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1 # Individual variation in the avian gut microbiota: the influence of host state
2 and environmental heterogeneity

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24 out the DNA extraction and library prep; S.E.S. analysed the data; S.E.S., G.L.D. and J.L.Q

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Abstract

The gut microbiome has important consequences for fitness, yet the complex, interactive nature of ecological factors that influence the gut microbiome has scarcely been investigated in natural populations. We sampled the gut microbiota of wild great tits (Parus major) at different life stages and across multiple conifer and mixed woodland fragments, allowing us to evaluate multiple factors that relate to within-individual gut microbiota acquisition, including habitat type, nest position and life history traits. The gut microbiota varied with both environment and life-history in ways that were largely dependent on age. Notably, it was the nestling, as opposed to the adult gut microbiota that was most sensitive to ecological variation, pointing to a high degree of developmental plasticity. Individual nestling differences in gut microbiota were consistently different (repeatable) from one to two weeks of life, driven entirely by the effect of sharing the same nest. Our findings point to important early developmental windows in which the gut microbiota are most sensitive to environmental variation and suggest reproductive timing, and hence parental quality or food availability, interact with the microbiome.

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51
52 **Key words:** Gut microbiome; gut microbiota; repeatability; early development;
53 environmental variation; host state
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76

77 **Introduction**

78

79 The gut microbiome—the enteric microbial community and their genes—is strongly
80 influenced by the environment (Rothschild et al., 2018), and plays a major role in host
81 ecology and evolution (Dethlefsen et al., 2007; Koskella et al., 2017). Although much
82 progress has been made in understanding the interplay between gut microbiome communities
83 and host phenotypes, this research has been primarily restricted to lab systems (Colston &
84 Jackson, 2016; G. L. Davidson et al., 2020). In nature, animal microbiomes are far more
85 variable and diverse than their captive counterparts because they are exposed to a much wider
86 variety of conditions (Hird, 2017; McKenzie et al., 2017), and because wild hosts are more
87 genetically, and therefore biologically diverse than lab-reared animals. Exploring how gut
88 microbiomes are acquired, their stability across life stages, and their interactions with
89 extrinsic and intrinsic traits in nature are important for understanding what drives host
90 phenotypic consequences associated with gut microbiome variation.

91

92 The gut microbiota can directly affect the development of key physiological and biological
93 traits of the host (Cox et al., 2014; J. A. Foster et al., 2017; Hansen et al., 2012). Although
94 most of our understanding of these processes comes from germ-free lab models and gut
95 microbiota interventions, evidence is emerging that the developing gut microbiome has
96 important consequences for hosts in natural systems. We previously showed in a wild-bird
97 population that nestling gut microbiota diversity predicts nestling weight (G. L. Davidson et
98 al., 2021), itself a key predictor of offspring survival and recruitment. Disruption to microbial
99 gut colonisation at critical developmental periods can affect host immunity, metabolism and

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100 physical development in other systems such as bees, birds and frogs (Knutie et al., 2017;
101 Schwarz et al., 2016; Simon et al., 2016; Warne et al., 2019). Early life is a critical time for
102 gut colonisation and the developing gut microbiota in young animals is generally more
103 sensitive to environmental variation than in the more established adult gut microbiota
104 (Derrien et al., 2019; Grond et al., 2017). Although it is well known that the gut microbiota
105 changes with development, the role that ecology plays in this process remains poorly
106 understood.
107
108 Many fundamental drivers of ecological variation have the potential to affect the gut
109 microbiota. Individual variation in the gut microbiota may be influenced by exposure to
110 different environmental pools of microbes, for example, those in the air, soil or diet (Grond et
111 al., 2017; Kartzinel et al., 2019; Liddicoat et al., 2020; Ren et al., 2017). The link with diet, in
112 particular, is likely to explain why gut microbiota can vary with habitat type (Drobniak et al.,
113 2021; Goossens et al., 2022; Teyssier, Rouffaer, et al., 2018). Vegetation and diet can also
114 vary dramatically within habitats, for example with respect to edge effects (J. Chen et al.,
115 1992; Wilkin et al., 2007), although it is unknown whether individual microbial communities
116 are affected by these fine-scale processes. Between-seasonal dietary change has been linked
117 to temporal variation in the gut microbiota (Baniel et al., 2021; Hicks et al., 2018; Maurice et
118 al., 2015; Ren et al., 2017). Within-season variation in the gut microbiota has yet to be
119 documented but is likely important because in many animals, reproductive timing is critical
120 to ensure breeding coincides with seasonal peaks in food abundance (Brommer et al., 2002;
121 Grüebler & Naef-Daenzer, 2010; Rodríguez et al., 2016; Rubenstein & Wikelski, 2003).
122 Hence, the timing of reproduction should dictate nestlings' exposure to dietary microbes,
123 although this has never been tested in the wild.
124

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125 Similarly indirect effects mediated by the host, for example, homeostatic responses to
126 environmental stressors may have knock-on effects on the gut microbiota (Noguera et al.,
127 2018; Stothart et al., 2019). Exercise intensity (Mailing et al., 2019) and energetics (reviewed
128 in Lindsay et al. (2020)) are associated with the microbiota, while stress is also directly
129 linked to the microbiota in general (Sudo et al., 2004), and in the context of reproduction
130 specifically (MacLeod et al., 2022). Similarly, the gut microbiota in adults is mediated by a
131 variety of sex hormones (Mallott et al., 2020), and can lead to sex-specific associations
132 between the gut microbiota and environmental sources of variation (Org et al., 2016). For
133 nestlings, larger brood sizes lead to increased competition for food (Smith et al., 1989), and
134 higher glucocorticoid hormone concentrations (Greggor et al., 2017; Smith et al., 1988), both
135 of which have been reported to influence their gut microbiota in the wild (David et al., 2014;
136 G. Davidson et al., 2020; Noguera et al., 2018; Teyssier et al., 2020). At the same time, brood
137 size is an indicator of parental effort (Pettifor et al., 2001), and alters glucocorticoid
138 concentrations in adults (Bonier et al., 2011), which likewise could affect the adult
139 microbiota.
140
141 Thus, the composition of gut microbes are expected to vary across time, in response to
142 environmental variation, and with respect to a whole variety of individual state variables.
143 Nevertheless, at the same time, individuality and stability in microbiota can also be
144 important, as observed in studies of human health (L. Chen et al., 2021; Fassarella et al.,
145 2021). Consistent among-individual variation indicates limited individual plasticity, that is,
146 major constraints caused by intrinsic differences among individuals in some causal
147 factor—for example, genetic, epigenetic, or early environment effects (Rajilić-Stojanović et
148 al., 2013; Zoetendal et al., 2001). Typically stability in phenotypic variation among
149 individuals is quantified by taking multiple measures from the same individual over time, and

