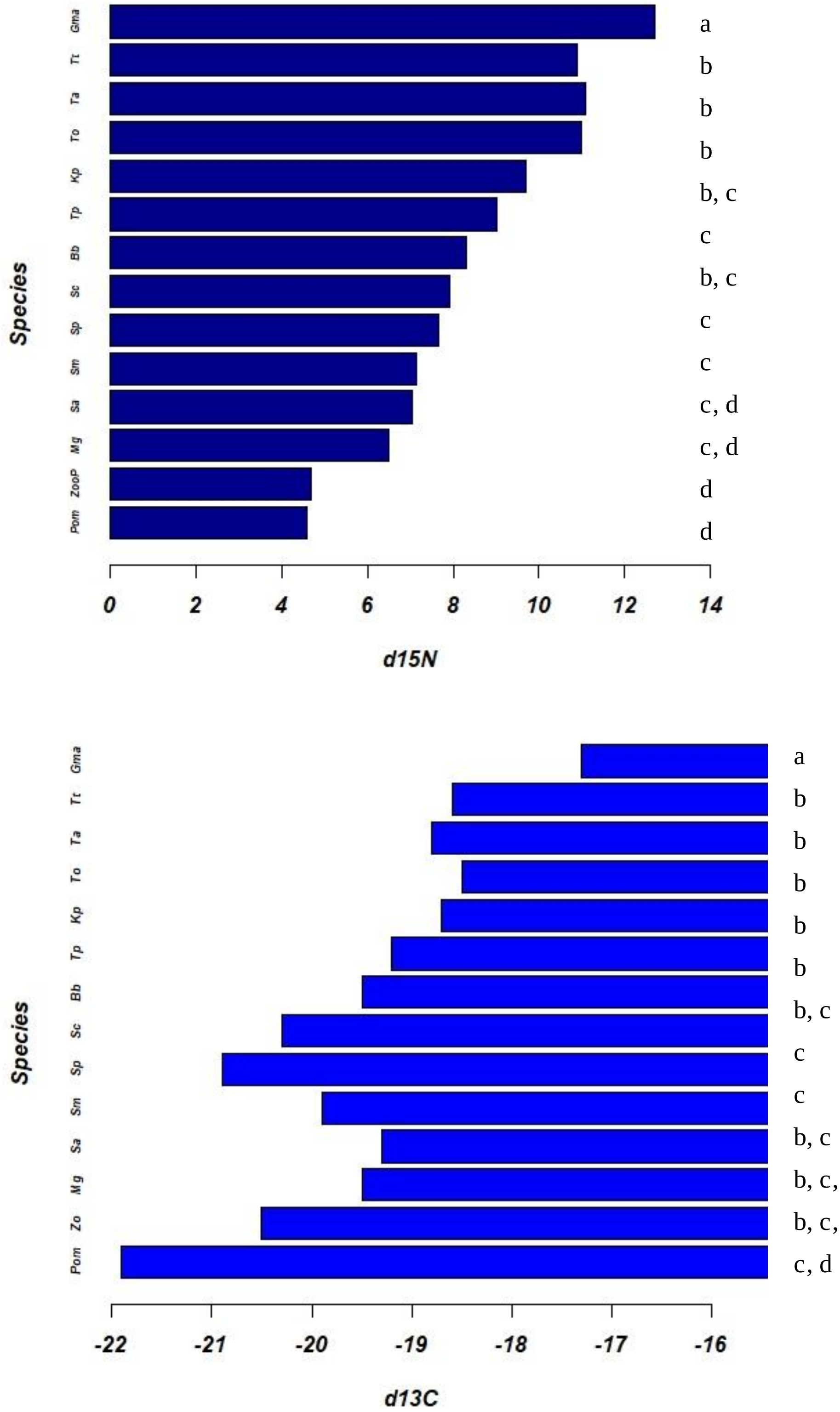

Fig 2.5 ***a)*** *δ^{15}N values in bottlenose dolphin and short-finned pilot whale skin tissue and in their potential preys muscles tissue from Madeira.* ***b)*** *δ13C values in bottlenose dolphin and short-finned pilot whale skin tissue and in their potential preys' muscles tissue from Madeira. Individuals with the same letters did not significantly differ, $p > 0.05$.*

Zo*= Zooplankton,* ***Mg****= snipefish,* ***Sa****= round sardinella,* ***Sm****= Madeiran sardinella,* ***Sp****= European pilchard,* ***Sc****= Atlantic chub mackerel,* ***Bb****= bogue,* ***Tp****= Blue jack mackerel,* ***Kp****= skipjack tuna,* ***To****= bigeye tuna,* ***Ta****= albacore tuna,* ***Tt****= bottlenose dolphin,* ***Gma****= short-finned pilot whales.*

Bottlenose dolphins and short-finned pilot whales Diet

Short-finned pilot whales, with an average $\delta^{15}N$ of 12.7 (range 11.9-14) had the highest value among the cetaceans, with bottlenose dolphins having an average of 10.9 (range 9.2–11.5). Preys selected for each consumer species were based on the results obtained from the biplots relating $\delta^{15}N$ and $\delta^{13}C$ (**Fig 3.6 and Fig 3.7**) and from literature. The biplot generated for bottlenose dolphins, showed that the consumer isotopic values fell within the range of the food source isotopic values. The plot showed that, albacore and bigeye tunas had very close stable isotope values and therefore, could be grouped as a single source in the mixing model. Zooplankton, snipefish, round and Madeiran sardinella diverged significantly in their isotopic values with bottlenose dolphins and as seen in the plot, their isotopic values seem to be too divergent to be taken into consideration in the mixing model. Blue jack mackerels and skipjack tunas seemed to be their own group in the model. Moreover, blue jack mackerels are known to contribute greatly to the diet of bottlenose dolphin and therefore, they were considered individually.

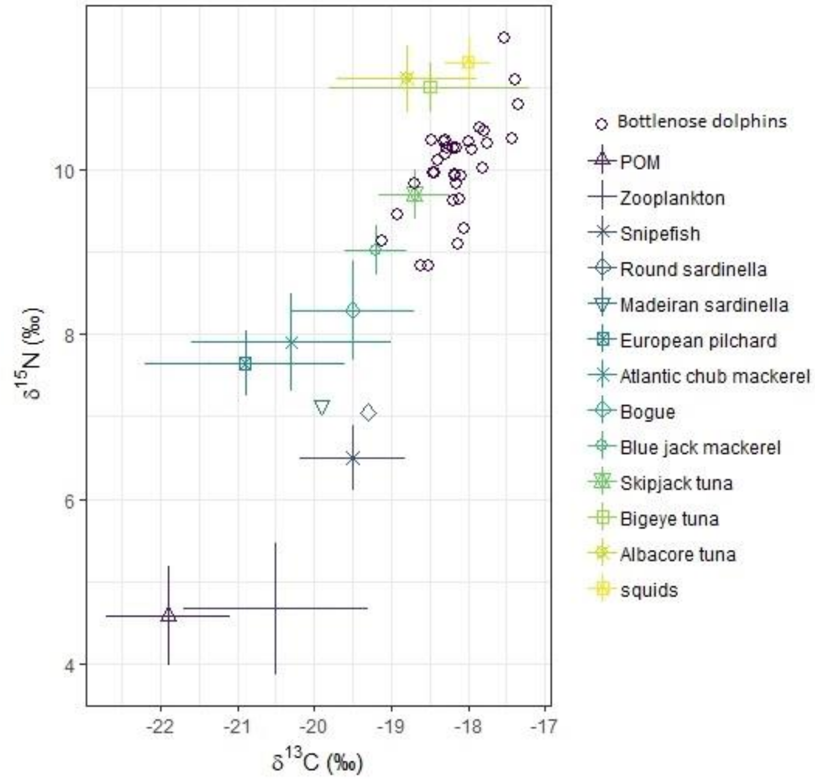

Fig 3.6 *Biplot of stable isotopic signatures for consumers (bottlenose dolphins) and sources from Madeira without the TEFs applied. This biplot was created using Bayesian mixing model SIAR (Parnell et al. 2010). Error bars represent 95% confidence intervals.*

The isotope mixing models showed that the contributions of small fishes (Bogue, Atlantic mackerel and European pilchard), blue jack mackerels, skipjack tunas, tunas (Albacore and bigeye) and squids varied greatly between short-finned pilot whales and bottlenose dolphins. From the Bayesian mixing model generated for bottlenose dolphins, small fishes, blue jack mackerels, skipjack tunas and squids were found to contribute from 33.8% to 76.9%, 0.2% to 42.2%, 0% to19.3%, and 5.6% to 22.8% to the diet of the species, based on 95% Confidence Interval. There was a difference in small fish and blue jack mackerels´ contribution to the diet between autumn and spring, which was not observed for other prey species. Pelagic and demersal fishes seemed to be the main prey sources contributing to the diet of bottlenose dolphins as it was observed in other studies (Walker *et al.* 1999, Kiszka *et al.* 2014). In autumn, the smallest estimate of small fish contribution to the diet of bottlenose dolphins was higher (33.8%) than the mode contribution of other potential sources, (Blue jack mackerels= 2.19% ± 95%, skipjack= 8.2% ± 95%, and squids= 13.7% ± 95%) (**Table 3.4** and **Fig 3.8**). In spring, the mode contribution of small fishes and blue jack mackerels were equal (39.5% ± 95% and 39.2% ± 95%, respectively), whereas other prey species kept the same contributions as autumn (**Table 3.4** and **Fig 3.8**).

The biplot for short-finned pilot whales showed that the consumer isotopic values did not fall within the range of the food source isotopic values (**Fig 3.7**). This data implies that other food sources should be included in the mixing model, or that the TEFs used for this species were not adequate. Group of sources were organised the same way as for bottlenose dolphins.

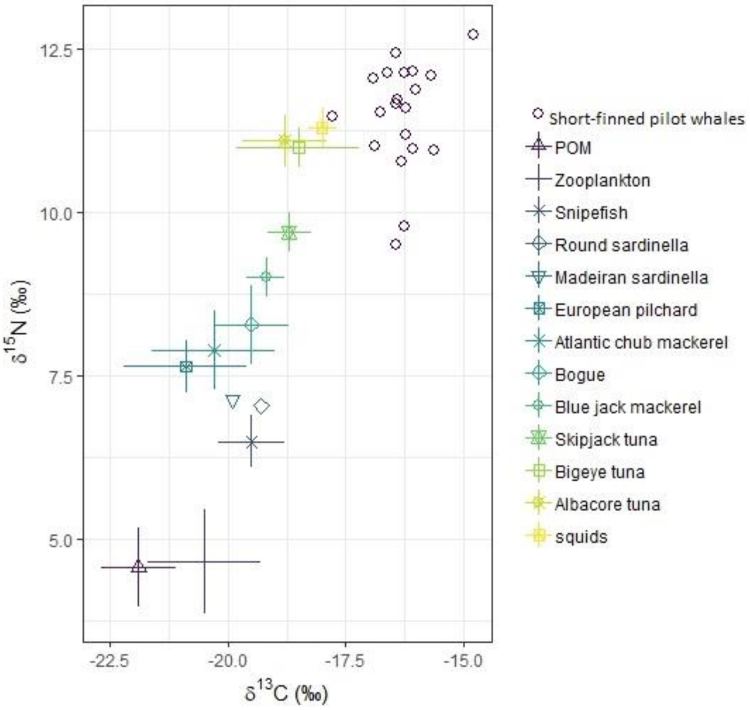

Fig 3.7 *Biplot of stable isotopic signatures for consumers (Short-finned pilot whales) and sources from Madeira without the TEFs applied. This biplot was created using Bayesian mixing model SIAR (Parnell et al. 2010). Error bars represent 95% confidence intervals.*

From the Bayesian mixing model generated for short-finned pilot whales, small fish (Atlantic mackerel, bogue, European pilchard), blue Jack mackerels, skipjack tunas and tunas (albacore, bigeye) were found to contribute from 0.4% to 39%, 5.3% to 51.5%, 8.5% to 53.5% and 3.9% to 39.7%, based on 95% confidence interval **(Table 3.4)**. Although, there were non-significant (Kruskal-wallis and posthoc test: p, 0.44; p, 0.06; p, 0.06; **Table 3.3**) differences between the stable isotope values of the 3 residency patterns of pilot whales, the model revealed different feeding habits between them. On overall, transient individuals were hypothesised to feed on smaller trophic level preys compared to the 2 other residency patterns (**Table 3.4 and Fig 3.9**). Small fishes and blue jack mackerels contributed the most in their diet (30.4% ± 95%, and 28.2% ± 95%, respectively). Squids were initially included in the model, bus as their contribution in the diet of pilot whales was almost negligible, we decided to exclude it. Visitor and resident individuals had lots of similarities in their diet composition. Skipjack tunas were hypothesized to have the highest contribution (32.7% ± 95%, and 31.6% ± 95%, respectively) in their diets, followed by blue jack mackerels (30.1% ± 95%, and 29.6% ± 95%, respectively) and then tuna species (23.8% ± 95%, 22.9% ± 95%, respectively), small fishes had a slight contribution to the diet of visitors and residents (13.4% ± 95%, 15.9% ± 95%, respectively) (**Table 3.4 and Fig 3.9**).

