APPENDICES

MESOPELAGIC DIET AS PATHWAY OF HIGH MERCURY LEVELS IN BODY FEATHERS OF THE ENDANGERED DIABLOTIN BLACK-CAPPED PETREL (DIABLOTIN) *PTERODROMA HASITATA*

Appendix 1. Detailed DNA metabarcoding methods and results

Amplification of barcoding genes

PCR reactions were performed in two stages, whereby the first-stage PCR amplified the target amplicon, while the second-stage PCR ligated sample-specific tags to the amplicons, allowing them to be combined for sequencing and traced back to the samples from which they came. The first-stage PCRs were performed in a dedicated clean S1-laboratory, with UV sterilization of surfaces and the inclusion of positive and negative controls to monitor for contamination during PCR set-up. The first-stage PCRs were also performed in duplicate to increase the likelihood of detecting rare sequences. PCR products were visualised via gel electrophoresis to confirm amplification and check that negative controls were clear, before products from each duplicate were combined and diluted in preparation for the second stage PCR.

To identify fish prey, we amplified a fragment of the 12S rRNA gene using the popular MiFish primers (Miya *et al.*, 2015; underlined below) to which we had added TruSeq tails (primer sequences were thus MiFish-U-F-TruSeq:
5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCGGTAAAACTCGTGCCAGC-3';
and MiFish-U-R-Truseq:
5'-TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCATAGTGGGGTATCTAATCCCAGTTTG-3').
First-stage PCRs were performed in 12 μL reactions, containing 6 μL of KAPA HiFi HotStart 2X Ready Mix, 0.7 μL of the forward and reverse primers at 5 μM concentrations, and 4.6 μL of template DNA or molecular-grade water for negative controls. For the PCR positive controls, we used a mock community, consisting of a mixture of DNA extracted from known fish species, allowing us to determine whether we were amplifying and detecting all species that we expected to see in the mock community. PCR thermocycling conditions were: 95 °C for 3 mins; 35 cycles of 98 °C for 30 secs; 65 °C for 30 secs; 72 °C for 30 secs; followed by a final extension period of 72 °C for 5 mins.

To identify other prey, we used the universal eukaryotic primers developed by McInnes *et al.* (2017) to amplify the v7 region of the small subunit rDNA (hereafter 18S), allowing us to theoretically identify

all prey to the family- or order-level. We added Nextera tails to the primer sequences from (McInnes, Alderman, Deagle, *et al.* 2017) such that our forward and reverse primers were:
18S_Nx_F 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGTCTGTGATGCCCTTAGATG-3'
and
18S_Nx_R 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGTGTGTACAAAGGGCAGGG-3',
respectively. The 12 µL PCR reaction included 6 µL of Amplitaq Gold 360 Master Mix, 0.84 µL of each of the forward and reverse primers at a 5 µM concentration, 0.5 µL of BSA at 20 mg/ml concentration, 2.82 µL of molecular grade water, and 1 µL of template DNA. Thermocycling conditions were: 95 ^{o}C for 10 min, 35 cycles of 95 ^{o}C for 30 seconds, 67.5 ^{o}C for 30 seconds, and 72 ^{o}C for 30 seconds, followed by a final extension at 72 ^{o}C for 7 minutes.

For both MiFish and 18S amplicons, the second-stage PCR was performed by the Hubbard Center for Genome Studies at the University of New Hampshire and used the diluted product from the first-stage PCR as template. The second-stage PCR added the flow cell binding sites and sequencing primer binding sites, in addition to i7 and i5 indexes used to identify samples. We used unique dual indexes, such that any reads affected by tag-jumping would be removed during demultiplexing. Sequencing was performed using a small percentage of a lane on a NovaSeq 6000 system, using 250bp paired-end chemistry.

