1 **Metabarcoding, Stables Isotopes and Tracking: unraveling the trophic ecology of a winter-breeding**

2 **storm-petrel (*Hydrobates castro*) with a multimethod approach.**

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17 **Key-words:** *Hydrobates castro*, diet, HTS, trophic ecology, stable isotopes.

18

19 **Abstract**

20   Detailed information on diet and foraging ecology is scarce for most small seabirds such as storm-petrels. In this

21 study, we used molecular techniques, stable isotope analysis, and geolocators to study the diet, trophic ecology, and at-sea

22 distribution of Madeiran storm-petrels (*Hydrobates castro*) breeding in Farilhões Islet, Portugal, in 2015 - 2017.

23   The diet of Madeiran storm-petrels was dominated by fish for both sexes and study years, with Gadidae representing

24 the main prey family. In 2017, females also fed on Aulopiformes, Stomiiformes and Myctophiformes, which were not

25 identified in the other groups, suggesting some degree of inter-annual and intersexual plasticity in their diet. The carbon

26 isotopic ratios of birds during 2017 were significantly higher when compared to 2015, which might be related to foraging

27 near coastal areas in 2017. Indeed, tracking data for 2017 show that birds foraged near the colony and near the West

28 African coast.

29   Overall, both sexes of this species exhibited a similar trophic ecology and diet during the breeding season. However,

30 intersexual differences occurred during the non-breeding season, when females showed significantly lower nitrogen

31   isotopic ratios than males (in 2016), and the lowest niche overlap between sexes occurred. This, together with the fact that

32   environmental conditions appeared less favourable in 2016 suggests that intersexual differences in the foraging ecology

33   of this species may be related with environmental conditions.

34

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

3

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.

94   This study investigates the diet, trophic ecology and at-sea distribution of the Madeiran storm-petrel *Hydrobates*

95   *castro* breeding in Farilhões Islet, Portugal. *H. castro* is a medium sized storm-petrel (Monteiro *et al*. 1996b) breeding in

96   oceanic islands from equatorial to subtropical latitudes, mostly in winter (Monteiro and Furness 1998). There are some

97   records of their distribution around the Portuguese coast and the archipelagos of Madeira and Azores throughout the year,

98   suggesting that this species does not migrate extensively (Meirinho *et al*. 2014). Very little is known about the feeding

99   ecology of this species; it is thought that their diet is based on zooplankton and small mesopelagic fishes, as are other

100   similarly sized storm-petrel species (Monteiro *et al*. 1996b), but so far there is no comprehensive information about the

101   diet of *H. castro.* A comparative study about the trophic ecology of Atlantic Procellariformes in several breeding sites at

102   the end of the breeding season showed that the Madeiran storm-petrel exhibits a small isotopic niche, displaying similar

103   isotopic ratios between different sites and years, with few spatial differences and little variability between years (Roscales

104   *et al*. 2011). Therefore, we expect Madeiran storm-petrels to: (1) show a generalist diet composition, not restricted to

105   zooplankton; (2) forage mainly over pelagic regions during the breeding period, with some individuals making longer

106   trips towards the African coast, as reported by at-sea census surveys (Meirinho *et al.* 2014) and the tracking of a single

107   individual (Oliveira *et al.* 2013). There are no clear expectations regarding sexual differences in trophic ecology and diet

108   composition, because most storm-petrel species do not exhibit such differences. However, given the close phylogenetic

109   proximity to the Monteiro's storm-petrel in which such differences occur (Paiva *et al.* 2018), our species may present

110   sexual segregation in its foraging ecology. To our knowledge, this is the most detailed and comprehensive study on the

111   foraging ecology of a winter breeding storm-petrel, as most studies are on summer breeding populations (e.g Pollet *et al*.

112   2014b). Overall, this study will not only present baseline information on the foraging ecology of this species, but also will

113   provide a comprehensive framework for the conservation and management of other winter breeding storm-petrels.

114

115   **Methods**

116   **Study area and species**

117   This study was conducted on Farilhão Grande Islet (39° 28' 31" N, 9° 32' 45" W), within Berlengas archipelago,

118   offshore Peniche, Portugal. Farilhão Grande Islet is characterized by rocky substrate, with steep and vertical cliffs, where

119   approximately 100 to 200 breeding pairs of Madeiran storm-petrels are estimated to breed (Mendes 2013). This species

120   arrives at the islet to breed between August and September, nesting in cavities, and departs around February (Granadeiro

121   *et al.* 1998).

