Weanling gut microbiota composition of a mouse model selectively bred for high voluntary wheel-running behavior

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Abstract

We compared the fecal microbial community composition and diversity of four replicate lines of mice selectively bred for high wheel-running activity over 81 generations (HR lines) and four non-selected control (C) lines. We performed 16S rRNA gene sequencing on fecal samples taken 24 hours after weaning, identifying a total of 2,074 bacterial Operational Taxonomic Units. HR and C mice did not significantly differ for measures of alpha diversity, but HR had a higher relative abundance of the family *Clostridiaceae*. These results differ from a study of rats, where a line bred for high forced-treadmill endurance and that also ran more on wheels had lower relative abundance of *Clostridiaceae*, as compared with a line bred for low endurance that ran less on wheels. Within the HR and C groups, replicate lines had unique microbiomes based on unweighted UniFrac beta diversity, indicating random genetic drift and/or multiple adaptive responses to selection.

INTRODUCTION

The mammalian gut microbiome plays an essential role in a host's biology, including immune system function, energy extraction, and protection from pathogens (Gilbert et al., 2018; Kohl and Carey, 2016). Within an individual, the microbiome is shaped by both host genetics and environmental factors (Benson et al., 2010; Carmody et al., 2015; Tamburini et al., 2016).

Both acute and chronic voluntary exercise can affect the gut microbiome in rodents and humans (Campbell and Wisniewski, 2017; Mailing et al., 2019; Mohr et al., 2020). For example, adult rats given wheels for 5 weeks had more butyrate-producing bacteria in their ceca compared to sedentary controls, and an increased amount of cecal n-butyrate, an essential short-chain fatty acid for intestinal epithelial cell health (Matsumoto et al., 2008). In human marathoners, fecal samples pre- versus post-event demonstrated rapid changes to their microbiome (Scheiman et al., 2019).

Conversely, the gut microbiome can affect both exercise ability and motivation to engage in exercise (Dohnalová et al., 2022). Among adult male C57BL/6N mice treated with antibiotics then gavaged with cecal microbial communities harvested from sedentary versus exercised mice, recipients of exercised microbiome ran more on wheels compared with recipients of the sedentary microbiome (Oyanagi et al., 2018). With respect to exercise ability, five hours after mice were gavaged with a lactate-metabolizing strain of *Veillonella atypica* cultured from post-marathon athletes, they had significantly longer run times to "exhaustion" versus those gavaged with *Lactobacillus bulgaricus*, a common bacterial symbiont that cannot metabolize lactate (Scheiman et al., 2019). However, the method of motivation was not stated and *Lactobacillus bulgaricus* is a lactate producer, complicating interpretation (Carmody and Baggish, 2019).

We compared the weanling gut microbiota of mice that differ in both exercise ability and motivation: four replicate High Runner (HR) lines selectively bred for high voluntary wheel-running behavior over 81 generations, and 4 non-selected Control (C) lines (Swallow et al., 1998). The selection criterion is revolutions on days 5+6 of a 6-day access period between 6-8 weeks of age. HR and C mice differ in several ways that potentially correlate with unique gut microbial communities. First, HR mice run ~2.5-3-

fold more revolutions per day (Careau et al., 2013; Copes et al., 2015) and also have higher activity when housed individually without wheels (Copes et al., 2015; Malisch et al., 2009). High activity is accompanied by elevated food consumption relative to body size (Copes et al., 2015; Hiramatsu and Garland, 2018; Swallow et al., 2001), which could directly affect gut microbial community composition via changes in luminal resources (Alcock et al., 2014). HR and C mice have not been found to differ in small or large intestine mass or length, thus suggesting a faster transit time in HR mice (Kelly et al., 2017). HR mice also have higher body temperatures when active (Rhodes et al., 2000), altered hormone levels (Garland et al., 2016; Malisch et al., 2009), and tend to be smaller at weaning (Cadney et al., 2021; McNamara et al., 2022; Swallow et al., 1999).

Previously, we reported that adult male HR and C mice differ in gut microbial community composition, regardless of diet or exercise manipulation during early life (McNamara et al., 2021). In addition, when the microbiome is reduced with oral antibiotics, running behavior of C mice is largely unaffected, whereas HR mice run significantly fewer revolutions per day (McNamara et al., 2022), suggesting the gut microbiome is an important component of the high wheel-running phenotype.