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150 estimating individual repeatability using individual as a random effect in a mixed model
151 framework (Nakagawa & Schielzeth, 2010). Mixed models also enable an examination of
152 whether individual differences are independent of other individual or environmental factors
153 that can also be included as random or fixed effects in the same model. Despite its
154 widespread use in animal personality and cognition literature (Bell et al., 2009; Cauchoix et
155 al., 2018), to our knowledge this approach has yet to be applied to identify the sources of
156 variation in the gut microbiota.
157
158 We explored how the gut microbiota varied with the environment, with development, and
159 with intrinsic factors using a generalist passerine bird species breeding in nest boxes across
160 heterogenous and fragmented woodlands. The great tit (*Parus major*) is a widely used model
161 organism in ecological and behavioural studies (Cole & Quinn, 2012; Drent et al., 2003;
162 O'Shea et al., 2018; Tinbergen & Boerlijst, 1990). Within a single breeding season, we
163 simultaneously investigated how the gut microbiota were related to host state (age, sex,
164 reproductive effort) and a range of environmental factors (habitat type, distance to forest
165 edge, number of siblings in the nest), and whether these explained consistent between-
166 individual differences in the gut microbiota. While we had clear *a priori* reasons to expect
167 environmental and life history traits to covary with gut microbiota variation, and to do so
168 differentially across life stages because the developing gut microbiota in young animals are
169 generally more sensitive to environmental variation than in the more established adult gut
170 microbiota (Derrien et al., 2019; Grond et al., 2017), we had no *a priori* predictions about the
171 direction of such effects in the context of gut microbiota community metrics (i.e. high vs low
172 diversity and relative taxonomic abundance), owing to the complexity of host-microbe
173 interactions (Douglas, 2018; K. R. Foster et al., 2017; Zaneveld et al., 2017). Finally, we
174 conducted a range of repeatability analyses at the individual, the nest, and the woodland

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175 levels to determine whether individual nestlings differed consistently over a short but critical
176 period of their development, from eight to 15 days old. We further examined whether any
177 consistency at the individual-level was driven by intrinsic differences among individuals, or
178 instead by shared nest or woodland effects, or indeed a range of other fixed effects as
179 discussed above. Our analyses suggest a diverse and complex relationship between individual
180 gut microbiota and the environment, the implications of which we suggest may well be
181 substantial for populations.
182
183 **Methods**
184
185 ***Field monitoring and microbiota sampling***
186 Birds were sampled from nine small woodland fragments across Co. Cork, Ireland, five of
187 which were mixed/deciduous and four coniferous woodlands (see O'Shea et al. (2018). We
188 collected 262 faecal samples from 204 great tits from 63 nests (see table S1 for final sample
189 sizes post bioinformatic processing) for 16S rRNA gene sequencing. Nest boxes were
190 monitored during April-June 2016 to determine lay dates, hatching dates and nestling
191 survival. Typically, all individuals in a nest were sampled. Individual nestlings were sampled
192 when they were 8 days old (+/- 1 day), and again, if they survived, when they were 15 days
193 old (D8 and D15 birds respectively), at which point parents were also sampled. Birds were
194 placed individually into sterile holding bags inside a heated holding case and naturally-
195 produced faecal samples were collected. Urea has the potential to affect downstream
196 sequencing and was removed through absorption by coffee filters placed as lining in the
197 sampling bags (Khan et al., 1991). The faecal matter was collected within 15-20 minutes of
198 placing birds in the sampling bag, after which birds were returned to the nest. Faecal sacks
199 were ruptured immediately using a sterile inoculation loop and placed in a microcentrifuge

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200 tube containing 500uL of 100% ethanol. Samples were stored at -20°C within 8 hours of
201 collection until DNA extraction. D8 nestlings were weighed, and individually identified by
202 clipping the tip of one of their nails, taking care to avoid the blood vessel. Nestling birds were
203 again weighed at D15 and ringed with a unique identifiable metal ring (British Trust for
204 Ornithology). Samples from nestlings for which we had multiple samples (i.e. for both D8
205 and Day-15) are referred to as 'repeat samples'. Adult birds were trapped on the nest and, if
206 not already ringed, were fitted with a British Trust for Ornithology ring, weighed, and aged
207 as either 'immature' (first year breeding) or 'mature' (second year/+ breeding) using plumage
208 indicators.
209
210 ***DNA Extractions***
211 DNA was extracted from the dried faecal contents of all birds using the Qiagen QIAamp
212 DNA Stool Kit, following the 'Isolation of DNA from Stool for Pathogen Detection' protocol
213 (June 2012 edition), with modifications described in Shutt et al. (2020) to accommodate dried
214 avian faeces. DNA was stored at -20°C. Full extraction methods are described in the
215 Supporting Information of Davidson et al. (2021).
216
217 ***Illumina MiSeq sequencing***
218 Full library preparation details are described in Supporting Information of Davidson et al.
219 (2021). Briefly, the V3-V4 variable region of the 16S rRNA gene was amplified from the
220 DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). The DNA
221 was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also
222 incorporates the Illumina overhang adaptor. Samples were sequenced on the MiSeq
223 sequencing platform (Clinical Microbiomics, Denmark), using a 2 x 300 cycle kit, following
224 standard Illumina sequencing protocols.

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225

226 ***Bioinformatics***

227 The DADA2 pipeline was used to process the raw sequencing data (Callahan et al., 2016) in
228 R version 3.5 (R Core Team, 2019). Sequence quality was visually inspected. Sequences
229 were trimmed to remove adapters and lower quality reads (median quality scores below 25-
230 30 threshold) at the extremities of the sequence and filtered to remove sequences with
231 expected errors above 1. Read errors were estimated before dereplication. Forward and
232 reverse reads were merged to construct 'contig' sequences, which were used to construct a
233 sequence table of Amplicon Sequence Variants (ASV's), which in turn counts the number of
234 times each unique sequence is detected. The previous steps were performed for each run
235 separately. Then the separate sequence tables were merged and chimeras removed using the
236 'consensus' method. Taxonomy was assigned to each ASV by RDP's Naive Bayes Classifier
237 (Wang et al., 2007) against the Silva reference database (version 132) (Quast et al., 2012).
238 This method groups sequences with 100% sequence identity in contrast to the lower
239 resolution OTU method which groups sequences at 97% identity. ASV's allow greater
240 sensitivity and specificity, better discrimination of ecological patterns than OTU's and are
241 reusable across studies (Callahan et al., 2017).

242

243 The DADA2 outputs were assembled into a single Phyloseq object (McMurdie & Holmes,
244 2013). Sequences identified as mitochondrial or chloroplast were removed. Sample
245 completeness curves were plotted using vegan (Oksanen et al., 2019) and helped determine
246 the lower cut-off for sample reads at 10,000 reads. Low read samples (<10,000 reads, 11
247 samples) were removed leaving 195 (adult=51, Day-8=81, Day-15=114) samples for the
248 analysis. Alpha diversity (both Shannon and Chao1 diversity) was calculated using the
249 '*estimate_richness*' function from the phyloseq package on the filtered dataset.

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250

251 After removing low read samples (<10,000 reads, n = 11), chloroplast sequences and
252 mitochondria sequences, there were 18,890,006 total reads clustered into 54,343 ASV's in
253 246 samples (see table S1 for sample breakdown). Reads ranged from 10,220 to 557,336,
254 with a mean of 76,789 reads per sample.

