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First description of Manx Shearwater *Puffinus puffinus* diet using DNA metabarcoding

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Abstract
Obtaining quantitative information on fundamental aspects of ecology such as diet can be challenging for pelagic seabird species. DNA metabarcoding of faecal samples is a non-invasive method of dietary analysis, with potential to identify prey at high taxonomic resolution. We apply this method to understand the diet of the Manx Shearwater *Puffinus puffinus*, a highly pelagic species where little quantitative information on diet is available. Using DNA metabarcoding analysis on faecal samples for this species, we identified six species of fishes: European Sprat *Sprattus sprattus*, Blue Whiting *Micromesistius poutassou*, Atlantic Herring *Clupea harengus*, Northern Rockling *Ciliata septentrionalis*, sandeel *Ammodytes* sp and a species of Cottoidei (sculpins and allies). Of 153 dietary samples, only 19 produced sufficient DNA to identify prey species. We speculate that our low success rate may be the result of long foraging trips early in the breeding season, where excretion prior to sampling may reduce the amount of prey matter in faecal samples and prey DNA degradation during prolonged residence in the gastrointestinal tract may reduce the detectability of prey matter. We discuss improvements in sample collection for future dietary studies of this and other highly mobile species with similar traits.

Introduction
Many seabird species face declines due to fish stocks being depleted via over-fishing, pollution, and climate change (Kowalczyk *et al.* 2014; Wanless *et al.* 2018). With recent reports suggesting almost half of UK seabird species have declined over the last 20 years (Burnell *et al.* 2023), it is becoming increasingly important to study diet—an often-overlooked but fundamental aspect of a species' ecology. This is especially true of wide-ranging pelagic seabirds, where direct observation of at-sea foraging behaviour is challenging, and the identification of prey in regurgitated food brought to offspring is often difficult (Weimerskirch *et al.* 2005; Kane *et al.* 2020). As seabirds return to land to breed, they are more accessible as study organisms than most pelagic foragers. Changes in seabird foraging behaviour can therefore provide insights into the productivity of remote marine ecosystems (Thompson *et al.* 1998; Piatt *et al.* 2007; Grémillet & Boulinier 2009).

Innovations in biotelemetry have allowed the remote observation of foraging behaviour (Dean *et al.* 2015; Whitford & Klimley 2019; Bolton 2021); however, until recently, only traditional methods of understanding diet itself were available (Weiser & Powell 2011; Nielsen *et al.* 2018; Whitford & Klimley 2019). For species that carry whole prey to their offspring, some prey identification can occur through field observation (Baillie & Jones 2003). In cases where feeding occurs through regurgitation, this requires forcing regurgitation (Thompson 1987; Weiser & Powell 2011), a method that is both invasive

and does not allow for clear identification of prey species (Nielsen *et al.* 2018). Stable isotope analysis is a less invasive method for investigating diet, but the results of which can only indicate the trophic position of prey, and approximate foraging locations (Navarro *et al*. 2007; Meier *et al.* 2017; Austin *et al.* 2019). Here we employ an alternative approach for sampling seabird prey, using DNA metabarcoding of faecal samples. This method has potential to provide both quantitative and qualitative information and can distinguish between morphologically similar prey types (Deagle *et al.* 2007, 2019). DNA metabarcoding has been applied to the faecal samples of multiple seabird species to understand diet and assess conservation risks (McInnes *et al.* 2017; Komura *et al.* 2018; Marcuk *et al.* 2024). For example, in Brown Boobies *Sula leucogaster* and Cape Verde Shearwaters *Calonectris edwardsii*, DNA metabarcoding was used to identify prey overlap with commercially targeted fish to assess bycatch risk (Carreiro *et al.* 2023). In Atlantic Puffins *Fratercula arctica,* DNA metabarcoding was applied to investigate how adult and chick diets differ and how foraging behaviour links to diet across colonies of differing productivity (Fayet *et al.* 2021; Kennerley *et al.* 2024).