Table 2.4 *Isotopic mixing models´ results based on* $\delta^{15}N$ *and* $\delta^{13}C$ *values for bottlenose dolphins and for short-finned pilot whales in function of their residency patterns according to SIAR;* $\delta^{15}N$ *TEF of 1.57 ± 0.52‰ and* $\delta^{13}C$ *TEF of 1.01 ± 0.3‰ for bottlenose dolphins based on Gimenez et al (2016).* $\delta^{15}N$ *TEF of 3.2 ± 1.0 ‰ and* $\delta^{13}C$ *TEF of 0.4 ± 1.2‰ based on* Vander Zandem and Rasmussen (2001).
(Small fish = Atlantic mackerel, bogue, european pilchard, Tuna= Albacore and bigeye). Round and Madeiran sardinella as well as bogue could not be used in the mixing model as the sample sizes were too small (minimum of 5).

Source	BOTTLENOSE DOLPHINS					
	Autumn			**Spring**		
	Low 95%	High 95%	Mode	Low 95%	High 95%	Mode
Small fish	33.8%	76.9%	56.1%	24.9%	54.4%	39.5%
Blue Jack mackerel	0.2%	42.2%	2.19%	18.4%	58.7%	39.2%
Skipjack	0%	19.3%	8.2%	0%	14.5%	5.5%
Squids	5.6%	22.8%	13.7%	10.5%	21.3%	15.7%
SD .ϱ 13C	0.000	0.000	0.000	0.000	0.000	0.000
SD .ϱ 15N	0.000	0.000	0.000	0.000	0.000	0.000

	SHORT-FINNED PILOT WHALES								
	Resident			**Transient**			**Visitor**		
	Low 95%	High 95%	Mode	Low 95%	High 95%	Mode	Low 95%	High 95%	Mode
Small fish	0.4%	30.9%	15.9%	10.9%	49.2%	30.4%	0.2%	26.5%	13.4%
Blue Jack mackerel	5.3%	51.5%	29.6%	2.3%	49.9%	28.2%	7.1%	51.5%	30.1%
Skipjack tuna	8.5%	53.5%	31.6%	1.4%	44.7%	24.8%	10.6%	53.8%	32.7%
Tuna	3.9%	39.7%	22.9%	0.1%	32.8%	16.6%	6.6%	39.8%	23.8%
SD .ϱ 13C	0.000	0.79	0.30	0.000	1.17	0.43	0.000	0.80	0.32
SD .ϱ 15N	0.000	1.21	0.45	0.000	1.38	0.50	0.000	0.81	0.31

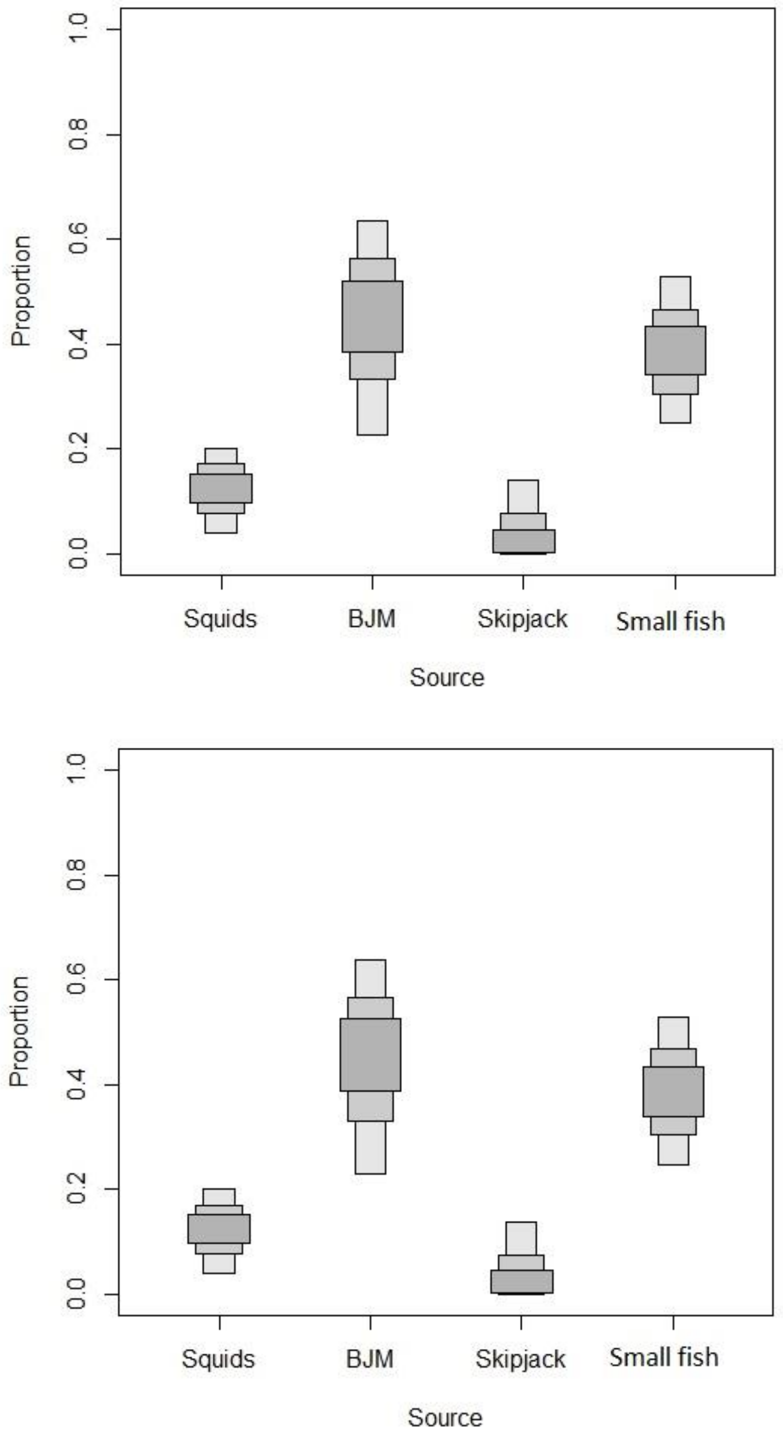

Fig 3.8 *Feasible composition of the diet of bottlenose dolphins in Madeira estimated with SIAR mixing model*
Median 50%, 75% and 95% credibility intervals (respectively dark grey, intermediate grey and light boxes). (Small fish = Atlantic mackerel, bogue, European pilchard, BJM= Blue Jack Mackerel)
Top= autumn, bottom= spring

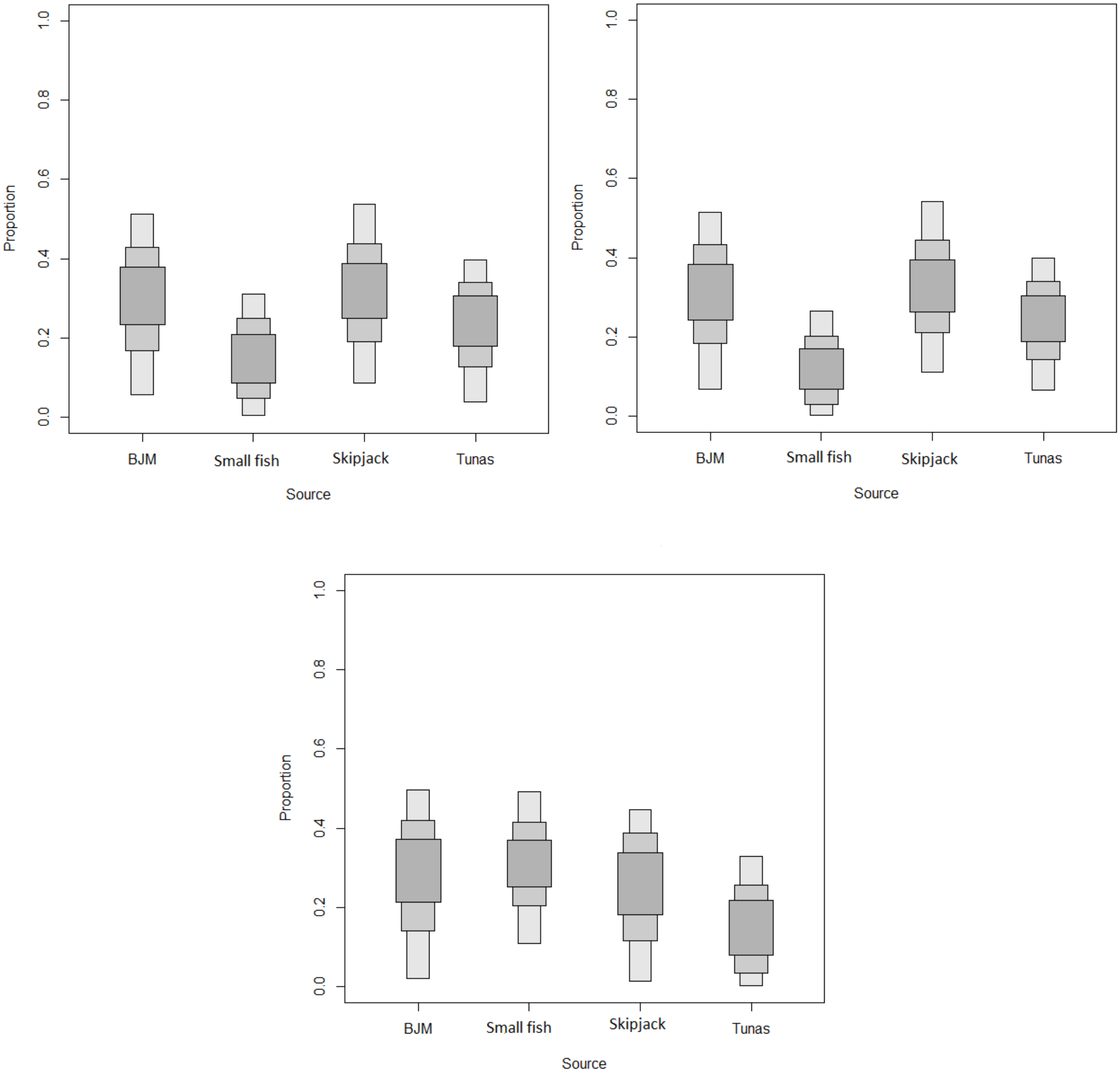

Fig 2.9 *Feasible composition of the diet of short-finned pilot whales in Madeira estimated with SIAR mixing model*
Median 50%, 75% and 95% credibility intervals (respectively light grey, intermediate grey and dark grey boxes). (Small fish = Atlantic mackerel, bogue, European pilchard, BJM= Blue Jack Mackerel, Tunas= albacore and bigeye tuna)
Top left= Visitor short-finned pilot whales, top right= Resident short-finned pilot whales, bottom= Transient short-finned pilot whales.

Trophic level determination of the species analysed in Madeira

The following equation was used to estimate trophic levels (TL):

TL = (δ^{15}Nconsumer - δ^{15}Nmean baseline)/ 3.2 + 2, where 3.2 is assumed to be the δ^{15}N trophic fractionation factor of the food web, from the mean values of Vander Zandem and Rasmussen, (2001). δ^{15}N mean baseline was the mean of the isotopic values of Zooplankton. δ^{13}C values helped to determine the origin of food source (Vander Zanden and Vadeboncoeur 2002, Sherwood and Rose 2005). 15 species were analysed in the present study and POM was identified as the only primary producer.

