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Diet analysis of Kittiwake and Shag using DNA metabarcoding of faeces

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Abstract

Many seabird species are in decline, both globally and nationally, largely attributed to anthropogenic changes in the environment. Understanding the drivers of these declines requires detailed knowledge of population demographics and diet. However, long-term, multi-colony studies of seabird diet remain scarce. This study aimed to evaluate the feasibility of using DNA metabarcoding to obtain colony-level diet data from seabird faeces collected opportunistically during the breeding season.

We collected 45 Black-legged Kittiwake *Rissa tridactyla* and 43 European Shag *Gulosus aristotelis* faecal samples from four locations on the west coast of Scotland (Colonsay, Canna, the Treshnish Isles and the Shiant Isles). Prey DNA was successfully extracted from 76 (86%) samples, providing diet data for a region with limited diet monitoring. Across species and locations, we identified 20 prey fish taxa and nine likely invertebrate prey taxa, indicating that a broad range of prey was consumed. Sample sizes from Canna and Colonsay were sufficient to detect evidence for differences in diet composition of Kittiwakes between locations, and between Kittiwakes and Shags on Canna. DNA metabarcoding offers a non-invasive, practical, complimentary approach to conventional diet monitoring methods, reducing biases associated with visual identification of prey while enabling higher taxonomic resolution. Opportunistically collecting faeces also increases the capacity of

obtaining spatially and temporally representative diet monitoring, even in logistically challenging locations. These findings demonstrate the use of faecal DNA metabarcoding for seabird diet monitoring and consequently its potential to inform effective management and conservation decisions.

Introduction

The UK holds internationally important populations of seabird species, many of which are declining, with further losses predicted with future likely climate change scenarios (Davies *et al.* 2023). Understanding the drivers of these declines requires an understanding of population demographics among regions, as well as diet (Barrett *et al.* 2007; Karnovsky *et al.* 2012). Prey availability is a major factor driving seabird breeding success (Frederiksen *et al.* 2005), and the greatest impact of climate change on seabirds is likely indirect through temperature-mediated changes on prey populations (Renner *et al.* 2024; Johnston *et al.* 2025). However, despite the known links between food availability, diet, and seabird demography, long-term and multi-colony studies on seabird diet are rare. Much of our understanding of the links between diet, behaviour, and demographic rates comes from studies focussed on the northern North Sea (e.g. from long-term studies on the Isle of May based on five main study species; Newell *et al.* 2024). Therefore, it is of high priority to collect long-term diet information across broader ranges of geography and species to better understand the impacts of changes in the marine environment, particularly those associated with climate change. The development of any method that has the capacity to augment data collection will also create the opportunity for the setting up of long-term, multi-colony studies.

Conventional diet monitoring during the breeding season has largely involved visual observations of prey being delivered to chicks, collecting regurgitates opportunistically when handling individuals or collecting pellets from nest sites (Barrett *et al.* 2007). Although these methods have their advantages, being relatively non-invasive and allowing large sample sizes to be collected, they can also result in identification biases. Identifying prey species being delivered to chicks by observation can result in misidentification and the overlooking of small items, although this can be reduced through the use of photographs (Gaglio *et al.* 2017). Observations and photographs are only useful for prey-loading species, such as terns and auks. On the other hand, determining diet from pellets or regurgitates can over-represent prey items with indigestible hard parts and therefore under-represent soft food items (Barrett *et al.* 2007). All these methods require specialist knowledge and can require considerable time in the colony or laboratory identifying prey items.

An alternative technique that can reduce biases in prey items identified is the use of DNA metabarcoding to detect prey DNA in regurgitates or faeces (Barrett *et al.* 2007). This technique is

increasingly being used to successfully study the diet of a range of seabirds; Macaroni Penguins *Eudyptes chrysolophus* (Deagle *et al.* 2007), Atlantic Puffins *Fratercula arctica* (Bowser *et al.* 2013; Fayet *et al.* 2021), and European Storm Petrels *Hydrobates pelagicus* (Carreiro *et al.* 2020). DNA metabarcoding can detect a broad range of prey items, including soft-bodied species that can be difficult to identify with conventional methods, and can identify prey items to a higher taxonomic resolution (Deagle e*t al.* 2007; McInnes 2016; Ceia *et al.* 2022). It also provides a relatively non-invasive method to study seabird diet, especially if sampling faeces from the environment (de Leeuw *et al.* 2024; Good *et al.* 2024). Given the logistics involved in monitoring seabird diet through more conventional approaches, the potential of using DNA metabarcoding to analyse seabird faeces collected opportunistically from colonies may provide a more feasible way of obtaining diet information from a wider range of locations and species (de Leeuw *et al.* 2024; Good *et al.* 2024).

The main aim of this study was to determine the feasibility of using DNA metabarcoding to obtain colony-level diet data through opportunistically collecting seabird faeces during the breeding season from several locations on the west coast of Scotland: a region of the UK where the collection of diet data is currently limited. We focused on two seabird species: the Black-legged Kittiwake *Rissa tridactyla* (hereafter Kittiwake) and European Shag *Gulosus aristotelis* (hereafter Shag), both of which are, respectively, on the Red and Amber list of the UK Birds of Conservation Concern (Stanbury *et al.* 2024). We selected these two species due to their contrasting foraging strategies, with Kittiwakes being surface feeders and Shags benthic feeders, providing a wide array of prey species consumed. While the diets of both species have been studied at a limited number of UK sites using conventional methods, their diet has not yet been examined using DNA metabarcoding. Where sample sizes allowed, we carried out statistical comparisons of diet between species and locations. For one location, where conventional diet sampling methods were also used (regurgitates and pellets on Canna), we compared those results with those obtained by DNA metabarcoding of the faecal samples to evaluate the consistency between methods in identifying diet composition.

Methods

Study species

Kittiwakes are surface feeders that predominantly prey on sandeels (Ammodytidae), especially in the North Sea (Harris & Wanless 1997; Bull *et al.* 2004). They also prey on gadoids and clupeids, especially European Sprat *Spratus spratus*, and particularly in the west of the UK (Swann *et al.* 2008, Chivers *et al.* 2012). Shags are benthic foragers and sandeels are also prominent in their diet in the UK along with Gadidae, Cottidae, Pleuronectidae and Gobiidae species, depending on location and year (Swann *et al*. 2008; Howells *et al.* 2018). Diet data for Kittiwake and Shag are currently routinely

collected from two 'Key Sites' in Scotland as part of the Seabird Monitoring Programme (SMP); Canna and the Isle of May (Swann *et al*. 2023, Newell *et al*. 2023). In addition, the diet of these two species has been examined through several shorter-term studies across the UK (e.g. Krystalli *et al.* 2019; Ruffino *et al.* 2023). Kittiwake diet data is typically obtained from regurgitates when handling chicks and adults, whilst Shag diet is typically obtained from regurgitates (mainly from chicks) and pellets (from adults; Swann *et al*. 2023; Newell *et al*. 2023).