255

256 **Statistical analysis**

257

258 ***Datasets***

259 The analyses detailed below were conducted on one of two subsets of the data, depending on
260 the questions being explored. The first subset, referred to as the '*all birds*' subset, contained
261 birds of all developmental ages (8 day-old nestlings (D8); 15 day-old nestlings (D15);
262 adults), and was used to examine the relationship between the gut microbiome,
263 developmental age, and the interaction between developmental age and other life-history and
264 environmental variables. Because it is generally expected that young animals are especially
265 sensitive to environmental variation (Derrien et al., 2019; Grond et al., 2017), our main focus
266 was on the interactions between developmental age and the other main effects. However we
267 also explored the possibility that all main effects might have had interacting effects on
268 microbiota. Analyses using the *all birds* dataset did not distinguish between birds breeding in
269 their first year of life (immature) and those that were older (mature) because of convergence
270 issues. The second subset of data contained adults only, and focussed on sex and its
271 interaction with other life-history and environmental variables. All analyses were conducted
272 in R version 3.6.3 (R Core Team, 2020).

273

274 ***Alpha diversity***

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275 Linear mixed models (LMM) were used to test the effect of host and environmental factors
276 on alpha diversity. Shannon diversity (Shannon, 1948) measured richness weighted by
277 abundance (the evenness of a community), and Chao1 (Chao, 1984) measured richness,
278 specifically estimating taxa abundance and rare taxa missed from under sampling. Models
279 were fit using the *lme4* package (Bates et al., 2015) on each data subset. Significance was
280 determined using Satterwaite's degrees of freedom method (Satterthwaite, 1946)
281 implemented using the *lmerTest* package (Kuznetsova et al., 2017). The distribution of each
282 alpha diversity metric was assessed graphically and transformed towards normality as
283 appropriate.
284
285 The following variables were included as main effects: age; sex; habitat-type:
286 coniferous/mixed; distance to edge, i.e. distance from the nest to the nearest woodland edge;
287 maximum brood size of nest; first-egg lay date of nest. In the *all birds* global models, all
288 pairwise interactions between these main effects (except for lay date × brood size, which
289 were strongly colinear; sex also excluded because it was unavailable for nestlings) were
290 included. In the *adults only* global models, all pairwise interactions were included, except for
291 lay date × brood size and any habitat or age interactions, due to an imbalance in these factors
292 in the dataset, (see table S1). Backwards difference coding was used, instead of the default
293 dummy coding, when fitting the age variable which allowed us to compare each age group to
294 the previous age group sequentially, rather than to a single reference level. This contrast
295 scheme gives sum contrasts, so coefficients reflect main effects rather than marginal effects.
296 Woodland site, nest ID and individual ID were fit to model as nested random intercepts (in
297 the form woodland site/nest ID/individual ID), to control for non-independence. Bird ID was
298 dropped from the *all birds* Chao1 model and woodland site dropped from both adult diversity
299 models due to singular fit warnings.

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300

301 ***Phylum-level-Relative-Abundance-GLMMs***

302 The two most abundant phyla (Proteobacteria, mean ± SE: 41.6% ± 2.1%; Firmicutes, mean
303 ± SE: 36.6% ± 2.2%) were modelled separately to determine which variables correlated with
304 changes in the phyla relative abundance, in order to develop a broad sense of how the
305 microbial community changed with varying ecological factors. Binomial Generalised Linear
306 Mixed Models (GLMMs) were used. The binomial response variable was 'phyla abundance'
307 (sequence reads) weighted by the total number of sequence reads, per sample. These data
308 were also subset into *all birds* and *adults only* subsets, and we used the same model formulas
309 as the alpha diversity models above. The *all birds* global models did not converge so
310 simplified models were refit only including pairwise interactions with age. The age variable
311 was coded using backwards difference coding, as in the alpha diversity models.

312

313 **Model averaging**

314 Model averaging was performed using the MumIn package (Bartoń, 2020) as outlined by
315 Grueber et al. (2011), on the alpha diversity models and the phylum level relative abundance
316 models. In each case, a full model was fit using *lmer* or *glmer*, variables were standardised
317 using the arm package (Gelman et al., 2021) and then a model set generated with the dredge
318 command. The top model set was selected from the complete model set, with models within
319 2AICc of the top model considered part of this top model set. If multiple models were within
320 the 2AICc cut-off, these were averaged, otherwise the single top model was reported. The
321 input variables were standardised using the Gelman procedure (Gelman, 2008) centring all
322 regression inputs on zero and dividing by 2SDs.

323

324 **Model checking and plotting**

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325 Model diagnostics for the alpha diversity and phylum level relative abundance models were
326 checked with *DHARMa* (Hartig, 2019). *DHARMa* simulates data based on the model
327 provided and is a better validator than simple residual vs fitted data plots for mixed effects
328 models. Simulated residual plots were made for each of the models in the top model set. In
329 cases where the simulated residuals showed a clear pattern, models were interpreted
330 cautiously. The *adults only* phyla level models showed overdispersion so these models were
331 refit with an observation level random term following Harrison (2015).
332
333 All plots were created using ggplot2 (Wickham, 2016). Alpha diversity and phylum-level
334 results were plotted using 'JTools' and 'Interaction' (Long, 2019, 2022) packages, which are
335 based on ggplot2. The model estimates for the top model in each model set were plotted, not
336 the model averaged results. These plots used partial residuals, which allow the plotting of
337 data accounting for the variables other than the predictor variable of interest and can be better
338 at showing relationships between variables in multivariate mixed models than plotting the
339 raw data. Plots of the raw data vs the response variable were also made to verify the
340 robustness of model results.
341
342 ***Beta-diversity***
343 Beta-diversity measures the dis-similarity between two or more communities, and in our
344 analyses consisted of the pairwise distances between all samples. Beta-diversity was analysed
345 using a compositional approach, which accounts for the proportional nature of high-
346 throughput sequencing data (Gloor et al., 2016; Gloor & Reid, 2016). Low prevalence taxa,
347 i.e. those with less than one copy in 5% of samples, were excluded to reduce possible
348 contaminants and sequencing artefacts (Bokulich et al., 2013). The filtered dataset was
349 centre-log ratio transformed and then the Euclidean (or Aitchison) distance between samples

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350 was calculated (Aitchison, 1983). The beta-diversity of samples was tested using
351 PERMANOVA (*adonis2* function from the vegan package (Oksanen et al., 2019)) after
352 checking variables for homogeneity of dispersion. Models were fit using the 'margin' option
353 which tests the marginal effect of each variable while accounting for the other variables in the
354 model. Nest was used as a blocking factor to control for the non-independence of samples
355 and sequence plate was included as a fixed effect to account for batch effects. Predictor
356 variables were centred and scaled. For the *all birds* dataset two models were run: one with
357 only non-interacting fixed effects, and another model which included the main interaction of
358 interest (age × habitat), as PERMANOVA cannot calculate the marginal effect of fixed
359 effects from the interactions in the model. For the *adults only* dataset a single model was run
360 with only non-interacting fixed effects. No variable, except habitat, had homogenous
361 dispersion when all birds were considered (table S2), so significant PERMANOVA results
362 could reflect differences in group variance rather than differences in group means, or could
363 reflect differences in both group variance and group means (Anderson & Walsh, 2013).
364 When adult birds were considered separately age, sex and habitat all had homogenous
365 dispersions (table S3). Beta diversity was visualised using PCA plots of the (pairwise)
366 Euclidian distance between samples, and plotted samples according to their first and second
367 principal component values.

