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USING MOLECULAR DIET ANALYSES AND STABLE ISOTOPES TO UNRAVEL THE FORAGING ECOLOGY OF STORM PETRELS IN THE NORTH ATLANTIC OCEAN

Dissertação de Mestrado em Investigação em Ecologia,
orientada pelo Professor Doutor Jaime Albino Ramos e pelo Doutor Vitor Hugo Paiva,
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Using molecular diet analyses and stable isotopes to unravel the foraging ecology of storm petrels in the North Atlantic Ocean.

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“I am a poor student sitting at the feet of giants, yearning for their wisdom and begging for lessons that might one day make me a complete artist, so that if all goes well, I may one day sit beside them.”

Rod Taylor

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Abstract

As marine top predators, seabirds reflect changes in the lower trophic levels of marine ecosystems, and ecologists have been using seabirds as sentinels of ocean conditions. However, there is very little information concerning the potential of smaller seabird species, such as the storm-petrels, as sentinels of the marine environment. The aim of this work was to study the diet and trophic ecology of Madeiran storm-petrel (*Hydrobates castro*) breeding in Farilhões islet, Portugal. Molecular techniques were used to analyze diet during the breeding seasons of 2015/2016 and 2016/2017, and stable isotopes of blood and feathers were used to assess the trophic ecology from 2015 to 2017. We assessed the existence of sexual dimorphism, and also deployed tracking devices in some birds to help us understand their at-sea distribution. Finally, information concerning stable isotopic values of other storm-petrels was compiled to understand the trophic ecology of the north Atlantic storm-petrels.

The diet of Madeiran storm-petrel was dominated by fish in both sexes and study years. In 2015 females were more generalists than males, while in 2017 the inverse situation occurred. This shows a shift in their diet, and suggests some degree of inter-annual plasticity in the diet of this species. Concerning the trophic ecology, we detected significant differences between years in carbon stable isotope values during the breeding season, with birds foraging nearer coastal areas in 2017, which was confirmed by tracking data. In the non-breeding season of 2016, females fed on prey of lower level than males, resulting in significant differences in nitrogen stable isotope values between sexes in this year. Despite such differences, there was no sexual segregation in the trophic ecology of this species, and overall males showed wider isotopic niches than females, except in the end of the 2015 breeding season, when the inverse situation occurred.

Overall, our results showed that during breeding, female and male Madeiran storm-petrels have similar foraging ecology, despite slight differences in diet preferences. During this period, both sexes alternate between short distance trips in the colony surroundings to feed their chicks and long-distance forays to the highly

productive area in the African coast, presumably to restore their body condition. This results in smaller niches than in the non-breeding season, in which this species widens its isotopic niche, since they are no longer constrained by their reproductive duties. The species seems to respond to environmental conditions, exhibiting intersexual differences in years with poorer environmental conditions, which are years of presumed food scarcity. Under such conditions, females tend to feed in prey of lower trophic levels when compared to males, perhaps to avoid intersexual competition. Comparing the feeding ecology of our study species with other storm-petrels from the north Atlantic, it was possible to notice that species breeding in northern areas prey at higher trophic levels, and most species seem to be generalists when compared to more specialist.

Key-words: trophic ecology, stable isotopes, diet, molecular techniques, Madeiran storm-petrel (*Hydrobates castro*).

Resumo

Como predadores de topo, as aves marinhas, nomeadamente a sua ecologia, refletem mudanças em níveis tróficos inferiores dos ecossistemas marinhos, e os cientistas têm vindo a usar as aves marinhas como sentinelas das condições oceânicas. No entanto, existe muita pouca informação relativamente ao potencial de pequenas aves marinhas, tais como os painhos, para virem a ser usadas como sentinelas marinhas. O objetivo deste trabalho foi estudar a dieta e ecologia trófica da população de Painho-da-Madeira (*Hydrobates castro*) que se reproduz no ilhéu dos Farilhões, Portugal. Analisamos a dieta desta espécie durante as épocas reprodutivas de 2015/2016 e 2016/2017 através de métodos moleculares, e a sua ecologia trófica através da análise de isótopos estáveis. Foi avaliada a existência de dimorfismo sexual nesta espécie, e foram implantados alguns dispositivos de seguimento individual para perceber a sua distribuição no mar. Finalmente, foi compilada informação relativa à ecologia trófica de outros painhos reprodutores no Atlântico Norte.

A dieta do Painho-da-Madeira foi dominada por peixe em ambos os sexos e anos do nosso estudo. Em 2015 as fêmeas foram mais generalistas que os machos, enquanto que em 2017 a situação se inverteu. Isso mostra uma mudança na sua dieta entre anos, revelando algum nível de plasticidade interanual para esta espécie. Relativamente à ecologia trófica, detetaram-se diferenças significativas entre anos nos níveis do isótopo estável do carbono durante a época reprodutiva, em que em 2017 as aves alimentaram-se mais em zonas costeiras, tal facto confirmado pelos resultados de seguimento. Na época não reprodutora de 2016 as fêmeas alimentaram-se de presas de níveis tróficos mais baixos do que os machos, resultando em diferenças significativas nos níveis do isótopo estável de azoto entre sexos. Apesar de tais diferenças, não se detetou segregação sexual na ecologia trófica desta espécie, e no geral os machos apresentam nichos isotópicos mais amplos do que as fêmeas, à exceção da época pós-reprodução de 2015, onde aconteceu o oposto.

De uma forma geral, os nossos resultados mostraram que durante a época reprodutora, ambos os sexos do Painho-da-Madeira têm uma ecologia trófica semelhante, apesar de ocorrerem ligeiras diferenças na dieta. Durante este período,

ambos os sexos alternam entre viagens de curta distancia nas redondezas da colónia para alimentar as crias, e viagens de longa distâncias para a zona altamente produtiva da costa Africana, provavelmente para reposição da sua condição corporal. Isto resulta em nichos mais pequenos do que na época não reprodutiva, na qual os indivíduos expandem o seu nicho trófico por não estarem restritos às responsabilidades reprodutoras. Esta espécie também aparenta responder a condições ambientais, exibindo diferenças intersexuais em anos de piores condições ambientais, os quais correspondem provavelmente a anos de escassez de alimento. Sob tais condições, as fêmeas tendem a alimentar-se de presas de níveis tróficos mais baixos relativamente aos machos, talvez para evitar competição intersexual. Comparando a ecologia trófica dos diversos painhos que reproduzem no Atlântico Norte, é possível concluir que as espécies que se reproduzem mais a norte alimentam-se de presas de níveis tróficos mais altos, e em geral os painhos parecem ser generalistas quando comparadas com espécies mais especialistas.

Palavras-chave: ecologia trófica, isótopos estáveis, dieta, técnicas moleculares, Painho-da-Madeira (*Hydrobates castro*).

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1. Introduction



1.1. Seabirds as sentinels of change in marine ecosystems

Oceans have an important role regulating global temperature, and are under strong pressure from human activities such as overfishing and pollution, with strong negative consequences for marine biodiversity and functioning of marine ecosystems (McCarty 2001). Thus, it is imperative to identify tools to monitor the status of marine ecosystems, and seabirds can be useful in that matter because many species spend their whole life at sea, coming to land only to breed (Lascelles *et al.* 2012). As marine top predators, their feeding ecology, breeding and behaviour reflect changes in the lower trophic levels of marine ecosystems (Montevecchi *et al.* 2006).

Several aspects of seabird ecology make them good bioindicators of marine ecosystem conditions, namely: 1) many species are relatively well studied and can be detected at distance from land, and/or are highly visible at sea; 2) seabirds feed in the ocean but breed in colonies on land, which provides the opportunity to obtain a large number of samples in a limited space and time period, and this characteristic, combined with the wide range distribution of breeding areas for the same species, provides a natural laboratory to test reactions to environmental changes at different sites; 3) as top predators, seabirds reflect the changes at lower trophic levels, since physical and biological changes in ecosystem also determine the distribution and abundance of the animals existing in that habitat, reflecting in diet changes and abundance of their predators (Springer *et al.* 1984). Many studies demonstrated that changes in seabirds' diet not only reflect changes in the distribution and abundance of their prey (Croxall *et al.* 2002; Scioscia *et al.* 2014), but also these changes can be found and studied in their tissues through the incorporation of stable isotopes assimilated from their prey (Hobson & Clark 1992; Hobson *et al.* 1994). Overall, seabirds' trophic ecology gives relevant information about the relationship with other seabirds and food availability in the oceans, comprising essential data for their conservation and ecosystem management (Iverson *et al.* 2007; Xavier *et al.* 2011). For all those reasons, for the last 20 years seabirds have been used as sentinels of oceans conditions (Gremillet & Charmantier 2010), and several studies discuss how to choose and use the most suitable sentinel species of the marine environment (Frederiksen *et*

al. 2007; Parsons *et al.* 2008; Mallory *et al.* 2010). Species with low resistance and resilience to environmental changes are more appropriate as sentinels (Gremillet & Charmantier 2010), since they usually have small ecological niches, and easily show shifts in their spatial, trophic and breeding ecology (Paiva *et al.* 2013a, b). Black-legged kittiwakes (*Rissa tridactyla*), a seagull from the family Laridae, have been classified as one of the most susceptible species concerning the effects of overfishing in the North Sea, because their breeding success reduces drastically when food availability decreases (Furness & Tasker 2000). Other studies proposed that seabirds with high resilience and plasticity to environmental changes may also be effective sentinels. Seabird species from the order procellariiform, such as the albatrosses, petrels and shearwaters, have extreme life-history characteristics: one egg clutch, slow chick growth and large fat storage (Warham 1990; Granadeiro *et al.* 1998b), and these parameters change rapidly as a response to marine environmental changes. As an example, in a population of Cory's shearwaters (*Calonectris borealis*) which normally reproduces and feeds along the Portuguese coast, the females changed their foraging behaviour during periods of adverse environmental conditions (i.e. lack of food resources), and foraged in pelagic remote areas of the North Atlantic basin. In fact, this change in behaviour led to a reduction of their reproductive success, which may also be used as an indicator of changes in environmental conditions (Paiva *et al.* 2013b). The Cape Verde Shearwater (*Calonectris edwardsii*) has been suggested recently as a sentinel species for marine conservation off West Africa by Paiva *et al.* (2015), because it has a wide oceanic range and feeds in areas that are heavily used by other top predators and by international fishery fleets. Thus, changes in their foraging distribution and diet may indicate changes in the wider marine ecosystem.