Despite being a well-studied bird, very little is known about the diet of the Manx Shearwater *Puffinus puffinus*, an apex predator that is believed to specialise in the active subsurface pursuit of small fish and some cephalopods (Brooke 1990). Most of the global population of Manx Shearwaters breeds on islands around the UK, with foraging efforts centred in the Irish and Celtic seas, both of which are sites for significant offshore wind development in the next decade (Guilford *et al.* 2008; Crown Estate 2024). It is unclear how large-scale modifications to foraging areas may impact seabirds, and it is therefore important to understand and quantify any dietary specificities for this species (Masden *et al.* 2010; Warwick-Evans *et al.* 2018).

The last investigation of Manx Shearwater diet was conducted in the 1980s on a small number of samples, the results of which suggested pre-laying diet might be different to other breeding season stages (Thompson 1987). However, as diet was investigated through forced regurgitation, most fish could not be identified to a species level. Other dietary studies in seabirds suggest that prior to laying, females may require specific nutrients for egg synthesis (Boersma *et al.* 2004; Sorensen *et al.* 2009; Phillips *et al.* 2011). Here, we aim to provide novel dietary information; establishing the prey species present in the diet of Manx Shearwater and comparing diet between pre- and post-laying periods to understand how diet may vary with breeding stage. To understand how diet links to foraging sites, we additionally compared pre- and post-laying foraging areas using geolocator (GLS) devices. We also aimed to investigate sex differences in diet between males and females.

Methods

Ethics approval

All data collection was conducted under British Trust for Ornithology (BTO) licensing, which included Special Methods for GLS deployments and C permit licenses for ringing and handling Manx Shearwaters (permit number: C/6922). The University of Oxford's Animal Welfare and Ethical Review Board also approved the long-term geolocator work and specific dietary collection methods. Additionally, permission was obtained from the Islands Conservation Advisory Committee (ICAC) and wardens on Skomer.

Diet sample collection

Fieldwork was carried out on Skomer, an island located in the southeast corner of the Irish Sea, less than 2 km off the west coast of Wales, UK (51.7358°N, 5.2964°W) from 2021 to 2023 under the relevant BTO licences. Sex was assigned through morphometric differences in the cloaca on the egg-laying date where females have larger, inflamed cloacas (N = 199 birds of both sexes). This method was validated for a subsample of birds (N = 57) using DNA sexing from five plucked feathers (Fayet *et al.* 2021). Over all years, 153 dietary samples were collected during pre-laying and incubation (29 April-17 June); 94 from males, 57 from females, and two from birds of unknown sex. To obtain a pre-laying

sample, burrows were checked for occupancy every day prior to laying. For incubation samples, birds were sampled 1-2 weeks after the start of the 7-week incubation period. We placed each bird individually in a closed cardboard box for up to one hour. The boxes were lined with non-absorbent materials, either parchment paper or tin foil, and the box was checked every 10 minutes. If the bird defecated, it was removed from the box and placed back in its burrow. Out of 199 attempts to collect faecal samples, 116 were successful. Twenty-five additional samples were obtained opportunistically from clothing or vegetation. Faecal material was collected using sterile sticks and placed in a 2-ml screw-cap tube containing 1 ml Zymo Research DNA/RNA Shield. Samples were stored at room temperature until DNA was extracted. Each substrate from which samples were collected was also swabbed away from the faecal sample to provide a blank sample, used to monitor for background contamination. As we found it difficult to obtain pre-laying samples from females, 11 samples were collected through swabbing the egg on the laying date to remove any faecal material present. Most (78%) sampled individuals (for both egg swabs and faecal samples) were still incubating their eggs when checked 2-3 weeks after sampling, consistent with the natural egg failure rates observed on Skomer island (Brooke, 1990).

