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Metabarcoding reveals selective dietary responses to environmental availability in the diet of a nocturnal, aerial insectivore, the European Nightjar (*Caprimulgus europaeus*)

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Many bird species are vulnerable to environmental change, so knowledge of their diet and its variation can help to understand population status and flexibility to respond to change. Insectivorous species are predicted to have a flexible diet within and between individuals, which can respond to naturally fluctuating prey abundance, thus allowing

opportunistic exploitation of available resources. We analysed the diet of a nocturnal, aerial insectivore, the European Nightjar *Caprimulgus europaeus*, using high-throughput metabarcoding. We quantified diet diversity and composition of 130 faecal samples from nests and roosts on a northern breeding site in the UK from 2015 – 2018, and compared differences among individuals and years. Although dominated by moths, diet varied significantly between individual faecal samples and between years and months. Prey species composition varied between years, and was more variable between samples in 2017, compared to other years. Faecal samples were significantly more likely to contain moth species with a wingspan of >60mm and less likely to contain moth species of <25mm wingspan. This indicated size-selective foraging, which also varied between months and years. Diet was driven by inter-individual variation, indicating population-level flexibility in prey choice. Metabarcoding provided a valuable tool in the exploration of insectivorous diets, but efforts are needed to build comprehensive reference libraries, in order to compile full prey species lists.

Keywords: avian ecology, diet, metabarcoding, faecal sampling, 16S rRNA gene, moth, Lepidoptera

Understanding variation in the composition and diversity of diet within a bird population can assist understanding of how a population or species is likely to fare in response to anthropogenic change (Howells *et al.* 2017). Environmental change is likely to affect certain groups of species more acutely than others; for example, specialist predators reliant on ephemeral insect prey may be more sensitive (Nocera *et al.* 2012, Stanton *et al*. 2017). Species that take flying insect prey on the wing ('aerial insectivores') are identified globally as being at risk (Nebel *et al.* 2010, Nocera *et al.* 2012) due in part to the significant decline in insect populations worldwide (Hallmann *et al.* 2017, van Strien *et al.* 2019). The diet composition of aerially-feeding, insectivorous birds may reflect localised spatial and temporal variation in prey abundance related to habitat type (Mills *et al.* 2020) or weather conditions (Imlay *et al.* 2017). Their diet may also reflect the ease of catching particular prey items, their nutritional content or habitat quality (Garlapow 2007, English 2009, Sharps 2013, Razeng & Watson 2015).

Difficulty observing feeding, and in finding and dissecting faecal matter (Mumma *et al.* 2016, Nielsen *et al.* 2018) mean that there is little information on the diet of adult, insectivorous birds, especially nocturnal species. Moreover, soft-bodied moths and flies that are primarily taken by this feeding guild are not readily identifiable in faecal matter (Razgour *et al.* 2011, Trevelline *et al.* 2016). Recently, rapid development of molecular techniques such as metabarcoding, has allowed researchers to acquire valuable information relevant to species conservation through diet analysis (Gillet *et al*. 2015, Kress *et al*. 2015, Gerwing *et al*. 2016). Metabarcoding facilitates diet analysis using faecal samples, by identifying short sequences from specific genes within the DNA of prey items that remain in the faecal matter. Commonly-used barcodes include the cytochrome c oxidase subunit I (COI)

and the 16S rRNA gene, and these can effectively distinguish between species (Metzker 2010, Alberdi *et al*. 2012, da Silva *et al*. 2019) where the barcodes have high interspecific but low intraspecific diversity. As well as allowing samples to be processed in bulk (Taberlet *et al.* 2012), these methods are non-invasive and can be more comprehensive than visual identification (Ji *et al.* 2013, De Barba *et al.* 2014, Krehenwinkel *et al.* 2017), aspects that are particularly beneficial for vulnerable or secretive species (Thalinger *et al*. 2018).

The European Nightjar *Caprimulgus europaeus* (henceforth, 'Nightjar') is a nocturnal insectivore of conservation concern in the UK (Amber listed - Eaton *et al.* 2015, IUCN classification is least concern, BirdLife International 2016). This species completes an annual migration from central Africa to the UK to breed from May until August, and feeds on arthropods (Cramp 1985, Sharps 2013). Limited diet data have been obtained through stomach content analysis and physical dissection of faeces from European Nightjars and the Common Nighthawk *Chordeiles minor (*Sierro *et al*. 2001, Sharps 2013, Knight *et al*. 2018). However, these methods result in a bias towards remaining hard parts of consumed arthropods such as beetle elytra, legs and antennae, which means that methods such as metabarcoding that are capable of identifying partially or fully digested remains are needed. Extracting sufficient DNA from avian faeces is challenging, especially after degradation by exposure to sun, rain and soil microbes (Deagle *et al*. 2006, Oehm *et al*. 2011, Kamenova *et al*. 2018). The high concentration of uric acid (Eriksson *et al*. 2017) in conjunction with environmental exposure inhibits extraction and amplification of DNA, making the extraction and clean-up stages of metabarcoding, along with choice of primer sequences, critical to success (Alberdi *et al*. 2017, Carneiro *et al*. 2020).

92 Here we have analysed the diet of a sample of Nightjar individuals at a key
93 breeding site in the UK. To understand variation in Nightjar diet within this population
94 we used metabarcoding techniques, methods which have had limited application to
95 the diet of this species prior to now (Evens *et al*. 2020). We used metabarcoding to
96 test the following hypotheses:

97 1) Diet composition and diversity varies among individuals (as in Alberdi *et al*.
98 2020), as individual Nightjars at this site have been shown to vary significantly in
99 their habitat preferences (Mitchell *et al.* 2020).

