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1                   **Metabarcoding reveals selective dietary responses to**  
2                   **environmental availability in the diet of a nocturnal, aerial**  
3                   **insectivore, the European Nightjar (*Caprimulgus europaeus*)**  
4

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

26 opportunistic exploitation of available resources. We analysed the diet of a nocturnal,  
27 aerial insectivore, the European Nightjar *Caprimulgus europaeus*, using high-throughput  
28 metabarcoding. We quantified diet diversity and composition of 130 faecal samples from  
29 nests and roosts on a northern breeding site in the UK from 2015 – 2018, and compared  
30 differences among individuals and years. Although dominated by moths, diet varied  
31 significantly between individual faecal samples and between years and months. Prey  
32 species composition varied between years, and was more variable between samples in  
33 2017, compared to other years. Faecal samples were significantly more likely to contain  
34 moth species with a wingspan of >60mm and less likely to contain moth species of  
35 <25mm wingspan. This indicated size-selective foraging, which also varied between  
36 months and years. Diet was driven by inter-individual variation, indicating population-  
37 level flexibility in prey choice. Metabarcoding provided a valuable tool in the exploration  
38 of insectivorous diets, but efforts are needed to build comprehensive reference libraries,  
39 in order to compile full prey species lists.

40

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

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

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

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

75         The European Nightjar *Caprimulgus europaeus* (henceforth, 'Nightjar') is a  
76 nocturnal insectivore of conservation concern in the UK (Amber listed - Eaton *et al.*  
77 2015, IUCN classification is least concern, BirdLife International 2016). This species  
78 completes an annual migration from central Africa to the UK to breed from May until  
79 August, and feeds on arthropods (Cramp 1985, Sharps 2013). Limited diet data have  
80 been obtained through stomach content analysis and physical dissection of faeces  
81 from European Nightjars and the Common Nighthawk *Chordeiles minor* (Sierro *et al.*  
82 2001, Sharps 2013, Knight *et al.* 2018). However, these methods result in a bias  
83 towards remaining hard parts of consumed arthropods such as beetle elytra, legs  
84 and antennae, which means that methods such as metabarcoding that are capable  
85 of identifying partially or fully digested remains are needed. Extracting sufficient DNA  
86 from avian faeces is challenging, especially after degradation by exposure to sun,  
87 rain and soil microbes (Deagle *et al.* 2006, Oehm *et al.* 2011, Kamenova *et al.* 2018).  
88 The high concentration of uric acid (Eriksson *et al.* 2017) in conjunction with  
89 environmental exposure inhibits extraction and amplification of DNA, making the  
90 extraction and clean-up stages of metabarcoding, along with choice of primer  
91 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

116 (JNCC 2015). It is also a European Special Protection Area (SPA) for its breeding  
117 Nightjar population (1.9% of the UK population; JNCC 2015). The NNR has  
118 undergone substantial habitat management since 2014, primarily the removal of 562  
119 km<sup>2</sup> of birch woodland and an increase in wet habitat through the damming of  
120 drainage channels. Both techniques will influence vegetation structure and insect  
121 prey availability; selective and patchy clearance of woodland has been shown to  
122 increase species richness of Lepidoptera (Summerville & Crist 2002), and should  
123 also provide an increased amount of breeding space for Nightjars (Sharps 2013).  
124 Use of wetland habitat (e.g. bogs, mires) by nightjars is little considered in the  
125 literature explicitly as a breeding habitat, but there is some indication that they act as  
126 suitable foraging areas, containing a high number of invertebrates (English *et al.*  
127 2017, Evens *et al.* 2017).

128

### 129 **Faecal sample collection**

130 Nightjar faecal samples were collected from June to August, 2015 to 2018 (2015:  $n =$   
131 9, 2016:  $n = 20$ , 2017:  $n = 35$ , 2018:  $n = 36$ ) and were obtained from previously  
132 located roosts and nests, during the day. Upon approaching the nest or roost, we  
133 used binoculars to view the bird in situ. If the bird was not visible, we slowly  
134 approached the location until the bird flew away, whereupon the sex of the bird was  
135 visible (male Nightjars possess bright white spots on primaries 3,4 and 5 whilst  
136 females have smaller, beige spots). Nests and roosts of Nightjars and the size and  
137 shape of their faeces are distinctive and identifiable, as they are discrete curled  
138 pellets of between 3 – 8mm in width, found individually or in small clusters (see  
139 image in supplementary information). Faeces were placed into sterile, 30ml screw-  
140 top tubes (Elkay labs, UK) with wooden toothpicks, then labelled with sample