Zooplankton, slender snipefish, round sardinella, Madeiran sardinella and European pilchards were the primary consumers of the food chain and stood at the lowest level (range, 2, 2.57, 2.74, 2.77, 2.93, respectively). They were followed up by the secondary consumers Atlantic chub mackerel, bogue, blue jack mackerel, skipjack tuna, bigeye tuna and bottlenose dolphins at the third level of the food chain (range, 3, 3.13, 3.36, 3.57, 3.98, 3.9). Finally, albacore tunas, squids and short-finned pilot whales (range, 4, 4.1, 4.5) stood at the highest level of the food chain which classifies the species as top predators (**Table 2.5, Fig 2.10**). Short-finned pilot whales had the highest trophic level (TL = 4.5). Therefore, the maximum length of the food web in this study was 4.5 (**Table 2.5**).

These results are surprising, because sardinellas and European pilchards are at the same trophic level as zooplankton. Those largest species are expected to be at higher trophic level due to the fact that they do not feed on phytoplankton but tend to feed on small fish species and crustaceans.

Table 2.5 *Diet and trophic level of fish species analysed in Madeira Archipelago.*

Species	Diet	Trophic level (TL) TEF= 3.2 ‰	Ref
POM	-	-	
Zooplankton	POM/Phytoplankton	2	
Slender snipefish	Small invertebrate/ zooplankton	2.57	Lopez *et al.* 2006
Round sardinella	Crustaceans/Phytoplankton/ fish larvae	2.74	Tsikliras *et al.* 2005
Madeiran sardinella	Crustaceans/Phytoplankton/ fish larvae	2.77	Tsikliras *et al.* 2005
European pilchard	Decapod/Copepod	2.93	Costalago *et al.* 2014
Atlantic chub mackerel	Zooplankton/copepod/small fish	3	Castro2010
Bogue	Crustacea/seagrass/mollusc	3.13	El-Maremie and El-Mor2015
Blue jack mackerel	Zooplankton/copepod/small fish	3.36	Castro2010
Skipjack tuna	Crustacea/Fish/mollusc	3.57	Matsumoto and skillman 1975
Bigeye tuna	Mullet/small mackerel/sardine/ squid/ crustaceans	3.98	Junior *et al.* 2012
Albacore tuna	fish/crustacea / cephalopod mainly	4	Consoli *et al.* 2008
Bottlenose dolphin	Fish/bottom-dwelling invertebrate/ squid	3.9	Kiszka *et al.* 2014
Squids	Small fish/ crabs/ shrimp/squids	4.1	Merten *et al.* 2017
Short-finned pilot whale	Fish/octopus/squid (mainly)	4.5	Monteiro *et al.* 2015

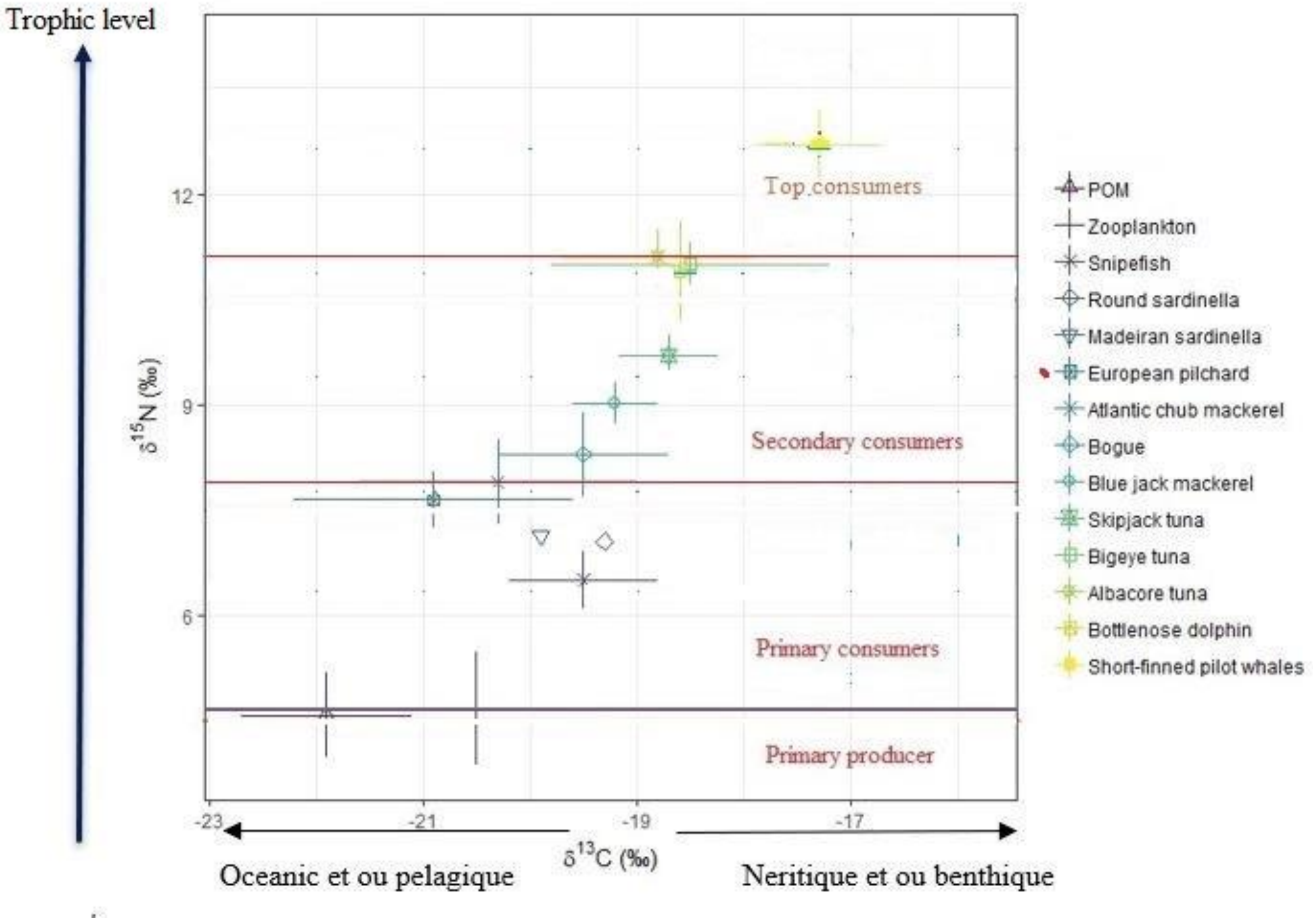

Fig 2.10 δ^{13}C *(%) and* δ^{15}N *(%) signatures (mean +/- SD) for bottlenose dolphins (n = 33), pilot whales (n=21) and their potential prey items in Madeira and their* trophic level positions.
In red, trophic level position estimated from TEF= 3.2 ‰; In blue trophic level position estimated from TEF= 1.57 ‰

Ecological niches and food partitioning

There were very small overlap in isotopic niche between short-finned pilot whales and other species sampled in Madeira (**Fig 2.11**). However, there were large overlaps in isotopic niches between bottlenose dolphins, the tuna species and blue jack mackerel **(Fig 2.11)**.

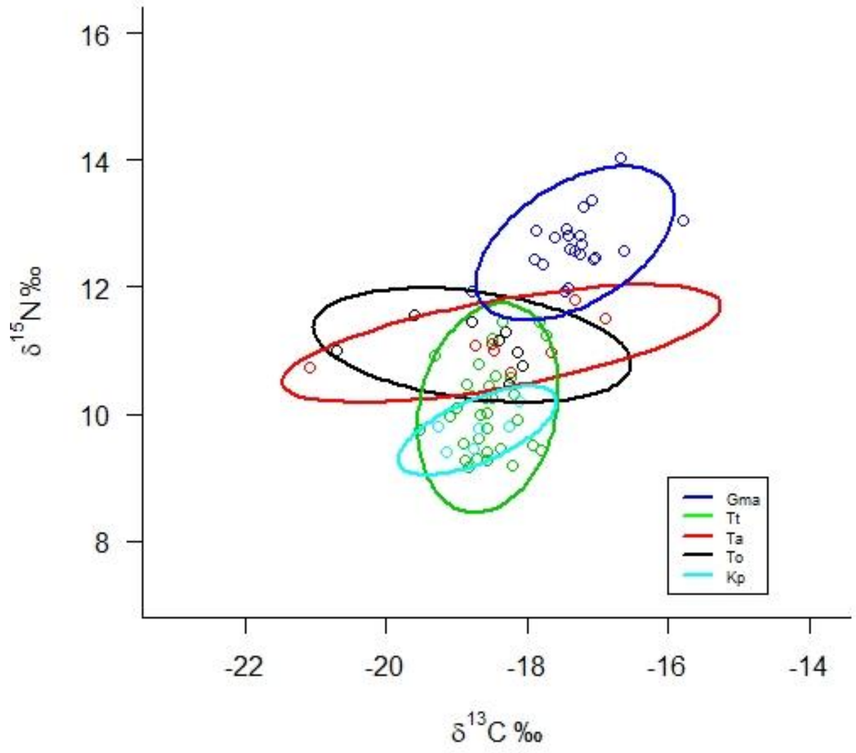

Fig 2.11 *Bi-plot of* $\delta^{15}N$ *and* $\delta^{13}C$ *values with Bayesian ellipses overlaid for co-occuring organisms in Madeira archipelago.*
Kp*= skipjack tuna,* ***To****= bigeye tuna,* ***Ta****= albacore tuna,* ***Tt****= bottlenose dolphin,* ***Gma****= short-finned pilot whales.*

The carbon and nitrogen stable isotope signatures of short-finned pilot whales and bottlenose dolphins skin tissues varied significantly between the two species. The $\delta^{15}N$ and $\delta^{13}C$ signatures of short finned pilot whales were significantly higher when compared with bottlenose dolphins (**Table 2.1** and **Fig 2.5**), which suggests that the species occupied a higher trophic level than bottlenose dolphin, feed on different preys and might not share the space, pilot whales relying further on seaward prey resources. The $\delta^{15}N$ enrichment was set at 2.3 with each trophic level in this study. Considering this parameter, the difference indicate that short-finned pilot whales are one third of a trophic level higher than bottlenose dolphins.

Between the two-cetacean species, there were no overlap in autumn, and a very slight overlap in spring (**Fig 2.12**). These results are in accordance with the significant differences observed for $\delta^{15}N$ and $\delta^{13}C$. The spatial niche segregation among the two species might diminish direct competition for habitat or prey resources and benefit them in a point of view of energy. From the SIBER plot, pilot whales seemed to feed on a smaller range of species than dolphins, because of smaller diversification in their δ^{15}N values. This pattern is accentuated in spring. In addition, both species had wider δ^{13}C values in spring than in autumn, which hypothesises that they travel more during this period of time.