100 2) Females have lower diet diversity than males (da Silva *et al*. 2020), as they
101 only spend a short time foraging due to higher investment in parental care.

102 3) Diet diversity and composition varies between years. Nightjars are
103 constrained in their breeding schedule due to migration, so their short window of
104 opportunity should coincide with peaks of preferred moth prey. However, weather,
105 and thus emergence and activity of moths, can vary substantially throughout the
106 breeding season and between years (Clare *et al.* 2011, 2014).

107 4) Nightjars select large moths more often than their relative abundance in the
108 environment, due to both nutritional value (Razeng & Watson 2015), and greater
109 ease of detecting large moths in low light (Bayne & Brigham 1995).

110

111 **METHODS**

112 This study took place at the Humberhead levels National Nature Reserve (NNR;
113 South Yorkshire, UK, comprising Thorne Moor (53.636N, 0.898W) and Hatfield Moor
114 (53.545N, -0.938W). The site is classified as a degraded raised bog, with 28% of the
115 site covered by bog or marsh, 10% by standing water, and 15% mixed woodland

(JNCC 2015). It is also a European Special Protection Area (SPA) for its breeding Nightjar population (1.9% of the UK population; JNCC 2015). The NNR has undergone substantial habitat management since 2014, primarily the removal of 562 km^2 of birch woodland and an increase in wet habitat through the damming of drainage channels. Both techniques will influence vegetation structure and insect prey availability; selective and patchy clearance of woodland has been shown to increase species richness of Lepidoptera (Summerville & Crist 2002), and should also provide an increased amount of breeding space for Nightjars (Sharps 2013). Use of wetland habitat (e.g. bogs, mires) by nightjars is little considered in the literature explicitly as a breeding habitat, but there is some indication that they act as suitable foraging areas, containing a high number of invertebrates (English *et al.* 2017, Evens *et al.* 2017).

Faecal sample collection

Nightjar faecal samples were collected from June to August, 2015 to 2018 (2015: n = 9, 2016: n = 20, 2017: n = 35, 2018: n = 36) and were obtained from previously located roosts and nests, during the day. Upon approaching the nest or roost, we used binoculars to view the bird in situ. If the bird was not visible, we slowly approached the location until the bird flew away, whereupon the sex of the bird was visible (male Nightjars possess bright white spots on primaries 3,4 and 5 whilst females have smaller, beige spots). Nests and roosts of Nightjars and the size and shape of their faeces are distinctive and identifiable, as they are discrete curled pellets of between 3 – 8mm in width, found individually or in small clusters (see image in supplementary information). Faeces were placed into sterile, 30ml screw-top tubes (Elkay labs, UK) with wooden toothpicks, then labelled with sample

number, date, location, and the sex of the bird. Because samples were taken from roosts and nests, some samples at nests may have been from juveniles, which cannot be sexed. Samples from nests were therefore excluded from comparative analyses between years, months and sexes. All samples were included in analyses of prey size. Birds were not individually identifiable in the field, so it is possible that some samples are repeats from the same bird. Roost and nest details were documented and these locations are held faithfully by individuals throughout the season, so we consider it unlikely that there is much duplication of individuals within the dataset. Where we have repeatedly sampled the same roost, or nest, we document these as likely to be from the same bird. Although defecations at roost and nest sites could potentially be from more than one bird (e.g. the male and the female at the nest), the male only visits the nest for short periods of time, and where we find fresh defecations during the day when the female is on the nest, we associate these with the female. Additionally, males were found by Berry (1979) not to roost with the female at the nest, but separately, often up to 100 metres away. This ties in with other information presented by Berry where strict fidelity to roost sites by individual male Nightjars was found within and between seasons.

Fresh faecal samples, identified by their soft consistency, were targeted. Where there were multiple faecal pellets at a location, we took softer, fresher pellets over dry samples. Where there were only drier, older pellets, we took a sample, but visited the site again in the next 2-3 days in order to find a fresher sample, both in order to improve the amount of DNA extracted and to better pinpoint deposition date. Samples were transferred to a -20°C freezer within four hours of collection, and then to a -80°C freezer in batches every seven days, and stored there until processing. All

laboratory work took place at the NERC Biomolecular Analysis Facility (NBAF) within the Department of Animal and Plant Sciences, at the University of Sheffield, UK.

DNA extraction

We extracted DNA from faecal samples using the QIAmp DNA Fast Mini Stool Kit (Qiagen, Germany), following the human DNA extraction protocol (Supplementary Material, Appendix I), adapted to suit more highly degraded DNA from bird faeces by a) increasing the amount of sample (300mg vs standard 220mg), b) using the inhibitex buffer provided with the kit at a higher volume than recommended (1.4ml vs standard 1ml), and c) incubating samples for 10 minutes longer than recommended after the lysis buffer was added to increase the number of cells broken down from the sample and increase the DNA yield. We quantified DNA concentration using a Fluostar Optima (BMG Labtech, Germany) and kept the DNA extracts in a -20°C freezer until PCR.