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

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



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

167

## 168 **DNA extraction**

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

179

## 180 **Polymerase Chain Reaction (PCR)**

181 Primer sets were selected based on their specificity and their ability to amplify short  
182 fragments of degraded DNA (fragments of between 100 – 180 base pairs (bp)),  
183 based on data from previous studies (Zeale *et al.* 2011, Clarke *et al.* 2014, Alberdi *et al.*  
184 2018). Two commonly-used primer sets targeting the mitochondrial cytochrome  
185 oxidase COI gene: the 'Uniminibar' primer set (Meusnier *et al.* 2008) and the 'ZBJ'  
186 primer set (Zeale *et al.* 2011) were tested, but not used because they produced  
187 inconsistent amplification across different groups of insects (also highlighted in  
188 Brandon-Mong *et al.* 2015), and amplified primer dimer (Brownie *et al.* 1997), when

189 specific products failed to amplify. We then tested the Ins16S\_1Short F and R primer  
190 set that amplifies a 156bp fragment of the mitochondrial 16S region (Clarke *et al.*  
191 2014), identified in the literature as providing wider taxonomic coverage across  
192 invertebrate orders. The use of these 16S rRNA primers resulted in more consistent  
193 amplification levels across all samples. A temperature-gradient PCR from 55 – 70°C  
194 (Supplementary Material, Appendix I) with a random subset of faecal samples ( $n =$   
195 4), moth ( $n = 4$ ), Nightjar control samples (DNA from egg albumen collected at nests,  
196  $n = 4$ ) and negative water samples ( $n = 2$ ). The optimal PCR annealing temperature  
197 was identified as 62°C. This temperature amplified moth tissue positive control  
198 samples but not Nightjar DNA. PCR products were visualised on a 1% agarose gel  
199 with a 100bp ladder (Invitrogen, Thermo Fischer Scientific, UK) to verify amplification  
200 success.

201

## 202 **Reference DNA library**

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

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

225

## 226 **Sequencing and bioinformatics**

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

232 Sequences were processed, trimmed and clustered into 'Molecular  
233 Operational Taxonomic Units' (MOTUs). The threshold at which sequences are  
234 clustered – i.e. their level of similarity – strongly influences how many MOTUs are  
235 produced. MOTUs are taxonomic proxies (Hemprich-Bennett *et al.* 2018), and are  
236 frequently interpreted as equating to species (da Silva *et al.* 2020). Depending on the  
237 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 Lepidoptera species (Rytönen  
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

263 thresholds were considered as noise, and removed (see Supplementary material,  
264 Appendix II for more detail). False positives can occur at multiple stages of the  
265 extraction and PCR procedures (Ficedola *et al.* 2015, Zepeda-Mendoza *et al.* 2016,  
266 Alberdi *et al.* 2018) and caution over such sequences is important (Froslev *et al.*  
267 2017).

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

271

## 272 **Statistical analyses**

273 To examine the diet of this population of Nightjars as a whole, we calculated the  
274 frequency of occurrence of sequences (FOO) – i.e. the proportion of samples in  
275 which each MOTU was found. Conversion of sequence data into a more quantitative  
276 estimate of diet, known as relative read abundance (RRA), has flaws (see discussion  
277 in Deagle *et al.* 2019). However, the use of FOO alone can be conservative, as it  
278 essentially equates one sequence that registers 10,000 reads, with another that only  
279 registers 10 reads, because the only criterion is to be present (Deagle *et al.* 2019).  
280 Thus, we use RRA to calculate all metrics, bearing in mind that numbers of  
281 sequence reads do not perfectly translate to percentage biomass (Rytönen *et al.*  
282 2019).

283           To examine variation in composition between samples, we used multivariate  
284 PERMANOVAs (using ‘*adonis*’ in ‘*vegan*’). These tested for differences in the means  
285 of Chao dissimilarity indices (R package ‘*vegan*’, function ‘*vegdist*’; Oksanen *et al.*  
286 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% vegetation 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

336 Simpson's dominance; Morris *et al.* (2014)) and allocated species to a size category  
337 (in mm; <25, 25-30, 30-40, 40-50, 50-60, or >60) based on Townsend and Waring  
338 (2014).

