

1 **Marked seasonal variation in the wild mouse gut microbiota**

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21

22 **Running title:** The wild mouse gut microbiota changes seasonally.

23 **Subject Category:** Microbe-microbe and microbe-host interactions.

24

25 **Abstract**

26 Recent studies have provided an unprecedented view of the microbial communities
27 colonizing captive mice; yet the host and environmental factors that shape the rodent
28 gut microbiota in their natural habitat remain largely unexplored. Here, we present
29 results from a two-year 16S rRNA gene sequencing-based survey of wild wood mice
30 (*Apodemus sylvaticus*) in two nearby woodlands. Similar to other mammals, wild mice
31 were colonized by 10 bacterial phyla and dominated by the Firmicutes, Bacteroidetes,
32 and Proteobacteria. Within the Firmicutes, the *Lactobacillus* genus was most abundant.
33 Putative bacterial pathogens were widespread and often abundant members of the wild
34 mouse gut microbiota. Among a suite of extrinsic (environmental) and intrinsic (host)
35 related factors examined, seasonal changes dominated in driving qualitative and
36 quantitative differences in the gut microbiota. In both years examined, we observed a
37 strong seasonal shift in gut microbial community structure, potentially due to the
38 transition from an insect- to a seed-based diet. This involved decreased levels of
39 *Lactobacillus*, and increased levels of *Alistipes* (Bacteroidetes phylum) and
40 *Helicobacter*. We also detected more subtle but statistically significant associations
41 between the gut microbiota and biogeography, sex, reproductive status, and co-
42 colonization with enteric nematodes. These results suggest that environmental factors
43 play a major role in shaping temporal variations in microbial community structure within
44 natural populations.

45

46 **Key words:** biogeography / gut microbiota / intestinal nematode / nutrition / wild mice

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48

49 **Introduction**

50 Mammals are home to trillions of microbes in their gastrointestinal tract (the gut
51 microbiota), which impact multiple aspects of host health and disease (Sommer and
52 Backhed 2013). Elucidating the ecological and evolutionary processes that shape host-
53 associated microbial communities remains a major outstanding goal (Costello et al
54 2012). Laboratory rodents are a valuable tool to dissect the relative contributions of
55 intrinsic and extrinsic factors (Carmody et al 2015); however, it remains unclear if these
56 interactions can be generalized to mammals in their natural habitat. Recent studies
57 have provided an initial view into the ecological factors linked to inter-individual
58 variations in the gut microbiotas of wild animals. Comparative analyses suggest that diet
59 is a major environmental factor contributing to gut microbial variation between
60 mammalian species (Muegge et al 2011). Diet also shapes the gut microbiota within a
61 species, as evidenced by longitudinal analyses of the black howler monkey gut
62 microbiota (Amato et al 2013, Amato et al 2015) and dietary perturbation experiments in
63 wild-caught mice and fish (Bolnick et al 2014, Wang et al 2014). Biogeographic variation
64 in the gut microbiota at large spatial scales has also been reported in house mice
65 (Linnenbrink et al 2013). Finally, host-specific factors like co-colonization with enteric
66 parasites (Hayes et al 2010, Keeney and Finlay 2011) and host genetics (Benson et al
67 2010, Goodrich et al 2014, McKnite et al 2012, Ochman et al 2010) may also contribute
68 to inter-individual and temporal variations in gut microbial community structure.

69 Yet the relative strengths of these various factors, and their interactions, remains
70 unclear due to the lack of systematic analyses that monitor both intrinsic and extrinsic
71 factors in natural populations. Such an analysis would require tractable systems

72 wherein host factors, environmental parameters, and temporal variations in the gut
73 microbiota can be monitored *in situ*. Here, we report findings from such a study in well-
74 characterized populations of wood mice (*Apodemus sylvaticus*) in the UK, which we
75 monitored for two years. We simultaneously measured multiple environmental (season,
76 location, population density) and host (age, sex, reproductive status, parasite infection
77 status) parameters, and repeatedly sampled multiple individuals over time. Using this
78 data, we examine the relative importance of environmental and intrinsic host factors in
79 shaping gut microbial community variation between and within individuals over time. We
80 discovered a notable seasonal variation in gut microbial community structure, which we
81 propose is due to changes in host dietary intake. We also found evidence for an impact
82 of spatial structure over a smaller scale than previously reported, reproductive status,
83 and nematode colonization. Together, our results provide an initial view of the wild wood
84 mouse gut microbiota and support the hypothesis that environmental factors such as
85 changes in food availability and subsequent dietary intake play a dominant role in
86 shaping wild mammal gut microbial communities.

87

88 **Materials and Methods**

89 *Sample collection*

90 In 2010 and 2011, *A. sylvaticus* were trapped on six grids in two mixed woodlands
91 (Manor and Haddon Wood; Figure S1) on the Wirral peninsula, UK. On each grid, two
92 live traps baited with grain and bedding material were placed every 10 meters in a 70m
93 x 70m square, and trapped monthly from May to November for three consecutive nights
94 in both years. In 2011, trapping was also performed for two consecutive nights during

95 one additional week in each of the months August, September, October, and
96 November, though no treatments were given. Trapped animals were tagged using
97 subcutaneous passive integrated transponder tags, so they could be individually
98 identified upon recapture. Fecal samples were collected from all traps containing a
99 single animal and stored in 10% buffered formalin for identification of gut parasites
100 (Knowles et al 2013). A sub-sample was also collected for characterization of the gut
101 microbiota, which was frozen at -80°C within 8 hours of collection. In order to assess the
102 potential effect of overnight temperature on gut microbial communities, we retrieved
103 temperature data for each sampling night from the Hawarden Chester airport weather
104 station near our field sites between the hours of 6pm and 12pm, the time from which
105 mice could enter traps, to when we collected fecal samples.

106

107 *Host phenotyping*

108 Animals were aged as either juvenile, sub-adult, or adult according to pelage in the first
109 instance, with body mass used as a secondary trait where pelage was inconclusive
110 (Juvenile<12g, Sub-Adult: 12-16g, Adult>16g). Body length, weight, sex, and
111 reproductive status were recorded. Animals were characterized as being either
112 reproductively active (descended or protruding testes for males, pregnant or with a
113 perforate vagina for females) or inactive. A subset of the mice were given anti-parasitic
114 treatments, including Ivermectin and Toltrazuril (2010), and Ivermectin, Fipronil,
115 Pyrantel pamoate, or two-drug combinations (2011). We did not detect any significant
116 impact of treatment on the gut microbiota (Table 1). Blood samples were tested for
117 *Bartonella* using a nested PCR assay (Knowles et al 2013).

118
119
120 *16S rRNA gene sequencing and analysis*
121 16S rRNA gene sequencing was performed on fecal samples collected from each trap
122 to characterize the distal gut microbiota (n=481 samples, 196,555±24,236 sequences
123 per sample; Table S1). DNA was extracted using the PowerSoil bacterial DNA
124 extraction kit (MoBio, Carlsbad CA), and the V4 region of the 16S rRNA gene was PCR-
125 amplified in triplicate using custom barcoded universal bacterial primers with the
126 following protocol: 94°C for 3 min, 35 cycles of 94°C for 45 sec, 50°C for 30 sec, and
127 72°C for 90 sec, with a final extension at 72°C for 10 min (Maurice et al 2013).
128 Triplicates were pooled, confirmed by gel electrophoresis, cleaned with the Ampure XP
129 kit (Agencourt, Danvers, MA), quantified using the Quant-iT Picogreen dsDNA Assay Kit
130 (Invitrogen, Carlsbad, CA), and sequenced on the Illumina HiSeq platform. 16S rRNA
131 gene sequences were analyzed using the QIIME software package (Caporaso et al
132 2010). All sequences were used for the comparison of the relative abundance of
133 bacterial taxa. Operational taxonomic units (OTUs) were assigned at 97% similarity
134 against the Greengenes database (DeSantis et al 2006), which we trimmed to span only
135 the 16S rRNA region flanked by our sequencing primers (positions 521-773). LefSe
136 (Segata et al 2012a) was run on sub-sampled datasets, after filtering out species-level
137 phylotypes with <100 sequences or found in only 1 sample. Statistical analysis of Bray-
138 Curtis dissimilarities calculated using the relative abundance of bacterial genera was
139 conducted using RStudio (ver. 0.98.1091) and the adonis function in the R package
140 "vegan" (Oksanen et al 2015). Only the first sample was included for each mouse to

141 avoid artifacts caused by within animal comparisons. Significance values were
142 computed using 10,000 permutations.