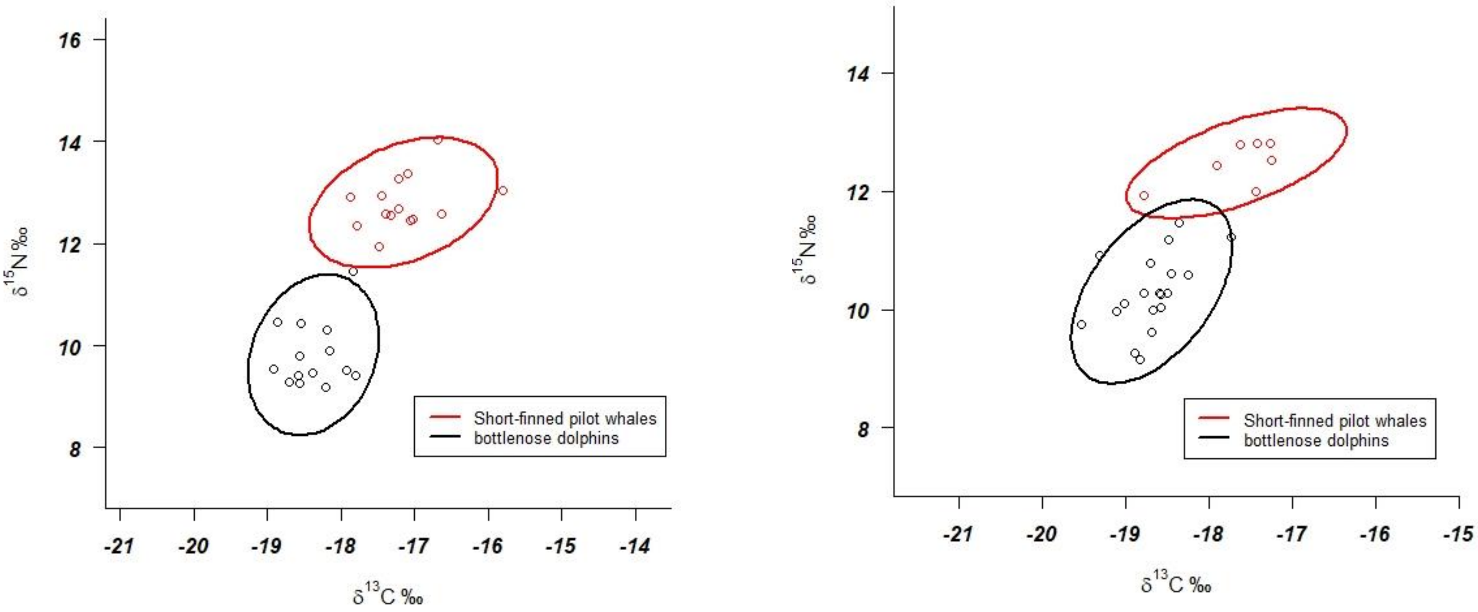

Fig 2.12 *Bi-plot of* δ^{15}*N and* δ^{13}C *values with Bayesian ellipses overlaid for bottlenose dolphins and pilot whales in Madeira archipelago in autumn (left) and spring (right).*

Bottlenose dolphins showed significant inter-season variations in their stable isotope values, although there was a big overlap between their isotopic niche in autumn and spring. In spring, nitrogen and carbon isotope values were more enriched than in spring (**Fig 2.13**). Pilot whales did not have fluctuations between seasons. Consequently, bottlenose dolphin had a wider isotopic niche than short finned pilot whale.

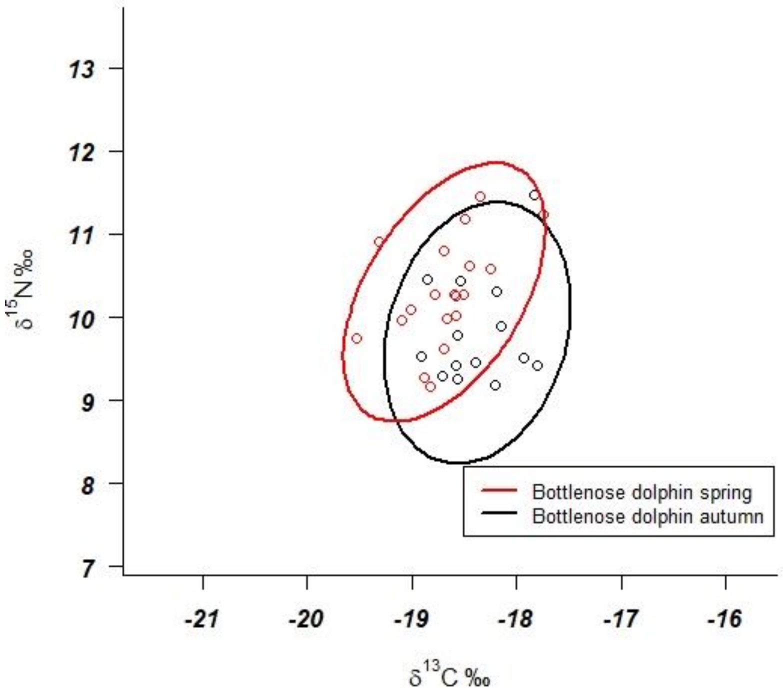

Fig 2.13 *Bi-plot of* δ^{15}*N and* δ^{13}C *values with Bayesian ellipses overlaid for bottlenose dolphins in Madeira archipelago in autumn and spring.*

Animals classified as resident showed very small variations in both δ^{15}N and δ^{13}C compared to animals classified as visitor or transient (**Fig 2.14**). These results, support a very specialised diet, based on high trophic level species inhabiting offshore/benthic waters. Visitor whales had less variations in their δ^{13}C values than transients (**Fig 2.14**), meaning that they tend to eat at same depth or same places, when compared to visitors. However, their δ^{15}N values were very variable, which suggests that they have a less specialised diet composed of various trophic levels species. Transient individuals as for visitors had a wide range of δ^{15}N values which suggests that they feed on a wide variety of species, although, preys tended to be at lower trophic levels when compared to residents and visitors (**Fig 2.14**).

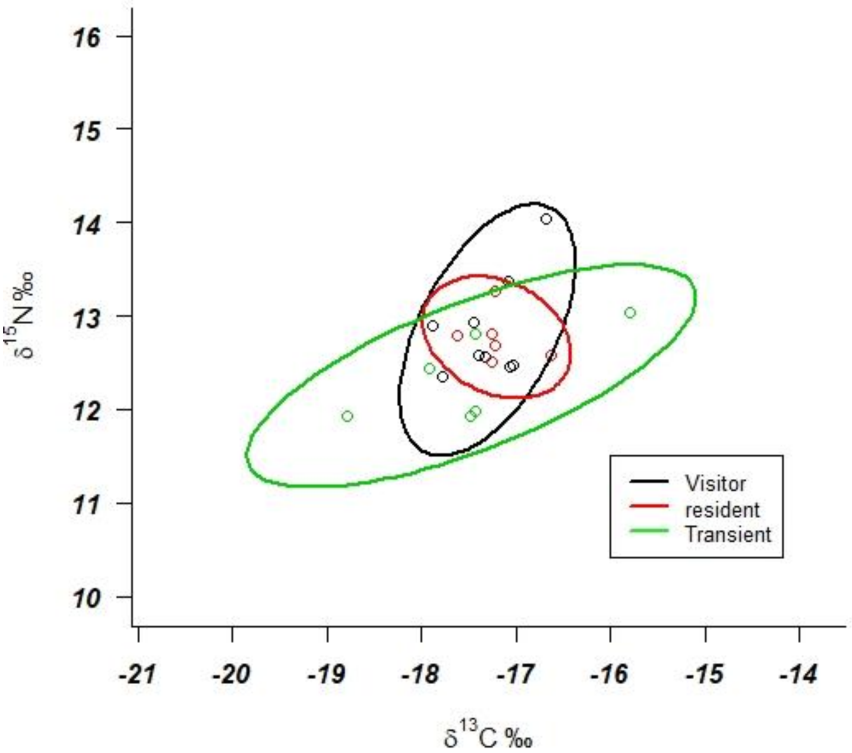

Fig 2.14 *Bi-plot of δ^{15}N and δ^{13}C values with Bayesian ellipses overlaid for short-finned pilot whales in Madeira with the residency patterns distinction*

RNA/DNA and stable isotopes

The relationship between the RNA/DNA values and the stable isotope signatures were tested. There was non-significant relationship between the RNA/DNA ratio and the δ^{13}C values for both species (Pearson´s correlation t-test: Tt: t, 0.55; p, 0.59, cor= 0.10, Gma: t, -0.1; p, 0.92, cor= -0.02). However, there was a significant positive correlation between the ratio and the δ^{15}N values for bottlenose dolphins (Pearson's correlation t-test: t2.18, p, 0.04, cor= 0.37) between the ratio and the δ^{15}N values for bottlen (**Fig 2.15**). However, there was no correlation for pilot whales (Pearson´s correlation t-test: t, 0.16; p, 0.87, cor= 0.04). In chapter 1, big disparities in the ratio values within the population of bottlenose dolphins were found. The relationship between the stable isotopic niches and the level of the RNA/DNA demonstrated that, at intermediate ratios, the isotopic niche was very much constraint in its carbon and nitrogen values, as it could be seen for short-finned pilot whale resident population. At high ratios, the isotopic niche was very wide and at low ratios, the isotopic niche was more constraint in its nitrogen values than high ratio but wider than for intermediate ratios, with a wide range of carbon values **(Fig 3.1**

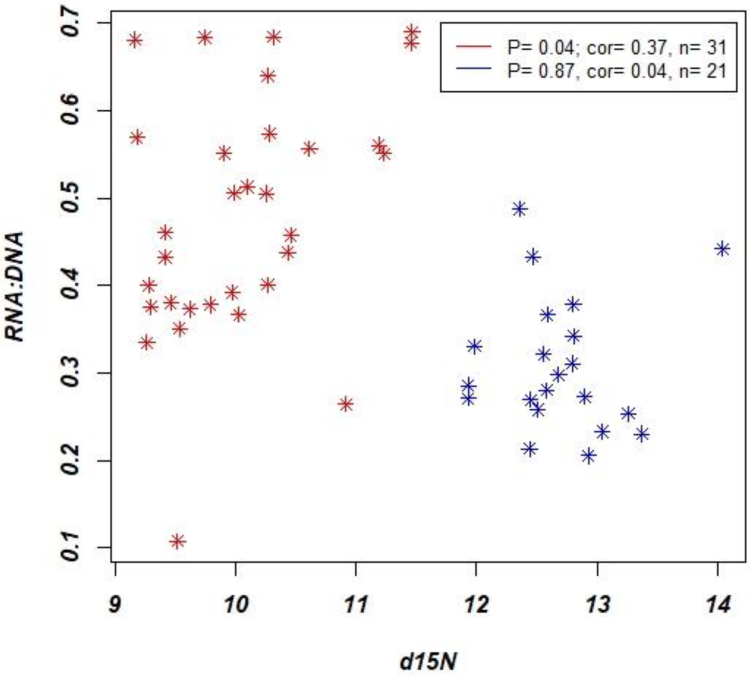

Fig 3.15 *δ^{15}N as a function of RNA/DNA in bottlenose dolphin (red dots) and short finned pilot whales (blue dots), sampled in Madeira in November 2017 and March-April 2018.*

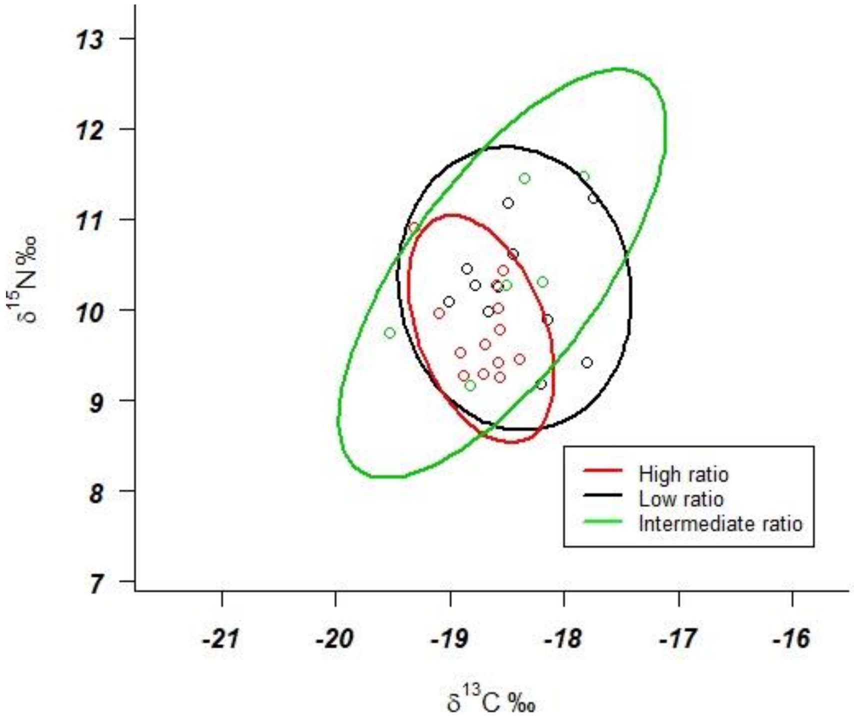

Fig 3.16 *Bi-plot of δ^{15}N and δ13C values with Bayesian ellipses overlaid for bottlenose dolphins in Madeira, in function of their RNA/DNA Low ratio= 0.24-0.44; Intermediate ratio= 0.44-0.64; High ratio= <0.64*

