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First Insights into the Diet Composition of Madeiran and Monteiro's Storm Petrels (*Hydrobates castro* and *H. monteiroi*) Breeding in the Azores

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Abstract. – Although studying the diet of threatened species is crucial in terms of conservation, the diet of the Madeiran Storm Petrel *Hydrobates castro* and the vulnerable and Azores-endemic Monteiro's Storm Petrel *H. monteiroi* is mostly unknown. The only information available to date comes from anecdotal observations, analysis of mercury levels and stable isotopes. Here is presented the first insights into prey consumption by adults and chicks from the two species breeding in the Azores. The rapidly developing field of metabarcoding was used to identify dietary items from fecal samples, to species level where possible. A total of thirteen fish, five cephalopod, one crustacean and two oligochaete operational taxonomic units (OTUs) were detected. Results suggest that both petrel species feed mainly on myctophid fish. However, differences were detected between the prey species consumed by (i) *H. monteiroi* and *H. castro,* (ii) two distinct *H. castro* populations (Vila and Praia islets), and (iii) chicks and adults within the same population.

Key Words. – Diet, Ecology, Metabarcoding, Hydrobatidae

Studying a species' diet is a key element for understanding its ecological and functional roles in the ecosystem as well as its conservation needs (Pauly *et al*. 1998; Shealer 2002). All methods currently available for the study of diet composition have important limitations: either the information that they can provide is not detailed enough, or the methods are invasive, or both (Barrett *et al*. 2007). Studying the diet of small, elusive animals such as the pelagic storm petrels (Hydrobatidae) is particularly challenging. Traditional sampling methods are too invasive for these small seabird species, and there is no satisfactory single method currently available to study their diet.

Over the past 15 years, molecular techniques have been extensively used to study seabird diet, through the identification of prey DNA from feces or regurgitations collected with minimal disturbance to the birds, and providing detailed information on the diet composition of penguins (e.g. Deagle *et al*. 2010; Jarman *et al*. 2013; Horswill *et al*. 2018; Xavier *et al*. 2018), cormorants (Thalinger *et al*. 2016; Oehm *et al*. 2017), shearwaters (Alonso *et al*. 2014) and storm petrels (Carreiro *et al*. 2020). Furthermore, molecular analysis of fecal samples allowed the first investigations of the diet of breeding and non-breeding adults, as well as chicks (McInnes *et al*. 2016; Horswill *et al*. 2018). This is important, since many studies assume that the food items regurgitated by breeding adults to feed the chicks are a good proxy for adults' diet (Waap *et al*. 2017; Bowser *et al*. 2013) but this is not always the case in seabirds (e.g. Davoren and Burger 1999; Wilson *et al*. 2004).

This study describes the use of a DNA-based method, metabarcoding, applied to fecal samples of two species of the genus *Hydrobates*, the Madeiran Storm Petrel (*H. castro*) and its sister species Monteiro's Storm Petrel (*H. monteiroi*), breeding in the Azores Archipelago, Portugal. The diet of both species is mostly unknown for the Azores populations, with the only information available to date coming from anecdotal observations (Monteiro *et al*. 1996b), analysis of mercury levels (Monteiro *et al*. 1995) and stable isotopes (Roscales *et al*.

2011; Bolton *et al*. 2008; Paiva *et al*. 2018); these suggest some degree of dietary segregation between the two species. In this study we analyze the diet from adults and chicks breeding in two different colonies: i) Praia Islet, where both species breed in allochrony (Monteiro's Storm Petrel between April and September; Madeiran Storm Petrel between September and March); and ii) Vila Islet, where only the Madeiran Storm Petrel breed (September to March).

Madeiran Storm Petrels are medium-sized storm petrels (~50g), that breed on oceanic islands from equatorial to subtropical latitudes (Monteiro and Furness 1998). A recent study of the diet of Madeiran Storm Petrels breeding on Farilhões Islet, off mainland Portugal (Carreiro *et al*. 2020), showed that they feed mainly on small gadid and myctophid fish species. This study also showed that the species, like many other Procellariiformes (e.g. Weimerskirch *et al*. 1994), seems to present a dual foraging strategy, with adults performing short foraging trips around the colony to feed their chicks, as well as long foraging trips, probably to restore their own body condition. This suggests there might be differences between the diet of adults and chicks, but no study to date has explored this.