However, there is very little information on the potential of smaller seabird species, such as the storm-petrels, to be used as sentinels of the marine ecosystem. There are 24 species of storm-petrels in the world, and although most of them are categorized as Least Concern in IUCN Red List, they are sensitive to human intrusion and the majority are threatened by invasive species (IUCN 2016). It is a priority to study such vulnerable species, not only for their conservation, but also to evaluate their potential as bioindicators of marine conditions. As lower trophic level consumers,

e.g. zooplankton, not only they can alert to changes in the environment at a faster speed than comparatively larger seabirds (Grémillet *et al.* 2015), but also because some of them have the peculiarity of reproducing in winter, they can be a sentinel for changes in environmental conditions during this specific season (Grémillet & Charmantier 2010).

1.2. How to study the diet and feeding ecology of seabirds

Observations of feeding behaviour have been by far the oldest method to identify seabird prey. This technique provides a direct study, but can be biased because observers may have difficulties in identifying prey and predators given the large distance and brevity of such observations (Duffy *et al.* 1986). A more accurate analysis of dietary habits is through stomach contents. In the past, stomach contents were obtained by killing the animals, but, for ethical reasons, this is now forbidden and was replaced by other non-lethal methods, such as the analysis of faecal or regurgitation samples (Deagle *et al.* 2007). Stomach contents can also be obtained from dead birds, but these samples should not be representative of healthy living animals. The technique to obtain the stomach content, by forcing regurgitation, is called stomach flushing, which involves pumping salt water through the animals esophagus (Wilson 1984; Barnett *et al.* 2010). This technique cannot be applied in small seabird species, as it is time consuming and invasive (Harris & Wanless 1993). Species that feed their offspring by regurgitating partially digested food can eventually do it spontaneously just by handling them, but this brings the issue of a differential diet between adults and offspring, making it difficult to understand which part of the sample was meant for the progenitor's meal and which part was meant for chick feeding (Wilson *et al.* 2004). Analyses of faecal samples are noninvasive, but the samples often do not present enough detectable prey items to allow complete reconstruction of diet composition, due to differential digestion of prey (Deagle *et al.* 2007). All these methods have been used mostly during the breeding season, giving poor information about trophic interactions for other periods of the reproductive cycle (Neves *et al.* 2012).

Because nutrients and their isotopic forms are assimilated through the diet and incorporated in predators' tissues, Stable Isotope Analyses (SIA) of those tissues have been used to study the trophic ecology of several seabird species (e.g. Roscales *et al.* 2011). The advantages of this method relies on the fact that the isotopic signatures are based on the assimilated food (not just the ingested), and also provides information on diet for a larger time scale than the traditional methods (Hobson & Clark 1992; Shealer 2002). Tissues with high turnover rates (e.g. blood cells) will integrate isotopic forms incorporated in a relatively recent past (4-6 weeks in the case of blood cells; Hobson and Clark 1992), while tissues that are formed over a specific period, but without turnover after their formation, like keratinous tissues (e.g. feathers, whiskers) are particularly advantageous, because they remain chemically inert after their formation, thus the isotopic forms of these tissues reflect the diet composition of the period when they were formed (Ramos & González-Solís 2012). However, SIA by itself gives an unclear response of precise trophic interactions, because it rarely provides a specific composition of the diet of the seabirds, giving instead information only about the trophic level of their prey (Iverson *et al.* 2007; Traugott *et al.* 2007).

Some complementary methods have been used, namely a tracking system of seabird movements over long periods of time, like Global Location Sensing devices (GLS). The information gathered by those devices, together with data obtained from SIA, makes it possible to build biogeographic patterns of stable isotopes in the marine ecosystem: isotopic signatures changes throughout different latitudes, depending on the distance to the shore or benthic habitats, but also on the productivity of the area, providing an estimated geographic gradient (i.e. isoscapes) of the oceans (Ramos *et al.* 2009b; Graham *et al.* 2010). The disadvantages of these techniques combined are that those isotopic gradients depend on different factors and may not be clear in some large oceanic areas (Ramos & González-Solís 2012). The profiles of fatty acids (FA), which allows to identify the origin of the lipids present in their adipose tissue (Budge *et al.* 2006), is another complementary method. The problem with the application of this method in seabirds is that it is not clear which is the turnover rates of fatty acids on seabirds, leading to unclear estimation of when their prey were ingested, and the variability of fatty acids between individuals of the same prey species can lead to

misinterpretations (Barrett *et al.* 2007). A more recent technique has been extensively used to study the diet of animals - the application of molecular methods to identify prey DNA from faeces, vomits or regurgitations - but there are very few studies applying this technique to study seabird diets (e.g. Deagle *et al.* 2007, Medeiros-Mirra 2010, Jarman *et al.* 2013). These molecular techniques can be particularly useful for smaller seabird species such as storm-petrels, because invasive methods like stomach flushing cannot be used with small species.

1.3. The use of molecular techniques to study the diet of seabirds

The greater advantage of a molecular approach to detect seabirds diet is that it gives a more diverse and complete data than SIA, since it relies on identifying DNA sequences that are unique to particular prey, from greatly degraded tissues (Jarman *et al.* 2013). Several approaches have been developed, each one with advantages and disadvantages (reviewed in King *et al.* 2008, Pompanon *et al.* 2012). One of the first DNA approaches to look at the diet was the DNA fingerprinting of digestive contents through amplification, using general or group-specific primers, followed by temperature or denaturing gradient gel electrophoresis (TGGE or DGGE) (e.g. Deagle *et al.* 2005). This method was effective to describe the diversity present in the diet, but presents some problems with cryptic bands, especially when analyzing the diet of generalist predators (Pompanon *et al.* 2012).

With the need to develop more robust methods, two main branches in the field of DNA analysis of diet were formed: both begin with the use of Polymerase Chain Reaction (PCR) to amplify DNA and obtain enough material for analyze. Then one approach is to use specific PCR primers designed to identify a few specific prey, and has been applied to study predator-prey systems (e.g. Meekan *et al.* 2009). However, this is only efficient once established and standardized in a laboratory, and limited to the range of prey whose primers have been designed (Pompanon *et al.* 2012). The second approach is to use a more general amplification of DNA, with general or group-specific primers, followed by the cloning and sequencing of PCR products to identify individual taxa. This has been used to study the diet of macaroni penguins (*Eudyptes*

chrysolophus) by Deagle *et al.* (2007), where it was detected DNA from several prey groups, such as euphausiids, fish, amphipods and cephalopods, and DNA from specific suborders and species of each prey group. Nevertheless, the problem in designing general primers is the risk of amplify non-target species, for example, when using faecal samples parasites and symbionts may also be amplified (King *et al.* 2008), and this process is poorly suited to the mass screening needed to obtain a comprehensive research of animal diets, because it is highly time-consuming due to the requirement of sequencing many clones (Pompanon *et al.* 2012). As the sequencing technology expanded, Next-Generation Sequencing (NGS) technologies have been developed, giving faster and cheaper tools. On seabirds, Deagle *et al.* (2010) detected DNA of several Osteichthyes and Cephalopod's species through NGS in faeces of captive little penguins (*Eudyptula minor*). For the first time it was possible to study the diet of breeding, chick and non-breeding Adélie penguins (*Pygoscelis adeliae*) using NGS (McInnes *et al.* 2016). Therefore, molecular techniques are increasingly being used by ecologists to unravel the feeding ecology of seabird species, and the sequencing database has been enlarged by more DNA barcoding (Pompanon *et al.* 2012).

1.4. Diet, trophic ecology and distribution of storm-petrels

The diet and feeding ecology of storm-petrels is perhaps the least known of all seabird groups, partly because traditional sampling methods are too invasive for these small seabird species. In addition, some storm-petrels reproduce during the winter, making it difficult to access their remote nesting colonies for sampling. Through behavioural observations, it is thought that the storm petrel's diet is largely based on zooplankton and small mesopelagic fishes, but for instance, regurgitations of Leach's storm-petrel (*Oceanodroma leucorhoa*) from the breeding season, detected mostly mesopelagic fish (Myctophidae and Gadidae), crustaceans and even cephalopods in the diet of chicks, going against the general idea of this storm-petrel's diet be based on zooplankton (Hedd & Montevecchi 2006; Hedd *et al.* 2009). Other study based on the analysis of regurgitations from European Storm Petrels (*Hydrobates pelagicus melitensis*) showed two feeding techniques: 1) the traditional long trip during the day,

feeding essentially on pelagic fish, and 2) short trips at night, very close to the colony, feeding mainly on Opossum Shrimps Mysidacea, which shows the adaptive behaviour of this species (Albores-Barajas *et al.* 2011). Because these methods could be invasive for most storm-petrel species, the use of molecular techniques can be a huge advantage to study their feeding ecology, and has been successfully used in the study of the diet of European storm-petrel (*Hydrobates pelagicus*), showing that this species has an opportunistic behaviour, feeding not only in the most abundant prey on their habitat, like fish, cephalopods, amphipods or isopods, but also on unexpected prey such as dolphins, through scavenging (Medeiros-Mirra 2010).