Faeces sample collection

Kittiwake and Shag faeces were collected from four locations along the west coast of Scotland during the breeding season, May – July 2023, coinciding with incubation and chick rearing: Colonsay (56.0951792, -6.2974108); Lunga, the Treshnish Isles (56.4946796, -6.4230933); Canna (57.0566943, -6.5837187); and the Shiant Isles (57.8962658, -6.3622937; Figure 1). On Colonsay, Kittiwake faeces were collected from an intertidal area of sand on Kiloran Bay where adults frequently roosted. As this area is swept clean daily by the tide, fresh faeces were collected on a low or falling tide. Samples were collected at least 1–2 m apart to increase the chance that the faeces were from different individuals. A field sample of sand was also collected to process along with the faecal samples to look at potential environmental contamination.

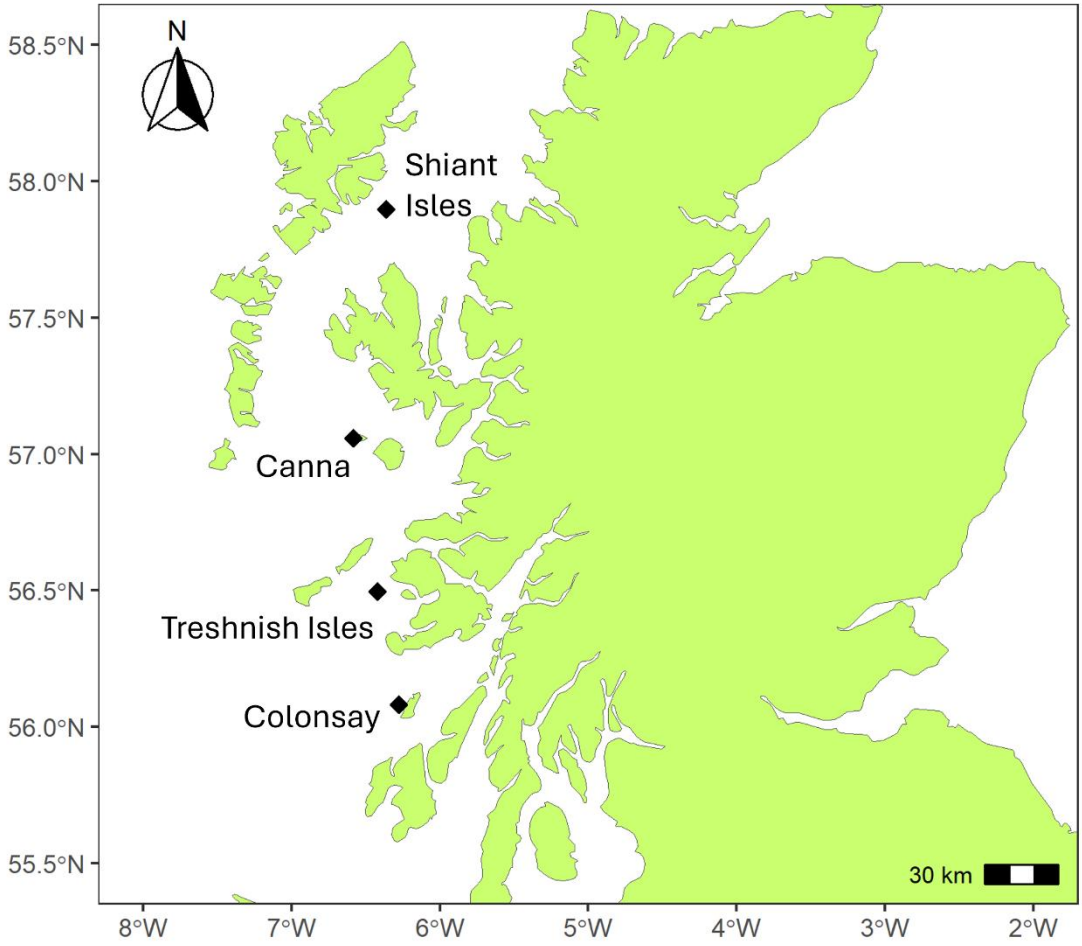

Figure 1. The four study locations on the west coast of Scotland where Kittiwake and Shag faecal samples were collected during the 2023 breeding season: Colonsay (56.0951792, -6.2974108; Kittiwake), the Treshnish Isles (56.4946796, -6.4230933; Kittiwake and Shag), Canna (57.0566943, -6.5837187; Kittiwake and Shag), and the Shiant Isles (57.8962658, -6.3622937; Shag).

At the remaining locations, faecal samples were obtained opportunistically whilst handling individuals during ringing activities at the colony. Faeces were scraped from the clothing worn by ringers or the substrate surrounding the nest, avoiding contamination from other faeces and dirt. Faeces were collected from both adults and chicks. Where feasible, faeces from adults were prioritised as most conventional diet data collected during the breeding season, especially of Kittiwakes, typically reflects what adults feed chicks (Swann *et al*. 2008). As we cannot fully rule out contamination between individuals when sampling faeces in this opportunistic way, we focused on the diet across all individuals of a species at the colony-level, and across chicks and adults where faeces from both were collected, rather than focusing on the diet of individuals.

At all locations, a disposable wooden spatula was used to obtain a small pea sized sample of the dark part of faeces. Fresh or wet faeces were targeted to increase the likelihood that samples contained good quality prey DNA (McInnes *et al.* 2017). A new spatula was used for each sample to prevent contamination between individuals. The faeces sample was then placed in a labelled Eppendorf tube containing 2 mL of RNA*later* solution and shaken gently to mix and stabilise the DNA without the need of freezing in the field. The aim was to have a volume ratio of solution to faeces of at least 5:1. All samples were sent to the UHI's Institute for Biodiversity and Freshwater Conservation (IBFC) following the breeding season where they were refrigerated until DNA extraction.