122

123   **Field Sampling**

124   We captured Madeiran storm-petrels using mist-nets placed along the rocky shore; birds were not captured on the nest

125   in order to avoid nest desertion (Rodway *et al.* 1996; Blackmer *et al.* 2004). Fieldwork was conducted over two breeding

126   seasons (2015/2016 and 2016/2017), hereafter referred to as 2015 and 2017. In the first breeding season, fieldwork for

127   sample collection was carried out on 10 November 2015 during the egg incubation period, and in the second breeding

128   season on 18 January 2017, during the chick-rearing period. A total of 30 and 21 individuals were captured in 2015 and

129   2017, respectively. Differences in sampling methodology were related to poor weather conditions, which prevented

130   access to the colony during the 2016 incubation period.

131   For all birds captured, their body mass, tarsus, and wing length were measured. Approximately 1 cm from the tip of

132   the first primary and the eighth secondary feather were also collected and stored in polythene bags for SIA. A blood

133   sample (~50 µL) was taken from the brachial vein and stored in 2-mL tubes with 70% ethanol for both stable isotope

134   analysis and molecular sexing. Birds were placed inside a box for a maximum of 15 minutes, in order to let the birds

135   defecate naturally, obtaining a total of 28 and 21 faecal samples from 2015 and 2017, respectively. Faecal samples were

136   stored in 2-mL tubes with 70% ethanol, and the bottom of the box was lined with plastic or tinfoil and replaced between

137   each individual.

138   Six birds that were ringed in previous years and were known to nest in this colony were instrumented with Global

139   Location Sensing (GLS) devices (model MK18L, BioTrack Lda.) in 2017 breeding season. Loggers were back-mounted

140   with a cotton harness, in January 2017, and when birds were re-captured, the logger information was downloaded without

141   taking the device off the bird. It was possible to get back the tracking information from four individuals during the early

142   chick-rearing period (January-February 2017). GLS devices represented less than 1% of the bird's body mass in order to

143   not impair the birds survival (Pollet et al. 2014c; Kürten et al. 2019).

144

145   **Sex and diet determination using molecular tools**

146   Molecular sex determination was carried out using an individual's whole blood sample using an adaptation of the

147   Chelex DNA extraction method (Medeiros-Mirra 2010, see Online Resource 1). DNA from storm-petrel faecal samples

148   was extracted using the QIAamp DNA Stool Mini Kit (Qiagen), following Zeale et al. (2011). Four primer sets (Online

149   Resource 2) were used to target different prey types in order to ensure good coverage and resolution of the range of

150   potential prey consumed by the birds: Osteichthyes (mtDNA 12S), Cephalopoda (nuclear 28S rDNA), Amphipoda

151   (nuclear 18S rDNA) and general invertebrates (mtDNA COI). The 18S, 28S and 12S primer sets have been previously

152   used for seabirds (Deagle *et al.* 2007; Medeiros-Mirra 2010), whereas the COI general invertebrate primer has not been

153   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

184   capsules and isotopic ratios of carbon (δ^{13}C (‰) $= \left(\frac{(13_C/12_C)_{sample}}{(13_C/12_C)_{V-PDB}} - 1 \right) x1000$) and nitrogen ($\delta^{15}$N (‰) $=$

185     $\left(\frac{(15_N/14_N)_{sample}}{(15_N/14_N)_{air}} - 1 \right) x1000$) (Libes 2009; Wada 2009), were determined by continuous-flow isotope-ratio mass

186   spectrometry (CF- IRMS).

187

188   **Tracking data processing**

189   GLS data was analyzed using the BASTrack software suite (British Antarctic Survey, Cambridge, UK), using a light

190   threshold of 10 and an elevation angle of −4.0 (derived from calibration devices left at an open site without shading at

191   Berlenga Island, located ~7km away from Farilhão Islet). The quality of the light curves checked with *TransEdit* was

192   high, so the geolocation error was assumed to be similar to that estimated by Phillips *et al.* (2004). Locations derived

193   from curves with apparent interruptions around sunset and sunrise were removed. Erroneous locations were also excluded

194   for a week around the equinoxes, when latitude estimates are unreliable. Predicted locations of each bird were examined

195   under the *adehabitatHR* R package (Calenge 2006) generating kernel Utilization Distribution (kernel UD) estimates. The

196   most appropriate smoothing parameter (*h*) was chosen via least squares cross-validation for the unsmoothed GLS data

197   and then applied as standard for the other data sets, and grid size was set at 0.25°. Following previous authors (Paiva et al.

198   2010b), we considered the 50 % and 95 % kernel UD contours to represent the core foraging areas (FA) and the home

199   range (HR), respectively.

