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1 **Title: Gradients in richness and turnover of a forest passerine's diet prior to**

2 **breeding: a mixed model approach applied to faecal metabarcoding data**

3

4 **Running title: Pre-breeding dietary gradients of a passerine**

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6 **Authors:** Jack D Shutt[1‡], James A Nicholls[1,2‡], Urmi H Trivedi[3], Malcolm D Burgess[4,5],

7 Graham N Stone[1], Jarrod D Hadfield[1#], Albert B Phillimore[1#*]

8

9 **Affiliations:**

10 1 Institute of Evolutionary Biology, The University of Edinburgh, The King's Buildings,

11 Edinburgh, EH9 3FL, UK;

12 2 Current address: Australian National Insect Collection, CSIRO, Clunies Ross Street, Acton,

13 ACT 2601, Australia;

14 3 Edinburgh Genomics, The University of Edinburgh, The King's Buildings, Edinburgh, EH9

15 3FL, UK;

16 4 Centre for Research in Animal Behaviour, University of Exeter, Exeter, EX4 4QG, UK;

17 5 RSPB Centre for Conservation Science, The Lodge, Sandy, Bedfordshire, SG19 2DL, UK

18

19 ‡ and # Contributed equally.

20 Correspondence: Albert Phillimore albert.phillimore@ed.ac.uk

21 Orcid ID's: JDS 0000-0002-4146-8748, MDB 0000-0003-1288-1231, ABP 0000-0002-6553-

22 1553

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Abstract

Little is known about the dietary richness and variation of generalist insectivorous species, including birds, due primarily to difficulties in prey identification. Using faecal metabarcoding we provide the most comprehensive analysis of a passerine's diet to date, identifying the relative magnitudes of biogeographic, habitat and temporal trends in the richness and turnover in diet of *Cyanistes caeruleus* (blue tit) along a 39-site, 2° latitudinal transect in Scotland. Faecal samples were collected in 2014-15 from adult birds roosting in nestboxes prior to nest building. DNA was extracted from 793 samples and we amplified COI and 16S minibarcodes. We identified 432 molecular operational taxonomic units (MOTUs) that correspond to putative dietary items. Most dietary items were rare, with Lepidoptera being the most abundant and taxon-rich prey order. We present a statistical approach for estimation of gradients and inter-sample variation in taxonomic richness and turnover using a generalised linear mixed model. We discuss the merits of this approach over existing tools and present methods for model-based estimation of repeatability, taxon richness and Jaccard indices. We find that dietary richness increases significantly as spring advances, but changes little with elevation, latitude or local tree composition. In comparison, dietary composition exhibits significant turnover along temporal and spatial gradients and among sites. Our study shows the promise of faecal metabarcoding for inferring the macroecology of food webs, but we also highlight the challenge posed by contamination and make recommendations of laboratory and statistical practices to minimise its impact on inference.

51

52

53 **Introduction**

54

55 Insectivorous passerine birds in temperate environments tend to be dietary generalists

56 feeding on a broad range of invertebrate taxa (Betts, 1955; Cholewa & Wesołowski,

57 2011). There is potential for the diet of such generalists to vary over geographic

58 gradients, among habitats and seasonally within a year. Such dietary variability within

59 generalist species is poorly understood and could have profound ecological

60 consequences. Spatial variation in resource availability has implications for

61 geographic patterns in population density, breeding productivity and the degree to

62 which local adaptation in resource use may evolve. Seasonal variation in resource

63 consumption has implications for the optimal scheduling of life history events, such

64 as reproduction (Charmantier et al., 2008; Durant et al., 2005) and seasonal movements

65 (Thorup et al., 2017).

66

67 Spatiotemporal trends in diet will arise from a combination of underlying trends in

68 invertebrate resource availability and the prey preferences of the consumer. Species

69 richness – or α-diversity – of temperate invertebrate taxa generally decreases with

70 increasing latitude (Baselga, 2008) and peaks at mid-elevations (Beck et al., 2017) in

71 the summer months (Thomsen et al., 2016). Within forests, invertebrate richness can

72   vary among tree taxa by more than an order of magnitude, and in the UK has been

73   found to be highest on *Salix*, *Quercus* and *Betula* (Kennedy & Southwood, 1984; Shutt,

74   Burgess, & Phillimore, 2019). In addition to changes in species richness, species

75   composition may change from one community to the next, which is quantified as β-

76   diversity (Baselga, 2010; Whittaker, 1972). While there is evidence that forest

77   invertebrate communities show turnover over biogeographic gradients (Novotny &

78   Weiblen, 2005) and among host tree taxa (Murakami, Ichie, & Hirao, 2008), the

79   relative magnitude of turnover along different gradients has received scant attention

80   (Novotny & Weiblen, 2005). Whether diet mirrors these gradients in resource

81   availability will largely depend on how much prey selection by the consumer departs

82   from random.

83

84   Forest-dwelling hole-nesting insectivorous birds, such as blue tits (*Cyanistes*

85   *caeruleus*), have been subject to decades of intensive study (C. M. Perrins, 1979).

86   While the diet of nestlings has proven relatively straightforward to quantify, either via

87   videos/cameras at the nest (Samplonius, Kappers, Brands, & Both, 2016), or neck

88   collars on nestlings (Burger et al., 2012), much less is known about the diet of adults

89   (but see Cholewa & Wesołowski, 2011; J. A. Gibb, 1954). The paucity of information

90   about adult diet arises because these birds often forage high in trees on small prey

91   items. To date most of our taxonomic information on adult tit diet has been derived

92   from dissections of the gizzard and gut contents of euthanised birds (Betts, 1955), a

93   method that precludes the identification of soft-bodied dietary items, has relatively

94   poor taxonomic resolution (e.g. order or family level) and is destructive. These studies

95   reveal that tits consume various insects (including Lepidoptera, Hemiptera, Diptera,

96   Coleoptera, Hymenoptera) and spiders, as well as some plant matter in winter (Betts,

97   1955; Cholewa & Wesołowski, 2011; Cramp & Perrins, 1993).

98

99   The advent of next-generation sequencing and faecal DNA metabarcoding now

100   provides a non-destructive means of obtaining diet information at a fine taxonomic

101   resolution (Pompanon et al., 2012; Symondson, 2002; Taberlet, Coissac, Pompanon,

102   Brochmann, & Willerslev, 2012). Where invertebrates comprise a large proportion of

103   the diet, DNA barcodes from the rapidly evolving cytochrome oxidase I (COI)

104   mitochondrial gene have become the standard and allow identification to species-level

105   in many cases (Kress, García-Robledo, Uriarte, & Erickson, 2015). To date, most

106   published faecal metabarcoding studies have examined variation in mammalian diet

107   (Bohmann et al., 2011; Clare, Symondson, Broders, et al., 2014; Clare, Symondson,

108   & Fenton, 2014; Razgour et al., 2011). In comparison to mammals in general, and bats

109   in particular, application of faecal metabarcoding for inference of the diet of avian

110   insectivores is a small but rapidly growing field. Progress has been hampered by the

111   challenge of extracting and successfully amplifying dietary DNA from avian faeces

112   (Jedlicka, Sharma, & Almeida, 2013; Vo & Jedlicka, 2014). As such, avian faecal

113   metabarcoding studies have sampled small numbers of individuals and/or locations

114   (Table 1) and the latter limitation has precluded detailed analysis of the drivers of

115   spatial or temporal variation in the diet of avian insectivores (for an exception see

116   Sullins et al., 2018).

117

118   To date the statistical tools employed by the nascent metabarcoding field have largely

119   borrowed from community ecology. In some studies the objective has been to describe

120   the diet composition of a taxon such that statistical analysis may be unnecessary (De

121   Barba et al., 2014). Metabarcoding studies that focus on patterns in taxon richness

122   commonly apply a two-step analysis, first using rarefaction to quantify diversity at a

123   focal sampling level and then using a statistical model to examine variation in taxon

124   richness among samples (Quéméré et al., 2013). Studies interested in how taxonomic

125   composition varies among samples have tended to rely on pairwise metrics, such as

126   the Jaccard index, and non-parametric methods, such as PERMANOVA and the

127   Mantel test (Alberdi, Aizpurua, Gilbert, & Bohmann, 2018; Mata et al., 2019;

128   Trevelline, Nuttle, Hoenig, et al., 2018). Generalised linear mixed models (GLMMs)

129   and their extensions provide a method for including structure in the data collection and

130   multiple predictors into an analysis (Warton et al., 2015), but few studies have utilised

131   them in diet metabarcoding to date (for exceptions see Mata et al., 2019; Nichols,

132   Åkesson, & Kjellander, 2016).

