

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

3

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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

35 **Introduction**

36 As marine top predators, seabirds reflect changes that occur at lower trophic levels. Physical and biological changes in
37 the ocean, such as differences in temperature and marine productivity, determine the distribution and abundance of
38 marine organisms, which can be reflected in dietary changes and abundance of predators (Springer *et al.* 1984). Overall, a
39 seabirds' trophic ecology gives relevant information about its relationship with lower trophic levels, providing essential
40 data for their conservation and ecosystem management (Iverson *et al.* 2007; Xavier *et al.* 2011). Seabird species from the
41 order Procellariiformes, such as the albatrosses, petrels, and shearwaters, have been used as sentinels of environmental
42 conditions (e.g. Paiva *et al.* 2015), since they present extreme life-history characteristics (Warham 1990; Granadeiro *et al.*
43 1998b), and their behaviour changes noticeably as a response to marine environmental variability. However, there is very
44 little information on the potential of smaller seabird species, such as the storm-petrels, to be used as sentinels of marine
45 ecosystems. As lower trophic level consumers, e.g. feeding on zooplankton, storm-petrels can alert to environmental
46 changes at a faster speed than comparatively larger seabirds (Grémillet *et al.* 2015). Additionally, some species of storm-
47 petrels reproduce in winter, which makes them a potential sentinel for changes in environmental conditions during this
48 specific season (Gremillet and Charmantier 2010).

49 The diet and feeding ecology of storm-petrels is perhaps the least known of all seabird groups, partly because
50 traditional sampling methods are too invasive for these small seabird species. Some non-invasive techniques, such as
51 Stable Isotope Analyses (SIA), have been used to study the trophic ecology of several seabird species (e.g. Roscales *et al.*
52 2011), including storm-petrels (e.g. Gladbach *et al.* 2007). However, SIA by itself gives an unclear response of precise
53 trophic interactions. SIA rarely indicates which specific prey species are consumed by seabirds, giving instead
54 information on the trophic level of their prey (Iverson *et al.* 2007; Traugott *et al.* 2007). Complementary methods have
55 been used, namely a tracking system of seabird movements over long periods of time, like Global Location Sensing
56 (GLS) devices. The information gathered by these devices, together with data obtained from SIA, make it possible to
57 build biogeographic patterns of stable isotopes in the marine ecosystem. It is now known that isotopic ratios change
58 throughout different latitudes, depending on the distance to the shore or benthic habitats, providing an estimated
59 geographic gradient (i.e. isoscapes) of the ocean (Ramos *et al.* 2009; Graham *et al.* 2010; Ceia *et al.* 2018). As for the
60 majority of Procellariiformes, which are known for having large foraging areas during the breeding season, with even
61 longer distant movements during migration, studies on storm-petrel distribution show that these birds, despite their small
62 size, also undergo long distance movements. For example, Leach's storm-petrels (*Hydrobates leucorhous*) breeding in
63 Canada, from two colonies located only 380 km apart, showed distinct foraging locations and ranges for each population
64 during the breeding season (Pollet *et al.* 2014b). Birds from these colonies were also tracked during the non-breeding

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

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

81 Several studies have reported sex-specific differences in seabird trophic ecology and behaviour (González-Solís *et al.*
82 2000; Kato *et al.* 2000; Lewis *et al.* 2005). Such differences normally occur in species with Sexual Size Dimorphism
83 (SSD), but in monomorphic species, where SSD does not occur, differences in trophic ecology between sexes are
84 expected to be smaller (Paiva *et al.* 2018). However, recent studies on monomorphic seabirds' species have shown sex-
85 specific foraging patterns to occur (Welcker *et al.* 2009; Elliott *et al.* 2010), explained by the "intersexual competition
86 hypothesis". This hypothesis suggests that one sex may forage more efficiently, outcompeting the other and originating
87 different foraging niches, or even resulting in sexual segregation in foraging areas (Lewis *et al.* 2002; Peck and Congdon
88 2006). Also, the "energetic constraint hypothesis" suggests that the parents invest differently throughout breeding stages,
89 resulting in different self-provisioning effort between sexes (Elliott *et al.* 2010). In monomorphic storm-petrels, Phillips
90 *et al.* (2009) did not find any significant sex-specific differences in two species' trophic ecology. However, more
91 recently, intersexual differences have been found in the trophic ecology and distribution of Monteiro's Storm-petrel
92 during incubation and chick-rearing periods (Paiva *et al.* 2018), where females fed on lower trophic levels and foraged in
93 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 Procellariiformes 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-