200

201   **Data analysis**

202   To test for possible intersexual differences in this population, body measurements (tarsus, wing and body mass) were

203   compared between sexes with two independent samples t-test. Results are described as mean ± SD. To analyse diet

204   detection, a similarity matrix was generated using the Bray–Curtis similarity measure. Adonis tests were run on the

205   matrices using 999 permutations to test for differences in diet screening between sexes and years. However, it was

206   detected that the amphipod primer pair was amplifying predator's DNA, therefore this set was withdrawn from this

207   analysis as it was impossible to distinguish through electrophoresis which samples were amplifying DNA from prey or

208   from predator.

209   The bioinformatic analysis of HTS data was carried out using a combination of USEARCH v10.0.240 (Edgar 2010)

210   and the Cutadapt package (Martin 2011) on a python script. All commands are provided in the GitHub repository:

211   https://github.com/AnaCarreiro/Carreiro_et_all_MSP. Paired-end reads were merged and then de-multiplexed based on

212   forward and reverse primers and MID tags, as well as stripped from all the adapters. Reads from 12S, 18S, 28S and COI

213   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

214   then merged into a master file for each prey target. All reads were filtered to a maximum of 1.0 Expected Errors (EE).

215   Reads were dereplicated, singletons removed, and clustered into OTUs (Operational Taxonomic Units). The UPARSE

216   pipeline was used for 12S and COI amplicons analysis with a 97% clustering (Edgar 2013) whereas 18S and 28S were

217   analyzed in the UNOISE algorithm (Edgar 2016) with a 99% clustering, as suggested in previous work for these target

218   groups (Bachy *et al*. 2013; Edgar and Flyvbjerg 2015). The total number of sequences retrieved, sequences lost, uniques,

219   singletons, and quimeras for each gene can be found in Online Resource 4.

220   To taxonomically classify OTUs, MegaBLAST from NCBI database was used (Zhang *et al*. 2000; Morgulis *et al*.

221   2008), and only results with 100% query cover were considered as matches. The resulting sequences were assigned to

222   taxonomic units using a cut-off of 90% sequence identity for 12S, 28S and COI genes, and 99% sequence identity for 18S

223   gene. These thresholds were based not only on each fragment size and their definitions in previous work using these

224   genes (e.g. Bachy *et al*. 2013), but also considering ecological data, since a lower sequence similarity threshold would

225   result in a mixing of different taxa with no ecological sense. For each OTU, all the reads matching the thresholds defined

226   were considered and analyzed together to classify each group to the lowest taxonomic level possible. Taxon was assigned

227   if the highest query sequences, with the same match, clustered monophyletically at that level. If the sequence matched

228   more than one species from the same genus or family, the lowest (most ancestral) common taxonomic rank was assigned.

229   Two multivariate analysis of variance (MANOVA; Wilk's lambda statistics) were used to compare differences in

230   both the carbon and nitrogen stable isotopic ratios, as response variables, of (1) blood and (2) feathers (P1 and S8). Such

231   differences were analyzed between (1) years (2015 vs 2017, $n= 30$ and $n= 21$ respectively), (2) sex (Female vs Male, $n=$

232   26 and $n= 25$ respectively), and (3) tissues for the comparison between feathers (P1 vs S8, $n= 51$ each), as independent

233   variables. MANOVAs were followed by separated factorial ANOVAs for each stable isotope and post-hoc multiple

234   comparisons Tuckey test. In order to compare isotopic niches between sexes, years and periods, we used recent metrics

235   based in a Bayesian framework (Stable Isotope Bayesian Ellipses in R: SIBER; Jackson *et al*. 2011). The standard ellipse

236   area drawn using the stable isotopic ratios of nitrogen and carbon, corrected for small sample sizes (SEA$_C$, an ellipse that

237   has 40% probability of containing a subsequently sampled datum) was used to quantify niche width and to compare it

238   between the two sexes among years and periods, and a Bayesian estimate of the standard ellipse and its area (SEA$_B$) to

239   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

240   10^4 replicates; see Jackson *et al*. 2011 for more details). All former computations were performed under R environment

241   (R Core Team 2018).

242

Results

244 **Sex determination and sexual dimorphism**

245   Sex determination using blood extracts was successful for 51 out of 52 samples, resulting in a total of 13 females and

246 17 males for 2015, and 13 females and eight males for 2017. Two males and one female were re-captured in 2017, and so

247 the first body measurements taken in 2015 were used in these analyses. Body measurements indicated that females had

248 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

249 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 ±

250 6.29 g, n = 25 vs 52.63 ± 6.30 g, n = 23, t_{46}= 1.47, P= 0.15).

