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Metabarcoding, Stables Isotopes and Tracking: unraveling the trophic ecology of a winter-breeding storm-petrel (*Hydrobates castro*) with a multimethod approach.

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Abstract

Detailed information on diet and foraging ecology is scarce for most small seabirds such as storm-petrels. In this study, we used molecular techniques, stable isotope analysis, and geolocators to study the diet, trophic ecology, and at-sea distribution of Madeiran storm-petrels (*Hydrobates castro*) breeding in Farilhões Islet, Portugal, in 2015 - 2017.

The diet of Madeiran storm-petrels was dominated by fish for both sexes and study years, with Gadidae representing the main prey family. In 2017, females also fed on Aulopiformes, Stomiiformes and Myctophiformes, which were not identified in the other groups, suggesting some degree of inter-annual and intersexual plasticity in their diet. The carbon isotopic ratios of birds during 2017 were significantly higher when compared to 2015, which might be related to foraging near coastal areas in 2017. Indeed, tracking data for 2017 show that birds foraged near the colony and near the West African coast.

Overall, both sexes of this species exhibited a similar trophic ecology and diet during the breeding season. However, intersexual differences occurred during the non-breeding season, when females showed significantly lower nitrogen

isotopic ratios than males (in 2016), and the lowest niche overlap between sexes occurred. This, together with the fact that environmental conditions appeared less favourable in 2016 suggests that intersexual differences in the foraging ecology of this species may be related with environmental conditions.

Introduction

As marine top predators, seabirds reflect changes that occur at lower trophic levels. Physical and biological changes in the ocean, such as differences in temperature and marine productivity, determine the distribution and abundance of marine organisms, which can be reflected in dietary changes and abundance of predators (Springer *et al*. 1984). Overall, a seabirds' trophic ecology gives relevant information about its relationship with lower trophic levels, providing essential data for their conservation and ecosystem management (Iverson *et al*. 2007; Xavier *et al*. 2011). Seabird species from the order Procellariformes, such as the albatrosses, petrels, and shearwaters, have been used as sentinels of environmental conditions (e.g. Paiva *et al*. 2015), since they present extreme life-history characteristics (Warham 1990; Granadeiro *et al*. 1998b), and their behaviour changes noticeably as a response to marine environmental variability. However, there is very little information on the potential of smaller seabird species, such as the storm-petrels, to be used as sentinels of marine ecosystems. As lower trophic level consumers, e.g. feeding on zooplankton, storm-petrels can alert to environmental changes at a faster speed than comparatively larger seabirds (Grémillet *et al*. 2015). Additionally, some species of storm-petrels reproduce in winter, which makes them a potential sentinel for changes in environmental conditions during this specific season (Gremillet and Charmantier 2010).

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. Some non-invasive techniques, such as Stable Isotope Analyses (SIA), have been used to study the trophic ecology of several seabird species (e.g. Roscales *et al.* 2011), including storm-petrels (e.g. Gladbach *et al.* 2007). However, SIA by itself gives an unclear response of precise trophic interactions. SIA rarely indicates which specific prey species are consumed by seabirds, giving instead information on the trophic level of their prey (Iverson *et al*. 2007; Traugott *et al*. 2007). Complementary methods have been used, namely a tracking system of seabird movements over long periods of time, like Global Location Sensing (GLS) devices. The information gathered by these devices, together with data obtained from SIA, make it possible to build biogeographic patterns of stable isotopes in the marine ecosystem. It is now known that isotopic ratios change throughout different latitudes, depending on the distance to the shore or benthic habitats, providing an estimated geographic gradient (i.e. isoscapes) of the ocean (Ramos *et al*. 2009; Graham *et al*. 2010; Ceia *et al*. 2018). As for the majority of Procellariformes, which are known for having large foraging areas during the breeding season, with even longer distant movements during migration, studies on storm-petrel distribution show that these birds, despite their small size, also undergo long distance movements. For example, Leach's storm-petrels (*Hydrobates leucorhous*) breeding in Canada, from two colonies located only 380 km apart, showed distinct foraging locations and ranges for each population during the breeding season (Pollet *et al*. 2014b). Birds from these colonies were also tracked during the non-breeding

season. They showed distinct wintering distributions: when storm-petrels from one colony migrated to the Brazilian coast, the others ventured to the African coast, surrounding Cape Verde (Pollet *et al*. 2014c). Although the combination of these techniques shows a strong potential to study the trophic ecology of seabirds, it has only been used for a small number of storm-petrel species (e.g. Pollet *et al*. 2014a; Halpin *et al*. 2018; Paiva *et al*. 2018).

Non-invasive molecular techniques such as DNA metabarcoding, have been used to study the diet of many vertebrate species, including seabirds over the past 16 years, where prey DNA has been identified from faeces, vomit, and regurgitations (Symondson 2002). This technique has been successfully used in the study of the diet of European storm-petrel's *Hydrobates pelagicus*, showing that this species has an opportunistic behaviour, feeding not only on abundant prey in its habitat, such as fish, cephalopods, amphipods or isopods, but also on unexpected prey such as dolphins, through scavenging (Medeiros-Mirra 2010). The great potential of these molecular techniques has led to rapid development of more efficient methods, such as high-throughput sequencing (HTS) technologies (Valentini *et al*. 2009). Most studies using HTS to infer seabird diet have been used on penguins, allowing DNA of several Osteichthyes and Cephalopod's species to be detected in faeces of several species, such as Little *Eudyptula minor*, Adelie *Pygoscelis adeliae*, Gentoo *Pygoscelis papua*, and Macaroni penguin *Eudyptes chrysolophus* (Deagle et al. 2010; Jarman et al. 2013; Horswill et al. 2018; Xavier et al. 2018). Despite its potential, HTS techniques have never been used to study storm-petrel diet, nor for detailed diet studies of winter breeding storm-petrels in the North Atlantic.