Studies on the trophic ecology of storm-petrels through SIA has showed that they generally feed on prey from lower trophic levels, but can easily shift their feeding habits and enlarge their trophic niche, showing some plasticity and opportunistic behavior. For example, Wilson's storm-petrel (*Oceanites oceanicus*) adjust their diet for prey of higher trophic levels in seasons when krill (*Euphausia superba*) is less abundant, especially during the chick provisioning period (Gladbach *et al.* 2007). Some trophic ecology' studies of storm petrels have been combined with a tracking system, complementing the information provided by isotopes. For example, Pollet *et al.* (2014b) studied two colonies of Leach's storm-petrels located only 380 km apart, and showed distinct foraging locations and foraging ranges for each colony during the breeding season. These same populations were tracked during the non-breeding season in other study, showing different wintering distribution: while one migrated to the Brazilian coast, the other went to the African coast, surrounding Cape-Verde (Pollet *et al.* 2014a). Although it was only used in few studies, with few species of storm-petrels, the combination of these techniques show a strong potential to study the trophic ecology of this group of seabirds.

Several studies reported sex-specific differences in seabird's trophic behaviour and ecology, namely in sexual segregation in foraging areas (e.g. González-Solís *et al.* 2000), diving behaviour (e.g. Kato *et al.* 2000), foraging trip duration (e.g. Lewis *et al.* 2005) or overall provisioning rate (e.g. Weimerskirch & Lys 2000). This normally occurs in species with Sexual Size Dimorphism (SSD), where one sex is larger than the other. In monomorphic species, where SSD does not occur, smaller differences in trophic

ecology between sexes are expected, but recent studies on monomorphic species showed that sex-specific foraging patterns can actually occur (Welcker *et al.* 2009; Elliott *et al.* 2010). These differences can be explained by the “intersexual competition hypothesis”, which suggests that, despite the lack of apparent intersexual morphological differences, one sex may forage more efficiently, outcompeting the other and showing different foraging niches, or even resulting in sexual segregation in foraging areas (Lewis *et al.* 2002; Peck & Congdon 2006). On the other hand, the “energetic constraint hypothesis” suggests that the parents invest differently during the different breeding stages, resulting in different energetic or nutritional requirements, and in different self-provisioning effort between sexes (Elliott *et al.* 2010). In small monomorphic procellariiforms, it was reported sex-specific differences in foraging distribution and behaviour in Barau’s petrel (*Pterodroma barau*) during the pre-laying period, but apparently there was no sexual segregation during chick rearing (Pinet *et al.* 2012). In monomorphic storm petrels, a study did not find any significant sex-specific differences in trophic ecology for Wilson’s storm petrel (*Oceanites oceanicus*), Grey-backed storm petrel (*Garrodia nereis*) or Black-bellied storm petrel (*Fregetta tropica*) (Phillips *et al.* 2009), but in other study there were intersexual differences in the trophic ecology and distribution of Monteiro’s Storm-petrel (*Hydrobates monteiroi*) during incubation and chick-rearing periods (Paiva *et al.* under review), where females preyed on lower trophic levels and foraged in significantly higher latitudes than males.

There is a large diversity within this group of seabirds, and only a few species have been studied. To fill these gaps, this study presents information on the diet, trophic ecology and distribution of the Madeiran storm-petrel (*Hydrobates castro*) breeding in Farilhões islet, Portugal. *H. castro* is a monomorphic medium size storm-petrel (Monteiro *et al.* 1996a), breeding in oceanic islands from equatorial to subtropical latitudes, mostly in winter (Monteiro & Furness 1998). This species is defined as presenting two breeding populations per year in some sites, breeding also in summer, but recently in the Azores these two populations were described as separated species; the “summer breeder” was classified as Monteiro’s Storm-petrel (*Hydrobates monteiroi*), presumably a speciation arising from temporal segregation

(Bolton *et al.* 2008). Like other storm petrels, the Madeiran storm petrel is a K-strategist, returning to nesting sites at night but avoiding bright nights of full moon (Bolton *et al.* 2004; Bolton 2007; Smith & Friesen 2007). Most of their breeding sites are well-studied, distributed all around the subtropical areas of Pacific and Atlantic coasts (Monteiro & Furness 1998), but their at-sea distribution is much less known (Lascelles *et al.* 2012). There are some records of their distribution around the Portuguese coast and the archipelagos of Madeira and Azores, suggesting that this species does not migrate extensively (Meirinho A *et al.* 2014), but these were only based in some at-sea observations; only one tracked individual with a GLS-logger was reported from the population of Farilhão Grande (Oliveira *et al.* 2013), which went close to the Moroccan Northwest African coast during the breeding season. Very little is known about the feeding ecology of this species: It is thought that its diet composition is based on zooplankton and small mesopelagic fishes, as other storm-petrel species (Monteiro *et al.* 1996a), but there is no comprehensive information about their diet composition. A comparative study about the trophic ecology of Atlantic procellariiforms in several breeding sites for the end of the breeding season showed that the Madeiran storm-petrel presents a small isotopic niche, displaying similar isotopic values between different sites and years, with few spatial differences and some variability between years (Roscales *et al.* 2011). Therefore, we expect Madeiran storm-petrels to: 1) exhibit a small isotopic niche when compared to other larger procellariiform species, as reported in a previous study (Roscales *et al.* 2011); 2) show a generalist diet composition, not restricted to zooplankton; 3) forage mainly over pelagic regions during the breeding period, with some individuals making longer trips towards the African coast, as reported by at-sea census surveys (Meirinho *et al.* 2014) and the tracking of one individual (Oliveira *et al.* 2013). There are no clear expectations regarding sexual differences in trophic ecology and diet composition, because most storm-petrel species do not exhibit such differences, but given the phylogenetic proximity with the Monteiro's storm-petrel in which such differences occur (Paiva *et al.* under review), our species may present sexual segregation in its foraging ecology.

2. Methods



2.1. Study area and Study species

This study was carried on Farilhão Grande Islet ($39^{\circ} 28' 31''$ N, $9^{\circ} 32' 45''$ W), within Berlengas archipelago, offshore Peniche, Portugal (Fig.1). This is an important seabird breeding site within the Berlenga Nature Reserve, and is the only territory in the Portuguese mainland where two procellariiform seabird species breed, *Calonectris diomedea borealis* and *Hydrobates castro* (Lecoq *et al.* 2011).

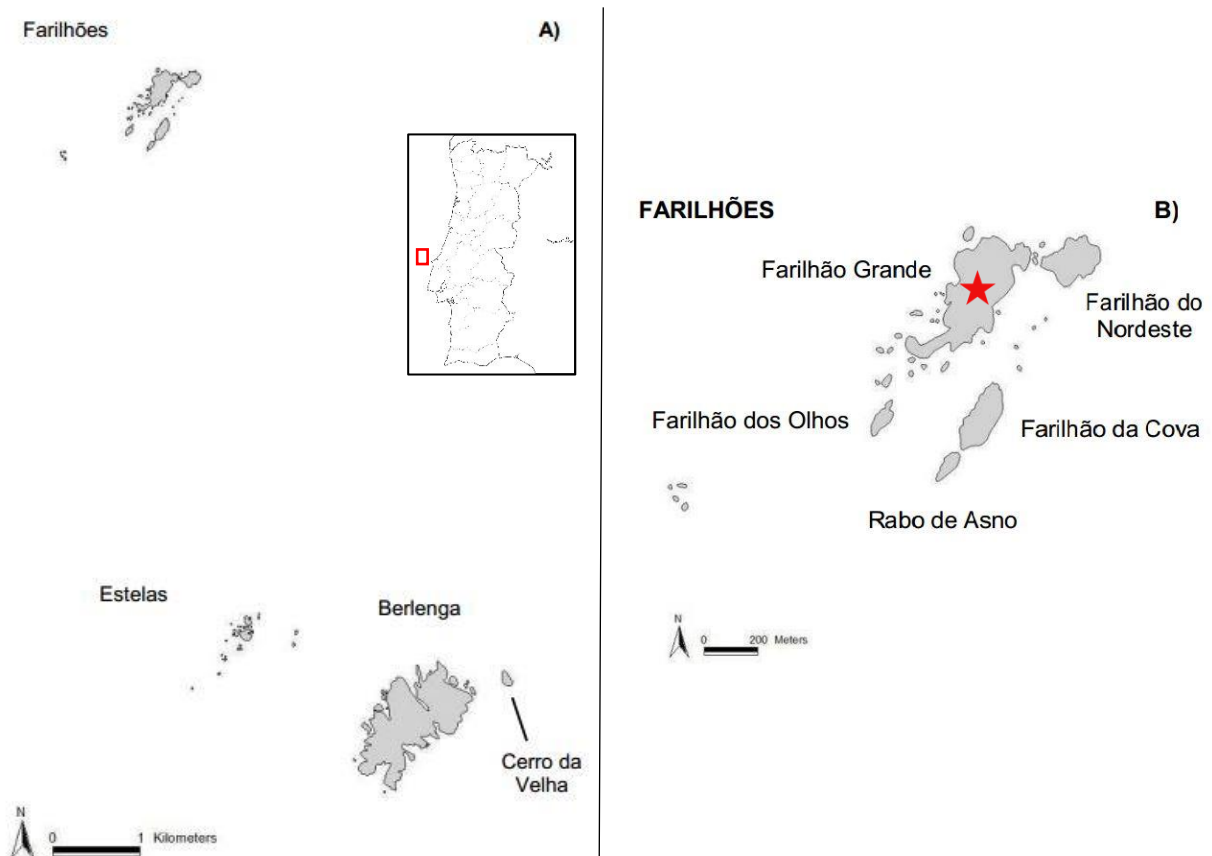


Figure 1 - Berlengas archipelago (a) with the Failhões islets in detail (b) (adapted from Lecoq *et al.* 2011). The ★ indicates the breeding site in Farilhão Grande islet.