251

252 **Diet determination**

253   DNA amplification was successful for all 49 faecal samples, amplifying in at least one of the primer sets. Since the

254 Amphipoda primer was not considered for this analysis, the percentage of samples that were only amplified by this

255 primer (13.0 to 31.0%, Fig. 1) were considered to contain no prey DNA since they also amplify predator DNA. DNA

256 amplification results showed a predominance of fish (Osteichthyes) in the samples of both sexes and years (Fig. 1), with

257 occurrence ranging from 60.0% to 69.2%. Males in 2017 were an exception, where both fish and invertebrates were

258 equally detected (62.5%). However, the sample size for males in 2017 was small (n= 8), which might have influenced

259 these results. The prey group with the lowest number of detections for both sex and year was Cephalopoda, ranging from

260 0% in 2017 males to 15.4% in 2015 females. There were no significant differences between the proportion of prey groups

261 between years (Adonis, R2 = 0.021, P= 0.444), sexes (Adonis, R2= 0.048, P= 0.156), nor an interaction between these

262 two variables (Adonis, R2= 0.041, P= 0.227). Regarding the results of the HTS, the 18S and COI primers failed to

263 provide any meaningful results due to the low quality of sequences. However, UPARSE detected 15 OTUs for the 12S

264 fish primers, and UNOISE detected 10 OTUs for the 28S Cephalopoda primers (Table 1). The fish OTUs were distributed

265 across two main families, Gadidae and Myctophidae, with five and four OTUs, respectively. The proportion of sequences

266 comprising each OTU varies between the groups, but the largest proportion of sequences was found for Gadidae, with an

267 unknown *Trisopterus* sp. being the most represented in 2015 females (48.80%), while the blue whiting (*Micromesistius*

268 *poutassou*) comprised the greatest number of prey sequences in the remaining groups (from 38.53 to 62.44%). In 2017

269 females, although the majority of sequences were represented by Gadidae, other families weighted almost as equally

270 (57.58% vs 42.42%), specially a non-identified family from Stomiiformes (25.06%), and the lanternfish species

271 *Myctophum punctatum* (13.65%). These, together with *Alepisaurus ferox* and other Myctophidae species, were detected

272 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.*

333   1998), and are also targeted by fisheries in Portugal. However, in the adult form these prey species are too large for

334   storm-petrels to consume, suggesting that Madeiran Storm-petrels either (1) prey on eggs or larvae of these species

335   (zooplankton), as previously described for this species (Monteiro *et al.* 1996b), or (2) they present an opportunistic and

336   scavenger behaviour by preying on leftovers by other predators or on fisheries discards, such as that described for the

337   European storm-petrel (Medeiros-Mirra 2010). It has been showed that other storm-petrel species feed primarily on adult

338   fish, as the Leach's storm-petrels in Canada (Hedd & Montevecchi 2006) which fed essentially on adult Myctophidae

339   fish. Furthermore, the high $\delta^{15}N$ values presented by our sample were similar to those reported for Cory's shearwaters

340   (*Calonectris borealis*, $\delta^{15}N$ values between 12.87 ± 0.26 and 13.34 ± 0.17) and the Macaronesian shearwaters (*Puffinus*

341   *baroli*, $\delta^{15}N$ values between 11.67 ± 0.5 and 12.97 ± 0.8) breeding in several Portuguese colonies, including in Berlengas

342   archipelago (e.g. Paiva et al. 2010a, 2016), and these species feed mainly on fish and cephalopod species. This suggests

343   that Madeiran storm-petrels may also feed on prey with higher nitrogen isotopic ratios and thus from higher trophic

344   levels, such as mesopelagic fish species and fisheries discards, rather than being an exclusively zooplanktivorous seabird.

345   This is further supported by $\delta^{15}N$ data of zooplankton in our study area, which is around 5-6‰ (Graham et al. 2010).

346   Considering trophic enrichment factors (3 - 5‰ enrichment from prey to predators tissues, Forero and Hobson 2003), a

347   zooplanktivorous seabird in this study area would be expected to present nitrogen isotopic values around 8-11‰ in its

348   tissues. Madeiran storm-petrels showed average $\delta^{15}N$ values of ~13‰, and such difference between zooplankton and our

349   species' tissues values lead us to assume that this is not a predominantly zooplancktivorous seabird species.

350   The diet of females for 2015 was similar to that of males for both years (Table 1), feeding mainly on Gadidae fish.