Bioinformatics

Bioinformatics were performed using Qiime2 v2021.2 (Bolyen *et al.* 2018). For both 18S and MiFish amplicons, forward and reverse primers were trimmed using the cutadapt plugin (Martin 2011). For the MiFish amplicons, we denoised and merged paired-end reads using the DADA2 plugin (Callahan *et al.* 2016), truncating the forward and reverse reads to 133 and 138 bp, respectively, and specified a minimum overlap of 30 bp between them. After denoising, taxonomy assignments were made using an iterative BLAST method against a custom reference database. To create the reference database, we used the RESCRIPt plugin (Robeson *et al.* 2021) to download any 12S or mitochondrial genomes from GenBank that originated from fish or birds that were studied in our laboratory. Downloaded database sequences were cleaned and dereplicated using RESCRIPt default parameters and finally, a human mitochondrial genome was added to the database, as this is a common source of contamination. The iterative BLAST method then took each representative sequence from our samples and blasted it 80 times against the reference database, increasing the percent identity incrementally from 70%–100 %, thus circumventing the limitation of the BLAST method, which keeps only the first hit that meets the search criteria, rather than the best hit. The script for the iterative

BLAST method is available from https://bitbucket.org/dwthomas/qiime2_tools/src/master/mktaxa.py. We chose this method to assign taxonomy rather than training a Naïve Bayes classifier, as this method identified all species in our mock community correctly, while a trained classifier mis-identified one species. Next, we filtered out unassigned reads, reads originating from human contamination or reads originating from the birds themselves. We then normalized all samples to match the sequencing depth of the sample with the lowest depth (10,872 reads) and manually checked all species assignments by blasting the representative sequences against the full GenBank database and checked that the fishes' ranges overlapped with the foraging areas used by Black-capped Petrels.

Results

For the 18S amplicons, we followed a similar pipeline with a few modifications. We truncated the forward and reverse reads to 150 bp during denoising. Then, to assign taxonomy to the sequences, we trained a Naïve Bayes classifier using the feature classifier plugin (Bokulich *et al.* 2018, Pedregosa *et al.* 2011) on a Qiime compatible version of the SILVA database (v132, released 10-Apr-2018, 99% clustered, downloaded from https://www.arb-silva.de/download/archive/qiime) after extracting the region bounded by our sequencing primers. Because the 18S gene is relatively conserved across eukaryotes, we did not attempt to assign taxonomy higher than the order-level. We excluded non-prey sequences including those from birds, mammals, parasites, and non-metazoan organisms. Sequencing of the 18S amplicons yielded 590,705 reads across all the samples, positive- and negative controls (mean 73,838 reads; Table S1). On average, 89% of the reads were retained after merging, denoising, and chimera removal. None of our extraction or PCR blanks contained any putative prey DNA, although they did contain contaminating mammalian (probably human), fungal, and algal DNA.

Sequencing of the 12S amplicons yielded a total of 7.7 million reads across all samples including positive and negative controls (mean 590,000 reads). After merging paired-end reads, denoising, and chimera removal, 61%–91% of reads were retained from the samples. No reads were retained in our PCR negative controls, while our extraction negative controls had either a small number of human or unassigned reads, indicating that contamination in our extraction and PCR pipeline was negligible. After filtering out non-fish sequences from the samples, we were left with between 10,872 and 1,556,058 reads per sample belonging to fish prey. To account for this variation in depth among samples, we normalized the read depth among samples to 10,872 (i.e. the minimum depth among all samples).

TABLE S1

Prey groups identified with 18S universal eukaryotic primers consumed by Black-capped Petrels captured at nest sites in the Dominican Republic in 2018 (n) and at sea off Cape Hatteras, USA, in 2019 (s).

	261 (n)		262 (n)		262* (n)		265 (n)		255 (s)	
Prey group	**Reads**	**Prop.**	**Reads**	**Reads**	**Prop.**	**Prop.**	**Reads**	**Prop.**	**Reads**	**Prop.**
Cephalopod	0	0	76	0	0	6.62	0	0	0	0
Teuthida sp.	59	5.60	0	0	0		0	0	0	0
Fish	994	94.4	1072	101482	100.0	93.4	57	100.0	229731	99.9

Petrels are identified by individual ID numbers, as in Table 1. Fecal samples were analysed for all individuals; for individual 262, an additional regurgitation sample was analysed (identified with *).