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

347

## 348 **RESULTS**

349 We retrieved a total of 13,900,616 sequence reads from 141 Nightjar faecal samples  
350 using the Illumina MiSeq Benchtop sequencer, which comprised 1631 MOTUs after  
351 trimming and clustering. After processing and filtering (i.e. removing 'impossible'  
352 reads likely to be a result of contamination and degradation: De Barba *et al.* 2014),  
353 we retained 625 unique MOTUs from 130 samples. Some samples ( $n = 11$ ),  
354 contained no reads once filtered, meaning that the amount of non-arthropod  
355 contamination was high, as has been found in other studies (Regnaut *et al.* 2006).  
356 65% of MOTUs were identified to species, 5% to genus, 12% to family and 18% only  
357 to order (all Lepidoptera). Lepidoptera were found in 99% of samples, followed by  
358 Diptera (27%), Coleoptera (9%), Neuroptera (7%) and <1% of both Hemiptera and  
359 Hymenoptera. Of the sequences identified to species level, the most common moth



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

#### 369 *Variation in composition and diversity among samples*

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

376 No significant differences were found in sample composition between sexes  
377 (Female:  $n = 26$ , Male:  $n = 43$ ;  $F_{1,68} = 0.36$ ,  $P = 0.99$ ), nor between the months in  
378 which the samples were collected (June:  $n = 21$ ; July:  $n = 32$ ; August:  $n = 15$ ;  $F_{2,70} =$   
379  $1.21$ ,  $P = 0.16$ ). Between-year differences in sample composition were significant  
380 (2015:  $n = 8$ , 2016:  $n = 17$ , 2017:  $n = 27$ , 2018:  $n = 27$ ;  $F_{3,75} = 3.3$ ,  $P = 0.001$ ;  $r^2 =$   
381  $0.12$ ), supported by a non-significant difference in the distribution of samples around  
382 the centroid ( $F_{3,75} = 1.37$ ,  $P = 0.26$ ; Figure 1), although some caution must be taken  
383 due to uneven sample sizes. Specific significant among-year differences in variance  
384 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

407 **DISCUSSION**

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

416         There was a dominance of Lepidoptera over other arthropods in Nightjar diet,  
417 which has been reported previously (Sierro *et al.* 2001, Sharps 2013, Evens *et al.*  
418 2020), although this does not hold true for all species of caprimulgid (Knight *et al.*  
419 2018). The prevalence of Lepidoptera in the diet of this population may reflect local  
420 availability of this prey type (exceedingly high numbers of larger moths, or very few  
421 Coleoptera) and an adjustment of the population to being almost entirely Lepidoptera  
422 specialists. It is also possible that due to our molecular optimisation procedures,  
423 there has been a lack of amplification of groups other than Lepidoptera. However,  
424 previous tests using the primers in our study showed very high taxonomic coverage  
425 (>96%) for 11 invertebrate orders, and showed higher success for Coleoptera than  
426 for Lepidoptera (Clarke *et al.* 2014).

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

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

446         Samples differed significantly from each other. Although some samples may  
447 have been taken from the same individual, intraspecific variation in diet is common  
448 and can reflect both resource availability and the avoidance of competition through  
449 resource partitioning (Kotler & Brown 1988, Maldonado *et al.* 2019, Alberdi *et al.*  
450 2020), or differences in habitat selection. We tracked a number of individuals in this  
451 population over the same four years of this study, and this tracking work highlighted  
452 a lack of overall population preference for any particular habitat type (Mitchell *et al.*  
453 2020). The lack of similarity in diet among samples potentially also indicates a lack of  
454 association with a particular habitat for foraging. For species exploiting patchy,  
455 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

481 foraging ranges. Again, their preference for large moths, which should provide higher  
482 energy gain per item than small moths is a strategy also recognised in other  
483 nocturnal insectivores (Clare *et al.* 2009, Vesterinen *et al.* 2016). Twilight foraging  
484 limits the time available for capturing prey, which should drive size bias upwards in  
485 line with the energy maximization – time minimization rule (Pyke 1984, Schoener  
486 2003), and our findings suggest that availability of larger moths might be a key  
487 aspect of Nightjar foraging strategy and requirements (Araújo *et al.* 2011, Schrimpf  
488 *et al.* 2012, Vesterinen *et al.* 2016, Evens *et al.* 2020).