Conventional diet data collection

During the 2023 breeding season, diet information was also collected for Shags and Kittiwakes on Canna following conventional methods; through collecting regurgitates and pellets (Table A1, see Supplementary Materials; see Swann *et al*. 2023 for details). For Shags, three chick regurgitates and 29 pellets from adults were collected during ringing activities between 25 May and 24 July (Swann *et al*. 2023). For Kittiwakes, 19 regurgitates were collected from chicks or adults returning to the nest site between 30 June and 5 July (Swann *et al*. 2023).

DNA extraction

Pre-PCR processes were performed in the IBFC eDNA facility, which has separate rooms for filtration, DNA extraction, and PCR preparation of sensitive environmental samples. DNA extraction was carried out in an amplified-DNA free room inside a laminar flow cabinet that was UV-sterilised for 30 minutes between extraction batches and cleaned with 10% bleach. The Qiagen PowerSoil Pro extraction kit was used to extract DNA from samples with no modifications. Either 0.25 g of sample was extracted or the whole sample if it weighed less than 0.25 g. Extractions were conducted in batches of either 23 or 11 samples and each batch included one extraction blank which was processed along with the samples to detect any contamination. Extracted DNA was stored at -20°C until downstream processing.

DNA amplification

The first step PCR for each of the markers (12s and cytochrome oxidase I (COI)) were carried out in a laboratory that did not contain amplified DNA inside a UV- and bleach- sterilised cabinet to prevent contamination from the main laboratory.

Fish-specific PCR primers

A region of 12S mitochondrial DNA was amplified with fish-specific PCR primers (MiFish as described in Miya *et al.* 2015, 2020). Primer sequences were: F: GTCGGTAAAACTCGTGCCAGC R: CATAGTGGGGTATCTAATCCCAGTTTG. The PCR positive control was Nile Tilapia *Oreochromis niloticus* or Eastern Happy *Astatotilapia calliptera* genomic DNA, positive controls had concentrations of 0.05 ng/µL. A nested two-step polymerase chain reaction (PCR) was used which adds two unique identifying tags to the sequences generated from each sample (Kitson *et al.* 2019); this minimises the risk of 'tag hopping' during sequencing which is a possible cause of false positives in genetic metabarcoding studies (Schnell *et al*. 2015). Briefly, samples (including field, extraction, and PCR blanks) were first amplified using the 12S specific primers with the identifying tags attached. The PCRs were carried out in a 15-µL reaction including 1.7 µL of DNA, 1.8 µL of primers, 7.5 µL of Q5 master mix (NEB), and 0.3 µL of bovine serum albumin (BSA). The PCR program included a 5 minute initial denaturation step at 98°C followed by 17 cycles of 10 seconds at 98°C, a touchdown step with a decrease in 0.5°C per cycle from 65°C to 58°C for 20 seconds and 30 seconds at 72°C. This was followed by 19 cycles of 98°C for 10 seconds, 58°C for 20 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 7 minutes.

Metazoan-specific PCR primers

We amplified a region of the COI mitochondrial region with primers designed to sequence metazoan diversity (Leray *et al.* 2013). Primer sequences were: F: GGWACWGGWTGAACWGTWTAYCCYCC, R:TANACYTCNGGRTGNCCRAARAAYCA. The COI primers were modified to include heterogeneity spacers, sequencing primers, and pre-adapters. A two-step polymerase chain reaction (PCR) was used. Briefly, samples (including field, extraction, and PCR blanks) were first amplified using the COI specific primers. The PCRs were carried out in 25-µL reactions including 2 µL of DNA, 1.5 µL of primers, and 12.5 µL of 2x Qiagen Type-It master mix. The PCR program included a 5 minute initial denaturation step at 95°C followed by 40 cycles of 20 seconds at 95°C, 49°C for 30 seconds, 72°C for 60 seconds, and a final extension step at 72°C for 60 seconds.

For both markers, each sample was amplified three times to compensate for random variation that arises when target DNA concentrations are low. The triplicate PCR products were pooled and Illumina indexes and sequencing tails added via a second, index PCR. The second PCR was carried out in a 25-µL reaction containing 2 L of DNA, 12.5 µL of NEB Q5 mastermix, 3 µL of primers, and 7.5 µL of nuclease-free water. The PCR program had an initial denaturing step of 3 minutes at 95°C followed by 10 cycles of 20 seconds at 98°C, 1 minute at 72°C, and a final extension stage of 5 minutes at 72°C. PCR products from all samples were then normalised and pooled into a single

sequencing library. The final libraries were paired-end sequenced on an Illumina MiSeq® using 2x 300 bp V3 chemistry (Illumina Inc., CA, USA) with 10% PhiX Control added.

DNA sequencing and taxonomic assignment

The sequencing data were analysed with a reproducible metabarcoding bioinformatic workflow, using Tapirs (https://github.com/EvoHull/Tapirs). Sequencing reads underwent a BLAST (Zhang *et al.* 2000) taxonomic assignment against a curated UK marine fish reference database, Meta-Fish Lib (Collins *et al.* 2021) for the fish 12s mitochondrial DNA and against a curated marine eukaryote reference database, MARES (Arranz et al. 2020) for the COI data. For 12S, a literature search found a list of known prey species and these were present in the database. Kittiwake and Shag sequences downloaded from Genbank were also added to the database (see Supplementary Materials). Following taxonomic assignment, a noise threshold of 0.1% of total reads per sample was applied to remove taxa with low frequency reads (Hänfling *et al.* 2016). Where the molecular marker was unable to distinguish amongst species some taxa were assigned to a higher taxonomic level.

Any samples with fewer than 500 total reads were considered to be of poor sequencing quality and removed. Reads assigned to controls, and samples with no taxonomically assignable reads, were also excluded from any analysis. Percentage frequency of occurrence (proportion of positive faecal samples; % FO), and relative read abundance (RRA; the average percentage of reads of a given taxa across positive faecal samples) for the fish data, were used to summarise prey species occurrence in Kittiwake and Shag diets.

The reads for each taxon were converted to percentages (excluding non-food sequences from total count) and a 1% minimum sequence percentage threshold was used to determine occurrences of prey species (Deagle *et al.* 2019). This cutoff was chosen as a trade-off between picking up rarer diet species and eliminating low-level background contamination (Deagle *et al.* 2019).

