Marked seasonal variation in the wild mouse gut microbiota

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Abstract

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

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

Introduction

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

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

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

Materials and Methods

Sample collection

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

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

Host phenotyping

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

16S rRNA gene sequencing and analysis

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

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

Parasite diagnosis

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

Linear mixed models

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

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

Spatial structuring of microbial communities

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

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

Results

The wild mouse gut harbours abundant Lactobacilli and putative enteric pathogens

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

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

Marked seasonal variation in microbial community structure

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

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

Multivariate modelling reveals associations with both host and environmental factors

We next used linear mixed models (LMMs; see *Methods*) to tease apart the relative influence of multiple environmental and host factors, and to determine their effects in isolation of confounding factors. We constructed 6 models for community dissimilarity metrics (principal coordinates 1 and 2 for Bray-Curtis, unweighted UniFrac, and weighted UniFrac) as well as separate models for the 10 most abundant bacterial genera (Table 1). Overall, these analyses suggest that the wood mouse gut microbiota is primarily shaped by environmental factors, with significant evidence for both temporal (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: $R^2_{GLMM(m)}$ =43.9% with month only vs.
312 48.7% with month and year; PC2: $R^2_{GLMM(m)}$ = 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 ($R^2_{GLMM(m)}$ =52% vs 49% variance explained), with a 2-fold increase in variance
316 explained for PC2 ($R^2_{GLMM(m)}$ =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

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

Discussion

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

In contrast to “specific pathogen free” laboratory mice, we detected widespread colonization by bacterial taxa that contain enteric pathogens, including *Helicobacter* and other Proteobacteria. However, given the resolution of our sequencing methods and the limited studies of wild mouse pathogens we cannot exclude the fact that these are 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 (Yatsunenko
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

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

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

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

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Conflict of Interest

The authors declare no conflict of interest.

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

References

Amato KR, Yeoman CJ, Kent A, Righini N, Carbonero F, Estrada A *et al* (2013). Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes. *ISME J* **7:** 1344-1353.

Amato KR, Leigh SR, Kent A, Mackie RI, Yeoman CJ, Stumpf RM *et al* (2015). The Gut Microbiota Appears to Compensate for Seasonal Diet Variation in the Wild Black Howler Monkey (Alouatta pigra). *Microb Ecol* **69:** 434-443.

Bates D, Maechler M, Bolker B (2013). lme4: Linear mixed-effects models using S4 classes R package version 0.999999-2. edn.

Benson AK, Kelly SA, Legge R, Ma F, Low SJ, Kim J *et al* (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci USA* **107:** 18933-18938.

Bolnick DI, Snowberg LK, Hirsch PE, Lauber CL, Knight R, Caporaso JG *et al* (2014). Individuals' diet diversity influences gut microbial diversity in two freshwater fish (threespine stickleback and Eurasian perch). *Ecol Lett* **17:** 979-987.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK *et al* (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7:** 335-336.

Carey HV, Walters WA, Knight R (2013). Seasonal restructuring of the ground squirrel gut microbiota over the annual hibernation cycle. *Am J Physiol Regul Integr Comp Physiol* **304:** R33-42.

Carmody RN, Gerber GK, Luevano JM, Jr., Gatti DM, Somes L, Svenson KL *et al* (2015). Diet dominates host genotype in shaping the murine gut microbiota. *Cell Host Microbe* **17:** 72-84.

Costello EK, Stagaman K, Dethlefsen L, Bohannan BJ, Relman DA (2012). The application of ecological theory toward an understanding of the human microbiome. *Science* **336:** 1255-1262.

Davenport ER, Mizrahi-Man O, Michelini K, Barreiro LB, Ober C, Gilad Y (2014). Seasonal variation in human gut microbiome composition. *PLoS One* **9:** e90731.

David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE *et al* (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505:** 559-563.

Degnan PH, Pusey AE, Lonsdorf EV, Goodall J, Wroblewski EE, Wilson ML *et al* (2012). Factors associated with the diversification of the gut microbial communities within chimpanzees from Gombe National Park. *Proc Natl Acad Sci USA* **109:** 13034-13039.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K *et al* (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72:** 5069-5072.