133

134   Here we employ faecal metabarcoding using COI minibarcodes to infer the diet of an

135   insectivorous woodland passerine, the blue tit, in early spring along a 220 km transect

136   in Scotland (Appendix 1 Fig. S1). We have three main aims: (i) to quantify dietary

137   taxon richness and composition at the molecular operational taxonomic unit (MOTU)

138   level; (ii) to quantify the magnitude of changes in both measures along gradients of

139   time (day of year), latitude, elevation and tree taxon composition; and (iii) to quantify

140   gradients in the contributions that six key invertebrate orders (Araneae, Coleoptera,

141   Diptera, Hemiptera, Hymenoptera, Lepidoptera) make to diet. We show that by

142   applying a GLMM to presence/absence data it is possible to estimate changes in taxon

143   richness and turnover among points and along gradients. We also demonstrate how

144   this mixed model approach can be used to estimate repeatability and control for some

145   types of systematic contamination.

146

147

148   **Material and Methods**

149

150   **Field data collection**

151   Fieldwork was conducted during the springs of 2014 and 2015 at 39 predominantly

152   deciduous woodland sites that together comprise a 220km latitudinal transect in

153   Scotland (Shutt, Bolton, Benedicto Cabello, Burgess, & Phillimore, 2018). At each

154   site there were six Schwegler 1B 26mm-hole nestboxes distributed at approximately

155   40m intervals. From mid-March the base of each nestbox was lined with greaseproof

156   paper – with the aim of slowing DNA degradation (Oehm, Juen, Nagiller, Neuhauser,

157   & Traugott, 2011) – which was replaced when damaged or heavily soiled, and

158   removed at the onset of nest building or once a bird had attempted removal. Each

159   nestbox was inspected on alternate days and faeces on the greaseproof paper were

160   removed with sterilised tweezers (after use they were wiped with lab tissue and

161   flamed), with up to a maximum of three faeces collected in a 2mL Eppendorf tube

162   containing pure ethanol. The total number of faeces in a nestbox was recorded

163   (excluding 129 samples from early 2014). Samples were stored at -18°C within a day

164   of collection and transferred to a -20°C freezer at the end of each spring. Faecal

165   samples were collected from 35 of the 39 sites from 19 March in 2014 and 18 March

166   in 2015 until nest building, giving a median sampling range of 20 days per site in 2014

167   and 24 days in 2015 (Appendix 1 Table S1).

168

169 Latitude (site range 55.98 – 57.88°N) and elevation (10 – 433m) were obtained for

170 each nestbox (Shutt et al., 2018). Site-level habitat metrics were derived from surveys

171 of numbers of trees of different genera belonging to three size classes (based on girth

172 at breast height) within 15m radius of each nestbox, as described in Shutt et al. (2018).

173 The site-level habitat variables we considered were total foliage, tree diversity

174 (Simpson's index), the amount of oak foliage and the amount of birch foliage (Shutt

175 et al., 2018).

176

177 **Molecular protocol**

178 We balanced sampling across nestboxes and dates as far as possible by imposing an

179 upper limit of 10 samples per nestbox per year and where this maximum was exceeded

180 we subsampled such that we maximised the range of dates per nestbox. If multiple

181 faeces (n = 2 – 3) were present within a sample tube, part of each individual scat was

182 used for the DNA extraction with the aim of sampling a broader range of diet. This

183 protocol resulted in processing of 793 of a total of 959 faecal samples.

184

185 Thirty samples were processed in duplicate to allow us to estimate technical

186 repeatability. The selected samples were evenly distributed throughout the sampling

187 period, including samples from multiple sampling locations in both 2014 and 2015.

188 The faeces for each of the 30 duplicated samples were evenly divided into two and

189 DNA extractions were performed on each subsample; although each subsample

190 contained sections from along the entire length of the original faeces, the faeces was

191 not completely homogenised before subsampling. Each duplicate extraction was

192 subsequently treated as an independent sample for all downstream processes. All

193 aspects of the laboratory protocol (DNA extraction, PCR amplifications, PCR clean-

194 up, sequencing on a MiSeq run) were performed at different times using different

195 aliquots of reagents for each replicate within a pair of subsamples. In addition we

196 included 24 controls (including extraction negatives, PCR negatives and *Dryocosmus*

197 *israeli* as a non-native invertebrate PCR positive).

198

199 DNA was extracted from faecal samples using the QIAamp DNA Stool Mini kit,

200 following the protocol for pathogen detection with a few custom modifications

201 designed to improve DNA yields (see online protocol for details;

202 dx.doi.org/10.17504/protocols.io.ve6e3he). Three loci were targeted for amplification

203 through PCR - the standard animal barcoding gene (COI), a secondary barcoding gene

204 to detect invertebrate prey DNA and confirm the faecal sample originated from a blue

205 tit and no other hole-roosting or -nesting passerine (16S rRNA), and a standard plant

206 barcoding gene (rbcL) (see online protocol for further details;

207 dx.doi.org/10.17504/protocols.io.2jdgci6). Given that DNA from dietary items is

208 expected to be very degraded, the primers used amplified a small 'minibarcode' region

209 of each gene (184-220 base pairs). Invertebrate primer sets were validated to ensure

210 that they would amplify DNA from the expected range of invertebrate taxa (two orders

211 of arachnids, isopods, nine insect orders).

212

213 We followed a two-stage PCR process, firstly to amplify the target regions, then

214 secondly to add indexed Illumina adaptors to the amplicons from each sample.

215 Amplicons were multiplexed into three pools, each containing between 273 and 276

216   samples (inclusive of 30 replicate samples) and 8 controls (3x PCR positives, 3x PCR

217   negatives and 2x extraction negatives; a total of 24 controls across the whole

218   experiment). Each pool was sequenced on an Illumina MiSeq, using 150 bp paired-

219   end reads.

220

221   **Bioinformatics processing**

222   Sequencing reads were initially de-multiplexed into sets corresponding to individual

223   faecal samples using the index combinations present within the adaptor sequences

224   using bcl2fastq (version v2.17.1.14). Reads were then de-multiplexed using fastq-

225   multx from ea-utils (version1.1.2-537) with parameter '-m 2' into sets corresponding

226   to each locus using the locus-specific primer sequences present at the beginning of

227   each read. Adaptor sequences, primer sequences and poor quality base calls were then

228   removed using cutadapt (version 1.8.3) with parameters: ' -m 50' , '-q 30', '-f fastq',

229   leaving only sequence corresponding to the targeted gene regions. Subsequent

230   processing of the sequences applied the UPARSE pipeline (initially developed for 16S

231   metabarcoding of bacteria, (Edgar, 2013)) to data for each locus separately.

232

233   The first step in the bioinformatics pipeline was to merge the paired reads derived from

234   either end of the sequenced fragment. This process was successful for all COI and rbcL

235   reads and many 16S reads; 16S reads derived from avian DNA did not overlap, but

236   comparison with known blue tit 16S sequences indicated that these reads could be

237   combined by adding four "N"s between the forward and reverse reads to produce a

238   composite sequence of the correct length (hereafter referred to as fused reads). Reads

239   were then filtered to ensure that within a locus they were all of the same length; this

240 process removed possible pseudogenes incorporating insertions/deletions from the

241 coding COI and rbcL data. The rbcL data were not used for subsequent analyses in this

242 study, and 16S data were only used to confirm the faeces were derived from blue tits.

243 The set of filtered COI sequences was then used for two purposes. Firstly, the set of

244 unique sequences present within the full data set derived from all samples was

245 determined, with counts made of their frequencies. Unique sequences represented by

246 only a single read were removed as they most likely represent sequencing errors. The

247 unique sequences were then clustered into molecular operational taxonomic units

248 (MOTUs), grouping sequences together that had an identity of 98% or more. The most

249 frequently occurring sequence within each MOTU was designated as the reference

250 sequence for that MOTU. The second use of the filtered reads involved mapping them

251 back to this reference set of MOTU sequences on a sample by sample basis, allowing

252 a mismatch of up to 2% between filtered reads and a reference sequence, to provide a

253 more accurate assessment of the frequency of each MOTU within each faecal sample.