Several studies have reported sex-specific differences in seabird trophic ecology and behaviour (González-Solís *et al*. 2000; Kato *et al*. 2000; Lewis *et al*. 2005). Such differences normally occur in species with Sexual Size Dimorphism (SSD), but in monomorphic species, where SSD does not occur, differences in trophic ecology between sexes are expected to be smaller (Paiva et al. 2018). However, recent studies on monomorphic seabirds' species have shown sex-specific foraging patterns to occur (Welcker *et al*. 2009; Elliott *et al*. 2010), explained by the "intersexual competition hypothesis". This hypothesis suggests that one sex may forage more efficiently, outcompeting the other and originating different foraging niches, or even resulting in sexual segregation in foraging areas (Lewis *et al*. 2002; Peck and Congdon 2006). Also, the "energetic constraint hypothesis" suggests that the parents invest differently throughout breeding stages, resulting in different self-provisioning effort between sexes (Elliott *et al*. 2010). In monomorphic storm-petrels, Phillips *et al.* (2009) did not find any significant sex-specific differences in two species' trophic ecology. However, more recently, intersexual differences have been found in the trophic ecology and distribution of Monteiro's Storm-petrel during incubation and chick-rearing periods (Paiva *et al*. 2018), where females fed on lower trophic levels and foraged in significantly higher latitudes than males.

This study investigates the diet, trophic ecology and at-sea distribution of the Madeiran storm-petrel *Hydrobates castro* breeding in Farilhões Islet, Portugal. *H. castro* is a medium sized storm-petrel (Monteiro *et al*. 1996b) breeding in oceanic islands from equatorial to subtropical latitudes, mostly in winter (Monteiro and Furness 1998). There are some records of their distribution around the Portuguese coast and the archipelagos of Madeira and Azores throughout the year, suggesting that this species does not migrate extensively (Meirinho *et al.* 2014). Very little is known about the feeding ecology of this species; it is thought that their diet is based on zooplankton and small mesopelagic fishes, as are other similarly sized storm-petrel species (Monteiro *et al*. 1996b), but so far there is no comprehensive information about the diet of *H. castro.* A comparative study about the trophic ecology of Atlantic Procellariformes in several breeding sites at the end of the breeding season showed that the Madeiran storm-petrel exhibits a small isotopic niche, displaying similar isotopic ratios between different sites and years, with few spatial differences and little variability between years (Roscales *et al*. 2011). Therefore, we expect Madeiran storm-petrels to: (1) show a generalist diet composition, not restricted to zooplankton; (2) 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 a single 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. However, given the close phylogenetic proximity to the Monteiro's storm-petrel in which such differences occur (Paiva *et al*. 2018), our species may present sexual segregation in its foraging ecology. To our knowledge, this is the most detailed and comprehensive study on the foraging ecology of a winter breeding storm-petrel, as most studies are on summer breeding populations (e.g Pollet *et al*. 2014b). Overall, this study will not only present baseline information on the foraging ecology of this species, but also will provide a comprehensive framework for the conservation and management of other winter breeding storm-petrels.

Methods

Study area and species

This study was conducted on Farilhão Grande Islet (39° 28' 31" N, 9° 32' 45" W), within Berlengas archipelago, offshore Peniche, Portugal. Farilhão Grande Islet is characterized by rocky substrate, with steep and vertical cliffs, where approximately 100 to 200 breeding pairs of Madeiran storm-petrels are estimated to breed (Mendes 2013). This species arrives at the islet to breed between August and September, nesting in cavities, and departs around February (Granadeiro *et al.* 1998).

Field Sampling

We captured Madeiran storm-petrels using mist-nets placed along the rocky shore; birds were not captured on the nest in order to avoid nest desertion (Rodway *et al.* 1996; Blackmer *et al*. 2004). Fieldwork was conducted over two breeding seasons (2015/2016 and 2016/2017), hereafter referred to as 2015 and 2017. In the first breeding season, fieldwork for sample collection was carried out on 10 November 2015 during the egg incubation period, and in the second breeding season on 18 January 2017, during the chick-rearing period. A total of 30 and 21 individuals were captured in 2015 and 2017, respectively. Differences in sampling methodology were related to poor weather conditions, which prevented access to the colony during the 2016 incubation period.

For all birds captured, their body mass, tarsus, and wing length were measured. Approximately 1 cm from the tip of the first primary and the eighth secondary feather were also collected and stored in polythene bags for SIA. A blood sample (~50 μL) was taken from the brachial vein and stored in 2-mL tubes with 70% ethanol for both stable isotope analysis and molecular sexing. Birds were placed inside a box for a maximum of 15 minutes, in order to let the birds defecate naturally, obtaining a total of 28 and 21 faecal samples from 2015 and 2017, respectively. Faecal samples were stored in 2-mL tubes with 70% ethanol, and the bottom of the box was lined with plastic or tinfoil and replaced between each individual.

Six birds that were ringed in previous years and were known to nest in this colony were instrumented with Global Location Sensing (GLS) devices (model MK18L, BioTrack Lda.) in 2017 breeding season. Loggers were back-mounted with a cotton harness, in January 2017, and when birds were re-captured, the logger information was downloaded without taking the device off the bird. It was possible to get back the tracking information from four individuals during the early chick-rearing period (January-February 2017). GLS devices represented less than 1% of the bird's body mass in order to not impair the birds survival (Pollet et al. 2014c; Kürten et al. 2019).

Sex and diet determination using molecular tools

Molecular sex determination was carried out using an individual's whole blood sample using an adaptation of the Chelex DNA extraction method (Medeiros-Mirra 2010, see Online Resource 1). DNA from storm-petrel faecal samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen), following Zeale et al. (2011). Four primer sets (Online Resource 2) were used to target different prey types in order to ensure good coverage 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 18S, 28S and 12S primer sets have been previously used for seabirds (Deagle *et al*. 2007; Medeiros-Mirra 2010), whereas the COI general invertebrate primer has not been used in prey detection of seabirds before, but has been shown to successfully amplify a wide range of target and non-

target species (Stockdale 2018). In this study, initial testing of the general invertebrate primer pair against reference marine invertebrate DNA and DNA from Madeiran storm-petrel showed positive results and confirmed that the primer was specific to invertebrates, with no amplification of predator's DNA (Online Resource 3). Each primer pair was labelled separately for males and females with a unique forward and reverse multiplex identifier (MID) tag. The PCR recipe and thermal profile were as described in Online Resource 2.