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

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

172

173 **Stable isotope analysis**

174 In Madeiran Storm-petrels, primary feather moult starts at the end of January (Monteiro *et al.* 1996a). The isotopic
175 ratios of these feathers taken during the breeding season represent the trophic ecology of the individuals when they were
176 formed (Ramos and González-Solís 2012), so during the end of the previous breeding period, thus early-2015 and early-
177 2016. Secondary feathers moult in August (Bolton *et al.* 2008), so they represent the end of the non-breeding season, thus
178 summer of 2015 and 2016. Blood regenerates quickly, representing the season when collected, i.e. the breeding season in
179 our study (October-November 2015 and December 2016-January 2017).

180 Feathers were cleaned of surface oils and contaminants using a 2:1 chloroform:methanol solution for 15 minutes
181 (three baths of 5 minutes each) and then oven dried at 60°C for 24 h. Once dried, feathers were cut into small fragments
182 using stainless steel scissors, avoiding the shaft. Blood samples were firstly air-dried to remove excess ethanol, then oven
183 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 ($\delta^{13}\text{C}$ (‰) = $\left(\frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{V-PDB}}} - 1\right) \times 1000$) and nitrogen ($\delta^{15}\text{N}$ (‰) =
185 $\left(\frac{(^{15}\text{N}/^{14}\text{N})_{\text{sample}}}{(^{15}\text{N}/^{14}\text{N})_{\text{air}}} - 1\right) \times 1000$) (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 $\sim 7\text{km}$ 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 \pm 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 Tukey 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

243 **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 ($55.31 \pm$
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, $R^2 = 0.021$, $P = 0.444$), sexes (Adonis, $R^2 = 0.048$, $P = 0.156$), nor an interaction between these
262 two variables (Adonis, $R^2 = 0.041$, $P = 0.227$). 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

273 the other groups. OTUs detected exclusively in the other groups, were the European pilchard (*Sardina pilchardus*) in
274 2015 females, *Trachurus* sp. in 2015 males and a non-identified Lampriform in 2017 males.

275 From the Cephalopoda primers, *Onykia* sp. from the Onychoteuthidae family represented the majority of sequences in
276 the 2015 male sequences (86.26%) while *Chiroteuthis* sp. had the greater number of sequences in 2017 females (90.07%).
277 Another unknown genus of Chiroteuthidae family, comprising four OTUs, was detected exclusively in 2017 females'
278 diet, as well as another non-identified family from Oegopsida, also comprising four OTUs, which was only detected in
279 2015 males' diet.

280

281 **Stable Isotopes**

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

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

296

297 **Tracking during the breeding season**

298 Tracking data of the four individuals during the breeding period of 2016-2017 showed that Madeiran storm-petrels
299 breeding in Farilhão have a large home range (95% kernel UD). Nevertheless, the tracked individuals concentrated their
300 foraging activity (50% kernel UD) in two main areas; the colony surroundings, and foraging up to 650km south, close to
301 the African coast (Fig. 3).

302

303 **Discussion**

304 This study is the most comprehensive work to date on the trophic ecology of winter-breeding storm petrels. It
305 integrated sexual, seasonal (breeding and non-breeding period), and temporal (two years) information on trophic
306 variability, to assess the foraging ecology of the Madeiran storm-petrel breeding on Farilhão islet, Berlengas archipelago,
307 Portugal. During the breeding season, males had a similar diet across both years, while a difference was detected in the
308 fish prey consumed by females between the two years of study, although no significant differences were detected in the
309 $\delta^{15}\text{N}$ values. This, together with the presence of a large overlap in the isotopic niche between sexes, suggests that the
310 foraging strategies of both males and females are rather similar during the breeding season. However, females had
311 significantly lower $\delta^{15}\text{N}$ values than males during the nonbreeding season of 2016.