3.5 Discussion

This study was the first work to study the trophic ecology of bottlenose dolphins and pilot whales in Madeira, via the usage of stable isotopes. In addition, the study focused on the species *Globicephala macrorhynchus,* which had very little attention before, when compared to the species *Globicephala melas (*Stephanis *et al.* 2008, Monteiro *et al.* 2015). Studies on Northeast Atlantic long-finned pilot whales have previously reported that skins´ isotopic ratios were around -17.7, SD= 0.7 in Scotland (Monteiro *et al.* 2015) and -16.30, SD= 0.30 in North Carolina (Stephanies *et al.* 2017) for δ^{13}C and were around 12, SD= 0.7 (Monteiro *et al.* 2015) and 12, SD= 0.34 (Stephanies *et al.* 2017) for δ^{15}N. The similarities in stable isotopes values with the present study (-17.3 δ^{13}C and 12.7 δ^{15}N) (**Table 2.1**), reveals that short-finned pilot whales might adopt similar feeding habits than long-finned pilot whales, although they inhabit different geographical areas. Carbon stable isotopes´ values also suggested that both species of pilot whales preferred to live offshore and in deep waters such as break shelves, were they can hunt on deep living or pelagic species such as squids and mackerels (Monteiro *et al.* 2015, Stephanies *et al.* 2017). Indeed, shelf breaks are providers of a high diversity of habitats, which attracts many fish species (Young *et al.* 2001, Colloca *et al.* 2004, Genin 2004), readily available for air-breathing predators. Our findings supports the idea that such environments should be carefully taken into consideration when envisaging conservation or management efforts for cetaceans like pilot whales (Thorne *et al.* 2017). When looking at the isotopic trophic models of each residency pattern in Madeira, it was observed differences in the proportion of prey consumed, although, their isotopic values were not statistically different (**Table 2.3** and **Table 2.4**). The non-significance of the results might be because of too little sampling sizes for each category, which increased the chance of assuming that there was no significant difference in stable isotopes values between residency patterns, while there was in fact one (Faber *et al.* 2014). Moreover, p-values were very close to express significant differences between transient and visitors (p-value, 0.06) and transient and resident (p-value, 0.06), which is clearly expressed by the SIBER plot (**Fig 2.14**). The models predicted that migratory individuals (transient and visitors) may have fed on a broad range of prey species, because of high variations in δ^{15}N values (Young *et al.* 2017) and that transient individuals consumed more of low trophic level species than visitors and residents. Thus, transient and visitor pilot whales could be qualified as less specialist than resident individuals, for whom nitrogen and carbon values were more constricted. Such results might be the evidence of energy saving during long travels for transient individuals, which expressed the widest dispersion among the whales. The $\delta^{13}C$ values of resident individuals expressed small geographical dispersion, limited to the offshore waters of Madeira, as did the $\delta^{13}C$ values of visitors (**Table 2.3** and **Fig 2.3**). Therefore, visitors seem to be more acquainted to the island and travel less than transients, which allow them to spend more time on hunting on higher preferential calorific preys, requiring higher energy expenditure. Resident individuals seemed to be rather specific in their diets and their $\delta^{15}N$ signatures (μ= 12.8) appeared like those of individuals consuming mainly squids in the Western and Eastern North Atlantic (Abend and Smith 1995, Stephanies *et al.* 2017, Monteiro *et al.* 2015) (**Table 2.3** and **Fig 2.3**). These results would be in concordance with a study on short-finned pilot whales from the Canary island, which found predominantly squids and mackerels´ species in their stomach content (Hernandez-Garcia and Martin 1994) and with a study from the North Iberian population of long-finned pilot whales (Mintzer *et al.* 2008). A possible explanation would be that because residents don´t need to travel long distance, they can spend more energy on foraging on higher calorific preys present in their region of subsistence. An alike behaviour was observed for resident killer whales off the coast of Alaska, who fed on the highest calorific prey present the all year long in the region (Bigg *et al.* 1990, Baird 1994, 2000). However, the results from the Bayesian mixing model did not support the idea that squids, were the main preys in pilot whales' diet in Madeira, but instead supported a big reliance on blue jack mackerels and tuna species (**Table 2.4** and **Fig 2.9**). Squids isotopic values of the region of Madeira have not been collected for this study. As a consequence, the importance of this species in the diet of pilot whales might have been underestimated due to wrong isotopic signatures assigned to the taxa. The deep waters of Madeira are favourable to the development of large pelagic fishes like bigeye, skipjack and albacore tunas and also small pelagic fishes like mackerels (Gouveia 2017). Bigeye tuna is a big, energetic fish in the region, which feeds on various food items in the water column (Moteki *et al.* 2001). It might benefit pilot whale's survival, as would do albacore tuna relatively smaller than bigeye but is still a high trophic level species, foraging on adult fish in epi to upper mesopelagic waters (Consoli *et al.* 2008). These two species are abundant in the region from April to September and may contribute to the high $\delta^{15}N$ values observed for pilot whales during spring season. Regarding skipjack and mackerels, they are epipelagic predators (Menezes *et al.* 2006), living by neritic zones of island shelves, banks, seamounts, and open waters around islands (Vasconcelos 2006). Skipjack tuna tends to be the most abundant from July until maximum

November (Gouveia *et al.* 2017), whereas mackerels live in school the all year long in Madeira (Vasconselos *et al.* 2006, 2012). The two species share very close ecological characteristics and may feed on the same prey species (small fishes and crustaceans), as their similarities in their isotopic values suggest. The results from the mixing models for pilot whales, agreed with a study of Overholtz and Waring (1991), who noticed that pilot whales tend to feed on mackerels via depredation during fishing operation. In addition, a study from Allen, (2016) observed that pilot whales occasionally interact with tuna long line fisheries and depredated on the species along the coasts of California. Pilot whales plausibly adopt the same behaviour in Madeira, which would explain the high proportion estimation of tuna species in their diets. Although, the SIBER plot demonstrated that resident individuals tended to have a rather specialist diet focused on high trophic level preys, the Bayesian mixing models estimated that they had same dietary habits as visitor individuals, which were estimated to feed on a mixed of blue jack mackerels, tuna fish species and at a lesser extend small pelagic fishes (**Table 2.4** and **Fig 2.9**). From stable isotope analyses, it is not possible to know if residents fed on same trophic level prey species at δ^{15}N of 12.8 or, if they fed equally on a mixed of lower trophic species and higher trophic level species, which gave the intermediate trophic level when analysing the isotopes signatures of resident pilot whales. Visitor animals, tended to always have a higher contribution of higher trophic level species (Blue jack mackerels, skipjack and other tunas) and a smaller contribution of lower trophic level species (Atlantic mackerels, bogue and European pilchard) in their diet than residents. These differences in contribution were of the order of 2%. This remark could be justified by the ability of visitors to dive for longer and in deeper waters to apprehend bigger preys at higher trophic levels, like it was observed for resident vs transient killer whales' animals in Alaska (Bigg *et al.* 1990, Baird 1994, 2000). Wells (1999), also exposed that offshore cetaceans have a tendency to have higher haemoglobin concentration than coastal cetaceans, which provides them with better aerobic capacities. Visitors might be bigger, more muscular, have greater stomach capacities and have different hunting skills than residents, resulting from travelling requirement and because of different social learning processes between population. These assets may enable them to feed on bigger and more challenging preys. Besides, there was a positive correlation between δ^{13}C and δ^{15}N values for pilot whales, which give the idea that at depth, pilot whales would be able to consume higher trophic level species or bigger individuals. The Bayesian mixing model demonstrated that there were differences in the feeding habit of transient individuals and other residency patterns (resident and visitor). Moreover, the SIBER plot demonstrated differences in trophic niche sizes between transient, resident and visitors. These variations in the relative proportions of each source consumed between transient individuals and other patterns can be explained by different preys' availability during their oceanic movements. Also, Estrada (2003), noticed that in inshore environments $\delta^{15}N$ values tended to be further fractionised due to longer food chain and provided higher $\delta^{15}N$ values to top predators. In addition, transient animals might respond to the optimal foraging theory, which predicts that animals may stop feeding on certain preys when they consider that the searching and handling time spent to consume them would not maximise their rate of energy intake (Stephens & Krebs 1986). Thus, they prefer to feed on alternative more valuable preys. Furthermore, when transient individuals reside in the Madeiran waters, it increases the inter and intra specific competition in the region, which limits further the availability of prey and drives certain individuals to rely on alternative prey types. A study from Svanback and Bolnick (2006), observed that resource competition led predators to add new prey items to their diets, as their preferred preys got scarce, which generated niche variations. This could be supported by the differences in the trophic niches between autumn and spring, as shown by the SIBER plot. Short-finned pilot whales tended to be more specialised in spring than in autumn (**Fig 2.12**), it could be related to the greater availability of their favourite food like squids, tunas and mackerels during spring (Gouveia *et al.* 2017). Another hypothesis would be that transient individuals might be quicker, more agile, more enduring and have different experience in face of hunting habits, which would provide them greater plasticity in their diets when compared to residents. In fact, Morton (1990), noticed that transient killer whales tended to travel and forage considerably more than their resident compatriots, which spent more time socializing and resting, which emphases that populations might adopt different behaviours taught via social learning. Finally, migrant individuals (transients and visitors) were sampled in Madeira, but their skin isotopic values may not be equilibrated with the local food web, and instead represent their feeding habits of the open ocean. For instance, transient animals had to travel through isotopically different oceanographic regions, which could drive to a change in the primary producer baseline and then, affect higher trophic levels (Hobson & Welch 1992). These variations come from different primary inorganic nitrogen (nitrogen, ammonium, nitrate) uptakes from phytoplankton and different biological and oceanographic processes, which modifies the rate of various form of nitrogen uptakes (Ambrose & DeNiro 1986). Therefore, there is a need to use tissues with quicker turnover rates for stable isotopes analysis, to more accurately reconstitute the diet of cetaceans at a precise moment. Muscle tissues´ analysis and turnover rate associated to it should be investigated for cetacean's trophic ecology studies.

Studies on Indo pacific bottlenose dolphins (Kiszka *et al.* 2014), central tropical pacific bottlenose dolphins (Young *et al.* 2017), and East Atlantic bottlenose dolphins (Fernandez *et al.* 2011) expressed very different isotopic signatures from the individuals in the present study. They all reported higher nitrogen stable isotope values (13.1+/-1.3; 16.3+/-0.8; 14.6+/-0.9, respectively) (**Table 2.1**), which could suggest a shorter food web in the Madeiran waters or a strong competition with sympatric species, which forces the bottlenose dolphins to feed on lower trophic levels. However, it could also suggest that isotopic values at the base of the food chains are very different from a place to another. Bottlenose dolphins were suggested to feed on intermediate trophic level pelagic and demersal fishes. Small fishes and blue jack mackerels were the main components of the species diet (**Table 2.4 and Fig 2.8**). The contribution of higher trophic level species, such as skipjack tunas and squids were way smaller in the predictions of bottlenose dolphins´ diet in Madeira. Chub mackerels, bogues and European pilchards are yearlong predators of Madeira, and feed on zooplankton, young cephalopods and small pelagic fishes (anchovies, sardines). They all recorded very similar isotopic signatures in Madeira. Therefore, the contribution of small fishes in the diet of bottlenose dolphins is a mixed of these 3 species. Chub mackerels tend to inhabit inshore waters during their autumn spawning season and migrate to deeper open waters for the spring feeding season (Pârcalaboiu 1971, Rabashi & Wojciechowski 1973, Castro 2010), where they get predated by bigger fishes such as tunas, sharks or sea birds. These movements are in common with the movements of bottlenose dolphins over the year. Indeed, there were small enrichments in the carbon and nitrogen isotopic signatures in spring for bottlenose dolphins (**Fig 2.12**), which suggests that they move seaward. There were small differences in the diet predictions from the mixing model, which hypothesises that bottlenose dolphins fed further on small fishes such as Atlantic mackerels, European pilchards and bogues in autumn, while they consumed a bigger proportion of blue jack mackerels in spring. (**Table 2.4** and **Fig 2.8**). The relatively high contribution of squids in the diet of bottlenose dolphins, as predicted by the mixing model, is rather surprising. Indeed, there was very little contribution of squids for short-finned pilot whales in Madeira, while it was supposed to be the species consuming the most squids. On overall, bottlenose dolphins exhibited a generalist behaviour toward food in Madeira, focused on locally available prey species as observed in previous studies (Barros and Odell 1990). However, bottlenose dolphins did not seem to consistently feed on higher trophic level species available when they were moving seaward, as the non-significant correlation between $\delta^{13}C$ and $\delta^{15}N$ demonstrated. This observation could be the evidence that dolphins do not necessarily depredate on whatever species is available but select them by preference. It could also underline that bottlenose dolphins are physically and physiologically unable to catch higher trophic level species due to their small size or because they are unable to dive deep or long enough to catch them. Finally, Walker *et al* (1999), showed that dolphins tended to select their preys as they grow old, as a result of increase experience, improved diving and prey catching abilities or increased stomach capacity; thus, feed on smaller trophic level species, which would benefit them better. The same scenario could be envisaged in Madeira, with adult bottlenose dolphins praising small fishes and mackerels rather than squids and skipjack tunas.