504 Di Rienzi SC, Sharon I, Wrighton KC, Koren O, Hug LA, Thomas BC *et al* (2013). The
505 human gut and groundwater harbor non-photosynthetic bacteria belonging to a new
506 candidate phylum sibling to Cyanobacteria. *Elife* **2:** e01102.
507 Duncan SH, Belenguer A, Holtrop G, Johnstone AM, Flint HJ, Lobley GE (2007).
508 Reduced dietary intake of carbohydrates by obese subjects results in decreased
509 concentrations of butyrate and butyrate-producing bacteria in feces. *Appl Environ*
510 *Microbiol* **73:** 1073-1078.
511 Eaton KA, Honkala A, Auchtung TA, Britton RA (2011). Probiotic Lactobacillus reuteri
512 ameliorates disease due to enterohemorrhagic Escherichia coli in germfree mice.
513 *Infect Immun* **79:** 185-191.
514 Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL *et al*
515 (2013). The long-term stability of the human gut microbiota. *Science* **341:** 1237439.
516 Godsall B, Coulson T, Malo AF (2014). From physiology to space use: energy reserves
517 and androgenization explain home-range size variation in a woodland rodent. *J Anim*
518 *Ecol* **83:** 126-135.
519 Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R *et al* (2014).
520 Human genetics shape the gut microbiome. *Cell* **159:** 789-799.
521 Gurnell J (1993). Tree seed production and food conditions for rodents in an Oak Wood
522 in southern England. *Forestry* **66:** 291-315.
523 Hayes KS, Bancroft AJ, Goldrick M, Portsmouth C, Roberts IS, Grencis RK (2010).
524 Exploitation of the intestinal microflora by the parasitic nematode Trichuris muris.
525 *Science* **328:** 1391-1394.

526 Kabir AM, Aiba Y, Takagi A, Kamiya S, Miwa T, Koga Y (1997). Prevention of
527 Helicobacter pylori infection by lactobacilli in a gnotobiotic murine model. *Gut* **41:** 49-
528 55.
529 Keeney KM, Finlay BB (2011). Enteric pathogen exploitation of the microbiota-
530 generated nutrient environment of the gut. *Curr Opin Microbiol* **14:** 92-98.
531 Knowles SC, Fenton A, Petchey OL, Jones TR, Barber R, Pedersen AB (2013). Stability
532 of within-host-parasite communities in a wild mammal system. *Proc Biol Sci* **280:**
533 20130598.
534 Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Backhed HK *et al* (2012). Host
535 remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell*
536 **150:** 470-480.
537 Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS *et al* (2008a).
538 Evolution of mammals and their gut microbes. *Science* **320:** 1647-1651.
539 Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI (2008b). Worlds within worlds:
540 evolution of the vertebrate gut microbiota. *Nat Rev Microbiol* **6:** 776-788.
541 Linnenbrink M, Wang J, Hardouin EA, Kunzel S, Metzler D, Baines JF (2013). The role
542 of biogeography in shaping diversity of the intestinal microbiota in house mice. *Mol*
543 *Ecol* **22:** 1904-1916.
544 Maurice CF, Haiser HJ, Turnbaugh PJ (2013). Xenobiotics shape the physiology and
545 gene expression of the active human gut microbiome. *Cell* **152:** 39-50.
546 McKnite AM, Perez-Munoz ME, Lu L, Williams EG, Brewer S, Andreux PA *et al* (2012).
547 Murine gut microbiota is defined by host genetics and modulates variation of
548 metabolic traits. *PLoS One* **7:** e39191.

549 Medellin-Pena MJ, Griffiths MW (2009). Effect of molecules secreted by Lactobacillus
550 acidophilus strain La-5 on Escherichia coli O157:H7 colonization. *Appl Environ*
551 *Microbiol* **75:** 1165-1172.
552 Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M *et al* (2008). The
553 metagenomics RAST server - a public resource for the automatic phylogenetic and
554 functional analysis of metagenomes. *BMC Bioinformatics* **9:** 386.
555 Moran PA (1950). Notes on continuous stochastic phenomena. *Biometrika* **37:** 17-23.
556 Muegge BD, Kuczynski J, Knights D, Clemente JC, Gonzalez A, Fontana L *et al* (2011).
557 Diet drives convergence in gut microbiome functions across mammalian phylogeny
558 and within humans. *Science* **332:** 970-974.
559 Nakagawa S, Schielzeth H (2013). A general and simple method for obtaining R2 from
560 generalized linear mixed-effects models. *Methods Ecol Evol* **4:** 133-142.
561 Nowell F, Higgs S (1989). Eimeria species infecting wood mice (genus Apodemus) and
562 the transfer of two species to Mus musculus. *Parasitology* **98 Pt 3:** 329-336.
563 O'Rourke JL, Grehan M, Lee A (2001). Non-pylori Helicobacter species in humans. *Gut*
564 **49:** 601-606.
565 Ochman H, Worobey M, Kuo CH, Ndjango JB, Peeters M, Hahn BH *et al* (2010).
566 Evolutionary relationships of wild hominids recapitulated by gut microbial
567 communities. *PLoS Biol* **8:** e1000546.
568 Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB *et al* (2015).
569 vegan: Community Ecology Package. R package version 2.2-1. *http://CRANR-*
570 *projectorg/package=vegan*.