368

369 **Repeatability**

370 The rptR package (Stoffel et al., 2019) was used to decompose the components of variance
371 underlying alpha diversity in nestlings measured across D8 and D15, and to examine the
372 extent to which consistent differences among nestlings were confounded by the fixed effects
373 of age and habitat, and the random effects of nest or woodland site. Specifically, repeatability
374 of Shannon and Chao1 diversity was calculated at the individual and group level in the

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375 following ways: (i) unadjusted repeatability for woodland site; (ii) unadjusted repeatability
376 for nest; (iii) unadjusted individual repeatability; (iv) individual repeatability adjusted for the
377 fixed effects of age × habitat, (v) repeatability of individual and nest adjusted for age ×
378 habitat; and (vi) repeatability of individual, nest and woodland site adjusted for age × habitat.
379 Adjusted repeatability is repeatability controlling for the fixed effect, where any variance
380 explained by fixed effects is excluded from the total variance used in the repeatability
381 estimate (Nakagawa & Schielzeth, 2010). Repeatability was adjusted for age, habitat and
382 their interaction because age × habitat was significant in the nestling only models of alpha
383 diversity (table S4.). Repeatability for phyla-level relative abundance was also calculated in
384 the same manner, adjusting for different fixed effects, according to a nestling only binomial
385 model (see table S5). Singleton nestling measurements were included to improve estimates
386 (Martin et al., 2011). Repeatability analyses were restricted to nestlings because no repeat
387 measures were available for the adults.
388
389
390 **Results** *(please see tables and figures at the end of the document)*
391
392 *Alpha diversity*
393 There was no relationship between Shannon diversity and age (table S6a). Chao1 diversity
394 was lower in D15 than in D8 nestlings (-0.35±0.15, p=0.02; table S6b), and there was no
395 difference between D15 nestlings and adult birds (-0.02±0.18, p=0.91; table S6b, figure 1).
396 The effect of habitat on alpha diversity depended on age (table S6a, b) - the decrease in
397 Chao1 diversity from D8 to D15 was more pronounced in mixed/deciduous than in
398 coniferous habitats (habitat × age: -0.73±0.32, p=0.025; table S6b, figure 1). As nest box
399 distance from woodland edge increased, so did both Shannon and Chao1 diversity, regardless

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400 of age (Shannon: 0.21±0.1, p=0.035; Chao1: 0.45±0.2, p=0.023; table S6, figure 2). Neither
401 brood size nor lay date predicted alpha diversity (table S6). None of the variables (age, sex,
402 habitat, distance to edge, brood size or lay date) predicted alpha diversity when adults were
403 analysed separately (table S7).
404

405 *Beta diversity*

406 Beta diversity differed across age (table 1a) where community composition became more
407 similar among individuals with age (figure 3a). Beta diversity varied with habitat as a main
408 effect (table 1a) and when interacting with age (table 1b). Visual inspection of the PCA plot
409 suggests that the distinction between the ages was most pronounced in the mixed/deciduous
410 woodland (figure S1), though dispersion tests indicate this result could be due to a difference
411 in group dispersions, group means or both (table S2). Beta diversity varied with brood size
412 and distance to edge, and there was also a tendency for beta diversity to vary with lay date
413 (table 1a). When adults were analysed separately, there was no difference in beta diversity
414 between mature and immature birds or between sexes (table 1c), and brood size was the only
415 factor to predict beta diversity (table 1).
416

417 *Phylum level relative abundance*

418 D8 nestlings had higher Proteobacteria abundance than D15 nestlings, who in turn had lower
419 Proteobacteria abundance than adults (table 2a and figure 4A). The main effect of age also
420 interacted with habitat. In both habitats, Proteobacteria decreased with nestling age (i.e.
421 between D8 and D15), but this effect was stronger for birds developing in mixed/deciduous
422 compared to coniferous habitats, mirroring the pattern above for alpha diversity (table 2,
423 figure 4A, 4B). Adult birds had greater abundances of Proteobacteria than D15 birds, and this
424 effect was especially pronounced in mixed/deciduous woodlands compared to conifer

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425 woodlands (table 2b; figure 4A). We note that although the age-habitat interactions are
426 supported by the model, they are not strongly supported by plots (Fig 5A and B) and should
427 be interpreted with caution. Brood size was negatively related to the abundance of
428 Proteobacteria in nestlings, especially for D15, but not for adults; the opposite was the case
429 for Firmicute abundance (table 2a and 2b; figure 4C and 4D). There was no effect of distance
430 to edge on phyla level abundance. Proteobacteria abundance was negatively related to lay
431 date in D8 nestlings, and positively in D15 nestlings and in adults. All of the patterns
432 described for Proteobacteria were found for Firmicutes in the inverse directions (figure 4,
433 table 2). Only lay date predicted phyla level relative abundance in the *adults only* models.
434 Proteobacteria increased with lay date (1.5±0.5, p=0.006), but did not relate to Firmicutes (-
435 0.7±0.6, p=0.223; table S8, figure 5).
436
437 *Nestling, nest and woodland site repeatability*
438 Microbiota were not repeatable across sites (model 1's, table S9) but were repeatable across
439 nests for Shannon, Chao1, Proteobacteria and Firmicutes abundance (R values from model
440 2's, respectively: 0.399; 0.421; 0.191; 0.261. See table S9 for credibility intervals and further
441 details). Individual gut microbiota were not significantly repeatable for Shannon diversity,
442 but was for Chao1 diversity, Proteobacteria abundance and Firmicutes abundance, when
443 unadjusted for any other effects (R values from model 3s, respectively: 0.378, n.s.; 0.46;
444 0.174; and 0.248). After controlling for fixed effects, individual repeatabilities for Shannon
445 and Chao1 diversity, but not for Proteobacteria or Firmicutes relative abundance, were
446 significant (R values from model 4's in table S9 respectively: 0.403; 0.50; 0.144, n.s.; 0.165,
447 n.s.). However, individual repeatabilities for all four gut microbiota metrics approached zero
448 when controlling for nest site as a random effect, which itself was repeatable in all four
449 variables (see R values from model 5's for both individual and nest in table S9), indicating

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450 that the majority of microbiota variability in nestlings was driven by nest identity, not
451 individual differences. The addition of woodland site as a random effect suggested that up to
452 a quarter of the nest site repeatability may have been driven by differences among sites (table
453 S9), though the credibility intervals for woodland site repeatability overlapped zero in all four
454 model 6's.
455
456 Given these repeatability results, we decided to carry out post-hoc analyses of how parent
457 traits correlated with offspring traits. We found that nestling microbiome was predicted only
458 by the mother's Shannon diversity (table S10a-d). Specifically, female Shannon diversity
459 negatively predicted offspring Shannon diversity (figure S2). None of mother's Chao1,
460 Proteobacteria abundance or Firmicutes abundance, or any of the father's microbiome traits
461 predicted the corresponding nestling trait (table S10e-h).
462
463
464 **Discussion**
465
466 We found that environmental links with the gut microbiota were dependent on age, pointing
467 to differential sensitivity of gut microbiota across life stages in response to habitat type, local
468 environmental gradients and life history traits (table 3), which is consistent with results from
469 human studies (Claesson et al., 2011). Notably, life history, but not environmental effects
470 predicted gut microbiota variation in adults; whereas nestling gut microbiota appeared
471 sensitive to a range of both environmental and life history variations (table 3). Repeated
472 measures showed that although nestlings appeared to show consistent individual differences
473 in gut microbiota diversity and phylum-level abundance, this was explained entirely by the
474 nest in which they developed. The post-hoc parent-offspring analyses found the microbiome