This study demonstrated that bottlenose dolphins and short-finned pilot whales inhabited dissimilar habitats and consumed different food sources, as the trophic niche of the two species suggested in the SIBER plot based on $\delta^{13}C$- $\delta^{15}N$ metrics (**Fig 2.12**). Bottlenose dolphins were predicted to rely more on coastal environments than short-finned pilot whales, which were predicted to be oceanic and spend time in depth as suggested by $\delta^{13}C$ values (-18.6 ± 0.4‰ and -17.3 ± 0.6‰, respectively) and noticed by Praca and Gannier, (2008). In this study, it was impossible to determine the gender of the individuals as genetic analyses were not possible at times. However, differential values in $\delta^{13}C$ contents are expected between females and males, as it is the case for some species such as blue whales. For blue whales, $\delta^{13}C$ values were higher for males, due to differences in metabolic activity, since females use more lipids than males (Busquets 2008). Both species seemed to feed on a wide range of species, with a predominance for small fishes and blue jack mackerels for dolphins and tuna species and blue jack mackerels for pilot whales. The less enriched nitrogen values observed for bottlenose dolphins, could be explained by a difference in size between the two cetaceans. Indeed, previous studies stressed the point on the correlation between high nitrogen stable isotope values and increase in body sizes (Hooker *et al.* 2001, Kiszka *et al.* 2009), linked to the ability of bigger predators to feed on bigger preys (Hooker *et al.* 2001). In addition, pilot whales are able to dive at deeper depth and for longer than bottlenose dolphins, which is directly related to their body size and metabolic rate (Mory 2002). This propriety allows them to hunt on high calorific preys present in deep waters. Nitrogen isotope values of Madeiran bottlenose dolphins were way smaller than other bottlenose dolphins worldwide. These results could be a consequence of the food partitioning occurring between pilot whales and dolphins in Madeira. Indeed, bottlenose dolphins might be able to feed on higher trophic level prey species or on bigger specimens at higher proportion if they could live further away from the coast. The coastal propriety of bottlenose dolphins may force them to feed on juvenile blue jack mackerels or other fishes, which tend to inhabit inshore areas when completing their developments. This emphasize a need to focus on the management of small pelagic fishes' fisheries in the island, as it is an important activity in the region. Short-finned pilot whales would then depredate on big adult blue jack mackerels or other fishes in more offshore waters. A small seaward shift in bottlenose dolphins' habitats during spring could be the evidence that with higher abundance of prey species; thus, lessening of inter-specific competition, bottlenose dolphins might be able to feed on bigger individuals, which would explain the nitrogen enrichment observed. Moreover, in spring there was a very small overlap of the 2 species isotopic niches (**Fig 2.12**), which shows a decline in the food partitioning pressure. In fact, the most important factor influencing the movement of bottlenose dolphins is the availability of their food sources (Shane *et al.* 1986). Yet, in spring, there is a phytoplankton bloom in Madeira, which supports the growth of zooplankton and consequently boosts the production of other organisms at higher trophic levels (Voipio 1981), which spread over the region. This event greatly modifies Madeiran ecosystems and commercial fisheries (tuna, mackerel and squids) in the region and benefit the most opportunistic cetaceans. Another reason that could explain the disparity in diet habits between seasons for bottlenose dolphins, would be that individuals sampled in spring could be migrants (transients and/ or visitors). Indeed, Dinis (2014), suggested from observation that spring and autumn seasons would gather bigger groups of bottlenose dolphins, directly linked to the pool of transient individuals arriving in the region. The hypothesis could not be checked with this study, as migrant and resident individuals of bottlenose dolphins could not be identified, due to poor quality of the pictures taken during the field sampling. Yet, offshore individuals are supposed to be bigger, with larger skulls and bigger rostrum as well as higher amount of haemoglobin in their blood than inshore individuals (Hersh and Duffield 1990, Mead and Potter 1995, Walker *et al.* 1999). These phenotypical features would enable them to feed on bigger prey species and depredate on deeper living species high in energy such as cephalopods. Besides, Walker (1999), noticed that there were significant isotopic signatures divergences between offshore and inshore bottlenose dolphins. Inshore dolphins were predicted to have a diet further based on fish than squids, while offshore dolphins were predicted the contrary. Finally, a change in primary producers at the base of the food chain in spring, could also be the factor of the shift in the isotopical niche of bottlenose dolphins between seasons (Lorrain *et al.* 2015). Indeed, the width niche for spring and autumn greatly overlapped for bottlenose dolphins, which suggests that the species does not drastically change habitat between seasons. In addition, the average isotopic values of POM were higher in spring (spring= 5.0 autumn= 4.3 for δ^{15}N and spring= -21.7; autumn= -2.19 for δ^{13}C, respectively), although no significant differences were detected. The same pattern was observed for Atlantic and blue jack mackerels (spring= 8 and 9.4 and autumn= 7.9 and 9 for δ^{15}N, respectively and spring= -20 and -19.1 and autumn= -20.5 and -19.2 for δ^{13}C, respectively), the only species suitable for comparison, due to enough individuals per season.

In the present study, bottlenose dolphins had a higher dietary niche area than short-finned pilot whales (**Fig 2.12**), which indicated that the intraspecific variability in diet between individuals for dolphins was stronger. This could be the evidence that bottlenose dolphins adapt to competition, by diversifying their diet habits in order to optimise resource availability (**Fig 2.12**). The results agreed with previous studies on bottlenose dolphins, which affirms that the species tend to be rather generalist (Leatherwood *et al.* 1975, Shane *et al.* 1986). Previous studies suggest that Delphinidae association in geographically close habitats can have a positive effect on at least one of the species. This benefit is manifested by better feeding, and/or decreased predation (Norris & Dohl 1980, Baraff & Asmutis-Silvia 1998, Scott & Cattanach 1998, Wilson 2000, Gygax 2001, Bearzi 2007). Moreover, there was a striking pattern in the isotopic niches between bottlenose dolphins and tuna species (albacore, bigeye and skipjack) in Madeira (**Fig 2.12**). Bottlenose dolphins and tuna species shared the same space and consumed the same prey species, surprising result as usually in stable isotopes studies, marine mammals display the highest δ^{15}N values (Hobson and Welch 1992, Ostrom *et al.* 1993). A strong interspecific competition between the four species is very likely. Short-finned pilot whales might indirectly benefit bottlenose dolphins via the consumption of bottlenose dolphins´ competitors. This information is valuable in the context of conservation and management of the two cetaceans in Madeira. If pilot whales became scarcer in Madeira, it could have a negative impact on the population of bottlenose dolphins with an increase in interspecific competition between tunas and dolphins.

The isotopic analyses of 12 dominant species living in Madeira allowed to reveal the basic structure of a partial food web in the region (**Table 2.5 and Fig 2.10**). Short-finned pilot whales were at the top of the food chain, and were followed by third consumers, bottlenose dolphins and tunas (albacore, bigeye and skipjack) then, secondary consumers, Madeiran sardinella, European pilchard, mackerels (Atlantic chub and blue jack) bogues and round sardinella, then primary consumers, slender snipefish and zooplankton, and to finish, POM was at the bottom of the food chain (**Fig 2.10**). The isotopic values of zooplankton were within the same δ^{13}C range as POM, which indicates that they might predominantly feed on detritus. At the basis of the food web, only POM was represented in this study. However, other various food sources like phytoplankton and macroalgae may have also contributed to the food chain. These primary producers would have then been consumed by filter feeding animals such as bivalves and gastropods not analysed in this work. Biofilm was another potential constituent of the food web but was not sampled in the study either. On overall, the food web in this study was relatively small, with a maximum TL of 4.1. The fact that higher trophic levels like other species of cetaceans (Sperm whales, sharks) were not incorporated in the analysis can explain this pattern.

Our stable isotope analyses place pilot whales and bottlenose dolphins as top predators in Madeira. This study highlights that potential conflicts with existing fisheries in Madeira are very likely. Indeed, the oceanographic and oligotrophic characteristics of Madeira allow only the catch of a small diversity of high trophic level, migratory fish species (Shon *et al.* 2015). Tunas represent a high proportion of short-fined pilot whales' diet in Madeira and constitute up to 48 % (Albacore: 15%, bigeye: 31%, skipjack: 2%) of total catches in the island (Shon *et al.* 2015). In the 1990s, tuna catches were around 9000 tonnes in the region, and dropped to 3000 tonnes in 2012 (Shon *et al.* 2015). These observations should trigger questions into the state and dynamic of tuna fish like species in these waters. Regarding mackerels, they represent a large portion of the bottlenose dolphins and pilot whales' diet in the island and represent 14 % (Atlantic mackerels: 6%; Blue jack mackerel: 8%) of total catches (Shon *et al.* 2015). Those last years, their catches declined (Shon *et al.* 2015), which hypothesises an overall drop in their abundance in the Madeiran ecosystem. There may be a need to improve fisheries sustainability in the region in order to avoid detrimental effects on the survival of the two-cetacean species at stake.

3.6 Conclusion

Bottlenose dolphins, short-finned pilot whales and other cetaceans, provide high socio-economic benefits through ecotourism in Madeira. Additionally, as top predators, they control smaller predators and avoid decimation of even smaller species, which maintains a good balance in the Madeiran ecosystem. Furthermore, as sentinel species, cetaceans warn us about the health of the ecosystem and prevent long lasting disruptions. In the present study, stable isotope analysis suggests that short-finned pilot whales inhabit offshore or feed on offshore or deep benthic prey species, whatever its residency pattern. The isotopic mixing model revealed that in fact, the diet habits of the whales were different between the residency patterns. The migratory behaviours of transients and visitors seem to promote the diversification of their diet, while a sedentary behaviour for residents would promote specialism. This finding, if verified with further stable isotope analyses and behavioural observations, could help to protect resident individuals by monitoring the few species on which they feed. The isotopic mixing model demonstrated that the main prey of transient pilot whales were small pelagic fishes such as Atlantic mackerels, bogues or European pilchards while for resident and visitor pilot whales the predominant prey species were either blue jack mackerel or Skipjack tuna. The results disagree with previous stomach contents or stable isotopes analysis from other studies. Although, the skin analysis of stable isotopes in cetaceans is the least invasive and provide rather accurate data on their feeding ecology, there is a need to complement this technic with results from stomach contents analysis to verify our assumptions, as they represent the finest scale resolution of prey taxa. The isotopic mixing model reveals that bottlenose dolphins feed on same prey species in autumn and spring but tend to feed on bigger specimens like blue jack mackerels in spring in Madeira. Like in other regions, in Madeira bottlenose dolphins also have a flexible diet. The assumptions claimed from the isotopic results on bottlenose dolphins, would be even more powerful if combined with observational studies. The isotopic analysis results of this study demonstrate that it exists a strong spatial and trophic partitioning between short-finned pilot whales and bottlenose dolphins in Madeira, when it comes to food. Although, one out of three pilot whales are seen coexisting with bottlenose dolphins (Alves 2013). These interactions could be brief and benefit animals on a short term, like for avoiding predation. It would be interesting to study the nature of the interactions between the two species, to understand the benefits they get from each other. Short-finned pilot whales feed on higher trophic level prey species, living in more seaward waters than bottlenose dolphins. Bottlenose dolphins are hypothesized to slightly shift their habitat occupancy across seasons, as competition pressure diminishes. Consequently, they would feed on bigger specimens present further offshore in spring. Prey availability, habitat structure and population density seem to be the main factors for resource and spatial segregation among the two marine mammals. Resource competition seems to have led to diversification of resources in Madeira. If investigated further, it will be possible to detect any disturbances from fisheries or whale watching activities in Madeira and at a wider extent, disturbance from pollution and climate change and allow us to predict futur changes in the ecosystem. Pilot whales, bottlenose dolphins, and tunas, seem to feed on the same resources as fisheries exploitation, especially on blue jack mackerels and tuna species, which represent 8% and 48%, respectively of total catches in Madeira. The two cetacean species and tuna species may be at risk from the blue jack mackerel depletion and short-finned pilot whales itself, from tuna depletion. A lack of squid species in the diet prediction of short-finned pilot whales is worrying and should be investigated in Madeira to discard any eventualities of disturbances on this taxon. Also, if the cetaceans take advantage of fisheries activities, they may be a risk of bycatch. The results of this study are particularly relevant in the context of fisheries and ecological impact assessment and can provide support for wildlife management decisions.