571 Parker SE, Malone S, Bunte RM, Smith AL (2009). Infectious diseases in wild mice
572 (Mus musculus) collected on and around the University of Pennsylvania
573 (Philadelphia) Campus. *Comparative medicine* **59:** 424-430.
574 Pena JA, Rogers AB, Ge Z, Ng V, Li SY, Fox JG *et al* (2005). Probiotic Lactobacillus
575 spp. diminish Helicobacter hepaticus-induced inflammatory bowel disease in
576 interleukin-10-deficient mice. *Infect Immun* **73:** 912-920.
577 Pike N (2011). Using false discovery rates for multiple comparisons in ecology and
578 evolution. *Methods Ecol Evol* **2:** 278-282.
579 Pritchard MH, Kruse GOW (1982). *The collection and preservation of animal parasites*,
580 vol. 1. University of Nebraska Press: Lincoln, NE.
581 Rausch S, Held J, Fischer A, Heimesaat MM, Kuhl AA, Bereswill S *et al* (2013). Small
582 intestinal nematode infection of mice is associated with increased enterobacterial
583 loads alongside the intestinal tract. *PLoS One* **8:** e74026.
584 Reynolds LA, Smith KA, Filbey KJ, Harcus Y, Hewitson JP, Redpath SA *et al* (2014).
585 Commensal-pathogen interactions in the intestinal tract: lactobacilli promote
586 infection with, and are promoted by, helminth parasites. *Gut Microbes* **5:** 522-532.
587 Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS *et al* (2012a).
588 Metagenomic biomarker discovery and explanation. *Genome Biol* **12:** R60.
589 Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C (2012b).
590 Metagenomic microbial community profiling using unique clade-specific marker
591 genes. *Nat Methods* **9:** 811-814.
592 Sommer F, Backhed F (2013). The gut microbiota--masters of host development and
593 physiology. *Nat Rev Microbiol* **11:** 227-238.

Walk ST, Blum AM, Ewing SA, Weinstock JV, Young VB (2010). Alteration of the murine gut microbiota during infection with the parasitic helminth Heligmosomoides polygyrus. *Inflamm Bowel Dis* **16:** 1841-1849.

Wang J, Linnenbrink M, Kunzel S, Fernandes R, Nadeau MJ, Rosenstiel P *et al* (2014). Dietary history contributes to enterotype-like clustering and functional metagenomic content in the intestinal microbiome of wild mice. *Proc Natl Acad Sci U S A* **111:** E2703-2710.

Wasimuddin, Cizkova D, Bryja J, Albrechtova J, Hauffe HC, Pialek J (2012). High prevalence and species diversity of Helicobacter spp. detected in wild house mice. *Appl Environ Microbiol* **78:** 8158-8160.

Watts CHB (1968). Foods eaten by wood mice (Apodemus sylvaticus) and bank voles (Clethrionomys glareolus) in Wytham Woods, Berkshire. *J Anim Ecol* **37:** 25-41.

Wolfe BE, Button JE, Santarelli M, Dutton RJ (2014). Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell* **158:** 422-433.

Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M *et al* (2012). Human gut microbiome viewed across age and geography. *Nature* **486:** 222-227.

Zenewicz LA, Yin X, Wang G, Elinav E, Hao L, Zhao L *et al* (2013). IL-22 deficiency alters colonic microbiota to be transmissible and colitogenic. *J Immunol* **190:** 5306-5312.

Figure legends

Figure 1. Taxonomic analysis of the wild mouse gut microbiota. Pie charts represent the relative abundance of bacterial **(a)** phyla and **(b)** orders (n=481 samples). The 10 most abundant phyla and orders are shown (phyla with a mean abundance <0.001% are not included; the remaining orders are represented by the "other" slice). Taxa are colored based on phylum. Sequences within the Cyanobacteria phylum could be attributed to chloroplasts (order Streptophyta), non-photosynthetic bacteria related to Cyanobacteria that are common in the mammalian gut (order YS2) (Di Rienzi et al 2013), and algae (order Chlorophyta, family Trebouxiophyceae). We did not detect any consistent seasonal changes in the abundance or prevalence of these three groups.

Figure 2. Seasonal variations in the wild mouse gut microbiota. (a) The first principle coordinate from a Bray-Curtis-based analysis of microbial community structure over time. Trend lines were generated by fitting a polynomial function to values from each year (GraphPad Prism version 6.0). Values are mean±sem (n=14-80 samples per group). Values from June and July were combined in 2011 due to limited available samples in July (n=2). **(b)** Association between average monthly microbial community structures between years. Values are mean (thick black line) and 95% CI (thin grey lines) from a linear regression. **(c)** The relative abundance of bacterial genera in spring and fall of both years. Values are mean±sem (n=24-123 samples per group; the first sample from each mouse was included). Asterisks represent significant differences (*p*-value<0.05, Wilcoxon rank-sum test).