Farilhão Grande Islet is characterized by rocky substrate, with vertical and steep cliffs, where about 100 to 200 breeding pairs of Madeiran storm-petrels are estimated to breed (Mendes 2013). This species arrives at the islet between August-September, nesting in cavities, then leaves around February (Fig. 2, Granadeiro *et al.* 1998).

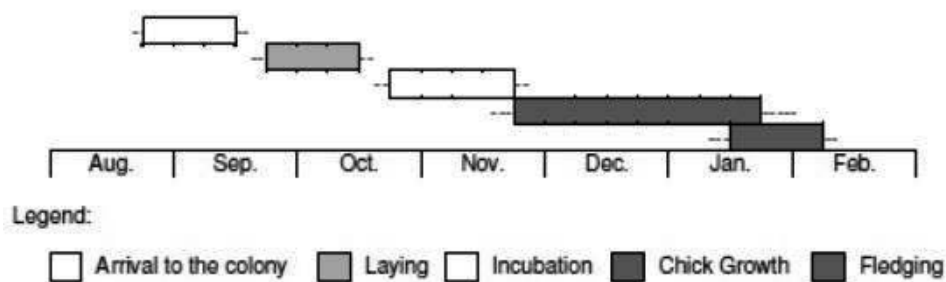


Figure 2 - Breeding phenology of Madeiran Storm-petrel in Farilhão Grande (adapted from Granadeiro *et al.* 1998a).

2.2. Field Sampling

To capture Madeiran storm-petrels in order to avoid nest desertion (Rodway *et al.* 1996; Blackmer *et al.* 2004), a mist net was placed along the rocky shore. In the first breeding season fieldwork was carried out in 10 November 2015, and in the second breeding season in 18 January 2017, where 30 and 21 individuals were captured, respectively. About 1cm from the first primary and eight secondary feathers were collected from all captured individuals and stored in polythene bags for stable isotope analysis. A blood sample (~50µl) was taken from the bird's brachial vein and stored in 2ml tubes with ethanol 70% for both stable isotope analysis and molecular sexing. After this, birds were placed inside a card box in which the bottom was lined with plastic or tinfoil, replaced between each individual. Birds were kept in the box for a maximum of 15 minutes to defecate naturally. Samples were then stored in 2ml tubes with 70% ethanol.

Four and six birds were instrumented with Global Location Sensing (GLS) devices (model MK18L, BioTrack Lda.) in 2015/2016 and 2016/2017 breeding seasons, respectively. Loggers were back mounted with a cotton harness to remain at least a year on the individuals. In 2017, when the tagged birds were recaptured during breeding the logger information was downloaded without taking the device out from the bird. Thus, it was possible to get back the tracking information from 4 individuals

during the incubation and early chick-rearing periods (November-February). Devices always represented less than 1% of the bird's body mass.

2.3. Molecular sexing

Molecular sex determination was done from individuals' whole blood samples using the Chelex DNA extraction method (Walsh *et al.* 1991). Approximately 15µl of blood was placed in a 1.5ml tube with 50µl water and 20µl of instagene matrix (Biorad) was added. The samples were mixed by vortex and heated at 50°C for 30 min followed by 8 min at 100°C. Sex specific markers (2550F and 2718R) were used for the DNA amplification (Fridolfsson & Ellegren 1999). DNA amplifications were carried out using the Multiplex PCR kit (Qiagen), in 5µl reactions containing 1x Multiplex PCR Master Mix, 0,2µM of each primer and 0.1 mg/ml of BSA (BioLabs), with 0.5µl of the DNA extract. Two negative controls (the extraction control, plus a PCR blank) were included in each set of PCR amplification. Thermal cycling conditions were: 95°C for 15 min, 35 cycles of [94°C for 30 sec, 50°C for 90 sec, and 72°C for 90 sec], concluding with 72°C for 10 min. PCR products were separated by electrophoresis in 1.5% agarose gels, after stained with Gel Loading Dye (BioLabs), and visualized by transillumination with UV light. Females are identified with two bands for the W and Z chromosomes while males have only one band for the Z chromosome.

2.4. Diet determination using Molecular Tools

DNA from storm-petrel faecal samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen), following Zaele *et al.* 2011 methodology. Blank extractions were included as a control to check for any contamination.

Four different primer sets were used to target different prey types and different genes to ensure a good cover and resolution of the range of potential prey consumed by the birds: Osteichthyes (mtDNA 12S), Cephalopoda (nuclear 28S rDNA), Amphipoda (nuclear 18S rDNA) and general invertebrates (mtDNA COI). The COI general invertebrates primer has not been used before in prey detection of seabirds

but has previously shown to successfully amplify a wide range of target and non-target species (Appendix A, Stockdale *et al.* In prep). Initial testing of general invertebrate primer pair against reference marine invertebrate DNA and DNA from the Madeiran storm-petrel confirmed that the primer was specific to invertebrates, with no amplification of predator's DNA (Appendix B). This primer sets amplify target prey sequences between 180 and 375 base pairs in size (Table 1). The 18S, 28S and 12S primer sets have been previously used for seabirds (Deagle *et al.* 2007; Medeiros-Mirra 2010). Since Amphipods' primer is known for amplification of the predator' DNA, the addition of the general invertebrates' primer was necessary to clarify these amplifications. This last primer was never used in seabird's diet detection before, and so the combination of the two primers contributes to detect more groups of invertebrates, obtaining the broader results as possible. The success of DNA extraction from fecal samples is presented by the number of samples that were positive in at least one of the prey group amplifications.

Table 1 - Primers used for prey DNA Screening.

Primer name	Sequence 5'-3'	Amplicon size (bp)	Annealing temp.	Reference
FishF1 FishR1	CGGTAAACTCGTGCC CCGCCAAGTCCTTTGGG	~300	56°C	Jarman unpubl., in Medeiros-Mirra 2010
Squid28SF Squid28SR	CGCCGAATCCCGTCGCMAGTAAAMGGCTTC CCAAGCAACCCGACTCTCGGATCGAA	~180	60°C	Deagle <i>et al.</i> 2007
AmphNSSF1 AmphNSSR1	CTGCGGTAAAAGGCTCGTAGTTGAA ACTGCTTTRAGCACTCTGATTTAC	204–375	52°C	Jarman <i>et al.</i> 2006
mICO1intF CI-N-2191	GGWACWGGWTGAACWGTWTAYCCYCC CCCGGTAAATATAAACTTC	333	50°C	Leray <i>et al.</i> 2013 Simon <i>et al.</i> 1994

Amplifications were performed separately for each primer pair, using the Multiplex PCR Kit (Qiagen) in 5µl reactions containing 1x Multiplex PCR Master Mix, 0.2µM of each primer and 0.1 mg/ml of BSA (New England Biolabs), with 1µl of the

DNA extract. A minimum of three negative controls (the extraction control, plus at least two PCR blanks) were included in each set of PCR amplifications. Thermal cycling conditions were: 95°C for 15 min, 35 cycles of [94°C for 30 sec, primer specific annealing temperature for 90 sec, and 72°C for 90 sec], concluding with 72°C for 10 min. PCR products were separated by electrophoresis in 1.5% agarose gels, after stained with Gel Loading Dye (BioLabs), and visualized by transillumination with UV light.

2.5. Trophic Ecology

In Madeiran Storm-petrels, primary feathers start moulting by the end of January, so their isotopic signatures represent the trophic ecology of the individuals during the end of the previous breeding period, thus early-2015 and early-2016. Secondary feathers moult in August so they represent the end of the non-breeding season (Monteiro *et al.* 1996b; Bolton *et al.* 2008), thus summer of 2015 and 2016. Blood regenerates quickly, representing the season when it is collected, i.e. the breeding season (October-November 2015 and December 2016-January 2017).

Feathers were cleaned from surface contaminants and oils using a 2:1 ratio solution of chloroform : methanol bath during 15 minutes (3 baths of 5 minutes each) and then oven dried at 60°C for 24h. After dried, feathers were cut into small fragments using stainless steel scissors, avoiding the shaft. Blood samples were firstly air dried from ethanol, then oven dried at 60°C for 24h as well. Approximately 0.35mg aliquots of each sample were weighed into tin capsules and isotopic ratios were determined by continuous-flow isotope-ratio mass spectrometry (CF- IRMS).