351   However, the diet of females for 2017 differed, because it also included Aulopiformes, Stomiiformes and

352   Myctophiformes, which suggests a certain level of inter-annual and intersexual plasticity in the diet of Madeiran storm

353   petrels. This can also be explained by an opportunistic foraging behaviour, taking advantage of the most common prey, a

354   strategy already described for other storm-petrel species such as the European storm-petrel, that seem to rely on sardines

355   and other common Cupleidae discarded from fisheries (Medeiros-Mirra 2010).

356

357   **Trophic ecology and isotopic niche**

358   Annual differences in the stable carbon isotope values of the birds for the breeding season, with higher values in 2017

359   than in 2015 could be a result of: 1) annual differences in marine productivity in the foraging area used by the birds (Ceia

360   et al. 2018, Graham et al. 2010), 2) the differential timing of collection of blood samples (2015 samples were collected

361   during incubation, while 2017 samples were collected during chick rearing) or 3) differences in their foraging grounds

362   between years. However, the higher stable carbon isotope values in 2017, together with tracking data for 2017, which

13

363   showed birds to forage near the colony and near the West African coast, suggests that annual differences in foraging

364   grounds may be important in explaining annual differences in stable isotope values.

365   A larger isotopic niche during the non-breeding season, compared to the breeding season, has already been reported

366   for several other seabirds (Hedd et al. 2010; Ceia et al. 2014; Ramos et al. 2015). This is related to the fact that when

367   seabirds are not breeding, and thus without the need to restrain their foraging area to the colony surroundings, they adopt

368   different foraging strategies and may forage in wider oceanic areas. This larger isotopic niche is then a result of either the

369   different individuals being spread out along different isotopic gradients in the ocean while foraging (Ceia et al. 2018), or

370   by foraging on prey of different trophic levels (Hedd *et al*. 2010).

371   In our study, nitrogen isotopic ratios showed differences between sexes for the non-breeding period, when females

372   showed lower nitrogen isotopic ratios. This might be related to 1) non-trophic level sources of $\delta^{15}N$ variation, i.e.

373   intersexual differences in distribution during the non-breeding season, or be a result of 2) differences in diet between

374   sexes or 3) differences in the relative amount of different prey taken, since the difference in nitrogen isotope values

375   between males and females was from 1 to 1.5‰ (i.e. <1 trophic level). We did not detect differences between sexes in the

376   carbon isotopic ratios, and with very limited tracking data during this season, we cannot conclude if such differences in

377   nitrogen isotopic ratios were influenced by spatial differences between sexes during the non-breeding season. Preliminary

378   data shows that some individuals of this population foraged around the Gulf of Mexico during the non-breeding season,

379   where nitrogen isotopic gradients are very variable, influenced by both the Loop Current, from the east, and by

380   Mississippi and Atchafalaya rivers discharges, up in the north (Nürnberg *et al*. 2008). This might play an important role

381   on the nitrogen isotopic values in our data, and also explain why P1 feathers (representing the end of breeding season)

382   have lower $\delta^{13}C$ and higher $\delta^{15}N$ values than S8 feathers (representing the non-breeding season). On the other hand, this

383   intersexual difference was observed for other storm-petrel species, the Monteiro's storm-petrel (Paiva *et al*. 2018). This

384   study showed that, when compared to females, males preyed on organisms of higher nitrogen isotopic ratios during the

385   non-breeding period, therefore Madeiran storm-petrels might also forage on prey with different levels of nitrogen isotopic

386   values. Paiva *et al*. (2018) further concludes that Monteiro's storm-petrel sexual segregation could be influenced by

387   poorer environmental conditions. In 2013, the year when these intersexual differences were detected in Monteiro's storm-

388   petrels, winter North Atlantic Oscillation index (wNAO) values were very low (-1.97). In 2015, the first year of our study

389   where no differences between sexes were detected, the wNAO was very high (3.56), while in 2016 it dropped to 0.98

390   (Hurrell, 2017). Around the Portuguese and African coastal areas, poor environmental conditions are depicted by negative

391   values of wNAO, which derives from storms and intense winds in these areas, leading to unusually strong upwellings in

392   these coasts (Sousa et al. 2008). This phenomenon drives plankton away from the shore, leading to its death (Robinson

393 2004; Santos *et al*. 2004), resulting in low abundance of prey for seabirds. These poor conditions may also lead to

394 differences between sexes in their foraging ecology (Phillips *et al*. 2011), since females and males might adopt different

395 feeding strategies to reduce competition. It seems that the feeding ecology of the Madeiran storm-petrel can be influenced

396 by environmental conditions as well, and this is further supported by the lowest niche overlap that was detected in this

397 season in 2016 (17,6%), where both sexes seem to avoid foraging in the same area, opposed to the previous year where a

398 complete niche overlap occurred (100%).