Unique gut microbial phenotypes in HR mice could result from acute effects of differences in HR and C traits and/or changes in the selective regime experienced by the microbiota. Our two previous studies cannot distinguish between these possibilities because adults will have experienced many weeks of differences in physical activity, food consumption, and other physiological differences that could acutely affect the microbiome. Although physical activity of HR and C pups prior to weaning has not been quantified, related aspects of pre-weaning behavior do not significantly differ, including first day of eye opening, moving, and feeding on solid food, and locomotor play (see Fig. 2A-C in Hiramatsu et al., 2017, 10-20 days old; Whitehead et al., in preparation, 15 days old). Therefore, we compared the gut microbiota at weaning to better discriminate whether microbial signatures are specific to the HR lines or a secondary response to acute phenotypes, such as wheel running, differences in physiology, etc.

METHODS

All experiments and methods were approved by the Institutional Animal Use and Care Committee of the University of California, Riverside.

Experimental animals

Females were sampled from generation 81 of the High Runner selection experiment, which began in 1993 with a population of 224 outbred Hsd:ICR mice (Swallow et al., 1998). Briefly, mice are weaned at 21 days of age and housed 4/cage separated by line and sex. At ~6-8 weeks of age, they are housed individually for six days in cages attached to a 1.12 m circumference wheel. Each generation, within each of the 4 replicate HR lines (lab designations 3,6,7,8), the highest-running male and female from within each of 10 families are chosen as breeders, based on the average revolutions on days 5 and 6. For the 4 replicate C lines (1,2,4,5), one male and one female are taken from each family without regard to running. Mice are paired within their line, and no sibling matings are allowed. Following weaning, mice are provided with Standard Laboratory Rodent Diet from Harlan Teklad (Envigo) (W-8604), which contains 24.3% kJ from protein, 4% kJ from fat, and 40.2% kJ from carbohydrate. Pregnant dams are given Harlan Teklad (Envigo) Lab Mouse Breeder Diet [S-2335] 7004 through weaning.

In the present study, mice were weaned at 21 days of age and housed individually for 24 hours prior to fecal sampling. Mice were checked for signs of distress (e.g. hunched posture) while individually housed, and no such signs were observed. Each was from a different litter, with six exceptions. From an initial sample of 100, five mice were excluded due to cage issues or premature death post-weaning, resulting in N=5 (11-12 from each line, except line 6 [polymorphic for the mini-muscle phenotype, described below], which had 14).

Fecal sampling

Fecal samples were collected from mice 20-24 hours after weaning. Mice were grasped at the nape until defecation occurred into a sterile tube, which was immediately placed on

dry ice and stored at -80°C. Fecal samples were shipped on dry ice to the Nutritional and Microbial Ecology Lab at Harvard University and stored at -80°C until processing.

DNA extraction

We used an established 16S ribosomal RNA (rRNA) gene sequencing pipeline to assess gut microbial community composition in each sample (Carmody et al., 2015; Carmody et al., 2019). We used the V4 region of the 16S rRNA gene because of its common use across gut microbiome research for 16S relative abundance analysis. The V4 regions is one of many "hypervariable" regions of the 16S gene (Shahi et al., 2017) and is recommended by the Earth Microbiome Project (https://earthmicrobiome.org/protocols-and-standards/16s/) to amplify the 16S gene. This protocol utilizes the widely sequenced 515F (forward) and 806R (reverse) primers from the conserved region to amplify the V4 region. Further, because microbiome research almost universally uses protocols from the Earth Microbiome Project, reference databases for the V4 region of the 16S gene are publicly available, highly covered, and constantly updated. Therefore, taxonomic identification is very accurate for the V4 hypervariable regions. Targeting a longer sequence and deeply sequencing a representative number of bacterial species would be cost prohibitive. We were able to deeply sequence each fecal sample using Illumina sequencing of a shorter marker gene (here 150 bp).

Briefly, we isolated DNA using the Qiagen PowerSoil DNA Isolation Kit (Catalog no. 12888). Next, we PCR amplified the V4 region of the 16S rRNA gene using custom barcoded 515F and 806R primers (Caporaso et al., 2011; Caporaso et al., 2012). PCR amplification was performed in triplicate using the following reaction recipe: 11 µl nuclease-free H_2O, 1 µl 25mM $MgCl_2$, 10 µl Quantabio 5Prime Hot MasterMix (Cat. No. 2200410), 2 µl primers (1 µl of forward primer and 1 µl of reverse primer), and 1 µl of template DNA. We included a negative control reaction per sample to ensure that primers and reagents were not contaminated. PCR was performed using BioRad T100 thermocyclers and the following protocol: 94°C for 3 minutes; 35 cycles of 94°C for 45 seconds, 50°C for 30 seconds, and 72°C for 90 seconds; and 10 minutes at 72°C. PCR amplicons were checked by running recombined triplicate reactions, negative controls, and a 100 bp DNA ladder on a 1.5% agarose gel in an electrophoresis chamber.