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

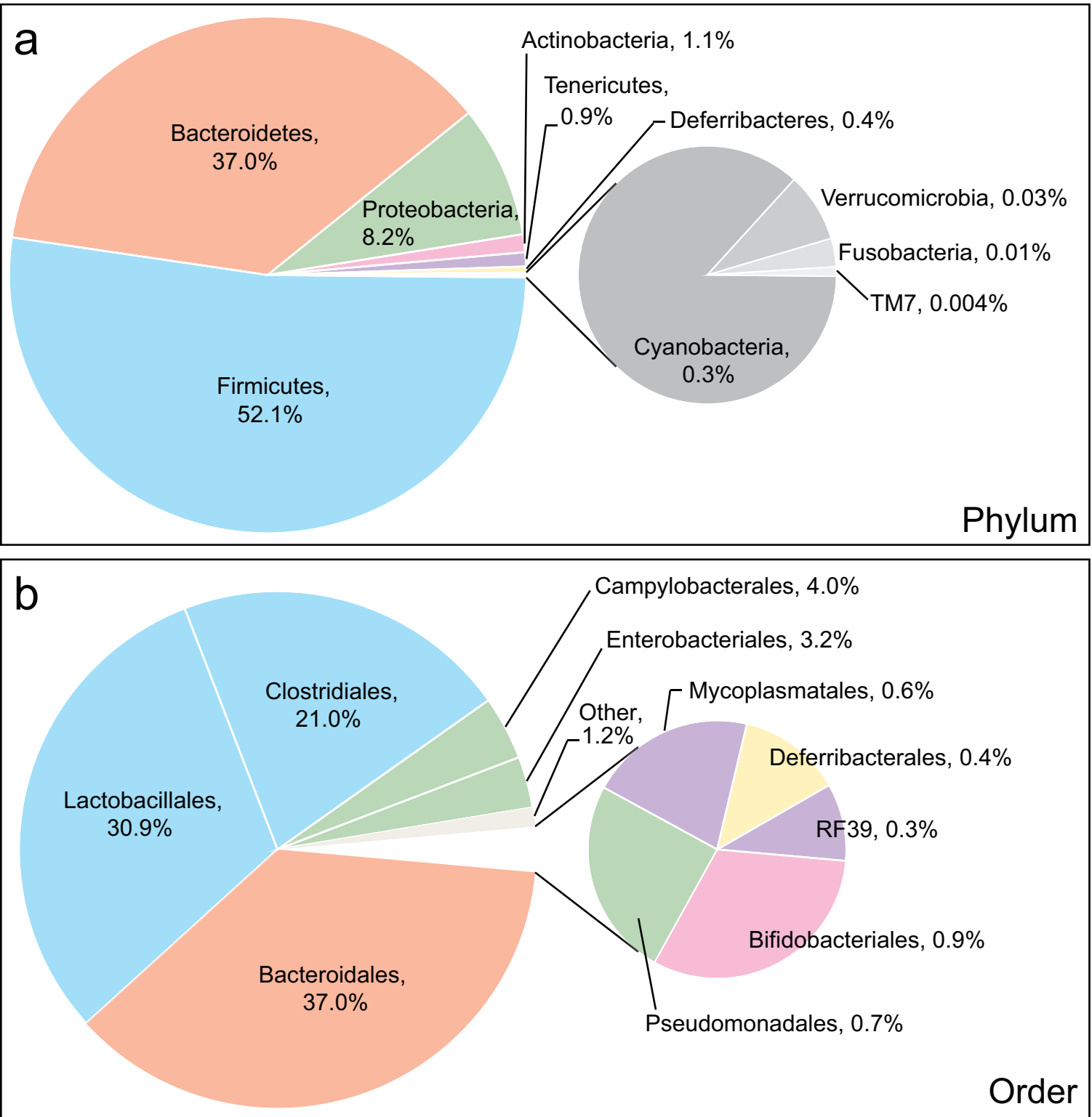
Figure 4. Spatial distribution of microbial community structure. Each circle represents the physical location of a given mouse at the time of sampling in Manor or Haddon Wood, which are subdivided into 2 and 4 fields, respectively. Shading is proportional to the percentile along unweighted UniFrac principal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