Polymerase Chain Reaction (PCR)

Primer sets were selected based on their specificity and their ability to amplify short fragments of degraded DNA (fragments of between 100 – 180 base pairs (bp)), based on data from previous studies (Zeale *et al.* 2011, Clarke *et al.* 2014, Alberdi *et al.* 2018). Two commonly-used primer sets targeting the mitochondrial cytochrome oxidase COI gene: the 'Uniminibar' primer set (Meusnier *et al.* 2008) and the 'ZBJ' primer set (Zeale *et al*. 2011) were tested, but not used because they produced inconsistent amplification across different groups of insects (also highlighted in Brandon-Mong *et al*. 2015), and amplified primer dimer (Brownie *et al.* 1997), when

specific products failed to amplify. We then tested the Ins16S_1Short F and R primer set that amplifies a 156bp fragment of the mitochondrial 16S region (Clarke *et al.* 2014), identified in the literature as providing wider taxonomic coverage across invertebrate orders. The use of these 16S rRNA primers resulted in more consistent amplification levels across all samples. A temperature-gradient PCR from 55 – 70°C (Supplementary Material, Appendix I) with a random subset of faecal samples (n = 4), moth (n = 4), Nightjar control samples (DNA from egg albumen collected at nests, n = 4) and negative water samples (n = 2). The optimal PCR annealing temperature was identified as 62°C. This temperature amplified moth tissue positive control samples but not Nightjar DNA. PCR products were visualised on a 1% agarose gel with a 100bp ladder (Invitrogen, Thermo Fischer Scientific, UK) to verify amplification success.

Reference DNA library

Although the INS16S 1Short primers provided good taxonomic coverage, there are unfortunately many fewer reference sequences available with which to compare sequence reads (Clarke *et al*. 2014, Tournayre *et al*. 2020). To improve the ability to identify sequences from faecal samples, we created a local reference 16S sequence library, representative of some of the assemblage present on the site. We extracted DNA from 81 specimens of 80 species of moths and beetles collected from the moth traps, to supplement those already available in the BLAST global database (Altschul *et al.* 1990). Specimens were identified to species based on visual and microscopic examination by LJM, using the criteria of Townsend and Waring (2014). Extractions followed an ammonium acetate precipitation method (Bruford *et al.* 1998), where a digestion solution, Proteinase K and ammonium acetate were added to each sample

of moth legs and incubated overnight, to penetrate the invertebrates' solid chitin-based exterior. Extracted insect DNA was Sanger-sequenced using di-dedoxy dye terminators (see Supplementary Material, Appendix I for protocol) using both the *ZBJ* COI primers (Zeale *et al.* 2011) and the INS16S 1Short primers (Clarke *et al.* 2014). Sequences were visualised using an ABI3730 DNA Sequencer (Applied Biosystems, ThermoFischer Scientific, USA). Due to the lack of British 16S moth sequences in the NCBI /EMBL /DDJB databases, we verified species identification by comparing the sequenced moth 16S genes against COI sequences, which are more frequently present in the BLAST database. Finally, 16S sequences from 78 invertebrate species were submitted to the NCBI GenBank database (sequence accession numbers: MK620910 – MK620988).

Sequencing and bioinformatics

Detailed sequencing preparation and procedures are provided in the Appendices. Briefly, samples were cleaned, individually labelled, quantified and then pooled for sequencing. We then used the high-powered computer clusters '*Iceberg'* and *'Sharc'* at the University of Sheffield, to process and quantify the raw reads generated by the Illumina Miseq Benchtop Sequencer.

Sequences were processed, trimmed and clustered into 'Molecular Operational Taxonomic Units' (MOTUs). The threshold at which sequences are clustered – i.e. their level of similarity – strongly influences how many MOTUs are produced. MOTUs are taxonomic proxies (Hemprich-Bennett *et al*. 2018), and are frequently interpreted as equating to species (da Silva *et al*. 2020). Depending on the level of clustering used however (most commonly between 96% and 99%; Clare *et*

238 *al*. 2016), sequences may be equated to family or order (Clare *et al*. 2016,
239 Hemprich-Bennett *et al*. 2018, Gordon 2019). We clustered our MOTUs at 97%,
240 based on our knowledge of the close-relatedness of Lepiodptera species (Rytkonen
241 *et al*. 2019), as well as an understanding that if degradation has taken place, as is
242 common with DNA within faecal matter, it may be better to take a more conservative
243 approach (Clare *et al*. 2016).

244 Sequences were then compared to those stored in the NCBI GenBank
245 nucleotide database (including those of the 78 potential prey species that we
246 submitted) using a Nucleotide BLAST (blastn; Altschul *et al.* 1990). Because of the
247 lack of 16S reference sequences of British Lepidoptera, some MOTUs were
248 automatically assigned to species not present in the UK or to multiple species with
249 an identical level of certainty. We therefore manually assigned such species to
250 genus or family, using the NCBI taxonomy browser. For all diversity analyses we
251 therefore used a matrix of MOTUs, rather than species and/or genera because of the
252 described discrepancy in assigning units (as in Razgour *et al*. 2011). Classifying
253 sequence reads this way allowed us to include all units identified through the
254 clustering process, whether or not we were able to assign them to a known taxa
255 (Clare *et al.* 2011, Hawlitschek *et al.* 2018, da Silva *et al*. 2020). MOTUs that had
256 been assigned to specific non-target species (*Homo sapiens*, *Sus scrofa*, *Columba*
257 *palumbus* and *Akkermansia sp*), were removed (da Silva *et al*. 2020).