399 The sexual dimorphism presented by this species, with females exhibiting a significantly longer wing-length than

400 males, might play a role on the dietary and trophic differences between sexes. Sexual dimorphism has also been reported

401 for European storm-petrels (Medeiros-Mirra 2010) and Monteiro's storm-petrels (Paiva *et al*. 2018), and is considered the

402 main driver of intersexual differences in the trophic ecology of Monteiro's storm-petrels during both the breeding (P1

403 feathers) and non-breeding (S8 feathers) periods. However, only collection of more data during subsequent years, along

404 with complementary information on diet will allow us to better understand intersexual stable isotopic differences in the

405 Madeiran storm-petrel.

406

407 **Distribution**

408 Regarding the distribution of this species during the breeding season, only four individuals with tracking data were

409 retrieved. The difficulties in retrieving more individuals with tracking data limited the possibilities of explaining the

410 intersexual and inter-annual differences obtained in the Madeiran storm-petrel' diet in any more detail. The data retrieved

411 from this small sample size is not enough to make population-level conclusions, however, the results were in accordance

412 with those reported by Oliveira *et al.* (2013) from November of 2011. This suggests that Madeiran storm-petrels breeding

413 in Farilhões islet might adopt two foraging strategies: short distance trips near the colony, probably to feed their chicks,

414 and longer distance trips near the African coast, probably to restore their body condition. This is a strategy commonly

415 seen in other Procellariformes (Weimerskirch 1998), and it is understandable why this population of Madeiran storm-

416 petrels could opt to forage in these main foraging areas. The West African coast is a hotspot of marine biodiversity,

417 exhaustively used by other top predators and by international fishery fleets, because it is an area with high marine

418 productivity (Paiva *et al*. 2015). On the other hand, the Portuguese coast is characterized by shallow foraging grounds,

419 with marine productivity being influenced either by cold northern or temperate southern winds (Sousa *et al*. 2008).

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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.