2.6. Trophic ecology of storm petrels in the North Atlantic

To understand how our species' trophic ecology compares with other storm-petrels in the North Atlantic Ocean, we compiled all published carbon and nitrogen SIA data from the first primary feathers as a proxy to infer prey consumed during the inter-

breeding season for storm-petrel species in the North Atlantic Ocean. It was possible to compare four storm-petrel species: Madeiran storm-petrel (*H. castro*), Leach's storm-petrel (*O. leucorhoa*), European storm-petrel (*H. pelagicus*) and Monteiro's storm-petrel (*H. monteiroi*). We plotted such data and added the expected range in prey isotope values for *H. castro*, based on the mean diet-feather trophic enrichment factors (see Meier *et al.* 2016). To understand potential food sources, we added isotopic values of prey species from Newfoundland, Canada (Hedd & Montevecchi 2006) as glacier lanternfish (*Benthosema glaciale*) and hyperiid amphipod (*Hyperia galba*), as well as prey species from Western Iberia (Meier *et al.* 2016) as European sardine (*Sardina pilchardus*), European anchovy (*Engraulis encrasicolus*), the Atlantic horse mackerel (*Trachurus trachurus*) and the Atlantic mackerel (*Scomber scombrus*).

2.7. Tracking data processing

GLS data was analyzed using the BASTrack software suite (British Antarctic Survey, Cambridge, UK), using a light threshold of 10 and with elevation angle of -4.0 (derived from calibration devices left at Berlenga). The quality of the light curves checked with *TransEdit* was high, so the geolocation error was assumed to be similar to that estimated by Phillips *et al.* (2004). Locations derived from curves with apparent interruptions around sunset and sunrise were removed. Erroneous locations were also excluded for a week around the equinoxes, when latitudes are unreliable.

Predicted geolocations of each bird were examined under the *adehabitatHR* R package (Calenge 2006) generating kernel Utilization Distribution (kernel UD) estimates. The most appropriate smoothing parameter (h) was chosen via least squares cross-validation for the unsmoothed GLS data and then applied as standard for the other data sets, and grid size was set at 0.25° . Following previous authors (Paiva *et al.* 2010), we considered the 50 and 95 % kernel UD contours to represent the core foraging areas (FA) and the home range (HR), respectively.

2.8. Data analysis

For the diet results, no weighting of the detection results was possible, so comparisons were carried out using the presence/absence detection data. Since it was detected the amplification of predator's DNA with the amphipods primer pair, and it is impossible to distinguish through electrophoresis which samples were detected, prey or predator's DNA, this set was withdrawn from this analysis. A similarity matrix was generated using the Bray–Curtis similarity measure. Adonis tests were run on the matrices using 999 permutations to test for statistically significant differences in diet composition between sexes and years. We also tested for intersexual differences in body measurements (tarsus, wing and body mass), using a t-test.

Three multivariate analysis of variance (MANOVA; Wilk's lambda statistics) were used to compare differences on both the carbon and nitrogen isotopic signatures (response variables) of (1) blood, (2) feather P1 and (3) feathers S8, between (1) sex and (2) years (independent variables). MANOVAs were followed by separated factorial ANOVAs for each stable isotope and post-hoc multiple comparisons Tuckey test.

In order to analyze stable isotope data in the context of isotopic niche between sexes, among years and periods, was used recent metrics based in a Bayesian framework (Stable Isotope Bayesian Ellipses in R: SIBER; Jackson *et al.* 2011). The standard ellipse area corrected for small sample sizes (SEA_C , an ellipse that has 40% probability of containing a subsequently sampled datum) was used to quantify niche width and to compare it between the two islands among years and periods, and a Bayesian estimate of the standard ellipse and its area (SEA_B) to test whether group 1 is smaller than group 2 (i.e. p , the proportion of ellipses in group 1 that were lower than group 2, for 10^4 replicates; see Jackson *et al.* 2011 for more details). Was used computational code to calculate the metrics from SIBER implemented in the package SIAR (stable isotope analysis in R; Parnell *et al.*, 2010) under R (R Core Team, 2014).

3. Results



3.1. Sex determination and sexual dimorphism

Sex determination was successful for 51 samples, resulting in a total of 13 females and 17 males for 2015, 13 females and 8 males for 2017. Body measurements indicated that females had significantly longer wings than males ($t_{46} = 3.86$, $P < 0.001$, Table 2), but similar tarsus size ($t_{46} = -0.03$, $P = 0.97$) and body mass ($t_{46} = 1.47$, $P = 0.15$).

Table 2 - Biometric measurements of Madeiran Storm-Petrels. Significant differences between sexes are represented in bold.

	Tarsus (mm; mean \pm SD)	Wing (mm; mean \pm SD)	Mass (g; mean \pm SD)
Females (N = 25)	23.33 \pm 0.79	160.40 \pm 3.89	55.31 \pm 6.29
Males (N = 23)	23.34 \pm 0.66	156.57 \pm 2.86	52.63 \pm 6.30

3.2. Diet determination

DNA amplification was successful in 100% of the samples, and the screening of prey DNA from fecal samples resulted in the predominance of fish (Osteichthyes) in the diet of both sexes and years (60.0% to 69.2%, Fig. 3), except for males in 2017, where the proportion of fish DNA was similar to that of general invertebrates (62.5%). However, sample size of males in 2017 was small ($n = 8$). The prey group with lower number of detections for both sexes and years was Cephalopoda, ranging from 0.0% in males 2017 to 15.4% in female 2015 (Fig. 3). There were no significant differences between the proportions of prey groups between years (Adonis, $R^2 = 0.021$, $P = 0.444$), sexes (Adonis, $R^2 = 0.048$, $P = 0.156$), nor in interaction between these two variables (Adonis, $R^2 = 0.041$, $P = 0.227$).

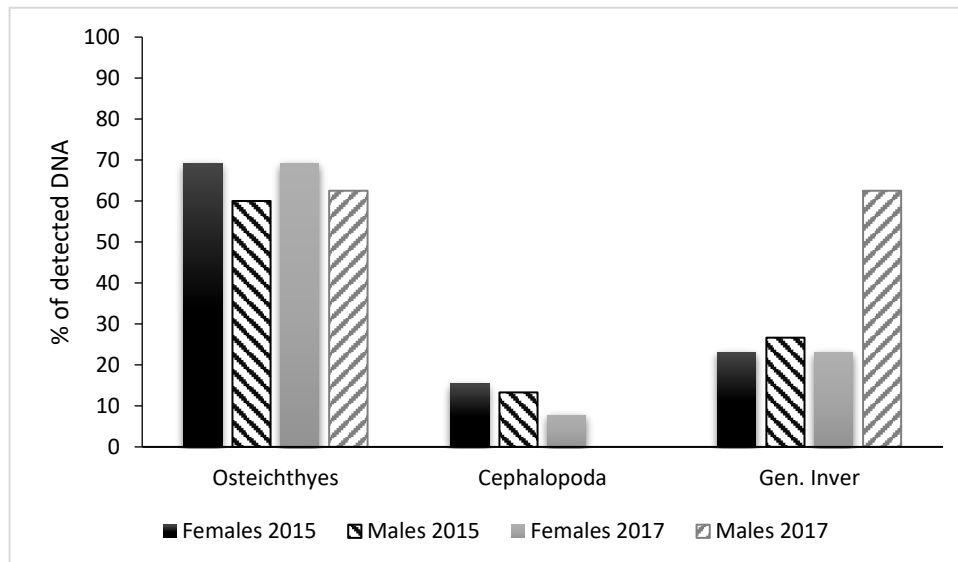


Figure 3 - Proportion (%) of detected fish, cephalopods and general invertebrate's DNA per sex and year for Madeiran storm-petrels in each year.

However, comparing the diet for each sampled Madeiran storm-petrel, we can see that in 2015 females present a larger proportion of individuals with mixture prey DNA than males, while males' diet presents larger detection of one prey type, such as general invertebrates DNA only and cephalopods DNA only (Fig. 4).

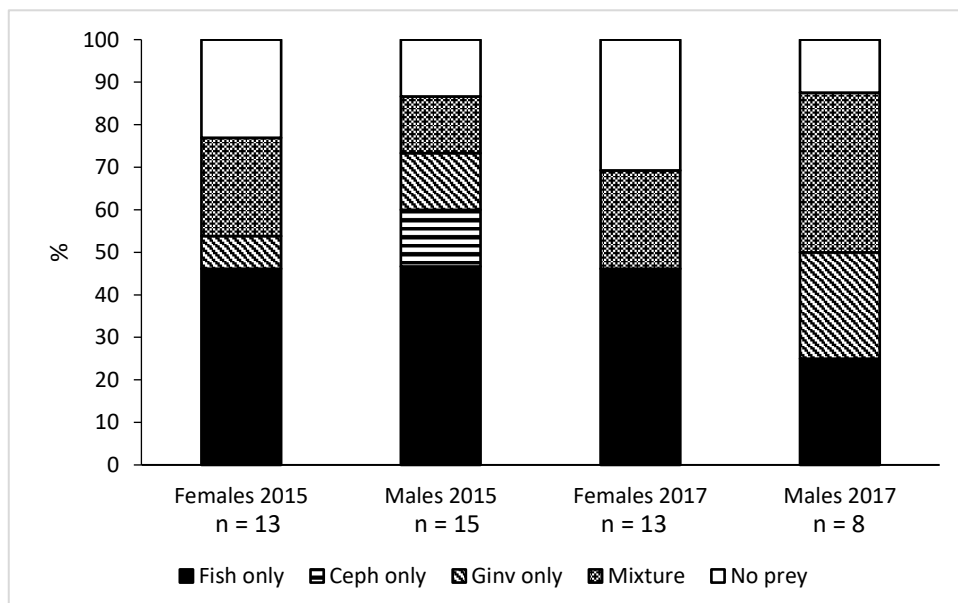


Figure 4 - Proportion (%) which tested positive for only fish DNA, only cephalopod DNA, only general invertebrates DNA, mixture (two or three types of DNA) or yielded no prey DNA per sex for Madeiran storm-petrels in each year.