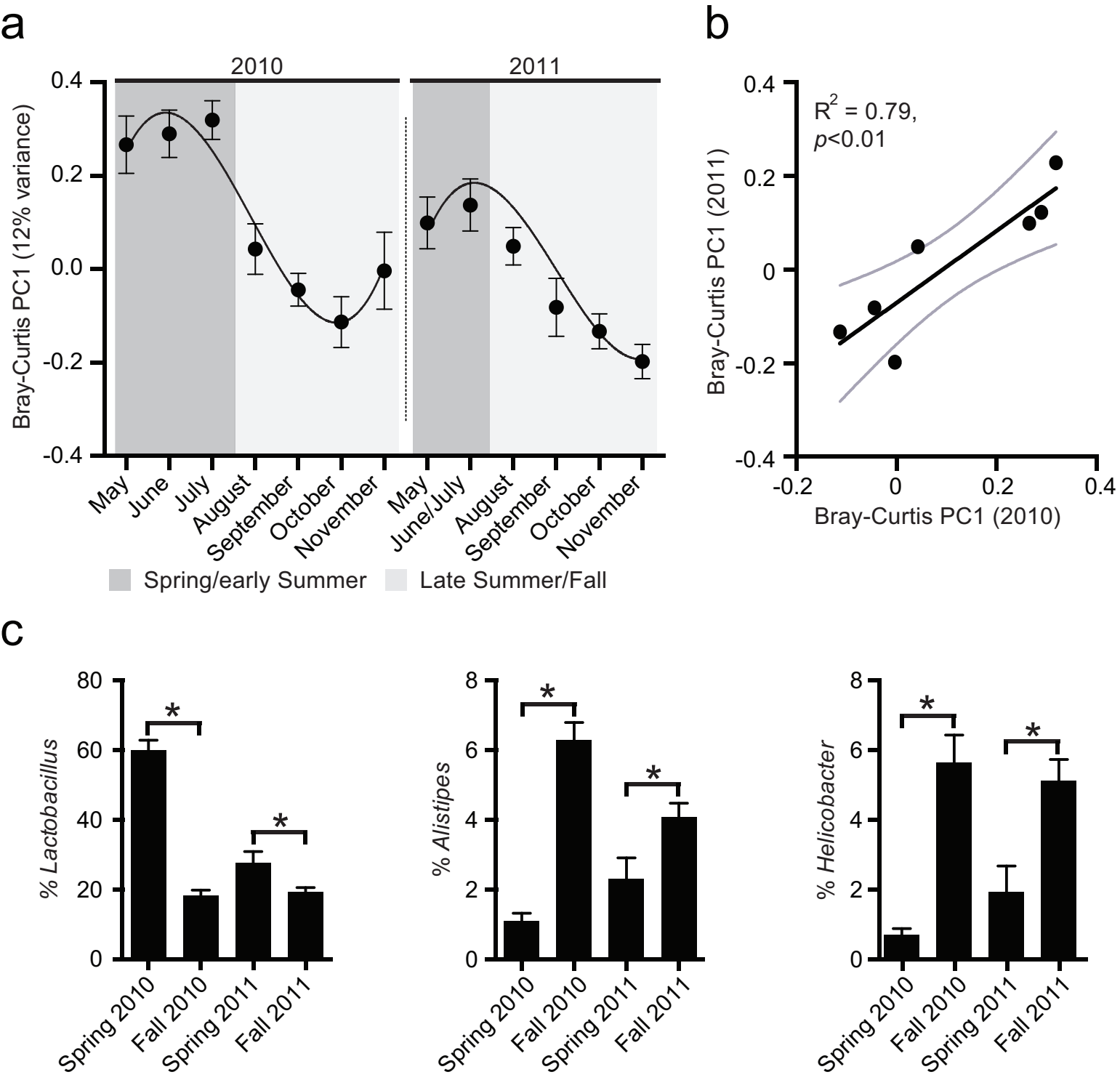
Figure 5. The gut microbiota is associated with intestinal helminth infection and reproductive state. (a) Values represent the relative abundance of *Lactobacillus* according to host sex and reproductive status. **(b)** Nematode infection status is positively associated with *Escherichia* and negatively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

Table legend

Table 1. Environmental and host factors associated with microbial community structure and membership in linear mixed models. Each model (response variable) is shown in a single row, with predictor variables in columns. Numbers indicate FDR-adjusted *p*-values (*i.e. q*-values; see *Methods*), and shading indicates significance level for each term in the minimal model following FDR-adjustment (red: $q<0.001$; orange: $0.001<q<0.01$; yellow: $0.01<q<0.05$; blank cells $q>0.05$. Grey cells indicate significant *q*-values for effects tested after removing interactions involving the component terms from the minimal model.