Amplicons were purified using Agencourt AMPure XP solution and resuspended in 40 µl of 1X TE buffer. Cleaned amplicons were quantified using the Quant-iT PicoGreen dsDNA Assay Kit, with fluorescence measured with a Spectramax Gemini XS Plate Reader set to 480 nm excitation / 520 nm emission. Cleaned amplicons were pooled at sample-specific volumes to obtain 80 ng DNA per sample. We purified 100 µl of the pooled solution using the Qiaquick MinElute kit (Cat. No. 28004). The eluted DNA was then gel-purified by 1.5% agarose gel electrophoresis. Band size was compared against a 100 bp DNA ladder, and the targeted 381 bp band was cut from the gel with a sterile razor and resuspended using the Qiaquick PCR Purification kit (Cat. No. 28104). The pool was diluted to 10 nM and submitted for sequencing on one lane of an Illumina HiSeq rapid flow cell (1 x 150 bp) at the Harvard Bauer Core.

Analysis of 16S rRNA Gene Sequences

Raw sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME) package version 1.8 (Caporaso et al., 2010). After quality filtering, we obtained a mean sequencing depth of 123,557 ± 50,871 (SEM) reads per sample. Operational taxonomic units (OTUs) were picked at 97% similarity (Caporaso et al., 2010). Bacterial relative abundances at taxonomic levels from phylum to genus were generated using the summarize_taxa.py script. Prior to alpha diversity analysis, we rarefied the dataset at 25,000 reads per sample. Alpha diversity (Shannon diversity index, Chao1, unique OTUs, and Faith's phylogenetic diversity) was analyzed using the alpha_diversity.py script. Prior to beta diversity analysis, we subsampled the dataset at 33,600 reads and used the beta_diversity_through_plots.py script to generate Bray-Curtis, unweighted UniFrac, and weighted UniFrac distance matrices and associated principal coordinates.

The bacterial 16S rRNA sequences will be deposited in the National Center for Biotechnology Information (NCBI)'s Sequence Read Archive (SRA) under SRA BioProject Accession PRJNA911624.

Statistical analyses

We used mixed models to analyze alpha diversity metrics and taxonomic relative abundances in SAS 9.4 Procedure Mixed (SAS Institute, Cary, NC, USA). Our models tested the effects of linetype (HR versus C lines) against the variance among replicate lines, nested as a random effect within linetype, with 1 and 6 d.f. We also tested the effect of mini-muscle status, a phenotype currently present in two of the four HR lines that is caused by a single base pair change in the *Myosin heavy polypeptide 4* gene (Kelly et al., 2013). The mini-muscle phenotype is characterized by a ~50% reduction in hindlimb muscle mass, larger internal organs, and a variety of other differences as compared to normal-muscle mice (e.g., see Garland et al., 2002; Swallow et al., 2009; Wallace and Garland, 2016). In the present study, 26 of 95 mice had the phenotype (all 12 in HR line 3, and 6 of 14 in HR line 6).

Bacterial relative abundances were log or arcsine square root transformed to improve normality of residuals (Brown et al., 2020; Kohl et al., 2016). In the present study, we limited analyses to taxa found with at least 50% prevalence among samples. We then used a targeted approach to test for differentially abundant bacteria between the HR and C linetypes. Specifically, we analyzed taxa previously associated with exercise in rodents (Campbell et al., 2016; Clarke et al., 2014; Codella et al., 2018; Hughes, 2020; Mach and Fuster-Botella, 2017; Munukka et al., 2018; Queipo-Ortuño et al., 2013): Proteobacteria (phylum), Bacteroidetes (phylum), Firmicutes (phylum), Tenericutes (phylum), Actinobacteria (phylum), *Rikenellaceae* (family), *Lactobacillaceae* (family), *Clostridiaceae* (family), *Clostridium (*genus within the family *Clostridiaceae)*, *Lactobacillus* (genus), *Bifidobacterium* (genus), *Akkermansia* (genus), and *Oscillospira* (genus). (Note: the genus *Clostridium* presently includes OTUs that are nested within multiple different families, reflecting a taxonomy that is not fully phylogenetic.) We were also interested in the genus *Veillonella* for its prior association with endurance exercise in humans (Scheiman et al., 2019), but *Veillonella* was not present. Statistical significance was judged at the P=0.05 level. For completeness, analyses of additional taxa with at least 50% prevalence among samples are presented in Table S1. A measure of effect size (Pearson's r) was calculated for all main and interactive effects for bacterial relative abundance and alpha diversity (Sullivan and Feinn, 2012).