In 2017, the proportion of fish-DNA only detected for females was maintained, but DNA for general invertebrates only was not detected, and the proportion of samples with no prey DNA increased in comparison to 2015. Males showed differences in diet between 2015 and 2017 because the proportion of samples with DNA of only fish decreased, and the proportion of samples with a mixture of DNA increased (Fig. 4).

3.3. Stable Isotopes

The blood stable isotope values differed between years (MANOVA, Wilk's λ , $F_{2,46}= 4.68$, $P= 0.01$), where carbon isotopic values were significantly lower in 2015 when compared to 2017 ($F_{1,46}= 9.38$, $P= 0.004$, Table 3). The stable isotope values for P1 feathers did not show any significant differences between years nor sexes, but the stable isotope values for S8 feathers differed in the interaction between sex and year (MANOVA, Wilk's λ , $F_{2,46}= 3.20$, $P = 0.0499$), where males presented significantly higher nitrogen isotopic values than females ($F_{1,46}= 6.40$, $P= 0.015$, Table 3), specifically females from 2017 showed lower values than the other groups ($F_{1,46}= 4.68$, $P=0.036$, Table 3).

Table 3 - Results of a factorial analysis of variance (ANOVA) showing multiple comparisons of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for female and male Madeiran storm-petrel for each year. Post-hoc multiple comparisons made with Tuckey test. Significant effects are shown in bold.

	$\delta^{13}\text{C}$ (‰)			$\delta^{15}\text{N}$ (‰)		
	F	P	Main effects	F	P	Main effects
Blood: breeding season						
Sex	$F_{1,46} = 1.52$	0.224		$F_{1,46} = 1.65$	0.206	
Year	$F_{1,46} = 9.38$	0.004	2017 > 2015	$F_{1,46} = 0.04$	0.852	
Sex*Year	$F_{1,46} = 0.02$	0.887		$F_{1,46} = 1.08$	0.304	
P1 Feathers: end of breeding period						
Sex	$F_{1,46} = 0.00$	0.985		$F_{1,46} = 0.24$	0.624	
Year	$F_{1,46} = 0.00$	0.955		$F_{1,46} = 0.03$	0.875	
Sex*Year	$F_{1,46} = 0.10$	0.759		$F_{1,46} = 0.22$	0.643	
S8 Feathers: non-breeding period						
Sex	$F_{1,46} = 0.15$	0.701		$F_{1,46} = 6.40$	0.015	males > females
Year	$F_{1,46} = 3.58$	0.065		$F_{1,46} = 0.95$	0.334	
Sex*Year	$F_{1,46} = 0.60$	0.443		$F_{1,46} = 4.68$	0.036	females 2017 < others

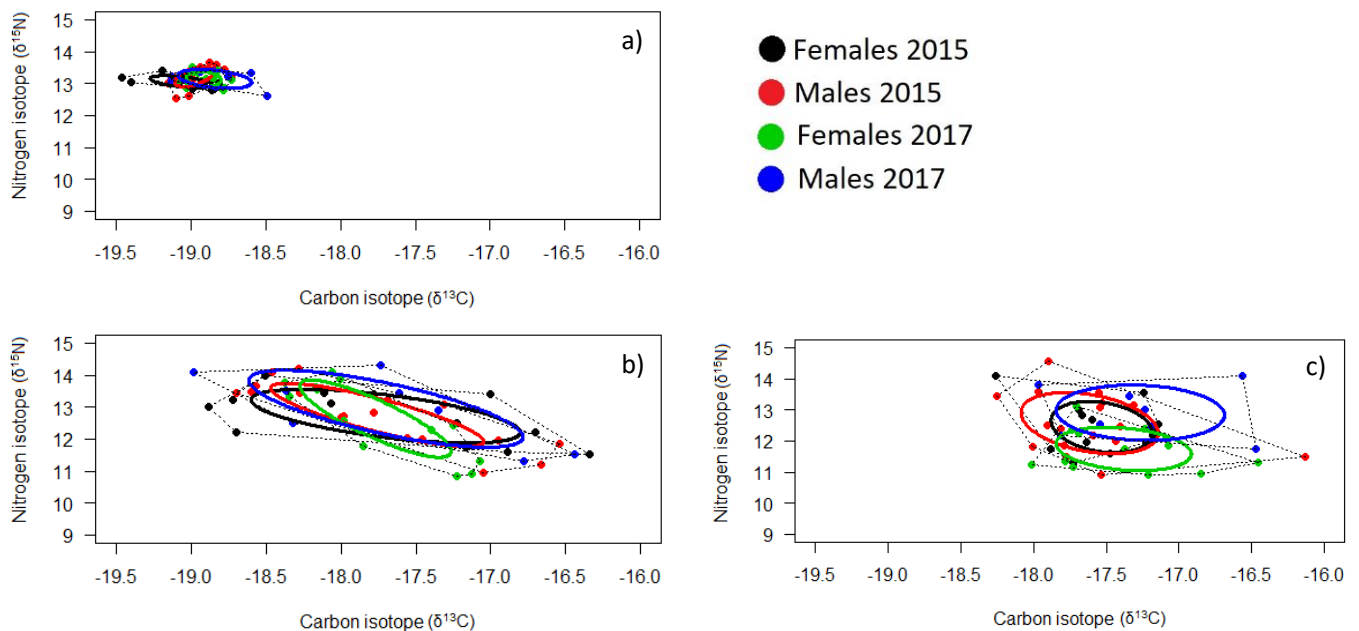


Figure 5 - Annual comparison of isotopic niche space of Madeiran Storm-petrel between (F) females and (M) males, using a) whole blood, b) 1st primary and c) 8th secondary.

During the breeding season, SIBER analysis showed that the narrower isotopic niches occurred in females for both years (Fig. 5-a, Table 4), while the widest isotopic niches occurred for males in 2017 for the end of the breeding season, followed by females in 2015 in the same season (Fig. 5-b, Table 4). For the non-breeding season, SIBER analysis showed the widest isotopic niches for males in both years (Fig. 5-c, Table 4). Niche width pairwise comparisons between sexes and years showed no significant differences in any season (SEAB; all $P > 0.14$, Table 4). However, males and females overlapped much more during the breeding season than in the non-breeding season (Table 4).

Table 4 - SIBER outputs: area of the standard ellipse (SEAC) for Female and Male Madeiran Storm-petrel for each year, and the overlap between these groups for each tissue. P-values based on Bayesian estimates of standard ellipses (SEAB) which assess possible niche width differences; and the of layman metric of convex hull area (TA).

	SEAc			SEAB	TA	
Blood: breeding season						
Year	Female	Male	Overlap	P	Female	Male
2015	0.10	0.11	0.04	0.62	0.21	0.25
2017	0.08	0.21	0.08	0.14	0.18	0.28
P1 Feathers: end of breeding period						
Year	Female	Male	Overlap	P	Female	Male
2015	1.89	1.18	1.08	0.77	3.72	2.85
2017	1.01	2.33	0.80	0.17	1.86	3.43
S8 Feathers: non-breeding period						
Year	Female	Male	Overlap	P	Female	Male
2015	0.87	1.37	0.38	0.27	1.85	3.80
2017	0.99	1.61	0.17	0.18	1.79	2.74

3.4. Tracking during the breeding season

Tracking data of four individuals during the breeding period of 2016-2017 (November 2016 - January 2017) shows that Madeiran storm-petrels have a wide home range (95% kernel UD). Nevertheless, the tracked individuals concentrated their foraging activity (50% kernel UD) in two main areas, the colony surroundings, and foraging up to 650km south, close to the African coast (Fig. 6).

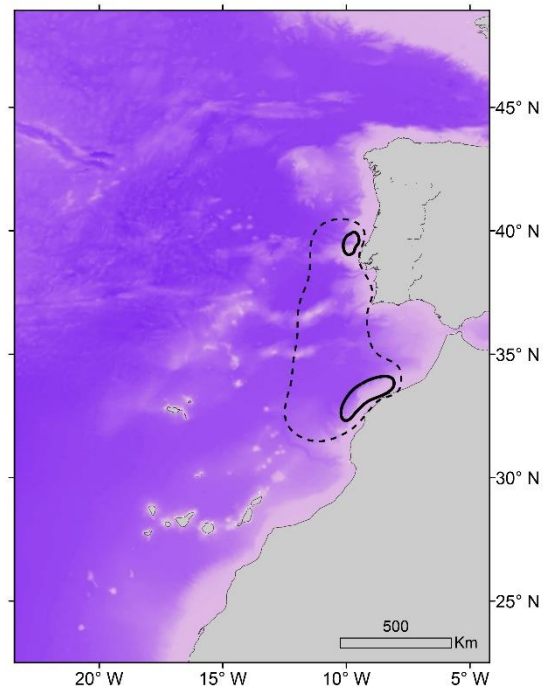


Figure 6 - Home range (95% kernel Utilization Distribution (UD); dashed line) and foraging area (50% kernel UD; filled line) of Madeiran storm-petrels from Farilhão Islet (Berlengas archipelago) during the incubation and early chick-rearing periods (November 2016 - January 2017). Bathymetry represented in the background varying from 1m (pink) to 3800m (blue) depth.