143

144 *Parasite diagnosis*

145 Gastrointestinal parasites (nematodes, cestodes and *Eimeria* protozoa) were detected
146 using the salt flotation technique (Pritchard and Kruse 1982). Saturated salt solution
147 was added to formalin-preserved fecal samples, such that eggs and oocysts in each
148 sample could be concentrated on a coverslip, and scanned for parasite detection at 10x
149 magnification. 40x magnification was used for parasite identification and making
150 parasite species-specific egg/oocyst counts. Coccidia (species belonging to the genus
151 *Eimeria*) were identified using unsporulated oocyst morphology (Nowell and Higgs
152 1989), and helminths using egg morphology. For each parasite species, the number of
153 eggs or oocysts per gram of feces was calculated for each sample. When multiple
154 samples were present for an individual within a 3-day trapping period, the arithmetic
155 mean egg/oocyst count was taken across these days. The dominant parasites detected
156 were nematodes (largely *Heligmosomoides polygyrus*) and coccidia, and thus our
157 analyses focus on these two parasite groups.

158

159 *Linear mixed models*

160 We performed linear mixed models (LMMs) using the lme4 package in R v.3.0.1 (Bates
161 et al 2013). We controlled for repeated sampling of individual mice by including
162 individual ID as a random intercept term. Model assumptions were checked by
163 examining the distribution of residuals and plotting fitted values against residuals;

164 response variables were square root or log-transformed where necessary to ensure
165 model assumptions were met. For models of individual genera, only samples with non-
166 zero abundance were included. In all starting models, the same set of predictors was
167 included: temperature, grid, month, year, age, sex, nematode infection status, *Eimeria*
168 infection status, drug treatment, and reproductive status. Several interaction terms were
169 included: year by month; reproductive status by sex; and parasite infection variables by
170 treatment. Only samples for which full metadata on all the above metrics were available
171 were included (Table S2). All models were initially simplified by backwards-stepwise
172 elimination of terms with p -value >0.10 , beginning with interactions, and the final minimal
173 model included only terms with p -value <0.05 . Adjusted p -values (q -values) were
174 calculated based on the 'Graphically Sharpened' False Discovery Rate (FDR) method
175 (Pike 2011).

176

177 *Spatial structuring of microbial communities*

178 Since wood mice are territorial and have home ranges smaller than our trapping grids
179 (Godsall et al 2014), fine-scale spatial variation in microhabitat and food availability
180 could influence gut microbial ecology, both within and across our trapping grids. To test
181 for biogeographic effects at this scale, we examined spatial autocorrelation in the gut
182 microbiota according to mouse capture location. Spatial autocorrelations were
183 measured using the Moran's I statistic (Moran 1950). Only the first sample was included
184 for each mouse to avoid artifacts caused by within animal comparisons. Genera found
185 in ≥ 10 samples (or mice) were analyzed, along with the first principal coordinates from
186 our Bray-Curtis, unweighted UniFrac, and weighted UniFrac analyses. We used a

187 binary spatial weights matrix, with spatial neighborhoods defined as being 0-50m apart.
188 Data from Manor and Haddon woods were analyzed both together and separately. To
189 control for temporal trends, we restricted our analysis to samples collected between
190 August and November and analyzed the two years separately. Spatial weight matrices
191 were row-standardized. The significance of Moran's I values was assessed with
192 permutation tests, coded using a Markov chain Monte Carlo algorithm. For each p -
193 value, ten chains of length 1,000,000 were run, each starting from a random initial
194 permutation. These settings were judged to give good chain convergence based on
195 examination of running mean plots. We used the software packages GeoDa and PySAL
196 (<https://geodacenter.asu.edu>). Batch scripts/code are available upon request.

197

198 **Results**

199 *The wild mouse gut harbours abundant Lactobacilli and putative enteric pathogens*
200 Consistent with results in captive and wild mammals (Ley et al 2008a), wild wood mice
201 were colonized by 10 bacterial phyla: Firmicutes (52.1±1.0% 16S rRNA gene
202 sequences; mean±stdev), Bacteroidetes (37.0±0.9%), Proteobacteria (8.2±0.5%),
203 Actinobacteria (1.1±0.2%), Tenericutes (0.9±0.1%), Defferibacteres (0.4±0.1%),
204 Cyanobacteria (0.3±0.03%), Verrucomicrobia (0.03±0.03%), Fusobacteria
205 (0.01±0.01%), and TM7 (0.004±0.0004%) (Figure 1a). Within the Firmicutes, the
206 dominant bacterial order was the Lactobacillales (genus: *Lactobacillus*) (Figures 1b,S2).
207 We also observed multiple α -, ϵ -, and γ -Proteobacterial genera that include potential
208 bacterial pathogens: e.g., *Bartonella*, *Helicobacter*, *Pseudomonas*, *Rickettsiella*, and
209 *Yersinia* (Figures 1b,S2, Table S3). All nine of the mice with detectable fecal *Bartonella*

210 also tested positive in time-matched blood samples, leading to a significant association
211 between blood and fecal detection of this genus (p -value <0.05 , χ^2 test). Many of these
212 genera were widespread, most notably *Helicobacter* (97.9% of samples), *Pseudomonas*
213 (73.2%), and *Yersinia* (44.5%). The same was true for intestinal parasites (Table S4),
214 including *Heligmosomoides polygyrus* (40%) and *Eimeria hungaryensis* (29.3%).

215

216 *Marked seasonal variation in microbial community structure*

217 Analysis of Bray-Curtis dissimilarity among samples revealed a clear seasonal pattern
218 differentiating samples collected in the spring/early summer (May through July) and
219 those collected in late summer/fall (August through November) [Figures 2a,S3; p -
220 value <0.001 , PERMANOVA of Bray-Curtis distances]. The observed seasonal shift in
221 the microbiota coincides with the expected timing of an annual transition to a seed-
222 based diet from a more insect-based diet (Watts 1968), and may therefore be driven by
223 a seasonal shift in food availability and diet. Consistent with this hypothesis, the mean
224 microbial community structure for each month was significantly correlated between the
225 two years (Figure 2b; $R^2=79\%$, p -value <0.01). The association between season and
226 microbial community structure was significant in both years when considered
227 independently, although the difference was more dramatic in 2010 [pseudo-F
228 value=31.2 (2010) versus 9.9 (2011), p -value <0.001 for both years; PERMANOVA test].
229 Statistical analysis with the LefSe software package revealed taxonomic groups ranging
230 from the phylum- to genus-level that were consistently associated with season in both
231 years (Table S5). *Lactobacillus* was found at a significantly higher abundance in the

232 spring of both years, whereas *Alistipes* and *Helicobacter* were consistently enriched in
233 the fall (Figure 2c).

234 Analysis of mice captured multiple times within a year confirmed that these
235 microbial changes occurred within individuals and were not simply due to mouse
236 population turnover (*i.e.* seasonal changes in the types of individual captured). We
237 observed within-individual shifts in microbiota structure in both years of the study that
238 followed the overall population trend (Figure S4). This was reflected by a strong positive
239 correlation between month-to-month differences in the mean population-wide value for
240 Bray-Curtis principal coordinates 1 and 2 (excluding repeat captured individuals) and
241 the mean within-individual change in these metrics (PC1 $R^2=65\%$; PC2 $R^2=56\%$; both p -
242 value <0.05 , linear regression; Figures 3a,b). Analysis of 25 mice captured in both
243 seasons confirmed that in nearly all cases there was a consistent direction of change
244 (Figure 3c; p -value <0.0001 , Wilcoxon rank-sum test).

245

246 *Limited spatial heterogeneity in community structure*

247 While microbial community structure differed significantly between the two woodlands
248 (p -value <0.001 , PERMANOVA of Bray-Curtis dissimilarities), this effect was noticeably
249 weaker than that of season: pseudo-F value=35.3 (season) versus 4.6 (wood) when
250 considering both years. Consistent with this weak effect, LefSe analysis only identified
251 two nested taxa that were significantly enriched in Manor Wood: the Clostridia class and
252 the Clostridiales order (LDA >2 , p -value <0.05). We did not detect any taxa that were
253 significantly enriched in Haddon Wood.

254 In order to quantify the spatial structure of the wild mouse gut microbiota in more
255 detail, we evaluated spatial autocorrelation at the genus level and using community
256 dissimilarity metrics (see *Methods*). In 2010, we detected significant spatial
257 autocorrelation for the Bray-Curtis and unweighted UniFrac metrics (Figure 4; q -
258 value <0.01). However, these patterns were weaker and only present for unweighted
259 UniFrac in 2011, and were absent in all cases when we only considered samples from
260 Haddon or Manor wood. Similarly, analyses of bacterial genera failed to detect
261 significant spatial autocorrelation for 117 of the 117 tested groups during either year (q -
262 value <0.01). Moreover, the maximum Moran's I value for this distance class was 0.138,
263 further indicating nonexistent or weak spatial associations. Together, these analyses
264 suggest that although the overall pattern of microbial community structure was distinct
265 between Haddon and Manor Wood, there was no evidence for finer spatial structure
266 within woods or between individual bacterial genera.