Beta diversity of the gut microbiome was assessed by calculating unweighted UniFrac, weighted UniFrac, and Bray-Curtis distance matrices and performing principal coordinate analyses (PCoA) to visualize the microbial community clustering based on distance. We used the adonis function within the vegan package in R to perform permutational analysis of variance (PERMANOVA) to determine significant clustering within the dataset (Anderson, 2001; Anderson, 2017). We permutated the distance matrix over linetype and mini-muscle status 999 times. We also permuted the distance matrix over line 999 times for separate analyses of the 4 replicate HR and 4 replicate C lines. Replicate line was not treated as a nested random effect because this feature is not available in the vegan package. One sample was removed from the unweighted UniFrac distance matrix based on its appearance as a strong outlier in the initial PCoA.

RESULTS AND DISCUSSION

Alpha diversity of the weanling gut microbiome

Based on mixed models, the average unique OTUs per mouse and other alpha diversity metrics did not statistically differ between weanling HR and C mice (Unique OTUs, $F_{1,6}$ = 0.01, P=0.7611; Shannon Index, $F_{1,6}$ = 028, P=0.6131; Chao1, $F_{1,6}$ = 0, P=0.9884; Faith's phylogenetic diversity, $F_{1,6}$ = 0.09, P=0.7777;) or between mini- and normal-muscle mice (Unique OTUs, $F_{1,86}$ = 0.52, P=0.4727; Shannon Index, $F_{1,86}$ = 0.0, P=0.9621; Chao1, $F_{1,86}$ = 0.81, P=0.3704; Faith's phylogenetic diversity, $F_{1,86}$ = 0.5, P=0.4797) (Table S1). Within the two linetypes, the 4 individual replicate C lines differed in the number of unique OTUs ($F_{3,41}$ = 3.14, P=0.0353) and Faith's phylogenetic diversity metric ($F_{3,41}$ = 3.06, P=0.0386), but not Chao1 ($F_{3,41}$ = 2.53, P=0.0707) or Shannon index ($F_{3,41}$ = 1.28, P=0.2954). The 4 individual replicate HR lines did not differ in the number of unique OTUs ($F_{3,45}$ = 0.93, P=0.4356), Faith's phylogenetic diversity ($F_{3,45}$ = 0.49, P=0.694), Chao 1 ($F_{3,45}$ = 0.96, P=0.4220), or Shannon index ($F_{3,45}$ = 0.62, P=0.6081).

The gut microbiota typically becomes more diverse with age in both humans and rodents (Koenig et al., 2011; Schloss et al., 2012; Yatsunenko et al., 2012). Consistent with this pattern, our weanling mice had fewer average OTUs (N = 373) compared with our previous study of adult HR and C mice (N = 430), although it is important to note that

these numbers cannot be strictly compared because they were based on different sequencing methods (McNamara et al., 2021).

Two prior rodent studies examined the gut microbiome in response to selective breeding for aspects of exercise capacity, although not in weanlings. Two lines of rats bred for either high (HCR) or low (LCR) endurance capacity during forced treadmill exercise did not differ in alpha diversity metrics at either 7 or 40 weeks of age (Pekkala et al., 2017: weaning occurs at 4 weeks of age). Four replicate lines of bank voles bred for oxygen consumption during swimming exercise at a temperature below the thermal neutral zone also did not differ in alpha diversity as compared with four control lines (mean age: 166 days) (Kohl et al., 2016).

Dominant phyla of the weanling gut microbiome

Across the entire sample of 95 mice, 2,074 OTUs were identified, representing 11 phyla, 21 classes, 38 orders, 108 families, and 218 genera of microbes. Typical for mice, composition was dominated by the phyla Firmicutes (48.4 ± 14%; mean ± S.D.) and Bacteroidetes (37.5 ± 17.1%) (Fig. S1). Based on mixed models, neither linetype (HR vs. C) nor mini-muscle status statistically affected the relative abundance of the phyla Bacteroidetes, Firmicutes, Proteobacteria, Tenericutes or Actinobacteria (Table S1).