312

313 **The diet of Madeiran storm-petrels**

314 The molecular techniques used in this study allowed the identification of many prey taxa to the genus and species
315 levels, some of which would have been unlikely to be identified through traditional methods. However, issues
316 encountered during HTS analysis, specially the unsuccessful test of a new primer pair to identify marine invertebrates, led
317 to conclude that more optimized primers for identification of marine biodiversity must be used. The proportions of
318 samples that were considered having no prey DNA can also be related with: (1) absence or very low concentration of
319 DNA in the sample, possibly indicating a period of fasting from these individuals, (2) failure in detecting prey DNA,
320 potentially due to DNA degradation or the presence of PCR inhibitors, (3) primers' taxonomic resolution: although we
321 used primers that targeted a wide range of prey groups, it is likely that they do not amplify all desired target prey species;
322 (4) lack of specific primer sets to detect other prey groups, (e.g. cartilaginous fish or mammals obtained through
323 scavenging). Issues with failure to detect prey DNA in faecal samples are common across dietary studies (Deagle et al.
324 2007). Primer choice is unlikely to be the main explanation for this since the primers' specificity was tested in a wide
325 range of prey, as well as mammals or cartilaginous fish are unlikely to be important prey for these birds (Medeiros-Mirra
326 2010).

327 Due to the challenges faced with the amphipods results, we cannot conclude precisely on the importance of this prey
328 group for Madeiran storm-petrels'. However, because the general invertebrates' primer also amplifies amphipods' DNA
329 (Online Resource 3), which was detected at a lower proportion than fish in all groups except in males of 2017 (Fig. 1), we
330 can conclude that fish is possibly the prey group with major importance for Madeiran storm-petrels during the breeding
331 season. Gadiformes (cod fishes) was the predominant fish order detected in our samples, particularly *Trisopterus* sp. and
332 *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 $\delta^{15}\text{N}$ values presented by our sample were similar to those reported for Cory's shearwaters
340 (*Calonectris borealis*, $\delta^{15}\text{N}$ values between 12.87 ± 0.26 and 13.34 ± 0.17) and the Macaronesian shearwaters (*Puffinus*
341 *baroli*, $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ values of ~13‰, and such difference between zooplankton and our
349 species' tissues values lead us to assume that this is not a predominantly zooplanctivorous 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

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 $\delta^{15}\text{N}$ 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 $\delta^{13}\text{C}$ and higher $\delta^{15}\text{N}$ 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 Procellariiformes (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).

420

421

422

423 **Conservation implications**

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

433

434 **Compliance with Ethical Standards**

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440 and/or institutional guidelines for the care and use of animals were followed. All animal handling and procedures in this
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445