Monteiro's Storm Petrel is classified as a vulnerable species (BirdLife International 2020), morphologically very similar to the Madeiran Storm Petrel, and has only recently been described as a separate species endemic to the Azores (Bolton *et al*. 2008). It nests on Praia and Baixo islets, off Graciosa Island, and potentially on the islands of Flores and Corvo (Meirinho *et al*. 2014). There is no comprehensive study of its diet, but from anecdotal observations and its phylogenetic closeness to the Madeiran Storm Petrel, it is assumed to feed on mesopelagic fish, especially myctophids (Monteiro *et al*. 1996a).

Here we describe for the first time the prey species consumed by these two storm petrel species breeding in the Azores, and compare the diets of (i) species that breed on the same islet at different times of the year (Madeiran and Monteiro's Storm Petrels on Praia Islet), (ii)

by two colonies of the same species (Madeiran Storm Petrels on Praia Islet, off Graciosa, and Vila Islet, off Santa Maria), and (iii) by adults and chicks of the two species and locations.

METHODS

Study Area

The Azores archipelago is situated in the mid-North Atlantic Ocean between 37º and 40º N, 25º and 32º W. It is comprised of nine volcanic islands, and numerous small islets (0.1 to 10 ha) distributed in three groups. Praia Islet is located off Graciosa Island (39° 2′ 35″ N, 27° 58′ 37″ W) in the central group, and Vila Islet is located off Santa Maria Island (36° 58′ 26″ N, 25° 10′ 16″ W) in the eastern group, *ca* 350 km from Praia Islet.

Fecal Collection

Fecal samples were collected in 2007 from both adults and chicks during the breeding season of each species: March to July (Monteiro's Storm Petrel) and September to December (Madeiran Storm Petrel). Fresh samples were collected opportunistically, during handling for banding (adults) or from the nest (chicks) and stored in 90% ethanol. A total of 103 fecal samples were collected across the two species/locations: 49 from Monteiro's Storm Petrels breeding on Praia (30 adults and 19 chicks), 37 from Madeiran Storm Petrels breeding on Praia (12 adults and 25 chicks) and 17 from Madeiran Storm Petrels breeding on Vila (12 adults and 5 chicks).

DNA Extraction and Amplification

DNA from feces was extracted using the QIAamp DNA Stool Mini Kit (Qiagen), following the manufacturer's standard protocol. For each extraction the whole sample was used, and hence, according to the manufacturer's instructions, the quantity of lysis buffer

added was adjusted depending on the weight of the sample. To test for any cross-over contamination, samples from each age group and population were extracted separately and two blank extractions were included for each batch of extractions.

All DNA extracts were screened using general primers for Bilateria organisms, as well as specific primers for Osteichthyes (bony fish) and Cephalopoda (cephalopods) (Table 1). All primers had previously been reported in the literature, including on previous studies in storm petrel species (Carreiro *et al*. 2020; Medeiros-Mirra 2010).

Amplifications were performed separately for each primer pair, using the Multiplex PCR Kit (Qiagen) in 20 µl reactions containing 1x Multiplex PCR Master Mix, 0.2 µM of each primer and 0.1 mg/ml of BSA (New England Biolabs). The template was 2 µl of the DNA extract. Thermal cycling conditions were as follows: 95ºC for 15 min, 35 cycles (94ºC for 30 sec, followed by the primer-specific annealing temperature for 90 sec, followed by 72ºC for 90 sec), concluding with 72ºC for 10 min. A minimum of three negative controls (two extraction controls, plus at least one distilled water blank) were included in each set of PCR amplifications. Initial PCR reactions were performed using non-modified primers, followed by PCR reactions with modified primers for sequencing (see below). PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and visualised by transillumination with UV light.