258 Finally, we created matrices of final read numbers and presence/absence
259 matrices in R (v. 3.6), which were used to filter out low numbers of reads (Galan
260 2018), that are likely to represent contamination rather than true sequences.
261 Following De Barba *et al.* (2014), we used our negative control samples to set
262 thresholds for each PCR amplification, sequences with numbers below these

thresholds were considered as noise, and removed (see Supplementary material, Appendix II for more detail). False positives can occur at multiple stages of the extraction and PCR procedures (Ficedola *et al*. 2015, Zepeda-Mendoza *et al*. 2016, Alberdi *et al*. 2018) and caution over such sequences is important (Froslev *et al*. 2017).

Sequencing data will be available online upon publication through the European Nucleotide Archive (ENA), part of the European Molecular Biology Laboratory (EMBL), as fastq files.

Statistical analyses

To examine the diet of this population of Nightjars as a whole, we calculated the frequency of occurrence of sequences (FOO) – i.e. the proportion of samples in which each MOTU was found. Conversion of sequence data into a more quantitative estimate of diet, known as relative read abundance (RRA), has flaws (see discussion in Deagle *et al*. 2019). However, the use of FOO alone can be conservative, as it essentially equates one sequence that registers 10,000 reads, with another that only registers 10 reads, because the only criterion is to be present (Deagle *et al*. 2019). Thus, we use RRA to calculate all metrics, bearing in mind that numbers of sequence reads do not perfectly translate to percentage biomass (Rytkönen *et al*. 2019).

To examine variation in composition between samples, we used multivariate PERMANOVAs (using '*adonis'* in '*vegan'*). These tested for differences in the means of Chao dissimilarity indices (R package '*vegan',* function *'vegdist'*; Oksanen *et al.* 2019) between sex, month and year (McClenaghan *et al*. 2019a). Because it was not

287 possible to sex chicks, these were removed from the data for calculation of the
288 PERMANOVAs, as were within-sample repeats. Sex, month and year in which the
289 sample was collected, were modelled separately as they could not be included in the
290 same PERMANOVA, because there were missing data from both the sex and month
291 categories. We used Chao dissimilarity indices within the PERMANOVAs in order to
292 account for unknown species, and because they were stable in our unbalanced
293 sample size scenario (Chao *et al*. 2005, Chao & Chiu 2016).

294 PERMANOVAs are a non-parametric version of a MANOVA and are stable
295 when faced with heterogeneity within groups (Anderson 2017). We used package
296 'pairwise Adonis' (Martinez Arbizu 2020) to conduct among-year pairwise
297 comparisons. As we had some unbalanced sample sizes, we calculated
298 homogeneity of group variances (function '*betadisper*' in vegan (Oksanen *et al.*
299 2019)), which calculates the distance from each sample to the group centroid
300 (Anderson, 2006) to estimate compositional variance within groups, using the Chao
301 dissimilarity indices again. We calculated 95% confidence intervals for all tests using
302 Tukey's HSD.

303 For a random subset of the samples, we undertook repeat extraction, PCR
304 and sequencing. Although most variation is attributed to between sample variation
305 (Mata *et al*. 2019), it is possible that due to a large number of variables such as size
306 of the dropping, digestion time and size of the prey items (Oehm *et al*. 2011), there
307 may be variation within a faecal sample as well (Ando 2020). We compared the
308 within-sample repeats using Chao dissimilarity indices, calculated from RRA as
309 above. The repeat samples are included in the supplementary information file,
310 marked with the suffix '_r'.

311 **Prey size variation and selection by Nightjars**

312 To understand if size-selective foraging took place, we used moth traps to obtain an
313 indication of flying insect prey availability on the breeding site. We placed 15W
314 actinic light traps (made in the electronics department at the University of York –
315 details and photos within the supplementary information), at eight locations within the
316 boundary of Thorne and Hatfield Moors (n = 16). Moths were trapped at four sites for
317 one night in each alternate week (i.e. sites 1-4 for one night (e.g. overnight Thursday
318 - Friday) in weeks 1, 3, 5 etc., followed by sites 5-9 for the same night in weeks 2, 4,
319 6 etc.) starting at the beginning of June until the middle of August. Traps were placed
320 at each site within two hours prior to dusk and emptied the following morning within
321 one hour of dawn. Trap locations were identified as grid squares on a map of the
322 site, and the traps placed in a randomly chosen but consistent location on the ground
323 within this square. Although the same grid squares were being sampled (each
324 square was sampled n = 4 times), the two-week interval between sampling
325 occasions should remove some concern about repeat sampling (Truxa & Fiedler
326 2012). The sites chosen for moth trapping included all habitat types present on the
327 nature reserve. Habitat types were classified as woodland (trees with canopy >2
328 metres high), scrub (trees with canopy <2 metres high), heather, wetland (rush-
329 dominated, <50% standing water), bog (>50% standing water, *Eriophorum*
330 dominated, bare-ground (bare peat areas with minimal (< 10% vegtation cover) and
331 'off site' – all areas outwith the boundary of the NNR). Habitat structure was also
332 incorporated, so as to include information about habitat manipulation (there were
333 three categories here: unmanaged, machine-manipulated (mass management using
334 flail/digger) or hand-manipulated (selective chainsawing, volunteers with handtools).
335 We recorded numbers of species and individuals, calculated diversity (Inverse

Simpson's dominance; Morris *et al.* (2014)) and allocated species to a size category (in mm; <25, 25-30, 30-40, 40-50, 50-60, or >60) based on Townsend and Waring (2014).