254 The taxonomic identity of MOTUs was determined using a BLAST search of the

255 reference set of MOTU sequences against public databases (GenBank and BOLD).

256

257 **Quality control and MOTU refinement**

258 Our analysis plan from this point on was pre-registered (osf.io/xgvm8). Some aspects

259 of our methods deviate from what was outlined in the pre-registration (see table S2 in

260 appendix 1 for an explanation of the motivation for these departures). We tested

261 whether samples were from blue tits by verifying the presence of blue tit fused 16S

262 sequences. The highest number of blue tit 16S reads from the 24 control samples was

263 58 and as a precaution all faecal samples that yielded fewer than 100 blue tit 16S

264   reads were excluded from further analyses as they were not conclusively confirmed

265   to be blue tit faeces (n = 9). Of the remaining avian faecal samples, blue tit was the

266   commonest of the fused 16S MOTU in all but one sample, but this sample still had

267   sufficient (n = 1465) blue tit reads to confirm its identity. No other avian DNA was

268   present in any sample.

269

270   COI reads were checked from control samples to confirm the presence of positive

271   control species and provide a baseline for background contamination. All nine PCR

272   positive control samples contained MOTUs attributable to *Dryocosmus israeli* (range

273   of reads = 7796 - 19115) and no more than 16 reads of any other MOTU identified as

274   belonging to the Metazoan kingdom. Eight out of nine PCR negative controls

275   contained no more than 19 reads of any MOTU. The ninth was highly contaminated

276   and contained 6798 reads arising from more than 20 MOTUs. Therefore, we checked

277   for contamination along rows or columns within plates by estimating Spearman's

278   correlations in the number of MOTU reads between samples in neighbouring cells in

279   the same PCR column or row. The row containing the contaminated negative sample

280   was found to have a substantially higher mean level of within row correlation (r = 0.37)

281   than other row and column correlations (mean r = 0.04). This was considered to be

282   most likely a systematic contamination event and this row (n = 11 focal samples + 1

283   negative control) was excluded from all analyses. In addition, closer inspection of the

284   contaminated plate revealed two wells (both faecal samples) in the neighbouring row

285   to the contamination event containing very similar MOTUs with the contaminated row

286   and these were also removed from further analysis. Of the six extraction negative

287   controls, four contained no MOTU at a higher read frequency than 3. The remaining

288   two contained contamination (maximum reads = 10037 and 1611) but on further

289   inspection there was no evidence for this being systematic. As there were few cases

290   where a control (positive or negative) had > 20 reads for any non-target MOTU, we

291   adopted 20 reads as the cut-off for identifying MOTU presence.

292

293   The above steps reduced the number of samples from 847 to 824 (772 focal) containing

294   2524 MOTUs. All MOTUs with fewer than 20 reads in any single sample were

295   removed as possible false positives (remaining n = 1432 MOTUs). All MOTUs

296   without any BLAST match, or identified as environmental contamination, were

297   removed (remaining n = 1323). Then, a full taxonomy was obtained for each remaining

298   MOTU and taxonomic reduction of the dataset began to eliminate non-prey items.

299   Firstly, only MOTUs belonging to the Metazoan kingdom were considered possible

300   prey items (remaining n = 1078). Then, all MOTUs not belonging to the phyla

301   Annelida, Arthropoda and Mollusca were discarded (remaining n = 1005). Finally, all

302   mites in the dataset of orders Astigmata (11), Mesostigmata (56), Oribatida (1),

303   Siphonoptera (2) and Trombidiformes (24) were removed, as they were likely to be

304   ectoparasites rather than actively foraged prey (remaining n = 911). For the MOTU

305   identification we required that the percentage match was at least 90% (remaining n =

306   785). Taxa identified to an identification match of 90% or more are considered correct

307   to a minimum of order level, and this is the level that is important to the analyses in

308   this study. Several MOTUs identified as ‘*Arachnida* sp’ were removed on finding that

309   these MOTUs were most closely matched to fungi (remaining n = 778). All

310   *Dryocosmus* (positive control) and waxworm (*Galleria mellonella* – from a feeding

311   experiment in 2014 that provided 10 waxworms in a plastic cup adjacent to two

312   nestboxes per site) MOTUs were removed (remaining n = 757). Then, all remaining

313   MOTUs belonging to the same best-hit taxon were merged (remaining n = 432).

314   Finally, due to the importance of Lepidoptera to tit diet we assessed the biological

315   plausibility of *Lepidoptera* identifications, which was possible due to comprehensive

316   UK occurrence data for this order (Sterling & Parsons, 2012; Waring & Townsend,

317   2017). Nineteen of 131 Lepidopteran MOTUs assigned species names were reassigned

318   to a British species when this species was within a 1% match of a geographically

319   implausible top hit. We assigned species status to taxa with a 99% or greater identity

320   match with the BLAST hit and a histogram of identity matches is provided (Fig S2).

321

322   **Statistical analyses**

323   Analyses focussed on the presence/absence of MOTUs in a sample, as read numbers

324   are not considered a reliable measure of the amount of a MOTU in a sample due to

325   biases in primer binding and amplification (Clare, 2014; Yu et al., 2012). Control

326   samples were excluded from analyses. DNA within a sample was often derived from

327   multiple faeces, and the effect of this on MOTU presence was controlled for by

328   including number of faeces as a four-level categorical fixed effect (1, 2, 3, unknown).

329

330   To examine geographic, habitat and temporal variation in blue tit diet (Shutt, Nicholls,

331   et al., 2019), we included the presence or absence of each MOTU in each sample as

332   the response variable in a Bayesian generalized linear mixed model (GLMM) with a

333   probit error structure (Hadfield, 2010). This analysis excluded the replicate samples

334   (for reasons discussed in Appendix 2). The effects of year and number of faeces in the

335   sample (treated as categorical) and the effects of ordinal date, latitude, elevation, total

foliage, birch foliage, oak foliage and tree diversity (treated as continuous) were treated as fixed. These fixed effects quantify trends in dietary richness. After accounting for these trends, variation in richness amongst sites, nest-boxes, days within year (categorical) and faecal samples were modelled by fitting each term as random. MOTU effects were fitted as random in order to capture differences amongst MOTUs in their overall prevalence. Variation in the prevalence of individual MOTUs amongst sites, nest-boxes, days within years (categorical) and faecal samples was modelled by interacting each term with MOTU. In the core model we also allowed the prevalence of individual MOTUs to vary with ordinal date, latitude and elevation effects, again by interacting each term with MOTU to form random regressions. The three slope terms were allowed to covary with each other and the main MOTU effect (the intercept). We also include plate by MOTU random interaction term to control for any plate-wide contamination by particular MOTUs present. To estimate and correct for any tendency for contamination of rows or columns within a plate we ran an additional model with row (within plate) and column (within plate) interacted with MOTU as random terms and this is the main model that we present in the results.

In addition to the core model, we also ran four additional models, each of which allowed the prevalence of individual MOTUs to vary across one of the four habitat variables. The additional random slope terms were allowed to covary with the original three slope terms and the intercept. However, because of the length of time that the core model took to run (three months) we excluded the day within year term and its interaction with MOTU. The importance of these effects are minor relative to other terms in the model (day in year variance = 0.003, day in year:MOTU variance = 0.036,

table S4A) and the interaction in particular contributed a lot to computation time because with 91 days and 432 MOTUS there are nearly 40,000 effects. All models were run for 260,000 iterations, with the first 60,000 removed as burn-in and thinning every 100. These models took two months to run on an iMac 10.13.6 with 3.4 Ghz Intel core i7, 16GB RAM and 4 cores.