Statistical analysis

All plots and analysis were carried out in the statistical programming environment R v. 4.4 (R Core Development Team 2024). We used both prey occurrence and relative read abundance (RRA) to summarise the diet data as recommended by Deagle *et al.* (2019). Occurrence data uses presence/absence of prey reads and ignores the number of reads so a prey species that has a low number of reads for a sample will be counted as equivalent to a prey species that makes up a high proportion of the reads of the same sample. Therefore, occurrence data can overestimate the importance of rare prey. In comparison, RRA values are based on the number of reads of each

identified prey species, which can provide a more accurate view of population-level diet (Deagle *et al.* 2019). Nonetheless, RRA summaries are more sensitive to recovery biases inherent in metabarcoding, including technical biases such as primer choice, or biological biases such as prey size (Deagle *et al.* 2019).

To determine whether our faeces sample sizes were adequate to make comparisons between the seabird species and locations, we used the package *iNext* (Chao *et al.* 2014; Hsieh *et al.* 2024). Where samples sizes were adequate, we conducted PERMANOVA tests using the *adonis2* function in the *vegan* package (Oksanen *et al*. 2020) to test for differences in the diet composition 1) between species and 2) among locations for each species, based on the presence and absence of taxa and RRA within faecal samples. We also compared the diet composition obtained from faeces and conventional methods for both Shag and Kittiwake on Canna (based on presence of occurrence only). PERMANOVA tests were run with the Jaccard method for presence of occurrence, and the Bray-Curtis method for RRA values, with 999 permutations. To determine which taxa were contributing most to the dissimilarity/similarity between groups we ran a similarity percentage analysis (SIMPER) tests using the *simper* function in the *vegan* package (Oksanen *et al*. 2020).

Table 1. Summary of Kittiwake and Shag faecal samples collected from locations along the west of Scotland during the 2023 breeding season. See Appendix 1 for additional information on individual samples.

Species	Location	Dates collected (day/month/year)	Number of samples collected	Number of samples with prey DNA[1]
Kittiwake	Canna	05/07/2023 – 26/07/2023	19	17 (14 adults, 3 chicks)
Shag	Canna	01/07/2023 – 05/07/2023	25	22 (all chicks)
Kittiwake	Kiloran Bay, Colonsay	28/05/2023	24	21 (all adults)
Shag	Shiant Isles	28/06/2023 – 07/07/2023	9	9 (6 adults, 3 chicks)
Kittiwake	Lunga, Treshnish Isles	06/2023	2	2 (both adults)
Shag	Lunga, Treshnish Isles	06/2023	9	5 (4 adults, 1 chick)

[1] The total number of faecal samples with prey DNA that were from chicks and adults, included in brackets.

Results

Across the four locations in the west of Scotland, we collected 45 Kittiwake and 43 Shag faeces, from both chicks and adults (Table 1). Prey DNA was successfully extracted from 76 (86%) of these 88 faecal samples (89% of Kittiwake and 84% of Shag samples; Table 1). Of the 12 faeces that did not reveal any prey DNA, two Shag and four Kittiwake samples were removed as they had a high proportion of reads from other seabird species or had no prey reads (Appendix 1). The remaining six (five Shag and one Kittiwake samples) were excluded as they contained fewer than 500 prey reads (Appendix 1). In addition to prey DNA, low levels of other seabirds were detected in some samples; Atlantic Puffin, Common Guillemot *Alca torda*, Razorbill *Uria aalge* and gull *Larus* species, likely due to cross contamination when the faeces were collected in the field (Appendix 1). The sand control sample collected from the beach on Colonsay contained no detectable DNA (Appendix 2) validating this approach for collecting faeces without environmental cross contamination.

Fish diet

From the fish 12S mitochondrial DNA sequencing data, we identified 34 fish taxa to the species level as well as to six fish families that that could not be identified to species level (Ammodytidae, Clupeidae, Gadidae, Triglidae, Pollachius and Pleuronectidae; Appendix 2). In total, 20 fish taxa were detected above the 1% minimum sequence percentage threshold used to determine occurrences of prey species (Table 2; Appendix 3).

A high proportion of Shag and Kittiwake individuals consumed sandeels, across all locations (Table 2). The proportion of individuals consuming other fish taxa varied by species and locations. For example, herrings and sprats (Clupediae) were important in the diet of Kittiwakes from Canna whilst, for Shags, a high proportion of individuals from Canna and the Shiant Isles also consumed cod (Gadidae) species. Fish species from the other families were typically only consumed by a small number of individuals (Table 2).

The 12S PCR negative controls were clear of contamination with three of the extraction blanks containing low numbers of reads of *Trisopterus minutus*, Triglidae or Ammodytidae (64 to 122 reads). These reads were in low numbers and not consistent amongst the extraction blanks and were therefore not likely to be a significant source of contamination (Appendix 2).

Marine invertebrate diet

From the COI sequencing data, we identified 75 eukaryote taxa from 68 (77%) of the faecal samples (Kittiwake = 35, Shag = 33); 59 of these were identified to genera, 13 to family, three to order, two to class, and one to kingdom (Appendix 4). However, only 34 of these taxa were detected above the 1% minimum sequence percentage threshold used to determine occurrences of prey taxa (Appendix 5). Of these only nine taxa (involving crab and bobtail squid species) were thought likely to be primary prey species (Table 2, Appendix 5). Of the remaining taxa, eight were thought more likely to be from secondary ingestion (i.e. prey species of the fish the Kittiwake and Shag consumed, including copepods and barnacles); 14 from environmental contamination when the faecal samples were collected in the field (including terrestrial invertebrates and fungi), and one from laboratory/environmental contamination (Arthropoda). Two of the COI PCR negative controls contained contamination from arthropod DNA and one contained Shag DNA. Therefore, for any samples that were in the same PCR batch that have reads identified as Arthopoda below 1,409 or Shag below 2867 had that taxon removed as contamination. Therefore, in total, marine eukaryote prey DNA was found in only six Kittiwake samples (three from Colonsay and three from Canna) and one Shag sample (from the Treshnish Isles; Table 2, Appendix 5). Given the limited data on marine

invertebrates found in the sampled Shag and Kittiwake faeces, we focused only on the fish data when comparing the diet between species and locations. We did include the invertebrate data when comparing the diet obtained from faeces and conventional methods on Canna.

Table 2. Frequency of occurrence (% FO) of prey taxa identified in the Kittiwake and Shag faeces across the four sampled locations along the west coast of Scotland in 2023. n = the number of faecal samples collected from each location. Only taxa detected above the 1% minimum sequence percentage threshold used to determine occurrences of prey are included. For the prey scientific names see Appendices 2 and 3.