DNA extraction of diet samples

DNA extractions and PCR set-up were performed in a clean lab dedicated to the handling of low DNA-content faecal samples. Prior to DNA extractions, we homogenised samples (including the field blanks, which were treated the same as the faecal samples throughout) via bead beating on a BioSpec Mini-BeadBeater-16. We added a combination of 0.5-mm and 1-mm diameter zirconia/silica beads to the samples, followed by bead beating for 2 minutes. We then centrifuged the samples at 10,000 g for 1 minute and transferred 200 µl of the supernatant to two 96-well plates for DNA extraction following a modified version of the Zymo Research Quick-DNA Faecal/Soil 96 Magbead Kit, run on an Opentrons OT-2 liquid handling robot (protocol at https://github.com/GemmaClucas/Opentrons_OT2_protocols/blob/main/Zymo_fecal_bead_extractions_12columns.py). The OT-2 was fitted with a HEPA filter to provide a sterile environment and positive pressure to reduce the chance of cross-contamination among samples. On each plate, we included six negative controls that were treated the same as samples throughout all subsequent steps, allowing us to monitor for contamination during the extraction process. We also included samples of sexes and breeding stages across the two plates to reduce batch effects.

We used a hierarchical approach to amplify prey DNA from the faecal samples. We used the universal eukaryotic primers developed by McInnes *et al*. (2017) to amplify the v7 region of the small subunit rDNA (hereafter 18S), theoretically allowing us to identify all prey to the family or order level. Then we used the MiFish primers developed by Miya *et al.* (2015) to amplify the 12S rRNA gene from any fish DNA found in the faecal samples, since these primers have been shown to allow species-level identifications in a diverse array of fishes. For both PCRs, we used a two-step approach whereby the target amplicon is amplified in the stage-one PCR, and then a second PCR is used to add sequencing adapters and indexes prior to pooling samples.

In the first stage PCR for the 18S amplicons, we added Nextera tails to the primer sequences from (McInnes *et al.* 2017) such that our forward and reverse primers were: 18S_Nx_F 5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGTCTGTGA TGCCCTTAGATG-3’ and 18S_Nx_R 5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGG GTGTGTACAAAGGGCAGGG-3’, respectively. We included two no-template controls on each plate to monitor for contamination during PCR set-up, and a positive control (mock community) containing DNA extracted directly from five known fish species. 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 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 °C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. Each PCR was performed in duplicate for each sample, and 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 before the second stage PCR.
The first stage PCR for the MiFish amplicons followed the same methods, with each plate of samples run in duplicate and the inclusion of positive and negative controls. We added TruSeq tails to the original primer sequences developed by Miya *et al.* (2015) such that our primers were: MiFish-U-F-TruSeq 5' ACACTCTTTCCCTACACGACGCTCTTCCGA
TCTGTCGGTAAAACTCGTGCCAGC-3 and MiFish-U-R-TruSeq 5' GTGACTGGAGT
TCAGACGTGTGCTCTTCCGATCTCATAGTGGGGTATCTAATCCCAGTTTG-3'. The 12-µL PCR reaction included 6 µL of KAPA HiFi HotStart ReadyMix 2x, 0.7 µL of each of the forward and reverse primers at 5 µM concentration, and 4.6 µL of template DNA (or molecular grade water for the no-template controls). For the mock community, we added 1 µL of template DNA and 3.6 µL of molecular grade water.

For both MiFish and 18S amplicons, the second stage PCR was performed by the Hubbard Centre for Genome Studies at the University of New Hampshire using 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 250-bp paired-end chemistry.