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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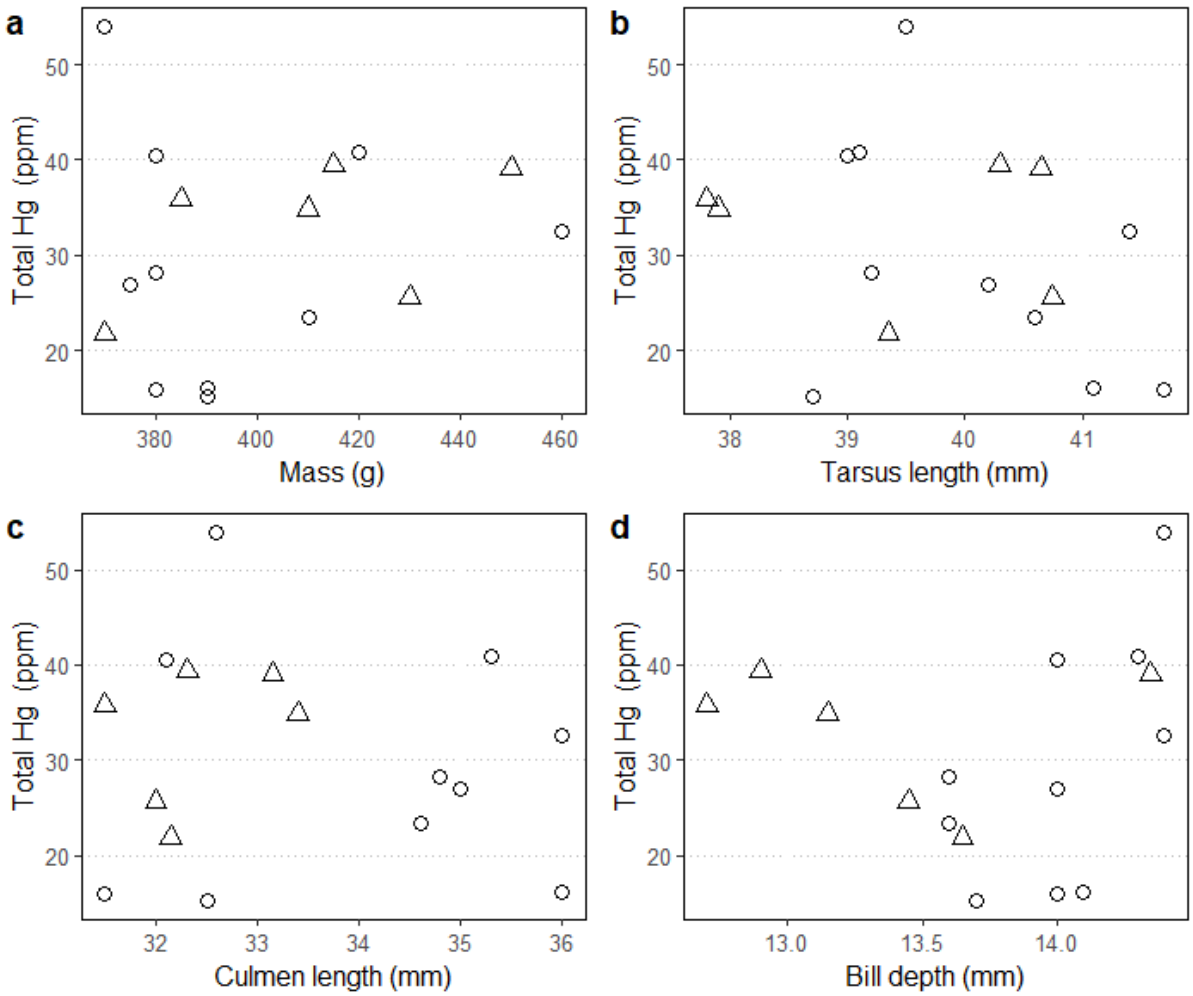

Appendix 2. Relationships between morphometrics and concentrations of total mercury (Hg) in Black-capped Petrel feathers. Triangles show samples collected at nesting sites in the Dominican Republic in 2018. Circles show samples collected at sea offshore North Carolina, USA in 2019.

Appendix 3. Published concentrations of Total mercury in feathers of *Pterodroma* species. Mean concentration, SD, SE, geometric mean, minimum, and maximum are given in ppm, although original studies may have provided values in different units.