3.5. Trophic ecology of storm petrels in the North Atlantic

After summarizing the isotopic data of our study species with that of other storm-petrels from the Atlantic (Fig. 6), we can see a geographical gradient of nitrogen isotopic values, decreasing from northern to southern breeding sites. The species breeding further north, the European storm-petrels from Iceland, foraged at the highest trophic levels. This species also showed the largest variation in isotopic values, in contrast with the values presented by the Monteiro's storm-petrel, a species that presented the smallest variability in isotopic data. In the carbon stable isotope axis, we can see an interspecies gradient, with European storm-petrels showing the highest values as well, followed successively by Madeiran, Monteiro's and Leach's storm-petrel.

The occupied prey space calculated for both studies of *H. castro* matched essentially with the isotopic values of glacier lanternfish and sardine, mesopelagic and pelagic fish respectively. For our study species, the occupied prey space also overlaps with the isotopic space of hyperiid amphipods.

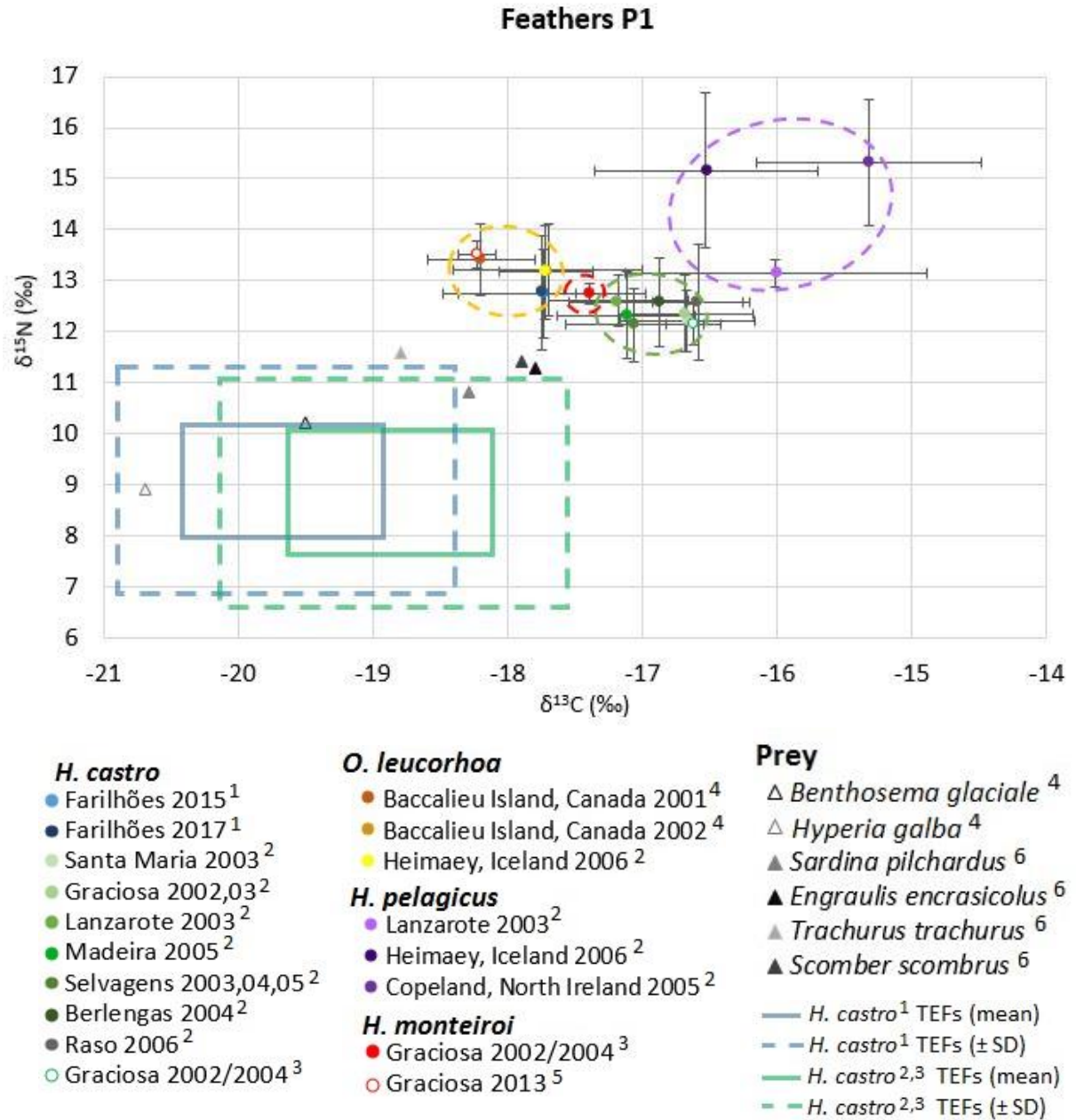


Figure 7 - Isotopic ratios (mean ± SD) from P1 feathers of Madeiran storm-petrel (*H. castro*), Leach's storm-petrel (*O. leucorhoa*), European storm-petrel (*H. pelagicus*) and Monteiro's storm-petrel (*H. monteiroi*). Values based on (1) this study; (2) Roscales et al. 2011; (3) Bolton et al. 2008; (4) Hedd & Montevecchi 2006 and (5) Paiva et al. under review. Prey isotopes values in (4) Hedd & Montevecchi 2006 and (6) Meier et al. 2016. Boxes represent the expected range in prey isotope values of *H. castro*, based on mean diet-feather trophic enrichment factors of 3.7‰ $\delta^{15}\text{N}$ and 1.9‰ $\delta^{13}\text{C}$ (solid boxes), and standard deviations of 1‰ $\delta^{15}\text{N}$ and 0.5‰ $\delta^{13}\text{C}$ (dotted boxes).

4. Discussion

«A thousand miles from land are we,
Tossing about on the roaring sea;
From billow to bounding billow cast,
Like fleecy snow on the stormy blast:
(...)
Up and down! Up and down!
From the base of the wave to the billow's crown,
And midst the flashing and feathery foam
The Stormy Petrel finds a home»

The Stormy Petrel
Barry Cornwall



Edited picture from João T. Vasconcelos

This study integrated sexual, seasonal (breeding and non-breeding period), and temporal (2 years) information on trophic variability, to assess the foraging ecology of the Madeiran storm-petrel breeding on Farilhão islet, Berlengas archipelago, Portugal. During the breeding season, there were no significant effect of sex and year on the diet of Madeiran storm-petrels, nor in the $\delta^{15}\text{N}$ values; and there was a significant effect of the interaction between year and sex on the $\delta^{15}\text{N}$ values during the non-breeding season, when females from 2017 exhibited significantly lower values than males in both years. Sexual dimorphism on this species, with females exhibiting significantly longer wings than males, might play a role on the former dietary and trophic differences. Sexual dimorphism have also been reported for European storm-petrels (Medeiros-Mirra 2010) and Monteiro's storm-petrels (Paiva et al. under review), and is considered the main driver of intersexual differences in the trophic ecology of Monteiro's storm-petrels during both the breeding (P1 feathers) and non-breeding (S8 feathers) periods.

4.1. Foraging ecology during the breeding season

Although there were no significant differences between sexes and years in the proportions of the various diet groups detected, our results showed an overall preference for fish, with females feeding on a more mixed diet than males. One of the limitations of the method applied is the impossibility to conclude in which development stage the prey is, since this method only detects prey DNA (Medeiros-Mirra 2010). In this sense, the large proportion of fish detected can still be fish larvae or eggs, in which zooplankton feeds, and thus it is not possible to conclude if its diet is based essentially on zooplankton, as expected for this storm-petrels (Monteiro *et al.* 1996a). Nevertheless, this species showed high $\delta^{15}\text{N}$ values, similar to those reported for the Leach's storm-petrels in Canada (Hedd & Montevecchi 2006) which fed essentially on adult Myctophidae fish, suggesting that Madeiran storm-petrels may also feed at higher trophic level prey (e.g. myctophidae fish) rather than being a zooplanktivorous seabird. Actually, one of handled birds during this study regurgitated a myctophid, which empirically, though not strongly, corroborates our assumption. A

further step in the detection of which species this seabird feeds on, through pyrosequencing, will allow a better understanding of its feeding ecology, which in future studies should be complemented with an analysis of isotopic values of prey samples from different trophic levels and from its foraging areas, or even complemented with analysis of fatty acids. The shift presented in the 2017 males' diet was probably influenced by the smaller sample size in 2017 ($n = 8$), compared to the other subsets. Finally, the substantial proportions of samples that resulted in no prey DNA can be related with two factors: a) failure in detecting prey DNA, and/or 2) lack of specific primer sets to detect other prey groups, e.g. for mammals, because storm-petrels can eventually have access to dead dolphins or whales through scavenging, as detected in the diet of European storm-petrel (Medeiros-Mirra 2010).

Concerning the distribution of this species during the breeding season, although only four individuals were tracked, the results were accordingly with the reported by Oliveira *et al.* (2013) from November of 2011. It seems that Madeiran storm-petrels breeding in Farilhões islet adopt two foraging strategies: short distance trips near the colony, probably to feed their chicks, and longer distance trips near the African coast, probably to restore their body condition. It is understandable why Madeiran storm-petrels opt to forage in these main foraging areas: the West African coast is an hotspot of marine biodiversity, exhaustively used by other top predators and by international fishery fleets, because it is an area with a high marine productivity (Paiva *et al.* 2015). On the other hand, the Portuguese coastal is characterized by its extensive shallow foraging grounds, influenced either by cold northern or temperate southern winds, which allows also high productivity (Pardal & Azeiteiro 2001). For this reason, this area is an essential migration corridor for seabirds reproducing in the North Atlantic or wintering further south (Stenhouse *et al.* 2012). Finally, maximum distance from colony were similar to values registered for the Leach's storm-petrels during the incubation period at Bon Portage Island in both 2012 and 2013 (Pollet *et al.* 2014b). This reinforces the idea that storm-petrels need long distance trips to build up their own body conditions, as is the case of other procellariiforms (Weimerskirch 1998).