		Kittiwake			Shag		
Taxa	Common name	Canna (n=17)	Colonsay (n=21)	Treshnish I. (n=2)	Canna (n=22)	Shiant I. (n=9)	Treshnish I. (n=5)
Fish prey							
Ammodytidae	Sandeel species	11 (65%)	17 (81%)	2 (100%)	19 (86%)	8 (89%)	4 (80%)
Ammodytidae	Smooth Sandeel	-	-	-	6 (28%)	1 (11%)	1 (20%)
Callionymidae	Common Dragonet	-	-	-	3 (14%)	-	-
Clupeidae	Herrings and sprats	6 (35%)	-	-	-	-	-
Clupeidae	Atlantic Herring	13 (77%)	2 (10%)	-	2 (9%)	-	-
Clupeidae	European Sprat	13 (77%)	4 (19%)	-	2 (9%)	1 (11%)	-
Gadidae	Cod species	3 (18%)	2 (10%)	1 (50%)	15 (68%)	8 (89%)	-
Gadidae	Atlantic Cod	-	-	-	1 (5%)	-	-
Gadidae	Blue Whiting	1 (6%)	-	-	-	-	-
Gadidae	Norway Pout	5 (29%)	1 (5%)	-	1 (5%)	6 (67%)	-
Gadidae	Poor Cod	3 (18%)	2 (10%)	-	5 (23%)	8 (89%)	1 (20%)
Labridae	Cuckoo Wrasse	-	-	-	-	1 (11%)	-
Labridae	Goldsinny Wrasse	-	-	-	2 (9%)	-	-
Lotidae	Ling	-	-	-	-	1 (11%)	-
Lotidae	Northern Rockling	-	1 (5%)	-	-	-	-
Pleuronectidae	Lemon Sole	1 (6%)	-	-	-	-	-
Pleuronectidae	Dab species	-	-	-	1 (5%)	-	-
Pollachius	Pollock species	-	-	-	-	1 (11%)	-
Scombridae	Atlantic Mackerel	1 (6%)	1 (5%)	-	2 (9%)	-	1 (20%)
Triglidae	Gurnard species	-	-	-	1 (5%)	-	-
Marine invertebrate prey							
Sepiolida	Bobtail Squid	-	1 (5%)	-	-	-	-
Crustacea	-	2 (12%)	-	-	-	-	-
Corystidae	Masked Crab	-	1 (5%)	-	-	-	-
Goneplacidae	Angular Crab	1 (6%)	-	-	-	-	-
Pilumnidae	Bristly Crab	1 (6%)	-	-	-	-	-
Polybiidae	Crab species	-	2 (10%)	-	-	-	-
Polybiidae	Dwarf Swimming Crab	-	1 (5%)	-	-	-	-
Polybiidae	Marbled Swimming Crab	1 (6%)	1 (5%)	-	-	-	-
Polybiidae	Velvet Crab	-	-	-	-	-	1 (11%)

Comparison of DNA metabarcoding and conventional methods

For Shags, we detected significant differences between diet composition (based on presence of occurrence) estimated by metabarcoding the faeces, and that from conventional methods (PERMANOVA; $F = 4.12$, $P = 0.001$, $R^2 = 0.07$). The SIMPER tests revealed that the dissimilarity between methods was driven by Smooth Sandeels *Gymnammodytes semisquamatus* and Poor Cod *Trisopterus minutus* ($P < 0.08$), with these being identified to species level within the faeces, whilst sandeel (Ammodytidae) and cod (Gadidae) species were only identified to family level within the pellets and regurgitates (Appendix 1). The majority of pellets and chick regurgitates contained the remains of gadoid and sandeel species, in agreement with the main taxa found within the Canna Shag faecal samples (Appendix 1; Figure 2). A range of additional fish and crustacea species were found in the Shag pellet and regurgitate samples, although these were generally only consumed by a small number of individuals (Appendix 1).

For Kittiwakes, we also detected significant differences between diet composition (based on presence of occurrence) estimated from metabarcoding the faeces, and that from regurgitates (PERMANOVA; $F = 16.66$, $P = 0.001$, $R^2 = 0.33$). This was driven by a greater range of fish and marine invertebrate taxa being identified in the faeces compared to regurgitates (12 versus 3 taxa, respectively; Appendix 1). However, the majority of chick and adult regurgitates contained the remains of sandeels and Clupied species, with a smaller number containing the remains of gadoid species, in agreement with the main taxa found within the Canna Kittiwake faecal samples (Appendix 1; Figure 3).

Comparison of fish diet between species and locations

Our sample sizes for Kittiwake and Shag faeces collected from the Treshnish and Shiant Isles were too small to include in further statistical analysis. However, the number of faecal samples from Colonsay and Canna were adequate for comparing diet composition.

There were significant differences in the fish prey of Kittiwakes and Shag (based on presence of occurrence) in faeces collected from Canna (PERMANOVA; $F = 11.59$, $P < 0.001$, $R^2 = 0.24$). The SIMPER tests revealed that the dissimilarity between diets was driven by European Sprat, Atlantic Herring *Clupea harengus*, herring/sprat (Clupeidae) species, cod (Gadidae) species, Norway Pout *Trisopterus esmarkii* and Blue Whiting *Micromesistius poutassou* (all $P < 0.04$). These species were found in a higher proportion of Kittiwake than Shag faeces, with the exception of cod (Gadidae) species (Table 2). There were also significant differences in the fish prey of Kittiwakes and Shag detected in faeces based on RRA values (PERMANOVA; $F = 7.88$, $P < 0.001$, $R^2 = 0.18$). The SIMPER tests revealed that the dissimilarity in diets was driven by European Sprat, Atlantic Herring,

herring/sprat (Clupeidae) species, Norway Pout and Blue Whiting (all $P < 0.05$). Again, these species had greater RRA in the Kittiwake than Shag faeces, with the exception of cod (Gadidae) species (Figure 2, 3).

We found significant differences in the fish prey of Kittiwakes (based on presence of occurrence) when comparing faecal samples from Canna and Colonsay (PERMANOVA; $F = 9.57$, $P < 0.001$, $R^2 = 0.21$). The SIMPER tests revealed that the dissimilarity between diets was driven by European Sprat, Atlantic Herring, herring/sprat (Clupeidae) species, Norway Pout, Blue Whiting and Lemon Sole *Microstomus kitt* (all $P < 0.04$). Specifically, these species were found in a higher proportion of Kittiwake faeces from Canna than from Colonsay (Table 2). We also found significant differences in the fish prey detected in Kittiwake faeces from Canna and Colonsay based on RRA values (PERMANOVA; $F = 4.80$, $P = 0.001$, $R^2 = 0.12$). The SIMPER tests revealed that this dissimilarity was driven by European Sprat, Atlantic Herring, herring/sprat (Clupeidae) species and Blue Whiting (all $P < 0.01$). As with presence of occurrence, these species were found to a greater extent in the Kittiwake faeces from Canna than Colonsay, with the diet of Canna Kittiwakes being more diverse than those from Colonsay (Figure 3).