4. FINAL REMARKS: RNA/DNA RATIO AND ITS RELATION TO STABLE ISOTOPES

The present study was the first attempt to combine results from an ecophysiological index, with results from a biological tracer in Cetaceans. When bottlenose dolphins fed on higher trophic levels, they tended to record better physiological conditions, characterised by higher RNA/DNAs (**Fig 2.13**). These results could not be applied to short finned pilot whales, since their δ^{15}N values were not correlated to the ratio, which undermines that other factors than the trophic level on which they feed, are responsible for fluctuations in the RNA/DNAs. In fact, the balance between the energy spent feeding and travelling is very likely to influence this parameter. High RNA/DNA ratios in visitors support that a diet composed of a wide variety of species with a predominance for high trophic level preys could be beneficial for short-finned pilot whales. Lower ratios in transients, support the idea that a diet composed of a wide variety of species, with a predominance for lower trophic level preys would not benefit as much individuals. The wider range of ratio values in migrants, when compared to residents, suggests that the prey species on which transient and visitor individuals are expected to feed, based on stable isotopes results, should be assessed for abundance and healthiness in Madeira and in other places where they travel to. For residents, a specialist diet based on rather high trophic level preys or a mixed diet with predominance for high trophic level species did not necessarily drive to higher ratio and thus, healthier individuals. The energy spent (diving and sprinting) trying to get high trophic level preys might overcome the energy intake provided by the preys themselves. Besides, an observation from Aguilar soto (2008), affirmed that there is a 40 % chance of failure when pilot whales try to get deep living cephalopods. These results infer that even though it exists animal associated island in Madeira, which evidence the good conditions of the island; the island does not necessarily provide better nutritional conditions but provide decent nutritional states for all residents. Fishing allowance and environmental conditions around the island should be checked regularly, to maintain the well-being of the whales. The exploration of the feeding ecology of the whales in function of their residency patterns, linked to the determination of their biochemical indexes could help to determine what part of the population is the most endangered among short-finned pilot whales in Madeira. This knowledge would allow to take measures for the protection of the species on a global scale. However, all the assumptions previously put forward cannot be certified, and there is a need to verify that the phylogenetic differences in the species do not exaggerate the variations in the RNA/DNA attributed to environmental factors such as food resources (Buckley and Szmant 2004). Therefore, the prehension of more samples, from various seasons on a prolonged period of time and the determination of parameters such as gender, size, and age of the individuals at a regional and global scale will allow more reliable and precise results. The positive correlation between $\delta^{15}N$ values and RNA/DNA ration for bottlenose dolphins may stress the point on the effect that food partitioning has on the species. If dolphins were able to feed on higher trophic level species or on bigger specimens praised by short-finned pilot whales, they could record higher RNA/DNA and be fitter. Besides, significantly higher values of stable isotopes and non-significantly higher RNA/DNAs for bottlenose dolphins in spring, could confirm the previous hypothesis. Therefore, activities such as fisheries, and whale watching should be more controlled in autumn. However, these differences could also be because of more migrant individuals' occurrence during this season, which feed on high trophic level species and might have different RNA/DNAs from residents, because of different physiological assets. None of the ratio or feeding habits of short-finned pilot whales were predicted to differ between seasons. When ratios were divided (low, intermediate and high ratios) and plotted against $\delta^{13}C$ and $\delta^{15}N$ values with SIBER (**Fig 2.14**) for bottlenose dolphins, the results were astonishingly similar to the plot generated with residency patterns of pilot whales. Therefore, variations in RNA/DNAs within the population of dolphins might be explain by different physiological characteristics between populations. This assumption must be verified. As no data on the indicator of nutritional state was available for the taxa Cetacea, it was not possible to compare our species between them and with species from cooler or warmer areas. The RNA/DNA should be further applied to other cetaceans´ species in order to have a base of comparison. The ratio, when combined with stable isotope analyses can assess the health of cetaceans´ population, monitor and forecast how mobile vertebrates will be affected by environmental and human disturbances

4.1 Limitations

Our study allowed to have a glimpse to the preliminary trophic roles, dietary habits and habitat occupancy of two important cetacean species in Madeira archipelago, thanks to the use of non-lethal biopsy sampling techniques, which prevailed skin and blubber material. The usage of skin isotopic values allowed to determine long term dietary changes thanks to the skin´s low turnover propriety (Hobson & Clark 1993, Gannes *et al.* 1997). However, it was challenging to determine the trophic ecology of bottlenose dolphins and short-finned pilot whales, as they turned up to be rather generalised; thus, opportunistic animals. In the present study, we tried to include as little sources as possible in the mixing model, to avoid variations in isotopic signatures (Di Beneditto *et al.* 2011). Nonetheless, a few prey species known for contributing to the diet of bottlenose dolphins and short-finned pilot whales were not yet taken into consideration. This is the case for anchovies, mullets and octopuses (Simoes-Lopez *et al.* 1998). These species might have had different isotopic signatures from the ones used in this study and produced different estimates (Philips *et al.* 2014). In addition, not many studies accurately determined TEFs for short-finned pilot whales. Regarding bottlenose dolphins, studies which used skin samples to determine the TEFs of the species were very different from a region to another (Vander Zandem and Rasmussen 2001, Giménez *et al.* 2016). Therefore, the study is based on literature values which might not be species or diet specific TEFs representative of the cetaceans and preys in the region at stake and may have engendered differences in preys´ predictions (Newsome *et al.* 2010, Caut *et al.* 2011, Borrell *et al. 2012*). In our study, we used different TEFs for short-finned pilot whales and bottlenose dolphins, which sometimes predicted different preys' proportions. The TEFs which generated the least error in the model were used. Another limits to this study are the multiple assumptions that were drawn and not verified at this date. The first, was to take isotopic values for squids based on literature values, because of lack of data for this taxon in Madeira. In fact, the oceanographic features of the island are known to produce very peculiar patterns concerning animal diversity and abundance (low productivity), this might have generated different isotopic signatures in island-associated squid species and other squids of the Atlantic. Moreover, from landing data, squids are mainly caught in August and September and sometimes, can be caught in May. Our sampling period did not match with these months supposed to have the highest occurrence of squids. Therefore, short-finned pilot whales might have relied on other taxa more readily available at this time. The second, was to assume, that bottlenose dolphins do not feed on Albacore and bigeye tunas. It would be worth to check this assumption via behavioural observations, as smaller or juvenile tunas might get predated. Furthermore, dolphins might depredate on tunas caught on longlines, although less likely given that in the region tunas are caught mainly with pole and sein. Third, in the present study, no equations for lipid normalisation was applied to the $\delta^{13}C$ values of top predators, since it was assumed that it would have no or detrimental effects on the Bayesian mixing model. However, the C/N ratios of bottlenose dolphins and pilot whales were very closed to the limit set by Post, (2010), which highlights a need to check this assumption. Fourth, all prey species were assumed to contribute equally to the diet of the two species through the year. However, isotopic signatures might be different in function of the seasons, like observed for Atlantic and blue mackerel as well as for POM, although differences were not significant. This assumption should be checked on all prey species considerate in this study and on numerous individuals to ensure on this change between seasons. Some preys like tunas, are also more abundant in spring than in autumn, this fluctuation in abundance should be taken into consideration when running the model. In the interpretation of the results, it was assumed that visitor and transient pilot whales morphologically differed from residents. This factor needs to be elucidated, as it could explain patterns in the dietary habits observed. Finally, Bayesian mixing models only include a mixed of species, whom isotopes values are reduced to a single value. This process ultimately limits discrimination of diet composition (Phillips *et al.* 2014). Quantitative data could not be established from this method.

4.2 Future works

Further work needs to investigate the specific discrimination factors of bottlenose dolphins and short-finned pilot whales in Madeira. The analysis of $\delta^{13}C$ after chemical lipid extraction complemented with the analysis of $\delta^{15}N$ without chemical lipid extraction can be envisaged to determine those parameters accurately. Then, the results can be compared to the results obtained from lipid normalisation equations, to check if equations can be reliable for futur works and save money and time. These TEFs can later be utilised as base for studies on the two species. The usage of different tissues like blubber, muscle, blood could give information on the diet of the two species over different period of time. Opportunistic sampling of muscles or blood on stranded animals could be considerate for more accurate data. The study should be complemented with analysis of important taxa, that could not be included in the estimation of the diet of the two species but were yet important. To determine what species should be added or dismissed in the model, a mixed of behavioural observations and analyses of stomach contents when available, should be envisaged. Potential taxa are octopuses, squids, mullets and anchovies as they have previously been proven to contribute noticeably to the cetaceans 'diet. Their addition in the model, will render the results from the Bayesian mixing models even more accurate and valuable for conservation policies. Cetaceans tend to have different feeding requirements in function of their gender, size and age (Walker *et al.* 1999). Therefore, these parameters should be determined and analysed for the individuals sampled. Genetic analysis can palliate this lack of information. It will improve the monitoring of the health state of the population on its whole and mitigate disturbances´ effects on the dynamic of the population in Madeira. In addition, such results could be exploited for other populations in different geographical areas. Continual stable isotope analyses for the two cetaceans in Madeira could be useful for their monitoring over time. It will provide accurate baseline of isotopic signatures, confirm data quality, harden the importance that these two cetaceans play in the ecosystem and reveal the potential effects of their removal in the region. Sampling on additional cetacean species in Madeira could investigate the relationship between them and investigate factors of diet flexibility, such as particularity of habitat structure of the island, high cetacean's density or poor prey availability. It could enlighten the ecosystem structure of the region, with top predators portioning their food and space in order to benefit everybody on a long run.

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ANNEXES

Standard method for nucleic acids analysis (Calderone 2001, 2008)

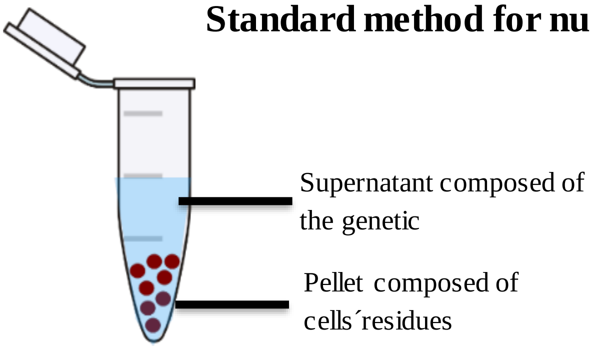

Fig.I.a. *Eppendorf with skin tissue material after vortexing and centrifuging*

1. Skin tissues of each individual were put in 600 µl of Sarcosine (0.5%) and sonicated 3 times for 10 secs at 70 A. It allowed to separate cells tissues from the genetic material.
2. Samples were vortexed (30 mins), then centrifuged (15 mins) at 12 000 r P. M. and 0 to 4 °C so that dense particles were separated from the lighter particles and the supernatant containing the genetic material was able to be analysed further.