Differences in the stable carbon isotope values between years for the breeding season, with higher values in 2017, meaning that this species foraged closer to coastal

areas during this year, were probably related with the different timing of collection of blood samples, because in 2015 samples were collected during incubation, while in 2017 it was during chick rearing. This is explained by the general foraging strategies of pelagic seabird species, because during the chick-rearing period breeders generally tend to forage in the colony surroundings, to regularly visit the nest and successfully raise the chick (Paiva *et al.* 2015). In 2015, birds may have foraged in more oceanic areas, instead of making long trips to the African coast as observed in the tracking data of 2017, thus exhibiting comparatively lower carbon isotopic values (France 1995; Quillfeldt *et al.* 2005). Because this species showed no significant differences between sexes in stable nitrogen isotope values, and presented a large overlap in their isotopic niche, we can assume that foraging strategies of both males and females are similar during the breeding season.

4.2. Foraging ecology during the non-breeding season

Seabirds' foraging ecology is normally influenced by spatial and temporal fluctuations in prey availability (Paiva *et al.* 2013a), and large-scale climatic events such as the ones depicted by the North Atlantic Oscillation (NAO) index play a huge part on these fluctuations (Sandvik & Einar Erikstad 2008). In more generalist and/or opportunistic species, its diet may vary in response to this variation in availability, but more specialized species, i.e. with lower plasticity, will be more severely affected by poor environmental conditions. An example of this is the Macaronesian shearwater (*Puffinus baroli*), which is sensitive to such fluctuations, changing its diet and foraging distribution in years of poor conditions (Ramos *et al.* 2015). Around the Portuguese and African coastal areas, poor environmental conditions are depicted by negative values of the winter North Atlantic Oscillation index (wNAO), which derives from storms and intense winds in these areas, leading to an unusually strong upwelling in these coasts. This phenomenon drives plankton away from the shore, leading to its death (Robinson 2004; Santos *et al.* 2004), resulting in low abundance of prey for seabirds. These poor conditions also may lead to differences between sexes in their foraging ecology (Phillips *et al.* 2011), since females and males adopt different feeding

strategies to reduce competition between them. This has been reported in years when the resources are scarcer and in species with evident sexual dimorphism, such as Cory's shearwater (Ramos *et al.* 2009a; Paiva *et al.* 2017).

In our study, nitrogen stable isotope values showed differences between sexes for the non-breeding period, most noticeably in 2016, when females fed on prey of lower trophic levels. Since it only occurred in one year, and the difference between males' and females' nitrogen isotope values was from 1 to 1.5‰ (i.e. <1 trophic level), this variation may come from differences in the relative amount of different prey taken. Nevertheless, this intersexual difference was observed for another storm-petrel species, the Monteiro storm-petrel, in which males also preyed at higher trophic levels than females during the non-breeding period (Paiva *et al.* under review). In 2013, the year when these intersexual differences were detected in Monteiro's storm-petrels, wNAO values were very low (-1.97). In 2015, the first year of our study where no differences between sexes were detected, the wNAO was very high (3.56), while in 2016 it dropped to 0.98 (Hurrell 2017). It seems that the feeding ecology of this species can be influenced by environmental conditions as well, and this is further supported by the lower niche overlap detected in this season, where both sexes seem to avoid foraging in the same areas. However, only a collection of more data during subsequent years, along with complementary information on distribution and diet will allow to understand these intersexual isotopic differences.

The larger isotopic niche during the non-breeding season has already been reported for several other seabirds and is related to the fact that when seabirds are not in their reproductive duties, and thus without the need to restrain their foraging area to the colony surroundings, they adopt different foraging strategies and may forage in wider oceanic areas, preying from higher to lower trophic level prey, thus showing a larger isotopic niche (Hedd *et al.* 2010).

4.3. Trophic ecology of storm petrels in the north Atlantic

The latitudinal gradient in nitrogen values observed in this study is in line with previous studies which found a decrease of $\delta^{15}\text{N}$ values in plankton, zooplankton and particulate organic matter (POM) from the north temperate regions to the tropical regions (Graham *et al.* 2010; Ramos & González-Solís 2012). Noticeably, Leach's storm-petrels migrate in the inter-breeding season to sub-tropical regions, and their primaries start to moult during this migration season, therefore the nitrogen isotope values may be lower than expected due to this factor (Hedd & Montevecchi 2006; Pollet *et al.* 2014a).

In both years of our study, seabirds presented nitrogen isotopic values similar to those reported by Roscales *et al.* (2011), showing a consistency with the trophic level's prey that they consume. However, comparing those values with the expected range in prey isotope values of *H. castro*, we can somehow infer that birds from our study may have fed in different quantities and/or different prey, since the isotopic prey space calculated for both studies differ substantially in $\delta^{13}\text{C}$ values. This is supported by the Leach's storm-petrel's diet in Bacallieu Island, where hyperiid amphipods were one of the main prey of their diet in the absence of myctophid fish (Hedd & Montevecchi 2006). Because the isotopic signatures of the Madeiran storm-petrel of our study were similar to those of Leach's storm-petrels, we guess that they were feeding in similar quantities of this trophic level prey. However, this extrapolation is limited to the few studies that presented prey isotopic signatures from lower trophic levels, and only a study of diet during the non-breeding season would be conclusive about the prey they ingest.

The detected interspecies gradient in $\delta^{13}\text{C}$ values between storm-petrel species can be related with the different capability of each species to reach different productive areas (Shealer 2002). This is also in line with the results of Roscales *et al.* (2011), where $\delta^{13}\text{C}$ values for several other procelariiform species provided an axis of variability, with each species occupying a range of carbon stable isotope, with almost no overlap among them. However, in Monteiro's storm-petrel in 2013 (Paiva *et al.* under review), and in both years of our study, birds went against this tendency in $\delta^{13}\text{C}$

values, fitting more closely to the Leach's storm-petrels. This might be related with a variation in the timing of migration and/or the moult location, or, comparing the calculated occupied prey space of Madeiran storm-petrels, in our study case, birds may had ingested more quantities of mesopelagic fishes.

Finally, it was also possible to observe that storm-petrels from northern areas of the Atlantic Ocean tend to be more generalists than species from southern sites, with European storm-petrels showing the highest variability in isotopic values. This species was already reported to have an opportunistic behaviour, feeding on prey of higher trophic levels through scavenging (Medeiros-Mirra 2010), while *H. monteiroi* exhibits a small variation in their isotopic values (Paiva et al. under review), and is somehow more specialized than the other storm-petrels.

4.4. Final considerations and limitations

The remoteness of the islet where our population breeds and the phenology of the study species (winter breeder) made islet visitation for sample collection extremely difficult and highly dependent on the sea state and weather conditions. That was the reason to collect samples in 2015 during the incubation period, while in 2017 the species was already chick-provisioning at sample collection. Tracking information is yet scarce for the same reason, though next September an extra effort will be made to retrieve the devices left year-round on the bird's, with researchers staying longer on the islet to increase chances of loggers' retrieval.

This was one of the first studies to use molecular techniques to detect diet of storm-petrels, and the next logic step is to use our molecular extractions on Next-Generation Sequencing (NGS). Indeed, samples are now being analyzed in that respect, which will allow the precise identification of each single taxa contained in each sample. Such data was not included on this thesis due to time constraints, but will integrate a future scientific publication of this work.

Appendix A - Some of the results of PCR tests for General Invertebrate primer pair (MICO1intF / CI-N-2191) performed on target and non-target species (Stockdale *et al.* In prep). Symbols indicate presence (+) or absence (-) of DNA amplification. Results represented with * indicates doubles banded results, where showed a non-specific banding as well as the expect band.

	Sample	Result
Target	Ant	+
	Crane fly	+
	Earwig	+
	Ladybird	+
	Slug	+
	Snail	+
	Spider	+
	Worm 1	-
	Worm 2	+
	Worm 3	+
	Worm 4	-
	Worm 5	+
	Worm 6	+
	Worm 7	+
	Worm 8	-
	Worm 9	+
	Worm 10	+
Non-target	Blackbird	+
	Crane	+*
	Robin	-
	Songthrush	+*
	Woodpigeon	+*

Appendix B - Results of PCR tests for General Invertebrate primer pair (MICO1intF / CI-N-2191) performed on marine invertebrates. Symbols indicate presence (+) or absence (-) of DNA amplification.

	Sample	Result
Amphipoda	<i>Apherusa jurinei</i>	+
	<i>Atylus swammerdami</i>	+
	<i>Dexamine spiniventris</i>	+
	<i>Echinogammarus planicrurus</i>	+
	<i>Jassa marmorata</i>	+
	<i>Jassa ocia</i>	+
	<i>Jassa pusilla</i>	-
	<i>Melita hergensis</i>	+
	<i>Pontocrates arenarius</i>	+
Decapoda	Larvae	+
	Megalopod	+
Hydrozoa	Medusa	+
Isopoda	<i>Dynamene</i> spp.	-
	<i>Eurydice pulchra</i>	+
	<i>Paragnathia formica</i>	+
	<i>Sphaeroma</i> spp.	+
	Unidentified spp (from Walles)	+
	Unidentified spp (from Walles)	+
	Unidentified spp (from Walles)	+
	Unidentified spp (from Walles)	+
	Unidentified spp (from Walles)	+
	Unidentified spp (from Walles)	+

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