267

268 *Multivariate modelling reveals associations with both host and environmental factors*

269 We next used linear mixed models (LMMs; see *Methods*) to tease apart the relative
270 influence of multiple environmental and host factors, and to determine their effects in
271 isolation of confounding factors. We constructed 6 models for community dissimilarity
272 metrics (principal coordinates 1 and 2 for Bray-Curtis, unweighted UniFrac, and
273 weighted UniFrac) as well as separate models for the 10 most abundant bacterial
274 genera (Table 1). Overall, these analyses suggest that the wood mouse gut microbiota
275 is primarily shaped by environmental factors, with significant evidence for both temporal
276 (see “Year” and “Month” columns) and spatial structuring (see “Grid” column). These

277 temporal trends could not simply be explained by seasonal variation in temperature
278 (Figure S5), since they were unaltered by inclusion of overnight temperature as a
279 covariate (Table 1). For all community dissimilarity metrics examined and most
280 individual genera, our minimal models included a significant year by month interaction
281 term, indicating seasonal differences that varied somewhat across the two years
282 investigated. If these interaction terms were dissolved into their component terms,
283 strong main effects of month were observed in nearly all models, with effects of year
284 also common though generally weaker. Consistent with our prior analysis of spatial
285 autocorrelation, there was a strong association between the community dissimilarity
286 metrics and trapping grid with weaker associations at the genus level. We also detected
287 association between some metrics and local population density at the time of capture
288 (Table 1).

289 To a lesser extent than extrinsic factors like season and year, host factors such
290 as reproductive status and sex were associated with microbial community structure,
291 sometimes in the form of an interaction between these two terms (Table 1). For
292 example, the abundance of *Lactobacillus* was higher in reproductively active than non-
293 active females, but did not depend on reproductive status for males (Figure 5a). We
294 also detected associations between the gut microbiota and intestinal parasites. In
295 particular, nematode infections were inversely associated with the abundance of the
296 most abundant *Lachnospiraceae* genus and positively associated with the genus
297 *Escherichia* (Figure 5b). However, no significant associations between coccidia infection
298 or anti-parasite treatment and the gut microbiota were found, possibly due to the
299 transient nature of the intervention (monthly treatment intervals; see *Methods*). Age-

300 related differences were rare, with *Alistipes* the only one of the ten most abundant
301 bacterial genera associated with host age, showing an increase across the age groups
302 from juvenile to adult (Table 1).

303 To illustrate how much variation in Bray-Curtis principal coordinates 1 and 2 was
304 explained by environmental factors like month and year, compared to host-related
305 factors, we calculated marginal R^2 statistics from our linear mixed models, using the
306 methods described by (Nakagawa and Schielzeth 2013). These are equivalent to
307 classic R^2 statistics for linear models, indicating the percentage of variation explained by
308 a given set of predictor variables (fixed effects). For both Bray-Curtis PC1 and PC2,
309 month (*i.e.* seasonal differences) explained a much larger proportion of variance than
310 year (Table S6). Inclusion of year when month was already present in the model
311 provided little additional explanatory power (PC1: $R^2_{\text{GLMM}(m)} = 43.9\%$ with month only vs.
312 48.7% with month and year; PC2: $R^2_{\text{GLMM}(m)} = 10.1\%$ with month only vs. 10.5% with
313 month and year). Furthermore, allowing the seasonal effect to vary among years (by
314 inclusion of a month*year interaction term) yielded limited additional explanatory power
315 for PC1 ($R^2_{\text{GLMM}(m)} = 52\%$ vs 49% variance explained), with a 2-fold increase in variance
316 explained for PC2 ($R^2_{\text{GLMM}(m)} = 19.5\%$ vs 10% variance explained).

317 Thus, seasonal differences in the gut microbiota appear to dominate the
318 differences between years and are largely consistent across years, in agreement with
319 our earlier analyses (Figure 2). Host-related factors (age, sex, reproductive state),
320 enteric parasite infections, and host density explained some additional variance (12%
321 more for PC1 and 8% more for PC2 than models with only month and year terms),
322 though their contribution was again smaller than the strong seasonal effects, particularly

323 for PC1 (Table S6). Individual identity explained 18% of the variation in Bray-Curtis PC1
324 even after including all other factors. We confirmed these trends by analyzing the entire
325 Bray-Curtis dissimilarity matrix according to season, host sex, and wood (see *Methods*).
326 Although all three factors showed a significant effect, seasonal effects explained more
327 variation ($R^2=13.3\%$, $p\text{-value}<10^{-4}$) than either host sex ($R^2=0.8\%$, $p\text{-value}<0.05$) or
328 spatial structure ($R^2=0.8\%$, $p\text{-value}<0.05$).

329

330 **Discussion**

331 At the phylum level, the wild mouse gut microbiota is comparable to that of other
332 mammals (including humans) with two major groups, the Firmicutes and Bacteroidetes,
333 accounting for ~90% of the 16S rRNA gene sequencing reads (Ley et al 2008a, Muegge
334 et al 2011). We also detected high levels of the *Lactobacillus* genus (phylum:
335 Firmicutes; order: Lactobacillales) constituting up to one-third of the community, similar
336 to other omnivorous mammals, such as bears, squirrels, and lemurs (Figure S6). These
337 results confirm that to a large degree the mammalian gut microbiota assembles in a
338 reproducible fashion regardless of the host species (Ley et al 2008a, Muegge et al
339 2011), reflective of the restricted set of microorganisms that have adapted to life in the
340 gastrointestinal tract (Ley et al 2008b).

341 In contrast to “specific pathogen free” laboratory mice, we detected widespread
342 colonization by bacterial taxa that contain enteric pathogens, including *Helicobacter* and
343 other Proteobacteria. However, given the resolution of our sequencing methods and the
344 limited studies of wild mouse pathogens we cannot exclude the fact that these are
345 commensal strains. Despite this important caveat, our results are consistent with

346 previous reports indicating that wild house mice can be reservoirs of diverse
347 *Helicobacter* strains capable of infecting humans and other vertebrates (O'Rourke et al
348 2001, Parker et al 2009, Wasimuddin et al 2012). We observed that *Helicobacter*
349 abundance increased in late summer/fall, when *Lactobacillus* levels are low. This might
350 suggest that *Lactobacillus* confers protection against infection as has been
351 demonstrated in laboratory mice (Eaton et al 2011, Kabir et al 1997, Medellin-Pena and
352 Griffiths 2009, Pena et al 2005). Alternatively, immune status (*i.e.*, IL-22 deficiency) has
353 been linked to the abundance of *Lactobacillus* (Zenewicz et al 2013), potentially
354 suggesting that these seasonal changes might be in part driven by the host response to
355 bacterial infection. Additional studies will be necessary to determine how the immune
356 system of these mice tolerates long-term enteric pathogen colonization and to
357 characterize the reciprocal interactions between these enteric pathogens and the
358 commensal gut microbiota.

359 The wild mouse gut microbiota underwent a consistent seasonal shift in both
360 years, with a decrease in *Lactobacillus* and concomitant increases in *Alistipes*,
361 *Helicobacter*, and the Lachnospiraceae family (phylum: Firmicutes). A possible
362 explanation is that mid-summer represents a transition from a diet rich in insects to a
363 diet primarily composed of seeds (Watts 1968), coincident with the annual seed fall,
364 which usually starts in late July in UK woodlands (Gurnell 1993). Thus, we propose that
365 seasonal patterns in dietary intake drive variations in the gut microbial community
366 structure of wild wood mice. Differences in the timing, extent, and tree species
367 composition of seed fall, which can vary markedly between years (Gurnell 1993), may
368 explain the observed variation between years in the magnitude of the seasonal

369 microbiota transition observed. Notably, a recent study of rural human subjects from
370 South Dakota revealed differences in the gut microbiota in summer relative to winter
371 (Davenport et al 2014), suggesting that seasonal reconfigurations may be a conserved
372 feature of host-associated microbial communities.