To explore size composition for the subset of MOTUs identified to species level within each sample, including those fed to chicks, we allocated each species identified within the faecal samples to a size category according to mean wingspan, using Townsend and Waring (2014). To test for significant differences in the frequency of occurrence of different size classes between years we used Kruskal-Wallis rank sum tests. To test for size selection of moth prey by Nightjars, we used proportional Z-tests to compare the frequency of occurrence of each size class found in the faecal samples, to those found in the traps, between years and months.

RESULTS

We retrieved a total of 13,900,616 sequence reads from 141 Nightjar faecal samples using the Illumina MiSeq Benchtop sequencer, which comprised 1631 MOTUs after trimming and clustering. After processing and filtering (i.e. removing 'impossible' reads likely to be a result of contamination and degradation: De Barba *et al.* 2014), we retained 625 unique MOTUs from 130 samples. Some samples (n = 11), contained no reads once filtered, meaning that the amount of non-arthropod contamination was high, as has been found in other studies (Regnaut *et al.* 2006). 65% of MOTUs were identified to species, 5% to genus, 12% to family and 18% only to order (all Lepidoptera). Lepidoptera were found in 99% of samples, followed by Diptera (27%), Coleoptera (9%), Neuroptera (7%) and <1% of both Hemiptera and Hymenoptera. Of the sequences identified to species level, the most common moth

species present were Poplar Hawk-moth *Lathoe populi* (in 43% of samples), Silver Y *Autographa gamma* (45%), Yellow-tail *Euproctis similis* (45%), True Lover's Knot *Lycophotia porphyrea* (49%), Smoky Wainscot *Mythimna impura* (50.4%), and Large Yellow Underwing *Noctua pronuba* (52%). Of the Coleoptera we were able to identify, we found *Harpalus* sp. (Carabidae*;* 2% of samples), *Melanotus villosus* and *Stenagostus rhombeus* (both Elateridae and in 6% of samples). Diptera sequences identified belonged to the families Chironomidae, Sciaridae, Culicidae and Tipulidae, but were not identifiable to any more detailed level than that of family. All identified species are listed in the Supplementary Information (Appendix III).

Variation in composition and diversity among samples

Samples contained an average of 15.6 MOTUs (+/- SD 9.7). Mean Chao dissimilarity of adult samples (2015: $n = 8$, 2016: $n = 17$, 2017: $n = 27$, 2018: $n = 27$; no chicks and no repeat samples, and a reduced total number of 429 MOTUs) was 0.91 (+/- SD 0.17), which shows relatively high individual variation. Mean Chao dissimilarity of the repeat samples was 0.85 (+/- SD 0.19), therefore making related samples slightly more similar.

No significant differences were found in sample composition between sexes (Female: $n = 26$, Male: $n = 43$; $F_{1,68}$: 0.36, $P = 0.99$), nor between the months in which the samples were collected (June: $n = 21$; July: $n = 32$; August: $n = 15$; $F_{2,70}$: 1.21, $P = 0.16$). Between-year differences in sample composition were significant (2015: $n = 8$, 2016: $n = 17$, 2017: $n = 27$, 2018: $n = 27$; $F_{3,75} = 3.3$, $P = 0.001$; r^2: 0.12), supported by a non-significant difference in the distribution of samples around the centroid ($F_{3,75}$: 1.37, $P = 0.26$; Figure 1), although some caution must be taken due to uneven sample sizes. Specific significant among-year differences in variance were shown between 2016 – 2017 and 2017 -2018 (Table 1).

385

386 **Moth species sizes within faecal samples**

387 96% of faecal samples contained moths with a wingspan of between 30 and 40mm
388 and 46% of samples contained the largest moths (>60 mm wingspan). The five most
389 frequent species in the samples all had a mean wingspan of more than 30mm; two of
390 these had a wingspan of more than 50mm (*N. pronuba, L. populi*). The largest moths
391 (>60mm) were present in significantly more samples in 2018 (66%), than all other
392 years (2015: 0%; 2016: 14%; 2017: 36%; X^2 = 27.08, df = 3, P <0.0001).

393

394 **Size selection in diet samples compared to prey availability**

395 Faecal samples were more likely than moth traps to contain the largest moths
396 (>60mm). This was significant in 2017 (diet samples: 0.4, moth traps: 0.18; X^2= 4.61,
397 df= 1, P = 0.03) and 2018 (diet samples: 0.67, moth traps: 0.34; X^2= 9.5, df =1, P =
398 0.002; Figure 2). In contrast, moth traps were more likely than faecal samples to
399 contain smaller moths (<25mm), but the difference was only significant in 2018 (diet
400 samples: 0.18, moth traps: 0.49; X^2= 9.55, df =1, P = 0.002; Figure 3).

401 Across all years, less than 18% of diet samples contained moths with the
402 smallest wingspan (<25mm). In June and August, faecal samples were more likely
403 than moth traps to contain the largest moths (June: 50-60mm: diet samples: 22%;
404 moth traps: 4%, X^2: 11.66, df =1, P = <0.001. August: >60mm: diet samples: 15%,
405 moth traps: 3%, X^2: 5.73, df = 1, P = 0.02).

406

DISCUSSION

We have demonstrated the use of high-throughput sequencing in investigating the diet of an insectivorous, avian migrant to the UK, joining a growing bank of literature exploring the use of metabarcoding to reduce the bias shown by physical dissection of faeces towards retained, undigested fragments of prey items (Evens *et al.* 2020, Mills *et al.* 2020). Our analyses showed strong intra- and inter-annual variation in diet richness and size-based prey selection, which varied annually and seasonally. Differences among years in Nightjar diet potentially reflected a response by Nightjars to environmental fluctuation influencing the annual life cycle of moth species.