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451

452

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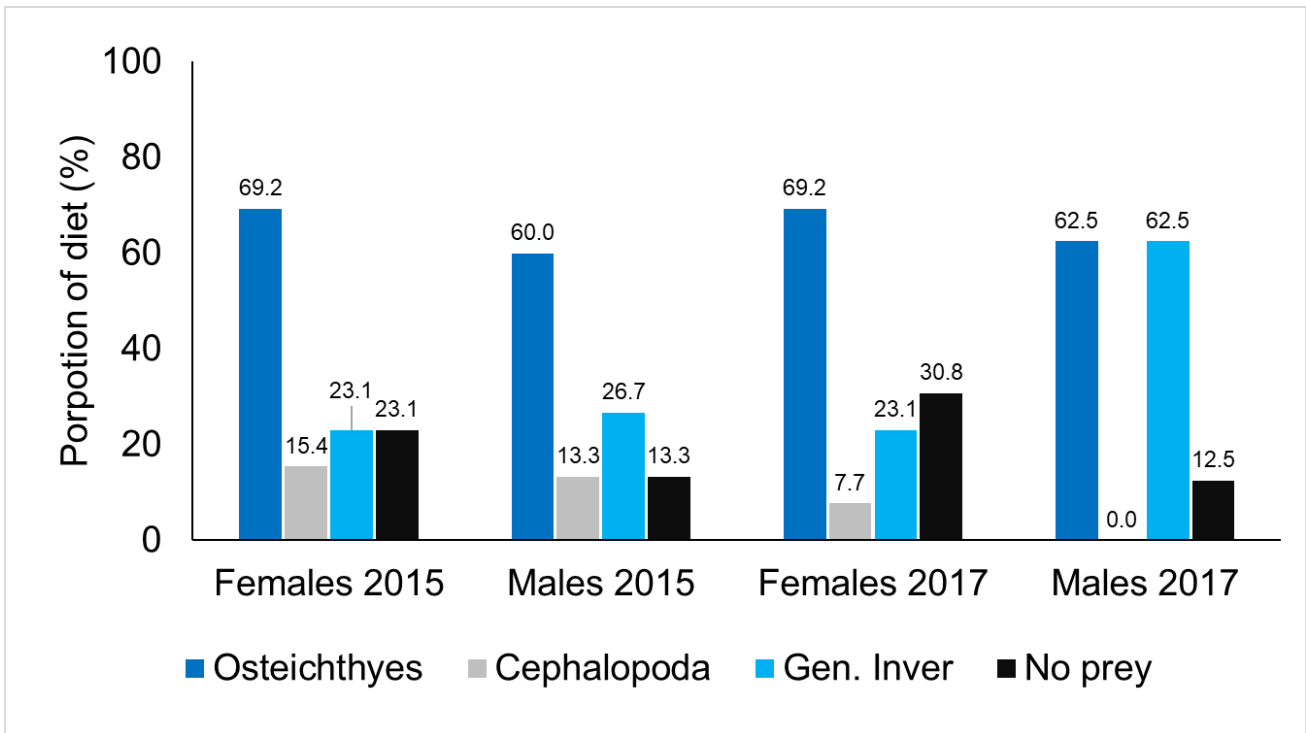
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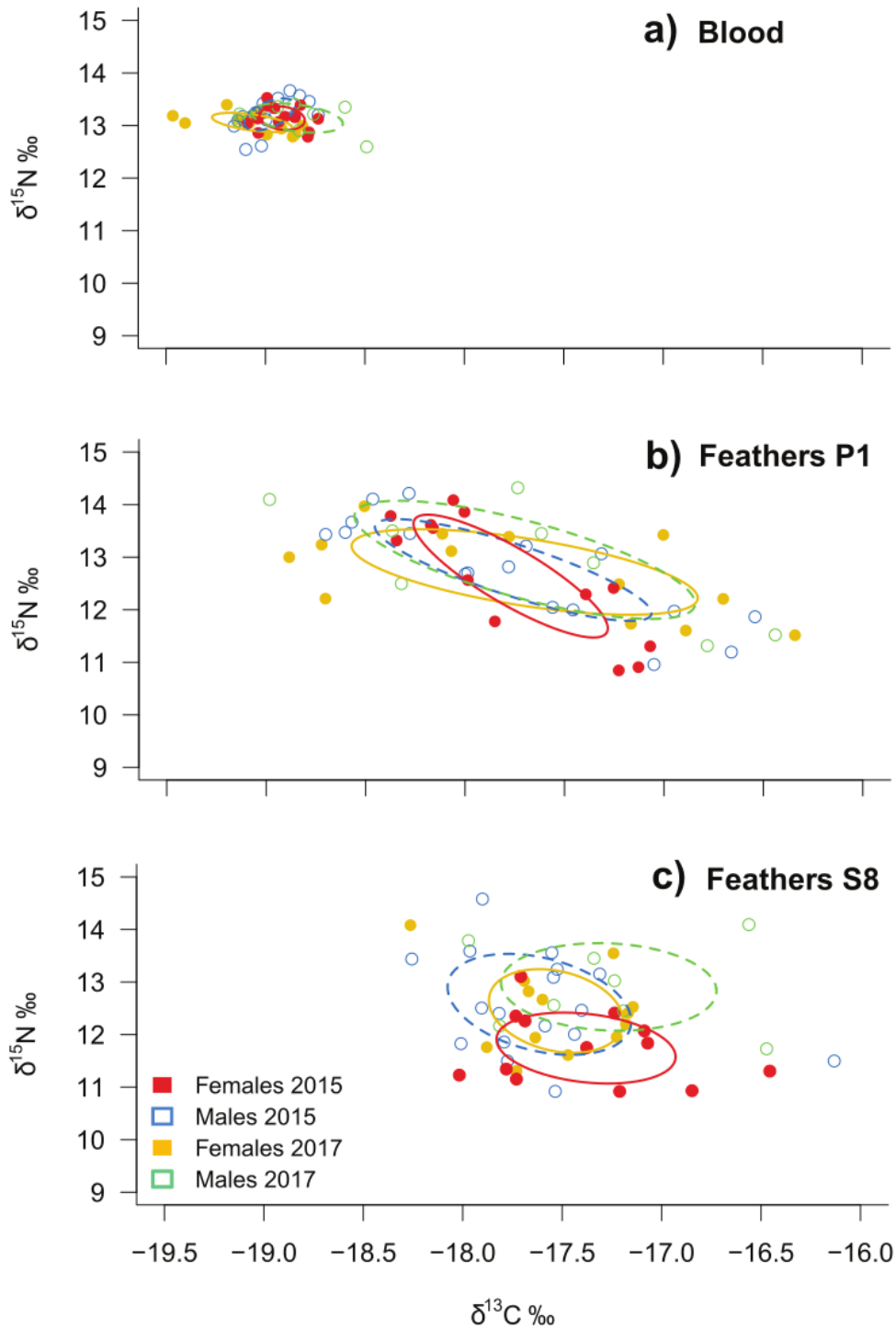
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647 **Figure 1** - Proportion (%) of detected fish, cephalopods, general invertebrate's and no DNA per sex and year for
648 Madeiran storm-petrels after DNA amplification.