373 If diet is indeed the dominant factor it still remains unclear what specific
374 components of the diet might drive the observed changes to gut microbial community
375 structure. The elevated levels of *Alistipes* in the fall may be reflective of increased bile
376 acid levels triggered by an increased consumption of fat, as seen in a recent human
377 dietary intervention study (David et al 2014). Members of the Lachnospiraceae family,
378 including *Eubacterium rectale* and *Roseburia*, have been linked to the fermentation of
379 dietary plant polysaccharides in human studies (David et al 2014, Duncan et al 2007),
380 and were also enriched in the fall coinciding with the increased access to plant seeds.
381 Similarly, the source and/or dietary trigger of *Lactobacillus* (often a minor member of the
382 mammalian distal gut microbiota) also remains unclear. *Lactobacillus* is often found in
383 fermented foods (Wolfe et al 2014), raising the possibility that their elevated abundance
384 early in the year may be driven by its cultivation in wood mouse food stores over winter.

385 Alternatively, seasonal changes in mouse physiology, including torpor and
386 reduced food consumption during winter, could also play a role in the observed
387 seasonal trends. Indeed, seasonal restructuring of the gut microbiota has recently been
388 observed in ground squirrels under controlled laboratory conditions (Carey et al 2013).
389 These shifts coincided with hibernation, suggesting they are driven by a shift from
390 dietary to host-derived substrates. We detected similar patterns in wild rodents,
391 including a decrease in the relative abundance of *Lactobacillus* and an increase in

392 *Alistipes* from spring/early summer to late summer/fall. Although wood mice do not
393 hibernate they are subject to daily torpor in conditions of low temperature and food
394 restriction. Thus, it is possible that the seasonal microbial shifts seen here may be
395 driven by the transition to a state of intermittent torpor.

396 What are the potential consequences of the observed seasonal shifts in gut
397 microbial community structure? Recent human intervention studies have shown rapid
398 and reproducible changes in microbial community structure and function upon
399 consumption of an animal- versus plant-based diet (David et al 2014). These results,
400 considered together with the current findings from wild wood mice, make it tempting to
401 speculate that the mammalian gut microbiota may provide a rapid way to optimize
402 caloric intake given volatile shifts in the availability of different foods. Microbial
403 communities that could rapidly shift their metabolic activity in response to changes in
404 host dietary intake could have enhanced dietary flexibility, likely increasing the fitness of
405 the host and its microbial consortia.

406 We also found significant but weak evidence for spatial structure, unlike the more
407 robust associations with geographic region found in recent studies of house mice
408 (Linnenbrink et al 2013), wild primates (Degnan et al 2012), and humans (Yatsunenکو
409 et al 2012). The significant spatial structure that we did find was evident only in
410 community-wide metrics when comparing between woods. Individual bacterial genera
411 showed no spatial structure, and no spatial structuring was evident within woods at
412 either the community or individual genus levels. These results emphasize that the gut
413 microbiota of these wild mouse populations is primarily shaped by factors that are not
414 spatially structured at the scales that we considered. These results suggest that either

415 (i) microbial dispersal occurs efficiently over distances far greater than the host range
416 evaluated here and/or (ii) the observed bacterial taxa are long-term and stable residents
417 of the wild wood mouse gut microbiota. Strain-level analyses of the gut microbiota (Faith
418 et al 2013, Segata et al 2012b) could help determine if there are finer differences
419 between woods, or among areas within each wood. Furthermore, surveying wild mice
420 across more distant sites could provide additional insight into broader biogeographical
421 patterns.

422 Our linear mixed models revealed significant associations with reproductive
423 status and intestinal parasites. Consistent with these findings, recent studies indicate
424 that the human gut microbiota is altered during pregnancy (Koren et al 2012), and
425 studies in laboratory mice have shown that infection by the nematodes *Trichuris muris*
426 depends on the gut microbiota (Hayes et al 2010). The associations between intestinal
427 nematodes and the bacterial genera *Escherichia* (positive) and *Lachnospiraceae*
428 (negative) support recent studies in humans and animal models (Rausch et al 2013,
429 Walk et al 2010), though we did not find the specific association between *H. polygyrus*
430 and relative *Lactobacillus* abundance, as recently reported in laboratory mice (Reynolds
431 et al 2014). Whether the associations found result from an altered immune response of
432 the host or from direct interactions between the intestinal parasites and the gut
433 microbiota remains to be elucidated. Determining the causal direction and underlying
434 mechanisms of these interactions will require more extensive longitudinal analyses of
435 wild mice before and after helminthic infection, as well as controlled studies using
436 captured and/or captive mice.

437 In conclusion, despite the common use of laboratory mice to study the
438 environmental and host factors that shape host-associated microbial communities, we
439 still know very little about their natural state. Our results provide an initial view of the
440 wild wood mouse gut microbiota, emphasizing commonalities between mammals, but
441 also the importance of considering temporal variations in nutritional status, enteric
442 pathogens, reproductive status, and parasite burden in setting the stage for host-
443 microbial interactions. Follow-up observational and interventional studies of wild mice,
444 paired with an in-depth analysis of dietary intake, are necessary to test the hypothesis
445 that the observed seasonal trends are due to changes in diet, and could provide a
446 complementary and tractable approach towards better understanding the causes and
447 consequences of inter-individual variations in the mammalian gut microbiota.

448

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459

460 **Conflict of Interest**

461 The authors declare no conflict of interest.

462

463 **Supplementary information is available at The ISME Journal's website.**

464

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615

616

617 **Figure legends**

618

619 **Figure 1. Taxonomic analysis of the wild mouse gut microbiota.** Pie charts

620 represent the relative abundance of bacterial **(a)** phyla and **(b)** orders (n=481 samples).

621 The 10 most abundant phyla and orders are shown (phyla with a mean abundance

622 <0.001% are not included; the remaining orders are represented by the “other” slice).

623 Taxa are colored based on phylum. Sequences within the Cyanobacteria phylum could

624 be attributed to chloroplasts (order Streptophyta), non-photosynthetic bacteria related to

625 Cyanobacteria that are common in the mammalian gut (order YS2) (Di Rienzi et al

626 2013), and algae (order Chlorophyta, family Trebouxiophyceae). We did not detect any

627 consistent seasonal changes in the abundance or prevalence of these three groups.

628

629 **Figure 2. Seasonal variations in the wild mouse gut microbiota. (a)** The first

630 principle coordinate from a Bray-Curtis-based analysis of microbial community structure

631 over time. Trend lines were generated by fitting a polynomial function to values from

632 each year (GraphPad Prism version 6.0). Values are mean±sem (n=14-80 samples per

633 group). Values from June and July were combined in 2011 due to limited available

634 samples in July (n=2). **(b)** Association between average monthly microbial community

635 structures between years. Values are mean (thick black line) and 95% CI (thin grey

636 lines) from a linear regression. **(c)** The relative abundance of bacterial genera in spring

637 and fall of both years. Values are mean±sem (n=24-123 samples per group; the first

638 sample from each mouse was included). Asterisks represent significant differences (*p*-

639 value<0.05, Wilcoxon rank-sum test).

640

641 **Figure 3. Seasonal patterns are detectable within individuals captured multiple**
642 **times.** Correlations between the mean month-to-month change in Bray-Curtis principle
643 coordinates 1 (panel **a**) and 2 (panel **b**) within-individuals, relative to the monthly
644 change observed at the population level (including only one pair of observations per
645 mouse; n=2-11 paired samples per datapoint). Dots represent monthly changes seen in
646 2010 (black) and 2011 (white). See Figure S4 for plots of individual animals over time.
647 **(c)** We calculated the mean value of Bray-Curtis principal coordinate 1 value for each
648 mouse in Season 1 (spring/early summer) and 2 (late summer/fall) (n=25 mice; ≤ 1
649 sample per mouse per month included). Nearly all mice exhibited a consistent direction
650 of change (black lines), with the exception of 3 animals (grey lines).

651

652 **Figure 4. Spatial distribution of microbial community structure.** Each circle
653 represents the physical location of a given mouse at the time of sampling in Manor or
654 Haddon Wood, which are subdivided into 2 and 4 fields, respectively. Shading is
655 proportional to the percentile along unweighted UniFrac principal coordinate 1 (an
656 indicated of overall microbial community membership). Between August and November
657 in 2010 there was a slight, but significant difference in community composition between
658 Haddon and Manor woods. However, this difference was absent in August-November
659 2011. Within woods, no significant spatial structuring of communities was observed in
660 either year.