There was a dominance of Lepidoptera over other arthropods in Nightjar diet, which has been reported previously (Sierro *et al.* 2001, Sharps 2013, Evens *et al*. 2020), although this does not hold true for all species of caprimulgid (Knight *et al.* 2018). The prevalence of Lepidoptera in the diet of this population may reflect local availability of this prey type (exceedingly high numbers of larger moths, or very few Coleoptera) and an adjustment of the population to being almost entirely Lepidoptera specialists. It is also possible that due to our molecular optimisation procedures, there has been a lack of amplification of groups other than Lepidoptera. However, previous tests using the primers in our study showed very high taxonomic coverage (>96%) for 11 invertebrate orders, and showed higher success for Coleoptera than for Lepidoptera (Clarke *et al*. 2014).

Moths present in the faecal samples were diverse in terms of their habitat preferences and population trends. The most common species found in faecal samples were all habitat generalists (e.g. *N. pronuba, E. similis*). True Lover's Knot, however, is a specialist of heathlands, its larvae feeding exclusively on heather (Townsend & Waring 2007, Thomsen *et al*. 2015). A number of heathland and wet

woodland specialist species (including for example, Oak Eggar *Lasiocampa quercus*, Map-winged swift *Hepialus fusconebulosa* and Gold Spot *Plusia festucae* were caught in the light traps, but most species found in both locations were generalists, found in such widespread habitats as hedgerows, woodlands and gardens. Although some such generalist species have more positive population trends than habitat specialists, large moths such as those preferentially selected by Nightjars, and another migrant insectivore the Common Cuckoo *Cuculus canorus* are, overall, in decline (Coulthard *et al.* 2019, Mills *et al*. 2020, Fox *et al*. 2021). The most commonly identified moth species within Nightjar faeces, the Large Yellow Underwing, has increased over the past 50 years by 72%, clearly thriving in generalist habitats (Harrower *et al*. 2020). Conversely, True Lover's Knot, Silver Y, Yellow-Tail, Poplar Hawkmoth and Smoky Wainscot, the other five most common components of Nightjar faecal matter, have all declined since 1968 by between 1.5 and 83% (the latter being the heathland specialist, True Lover's Knot).

Samples differed significantly from each other. Although some samples may have been taken from the same individual, intraspecific variation in diet is common and can reflect both resource availability and the avoidance of competition through resource partitioning (Kotler & Brown 1988, Maldonado *et al.* 2019, Alberdi *et al*. 2020), or differences in habitat selection. We tracked a number of individuals in this population over the same four years of this study, and this tracking work highlighted a lack of overall population preference for any particular habitat type (Mitchell *et al.* 2020). The lack of similarity in diet among samples potentially also indicates a lack of association with a particular habitat for foraging. For species exploiting patchy, ephemeral resources as the Nightjars are here, it is beneficial to be flexible to be

456 able to adjust to prey availability and abundance (Maldonado *et al.* 2019, Szigeti *et*
457 *al.* 2019).

458 Contrary to expectation, no significant difference in sample composition or
459 diversity was found between male and female Nightjars (Mata *et al.* 2016, Knight *et*
460 *al.* 2018). We expected to find that females had less diverse diets than males,
461 because of their limited foraging opportunity (Houston 1997). However, not all
462 females were incubating during the period when the faecal samples were acquired,
463 so perhaps were not restricted in their foraging duration. Differences between years
464 in sample variance and prey species richness may indicate that the population could
465 respond to variation in prey availability, by taking advantage of emergences of
466 particular species, perhaps including influxes of migratory moths, several species of
467 which (e.g. Silver Y) were identified within our Nightjar samples (Lee & McCracken
468 2005) . Annual and seasonal variation in moth community diversity and overall
469 abundance have been shown to be related to variation in climate, as well as habitat
470 management (Summerville & Crist 2004, Summerville *et al*. 2007).

471 By comparing diet and moth trap samples, we examined consumption
472 compared to local availability, bearing in mind that not all the MOTUs identified in the
473 diet samples could be translated into species and that moth trap catches do not
474 sample moths representatively (see Truxa & Fiedler 2012, Jonason *et al*. 2014 for
475 information on bias in trapping and Evens *et al*. 2020 for use of the same type of
476 trap). It is also pertinent to note that some Nightjars make trips away from their
477 nesting areas to separate foraging locations, in this and other studies (Alexander &
478 Cresswell 1992, Evens *et al*. 2020, Mitchell *et al.* 2020), which means that
479 depending on habitat type at any distant foraging locations, we will not have
480 completed accounted for all prey available to Nightjars across their individual

foraging ranges. Again, their preference for large moths, which should provide higher energy gain per item than small moths is a strategy also recognised in other nocturnal insectivores (Clare *et al.* 2009, Vesterinen *et al.* 2016). Twilight foraging limits the time available for capturing prey, which should drive size bias upwards in line with the energy maximization – time minimization rule (Pyke 1984, Schoener 2003), and our findings suggest that availability of larger moths might be a key aspect of Nightjar foraging strategy and requirements (Araújo *et al.* 2011, Schrimpf *et al.* 2012, Vesterinen *et al.* 2016, Evens *et al*. 2020).