Previously (as consistently observed in murine studies), we reported that the adult gut microbiota in these mice was dominated by Bacteroidetes (~68%), with Firmicutes (~28%) being second most abundant (McNamara et al., 2021). For the present sample from weanlings, the phylum Proteobacteria comprises a much larger portion of the weanling (11.8 ± 8.7%) compared to the adult (~1%) gut microbiome. Although our weanling and adult datasets were based on different sequencing methods, these trends are consistent with previous studies in lab rodents showing that the weanling gut microbiome is initially dominated by Firmicutes (Cox et al., 2014; Pantoja-Feliciano et al., 2013) followed by a shift towards Bacteroidetes (Cox et al., 2014; Nagpal et al., 2018).

Targeted taxonomic comparisons

HR mice had significantly higher relative abundance of family *Clostridiaceae* compared to C mice (LS Means for arcsine square root transformed values ± SE: HR = 0.0698 ± 0.0082; C = 0.0279 ± 0.0117) ($F_{1,6}$ = 10.54, P=0.0175, Fig. 1), with no statistical difference for the families *Rikenellaceae* or *Lactobacillaceae,* or genera *Clostridium, Bifidobacterium, Lactobacillus, Akkermansia,* and *Oscillospira* (Table S1). In our previous study of adults, HR mice also had a higher relative abundance of the family *Clostridiaceae* compared to controls, although the difference was not statistically significant (P=0.0750: Table S2 in McNamara et al., 2021).

The HCR and LCR lines of rats (see above) also differed in gut microbiome community composition (Liu et al., 2015; Pekkala et al., 2017). HCR rats have higher maximal aerobic capacity (VO_2max) and higher voluntary wheel running compared to the LCR line (Karvinen et al., 2015; Park et al., 2016; Swallow et al., 2010), paralleling the elevated endurance capacity (Meek et al., 2009) and VO_2max (Cadney et al., 2021) of HR mice. In addition, HCR are smaller than LCR rats (Pekkala et al., 2017; Wisløff et al., 2005) and HR are smaller than C mice (Dumke et al., 2001; Kelly et al., 2017). Thus, we anticipated some similar patterns of differentiation in the gut microbiome. However, whereas adult HCR rats had significantly lower relative abundance of *Clostridiaceae* compared to LCR (Liu et al., 2015), HR mice had a higher relative abundance of *Clostridiaceae* compared to the non-selected C lines for both adults (McNamara et al., 2021) and weanlings (present study). One obvious explanation for this difference is that they are different species, and rats and mice are known to differ in various ways with respect to exercise physiology and responses to exercise training (e.g., see Dumke et al., 2001; Kowalski and Bruce, 2014). Future studies in rodents bred for exercise-related traits, including our own HR mice, should examine the gut microbiome community across various timepoints and generations to illuminate if the abundance of *Clostridiaceae* changes during development and/or across generations.

Beta diversity of the weanling gut microbiome

Principal coordinate analysis based on unweighted UniFrac distances did not indicate separation between HR and C mice along the first two principal coordinate axes (Fig. 2A: although together they account for only 14% of the total variance in the data).

PERMANOVA of the unweighted UniFrac distance matrix also indicated no significant differentiation between HR and C mice (R^2=0.013, P=0.080), nor did reanalysis based on Bray-Curtis or weighted UniFrac distances (Fig. S2). This lack of differentiation contrasts with our previous results for adults, where HR and C mice clustered separately, regardless of diet and/or exercise treatment during early life, based on unweighted UniFrac distances (PERMANOVA P=0.009: McNamara et al., 2021).

Mini- and normal-muscle mice separated somewhat on the third PCoA axis for unweighted UniFrac distances, and PERMANOVA indicates statistically significant separation for unweighted UniFrac (Fig. 2B; R^2=0.014, P=0.047). Thus, weanling mice have distinct bacterial communities based on mini-muscle status. Mini-muscle individuals differ from wildtype in several ways that might affect the microbiome fairly directly, including higher mass-adjusted food consumption (Meek et al., 2014), larger stomachs, a trend for longer small intestines, and a trend for heavier caecum dry masses (Kelly et al., 2017). In addition, their smaller muscles but larger internal organs (heart, soleus, spleen, liver, kidney, lung: Kelly et al., 2017) could impact energetic demands and also the microbiome. However, no significant separation was detected when measured by Bray-Curtis or weighted UniFrac distances, suggesting that the effects of mini-muscle may be limited to differences in phylogenetic representation in the microbiome and not differences in relative abundance (Fig. S2).