661

662 **Figure 5. The gut microbiota is associated with intestinal helminth infection and**
663 **reproductive state. (a)** Values represent the relative abundance of *Lactobacillus*
664 according to host sex and reproductive status. **(b)** Nematode infection status is
665 positively associated with *Escherichia* and negatively associated with an unclassified
666 genus within the Lachnospiraceae family. All samples with non-zero abundance were
667 included. Values are mean±sem (n=92-205 samples per group).
668
669

670 **Table legend**

671

672 **Table 1. Environmental and host factors associated with microbial community**

673 **structure and membership in linear mixed models.** Each model (response variable)

674 is shown in a single row, with predictor variables in columns. Numbers indicate FDR-

675 adjusted p -values (*i.e.* q -values; see *Methods*), and shading indicates significance level

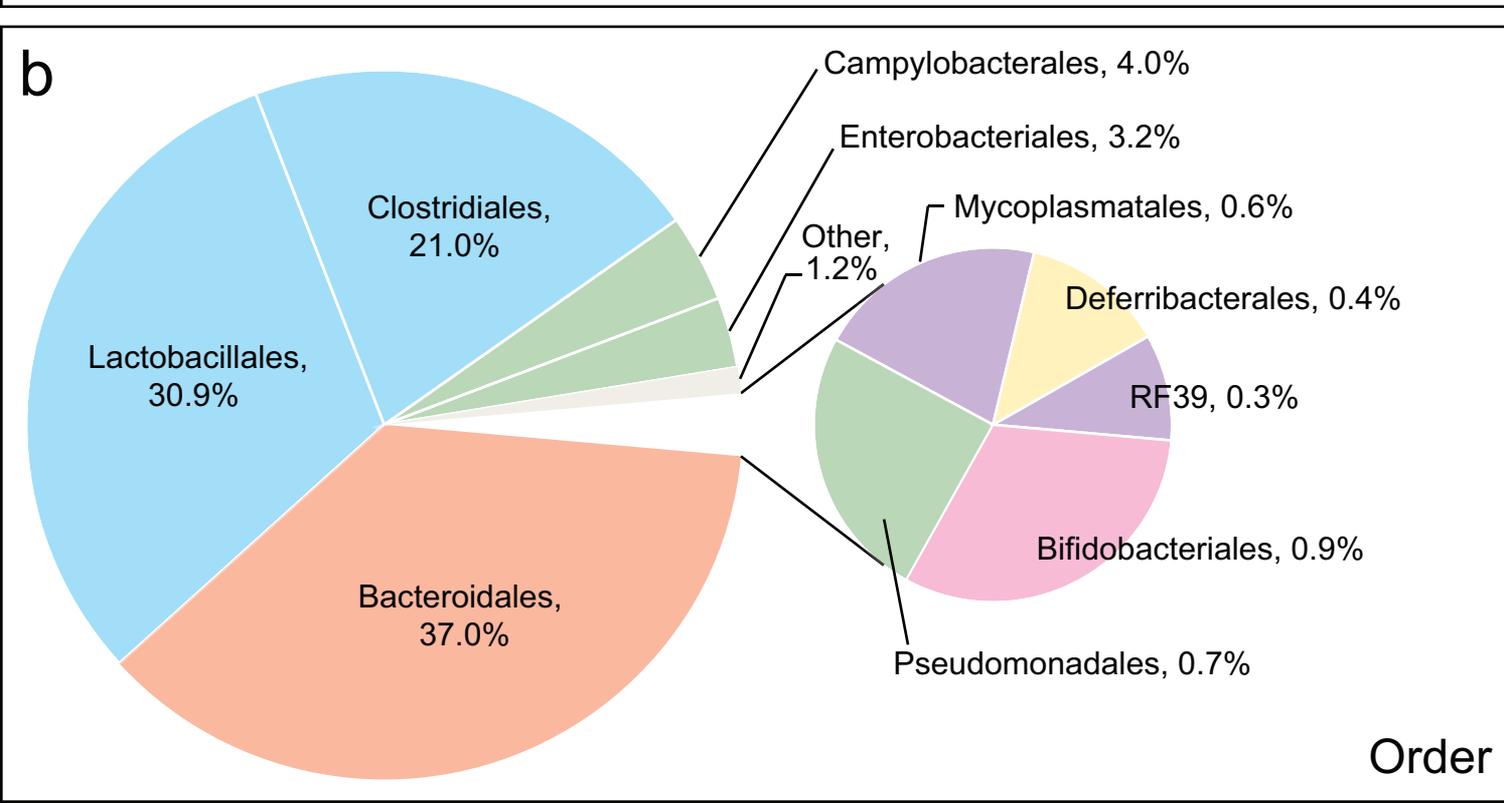
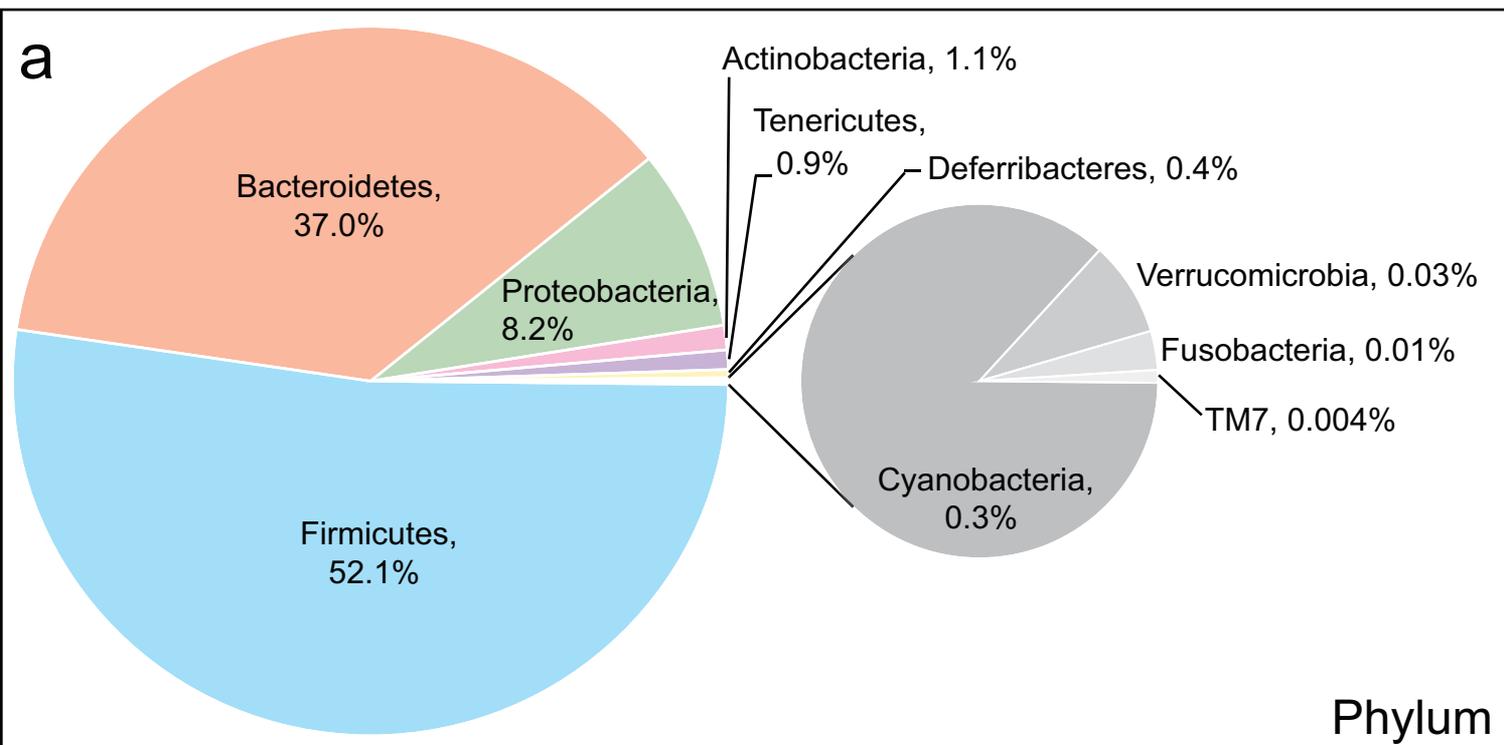
676 for each term in the minimal model following FDR-adjustment (red: $q < 0.001$; orange:

677 $0.001 < q < 0.01$; yellow: $0.01 < q < 0.05$; blank cells $q > 0.05$. Grey cells indicate significant q -