Bioinformatics of diet samples

Bioinformatics were performed using Qiime2 v2021.4 (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 specifying a minimum overlap of 50 bp between them. After denoising, data from both sequencing plates were merged, and 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 lab. Downloaded database sequences were cleaned and dereplicated using *RESCRIPt* default parameters. 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 correctly identified all species in our mock community, while a trained classifier mis-identified one species. Next, we filtered out unassigned reads, reads originating from human contamination, and reads originating from the birds themselves. We then created rarefaction curves using the *diversity* plugin from Qiime2 to determine the sequencing depth needed to adequately capture the fish diversity in each sample. We found that 400 reads were sufficient, so we rarefied all samples to a sequencing depth of 400, discarding samples that had fewer than 400 reads. We then manually checked all species assignments in the remaining samples by blasting the representative sequences against the full GenBank database and checked that the fish species' ranges overlapped with the foraging areas used by Manx Shearwaters.

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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). We downloaded a Qiime compatible version of the SILVA database (v132, released 10 Apr 2018) downloaded from https://www.arb-silva.de/download/archive/qiime and, using the 99% clustered version of the database, we extracted the region bounded by our sequencing primers and trained the classifier on this region. We then filtered out non-metazoan, avian, and mammalian DNA.

Movement behaviour

In 2021, 51 migratetech C65 Super geolocator devices (from Migrate Technology) were deployed on Manx Shearwaters to record their movements during the subsequent pre-laying and incubation periods. Devices weighed 1 g, ~0.25% of the average shearwaters' body mass (400 g). GLS devices were mounted onto a custom made Darvic leg ring, using cable ties and a small amount of super glue. GLS devices were set to record light and immersion, with immersion recorded every 6 seconds to generate an immersion score; with 50 representing total immersion and 0 being completely dry. Thirty-six pf those devices were retrieved in 2022; of these 13 complete incubation periods were available.

All processing and statistical analysis was carried out in R version 4.0.2 (R Core Team 2024). Light data were processed using the *geolight* package (version 2.0) to calculate position from twilight events defined by a light intensity threshold of 10 (Livoski & Hahn 2012). Day length was used to estimate latitude, whilst the timing of midday/midnight was used to estimate longitude. The sun elevation angle used to define twilight was set to -4.5°, determined after inspecting latitude versus time plots across a range of sun elevation angles to identify the one that best matched the calculated latitudes with those of Skomer (Bråthen *et al.* 2021). A rolling 3-day mean was calculated for both longitude and latitude to smooth variation in the data and reduce error. The pre-laying period was defined as the period between migration's end and the egg-laying date obtained from colony monitoring. Migration end was determined from the GLS twilight defined longitude, as it is not subject to equinox error. Homewards migration could clearly be visualised by a steep increase in longitude towards the colony, and migration end was defined as the first date past a threshold of 6°W. Kernel density estimation plots were used to visually assess sex differences in pre-laying and incubation positions in the R package *adehabitatHR* (version 0.4.19; Calenge 2006). Given the associated error with geolocation (100-200 km), only 50% kernels were applied to represent core area (h=0.1; grid=2000) (Phillips *et al.* 2004).