Samples were pooled by sample group (males and females for 2015 and 2017) and primer pair according to intensity of the PCR product on a 1.5 % agarose gel stained with SYBR®Safe (Thermo Fisher Scientific, Paisley, UK) when compared to a standardized 100-bp ladder. Only samples where a clear band was visible following electrophoresis were processed further and thus purified using Qiagen kit (QIAquick PCR Purification Kit). Therefore, four pools (from the four sample groups) were produced for all primer pairs, except for the Cephalopoda primers, where we only obtained samples with clear bands for 2015 males and 2017 females, thus resulting in only two pools for this primer pair. The DNA concentration of each pool was quantified using a Qubit (ThermoFisher Scientific, Waltham, MA), and pools were subsequently combined in order to provide four final overall pooled samples with an approximately equal amount of amplicon DNA from each faecal sample. Pooled samples of similar DNA concentration were purified using Agencourt AMPure XP purification beads (Beckman Coulter, Pasadena, CA), and again quantified using a Qubit (ThermoFisher Scientific, Waltham, MA). The four pools of tagged amplicons were used to prepare the libraries for paired-end sequencing using the NEXTFlex Rapid DNA-seq Library Prep Kit for Illumina (Bioscientific, Austin, TX) and sequenced on a MiSeq desktop sequencer (Illumina, San Diego, CA, USA).

Stable isotope analysis

In Madeiran Storm-petrels, primary feather moult starts at the end of January (Monteiro *et al*. 1996a).The isotopic ratios of these feathers taken during the breeding season represent the trophic ecology of the individuals when they were formed (Ramos and González-Solís 2012), so during the end of the previous breeding period, thus early-2015 and early-2016. Secondary feathers moult in August (Bolton *et al*. 2008), so they represent the end of the non-breeding season, thus summer of 2015 and 2016. Blood regenerates quickly, representing the season when collected, i.e. the breeding season in our study (October-November 2015 and December 2016-January 2017).

Feathers were cleaned of surface oils and contaminants using a 2:1 chloroform:methanol solution for 15 minutes (three baths of 5 minutes each) and then oven dried at 60°C for 24 h. Once dried, feathers were cut into small fragments using stainless steel scissors, avoiding the shaft. Blood samples were firstly air-dried to remove excess ethanol, then oven dried at 60°C for 24 h. Approximately 0.35 mg aliquots of each sample, both feather and blood, were weighed into tin

capsules and isotopic ratios of carbon ($\delta^{13}C$ (‰) = $\left(\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{V-PDB}} - 1\right) x1000$) and nitrogen ($\delta^{15}N$ (‰) = $\left(\frac{(^{15}N/^{14}N)_{sample}}{(^{15}N/^{14}N)_{air}} - 1\right) x1000$) (Libes 2009; Wada 2009), were determined by continuous-flow isotope-ratio mass spectrometry (CF- IRMS).

Tracking data processing

GLS data was analyzed using the BASTrack software suite (British Antarctic Survey, Cambridge, UK), using a light threshold of 10 and an elevation angle of −4.0 (derived from calibration devices left at an open site without shading at Berlenga Island, located ~7km away from Farilhão Islet). 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 latitude estimates are unreliable. Predicted locations 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. 2010b), we considered the 50 % and 95 % kernel UD contours to represent the core foraging areas (FA) and the home range (HR), respectively.

Data analysis

To test for possible intersexual differences in this population, body measurements (tarsus, wing and body mass) were compared between sexes with two independent samples t-test. Results are described as mean ± SD. To analyse diet detection, a similarity matrix was generated using the Bray–Curtis similarity measure. Adonis tests were run on the matrices using 999 permutations to test for differences in diet screening between sexes and years. However, it was detected that the amphipod primer pair was amplifying predator's DNA, therefore this set was withdrawn from this analysis as it was impossible to distinguish through electrophoresis which samples were amplifying DNA from prey or from predator.

The bioinformatic analysis of HTS data was carried out using a combination of USEARCH v10.0.240 (Edgar 2010) and the Cutadapt package (Martin 2011) on a python script. All commands are provided in the GitHub repository: https://github.com/AnaCarreiro/Carreiro_et_all_MSP. Paired-end reads were merged and then de-multiplexed based on forward and reverse primers and MID tags, as well as stripped from all the adapters. Reads from 12S, 18S, 28S and COI

amplicons were filtered to lengths from 260 to 310 bp, 160 to 220 bp, 110 to 160 bp and 290 to 340 bp respectively, and then merged into a master file for each prey target. All reads were filtered to a maximum of 1.0 Expected Errors (EE). Reads were dereplicated, singletons removed, and clustered into OTUs (Operational Taxonomic Units). The UPARSE pipeline was used for 12S and COI amplicons analysis with a 97% clustering (Edgar 2013) whereas 18S and 28S were analyzed in the UNOISE algorithm (Edgar 2016) with a 99% clustering, as suggested in previous work for these target groups (Bachy *et al*. 2013; Edgar and Flyvbjerg 2015). The total number of sequences retrieved, sequences lost, uniques, singletons, and quimeras for each gene can be found in Online Resource 4.

To taxonomically classify OTUs, MegaBLAST from NCBI database was used (Zhang *et al*. 2000; Morgulis *et al*. 2008), and only results with 100% query cover were considered as matches. The resulting sequences were assigned to taxonomic units using a cut-off of 90% sequence identity for 12S, 28S and COI genes, and 99% sequence identity for 18S gene. These thresholds were based not only on each fragment size and their definitions in previous work using these genes (e.g. Bachy *et al*. 2013), but also considering ecological data, since a lower sequence similarity threshold would result in a mixing of different taxa with no ecological sense. For each OTU, all the reads matching the thresholds defined were considered and analyzed together to classify each group to the lowest taxonomic level possible. Taxon was assigned if the highest query sequences, with the same match, clustered monophyletically at that level. If the sequence matched more than one species from the same genus or family, the lowest (most ancestral) common taxonomic rank was assigned.

Two multivariate analysis of variance (MANOVA; Wilk's lambda statistics) were used to compare differences in both the carbon and nitrogen stable isotopic ratios, as response variables, of (1) blood and (2) feathers (P1 and S8). Such differences were analyzed between (1) years (2015 vs 2017, n= 30 and n= 21 respectively), (2) sex (Female vs Male, n= 26 and n= 25 respectively), and (3) tissues for the comparison between feathers (P1 vs S8, n= 51 each), as independent variables. MANOVAs were followed by separated factorial ANOVAs for each stable isotope and post-hoc multiple comparisons Tuckey test. In order to compare isotopic niches between sexes, years and periods, we used recent metrics based in a Bayesian framework (Stable Isotope Bayesian Ellipses in R: SIBER; Jackson *et al*. 2011). The standard ellipse area drawn using the stable isotopic ratios of nitrogen and carbon, 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 sexes 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). All former computations were performed under R environment (R Core Team 2018).