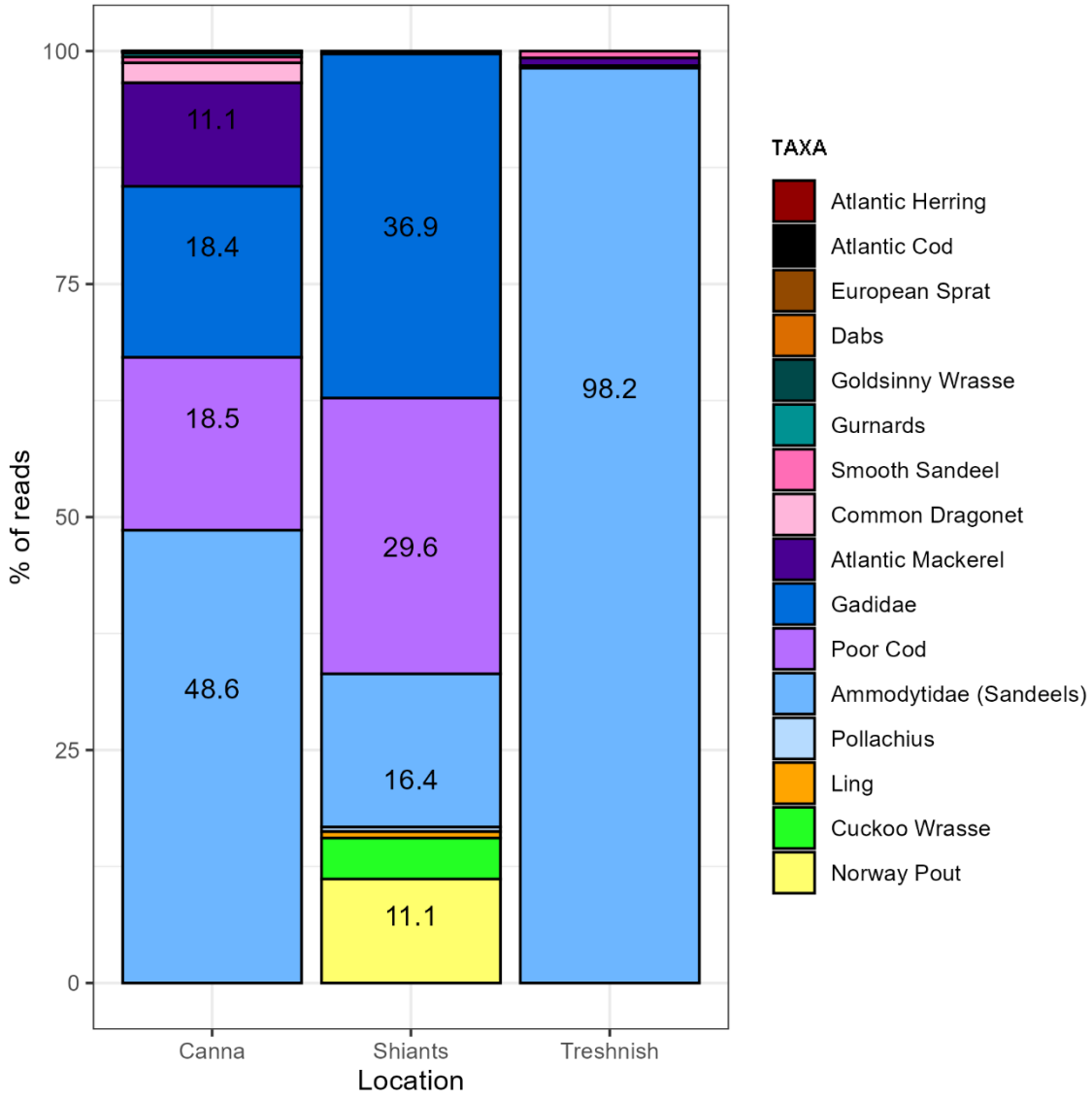

Figure 2. Percentage of reads for each prey taxon from Shag faeces at each location (Canna, n = 22; Shiant Isles, n = 9; and Treshnish Isles, n = 5) during the 2023 breeding season. Colours are specific to taxa detected and show the percent of relative read abundance (RRA). Only read values that made more than 10% of the total for that location are shown in the figure.