To examine trends in the presence/absence of prey orders in blue tit diet, the dataset was reduced down to presence/absence of the six most common orders (Araneae, Coleoptera, Diptera, Hemiptera, Hymenoptera and Lepidoptera), termed 'focal orders', which together comprise over 91% of all prey taxa identified. A similar GLMM to that described above was then employed, but with focal order and date, latitude, elevation and tree diversity individually and interacted with focal order as fixed effects. Site, nest-box, day and faecal samples were fitted as random main effects and as random interactions with focal order. These models were run for 195,000 iterations, with the first 45,000 removed as burn-in and thinning every 75.

To assess the repeatability of the approach we used a similar analysis to that described above with the presence/absence of each MOTU as a response for the faecal samples for which extraction, PCR and metabarcoding had been replicated (29 samples x 432 MOTUs). Fixed effects were year and the number of faeces in the sample, both as factors, with random terms limited to MOTU, faecal sample ID, faecal sample ID by MOTU interaction, extraction sample ID and residual. This model was run for 13 million generations with the first 3 million removed as burn-in and thinning every 5000.

384

385   All numeric predictor variables in all analyses were scaled to have a mean of 0 and a

386   variance of 1 to provide direct comparability of results. We used parameter expanded

387   priors for the variances such that the marginal priors on all variances followed a scaled

388   (1000) $F_{1,1}$ distribution. Traces of posteriors were visually inspected to check for

389   convergence and adequate sampling. For the main model, the effective sample sizes

390   (ESS) were a bit low for some variances (< 500), but in all cases the ESS were adequate

391   to provide a reliable point estimate (>100) even if in some instances the accuracy of

392   the credible intervals is poor. As a test of model adequacy we conducted posterior

393   predictive simulations to assess whether key features of the data were captured (Fig.

394   S3). We opted to use an MCMCglmm approach rather than much faster numerical

395   integration approaches, such as lme4 (Bates, Maechler, & Bolker, 2012) or glmmTMB

396   (Brooks et al., 2017), because posterior predictions revealed that parameter estimates

397   from MCMCglmm provided an accurate description of the data, whereas those from

398   lme4 and glmmTMB were highly inconsistent (Appendix 2). Additional simulations

399   confirmed that parameter estimates from lme4 were highly biased, most likely because

400   with rare-outcome data the approximations used for integrating over the random

401   effects break down.

402

403   In order to get a quantitative understanding of how α and β diversity change across

404   different levels of biological organisation (e.g., nestbox or site) and as a function of

405   continuous biogeographic variables (e.g., elevation or tree diversity) we develop a

406   framework for *focussing* repeatability metrics at the appropriate biological level (see

407   Appendix 2). The two-way dichotomy into between-group and within-group that

408 forms the basis of standard repeatability calculations (see Nakagawa & Schielzeth,

409 2010 for a review) can be seen as a special case. The quantities required for these

410 calculations also appear in many indices developed by ecologists to quantify similarity

411 in community structure. Given this, we show how such indices can also be derived

412 directly from a GLMM which has the advantages that credible intervals can easily be

413 computed, incomplete sampling is naturally dealt with (Chao, Chazdon, Colwell, &

414 Shen, 2006) and changes in the indices as a function of differences in a continuous

415 variable (such as latitude) can be handled. The main disadvantage of the approach is

416 that between-species correlation structures may typically be richer than what a fitted

417 GLMM assumes, such that variation in community structure may be greater than the

418 model allows. However, posterior predictive checking allows model inadequacies to

419 be detected, and richer correlation structures are available, for example through a

420 phylogeny (Hadfield & Nakagawa, 2010) or through factor analysis (Niku, Hui,

421 Taskinen, & Warton, 2019; Warton et al., 2015).

422

423 In Appendix 2 we also present methods for using model outputs to generate

424 expectations for the taxon richness of a faecal sample and Jaccard index (often used in

425 studies of β-diversity) that quantifies the similarity of facecal samples. This allows us

426 to relate model coefficients back to effect sizes that are more often used in community

427 ecology. However, as the Jaccard index captures both turnover and community

428 nestedness (Baselga, 2010), in the results we mainly use repeatability to quantify

429 turnover.

430

431

Results

Read quality

The three MiSeq runs combined generated 34.04 million raw paired-end reads, of which 9.8 million were classified as COI amplicons after de-multiplexing based on the primer sequences. Amplicons for 16S and rbcL were also generated, but our diet analysis focuses only on COI. 8.9 million merged sequences passed all the quality filters. Out of these, 8.7 million sequences were retained after alignment against the reference OTU sequences.

Diet Composition

After identifying samples that tested positive for blue tit 16S DNA, excluding non-prey taxa and collapsing similar sequences, we identified 432 prey MOTUs across 772 faecal samples. Of these MOTUs, 57% could be matched to candidate species on the basis of > 99% sequence identity and a voucher/reference specimen identified to species level. A further 4% were >99% matched and therefore identifiable to species level, but lacking a reference initially identified to species level. The remainder of MOTUs are not identifiable to species level but are diagnostically distinct dietary items at minimum within the order identified by the best hit. (Appendix 1 Fig. S2, Table S3). The mean number of MOTUs per sample was 5.06, with mode = 3, median = 5 and range = 0 - 20. The MOTU abundance distribution was highly right-skewed, with 42.4% recorded in only one sample and 74.3% recorded in five or fewer samples (Fig. 1A).

456   Only 15 MOTUs were recorded in more than 50 samples (five Lepidoptera, four

457   Hemiptera, three Diptera and one each of Collembola, Coleoptera and Hymenoptera).

458   Eleven of these MOTUs were identified to species level, with *Argyresthia goedartella*

459   (Lepidoptera: Yponomeutidae) most common (34.6% of samples, Fig. 1A inset). Most

460   of these species are associated with resources available early in spring (Table S4), such

461   as catkins on birch (*Betula pendula/pubescens*) or alder (*Alnus glutinosa*) or buds of

462   birch or sycamore (*Acer pseudoplatanus*). We also found winter moth (*Operophtera*

463   *brumata*) in 27 samples (3.5%), the larvae of which comprise a major component of

464   nestling diet later in the spring but were not known to occur in the diet in early spring.

465

466   Eighteen invertebrate orders were encountered in at least one sample, with Insecta

467   contributing 86.1% of MOTUs. Within insects, MOTUs matched to the order

468   Lepidoptera were the most commonly recorded (present in 73.6% of samples, Fig. 1B)

469   and taxon-rich (131 taxa, Fig. 1C). Other commonly recorded orders were Hemiptera,

470   Diptera, Hymenoptera, Coleoptera, Araneae and Collembola.

471

472   **Technical Repeatability**

473   The value of faecal metabarcoding as a tool to infer diet depends on how reliable it

474   proves to be and a key measure of this is repeatability. Our protocol included 30 paired

475   replicate extractions from a different portion of the same faecal sample (although note

476   that the sample was not homogenised prior to extraction), 29 of which remained after

477   quality control and which we used to estimate technical repeatability (Appendix 1

478   Table S5G). The repeatability estimate is highly sensitive to the quantity being

479   measured (measurand), the definition of within and between group, the reference

480   population and whether it is considered on the latent or data scale (Appendix 2). The

481   technical repeatability of a MOTU within a faeces (with only faeces and faeces:MOTU

482   contributing to the between-group variance) had a posterior mode of 0.305 (95%

483   credible interval = 0.223 – 0.408) on the data (0,1) scale and 0.783 (0.712 – 0.845) on

484   the latent (threshold) scale. Variation in MOTU richness at the sample level was

485   reasonable (0.325 (0.118 – 0.770)) but the richness of samples within faeces are not

486   strongly correlated and so the technical repeatability of richness for a faeces is low

487   (Data; 0.003 (0 – 0.714), Latent; 0.003 (0 – 0.676)). However, the credible intervals

488   are large, and the main analysis (see below) shows non-zero correlations between the

489   richness of faeces from the same nestbox suggesting the true technical repeatability of

490   richness must be non-zero.