Results

Sex determination and sexual dimorphism

Sex determination using blood extracts was successful for 51 out of 52 samples, resulting in a total of 13 females and 17 males for 2015, and 13 females and eight males for 2017. Two males and one female were re-captured in 2017, and so the first body measurements taken in 2015 were used in these analyses. Body measurements indicated that females had significantly longer wings than males (160.40 ± 3.89 mm, n = 25 vs 156.57 ± 2.86 mm, n = 23, t_{46}= 3.86, P< 0.001,), but similar tarsus length (23.33 ± 0.79 mm, n = 25 vs 23.34 ± 0.66 mm, n = 23, t_{46}= -0.03, P= 0.97) and body mass (55.31 ± 6.29 g, n = 25 vs 52.63 ± 6.30 g, n = 23, t_{46}= 1.47, P= 0.15).

Diet determination

DNA amplification was successful for all 49 faecal samples, amplifying in at least one of the primer sets. Since the Amphipoda primer was not considered for this analysis, the percentage of samples that were only amplified by this primer (13.0 to 31.0%, Fig. 1) were considered to contain no prey DNA since they also amplify predator DNA. DNA amplification results showed a predominance of fish (Osteichthyes) in the samples of both sexes and years (Fig. 1), with occurrence ranging from 60.0% to 69.2%. Males in 2017 were an exception, where both fish and invertebrates were equally detected (62.5%). However, the sample size for males in 2017 was small (n= 8), which might have influenced these results. The prey group with the lowest number of detections for both sex and year was Cephalopoda, ranging from 0% in 2017 males to 15.4% in 2015 females. There were no significant differences between the proportion of prey groups between years (Adonis, R2 = 0.021, P= 0.444), sexes (Adonis, R2= 0.048, P= 0.156), nor an interaction between these two variables (Adonis, R2= 0.041, P= 0.227). Regarding the results of the HTS, the 18S and COI primers failed to provide any meaningful results due to the low quality of sequences. However, UPARSE detected 15 OTUs for the 12S fish primers, and UNOISE detected 10 OTUs for the 28S Cephalopoda primers (Table 1). The fish OTUs were distributed across two main families, Gadidae and Myctophidae, with five and four OTUs, respectively. The proportion of sequences comprising each OTU varies between the groups, but the largest proportion of sequences was found for Gadidae, with an unknown *Trisopterus* sp. being the most represented in 2015 females (48.80%), while the blue whiting (*Micromesistius poutassou*) comprised the greatest number of prey sequences in the remaining groups (from 38.53 to 62.44%). In 2017 females, although the majority of sequences were represented by Gadidae, other families weighted almost as equally (57.58% vs 42.42%), specially a non-identified family from Stomiiformes (25.06%), and the lanternfish species *Myctophum punctatum* (13.65%). These, together with *Alepisaurus ferox* and other Myctophidae species, were detected exclusively in 2017 females, showing an evident difference between the fish prey consumed by this group compared to

the other groups. OTUs detected exclusively in the other groups, were the European pilchard (*Sardina pilchardus*) in 2015 females, *Trachurus* sp. in 2015 males and a non-identified Lampriform in 2017 males.

From the Cephalopoda primers, *Onykia* sp. from the Onychoteuthidae family represented the majority of sequences in the 2015 male sequences (86.26%) while *Chiroteuthis* sp. had the greater number of sequences in 2017 females (90.07%). Another unknown genus of Chiroteuthidae family, comprising four OTUs, was detected exclusively in 2017 females' diet, as well as another non-identified family from Oegopsida, also comprising four OTUs, which was only detected in 2015 males' diet.

Stable Isotopes

The blood stable isotope values differed between years (MANOVA, Wilk's λ, $F_{2,46}$= 4.68, P= 0.01), with carbon isotopic ratios significantly lower in 2015 compared to 2017 (ANOVA, $F_{1,46}$= 9.38, P= 0.004, Table 2). The stable isotope values for feathers showed a significant sex and year interaction (MANOVA, Wilk's λ, $F_{2,93}$= 3.44, P = 0.0363). Males presented higher nitrogen isotopic ratios than females (ANOVA, $F_{1,93}$= 3.96, P= 0.0495, Table 2), and P1 feathers presented lower carbon isotopic ratios (ANOVA, $F_{1,93}$= 4.80, P= 0.031, Table 2), and higher nitrogen isotopic ratios (ANOVA, $F_{1,93}$= 4.00, P= 0.048, Table 2) when compared to S8 feathers.

SIBER analysis showed that the narrower isotopic niches occurred during the breeding season (Fig. 2a, Table 3), while the widest isotopic niches occurred at the end of the breeding season (Fig. 2b, Table 3). Niche width pairwise comparisons between sexes and years showed no differences in area during breeding season (SEA_B; all P> 0.217, Online Resource 5). However, when comparing the end of breeding season (feather P1) with the non-breeding season (feather S8), differences in area were found between the two seasons, namely 1) for females in 2015, 2) between females 2015 and males 2017 and 3) between females and males in 2017 (SEA_B; all P< 0.021, Online Resource 5). The highest niche overlap between sexes occurred during the 2015 non-breeding season (Overlap: 100%, Online Resource 5), while the opposite occurred in 2017, in the same season (Overlap: 17.6%, Online Resource 5).

Tracking during the breeding season

Tracking data of the four individuals during the breeding period of 2016-2017 showed that Madeiran storm-petrels breeding in Farilhão have a large 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. 3).

Discussion

This study is the most comprehensive work to date on the trophic ecology of winter-breeding storm petrels. It integrated sexual, seasonal (breeding and non-breeding period), and temporal (two 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, males had a similar diet across both years, while a difference was detected in the fish prey consumed by females between the two years of study, although no significant differences were detected in the $\delta^{15}N$ values. This, together with the presence of a large overlap in the isotopic niche between sexes, suggests that the foraging strategies of both males and females are rather similar during the breeding season. However, females had significantly lower $\delta^{15}N$ values than males during the nonbreeding season of 2016.