Finally, we considered potential separation among the four replicate HR lines and among the four replicate C lines (Fig. 3). Differentiation was significant within both linetypes based on PERMANOVAs on unweighted UniFrac distances (HR lines with one outlier removed: R^2=0.028, P=0.038; C lines: R^2=0.037, P=0.006). Based on Bray-Curtis distances, separation among C lines was statistically significant, but not among HR lines, and weighted UniFrac distances indicated no separation among the replicate lines for either linetype (Fig. S3). Jointly, these data suggest that drift has been a stronger force on the gut microbiome in C lines than in HR lines, where, as expected, selection has been more important in HR lines.

When measured as adults (37 weeks of age after 11 weeks of individual housing with or without wheel access), the HCR and LCR lines of rats mentioned above clustered separately, based on unweighted and weighted UniFrac distances (Liu et al., 2015). In

the bank vole selection experiment, a total of 16 lines were included: four bred for aerobic capacity, four for ability to maintain body weight when fed a low-quality diet for four days, and four for predatory behavior towards crickets. As adults, none of the sets of selected lines had gut microbial profiles that differed from those of the control lines using weighted UniFrac distances (Kohl et al., 2016). However, unweighted UniFrac distances indicated the herbivorous lines differed in gut microbial profiles compared to the other groups.

Concluding remarks and future directions

Previously, we reported that selective breeding for voluntary wheel running resulted in higher relative abundance of *Clostridiaceae* (P=0.0750) among adults in 4 replicate HR lines as compared with their 4 non-selected C lines, in addition to substantial differentiation in beta diversity. In our previous study, we sequenced the Internal Transcribed Spacer region. Here, using 16S rRNA sequencing, we show that the HR lines also have higher relative abundance of *Clostridiaceae* at weaning. Notably, our study only examines how gut microbial community structure differs between mouse lines at weaning and does not examine function. Future studies should test for differences in levels of physical activity between the HR and C lines before weaning, as that could contribute to microbiome differences. In addition, transplantation of the HR microbiome into C mice, and vice versa, following ablation via antibiotics (McNamara et al., 2022), or into germ-free animals, would shed further light on the contribution of the gut microbiome to increased aerobically supported activity among HR mice. Reanalysis of data from previous studies in other systems could indicate if *Clostridiaceae* may be associated with exercise behavior and/or physiology.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.P.M, T.G., R.N.C. Methodology: M.P.M, T.G., R.N.C. Software: R.N.C. Validation: T.G., R.N.C. Formal analysis: M.P.M, E.V., T.G., R.N.C. Investigation: all coauthors. Resources: T.G., R.N.C. Data curation: M.P.M, E.V. T.G., R.N.C. Writing-original draft: M.P.M, E.V. T.G., R.N.C. Writing-review & editing: all coauthors. Visualization: M.P.M, E.V. T.G., R.N.C. Supervision: T.G., R.N.C. Project administration: T.G. Funding acquisition: T.G., R.N.C.

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Supplementary information

Supplementary information available online at:

Fig. S1 = phyla comparison bar chart.

Table S1 = taxa and alpha diversity p values.

Fig. S2 = Bray-Curtis and Weighted UniFrac Beta Diversity.

Fig. S3 = Bray-Curtis and Weighted UniFrac Beta Diversity Line plots.