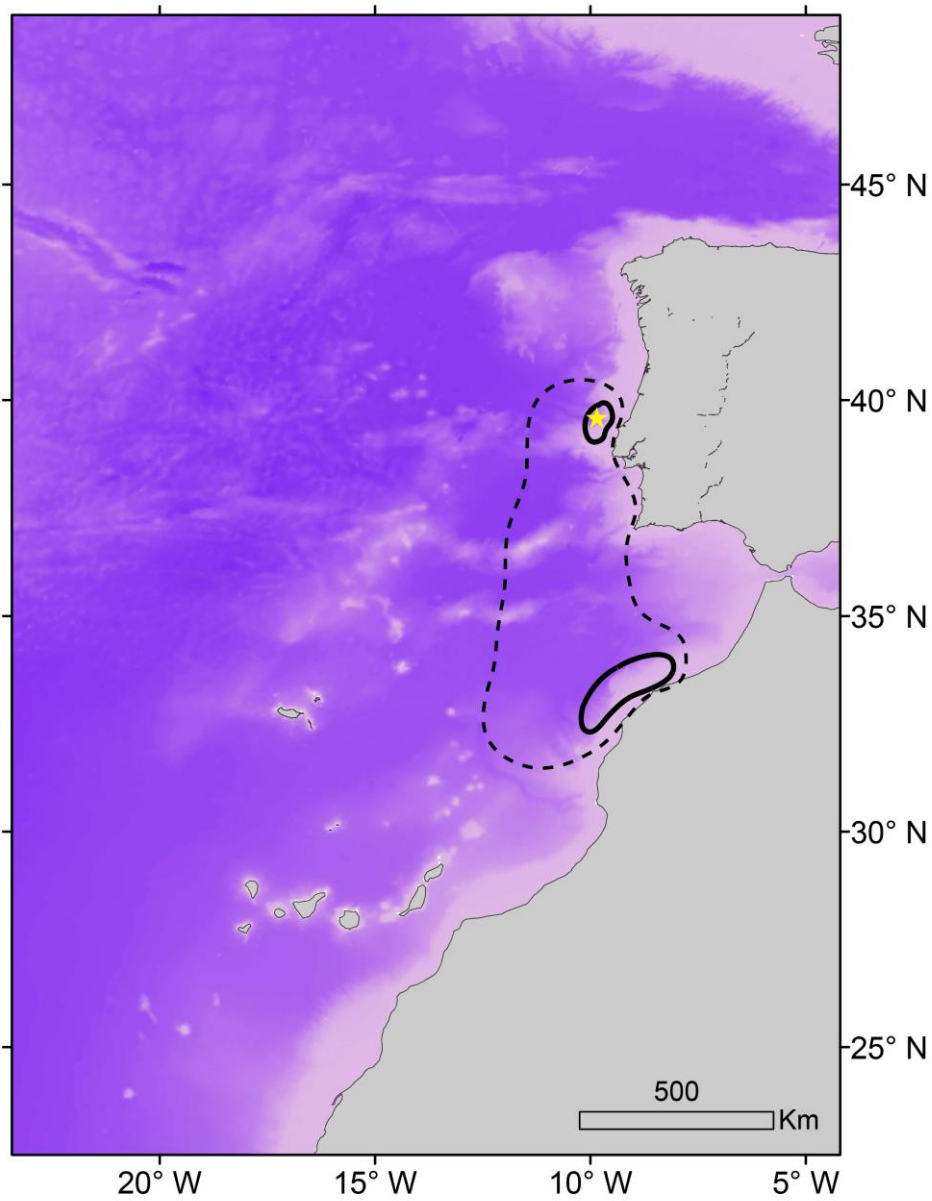
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652 **Figure 2** - Annual comparison of isotopic niche space of Madeiran storm-petrel between females (filled lines and
 653 symbols) and males (dotted lines and empty symbols), using a) whole blood, b) 1st primary feather and c) 8th secondary
 654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