The diet of Madeiran storm-petrels

The molecular techniques used in this study allowed the identification of many prey taxa to the genus and species levels, some of which would have been unlikely to be identified through traditional methods. However, issues encountered during HTS analysis, specially the unsuccessful test of a new primer pair to identify marine invertebrates, led to conclude that more optimized primers for identification of marine biodiversity must be used. The proportions of samples that were considered having no prey DNA can also be related with: (1) absence or very low concentration of DNA in the sample, possibly indicating a period of fasting from these individuals, (2) failure in detecting prey DNA, potentially due to DNA degradation or the presence of PCR inhibitors, (3) primers' taxonomic resolution: although we used primers that targeted a wide range of prey groups, it is likely that they do not amplify all desired target prey species; (4) lack of specific primer sets to detect other prey groups, (e.g. cartilaginous fish or mammals obtained through scavenging). Issues with failure to detect prey DNA in faecal samples are common across dietary studies (Deagle et al. 2007). Primer choice is unlikely to be the main explanation for this since the primers' specificity was tested in a wide range of prey, as well as mammals or cartilaginous fish are unlikely to be important prey for these birds (Medeiros-Mirra 2010).

Due to the challenges faced with the amphipods results, we cannot conclude precisely on the importance of this prey group for Madeiran storm-petrels'. However, because the general invertebrates' primer also amplifies amphipods' DNA (Online Resource 3), which was detected at a lower proportion than fish in all groups except in males of 2017 (Fig. 1), we can conclude that fish is possibly the prey group with major importance for Madeiran storm-petrels during the breeding season. Gadiformes (cod fishes) was the predominant fish order detected in our samples, particularly *Trisopterus* sp. and *Micromesistius poutassou.* These are two species highly abundant in the Northeast Atlantic (Cunha 1992; Rogers *et al.*

1998), and are also targeted by fisheries in Portugal. However, in the adult form these prey species are too large for storm-petrels to consume, suggesting that Madeiran Storm-petrels either (1) prey on eggs or larvae of these species (zooplankton), as previously described for this species (Monteiro *et al.* 1996b), or (2) they present an opportunistic and scavenger behaviour by preying on leftovers by other predators or on fisheries discards, such as that described for the European storm-petrel (Medeiros-Mirra 2010). It has been showed that other storm-petrel species feed primarily on adult fish, as the Leach's storm-petrels in Canada (Hedd & Montevecchi 2006) which fed essentially on adult Myctophidae fish. Furthermore, the high $\delta^{15}N$ values presented by our sample were similar to those reported for Cory's shearwaters (*Calonectris borealis*, $\delta^{15}N$ values between 12.87 ± 0.26 and 13.34 ± 0.17) and the Macaronesian shearwaters (*Puffinus baroli*, $\delta^{15}N$ values between 11.67 ± 0.5 and 12.97 ± 0.8) breeding in several Portuguese colonies, including in Berlengas archipelago (e.g. Paiva et al. 2010a, 2016), and these species feed mainly on fish and cephalopod species. This suggests that Madeiran storm-petrels may also feed on prey with higher nitrogen isotopic ratios and thus from higher trophic levels, such as mesopelagic fish species and fisheries discards, rather than being an exclusively zooplanktivorous seabird. This is further supported by $\delta^{15}N$ data of zooplankton in our study area, which is around 5-6‰ (Graham et al. 2010). Considering trophic enrichment factors (3 - 5‰ enrichment from prey to predators tissues, Forero and Hobson 2003), a zooplanktivorous seabird in this study area would be expected to present nitrogen isotopic values around 8-11‰ in its tissues. Madeiran storm-petrels showed average $\delta^{15}N$ values of ~13‰, and such difference between zooplankton and our species' tissues values lead us to assume that this is not a predominantly zooplancktivorous seabird species.

The diet of females for 2015 was similar to that of males for both years (Table 1), feeding mainly on Gadidae fish. However, the diet of females for 2017 differed, because it also included Aulopiformes, Stomiiformes and Myctophiformes, which suggests a certain level of inter-annual and intersexual plasticity in the diet of Madeiran storm petrels. This can also be explained by an opportunistic foraging behaviour, taking advantage of the most common prey, a strategy already described for other storm-petrel species such as the European storm-petrel, that seem to rely on sardines and other common Cupleidae discarded from fisheries (Medeiros-Mirra 2010).

Trophic ecology and isotopic niche

Annual differences in the stable carbon isotope values of the birds for the breeding season, with higher values in 2017 than in 2015 could be a result of: 1) annual differences in marine productivity in the foraging area used by the birds (Ceia et al. 2018, Graham et al. 2010), 2) the differential timing of collection of blood samples (2015 samples were collected during incubation, while 2017 samples were collected during chick rearing) or 3) differences in their foraging grounds between years. However, the higher stable carbon isotope values in 2017, together with tracking data for 2017, which

showed birds to forage near the colony and near the West African coast, suggests that annual differences in foraging grounds may be important in explaining annual differences in stable isotope values.

A larger isotopic niche during the non-breeding season, compared to the breeding season, has already been reported for several other seabirds (Hedd et al. 2010; Ceia et al. 2014; Ramos et al. 2015). This is related to the fact that when seabirds are not breeding, 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. This larger isotopic niche is then a result of either the different individuals being spread out along different isotopic gradients in the ocean while foraging (Ceia et al. 2018), or by foraging on prey of different trophic levels (Hedd *et al*. 2010).

In our study, nitrogen isotopic ratios showed differences between sexes for the non-breeding period, when females showed lower nitrogen isotopic ratios. This might be related to 1) non-trophic level sources of $\delta^{15}N$ variation, i.e. intersexual differences in distribution during the non-breeding season, or be a result of 2) differences in diet between sexes or 3) differences in the relative amount of different prey taken, since the difference in nitrogen isotope values between males and females was from 1 to 1.5‰ (i.e. <1 trophic level). We did not detect differences between sexes in the carbon isotopic ratios, and with very limited tracking data during this season, we cannot conclude if such differences in nitrogen isotopic ratios were influenced by spatial differences between sexes during the non-breeding season. Preliminary data shows that some individuals of this population foraged around the Gulf of Mexico during the non-breeding season, where nitrogen isotopic gradients are very variable, influenced by both the Loop Current, from the east, and by Mississippi and Atchafalaya rivers discharges, up in the north (Nürnberg *et al*. 2008). This might play an important role on the nitrogen isotopic values in our data, and also explain why P1 feathers (representing the end of breeding season) have lower $\delta^{13}C$ and higher $\delta^{15}N$ values than S8 feathers (representing the non-breeding season). On the other hand, this intersexual difference was observed for other storm-petrel species, the Monteiro's storm-petrel (Paiva *et al*. 2018). This study showed that, when compared to females, males preyed on organisms of higher nitrogen isotopic ratios during the non-breeding period, therefore Madeiran storm-petrels might also forage on prey with different levels of nitrogen isotopic values. Paiva *et al*. (2018) further concludes that Monteiro's storm-petrel sexual segregation could be influenced by poorer environmental conditions. In 2013, the year when these intersexual differences were detected in Monteiro's storm-petrels, winter North Atlantic Oscillation index (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). Around the Portuguese and African coastal areas, poor environmental conditions are depicted by negative values of wNAO, which derives from storms and intense winds in these areas, leading to unusually strong upwellings in these coasts (Sousa et al. 2008). 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 may also lead to differences between sexes in their foraging ecology (Phillips *et al*. 2011), since females and males might adopt different feeding strategies to reduce competition. It seems that the feeding ecology of the Madeiran storm-petrel can be influenced by environmental conditions as well, and this is further supported by the lowest niche overlap that was detected in this season in 2016 (17,6%), where both sexes seem to avoid foraging in the same area, opposed to the previous year where a complete niche overlap occurred (100%).