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Figures

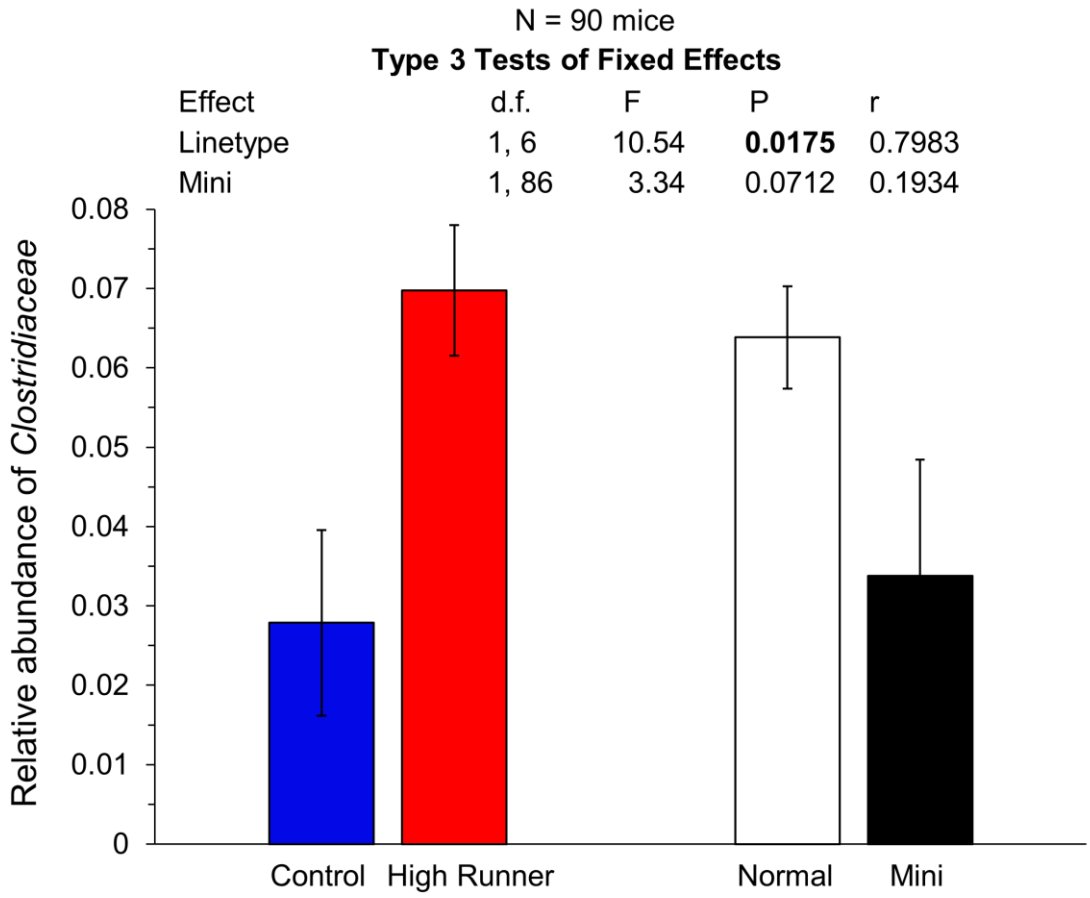

Fig. 1. Relative abundance of the family *Clostridiaceae.* Based on mixed models simultaneously comparing HR and C lines, as well as mini-muscled and normal-muscle mice, HR mice had significantly higher relative abundance of *Clostridiaceae* compared to Control mice (ANOVA, $F_{1,6}$ = 10.54, P = 0.0175), with no effect of mini-muscle status. Shown are least squares means ± SEs for arcsine square root transformed values. Pearson's r is a measure of effect size.