In order to determine the DNA and RNA concentrations of the samples via the reading of absorbances, standard curves need to be established. In this study, DNAstock and RNAstock produced from bovine material were used.

Fluorescent plate, each cell represents a well.

RNAstock ([0])	DNAstock ([0])	Sample 1	Sample 2	Sample 3
RNAstock ([10])	DNAstock ([10])	Sample 4	Sample 5	Sample 6
RNAstock ([20])	DNAstock ([15])	Sample 7	Sample 8	Sample 9
RNAstock ([30])	DNAstock ([20])	Sample 10	Sample 11	Sample 12
RNAstock ([0])	DNAstock ([0])	Sample 1	Sample 2	Sample 3
RNAstock ([10])	DNAstock ([10])	Sample 4	Sample 5	Sample 6
RNAstock ([20])	DNAstock ([15])	Sample 7	Sample 8	Sample 9
RNAstock ([30])	DNAstock ([20])	Sample 10	Sample 11	Sample 12

Table.I. a. *Organisation of the fluorescent plates for the establishment of standard curves.*

3. 50 µl of Aliquot of supernatant of each sample was pipeted into the wells of fluorescent plates (FP).
4. 120 µl of Tris buffer was added in each well in order to maintain a stable PH as DNA is PH sensitive.
5. The first line of the FP was filled with various RNAstock concentration (0, 10, 20 and 30 µl * 2 times).
6. The second line of the FP was filled with various DNAstock concentration (0, 10, 15, and 20 µl *2 times).
7. RNAstock wells were completed with complementary concentrations of Sarcosine (0.05 %), 85, 75, 65, and 55 µl). DNAstock wells were also completed with complementary concentration of Sarcosine (0.05 %), 70, 60, 55 and 50 µl.
8. 30 µl of RNAase was pipeted into the DNAstock wells and in all samples. The enzyme RNAase allowed later, after warming up at 37 °C to remove the RNA from the DNA.
9. 30 µl of GelRED, a fluorescent nucleic acid was added into each wells of the FP.
10. The plates were then read in a multiple reader for absorbance measures at 360 nm and 590 nm to determine the total amount of DNA and RNA in each sample.
11. After reading, RNAase was activated by incubating the FPs at 37 °C for 30 mins.
12. Samples were then read at 360 nm and 590 nm again for determination of the DNA concentrations only, within samples and DNAstock.
13. Once DNA concentrations were determined, RNA concentrations were calculated via the equation: [RNA] = Total fluorescence – fluorescence after RNAase activation.

Table 2.1 *Summary of sampling collection. Individuals ID, date, location and residency pattern of bottlenose dolphins.*

Individuals ID	Date	Latitude (°N)	Longitude (°W)	Residency pattern
Tt 3	15/11/2017	32.614	-16.905	
Tt 4	15/11/2017	32.614	-16.905	
Tt 5	15/11/2017	32.614	-16.905	
Tt 6	15/11/2017	32.607	-16.876	
Tt 7	15/11/2017	32.549	-16.880	
Tt 8	15/11/2017	32.596	-16.848	
Tt 9	15/11/2017	32.596	-16.848	
Tt 10	15/11/2017	32.596	-16.848	
Tt 11	17/11/2017	32.558	-16.903	
Tt 12	17/11/2017	32.558	-16.903	
Tt 13	17/11/2017	32.558	-16.903	
Tt 14	17/11/2017	32.558	-16.903	
Tt 15	19/11/2017	32.618	-17.031	
Tt 16	20/11/2017	32.605	-16.938	
Tt 18	16/03/2018	32.624	-16.917	
Tt 19	22/03/2018	32.611	-16.972	
Tt 20	22/03/2018	32.611	-16.972	
Tt 21	22/03/2018	32.611	-16.972	
Tt 22	22/03/2018	32.611	-16.972	
Tt 23	22/03/2018	32.611	-16.972	
Tt 24	22/03/2018	32.647	-17.131	
Tt 25	22/03/2018	32.647	-17.131	
Tt 26	16/04/2018	32.630	-16.909	
Tt 27	16/04/2018	32.630	-16.909	
Tt 28	16/04/2018	32.633	-16.903	
Tt 29	16/04/2018	32.632	-16.902	
Tt 30	16/04/2018	32.633	-16.897	
Tt 31	16/04/2018	32.640	-16.812	
Tt 32	17/04/2018	32.631	-16.899	Resident
Tt 33	18/04/2018	32.631	-16.899	
Tt 34	19/04/2018	32.630	-16.9 19	
Tt 35	20/04/2018	32.621	-16.933	
Tt 36	21/04/2018	32.621	-16.933	

Table 2.2 ***Summary*** *of sampling collection. Individuals ID, date, location and residency pattern of short-finned pilot whales.*

Individuals ID	Date	Latitude (°N)	Longitude (°W)	Residency pattern
Gma 2	03/11/2017	32.574	-16.782	Visitor
Gma 3	03/11/2017	32.574	-16.782	Visitor
Gma 4	03/11/2017	32.620	-16.757	Transient
Gma 5	03/11/2017	32.620	-16.757	Transient
Gma 6	06/11/2017	32.599	-16.952	Visitor
Gma 7	06/11/2017	32.599	-16.952	Visitor
Gma 8	13/11/2017	32.610	-17.053	Visitor
Gma 9	13/11/2017	32.610	-16.844	Visitor
Gma 10	15/11/2017	32.585	-16.903	Visitor
Gma 12	17/11/2017	32.558	-16.903	Visitor
Gma 13	17/11/2017	32.558	-16.751	Visitor
Gma 14	18/11/2017	32.627	-16.751	Resident
Gma 15	18/11/2017	32.627	-16.751	Resident
Gma 17	18/11/2017	32.627	-16.751	Resident
Gma 18	20/03/2018	32.627	-16.922	Transient
Gma 19	20/03/2018	32.600	-16.922	Transient
Gma 20	20/03/2018	32.600	-16.947	Transient
Gma 21	20/03/2018	32.585	-16.947	Transient
Gma 23	23/03/2018	32.585	-16.897	Resident
Gma 24	23/03/2018	32.581	-16.897	Resident
Gma 25	18/04/2018	32.581	-16.727	Resident
Gma 26	18/04/2018	32.546	-16.727	Resident

Table 2.3 *Biochemical conditions (mean± SD; RNA± SD; DNA± SD) of 30 bottlenose dolphins sampled during November 2017 and March-April 2018, in Madeira.*

Individual ID	RNA: DNA			Type
	Mean ± SD	RNA ± SD	DNA ± SD	
Tt 3	0.458 ± 0.002	2.89 ± 0.076	3.20± 0.104	
Tt 4	0.376 ± 0.028	194 ± 0.164	2.61± 0.026	
Tt 5	0.108 ± 0.11	0.18 ± 0.179	0.85± 0.006	
Tt 6	0.351 ± 0.039	6.03 ± 0.608	8.73± 0.098	
Tt 7	0.335 ± 0.037	2. 19 ± 0.365	3.29± 0. 192	
Tt 8	0.552 ± 0.066	6.78 ± 0.280	6.29± 0.496	
Tt 9	0.690 ± 0.24	11.02 ± 3.646	8.14± 0.160	
Tt 10	0.570 ± 0.090	5.14 ± 0.748	4.58± 0.056	
Tt 11	0.460 ± 0.057	2.50 ± 0.369	2.74± 0.070	
Tt 12	0.432 ± 0.006	2.71 ± 0.065	3.18± 0.122	
Tt 13	0.438 ± 0.79	5.60 ± 0.633	6.55± 0.444	
Tt 14	0.381 ± 0.07	3.08 ± 0.567	4.11± 0.033	
Tt 15	0.379 ± 0.07	4.49 ± 0.629	6.07± 0.286	
Tt 16	0.684 ± 0.072	6.49 ± 0.626	4.81± 0.040	
Tt 18	0.678 ± 0.11	7.94 ± 1.259	5.94± 0.028	
Tt 19	0.561 ± 0.52	6.08 ± 0.564	5.49± 0.003	
Tt 20	0.401 ± 0.12	6.22 ± 1.803	7.92± 0.143	
Tt 21	0.371 ± 0.02	5.58 ± 0.282	7.58± 0.112	
Tt 22	0.551 ± 0.03	5.54 ± 0.270	5.09± 0.003	
Tt 23	0.367 ± 0.13	1.56 ± 0.587	2.13± 0.055	
Tt 24	0.574 ± 0.17	5.62 ± 1.411	5.03± 0.244	
Tt 25	0.640 ± 0.004	7.92 ± 0.051	6.27± 0.003	
Tt 26	0.505 ± 0.025	3.83 ± 0.186	3.84± 0.003	
Tt 27	0.513 ± 0.17	5.54 ± 1.659	5.55± 0.222	
Tt 28	0.392 ± 0.03	2.89 ± 0.109	3.74± 0.112	
Tt 29	0.684 ± 0.11	7.52 ± 1.0 19	5.59± 0.121	
Tt 30	0.557 ± 0.11	3.79 ± 0.679	3.47± 0.092	
Tt 31	0.681 ± 0.09	9.02 ± 1.272	6.71± 0.037	
Tt 32	0.264 ± 0.03	1.33 ± 0.164	2.55± 0.053	Resident
Tt 33	0.506 ± 0.10	7.43 ± 1.276	7.47± 0.156	
Tt 34	0.401 ± 0.05	5.50 ± 0.506	6.98± 0.272	

Table 2.4 *Biochemical conditions ((mean± SD; RNA± SD; DNA± SD) of 21 short-finned pilot whales sampled during November 2017 and March-April 2018, in Madeira.*

Individual ID	**RNA : DNA**			**Type**
	Mean ± SD	**RNA ± SD**	**DNA ± SD**	
Gma 2	0.230 ± 0.111	2.68 ± 1.180	4.04 ± 0.213	Visitor
Gma 3	0.442 ± 0.079	4.51 ± 0.521	3.49 ± 0.229	Visitor
Gma 4	0.271 ± 0.028	3.37 ± 0.280	4.20 ± 0.090	Transient
Gma 5	0.233 ± 0.009	2.32 ± 0.109	3.37 ± 0.024	Transient
Gma 6	0.367 ± 0.115	6.18 ± 1965	5.68 ± 0.033	Visitor
Gma 7	0.205 ± 0.011	1.74 ± 0.081	2.87 ± 0.023	Visitor
Gma 8	0.488 ± 0.128	4.94 ± 1.025	3.47 ± 0. 198	Visitor
Gma 9	0.433 ± 0.116	3.52 ± 0.840	2.77 ± 0.087	Visitor
Gma 10	0.273 ± 0.039	3.27 ± 0.483	4.05 ± 0.018	Visitor
Gma 12	0.321 ± 0.028	6.21 ± 0.260	6.56 ± 0.291	Visitor
Gma 13	0.269 ± 0.050	1.74 ± 0.608	2.11 ± 0.372	Visitor
Gma 14	0.253 ± 0.067	2.73 ± 0.684	3.66 ± 0.050	Resident
Gma 15	0.298 ± 0.065	2.93 ± 0.716	3.30 ± 0.092	Resident
Gma 17	0.280 ± 0.130	3.23 ± 1.496	3.89 ± 0.000	Resident
Gma 18	0.214 ± 0.091	1.52 ± 0.590	2.46 ± 0.118	Transient
Gma 19	0.378 ± 0.073	5.91 ± 0.818	5.34 ± 0.293	Transient
Gma 20	0.331 ± 0.091	4.39 ± 0.982	4.56 ± 0.247	Transient
Gma 21	0.286 ± 0.107	5.73 ± 2.080	6.81 ± 0.099	Transient
Gma 23	0.342 ± 0.046	3.65 ± 0.608	3.59 ± 0.116	Resident
Gma 24	0.082 ± 0.063	1.22 ± 0.912	5.14 ± 0.170	Resident
Gma 25	0.311 ± 0.066	2.75 ± 0.627	2.98 ± 0.052	Resident
Gma 26	0.258 ± 0.105	1.89 ± 0.751	2.49 ± 0.031	Resident