489           Understanding the demographic consequences of variation in prey availability  
490 is important in order to make practical decisions about future management. The  
491 habitat management that occurred on our study area has resulted in a reduction in  
492 birch woodland, a rich invertebrate prey source. However, the outcome of this is not  
493 necessarily negative, if regenerating, herbaceous, understory vegetation that  
494 results from the clearance sustains sufficient numbers of moths (Summerville & Crist  
495 2004) and continuing low-intensity management supports large moth species (Mills  
496 *et al.* 2020). Whilst Nightjars may be resilient to variation in prey availability on an  
497 annual basis, it remains especially important to encourage habitat types that provide  
498 larval food for moths during the Nightjar chick raising period to allow offspring to  
499 fledge.

500

### 501 **Use of metabarcoding for insectivorous diets**

502 Metabarcoding has made it possible to identify physically unrecognisable remains of  
503 prey specimens for many taxa (e.g. Kartzinel & Pringle 2015, Hawlitschek *et al.*  
504 2018, McClenaghan *et al.* 2019a). Despite considerable progress, such methods are

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

511         Additionally, some studies have found within-sample variation in MOTU  
512 composition and diversity (Alberdi *et al.* 2018, Mata *et al.* 2018), although between-  
513 sample variation was always stronger. We did find lower within-sample variation than  
514 between-sample variation, but both measures were higher than in these other  
515 referenced studies. We do not know a sufficient amount about Nightjar gut transit  
516 time so we are unable to quantify how many specimens or prey capture events were  
517 contained in each faecal sample, but high within-sample variation could relate to a  
518 high number of specimens per defaecation, or may relate more to the methods used  
519 to extract the DNA. This does indicate that there are several steps that may be  
520 advised in order to ensure maximum information and variation is obtained, such as  
521 multiple extracts and PCR replicates of the same sample, as well as repeated  
522 sampling from the same individuals across the season, where known (Alberdi *et al.*  
523 2018). The latter would ensure that individual specialisation in diet could be recorded  
524 through longitudinal data collection as opposed to a 'snapshot' of an individual's diet  
525 (Araujo *et al.* 2011). Although there is concurrence across general metabarcoding  
526 protocols, the specific details such as number of replicates and use of controls are  
527 not standardised, which can limit utility and cross-study comparison (Deagle *et al.*  
528 2019, Ando *et al.* 2020).