The sexual dimorphism presented by this species, with females exhibiting a significantly longer wing-length than males, might play a role on the dietary and trophic differences between sexes. Sexual dimorphism has also been reported for European storm-petrels (Medeiros-Mirra 2010) and Monteiro's storm-petrels (Paiva *et al*. 2018), 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. However, only collection of more data during subsequent years, along with complementary information on diet will allow us to better understand intersexual stable isotopic differences in the Madeiran storm-petrel.

Distribution

Regarding the distribution of this species during the breeding season, only four individuals with tracking data were retrieved. The difficulties in retrieving more individuals with tracking data limited the possibilities of explaining the intersexual and inter-annual differences obtained in the Madeiran storm-petrel' diet in any more detail. The data retrieved from this small sample size is not enough to make population-level conclusions, however, the results were in accordance with those reported by Oliveira *et al.* (2013) from November of 2011. This suggests that Madeiran storm-petrels breeding in Farilhões islet might 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. This is a strategy commonly seen in other Procellariformes (Weimerskirch 1998), and it is understandable why this population of Madeiran storm-petrels could opt to forage in these main foraging areas. The West African coast is a hotspot of marine biodiversity, exhaustively used by other top predators and by international fishery fleets, because it is an area with high marine productivity (Paiva *et al*. 2015). On the other hand, the Portuguese coast is characterized by shallow foraging grounds, with marine productivity being influenced either by cold northern or temperate southern winds (Sousa *et al*. 2008).

Conservation implications

This work enabled us to describe the Madeiran storm-petrel diet and trophic ecology for the first time, and to our knowledge, is the first detailed work studying trophic ecology of winter-breeding storm petrels. It seems that this species uses highly productive at-sea areas for foraging, which may also be targeted by fisheries. This is a concern considering that not only does this species seem to feed on higher trophic level prey than previously considered, and thus might forage on prey discarded by fisheries, but also has obvious implications for the at-sea conservation of this species within national and international waters. Such findings are important for the conservation of such small seabirds that reproduce in winter, which when compared to summer breeders, might rely on different prey and experience different environmental conditions. Furthermore, the combination of techniques applied in this work is a suitable framework to study the trophic ecology of other storm-petrels during both the breeding and non-breeding periods.

Compliance with Ethical Standards

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Figures

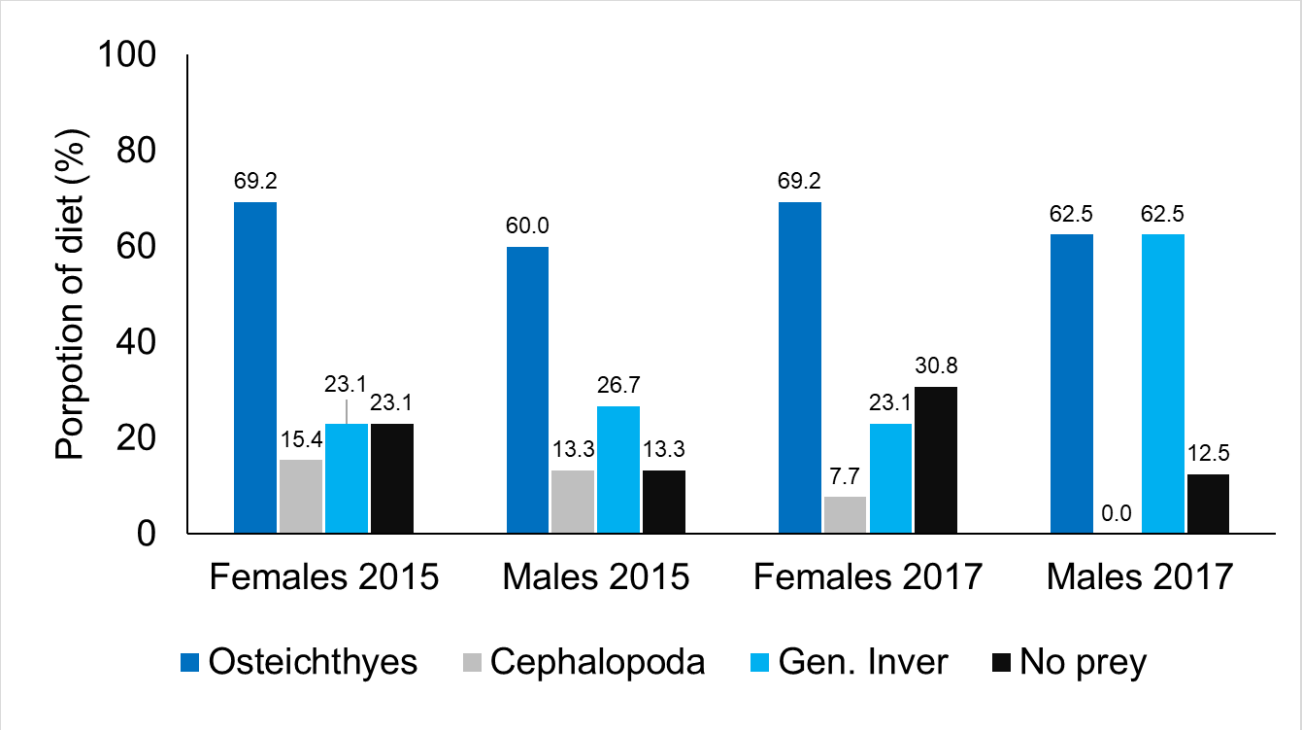

Figure 1 - Proportion (%) of detected fish, cephalopods, general invertebrate's and no DNA per sex and year for Madeiran storm-petrels after DNA amplification.