Understanding the demographic consequences of variation in prey availability is important in order to make practical decisions about future management. The habitat management that occurred on our study area has resulted in a reduction in birch woodland, a rich invertebrate prey source. However, the outcome of this is not necessarily negative, if regenerating, herbaceous, understorey vegetation that results from the clearance sustains sufficient numbers of moths (Summerville & Crist 2004) and continuing low-intensity management supports large moth species (Mills *et al.* 2020). Whilst Nightjars may be resilient to variation in prey availability on an annual basis, it remains especially important to encourage habitat types that provide larval food for moths during the Nightjar chick raising period to allow offspring to fledge.

Use of metabarcoding for insectivorous diets

Metabarcoding has made it possible to identify physically unrecognisable remains of prey specimens for many taxa (e.g. Kartzinel & Pringle 2015, Hawlitschek *et al.* 2018, McClenaghan *et al.* 2019a). Despite considerable progress, such methods are

still hindered by a few key challenges. Firstly, contamination of samples with non-target DNA means that caution must be taken when conducting laboratory work and the post-sequencing bioinformatics (Zepeda-Mendoza *et al*. 2016). Although one of the benefits of metabarcoding is that less common prey species can be identified, the post-sequencing filtering thresholds that must be established to produce confident results, can sometimes remove rare sequences.

Additionally, some studies have found within-sample variation in MOTU composition and diversity (Alberdi *et al*. 2018, Mata *et al*. 2018), although between-sample variation was always stronger. We did find lower within-sample variation than between-sample variation, but both measures were higher than in these other referenced studies. We do not know a sufficient amount about Nightjar gut transit time so we are unable to quantify how many specimens or prey capture events were contained in each faecal sample, but high within-sample variation could relate to a high number of specimens per defaecation, or may relate more to the methods used to extract the DNA. This does indicate that there are several steps that may be advised in order to ensure maximum information and variation is obtained, such as multiple extracts and PCR replicates of the same sample, as well as repeated sampling from the same individuals across the season, where known (Alberdi *et al*. 2018). The latter would ensure that individual specialisation in diet could be recorded through longitudinal data collection as opposed to a 'snapshot' of an individual's diet (Araujo *et al*. 2011). Although there is concurrence across general metabarcoding protocols, the specific details such as number of replicates and use of controls are not standardised, which can limit utility and cross-study comparison (Deagle *et al*. 2019, Ando *et al*. 2020).

For our study, by far the biggest of these challenges was a lack of reference sequences for particular groups of species in global databases (such as NCBI Genbank used for BLAST processing). This lack of reference sequences meant that species could not be identified for around 35% of our MOTUs, even after the addition of 78 species of moth to the 16S reference database (compared to 90% of COI sequences identified to species in Evens *et al*. 2020). This means that despite being able to assess sample richness, variation and change between months and years, understanding the ecological information that comes with a species-level assignment, which we are lacking, is invaluable (Hebert *et al.* 2003). Although we did contribute several dozen 16S sequences to GenBank, we know from a comprehensive compilation of Lepidoptera records on Thorne Moors alone (not both Moors that constitute the Humberhead Peatlands NNR), that there have been 717 moth species recorded (382 macro-, 335 micromoths; Moat 2014). This indicates that much more structured, systematic barcoding of the 16S rRNA gene of British moth species needs to take place, to further this type of work.

Conclusions

Nightjars showed within-population and annual diet variation, as well as size selection. The population appears able to adjust its diet in response to variation in prey availability, reflecting ecological theory. This has consequences for Nightjars' ability to withstand habitat change, with populations and species exhibiting diverse diets often able to adapt better. For cryptic, insectivorous species such as the Nightjar, metabarcoding provides a very useful tool to identify diet components, despite continuing challenges related to extraction through to sequencing.

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Data availability statement

Raw Fastq sequencing files will be openly-available upon publication through the ENA, part of the EMBL under the primary accession number PRJEB44974. Summary files for statistical analysis are available as an excel file in SOM 4.

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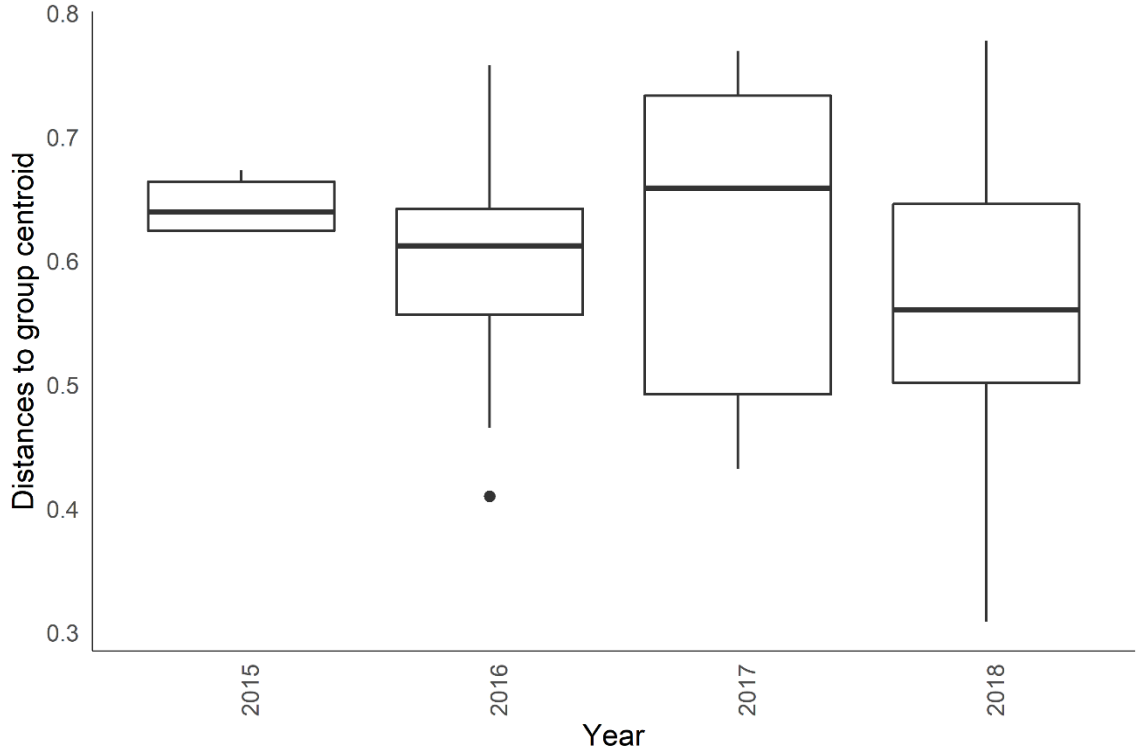