English name	Code	Species	Age class	Sex	Type	Sample preparation	n	Mean concentration	SD	SE	Geo-metric mean	Min.	Max.	Original units	Years sampled	Site	Reference
Cook's petrel	COPE	*cookii*	Adult	UNK	Body	fresh weight	1	12.4						µg/g	1975-1983	New Zealand	Lock *et al.* 1992
			Young	UNK	Body	fresh weight	1	0.7						µg/g	1975-1983	New Zealand	Lock *et al.* 1992
Mottled petrel	MOPE	*inexpectata*	Young	UNK	Body	fresh weight	1	7.5						µg/g	1975-1983	New Zealand	Lock *et al.* 1992
White-headed petrel	WHPE	*lessonii*	Adult	UNK	Body	fresh weight	1	24.4						µg/g	1975-1983	New Zealand	Lock *et al.* 1992
			Young	UNK	Body	fresh weight	10	1.54	0.34			1.07	1.99	µg/g	2003	Kerguelen Island	Blévin *et al.* 2013
			Adult	UNK	Breast	dry weight	10	12.4	2.00			9.2	17.1	µg/g	2003-2011	Kerguelen Island	Carravieri *et al.* 2014
Black-capped petrel	BCPE	*hasitata*	Adult	UNK	UNK	UNK	22	18						mg/kg	1980	Offshore NC, USA	Simons *et al.* 2013
			Adult	UNK	Breast	dry weight	10	29.363	12.13			15.196	53.942	ng/g	2019	Offshore NC, USA	This study
			Adult	UNK	Breast	dry weight	10	31.250	9.14			15.276	43.039	ng/g	2018	Dominican Rep.	This study
Atlantic petrel	ATPE	*incerta*	Adult	UNK	Breast	dry weight	23	13.9	3.60					ng/g	1983	Gough Island	Thompson *et al.* 1990
			Adult	UNK	Breast	dry weight	15	13.52	4.14			3.92	20.09	ng/g	1985	Gough Island	Thompson *et al.* 1993
			Adult	UNK	Breast	dry weight	10	20.118	5.01			12.658	27.757	ng/g	2009	Gough Island	Becker *et al.* 2016
			Adult	UNK	Breast	dry weight	10	20.118	5.01			12.658	27.757	ng/g	2009	Gough Island	Becker *et al.* 2016
Soft-plumaged petrel	SPPE	*mollis*	Adult	UNK	Breast	dry weight	21	10.3	2.30					ng/g	1983	Gough Island	Thompson *et al.* 1990
			Adult	UNK	Breast	dry weight	17	9.82	2.32			5.36	13.4	ng/g	1985	Gough Island	Thompson *et al.* 1993
			Adult	UNK	Breast	dry weight	19	12.21	4.23			4.67	25.48	ng/g	2003-2011	Kerguelen Island	Carravieri *et al.* 2014
			Adult	UNK	Breast	dry weight	10	15.063	3.51			9.18	19.983	ng/g	2009	Gough Island	Becker *et al.* 2016
			Adult	UNK	Breast	dry weight	5	7.202	4.98			1.715	11.546	ng/g	2011	Marion Island	Becker *et al.* 2016
Juan Fernandez petrel	JFPE	*externa*	Adult	Female	Contour	dry weight	5	4.2	0.30					µg/g	1995	Chilean coast	Ochoa-acuña *et al.* 2002
			Adult	Male	Contour	dry weight	11	3.9	0.20					µg/g	1995	Chilean coast	Ochoa-acuña *et al.* 2002
Stejneger's petrel	STPE	*longirostris*	Adult	Female	Contour	dry weight	2	7.3	0.80					µg/g	1995	Chilean coast	Ochoa-acuña *et al.* 2002
Kermadec petrel	KERP	*neglecta*	Adult	Female	Contour	dry weight	1	11						µg/g	1995	Chilean coast	Ochoa-acuña *et al.* 2002
			Adult	Male	Contour	dry weight	1	13						µg/g	1995	Chilean coast	Ochoa-acuña *et al.* 2002