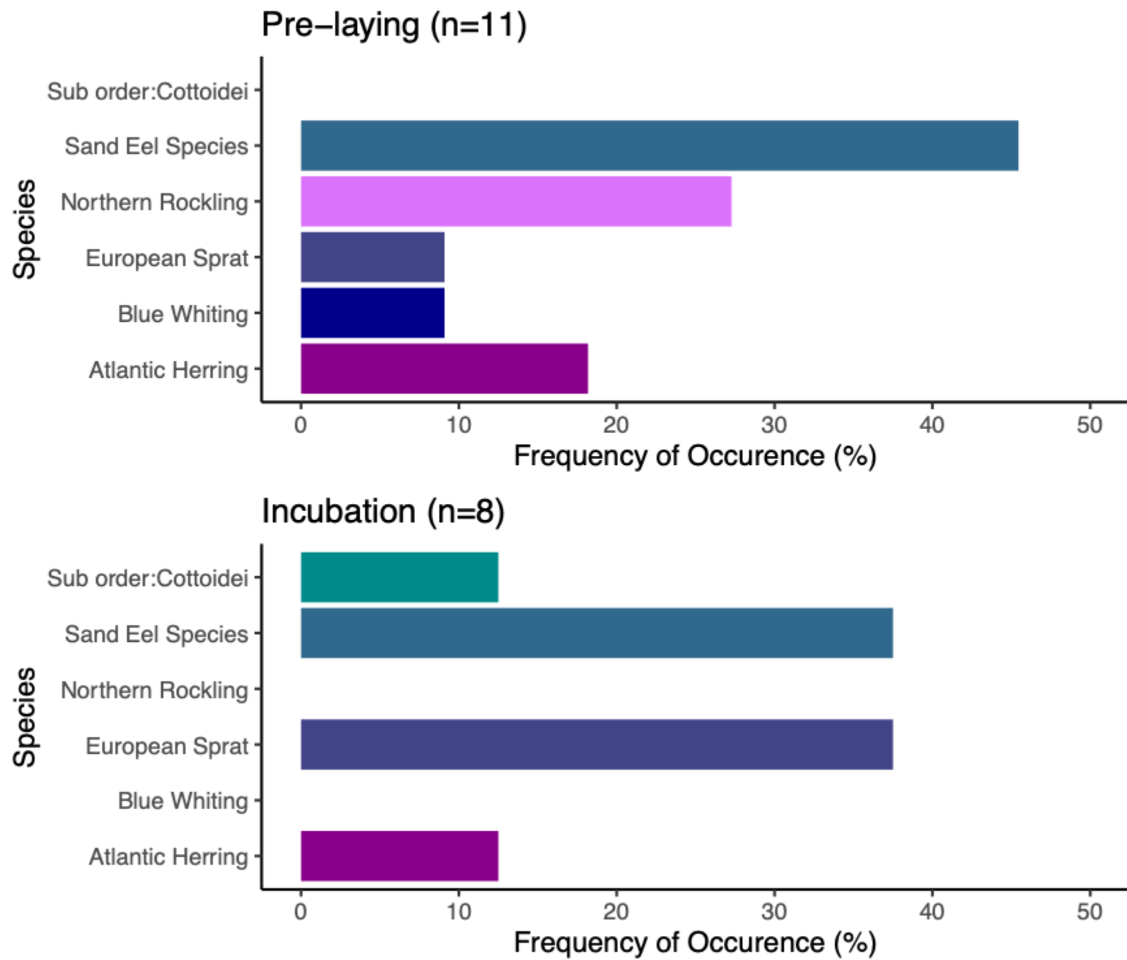

Figure 1. Results from the DNA metabarcoding of faecal samples from Manx Shearwaters *Puffinus puffinus* of Skomer Island to assess the diet of pre-laying (N = 11) and incubating birds (N = 8). The frequency of occurrence represents the percentage of individuals that had each prey species present in their diet. Both sexes were pooled given the small sample sizes (N = 3) available for females.