885

886 **Figure 1.** Mean and variance of faecal sample distances to group centroids for each year samples
887 were collected, produced by the '*betadisper*' function in r. Sample sizes vary between groups and are
888 presented in the text.

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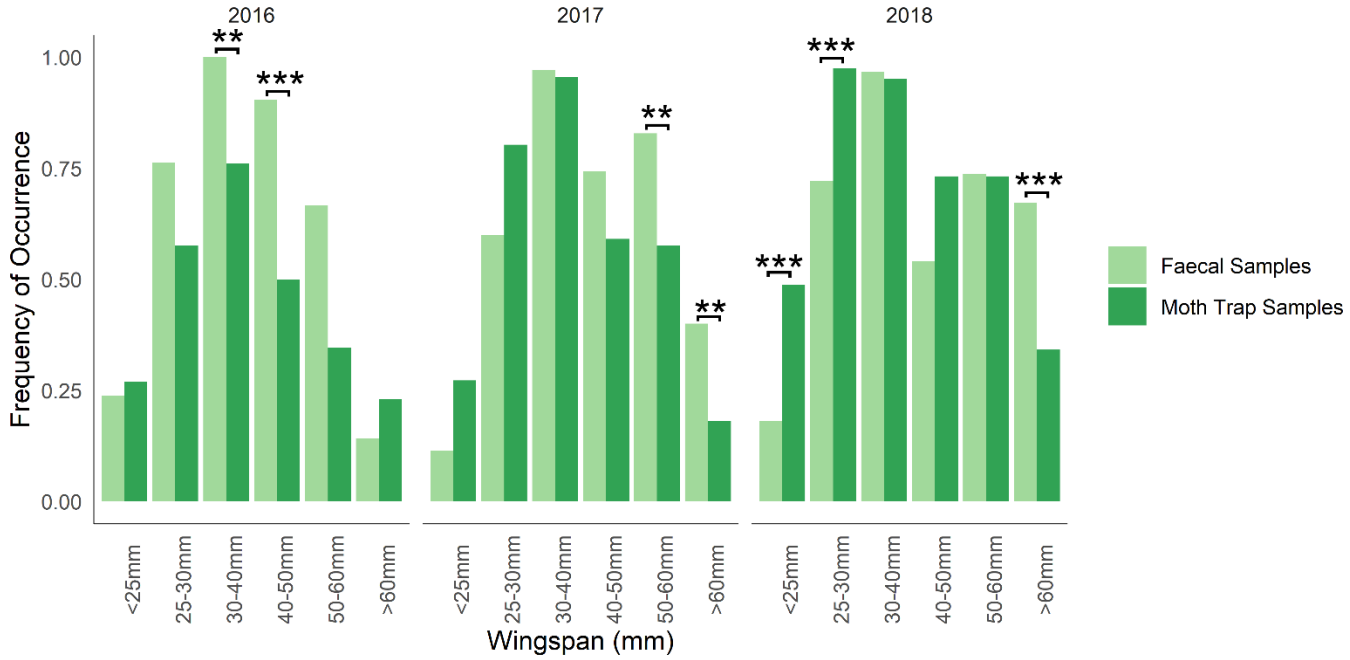

891

892 **Figure 2.** Proportion of each moth size class present in diet samples and moth trap catches, for each
893 year. Differences between faecal samples and moth trap samples were calculated using proportional
894 Z-tests and significance levels are denoted using: ** = <0.05, *** =<0.01. Note: No moth trapping took
895 place in 2015.

896

Table 1. Pairwise comparisons of among-year differences in mean sample composition using the function pairwise.Adonis in R which takes the Chao dissimilarity values as input. Significant adjusted *P* values (< 0.05) in bold.

Pairs	df	Sums of Sq	*F*	R^2	*P*	*P* adjusted
2015 vs 2016	1	0.663	1.587	0.065	0.034	0.204
2015 vs 2017	1	0.623	1.452	0.042	0.089	0.534
2015 vs 2018	1	0.872	2.301	0.065	0.006	**0.036**
2016 vs 2017	1	1.656	4.064	0.088	0.001	**0.006**
2016 vs 2018	1	2.495	6.775	0.139	0.001	**0.006**
2017 vs 2018	1	1.061	2.758	0.050	0.006	**0.036**