English name	Code	Species	Age class	Sex	Type	Sample preparation	n	Mean concentration	SD	SE	Geo-metric mean	Min.	Max.	Original units	Years sampled	Site	Reference
Bonin petrel	BOPE	*hypoleuca*	Adult	UNK	Breast	dry weight	27	19.7		0.001	19			ng/g	?	Midway Island	Burger and Gochfeld 2000
			Young	UNK	Breast	dry weight	20	3.87		0.315	3.7			ng/g	?	Midway Island	Burger and Gochfeld 2000
			Young	UNK	Breast	dry weight	42	2.13	0.95			0.241	6.05	ng/g	2014-2015	Midway Island	Shaw 2019
			Young	UNK	Primaries	dry weight	42	1.47	0.71			0.181	2.88	ng/g	2014-2015	Midway Island	Shaw 2019
			Adult	UNK	Breast	dry weight	42	9.88	4.71			0.457	16	ng/g	2014-2015	Midway Island	Shaw 2019
			Adult	UNK	Primaries	dry weight	42	8.8	3.45			1.74	14.3	ng/g	2014-2015	Midway Island	Shaw 2019
Barau's petrel	BAPE	*baraui*	Young	UNK	Breast	dry weight	32	0.3	0.07					µg/g	2001-2004	Reunion Island	Kojadinovic *et al.* 2007
			Adult	UNK	Breast	dry weight	20	0.96	0.31					µg/g	2001-2004	Reunion Island	Kojadinovic *et al.* 2007
Great-winged petrel	GWPE	*macroptera*	Young	UNK	Breast	dry weight	10	1.64	0.48			0.96	2.68	µg/g	2005	Kerguelen Island	Blévin *et al.* 2013
			Adult	UNK	Breast	dry weight	14	15.82	4.44			9.76	27.13	ng/g	2003-2011	Kerguelen Island	Carravieri *et al.* 2014
			Adult	UNK	Breast	Dry weight	5	28.038	9.98			16.617	39.15	ng/g	2011	Marion Island	Becker *et al.* 2016
Grey-faced petrel	GFPE	*gouldi*	Adult	UNK	Breast	dry weight	220	36.48	9.59			18.06	64.22	ppm	2006-2012	North Island, NZ	Lyver *et al.* 2017
Chatham petrel	CHPE	*axillaris*	Adult	UNK	Undertail	dry weight	10	9.56	2.38			6.53	13.23	µg/g	2015	Chatham Islands	Thébault *et al.* 2020
Magenta petrel	MAPE	*magentae*	Adult	UNK	Flank	dry weight	8	34.14	6.83			27.34	45.69	µg/g	2015	Chatham Islands	Thébault *et al.* 2020

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Appendix 4. List of Black-capped Petrel diet samples collected at nest sites (2018) and at sea (2019)

Individual ID [a]	Sample ID	Sampling date (dd-mm-yyyy)	Sample age	Sample type	DNA amplification [b]		DNA sequencing
					MiFish	18S	
Breeding site (Loma del Toro, Dominican Republic)							
301	TRO11	15-04-2018	Fresh	Chick regurgitate (mostly fish oil)	N	N	N
261	TRO2A	15-04-2018	Fresh	Feces	Y	Y	Y
302	TTRO1	16-04-2018	>1 day old (still humid)	Feces	N	N	N
302	TRO15	17-04-2018	Fresh	Feces	N	N	N
263	TRO1	17-04-2018	Fresh	Feces	N	N	N
262	TRO102A	17-04-2018	Fresh	Adult regurgitate	Y	Y	Y
262	TRO102B	17-04-2018	Fresh	Feces (composed of little black "grains" ~ 1mm diameter)	N	N	N
262	TRO102C	17-04-2018	Fresh	Feces	Y	N	Y
304	TTRO9	18-04-2018	Fresh from night	Feces	N	N	N
305	CLH14	19-04-2018	Fresh	Feces	N	N	N
306	CLH109	20-04-2018	Fresh	Feces	N	N	N
265	CLH13	21-04-2018	Fresh	Feces	Faint	N	Y
307	TTRO8	23-04-2018	Fresh from night	Feces	N	N	N
Non-breeding site (Offshore Cape Hatteras, North Carlina, USA)							
249	HA05	05-09-2019	Fresh	Feces	Faint	Faint	Y
255	HA06	05-09-2019	Fresh	Feces	Y	Faint	Y

[a] Individuals with IDs in bold represent individuals for which mercury levels are available.