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475 traits of nestlings were not typically correlated with their parents' traits, although nestlings'
476 Shannon diversity was negatively predicted by their mother's Shannon diversity. We discuss
477 differential gut microbiota sensitivities to environmental and life history variation across age
478 groups and place these correlative findings in the context of future directions for
479 understanding the ecological and evolutionary consequences of these patterns in wild
480 systems.
481
482 *Environment*
483 Only nestling, not adult, gut microbiota varied in response to woodland type (table 3). This is
484 not surprising given microbial community assembly theory (Costello et al., 2012; Coyte et
485 al., 2021) where source microbes vary according to the external environment (e.g. diet,
486 nesting material, habitat) (C.-Y. Chen et al., 2020; Goodenough et al., 2016; Goossens et al.,
487 2022; Koenig et al., 2010) and compete for available niches as the host physiological
488 environment develops (Costello et al., 2012). Diet has a strong seasonal component during
489 this time, as the availability of food items changes throughout the breeding season and varies
490 with habitat type, habitat quality and parental preferences (Wilkin et al., 2009). Regardless of
491 age, the alpha diversity of the microbiota was higher for birds in nests further from the edge
492 of the woodland (figure 2), with a trend for the strength of this association to diminish with
493 age, which we hypothesise could be due to differences in the exposure to microbes via
494 nesting materials (Goodenough et al., 2016) and diet (G. Davidson et al., 2020) during a time
495 when the microbiota are particularly sensitive to environmental variation. Similarly, D8
496 nestlings were especially sensitive to the woodland habitat (mixed/deciduous or coniferous),
497 whereas the effect of woodland on alpha diversity was not present in adults, nor did it differ
498 between adults and D15 nestlings, suggesting that the effects of habitat variation on alpha
499 diversity stabilised early in development. However, for phylum-level abundance, this stability

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500 was not observed until adulthood. Future studies should collect microbiota community data
501 and with contemporary DNA metabarcoding data across environmental gradients to
502 determine whether the microbiota covaries with diet across habitats.
503
504 *Life history and age*
505 Lay date was positively correlated to Proteobacteria in D15 nestlings and in adults, but
506 negatively in D8 nestlings, while the exact reverse was true for Firmicute abundance. The
507 abundance of caterpillars, the primary food source for developing great tits, peaks mid
508 breeding season, the precise timing of which could affect the gut microbiota directly, or
509 indirectly through the effects of food availability on stress, immunity or growth (Hooper et
510 al., 2012; Potti et al., 2002; Stothart et al., 2019). Indeed, nestlings with an early or late lay-
511 date may experience stress from lower food availability and higher predation pressure (Naef-
512 Daenzer et al., 2001), and experimentally manipulated glucocorticoids have been reported to
513 affect nestling bird gut microbial diversity (Noguera et al., 2018). Furthermore, the effect of
514 lay date on stress may depend on nestling age because of the increasing nutritional demands
515 of older nestlings, so that young nestlings from early-nesting parents could face similar
516 conditions to old nestlings from late-nesting parents. If, hypothetically, the positive
517 correlation observed between adult Proteobacteria and lay date was driven by stress, this
518 could explain why the effect of lay date on phylum-level abundance was in opposing
519 directions for D8 and D15 nestlings: D15 nestlings from early nests may have been equally
520 stressed to D8 nestlings from late nests (figure 4G).
521
522 Brood size also correlated with microbiota variation, which we suggest is likely due to larger
523 brood sizes leading to higher stress as a consequence of increased sibling competition
524 (Neuenschwander et al., 2003) and lower food availability (Smith et al., 1988). The effect of

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525 brood size on microbiota was especially pronounced for D15 birds, when demand for food is
526 highest and sibling-sibling conflict greatest. It is possible that larger brood sizes simply have
527 a different reservoir of bacteria due to higher levels of provisioning. Cross fostering
528 experiments that manipulate the size of broods, and compares broods with nestlings from
529 multiple source nests against broods with nestlings from a single source, would test the
530 relative importance of this reservoir and competition hypotheses.
531
532 Contrary to our predictions, in the *adults only* dataset, we did not detect any effects of lay
533 date on gut microbiota, nor any sex-dependent interactions, despite lay date being strongly
534 determined by female condition (Brown & Brown, 1999). Lay date could also potentially
535 affect the adult gut microbiota indirectly, via increased glucocorticoids related to
536 provisioning effort (Bonier et al., 2011), because provisioning the young is more challenging
537 outside of the peak of food abundance. Although stress hormones have been reported to alter
538 gut microbiota in reproductive female lizards (*Sceloporus undulatus*), these effects were only
539 observed at specific reproductive stages (MacLeod et al., 2022). In the current study, adults
540 were sampled for gut microbiota near the end of their reproductive cycle (i.e. when chicks
541 were near fledging), and therefore it is possible we missed key reproductive time points
542 where gut microbiota were sensitive to physiological influences associated with lay date.
543 Nevertheless, brood size, which may also reflect parental quality (Pettifor et al., 2001) and/or
544 higher glucocorticoid demands associated with provisioning (Bonier et al., 2011), predicted
545 gut microbiota community composition in adults of both sexes. Overall, these findings
546 highlight a potentially exciting area of research on reproductive-dependent physiology and
547 parental care, and its interaction with the function of the gut microbiome. Brood size
548 manipulation in conjunction with glucocorticoid measurements would determine whether the
549 effect of brood size on the microbiota was related to parental quality or glucocorticoid levels.

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550

551 *Repeatability*

552 The repeatability analysis revealed the importance of the immediate nest environment in
553 structuring the microbiota, whereas the larger-scale effect of woodland site was negligible.
554 Human parents provide their offspring with beneficial microbes acquired over their own
555 lifespan and hence provide their offspring a fitness advantage (Sprockett et al., 2018). These
556 microbes prime them for their environment during early life and are particularly influential
557 due to priority effects, where the timing of colonisation influences future species interactions
558 (Sprockett et al., 2018). Notably, individual-level repeatability of microbial diversity was
559 zero when nest identity was controlled, where individual's gut microbiota were not following
560 individual trajectories but were converging on their nestmates. The importance of the nest in
561 determining microbiota diversity and structure has been highlighted in this species and others
562 previously (Campos-Cerda & Bohannan, 2020; Teyssier, Lens, et al., 2018). If genetics were
563 an important driver of the microbiota in nestlings we would expect to see some level of
564 repeatability, even when controlling for nest as siblings are expected to share only 50% of
565 their DNA on average. These results point to considerable plasticity in microbiota assembly
566 during early development, and given the negligible evidence for positive correlation between
567 any parent and offspring microbiome trait, is further support to the greater influence of local
568 environmental factors, most likely linked to local food availability, over intrinsic or host
569 factors, in avian microbiota (Song et al., 2020). Despite these findings, the negative
570 correlation for the mother's Shannon diversity raises the possibility of a maternal effect on
571 the gut microbiota, which has been demonstrated in wild North American red squirrels
572 (Tamiasciurus hudsonicus) using a quantitative genetic approach (Ren et al., 2017).