Preparation of DNA Libraries for Sequencing

Three different libraries were made for each set of fecal samples: 1) a general prey library using Bilateria primers; 2) a fish library using Osteichthyes primers and 3) a squid library using cephalopod primers. Each primer was modified by the addition of the 454 fusion sequence (Roche 2012) and a unique three base pair long label tag (MID tags) so that the different sample group had a unique combination of tags and could run together in the same

platform. MID tags were chosen from the list of 454 Standard MID set sequences recommended by Roche (2012).

Only samples that showed gel bands with each respective primer set were used for that particular library. General Bilateria primers were expected to have a low resolution in terms of prey identification (Phylum or Class; Jarman *et al*. 2004). Therefore, they were used mainly to confirm the major groups detected with the other primers, as well as identify the presence of other potential prey types that were not specifically screened for in this study. For each primer pair and sample set, the concentration of each individual sample was measured from the gel using a reference ladder and pooled at equimolar concentrations so that the contribution from each individual bird was similar. The DNA concentration of each pool was measured using Qubit (ThermoFisher Scientific, Waltham, MA, USA), and all pools were subsequently combined according to their concentration so that each one contributed equally to the final pool. The overall pool sample was sent to Eurofins MWG Operon for amplicon sequencing with the Roche GS-FLX Titanium series chemistry (454).

Data Analysis

For the sequencing data, the 'cutadapt' python package (Martin 2011) was used to de-multiplex the pooled sequences based on the forward and reverse primers and MID tags and to remove all the adapters (including primers and MID tags). Sequences missing any of the adapters were discarded. Reads from 12S, 18S and 28S amplicons were filtered to a length of 260 to 280 base pairs (bp), 180 to 212 bp and 105 to 135 bp respectively (minimum and maximum) and merged into a master file for each target group.

Reads were dereplicated using -fastx_uniques in USEARCH v10.0.240 (Edgar 2010) and singletons were removed. The UPARSE pipeline was used for 12S and 28S amplicons analysis with a 97% clustering (Edgar 2013). 18S were analyzed in the UNOISE algorithm

(Edgar 2016) with a 99% clustering, as suggested in previous works for these target groups (Bachy *et al*. 2013; Edgar and Flyvbjerg 2015). The total number of unique sequences, singletons, sequences lost, and sequences retrieved for each gene can be found in Table 2.

All commands and associated python scripts in the analysis are provided in the GitHub repository: https://github.com/AnaCarreiro/AzoresMSP2007. The NCBI database (Morgulis *et al*. 2008; Zhang *et al*. 2000) was used to taxonomically classify Operational Taxonomic Units (OTUs) through MegaBLAST, and only results with 100% query cover were considered as matches. Each primer pair's results were analyzed using different analysis parameters: i) for the 12S gene a minimum 90% identity and E-value of 1^{-100}; ii) for the 18S gene a minimum of 99% identity and E-value of 1^{-94} and iii) for the 28S a minimum of 95% identity and E-value of 1^{-47}. These thresholds were defined not only considering each fragment size, but also based on previous works using these genes (e.g. Bachy *et al*., 2013). Lowering these sequence similarity thresholds would result in a mixing of different taxa with no ecological sense for the study area and species. For each OTU, all the sequences matching the thresholds defined were considered and analyzed together in order to classify each group to the lowest taxonomic level possible. Low quality OTUs (i.e. sequences with lower similarity thresholds) and sequences from predator, parasites or contamination from the lab (Table 2) were excluded from the analysis. Taxon (e.g. species, genus, family) was assigned if the query sequences clustered monophyletically at that level, producing an identical match in BLAST, higher than any other taxa. Moreover, despite meeting the previous criteria, species- or genus-level identifications were not assigned if the identity match was below 99%. Therefore, those cases were discussed as probable genus-level identifications.

RESULTS

Very few samples presented visible bands in gel electrophoresis (Table 3). The number of samples with no visible bands, i.e. which were considered not to contain DNA, might have been a result of: (i) actual absence or very low DNA concentration in the sample, (ii) DNA degradation or the presence of PCR inhibitors, or (iii) being false negatives. The latter phenomenon has been reported by recent studies, which found that negatives in the gel do not necessarily mean lack of DNA (Zinger *et al*. 2019). This might also explain why cephalopod OTUs were detected with 18S primers in samples that did not show any clear bands in the gel of 28S primer amplifications (Table 3).