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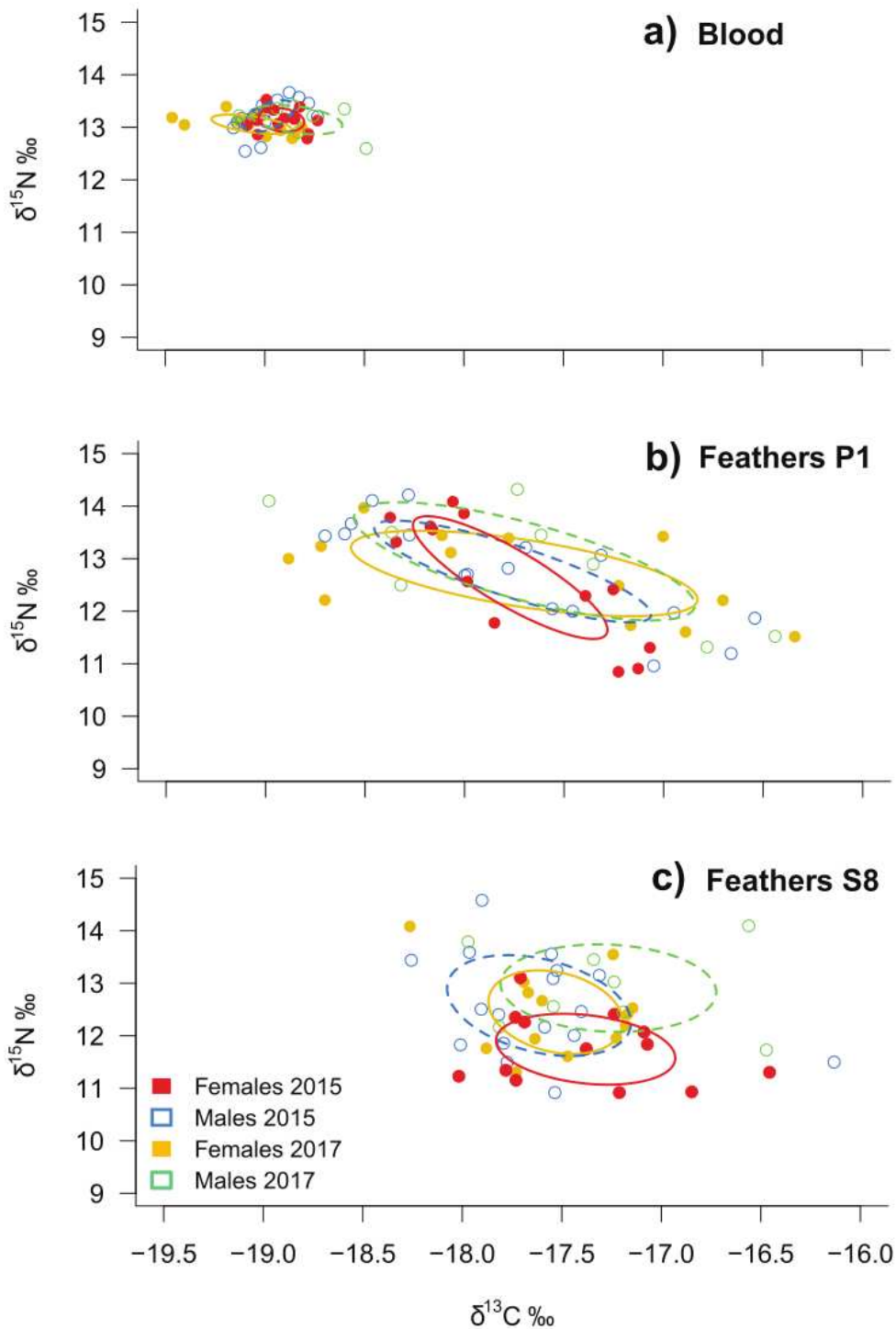
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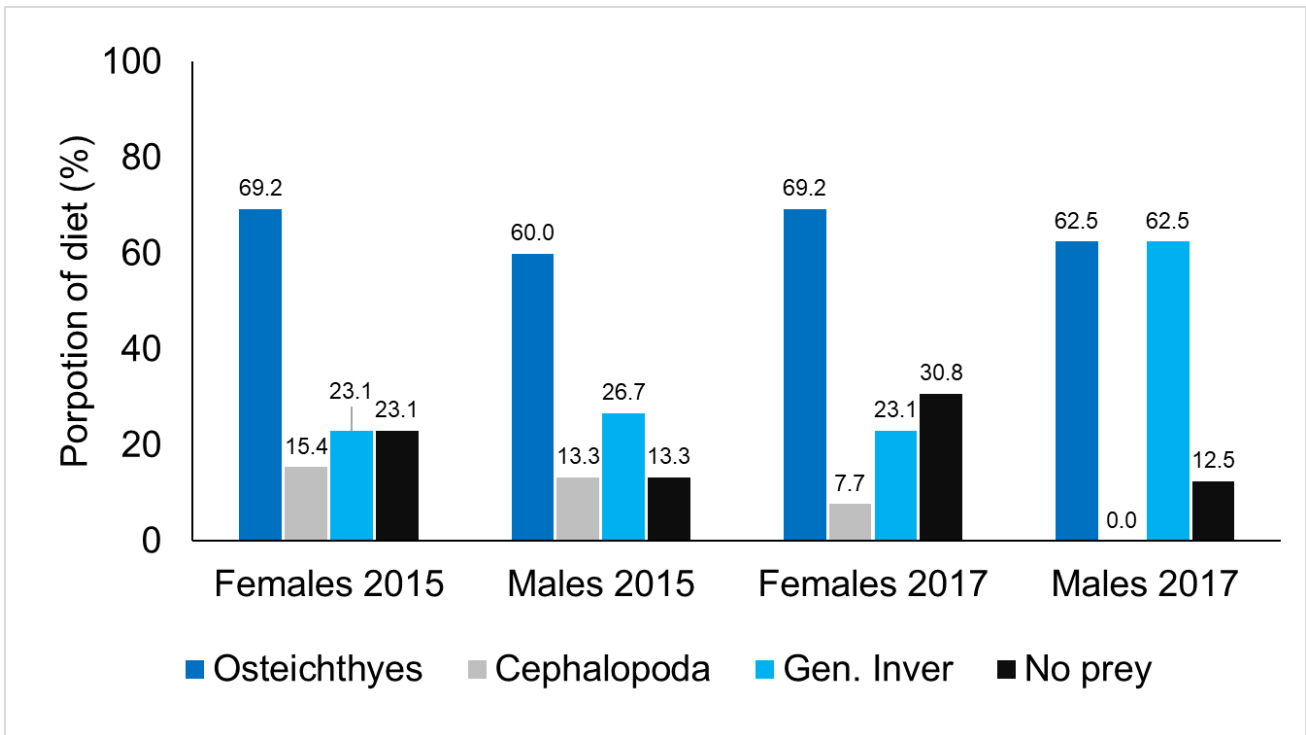
Figure 1 - Proportion (%) of detected fish, cephalopods, general invertebrate's and no DNA per sex and year for Madeiran storm-petrels after DNA amplification.