Figure 1


a
Actinobacteria, 1.1%
Tenericutes, 0.9%
Deferribacteres, 0.4%
Verrucomicrobia, 0.03%
Fusobacteria, 0.01%
TM7, 0.004%
Cyanobacteria, 0.3%
Bacteroidetes, 37.0%
Proteobacteria, 8.2%
Firmicutes, 52.1%
Phylum
b
Campylobacterales, 4.0%
Enterobacteriales, 3.2%
Mycoplasmatales, 0.6%
Other, 1.2%
Deferribacterales, 0.4%
RF39, 0.3%
Bifidobacteriales, 0.9%
Pseudomonadales, 0.7%
Clostridiales, 21.0%
Lactobacillales, 30.9%
Bacteroidales, 37.0%
Order


a
2010
2011
Bray-Curtis PC1 (12% variance)
0.4
0.2
0.0
-0.2
-0.4
May
June
July
August
September
October
November
May
June/July
August
September
October
November
Spring/early Summer
Late Summer/Fall
b
R^2 = 0.79,
p<0.01
Bray-Curtis PC1 (2011)
Bray-Curtis PC1 (2010)
c
% Lactobacillus
% Alistipes
% Helicobacter
Spring 2010
Fall 2010
Spring 2011
Fall 2011
*

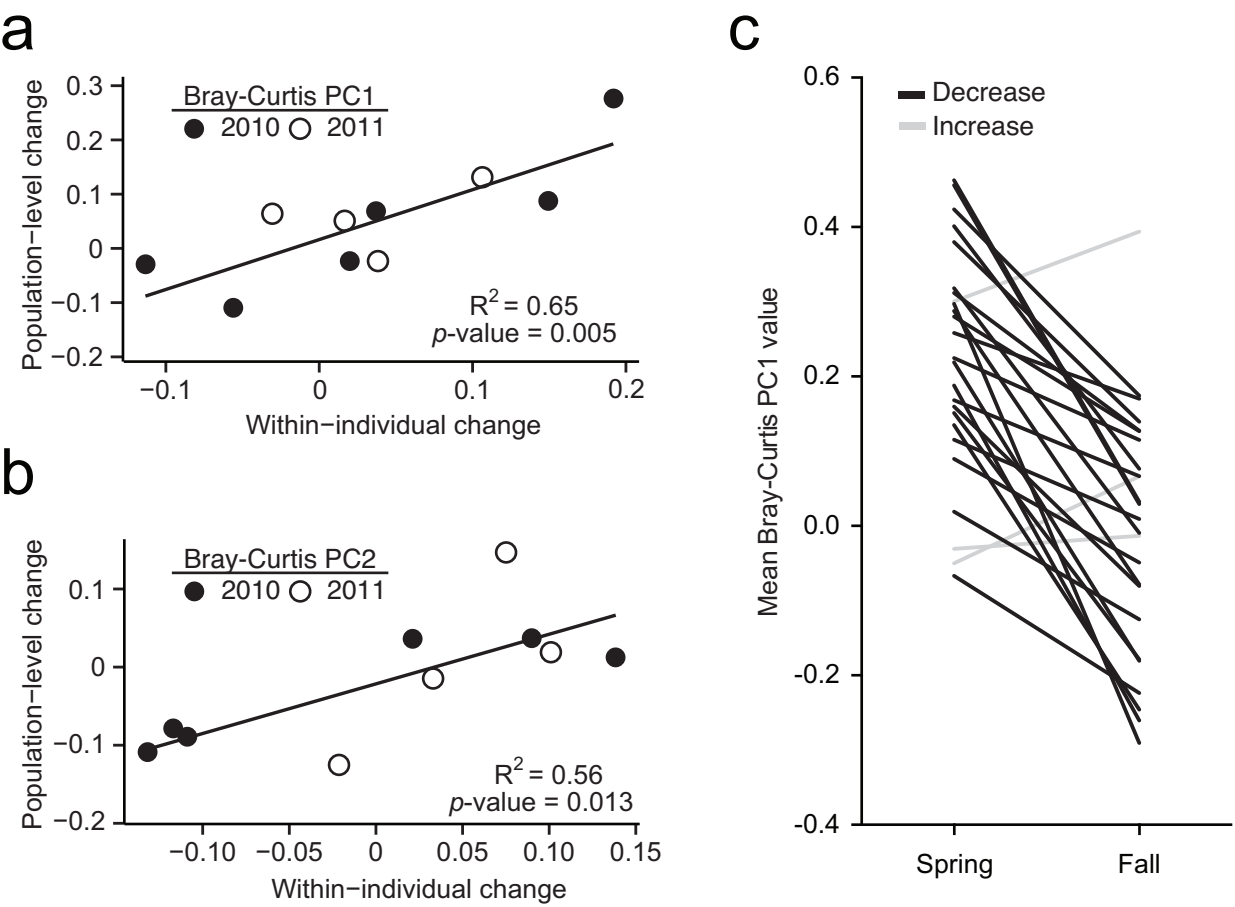

a
Bray-Curtis PC1
2010 2011
R^2 = 0.65
p-value = 0.005
Population−level change
Within−individual change
b
Bray-Curtis PC2
2010 2011
R^2 = 0.56
p-value = 0.013
Population−level change
Within−individual change
c
Decrease
Increase
Mean Bray-Curtis PC1 value
Spring
Fall