573

574 *Conclusions*

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575 Studies of environmental associations with the gut microbiome in natural systems typically
576 focus on a single ecological feature (e.g. habitat type, season), when in fact multiple
577 interacting ecological factors likely shape the gut microbiome simultaneously. Our results
578 point to links between different aspects of the gut microbiota and multiple aspects of the
579 environment and life history variation: the local nest, sibling interactions, location within
580 woodland plots, the woodland plots themselves, and potentially changes in food availability
581 during the season. These effects are to be expected since many of the factors involved are
582 fundamental drivers of ecological processes within populations, and yet notably, it was the
583 natal, as opposed to the adult gut microbiota that was most sensitive to ecological variation,
584 pointing to a high degree of developmental plasticity. Unpicking the mechanisms, causes and
585 consequences of gut microbiome variation among wild populations is an exciting avenue of
586 future research that remains largely unexplored.

587

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945

946 **Data accessibility statement:** Metadata is available at DataDryad
947 https://datadryad.org/stash/dataset/doi:10.5061/dryad.bk3j9kd9g (Davidson, et al. 2020).
948 Sequence data are available in the European Nucleotide Archive under access number
949 PRJEB42330, and ERS5506097 - ERS5506338.

Tables and figures

Table 1. Beta-diversity PERMANOVA. Results are marginal effects and significance is calculated from 999 permutations. (a) model with only main effects, (b) model which additionally includes age × habitat interaction term (see methods for explanation) and (c) adults only model. Cells blank where variable not tested.

Independent variables	(a) All birds				(b) All birds (with interaction)				(c) Adults only			
	SS	R^2	F	P	SS	R^2	F	P	SS	R^2	F	P
Sex									212.96	0.02	0.82	0.83
Age	1062.07	0.02	2.31	0.001					271.04	0.02	1.05	0.63
Habitat	307.08	0.01	1.34	0.013					290.32	0.02	1.12	0.44
Lay date	423.03	0.01	1.84	0.07	419.75	0.01	1.83	0.08	323.59	0.02	1.25	0.13
Brood size	318.83	0.01	1.39	0.006	305.34	0.01	1.33	0.018	223.30	0.02	0.86	0.004
Distance to edge	496.18	0.01	2.16	0.001	502.35	0.01	2.19	0.001	327.04	0.02	1.26	0.34
Sequence plate	3653.08	0.06	3.97	0.001	3634.14	0.06	3.96	0.001	1462.09	0.11	1.88	0.01
Age × Habitat					594.52	0.01	1.30	0.015				

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Table 2. Phylum level, binomial model results for the *all birds* (nestlings and adults) subset. Only a single model was retained in the top model sets (<2AICc) so results are not model averaged. The reference level for habitat is ‘coniferous’ which is compared here to ‘mixed/deciduous’. The age variable is backwards difference coded (see methods for explanation).

	(a) Proteobacteria				**(b) Firmicutes**			
Independent variables	**Estimate**	**S.E.**	**z**	**P**	**Estimate**	**S.E.**	**z**	**P**
(Intercept)	0.16	0.67	0.24	0.81	-2.17	0.70	-3.12	0.002
Age (D15/D8)	-1.52	0.00	-342.63	<0.001	1.30	0.00	315.88	<0.001
Age (Adult/D15)	2.45	0.31	7.88	<0.001	-2.94	0.35	-8.47	<0.001
Habitat	-0.08	0.72	-0.12	0.91	0.08	0.89	0.09	0.93
Brood size	-0.72	0.20	-3.58	<0.001	0.97	0.22	4.36	<0.001
Distance to edge	0.66	0.41	1.62	0.11	0.12	0.48	0.25	0.81
Lay date	0.49	0.44	1.11	0.27	-0.38	0.53	-0.70	0.48
Age (D15/D8) × Habitat	-0.87	0.01	-129.32	<0.001	1.92	0.01	302.68	<0.001
Age (Adult/D15) × Habitat	1.90	0.84	2.26	0.024	-1.67	0.91	-1.84	0.07
Age (D15/D8) × Brood size	-1.19	0.01	-174.16	<0.001	1.36	0.01	211.54	<0.001
Age (Adult/D15) × Brood size	1.90	0.61	3.14	0.002	-1.28	0.66	-1.93	0.054
Age (Day15/Day8) × Distance to edge	-0.63	0.01	-115.05	<0.001	0.73	0.01	129.92	<0.001

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Age (Adult/D15) × Distance to edge	-0.66	0.65	-1.01	0.31	0.34	0.71	0.47	0.64
Age (D15/D8) × Lay date	1.77	0.01	319.66	<0.001	-1.67	0.01	-309.36	<0.001
Age (Adult/D15) × Lay date	0.67	0.79	0.85	0.40	-0.34	0.84	-0.40	0.69

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Table 3. Summary of results. Arrows show the direction of the effect on the microbiome trait (not applicable for β diversity as effects do not have directionality). Paired arrows indicate the direction of effect for Proteobacteria and Firmicutes, respectively. Asterisks show significant results for beta diversity. 'Coniferous' is the reference level for habitat and bracketed term with 'Age' indicates age level which is being compared to the proceeding level.

	α diversity	β diversity		Proteobacteria-Firmicutes diversity	
	All birds	All birds	Adults	All birds	Adults
Age (D15)	↓	*		↓ ↑	
Age (Adult)				↑ ↓	
Distance	↑				
Lay date					↑ n.s.
Habitat		*			
Brood size		*	*	↓ ↑	
Age (D15) x Habitat	↓	*		↓ ↑	
Age (Adult) x Habitat				↑ ↓	
Age (D15) x Brood size				↓ ↑	
Age (Adult) x Brood size				↑ n.s.	
Age (D15) x Distance				↓ ↑	
Age (D15) x Lay date				↑ ↓	