[b] Y = yes, and N= no.

Appendix 5. Fish identified using MiFish primers on the 12S rRNA gene from samples collected from by Black-capped Petrels captured at nest sites in the Dominican Republic in 2018 (n) and at sea off Cape Hatteras, USA, in 2019 (s)

Genus	Species	Known distribution [a]	Habitat [b]	Diel migr.	249 (s)		255 (s)		261 (n)		262 (n)		262* (n)		265 (n)		Total reads	Total prop.
					Reads	Prop.	Reads	Prop.	Reads	Prop.	Reads	Prop.	Reads	Prop.	Reads	Prop.		
Anguilliformes																		
Synaphobranchidae																		
Synaphobranchus	*kaupii*	Atl.	1		3279	30.2											3279	5.0
Serrivomeridae																		
Serrivomer	*beanii*	Atl., Car., GoM	2	Y			10872	100.0									10872	16.7
Myctophiformes			2	Y														
Myctophidae			3												498	4.6	498	0.8
Diaphus	*dumerilii*	Atl., Car., GoM	3	Y	6316	58.1											6316	9.7
Diaphus	*thermophilus*	Atl., Car.	3	Y					261	2.4					166	1.5	427	0.7
Stomiiformes																		
Gonostomatidae																		
Zaphotias	*pedaliotus*	Car.	4										1582	14.6			1582	2.4
Sternoptychidae																		
Polyipnus		Atl., Car., GoM	5						298	2.7							298	0.5
Lampriformes																		
Trachipteridae																		
Desmodema	*polystictum* [c]	GoM., Florida	3								460	4.2	192	1.8			652	1.0
Zeiformes																		
Grammicolepididae																		
Xenolepidichthys	*dalgleishi*	Atl., Car., GoM	6						370	3.4							370	0.6
Zeniontidae		Car., GoM.	5						1619	14.9							1619	2.5
Ophidiiformes																		
Ophidiidae		Atl., Car., GoM	6		1277	11.7											1277	2.0
Scombriformes			2						175	1.6					6957	64.0	7132	10.9
Trichiuridae																		

Benthodesmus [c]		Atl., Car., GoM	6								879	8.1	879	1.3
Chiasmodontidae			1					8317	76.5	6893	63.4		15210	23.3
Pseudoscopelus [c]		Atl., Car., GoM	4			688	6.3	734	6.8	2202	20.3	2372	21.8	5996	9.2
Caproiformes															
Caproidae															
Antigonia	*capros* [c]	Atl., Car.	1			5160	47.5							5160	7.9
Carangiformes															
Carangidae															
Caranx	*crysos* [d]	Atl., Car., GoM	7					347	3.2					347	0.5
Decapterus	*macarellus* [e]	Atl., Car.	3					1014	9.3					1014	1.6
Perciformes															
Lutjanidae															
Pristipomoides [f]		Atl., Car., GoM	6			2248	20.7							2248	3.4

Petrels are identified by individual ID numbers, as in Table 1. Fecal samples were analysed for all individuals; for individual 262, an additional regurgitation sample was analysed (identified with *). Numbers of sequence reads (Reads) and proportions (Prop.) are given for prey items present in samples. Information on distribution, depth range, habitat, and evidence of diel migration (Diel migr.: Y = yes) were compiled from fishbase.org.

[a] Known distribution of prey species in Black-capped Petrel range: Atl. = western North Atlantic, Car. = Caribbean Sea, GoM = northern Gulf of Mexico.

[b] Habitat of prey species: 1 = Mesopelagic to benthic; 2 = Epi- to bathy-pelagic; 3 = Epi- to meso-pelagic; 4 = Meso- to bathy-pelagic; 5 = Bathydemersal; 6 = Benthopelagic; 7 =.

[c] Juveniles of this prey are mesopelagic.

[d] Commercially fished; Associated with reefs; Juveniles associated with Sargassum.

[e] Commercially fished (purse-seine).

[f] Commercially fished (trawled)