491

492   **Dietary MOTU richness**

493   We used a generalized linear mixed model (GLMM) with a binary (threshold) response

494   to examine the predictors of MOTU presence. From the main effects we can gain

495   insights into how dietary MOTU richness (related to α-diversity) varies across time

496   and space. Day of year predicted a small but significant increase in dietary richness

497   over the course of the spring (b = 0.082 (0.024 – 0.135), Fig. 2C), with the expected

498   number of MOTUs per faecal sample increasing from 1.981 to 3.933 from the first to

499   last date (Table 2). For elevation (b = -0.022 (-0.131 – 0.104)) and latitude (b = 0.058

500   (-0.015 – 0.144)) gradients in dietary richness were non-significant (Fig. 2A-B, Table

501   2), as were the metrics describing among-site variation in woodland habitat (total

502   foliage, foliage diversity, amount of oak, amount of birch, Table S5B). The

503   repeatability of species richness within nestboxes at a site was moderate (Data; 0.140

504 (0.041-0.264), Latent; 0.158 (0.046-0.296), Appendix 2) but we found little evidence

505 that richness varied among sites or among days within a year (after controlling for the

506 linear increase). The effect of including more than one faeces in the sample was

507 positive, but non-significant.

508

509 **Dietary MOTU turnover**

510 The probability of being present in a sample varied substantially across MOTUs

511 (variance on probit scale = 0.574 (0.475 – 0.696), Appendix 1 Table S5B). From the

512 interactions between MOTU identity and other terms we can gain insights into how

513 the probability of sampling individual MOTUs changes over time and space, providing

514 a measure of turnover and its significance. There was significant among MOTU

515 variation in the slope of presence/absence on day of year, elevation and latitude

516 (Appendix 1 Table S5B, Fig. 2D-F), with MOTU turnover more pronounced over

517 elevation and day of year. However, the predicted repeatabilities for MOTUs in faeces

518 sampled at the same elevation (but at different sites) were rather low (Data; 0.002

519 (0.001-0.003), Latent; 0.041 (0.028-0.059)). Due to the substantial between- faeces

520 and between nest-box variation in MOTU presence the repeatability for the site-level

521 probability of a MOTU at the same elevation was higher (Data; 0.066 (0.041 – 0.095),

522 Latent; 0.148 (0.106 – 0.215)), but still modest. The effect of date was similarly low

523 and even within nestboxes the repeatability of a MOTU in faeces from the same day

524 was small (Data; 0.002 (0.001-0.004), Latent; 0.081 (0.057-0.114)). See Appendix 2

525 for further analysis of repeatabilities. As an alternative measure of how environmental

526 variables affect community composition we calculated the expectation for the Jaccard

527 index and standardised Jaccard index (Appendix 2) between two sites at (i) the mean

528   and (ii) sampled extremes of latitude, elevation and day of year (Table 2). For all three

529   environmental variables communities are less similar (lower Jaccard index) at the

530   extremes than they are at the mean, but this effect is most pronounced for elevation

531   and day of year.

532

533   We considered among-MOTU variation in the relationship between the four

534   continuous habitat variables and probability of occurrence in four additional models

535   (Tables S5C–F). For three habitat metrics (total foliage, tree diversity and oak

536   availability) among-MOTU variation in habitat slopes was small and non-significant,

537   implying no discernible MOTU turnover along these gradients. The slope of MOTU

538   presence/absence on birch availability exhibited significant among-MOTU variation,

539   but turnover along this gradient is less than found for biogeographic and temporal

540   gradients (Appendix 1 Table S5F, Fig. S4) indicating a weak relationship.

541

542   The variance in the MOTU identity by site effects was large (0.474 (0.394 – 0.551)),

543   revealing that even after controlling for biogeographic trends in turnover gradients

544   there is substantial MOTU turnover among sites (Table S5B). Indeed, the

545   biogeographic and habitat variables in aggregate only explained a small fraction of the

546   between site variance (Data; 0.101 (0.069 – 0.142), Latent; 0.236 (0.174 –0.296),

547   Appendix 2). The total within-site (due to both biogeographic variation and random

548   site variation) repeatability was small if assessed at the level of faeces (Data; 0.023

549   (0.016 – 0.029), Latent; 0.275 (0.242 – 0.306)) but larger if assessed at the level of

550   nestboxes (Data; 0.270 (0.223 – 0.334), Latent; 0.568 (0.520 – 0.628)). This arises

551   because of the considerable variance amongst faeces within a nestbox. The variance

552 in MOTU identity by nestbox effects was comparable to the site effects (0.434 (0.376

553 –0.529)), but the within-nestbox repeatability at a single site was small (Data; 0.016

554 (0.012 – 0.022), Latent; 0.275 (0.247 – 0.312)), again because of the large between-

555 faeces variance. The within-nestbox repeatability across sites (where site and nestbox

556 effects contribute to the between group variance) was greater (Data; 0.069 (0.057 –

557 0.083), Latent; 0.474 (0.445 – 0.502)).

558

559 Interactions between MOTU and plate, plate-row and plate-column were also

560 significant (Appendix 1 Table S5B), which may reflect within plate contamination.

561 However, our placing of samples on the plate in the order in which samples were

562 collected in the field (spatially and temporally structured) could also contribute to this

563 signature if there is spatiotemporal structure in MOTU presence/absence that is not

564 accounted for by the day of year:MOTU and site:MOTU terms.

565

566 **Order level trends**

567 Lepidoptera showed a significant increase in probability of occurrence with increasing

568 latitude (b = 0.236 (0.044 – 0.430)) and elevation (b = 0.309 (0.073 – 0.583), Fig.

569 3AB, Appendix 1 Table S6). Other than Lepidoptera, only Diptera also showed a

570 significant increase with latitude (b = 0.252 (0.058 – 0.446)). Hymenoptera showed a

571 significant increase in probability of occurrence with increasing elevation (b = 0.319

572 (0.061 – 0.557)), with positive trends also apparent for Diptera, Hemiptera and

573 Coleoptera.

574

575   The probability of sampling a hemipteran increases very steeply through time over the

576   course of the spring (b = 0.422 (0.259 – 0.590)), with significant positive relationships

577   also apparent for Lepidoptera (b = 0.174 (0.006 – 0.341)) and Coleoptera (b = 0.269

578   (0.113 – 0.424)) (Fig. 3C). Increasing site level tree diversity had a significant positive

579   effect on the probability of sampling Diptera (b = 0.344 (0.095 – 0.586)) and a

580   significant negative effect on the probability of sampling Hymenoptera (b = -0.283 (-

581   0.528 – -0.037), Fig. 3D).

582

583

584   **Discussion**

585

586   We demonstrate that faecal metabarcoding can provide deep insights into the diet of a

587   generalist woodland bird, and provide the first in-depth analysis of the natural diet of

588   a passerine bird prior to breeding. We show that across Scottish woodlands in early

589   spring - when overall food availability is low - blue tits are able to locate and harvest

590   over 400 prey taxa. Further, we show strong temporal patterns in the taxonomic

591   richness and composition of the invertebrate prey items.

592

593   **Diet Composition**

594   Our findings on blue tit diet composition broadly agree with previous work on this

595   species (Betts, 1955; J. Gibb & Betts, 1963). As for previous faecal metabarcoding

596   studies on generalist insectivores (Clare, Fraser, Braid, Fenton, & Hebert, 2009;

597   Jedlicka, Vo, & Almeida, 2016; Sedlock, Krüger, & Clare, 2014), we found most

598   dietary taxa to be rare. The six most common orders were also detected using

599 morphology-based identification of gizzard contents by Betts (1955). For a fuller

600 discussion of the commonest taxa see the extended discussion in Appendix 1.

601

602 One surprise in our data was the prevalence of winter moth early in the spring. The

603 larvae of this species are one of the main foods provisioned to nestling tits (Betts, 1955;

604 C. Perrins, 1991) and whilst they are the most common spring Lepidopteran larvae on

605 our transect, their availability peaks in late May/early June (Shutt, Burgess, et al.,

606 2019), and so we did not anticipate finding them in the diet in March/April. A *post hoc*

607 analysis (GLMM with threshold response, site and nestbox effects as random and year

608 effects as fixed) revealed that the probability of occurrence in a sample increases

609 significantly in the days running up to the site-average first egg laying date (b = 0.039,

610 CI = 0.023 – 0.055), from around a 2% chance at 30 days prior to laying to 17% at the

611 average site-level blue tit first egg date. This increase in the incidence of winter moth

612 in the diet most likely corresponds with a change in the availability of early instar

613 larvae, rather than eggs, which would be available throughout the period (Waring &

614 Townsend, 2017). This finding raises the possibility that tits might use early instars of

615 winter moth and other foliar caterpillar larvae as a cue of when to breed.