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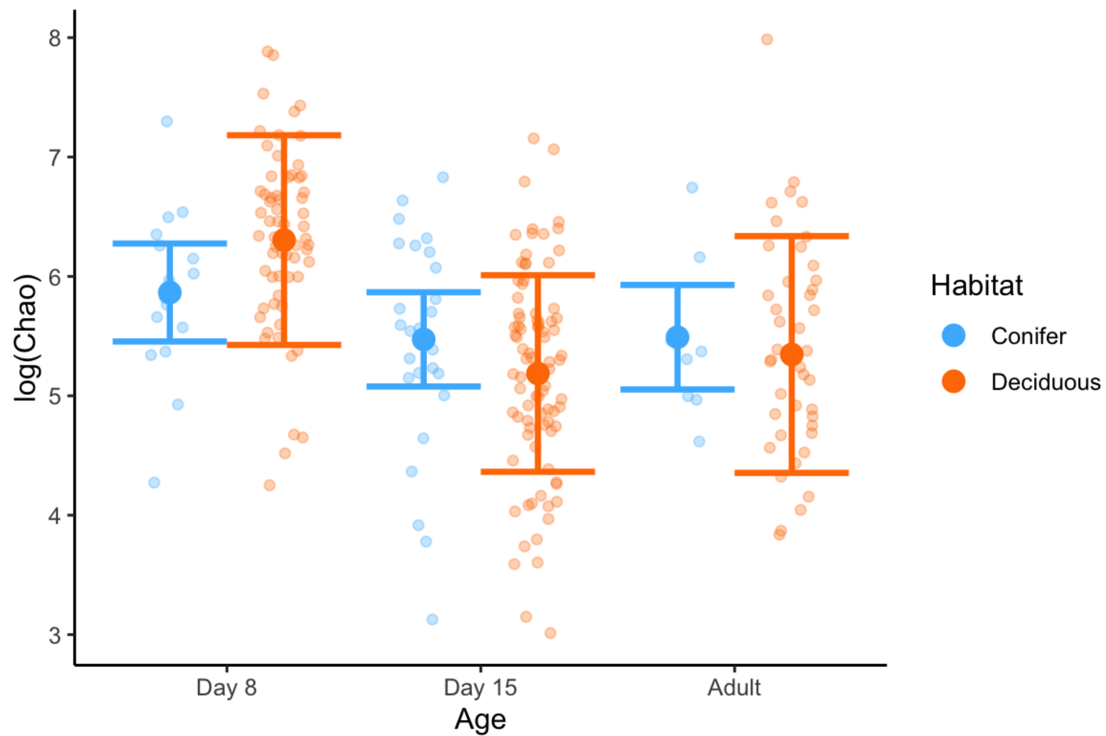
973

974 Figure 1. Partial residuals plot for Chao1 diversity regressed on age across habitats (*all birds*
975 model), means with 95% confidence intervals.



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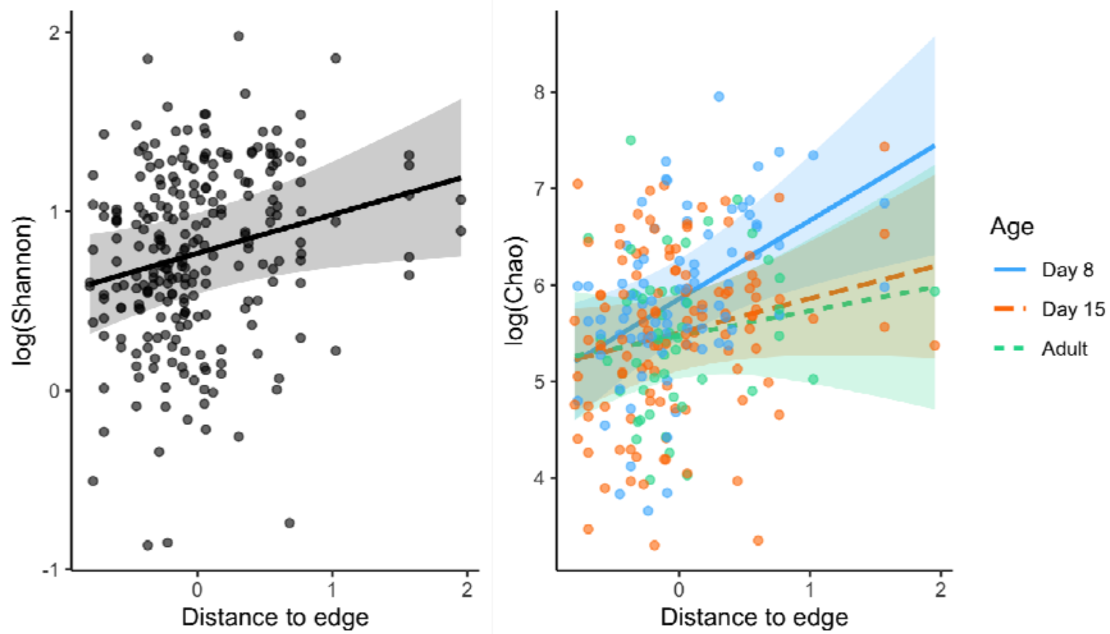
977 Figure 2. Fitted line on partial residuals plot with 95% confidence intervals for logged
978 diversity regressed on distance to edge from the *all birds* diversity models. Distance to edge
979 variable was centred and scaled so some values are below zero. Interaction trend in chao
980 model so slopes plotted separately by age.



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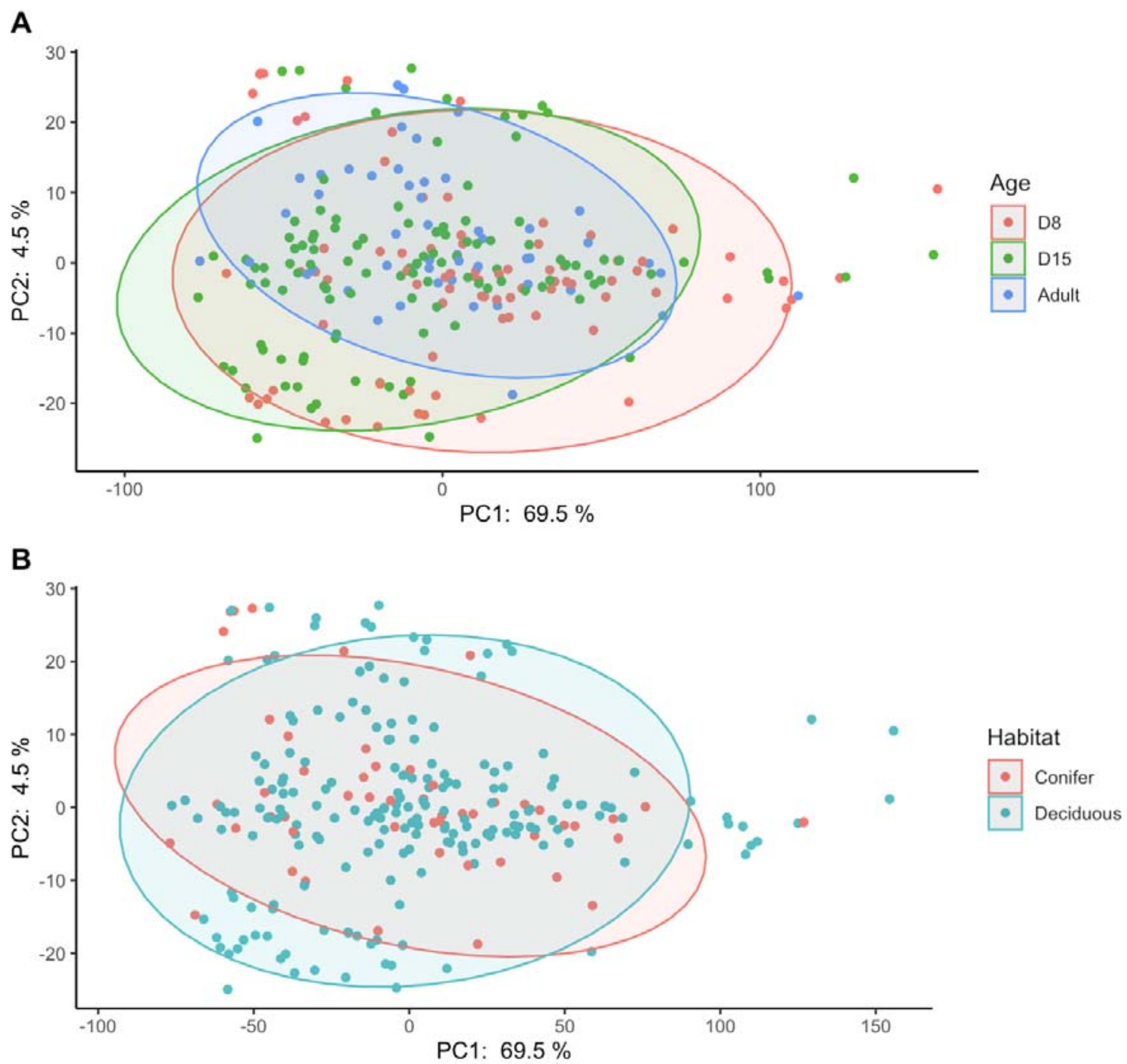
981 Figure 3. PCA plot of beta diversity with ellipses around (A) age groups, and (B) habitat
982 types.