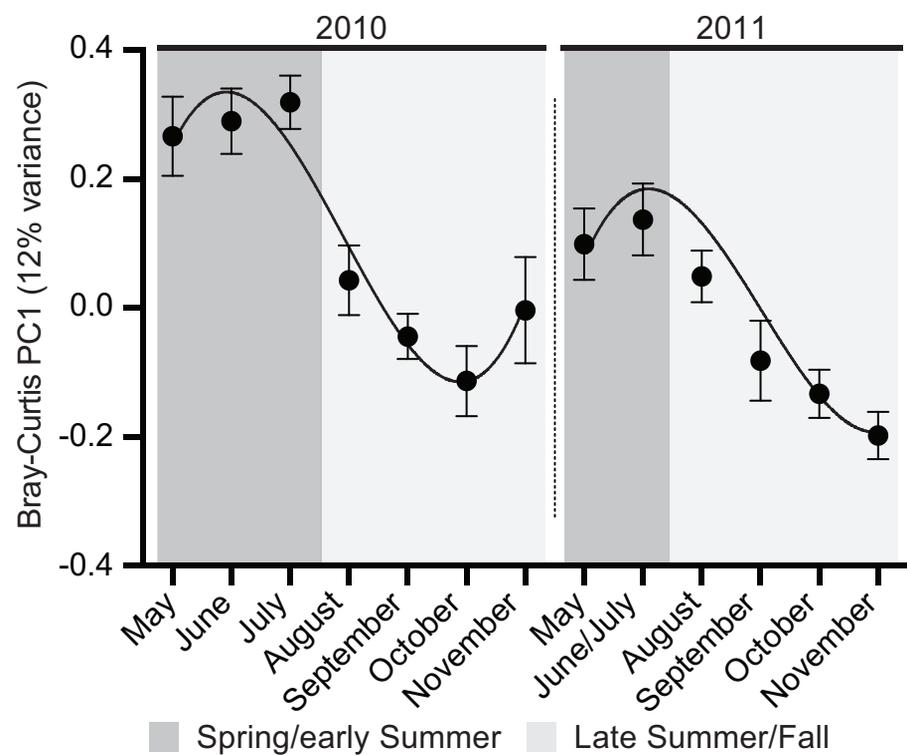
678 values for effects tested after removing interactions involving the component terms from

679 the minimal model.

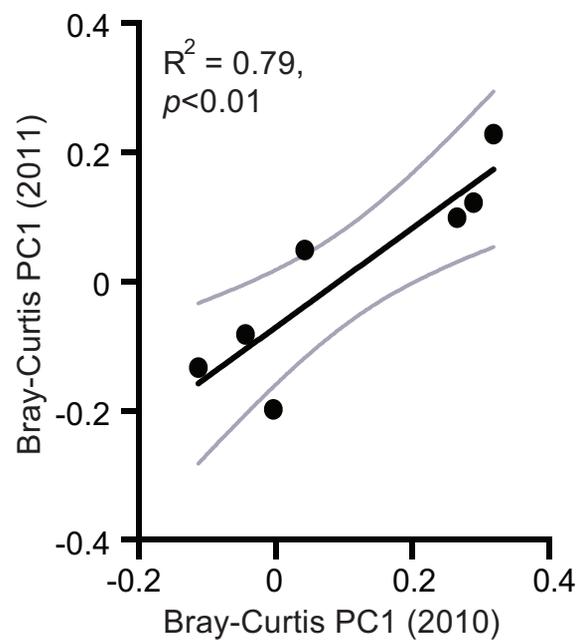
Figure 1



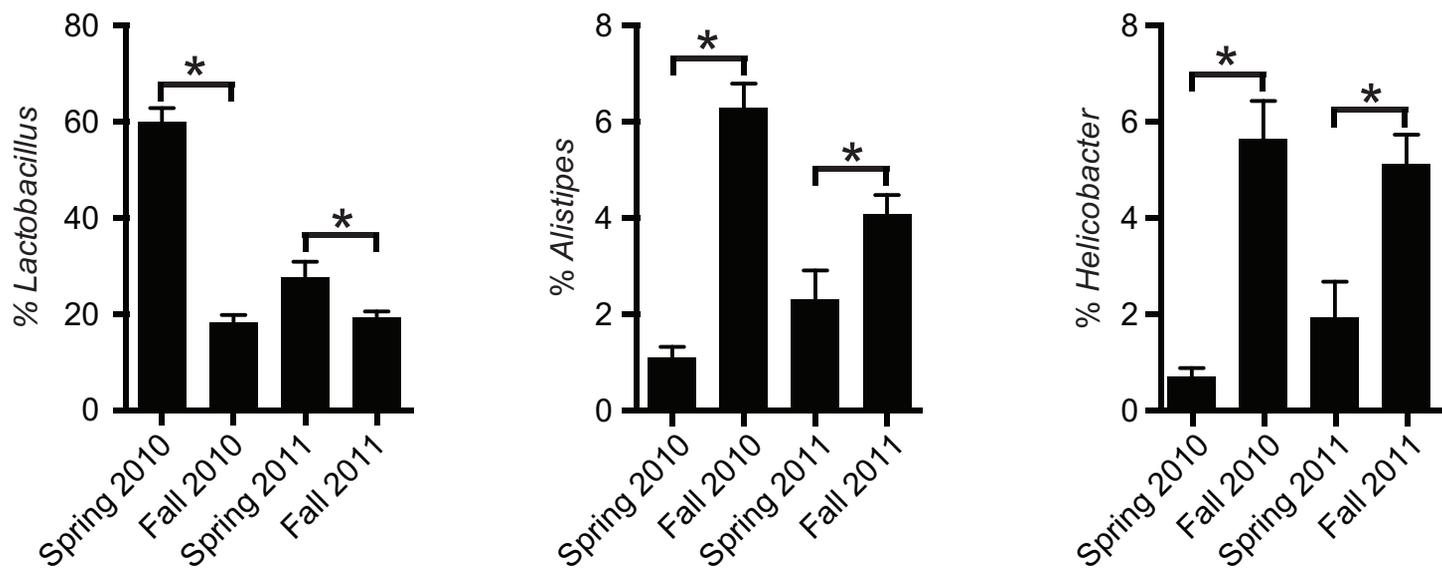
a

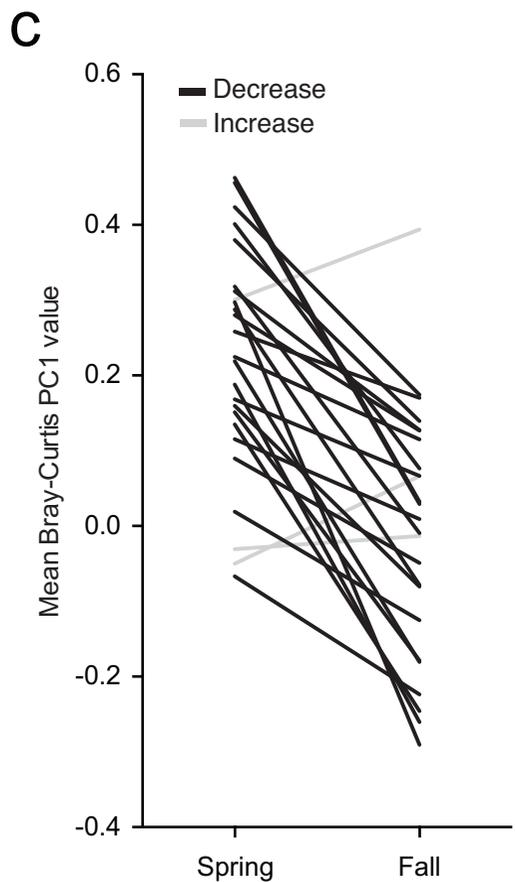
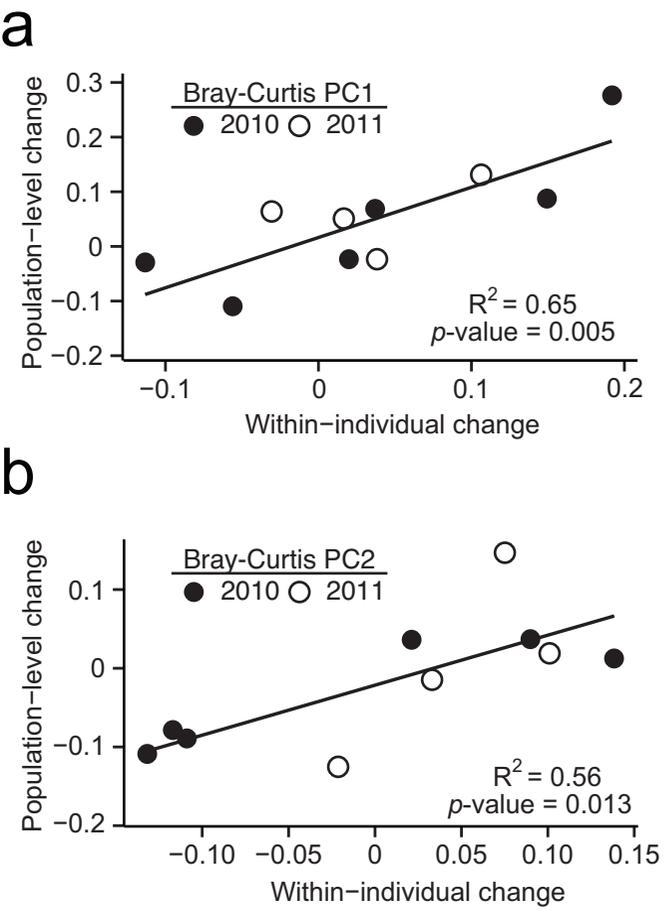