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

544

## 545 **Conclusions**

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



553

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560

561 Data availability statement

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

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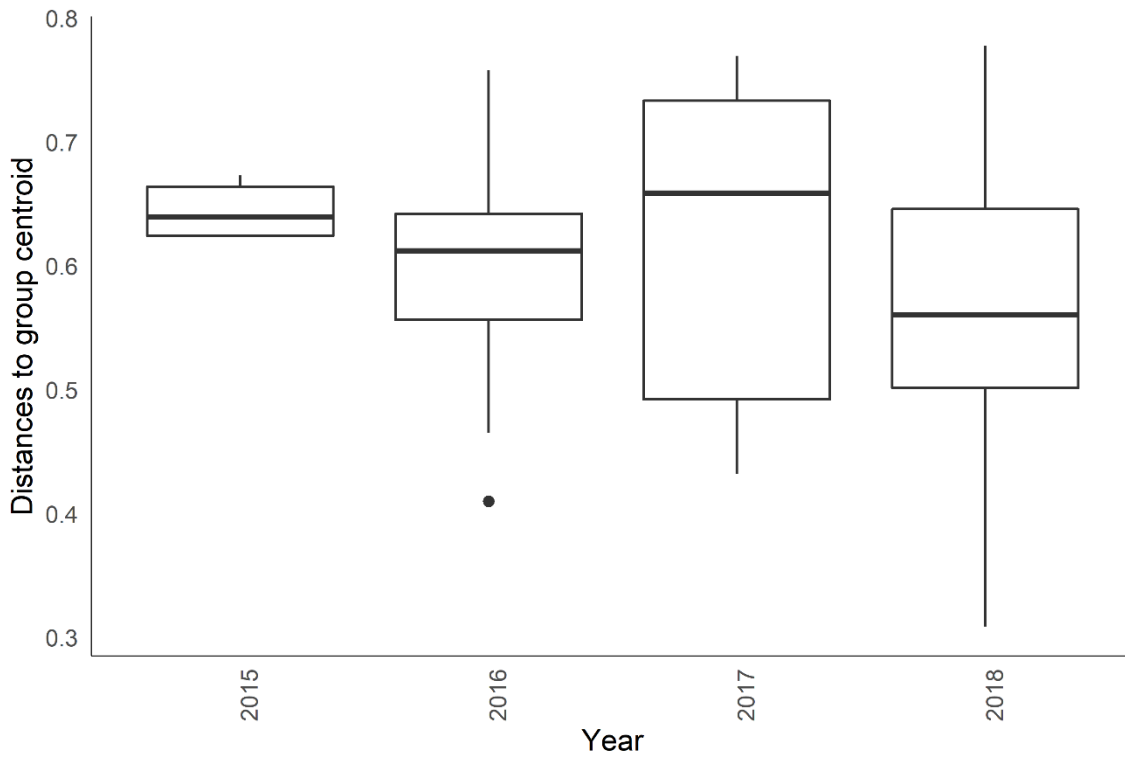
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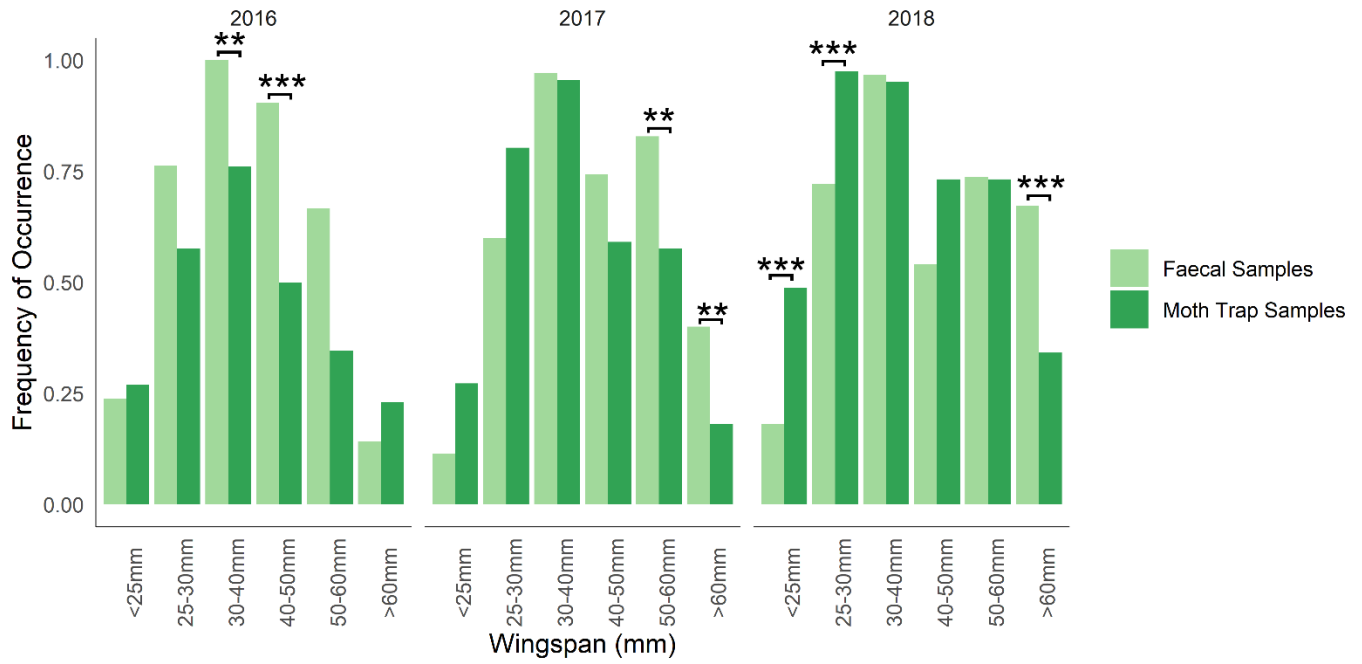
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886 **Figure 1.** Mean and variance of faecal sample distances to group centroids for each year samples  
887 were collected, produced by the *'betadisper'* function in r. Sample sizes vary between groups and are  
888 presented in the text.

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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.

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

| <b>Pairs</b> | <b>df</b> | <b>Sums of Sq</b> | <b><i>F</i></b> | <b><i>R</i><sup>2</sup></b> | <b><i>P</i></b> | <b><i>P</i> adjusted</b> |
|--------------|-----------|-------------------|-----------------|-----------------------------|-----------------|--------------------------|
| 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           | <b>0.036</b>             |
| 2016 vs 2017 | 1         | 1.656             | 4.064           | 0.088                       | 0.001           | <b>0.006</b>             |
| 2016 vs 2018 | 1         | 2.495             | 6.775           | 0.139                       | 0.001           | <b>0.006</b>             |
| 2017 vs 2018 | 1         | 1.061             | 2.758           | 0.050                       | 0.006           | <b>0.036</b>             |

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