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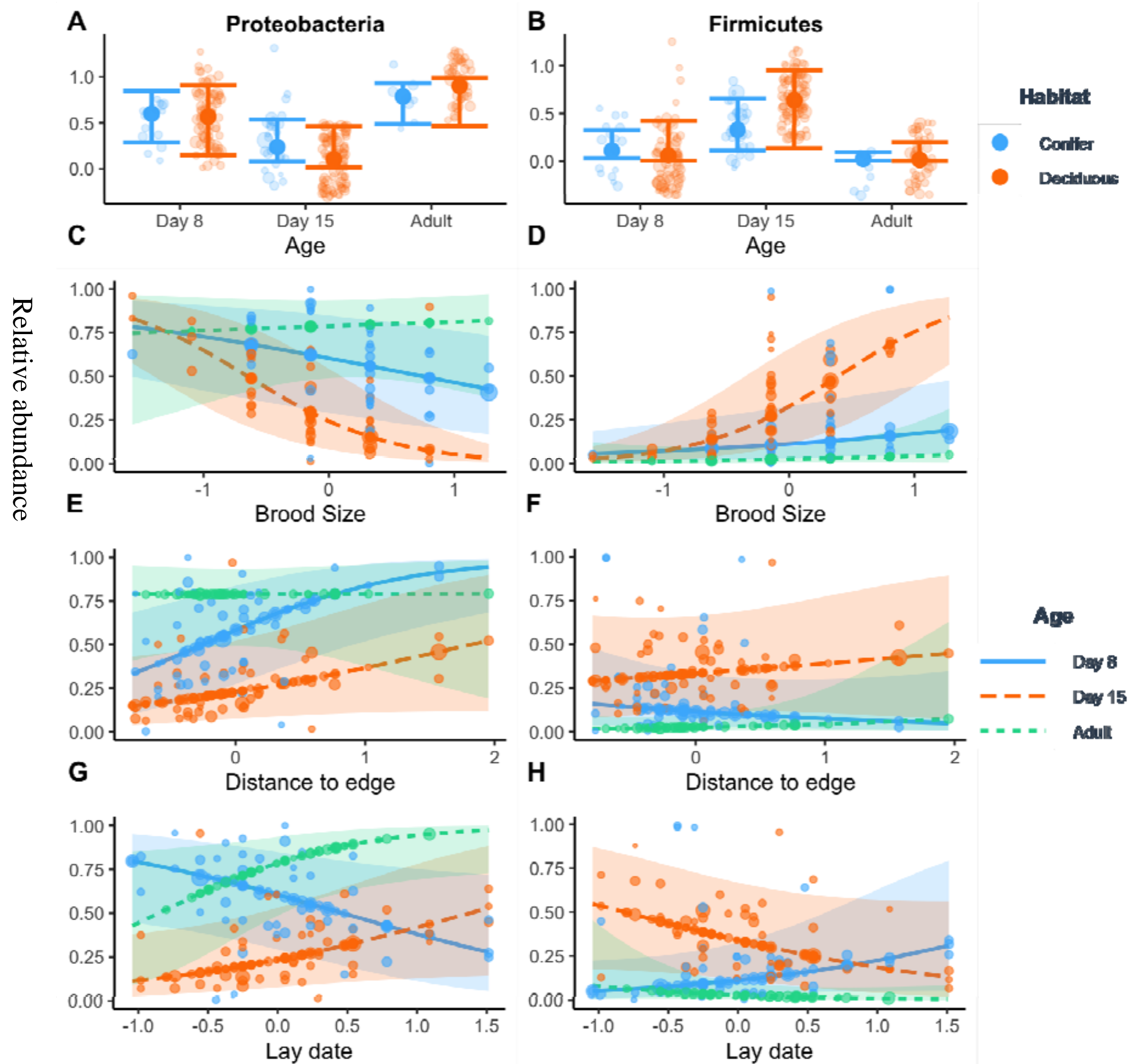
985 Figure 4. Partial residual plots for relative abundance of Proteobacteria (left column) and
986 Firmicutes (right column) for *all birds* phyla-level models showing means with 95%
987 confidence intervals. Point size reflects the weight of that observation, i.e. the 'number of
988 sequence reads' of the sample. Some points may appear out of range due to 'jitter' function
989 which separates overlapping points.



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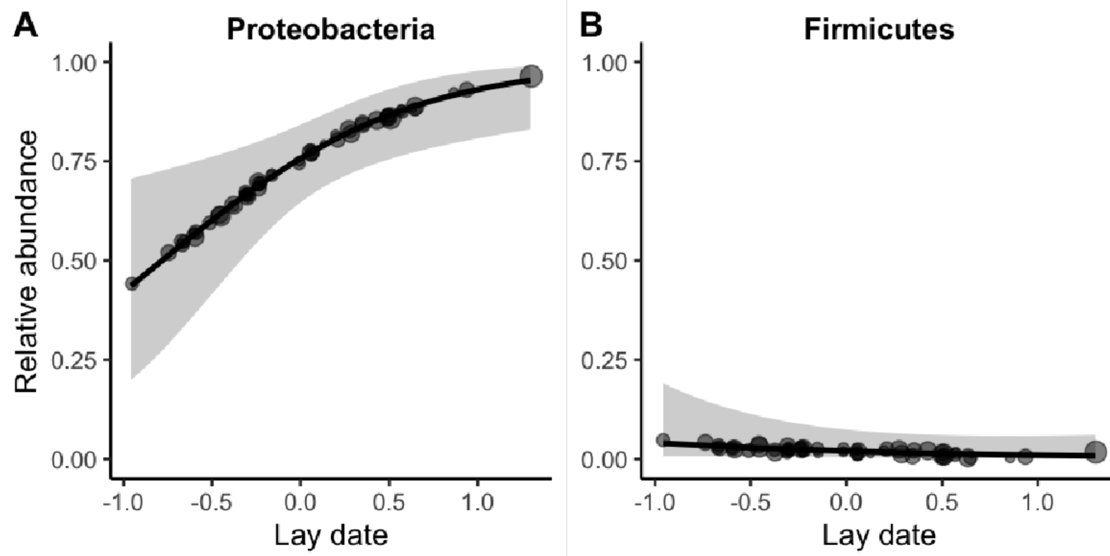
991 Figure 5. Partial residual plots for relative abundance of Proteobacteria and Firmicutes
992 against lay date (adult birds models), means with 95% confidence intervals.



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