b



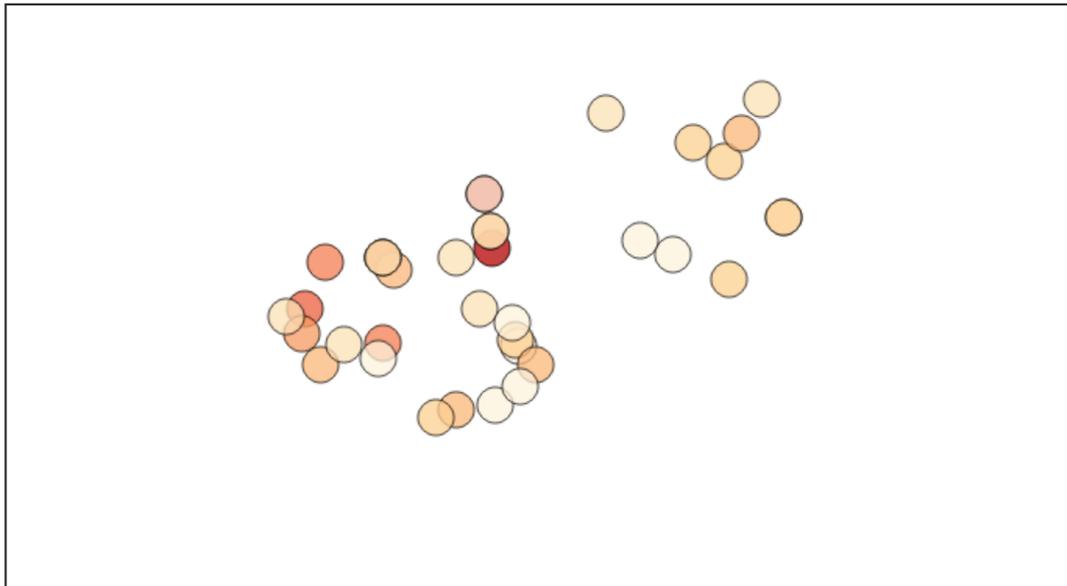
c



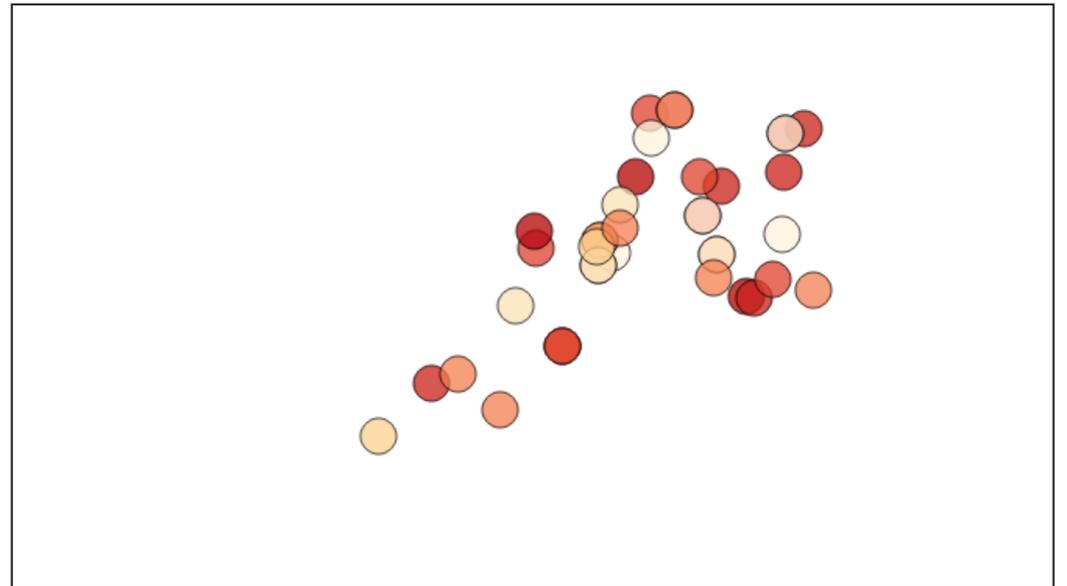


Manor Wood

2010



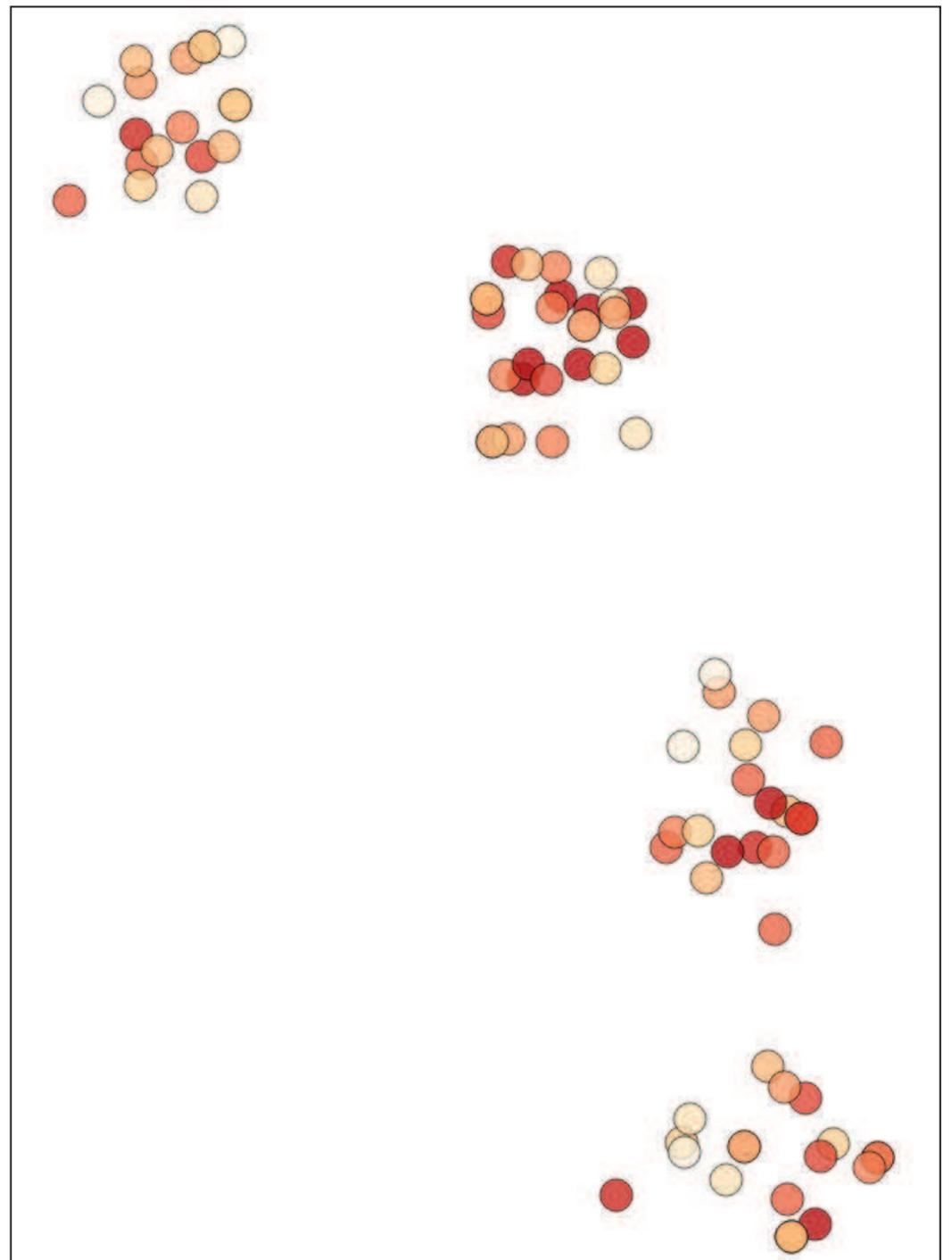
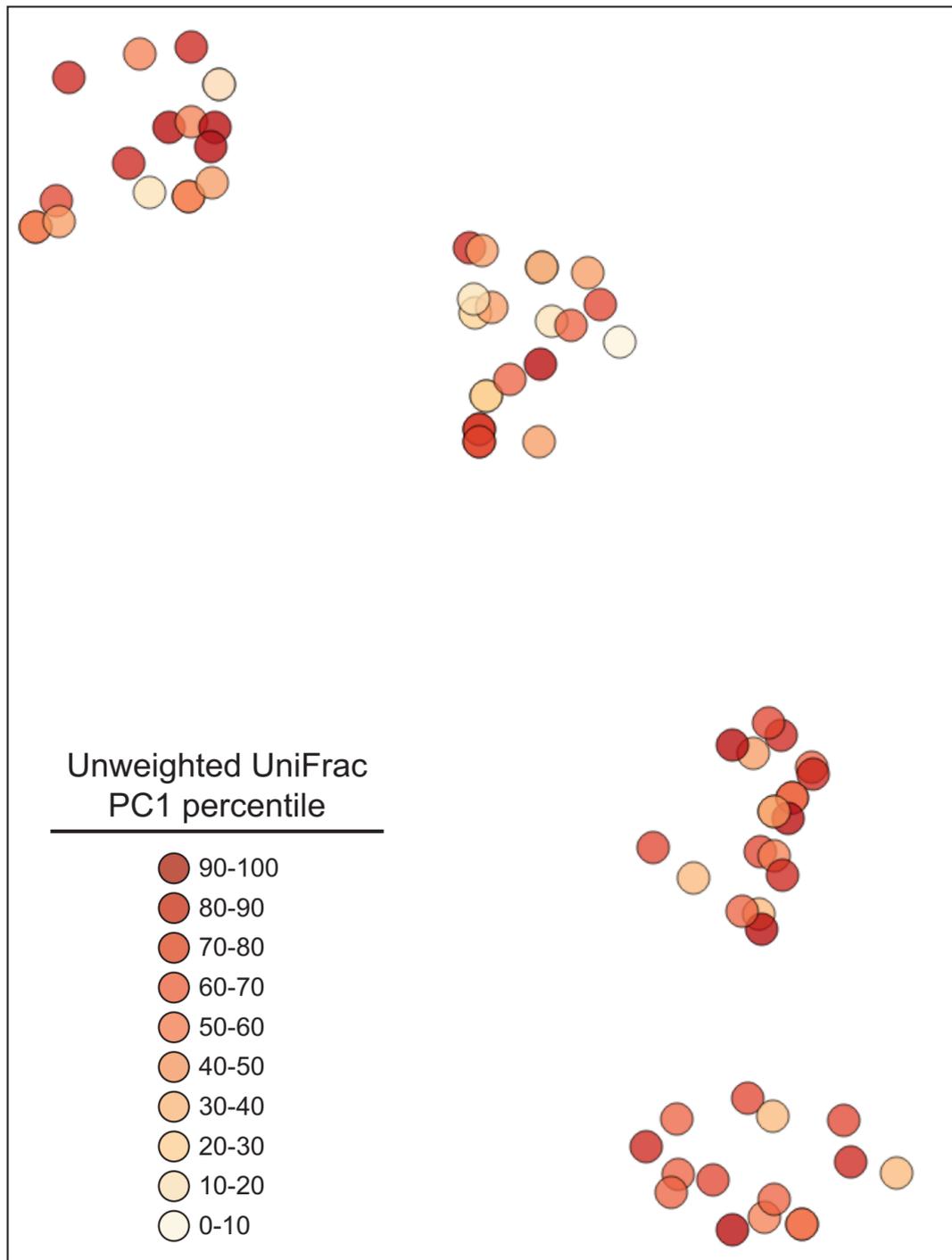
2011