616

617 **Dietary Richness and Turnover**

618 The biogeographic variables that we considered, latitude and elevation, had no

619 significant effect upon dietary MOTU richness, but a significant effect upon dietary

620 turnover. This reveals that whilst the total richness of prey eaten may not vary

621 geographically (see also the very low site variance), the taxa comprising the diet vary

622 along biogeographic clines (more so over elevation than latitude) and also from site to

site, as revealed by the significant site by MOTU interaction component. These findings are consistent with those from faecal metabarcoding of insectivorous bats (Clare, Symondson, Broders, et al., 2014; Sedlock et al., 2014) and could indicate local dietary specialisation. However, we suspect that a more likely explanation for this apparent specialisation is that it arises from patterns in prey availability (V. Moran & Southwood, 1982) and that the birds are flexible in their prey.

The increase in dietary MOTU richness as spring progresses parallels seasonal increases in the abundance and availability of herbivorous insects in European forests (Bale et al., 2002; Southwood, Wint, Kennedy, & Greenwood, 2004). Whilst dietary richness generally increases during spring, some taxa become less likely to occur and others more so, arising from the distinct phenologies of individual prey taxa (Forrest, 2016; Southwood et al., 2004). All of the main orders showed a tendency toward increasing as spring progressed, though on the data scale the increase was steepest for Hemiptera, which may be attributable to a pronounced spring phenology in the abundance of aphids on buds and leaves (Bell et al., 2015).

The habitat indices that we consider were non-significant predictors of blue tit dietary richness, and MOTU turnover along such gradients was much weaker than estimated for the biogeographic and temporal variables. One potential explanation for our low estimate of turnover along such habitat gradients is that most invertebrate prey species may not be entirely restricted to a particular tree species. Alternatively, perhaps our 'territory' based habitat metrics are inadequate measures of the availability of different tree species to each bird at this time. At face value our results are consistent with the

647   greater importance of larger-scale geographic clines (i.e. latitude, elevation) as

648   determinants of prey presence/absence, presumably because they act as a proxy for

649   other environmental variables that limit invertebrate distributions, such as temperature.

650   However, substantial spatial turnover remained even after controlling for

651   spatiotemporal gradients, which suggests that there are important drivers of prey

652   turnover that we have overlooked.

653

654   **Model based inference of richness and turnover**

655   Describing and explaining temporal and geographical variation in components of

656   diversity is a mainstay of community ecology (Dornelas et al., 2014; Li et al., 2018;

657   Magurran, 2013). α-diversity can be calculated for the sampled community scale (be

658   that a location or point in time), which has made its statistical analysis relatively

659   straightforward. In comparison, β-diversity is often calculated as a pairwise

660   similarity between communities (Koleff, Gaston, & Lennon, 2003), and where

661   multiple communities are considered the non-independence of comparisons presents

662   a challenge to statistical inference (Baselga, 2010). In an important development

663   Baeten et al. (2014) explained how a generalized linear model with taxon

664   presence/absence as a binomial response could be used to estimate changes in

665   richness and turnover between points and crucially determine statistical significance.

666   Here we have extended their framework to a generalized linear mixed model and we

667   show that the interaction of taxon (MOTU) with categorical (random intercepts) and

668   continuous (random slopes) variables estimates turnover between points (in space or

669   time) and along gradients, respectively. We also show that it is possible to predict the

670   Jaccard index (measure of β-diversity) between a pair of communities sampled at

671   points in space or time as a measure of effect size (Appendix 2). The principal

672   benefits of this new model-based approach over existing pair-wise approaches are

673   that (i) it allows estimation of confidence intervals and p values for turnover and

674   richness along gradients and among samples without such calculations being

675   complicated by non-independence; (ii) hierarchical structure in the sampling can be

676   included, and turnover can be assessed at each level explicitly taking into account

677   heterogeneity in sampling effort at lower levels; (iii) multiple covariates can be

678   included; (iv) inferences can be made including or excluding a control for taxon

679   abundance and (v) model based inference of repeatability is possible (see Appendix

680   2). The model coefficients can also be used to derive predictions of the total number

681   of taxa in a community and the Jaccard index (or alternative β-diversity metric)

682   between communities. Our model is defined in the context of the probability of a

683   taxon being present in a faecal sample, and as the number of samples (n) increases

684   total taxon richness is predicted to increase monotonically (with a decelerating

685   slope), such that when $n = \infty$, every taxon will be present. There are similarities

686   between this curve and rarefaction curves that are often used to standardise for

687   heterogeneity in sampling in ecology (Gotelli & Colwell, 2011), with both methods

688   requiring inference of the probability of each taxon being in a sample. In addition,

689   the Jaccard index will increase monotonically and with an accelerating function with

690   increasing species richness (Appendix 2) and monotonically and with a decelerating

691   function with sampling effort. Given that community level diversity metrics are

692   highly sensitive to the choice of n, we suggest that when using our framework an $n =$

693   1 represents the most natural level at which to report community-level metrics (see

694   Appendix 2) and requires no extrapolation.

695

696   A limitation of our approach is that by imposing a parametric correlation structure on

697   the data, that correlation structure is relatively simple and probably doesn't catch the

698   full complexity of species associations. For example, if there was a patchily

699   distributed species of herb on which three prey taxa were specialised on, then these

700   three species would co-occur with higher probability than our model would suggest.

701   Rectifying these problem would require a) identifying the herb that generates these

702   correlations, measuring its prevalence and incorporating that data into the model b)

703   use more complex correlation structures to be modelled in situations where the

704   number of taxa is large (Runcie & Mukherjee, 2013; Warton et al., 2015) or c)

705   develop sandwich type estimators (Huber, 1967; Zeger, Liang, & Albert, 1988) that

706   would allow robust inferences to be made even when unmodelled correlations exist.

707

708   **Methodological Considerations**

709   In this study we have demonstrated that faecal metabarcoding can provide a robust and

710   powerful method for assessing passerine diet, allowing greater sample sizes and

711   taxonomic resolution than direct assessment (Betts, 1955). Inclusion of positive and

712   negative controls and repeat samples are part of the standard laboratory practice

713   (Alberdi et al., 2018) – though few previous metabarcoding studies have included any

714   of these (but see De Barba et al., 2014; Jedlicka et al., 2016) – and have proven

715   invaluable in informing this work. Our protocol yielded fourteen MOTUs for the

716   positive control taxon, suggesting that the 2% divergence rule of thumb used in early

717   barcoding studies to group conspecific COI barcode sequences in Metazoa (Hebert,

718   Cywinska, & Ball, 2003 and http://www.barcodinglife.com) is likely to produce

719   spurious taxa, potentially misleading naïve analyses and underlining the necessity for

720   subsequent quality control steps. Negative controls (extraction and PCR) allowed us

721   to identify a case of systematic contamination and also informed our cut-off number

722   of reads (but see Deagle et al., 2018 for a critique of thresholds). After strict removal

723   of samples that appeared likely to have been affected by systematic contamination,

724   some residual contamination on plates was evident and we were able to control for this

725   to some degree by including row:MOTU, column:MOTU and plate:MOTU as random

726   terms. We recommend that future studies adopt the plate:MOTU random term and

727   randomise samples across plates, such that samples from a single year, site or time of

728   year do not all appear on one plate. Although the maximum number of taxa in a sample

729   was high (n = 20), PCR competition and the methodological maximum reads per

730   metabarcoding plate presumably place a limit on detecting very rare dietary items.

731   Reducing the number of target loci (three in this study, see methods) or level of

732   multiplexing (i.e. the number of samples per sequencing run) could increase the reads

733   available per locus per sample and increase detectability. However, reducing

734   multiplexing may come at an increased financial cost for sequencing.

735

736   From our repeat samples we were able to estimate technical repeatability and several

737   measures of biological repeatability (Appendix 2). Repeatability of MOTU

738   presence/absence was rather low, consistent with low repeatability estimates found by

739   another study that subsampled avian faecal samples (Jedlicka et al., 2016). An

740   implication is that if the focus of an avian faecal metabarcoding study is on the

741   detection of the presence/absence of a specific taxon, then multiple repeat DNA

742   extractions, amplifications and metabarcoding runs are advisable. Homogenisation of

743   faecal samples prior to DNA extraction may increase both the ability to detect a

744   particular taxon and repeatability given the possible heterogeneity within single faeces.