Despite the small sample sizes, a total of seven prey OTUs were detected for the 18S Bilateria primers, four prey OTUs were detected for the 28S cephalopod primers, and 10 prey OTUs were detected for the 12S fish primers (Table 3). As expected, it was difficult to achieve a high taxonomic resolution for prey identification from general Bilateria primers, but it was possible to identify major prey sub-classes, some orders and families, namely from fish, cephalopods, crustaceans, and oligochaetes. For cephalopod primers, it was only possible to detect one OTU at the genus level, namely the squid *Moroteuthis* sp., which comprised the majority of the sequences in all groups analyzed (from 55% to 100%). Three other genera from the Order Oegopsida were also detected, with a smaller proportional detection in each group.

Fish primers detected several OTUs from the Myctophidae family (Laternfish), and single OTUs from the Sparidae and Regalecidae families. Two OTUs were defined to the genus level, namely a Jack Mackerel *Trachurus* sp. and Deep-sea Barreleye *Monacoa* sp., and one to the species level, specifically the Madeira Lanternfish *Ceratoscopelus maderensis*. The proportion of sequences comprising each fish OTU was very well distinguished between storm petrel species, locations and age groups, with most OTUs being detected in only one of the groups. Lanternfish were the most abundant OTUs found in the diet of adult birds,

215 comprising from 97.8% to 100% of the sequences detected for both species and breeding
216 locations. Madeiran Storm Petrel chicks from Praia Islet had a diet similar to that of their
217 parents, with samples exclusively containing lanternfish. However, the other chick groups had
218 a very distinct diet from the adults: Jack Mackerel was exclusively detected in the diet of the
219 Madeiran Storm Petrel chicks from Vila Islet and comprised the total of its sequences, and a
220 Sparidae OTU comprised 97.4% of the sequences detected for Monteiro's Storm Petrel
221 chicks.
222
223 DISCUSSION
224 This study is the first work to date to identify prey species in the diet of the Madeiran and
225 Monteiro's Storm Petrels in the Azores archipelago, and the first description of the diet of the
226 Azores-endemic Monteiro's Storm Petrel. It integrated both spatial and age-related
227 comparisons of the diet of both species during the breeding season of 2007. Although the
228 number of initial samples was 103, the final sample sizes were very small, and comparisons
229 must be interpreted only as indicative.
230 Overall, the diet of these two seabird species seems dominated by myctophid fish,
231 although with possible dietary segregation among all groups. For example, although the adults
232 of both species breeding in Praia Islet rely mostly on Myctophidae fish (Lanternfish), they
233 seem to prey on different species of myctophids. Our results are in line with previous studies
234 of mercury and stable isotopes in feather samples of these same species (Monteiro *et al*. 1995;
235 Bolton *et al*. 2008). Since myctophids are mesopelagic and migrate to the sea surface
236 nocturnally, the presence of myctophid species in the diet of the two species and in the two
237 populations of Madeiran Storm Petrels suggests that the birds feed at night, and possibly over
238 bathymetric features which cause upwelling to occur, making mesopelagic prey more
239 available at the surface (Watanuki and Thiebot 2018). Prey from commercial fish groups were

found for the chicks of Madeiran Storm Petrels on Vila Islet and the chicks of Monteiro's Storm Petrels on Praia Islet, suggesting that birds might consume fishery discards, as previously suggested by other storm petrel dietary studies (Medeiros-Mirra 2010; Carreiro *et al*. 2020). Segregation in the diets of Madeiran and Monteiro's Storm Petrels at different colonies may result in differential breeding success (e.g. Ramírez *et al*. 2016) or adult survival between species and between colonies (Ramos *et al*. 2012), especially if climate change and changes in discard legislation affect the relative availability of different prey species. Seabirds are particularly vulnerable to stochastic changes in prey availability during the breeding season, as they are constrained to forage relatively close to the colony in order to regularly return with food to the nest. Therefore, further understanding the precise diet of Monteiro's Storm Petrel during the breeding season, and how this compares and contrasts to its allochronous sister species, the Madeiran Storm Petrel, will improve our understanding of how climate change or other human impacts may differentially influence populations of closely related seabird species.