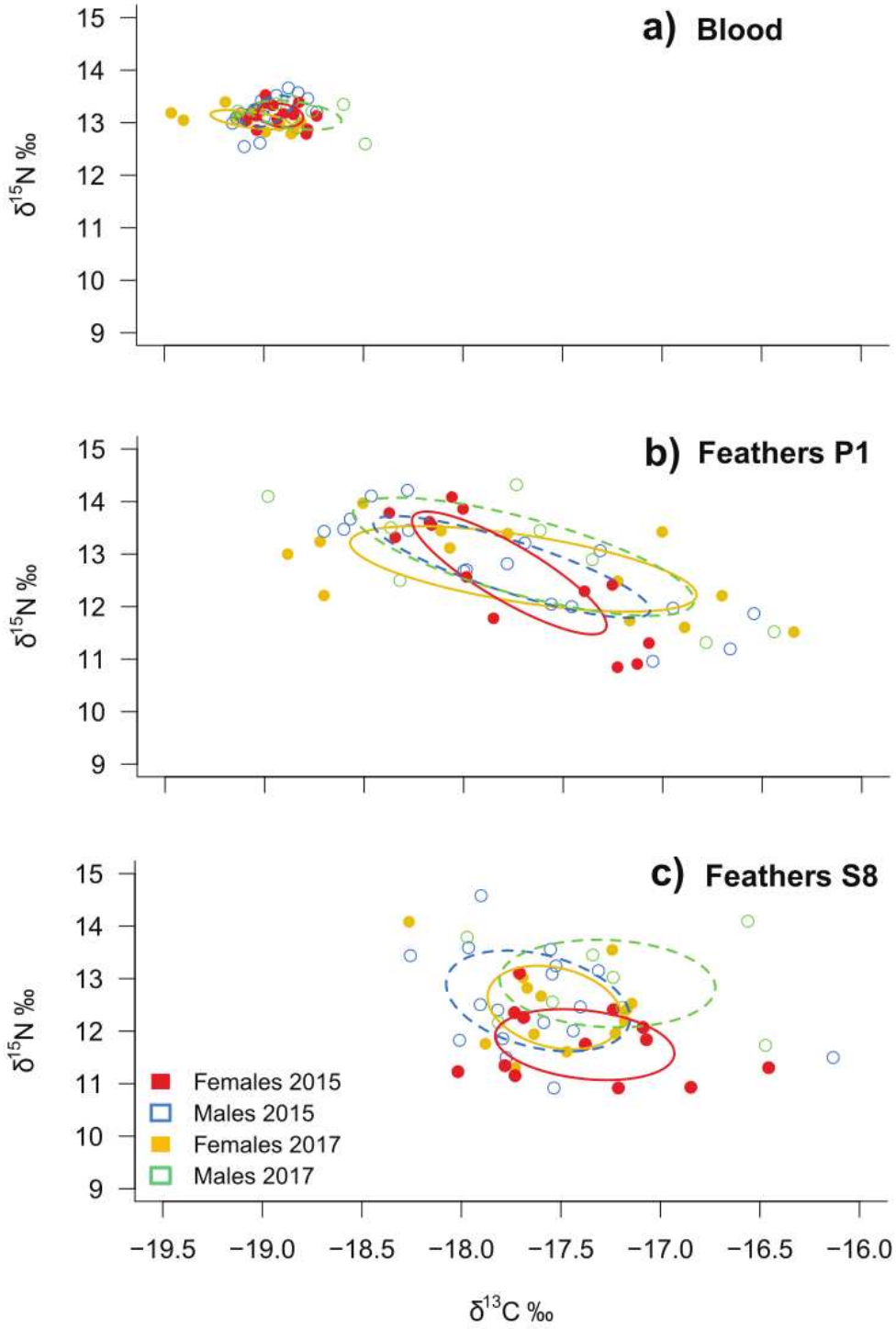

Figure 2 - Annual comparison of isotopic niche space of Madeiran storm-petrel between females (filled lines and symbols) and males (dotted lines and empty symbols), using a) whole blood, b) 1st primary feather and c) 8th secondary feather. Ellipses represent the standard ellipses areas corrected for small sample size (SEAc), constructed using the Stable Isotopes Bayesian Ellipses package in R (SIBER, Jackson et al. 2011).