Figure 4

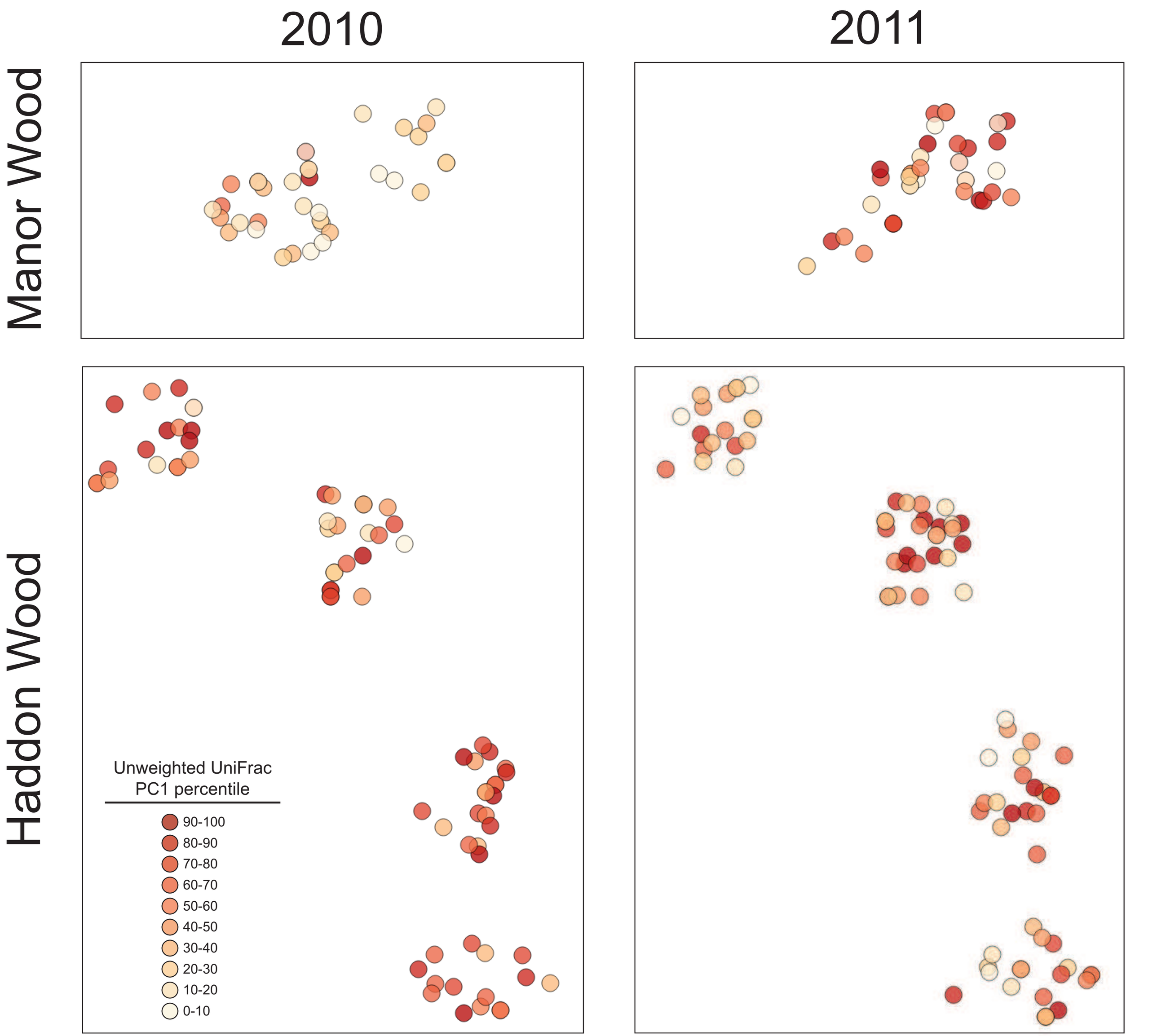

2010
2011
Manor Wood
Haddon Wood
Unweighted UniFrac
PC1 percentile
90-100
80-90
70-80
60-70
50-60
40-50
30-40
20-30
10-20
0-10