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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).

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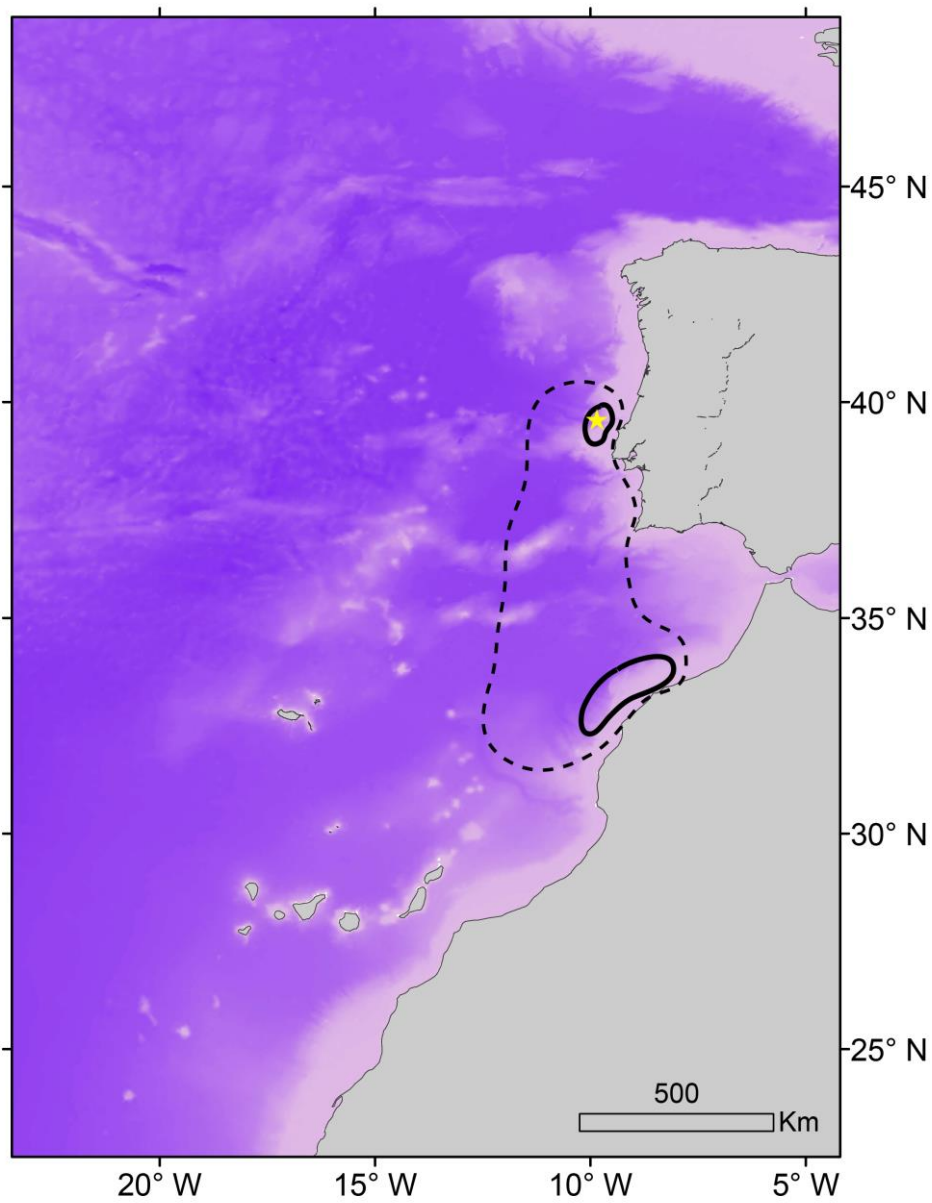
Figure 3 - Home range (95% kernel Utilization Distribution (UD); dashed line) and foraging area (50% kernel UD; filled line) of Madeiran storm-petrels from Farilhão Islet (Berlengas archipelago – identified with a star) during the early chick-rearing periods (January - February 2017). Bathymetry represented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

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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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672 **Table 2** - Results of a factorial analysis of variance (ANOVA) showing multiple comparisons of δ^{13}C and δ^{15}N values for

673 female and male Madeiran storm-petrel for each year. Feathers were pooled together in the analysis. Post-hoc multiple

674 comparisons made with Tukey test. Significant effects are shown in bold.

	δ^{13}C			δ^{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	

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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).

	SEA$_C$		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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