745

746   **Conclusion**

747   Using a metabarcoding approach, we reveal the diet of a generalist passerine at a finer

748   resolution than any previous study and quantify dietary richness and turnover across

749   space and time. At the scale of our study, blue tit dietary richness increases as spring

750   progresses, but is unaffected by latitude, elevation and habitat, whilst dietary turnover

751   is most pronounced over temporal (day of year) and elevational gradients.

752

753

762

763

764   **References**

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980   estimating equation approach. *Biometrics*, 1049-1060.
981

Data Accessibility Statement

982 **Data Accessibility Statement**

983 The MOTU presence/absence data for COI are available

984 from https://doi.org/10.5061/dryad.hhmgqnkd3. The data and model outputs used in

985 appendix 2 are available from

986 https://github.com/allyphillimore/faecalmetabarcoding-adults

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988

989 **Author contributions**

990 JDS, JAN, ABP and JDH were the main contributors to study conceptualization and

991 methodology, with JDS and ABP responsible for fieldwork, JAN responsible for

992 designing and conducting the molecular work and JDH designing the statistical

993 methods, developing the theory and writing Appendix 2. JDS and UHT contributed to

994 data curation. Statistical analysis was conducted by JDS, ABP and JDH. ABP was

995 responsible for project administration and ABP and JDH for funding acquisition. JDS

996 wrote the original draft, and all authors contributed to further writing and editing.

997

998

Tables and Figures

Table 1. Sampling and laboratory protocols employed by published faecal barcoding
studies focusing on the invertebrate component of diet. An entry of 'None' means that
while steps may have been taken in the study, no specific method was detailed.

Number of study species (most common species)	Total number of samples (maximum number of samples per species)	Number of sites (region)	Controls	Measures taken to assess repeatability	Reference
1 (Lesser Prairie-Chicken)	314	4 (Kansas and Colorado, USA)	None	None	(Sullins et al., 2018)
1 (Western Bluebird)	210	3 (neighbouring vineyards, California, USA)	None	Ten faeces subsampled.	(Jedlicka et al., 2016)
3 (Wood Thrush)	137 (51)	1 (Pennsylvania, USA)	PCR negatives and positives	None	(Trevelline, Nuttle, Hoenig, et al., 2018)
1 (Louisiana Waterthrush)	130	2 (Arkansas and Pennsylvania, USA)	None	None	(Trevelline, Latta, Marshall, Nuttle, & Porter, 2016)
1 (Louisiana Waterthrush)	92	3 (headwater streams, Pennsylvania, USA)	None	None	(Trevelline, Nuttle, Porter, et al., 2018)
(Rufous hummingbird)	30	1 (Vancouver Island, Canada)	1 x extraction negative	None	(A. J. Moran, Prosser, & Moran, 2019)
13 (Lewin's Honeyeater)	82 (29)	1 (Bundaberg, Australia)	Extraction negatives	PCR run twice to test amplification repeatability	(Crisol-Martínez, Moreno-Moyano, Wormington, Brown, & Stanley, 2016)

1 (Western Bluebird)*	16	2 (neighbouring vineyards, California, USA)	None	Faecal sample was subdivided and run on two extraction kits.	(Jedlicka et al., 2013)
4 (Blue tit, Great Tit, Willow Tit)	14 (4)	2 (Oulu and Kuusamo, Finland)	Extraction negative	None	(Rytkönen et al., 2019)
3 (Sedge Warbler)‡	11 (6)	3 (South Wales, UK)	None	None	(King, Symondson, & Thomas, 2015)

1005   ‡ Study employed Sanger sequencing rather than metabarcoding.

1006

1007 Table 2. Expectations for the MOTU richness of - and Jaccard indices between -

1008 samples of communities at (i) the same and (ii) extreme points along latitude,

1009 elevation and day of year gradients. Expectations are calculated for a random sample,

1010 nestbox, day and site averaging over variation in other predictor variables (for further

1011 details see Appendix 2). Expectations were generated for 2014 and a single faecal

1012 sample.

1013

Predictor	Sampling position	MOTU richness at mean	MOTU richness at minimum	MOTU richness at maximum	Jaccard index	Standardised Jaccard index[1]
Latitude	Mean	2.339 (1.665 - 3.271)			0.011 (0.008 - 0.015)	5.255 (4.238 - 6.296)
Latitude	Extremes		2.607 (1.476 - 3.831)	2.213 (1.266 - 3.444)	0.009 (0.006 - 0.012)	3.569 (2.776 - 4.423)
Elevation	Mean	2.340 (1.669 - 3.277)			0.013 (0.009 - 0.017)	5.966 (4.814 - 7.179)
Elevation	Extremes		2.435 (1.524 - 3.647)	2.138 (1.084 - 3.700)	0.008 (0.005 - 0.011)	2.344 (1.727 - 3.047)
Day of year	Mean	2.464 (1.653 - 3.252)			0.013 (0.008 - 0.017)	5.922 (4.811 - 7.103)
Day of year	Extremes		1.981 (1.352 - 2.848)	3.933 (2.459 - 5.603)	0.007 (0.005 - 0.010)	1.973 (1.431 - 2.592)

1014

1015 [1] The standardised Jaccard index is the ratio of the observed index to that expected if the same number

1016 of species were sampled at random from two communities (see Appendix 2). It will tend to be > 1 as

1017 common/widespread species will be over-represented in both communities. The expectation for the

1018 Jaccard index and standardised index for two samples taken entirely at random from the transect is

1019   0.01 (0.007 – 0.014) and 4.727 (3.891 – 5.732), respectively, and these values can be taken as a

1020   baseline that captures the effect of common/widespread species on measures of community similarity.
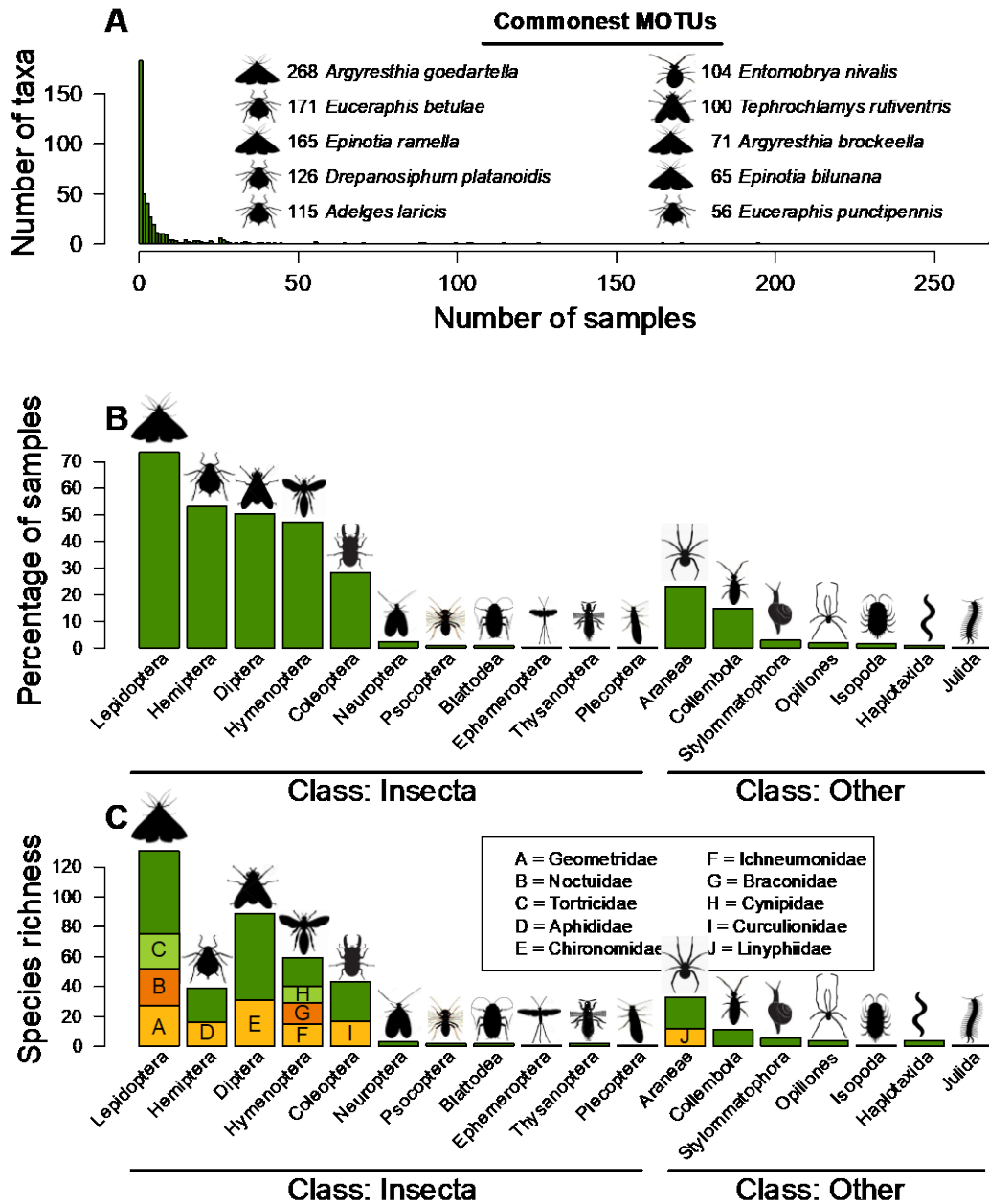
Fig. 1 A Histogram of the abundance distribution of prey MOTUs. Inset details the most prevalent MOTUs identified to species level (those recorded in more than 50 samples), with the number of samples they were recorded in. **B** Relative abundance of prey orders in the spring diet of blue tits. **C** Number of MOTUs within prey orders (families comprising > 10 MOTUs are highlighted individually within their respective orders). In **B** and **C** orders within Insecta (left) are split from orders within other classes (right). Images are used to indicate taxonomic order rather than the life-stage or species that is preyed upon.