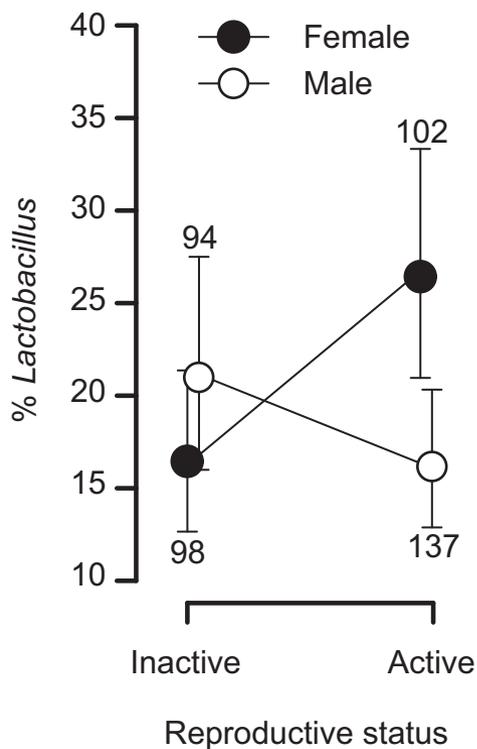
Haddon Wood

Unweighted UniFrac
PC1 percentile

- 90-100
- 80-90
- 70-80
- 60-70
- 50-60
- 40-50
- 30-40
- 20-30
- 10-20
- 0-10



a



b

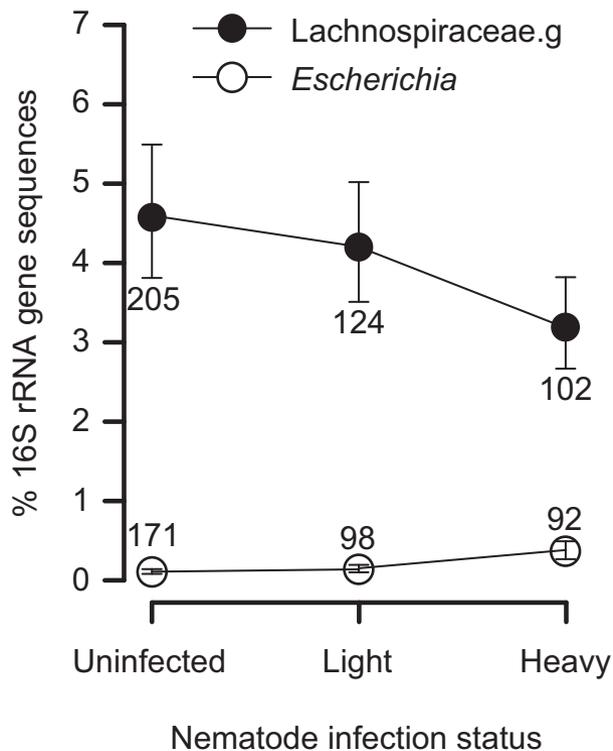


Table 1. Environmental and host factors associated with microbial community structure and membership in linear mixed models.

Model	Response variable	n	Extrinsic factors					Intrinsic factors				Parasites and treatment						
			External temperature	Year	Month	Year: Month	Grid	Density	Age	Sex	Reprod	Reprod: Sex	Nematodes	Coccidia	Treated	Nematodes: Treated	Coccidia: Treated	
Community dissimilarity metrics																		
1	Bray-Curtis PC1	431		<0.0001	<0.0001	<0.0001	<0.0001	0.0064		0.0198		0.0140						
2	Bray-Curtis PC2	431		<0.0001	<0.0001	<0.0001	<0.0001	0.0175				0.0193	0.0161					
3	Unweighted Unifrac PC1	431		<0.0001	<0.0001	0.0001	<0.0001	<0.0001		0.0054								
4	Unweighted Unifrac PC2	431		0.0146	<0.0001	<0.0001	0.0090		0.0043									
5	Weighted Unifrac PC1	431		0.0805	<0.0001	<0.0001	0.0155					0.0048						
6	Weighted Unifrac PC2	431		<0.0001	<0.0001	0.0032	<0.0001	0.0047		0.0174								
Individual genera																		
7	Bacteroidales (unknown family & genus)	431			0.0012	0.0054	0.0266					0.044						
8	<i>Lactobacillus</i>	431		0.0097	<0.0001	<0.0001	0.0002					0.002						
9	Lachnospiraceae (unknown genus)	431		<0.0001	0.0007			0.0335		0.006			0.013					
10	<i>Alistipes</i>	431			<0.0001	0.0008	0.0012		0.001									
11	<i>Helicobacter</i>	422			<0.0001	0.0167												
12	Ruminococcaceae (unknown genus)	431		<0.0001	<0.0001	0.0001	0.0003	0.0048		0.014								
13	<i>Streptococcus</i>	410			<0.0001					0.024								
14	<i>Escherichia</i>	361		0.0033									0.043					
15	Catabacteriaceae (unknown genus)	427		<0.0001	<0.0001	<0.0001		0.0038					0.036					
16	<i>Clostridium</i>	431	0.0434	<0.0001						0.046								
												q < 0.001		0.001 ≤ q ≤ 0.01		0.01 ≤ q < 0.05		