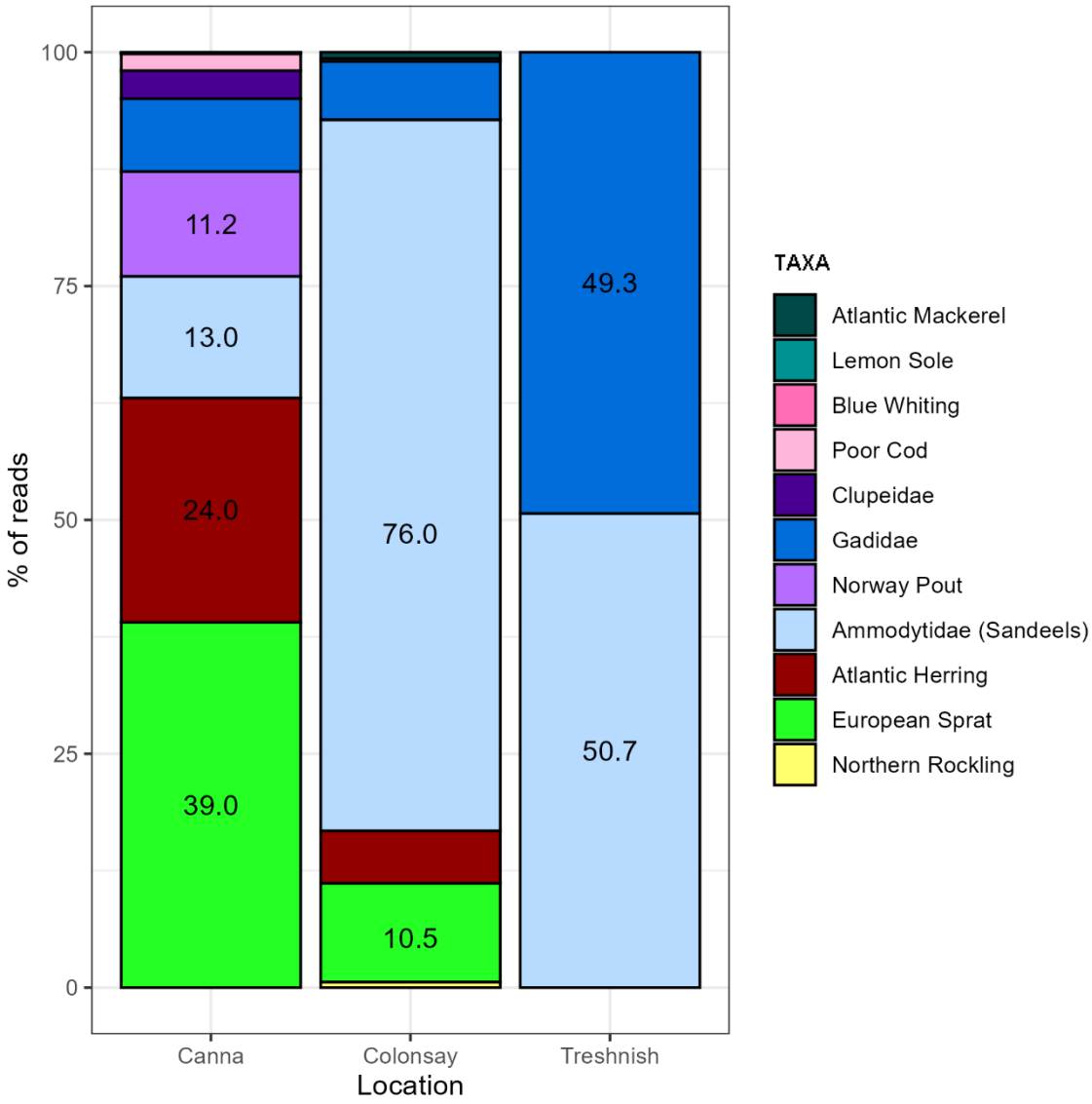

Figure 3. Percentage of reads for each prey taxon from Kittiwake faeces at each location (Canna, n = 17; Colonsay, n = 21; and Treshnish Isles, n = 2) during the 2023 breeding season. Colours are specific to taxa detected and show the percent of relative read abundance (RRA). Only read values that made more than 10% of the total for that location are shown in the figure.

Discussion

We successfully extracted marine fish and invertebrate prey DNA from Shag and Kittiwake faeces collected opportunistically during the breeding season. This allowed us to obtain preliminary diet data for a region of west Scotland where seabird diet monitoring has largely been restricted to a single location, Canna, during the chick-rearing period.

The results provided by the DNA metabarcoding analyses were consistent with other studies on Kittiwake and Shag diet elsewhere in the UK, with sandeels being a major component (Harris & Wanless 1997; Bull *et al.* 2004; Swann *et al.* 2008; Howells *et al.* 2018). As found in previous studies, clupeids (mainly Atlantic Herring and European Sprat) were also important in the diet of Kittiwakes (Swann *et al*. 2008; Chivers *et al.* 2012), whilst gadoids (including Poor Cod and to a lesser extent Norway Pout) were more prominent in the diet of Shags (Swann *et al*. 2008; Howells *et al.* 2018). The wide range of prey taxa detected in this study further agrees with previous studies in that the diet of seabirds on the west of the UK is typically diverse, in contrast to the North Sea where sandeels generally dominate (Swann *et al*. 2008; Chivers *et al.* 2012). However, long-term studies on the Isle of May have shown that the proportion of sandeels in seabird diets can vary annually, and has largely declined over time attributed to changes in sea surface temperatures in the North Sea (Howells *et al.* 2018; Wanless *et al.* 2018).

Comparison of DNA metabarcoding and conventional methods

Collecting Shag and Kittiwake faecal samples from Canna provided an opportunity to compare diet information obtained through DNA metabarcoding to that obtained from more conventional methods; regurgitates (Shag and Kittiwakes) and pellets (adult Shags). The overall diet composition of both species differed statistically between the two methods; this was largely driven by metabarcoding identifying taxa to a higher resolution than could be identified from the remains found in regurgitates and pellets, as has been observed previously (Thalinger *et al*. 2022). However, the main fish prey found in the diet of both species identified from regurgitates and pellets – sandeels, gadoids and clupeids– were also the main fish families found in the faeces. Clupeids frequently found in regurgitates on Canna were thought to be mostly European Sprat based on otoliths (Swann *et al*. 2008; Swann *et al*. 2023), whereas the DNA metabarcoding of faeces revealed that Atlantic Herring was also prominent. Most Kittiwake faeces contained both European Sprat and Atlantic Herring DNA, which may indicate that these individuals were feeding on mixed shoals of clupeids (Waggitt *et al.* 2018). Similar to other studies comparing DNA metabarcoding to more conventional diet sampling, we found a greater diversity of species in the diet of Kittiwakes faeces compared to the regurgitates (Deagle *et al*. 2007). This was less obvious for Shags as although a range of fish species were detected within the faeces, there were also several invertebrate prey species identified in the pellets (namely molluscs but also, in a small number of pellets, shrimp, cephalopod, sea urchin, and hermit crab species) that were not detected in the faeces. It is important to note that the diet data obtained from the methods are not directly comparable, especially given that the pellets/regurgitates and faeces were collected from different individuals;

with all samples providing only a snapshot of an individual's diet (Barrett et al. 2007). Given how localised Shag foraging areas can be, the observed differences may reflect variation in where individuals were foraging and/or what they are consuming (Bogdanova et al. 2014; Morgan et al. 2019). Furthermore, for Shags, all faeces were collected from chicks whereas most conventional diet samples were pellets from adults. Shag pellets and regurgitates were also collected over a wider range of dates across incubation and chick rearing (from late May to late July) compared to the faeces, which were collected in early July, during chick rearing, which may influence what prey were being targeted. However, the importance of sandeel and gadoid species reflected by both the pellets/regurgitates and faeces indicate that these are important components of both adult and chick diet in Shags on Canna.

For Kittiwakes, most faeces and regurgitates were collected in early July, during chick rearing. Most faeces (14 of 17 samples) were from adults, reflecting adult diet unlike the regurgitates which reflect chick diet. Differences can occur in the prey adult seabirds consume compared to what they feed their chicks (Barrett *et al.* 2007). However, the similarity of key prey species found by both diet sampling approaches within this study may suggest that adult and chick diet were similar for Kittiwakes on Canna. Most Kittiwake diet studies are based on chick regurgitates or regurgitates from adults that are destined for chicks (Swann *et al*. 2008; Chivers *et al.* 2012; Hatch 2013). DNA metabarcoding of adult faeces therefore provides an opportunity to better monitor the diet of adult Kittiwakes in addition to chicks.