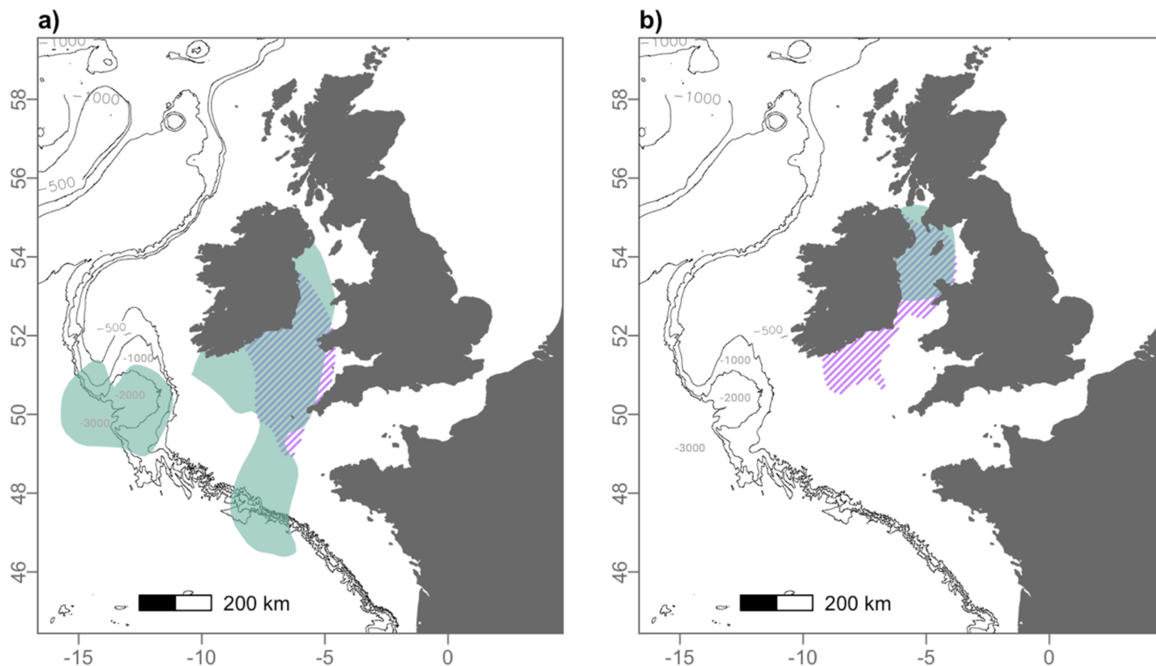

Figure 2. Kernel density estimation plots representing sex differences in movements of Manx Shearwaters *Puffinus puffinus* from Skomer Island during the pre-laying and incubation periods. a) Pre-laying movements (50% kernels) from GLS derived position (19 females; 17 males). b) Post-laying movements (50% kernels) from GLS derived position (6 females; 7 males). Females are plotted in green, whilst males are in hatched purple. Bathymetry was plotted through the *marmap* package (version 1.0.10) in R using depth contours at -500, -1000, -2000, and -3000 meteres, with increasing intervals at greater depths to highlight major topographic features (Pante & Simon-Bouhet, 2013).

Results

For the MiFish primers, 19 of the 153 dietary samples retained prey information and 7,600 sequences were retained. Of those 19 samples, 14 were from individuals sampled in boxes (N = 123 box samples), four were collected opportunistically in the field (N = 19 opportunistic samples) and one was collected from egg swabbing (N = 11 egg swab samples). The other 134 samples did not contain identifiable prey material, containing only DNA from the bird itself. The 18S primers for broader invertebrate identification contained some information on secondary predation (copepods), but mostly contained bird DNA, fungal DNA, algal or parasite DNA. Here we present only the results of the fish diet (Figure 1).

Manx Shearwaters consumed six different types of identifiable prey. Northern Rockling *Ciliata septentrionalis* and Blue Whiting *Micromesistius poutassou* were found only in the pre-laying diet, whilst fish of the suborder Cottoidei (sculpins and allies) were found only during incubation. Sandeel *Ammodytes* sp., Sprat *Sprattus sprattus*, and Herring *Clupea harengus* were found in both breeding stages with a greater proportion of Sprat present in the post-laying diet. We were unable to make any meaningful quantitative comparisons of the sexes, as only three of the successful samples were from females. However, Blue Whiting *Micromesistius poutassou* and Cottoidei species were only present in female diets, whilst Sprat and Herring were only detected in male diets.

From GLS-derived 50% kernels, pre-laying males appear to occupy a smaller range than females, extending towards the edge of the continental shelf of the Bay of Biscay (Figure 2). During post-laying movements, kernels indicate that birds foraged in an area more local to the colony, with foraging

primarily located in the Irish and Celtic Seas. Males foraged more widely, with some males foraging occurring in the Bay of Biscay.