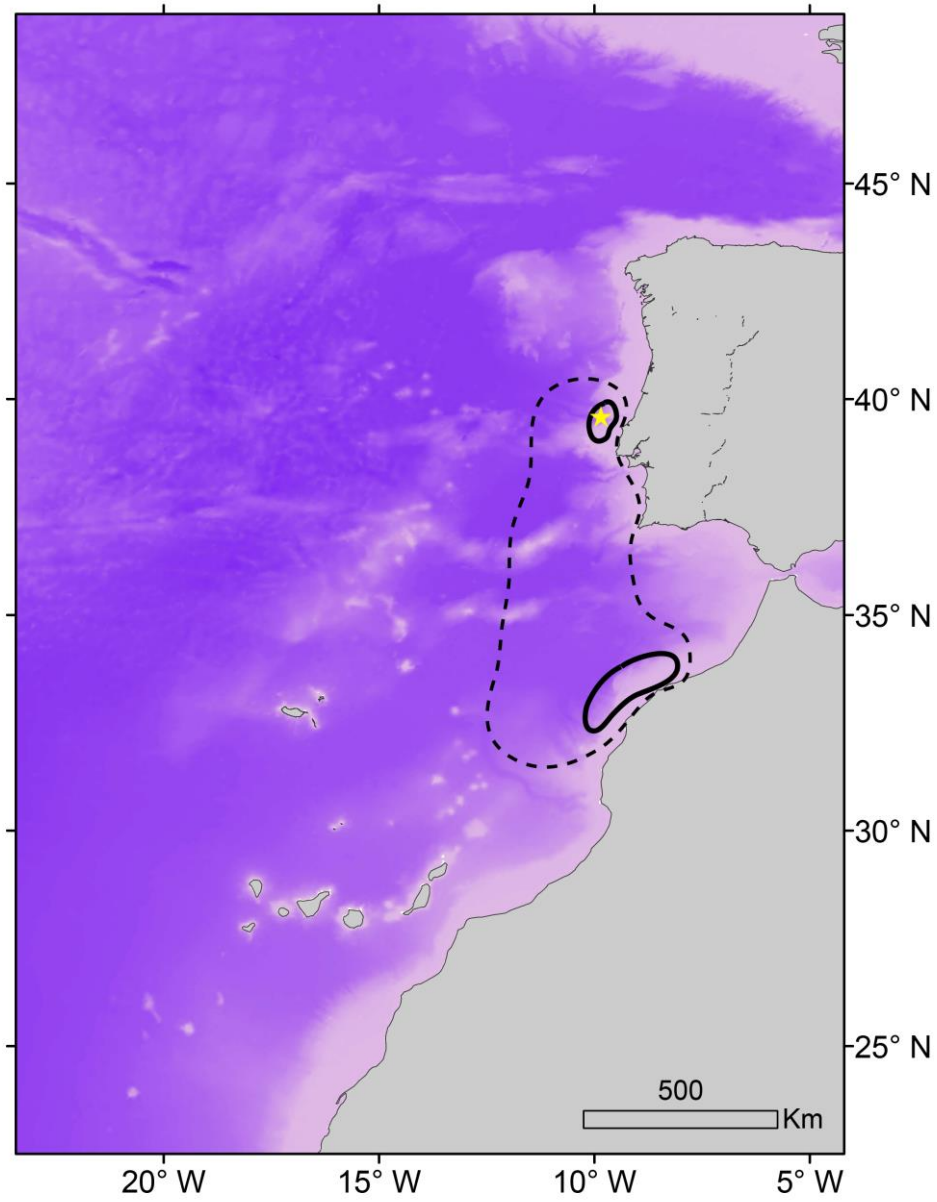

Figure 3 - Home range (95% kernel Utilization Distribution (UD); dashed line) and foraging area (50% kernel UD; filled line) of Madeiran storm-petrels from Farilhão Islet (Berlengas archipelago – identified with a star) during the early chick-rearing periods (January - February 2017). Bathymetry represented in the background varying from 1m (pink) to 3800m (blue) depth.

665 **Table 1** – Taxa identified from high-throughput sequencing of both (F) female and (M) male scats of two years of
666 data (2015 and 2017) from Madeiran Storm-Petrels, using DNA fragments from two different genes. Percentages refer to
667 proportion of sequences that comprise each prey type. Grey shading represent positive values, '-' represents groups that
668 were not analysed.

Target Gene	Class	Order	Family	Genus/Species	2015 F (%)	2017 F (%)	2015 M (%)	2017 M (%)
12S	Actinopterygii	Gadiformes	Gadidae	*Trisopterus minutus*	6.21	0.00	1.41	0.53
				other *Trisopterus* sp.	48.80	17.87	25.53	16.41
				Micromesistius poutassou	22.33	38.53	38.68	62.44
				Gadus sp.	7.91	1.19	12.15	10.52
				Gadiculus argenteus thori	1.48	0.00	14.87	0.00
		Clupeiformes	Clupeidae	*Sardina pilchardus*	11.43	0.00	0.00	0.00
		Perciformes	Sparidae	*Pagellus acarne*	1.84	0.00	0.00	0.00
			Carangidae	*Trachurus* sp.	0.00	0.00	7.35	0.00
		Lampriformes	unknown family		0.00	0.00	0.00	9.57
		Aulopiformes	Alepisauridae	*Alepisaurus ferox*	0.00	1.50	0.00	0.00
		Stomiiformes	unknown family		0.00	25.06	0.00	0.00
		Myctophiformes	Myctophidae	*Myctophum punctatum*	0.00	13.65	0.00	0.00
				Ceratoscopelus maderensis	0.00	1.61	0.00	0.00
				Protomyctophum sp.	0.00	0.60	0.00	0.00
				unknown genus	0.00	0.00	0.00	0.53
28S	Cephalopoda	Oegopsida	Onychoteuthidae	*Onykia* sp.	-	4.91	86.26	-
			Chiroteuthidae	*Chiroteuthis* sp.	-	90.07	7.96	-
				unknown genus[a]	-	5.02	0.00	-
			unknown Family[a]		-	0.00	5.77	-

669 [a]Classification contains 4 OUTs.

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Table 2 - Results of a factorial analysis of variance (ANOVA) showing multiple comparisons of $\delta^{13}C$ and $\delta^{15}N$ values for female and male Madeiran storm-petrel for each year. Feathers were pooled together in the analysis. Post-hoc multiple comparisons made with Tukey test. Significant effects are shown in bold.

	$\delta^{13}C$			**$\delta^{15}N$**		
	F	**P**	**Main effects**	**F**	**P**	**Main effects**
Blood						
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	
Feathers						
Sex	$F_{1,93}$= 0.05	0.828		$F_{1,93}$= 3.96	**0.050**	Males > Females
Year	$F_{1,93}$= 0.88	0.350		$F_{1,93}$= 0.25	0.620	
Tissue	$F_{1,93}$= 4.80	**0.031**	S8 > P1	$F_{1,93}$= 4.00	**0.048**	S8 < P1
Sex*Year	$F_{1,93}$= 0.45	0.506		$F_{1,93}$= 3.01	0.086	
Sex*Tissue	$F_{1,93}$= 0.03	0.853		$F_{1,93}$= 1.51	0.223	
Year*Tissue	$F_{1,93}$= 1.07	0.304		$F_{1,93}$= 0.55	0.459	
Sex*Tissue*Year	$F_{1,93}$= 0.02	0.889		$F_{1,93}$= 1.03	0.313	

678 **Table 3** - SIBER outputs: area of the standard ellipse (SEA_C) for female and male Madeiran Storm-petrel for each

679 year.and the layman metric of convex hull area (TA).

	SEAC		TA	
Blood: breeding season				
Year	**Female**	**Male**	**Female**	**Male**
2015	0.10	0.11	0.21	0.25
2017	0.08	0.21	0.18	0.28
P1 Feathers: end of breeding period				
Year	**Female**	**Male**	**Female**	**Male**
2015	1.89	1.18	3.72	2.85
2017	1.01	2.33	1.86	3.43
S8 Feathers: non-breeding period				
Year	**Female**	**Male**	**Female**	**Male**
2015	0.87	1.37	1.85	3.80
2017	0.99	1.61	1.79	2.74

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