Within the faecal samples we detected limited prey DNA from marine invertebrates, which Shag and Kittiwakes are known to feed on (Swann *et al*. 2008; Howells *et al.* 2018). Only a small number of individuals, mostly Kittiwakes, were found to have consumed marine invertebrate prey. The swimming crabs and squid found in the diet of Kittiwakes from Colonsay may be due to these individuals foraging in tidal upwellings (such as the Gulf of Corryvreckan, north of Jura to the northeast of Colonsay), based on observations and previous tracking data of seabirds at this site (D. C. Jardine pers. obs., E. Owen pers. comm.). The lack of invertebrate prey may be due to these species not being important in the birds' diets when sampled, particularly during chick rearing. Alternatively, it could be that there were issues identifying DNA using the COI universal metazoan primer. For example, there was a high percentage of unassigned reads in many of the faecal samples, likely due to the proportion of marine species represented in reference databases still being low (Arranz *et al*. 2020; Casey *et al.* 2021). Attempts were made to resolve this by decreasing the similarity threshold from 98% to 90%, which gives fewer accurate species level identifications but could possibly lead to further reads being identified to a higher taxonomic level. However, relaxing the similarity threshold did not significantly decrease the number of unassigned reads.

Comparison of fish diet between species and locations

We initially aimed to collect 25 faecal samples from each location and species. However, there were difficulties in obtaining samples from some locations. On the Shiant Isles, only nine provided adequate faecal samples although over 50 individual Shags were handled during the ringing trip. On the Treshnish Isles, the ringing team was hampered by bad weather, as well as individual birds not providing faeces. This is useful to know for future plans for data collection as a large number of birds may need to be handled to obtain adequate sample sizes. It was also difficult to predict the timing of defecation when handling individual birds, so having a dedicated person to collect the faeces from clothing or the substrate would be beneficial. The use of a plastic ground sheet for the ringing area could also help in collecting fresh samples. Collecting faeces from roosting individuals on the beach on Colonsay alleviated some of these issues and had several advantages over opportunistically collecting faeces during ringing activities: it was a quick process (less than an hour) that does not require a ringing licence, it is particularly useful for Kittiwakes that are less likely to defecate during handling than Shags, and the faeces collected from the beach was not contaminated by DNA from contamination by other seabird species or the environment.

Although the number of faecal samples collected from the Treshnish and Shiant Isles were too low for useful statistical comparisons, these data still provide an indication of the diet of these species as no previous diet data has been published from these two colonies. We did have adequate sample sizes to compare the diet of Kittiwakes between Canna and Colonsay, which revealed differences in fish composition, with sandeels being more prominent in the diet of the latter. However, faeces from Colonsay were collected in late May, coinciding with late incubation, whilst those from Canna were collected in early July, during early chick rearing. Therefore, we cannot disentangle whether the dissimilarity in diet observed among locations was due to spatial or temporal differences in where or when the samples were collected. Collecting faeces throughout the breeding season would provide a better understanding of how diet may change over time. It should also be noted that we assumed that all Kittiwake faeces collected from the beach roost site were from local breeding individuals. Although this is likely, with colour-ringed Kittiwakes from nearby breeding colonies sighted roosting on the beach (D. C. Jardine pers. obs.), we cannot completely rule out that our samples were from birds that were breeding elsewhere or were non-breeders. In comparison, all adults handled during ringing activities were known to be breeding at that colony.

Although there are benefits to DNA metabarcoding, such as providing higher taxonomic resolution of consumed prey species and allowing the detection of soft-bodied prey often overlooked in conventional analyses, there are also limitations that are important to consider. These have been

discussed extensively elsewhere (Ando *et al.* 2020; Hoenig *et al.* 2022), so we focus here on those that are most relevant to the results of this study. An important consideration is that diet information from faeces provides no information on the size or quality of the prey consumed, which can be important to understand demographic responses to changes in diet (Wanless *et al.* 2005; Gutowsky *et al.* 2009), and which may bias our understanding of which prey species are most important (Deagle *et al.* 2019). For example, it is known that in the North Sea seabirds will transition from a diet of 1+ group sandeels (one year old or older) to 0-group (young-of-the-year) sandeels during the breeding season (Harris & Wanless 1997). Combining DNA metabarcoding with other methods of diet collection can therefore be beneficial, if a broader understanding of seabird diet is required (Marcuk *et al.* 2024).

In this study, the faeces from ringing activities also contained DNA from non-prey species. This included DNA from other seabird species and terrestrial invertebrates and fungi, which were assumed to be from environmental contamination when the faeces were collected in the field. Furthermore, we detected taxa that are likely secondarily consumed, as they were prey that the fish were eating. In most cases, DNA from these non-prey sources were filtered out due to low prey reads as a result of lower total biomass and a higher level of degradation. Where this did not occur, it was relatively straightforward to identify which species were unlikely to be primary prey of the seabirds that we studied. It is important to acknowledge that environmental contamination and secondary consumption of some taxa might not be so obvious (Ando *et al.* 2020).

A final consideration is that the DNA in faecal samples will be degraded to various extents through the process of digestion within the individual being sampled (de Sousa *et al.* 2019, Silva *et al.* 2024). This degradation may continue if the faeces is exposed to high levels of sunlight (Oehm *et al.* 2011). We tried to minimise the degradation of DNA within faeces by collecting fresh faeces and storing the samples in RNA*later*. We believe this was successful given the high proportion of faecal samples that we were able to extract prey DNA from.

Despite these limitations, this study builds on evidence that DNA metabarcoding can successfully be used to obtain diet data from faeces collected opportunistically from species and locations where existing information is currently lacking or non-existent (de Leeuw *et al.* 2024; Good *et al.* 2024); particularly where conventional methods are impractical due to logistical challenges or time constraints. Collecting faeces from roosting individuals eliminates the need to handle individuals and opens up the possibility of collecting diet data across a greater proportion of the annual cycle, particularly outside the chick-rearing period where diet data are typically lacking. By collecting diet data throughout the breeding season, our temporal understanding of seabird diet can be improved

substantially, allowing us to explore the influence of diet and prey availability on phenology and demographic rates during this period.

Data Availability

All raw data analysed in this study is publicly available at https://zenodo.org/uploads/15125968.

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