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659 **Figure 3** - Home range (95% kernel Utilization Distribution (UD); dashed line) and foraging area (50% kernel UD; filled
 660 line) of Madeiran storm-petrels from Farihão Islet (Berlengas archipelago – identified with a star) during the early chick-
 661 rearing periods (January - February 2017). Bathymetry represented in the background varying from 1m (pink) to 3800m
 662 (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	<i>Trisopterus minutus</i>	6.21	0.00	1.41	0.53
				other <i>Trisopterus</i> sp.	48.80	17.87	25.53	16.41
				<i>Micromesistius poutassou</i>	22.33	38.53	38.68	62.44
				<i>Gadus</i> sp.	7.91	1.19	12.15	10.52
				<i>Gadiculus argenteus thori</i>	1.48	0.00	14.87	0.00
		Clupeiformes	Clupeidae	<i>Sardina pilchardus</i>	11.43	0.00	0.00	0.00
		Perciformes	Sparidae	<i>Pagellus acarne</i>	1.84	0.00	0.00	0.00
			Carangidae	<i>Trachurus</i> sp.	0.00	0.00	7.35	0.00
		Lampriformes	unknown family		0.00	0.00	0.00	9.57
		Aulopiformes	Alepisauridae	<i>Alepisaurus ferox</i>	0.00	1.50	0.00	0.00
		Stomiiformes	unknown family		0.00	25.06	0.00	0.00
		Myctophiformes	Myctophidae	<i>Myctophum punctatum</i>	0.00	13.65	0.00	0.00
				<i>Ceratoscopelus maderensis</i>	0.00	1.61	0.00	0.00
				<i>Protomyctophum</i> sp.	0.00	0.60	0.00	0.00
unknown genus	0.00			0.00	0.00	0.53		
28S	Cephalopoda	Oegopsida	Onychoteuthidae	<i>Onykia</i> sp.	-	4.91	86.26	-
			Chiroteuthidae	<i>Chiroteuthis</i> sp.	-	90.07	7.96	-
				unknown genus ^a	-	5.02	0.00	-
			unknown Family ^a	-	0.00	5.77	-	

669 ^aClassification contains 4 OUTs.

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672 **Table 2** - Results of a factorial analysis of variance (ANOVA) showing multiple comparisons of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ 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.

		$\delta^{13}\text{C}$			$\delta^{15}\text{N}$		
		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 (SEAC) 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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