1031
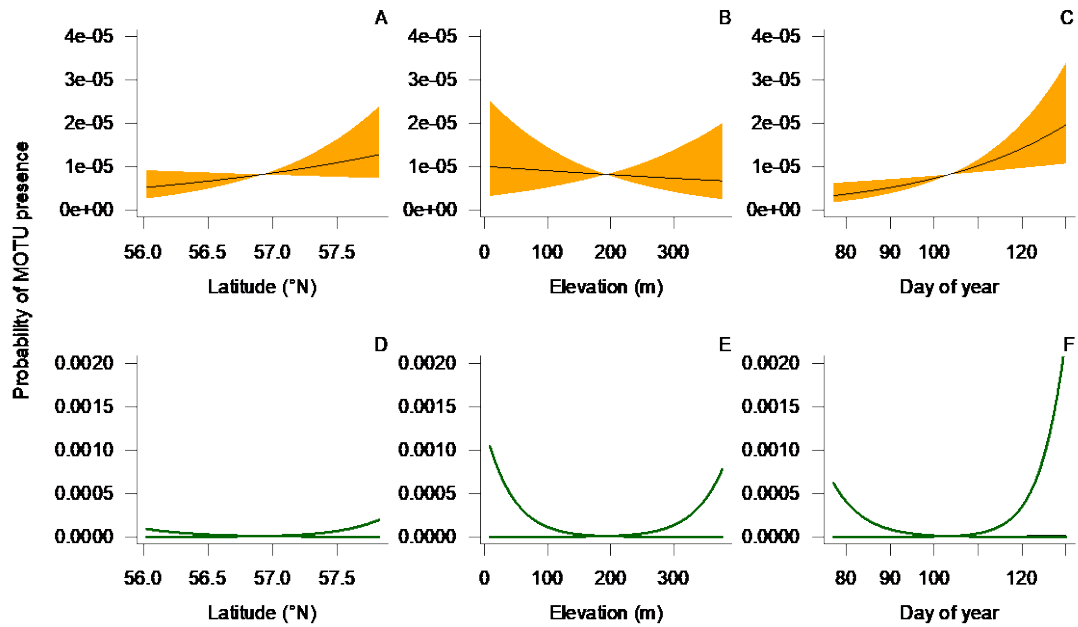
Fig. 2 Dietary richness (A – C) and turnover (D – F) along latitudinal (A, D), elevational (B, E) and temporal (C, F) gradients. In A - C the solid black lines indicates the model prediction of dietary MOTU occurrence (related to richness), with the solid orange area illustrating the 95% credible intervals in the slope. In D – F the green lines correspond to the 95% upper and lower bounds of the estimated distribution of among-MOTU slopes. The wider the difference between the upper and lower line the greater the turnover along the gradient. Predictions are made from the core model (Table S4B).
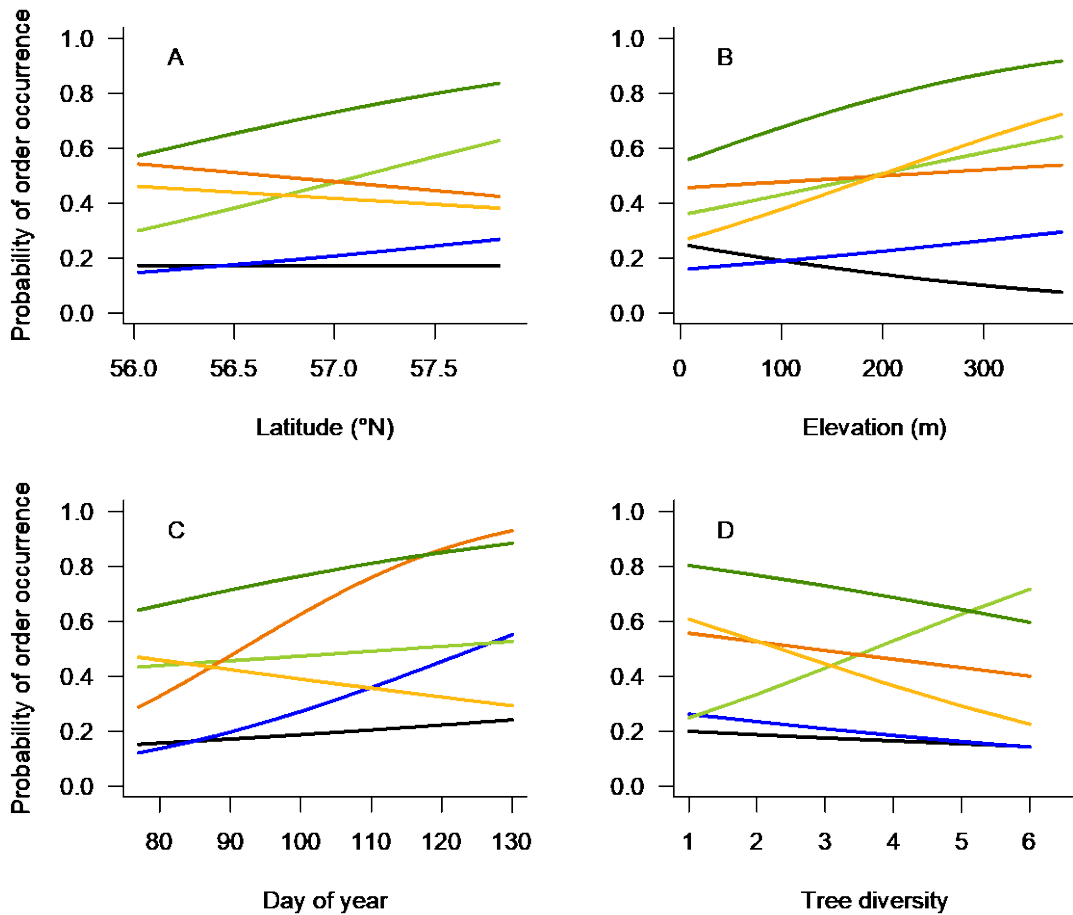
Fig. 3 Model predictions for the occurence of six prey orders across A. latitude, B. elevation, C. day of year and D. tree diversity. Predictions are made based on the intercept of the model reported in Table S5.