Discussion

This study provides the first description of diet for Manx Shearwaters using DNA metabarcoding. Although there was a limited number of samples (N = 19) with dietary material suitable for analysis, we identified six fish species consumed during the pre-laying and incubation periods (Figure 1). Sandeels were the most frequently observed prey species across dietary samples. Sandeel is a critical prey item for many breeding seabirds but is largely declining due to oceanic warming (Fayet *et al.* 2021). A small number of samples (N = 3) also had Northern Rockling species present. It was not previously considered that Manx Shearwaters might feed on benthic species (Thompson 1987). However, the presence of Northern Rockling in the diet could provide evidence for benthic foraging or alternatively occur via feeding on pelagic juveniles or on fisheries discards (Furness *et al.* 2007). Unlike other closely related shearwaters, Manx Shearwaters are not known to follow fishing vessels.

Understanding seabird diets is fundamental given the current changes to marine habitat, especially during pre-laying where diet may influence egg formation (Jouanin *et al.* 2001; Fayet *et al.* 2021). Whilst DNA metabarcoding has proven to be a successful method of identifying prey in multiple pelagic seabirds, sampling shearwaters may present a new set of challenges (McInnes *et al.* 2017; Deagle *et al.* 2019; Carreiro *et al.* 2020). Shearwaters take long trips out to sea and may have excreted dietary material prior to returning to the colony to be sampled. Gut passage time is on the order of hours in seabirds (Hilton *et al*. 2000) and experiments in crows *Corvus sp.* have shown that dietary DNA may only be detectable up to 4 hours after feeding (Oehm *et al.* 2011). This is likely due to a combination of excretion and DNA degradation. With prey material spending time in the acidic environment of the gastro-intestinal tract, DNA degradation is likely to be part of the reason why many of our samples did not have amplifiable prey DNA. Given the collected samples were often of liquid consistency and green colouration, we suggest a lack of material is more likely to be the cause. Whilst false negatives are possible, they were likely minimal in this study, and unlikely to have caused the majority of samples to have no prey DNA. Similar challenges have arisen when using faecal samples from other procellariform seabirds. For example, in faecal samples from Shy Albatrosses *Thalassarche cauta*, breeding stage impacted the amount of dietary DNA present in faecal samples, with birds fasting during incubation retaining less dietary material (McInnes 2017). In Wedge-tailed Shearwaters *Ardenna pacifica*, a higher proportion of samples retained dietary DNA from the chick-rearing period than the pre-breeding period (Komura *et al.* 2018). It therefore appears that longer trips or incubation stint lengths may reduce the amount of prey material available for DNA amplification.

Owing to the small number of successful samples, especially for females, in this study, we were unable to compare sexes quantitatively to understand whether specific prey might be needed for egg synthesis. Female foraging trips during pre-laying can be especially long as females undertake a 'pre-laying exodus' (Brooke 1990; Jouanin *et al.* 2001). Kernel density plots (Figure 2) demonstrate that females disperse over a wider area during the pre-laying period, with foraging areas including the continental shelf around the Bay of Biscay, a spatial pattern that is consistent with previous work tracking this species (Guilford *et al.* 2009; Dean *et al.* 2013). We think our small sample size for females might be a consequence of pre-laying behaviour itself, with females taking long trips out to sea and potentially excreting dietary material before returning to the colony where they can be sampled. We obtained a lower number of faecal samples from females than males, with 45% of female sampling attempts resulting in sample collection whilst 63% of male sampling attempts were successful. Of the 19 samples that retained prey material, only three of these were from females (one of which was an egg swab).

For future dietary studies, we suggest sampling during periods of high colony visitation such as during chick rearing, where birds return more frequently to feed their chicks. Sampling birds immediately upon their return to the colony may also increase success rates, as opposed to sampling them on the following day, as we did here. Blocking primers, which prevent the amplification of host DNA in favour of dietary DNA, will also likely improve success rates (Pertoldi *et al.* 2021). Finally, egg swabbing could also be used to obtain the pre-laying diet of females alongside faecal sampling.

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