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395 **Table 1. Primer pairs used in the analysis of the diet of *Hydrobates castro* and *H. monteiroi*.**

Target	Primer name	Sequence 5'-3'	Product size (bp)	Annealing temp. (ºC)	Reference
Actinopteriigy mitochondrial 12S	FishF1	CGGTAAAACTCGTGCC	~300	56	Jarman unpubl. *in* Medeiros-Mirra (2010)
	FishR1	CCGCCAAGTCCTTTGGG			
Bilateria nuclear 18S	BilSSU1100_F	AGAGGTGAAATTSTTGGAYCG	~245	62	Jarman *et al.* (2004)
	BilSSU1300_R	CCTTTAAGTTTCAGCTTTGCA			
Cephalopoda nuclear 28S	Squid28SF	CGCCGAATCCCGTCGCMAGTAAAMGGCTTC	~180	60	Deagle *et al.* (2005)
	Squid28SR	CCAAGCAACCCGACTCTCGGATCGAA			

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Table 2. Total number of uniques, singletons, chimeras and sequences lost for each primer, as well as total sequences retrieved per group.

	12S	28S	18S
Sequences after filtering	1598	3617	17019
Uniques	603	458	3187
Singletons	456	246	2168
Chimeras	0	0	0
Total sequences lost	682	62	14083
Low quality querys	334	62	2705
Contamination	348	0	1337
Predator DNA	0	0	9119
Parasite DNA	0	0	922
OTUs/ZOTUs	12	4	7
Sequences retrieved	916	3555	2936
VA	197	0	256
VC	5	0	46
PA	376	2282	1436
PC	58	0	297
MA	91	878	158
MC	189	395	743

Table 3. Taxa identified from high-throughput sequencing of fecal samples obtained from Madeiran Storm Petrel (*Hydrobates castro*) breeding on Vila Islet (VA – Adults; VC – Chicks) and Praia Islet (PA – Adults; PC – Chicks), and of Monteiro's Storm Petrel (*H. monteiroi*) breeding on Praia Islet (MA – Adults; MC – Chicks), using DNA fragments from three different genes. Values represent the percentage of sequences for each sample set that comprise each prey type.

Target Gene	Classification	VA	VC	PA	PC	MA	MC
		N=1	N=1	N=5	N=4	N=2	N=4
12S	Fish						
	Trachurus sp.		100.0				
	Myctophidae 1 (Family)	100.0		28.7			
	Myctophidae 2 (Family)			6.4			
	Myctophidae 3 (Family)			10.4			
	Monacoa sp.			5.3			
	Myctophidae 4 (Family)			49.2	100.0		
	Ceratoscopelus maderensis					97.8	
	Regalecidae (Family)					2.2	
	Sparidae (Family)						97.4
	Myctophidae 5 (Family)						2.6
		-	-	N=2	-	N=2	N=1
28s	Squid						
	Oegopsida 1 (Order)			45.0			
	Moroteuthis sp.			55.0		96.7	100.0
	Oegopsida 2 (Order)					0.6	
	Oegopsida 3 (Order)					2.7	
		N=8	N=4	N=7	N=12	N=11	N=7
18S	Fish						
	Gadiformes (Order)		17.4		6.7		
	Actinopterygii (Class)	79.3	39.1	82.7	58.2	38.6	63.4
	Teleost (Infraclass)	11.7	43.5	14.8	15.5	15.2	34.3
	Oligochaetes						
	Naididae (Family)	0.4		0.5	4.4	13.3	2.3
	Naididae 2 (Family)			0.3	7.4	19.0	
	Cephalopods						
	Coleoidea (Subclass)	8.6		1.7	3.0	13.9	
	Crustaceans						
	Chydoridae (Family)				4.7		