Figure 5

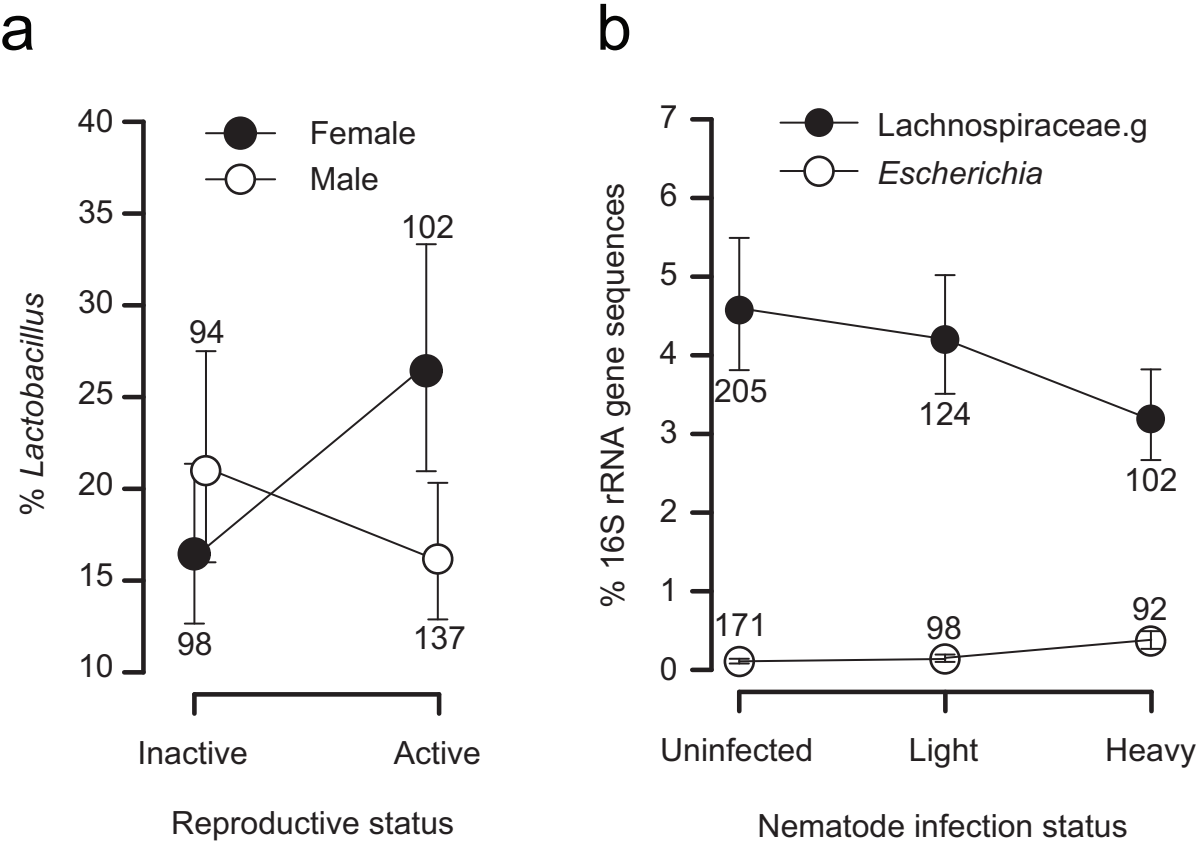

a
b
40
35
30
25
20
15
10
% Lactobacillus
Female
Male
102
94
98
137
Inactive
Active
Reproductive status
7
6
5
4
3
2
1
0
% 16S rRNA gene sequences
Lachnospiraceae.g
Escherichia
205
124
102
171
98
92
Uninfected
Light
Heavy
Nematode infection status

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

Model	Response variable	Extrinsic factors						Intrinsic factors				Parasites and treatment				
		n	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		0.0175				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	*Lactobacillus*	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	*Alistipes*	431			<0.0001	0.0008	0.0012		0.001								
11	*Helicobacter*	422			<0.0001	0.0167											
12	Ruminococcaceae (unknown genus)	431		<0.0001	<0.0001	0.0001	0.0003	0.0048		0.014							
13	*Streptococcus*	410			<0.0001					0.024							
14	*Escherichia*	361		0.0033									0.043				
15	Catabacteriaceae (unknown genus)	427		<0.0001	<0.0001	<0.0001		0.0038					0.036				
16	*Clostridium*	431	0.0434	<0.0001						0.046							

q < 0.001 **0.001 ≤ q ≤ 0.01** **0.01 ≤ q < 0.05**