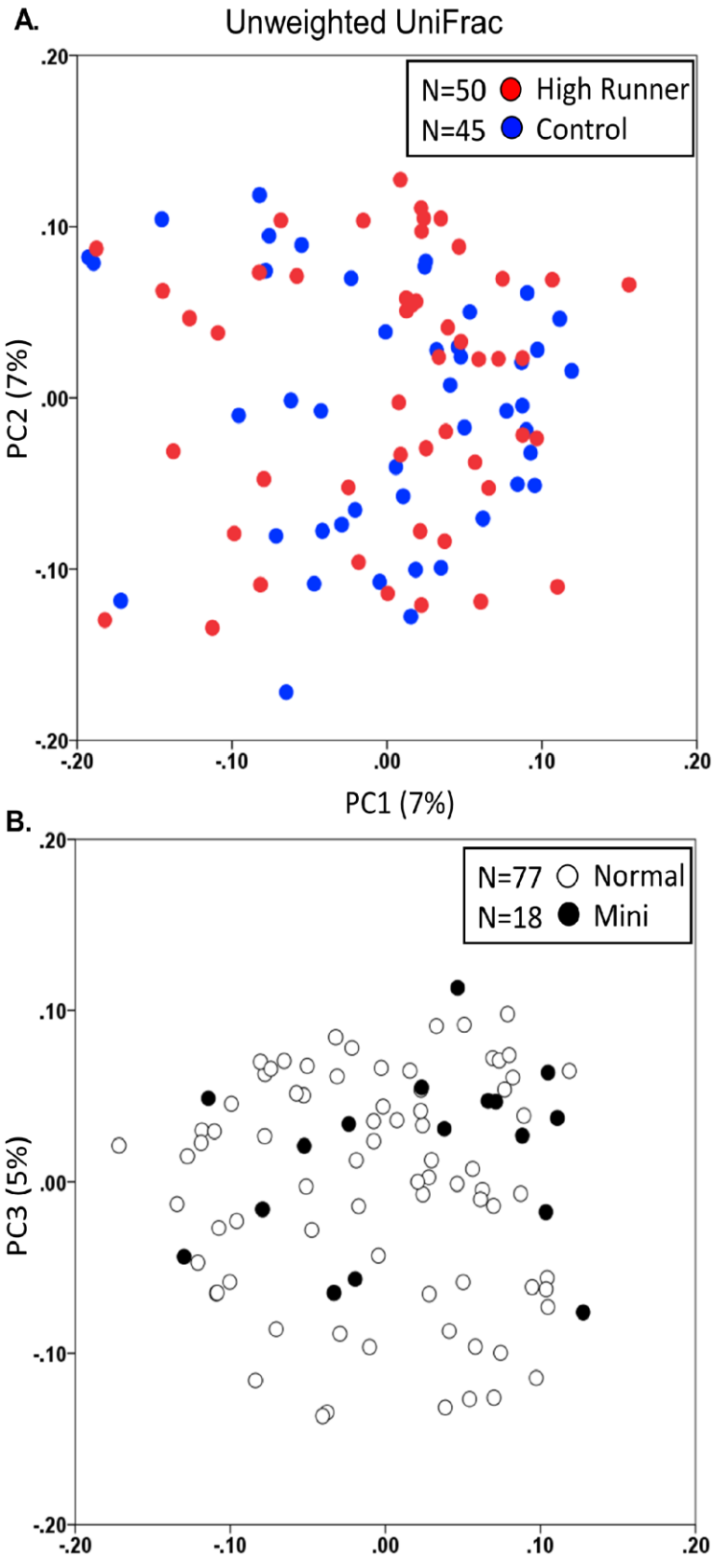

Fig. 2. Principal coordinate analysis of unweighted UniFrac distances between the 16S rRNA gene sequencing-based profiles of weanling fecal microbiomes in relation to (A) linetype and (B) mini-muscle status (one outlier removed: see text). PERMANOVAs indicated significant separation based on mini-muscle status mice (R^2=0.014, P=0.047), but not between linetypes (R^2=0.013, P=0.080).

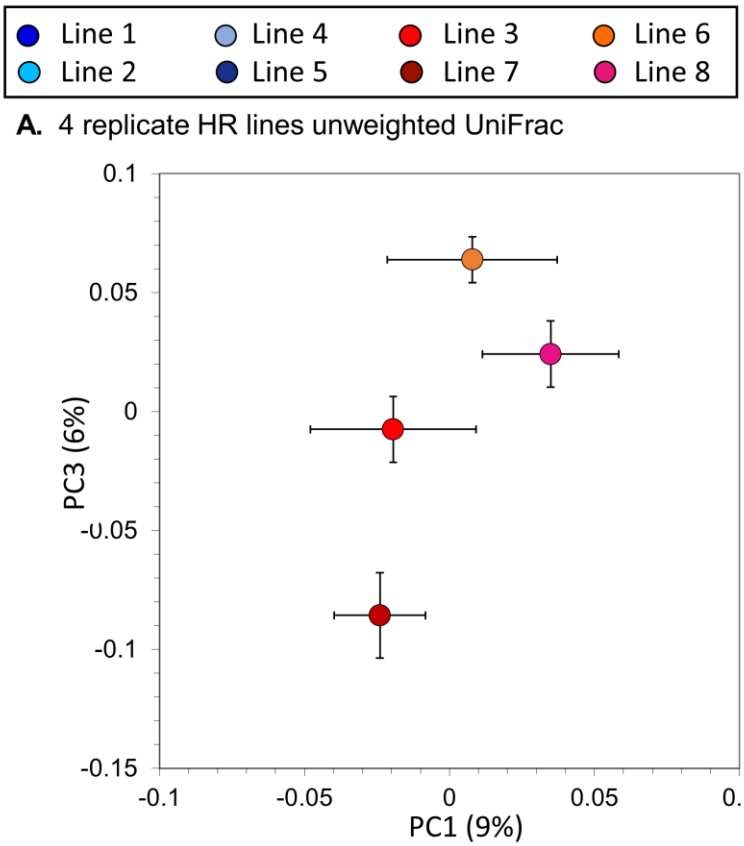

Fig. 3. Principal coordinate analysis of unweighted UniFrac distances between the 16S rRNA gene sequencing-based profiles of the 4 HR lines (A) and the 4 C lines (B) (performed for each linetype separately). Values are means and SEs for scores on PCoA axes. PERMANOVAs indicated significant separation among the 4 HR lines and among the 4 C lines (HR lines: R^2=0.028, P=0.038; C lines: R^2=0.037, P=0.006).

Figure S1

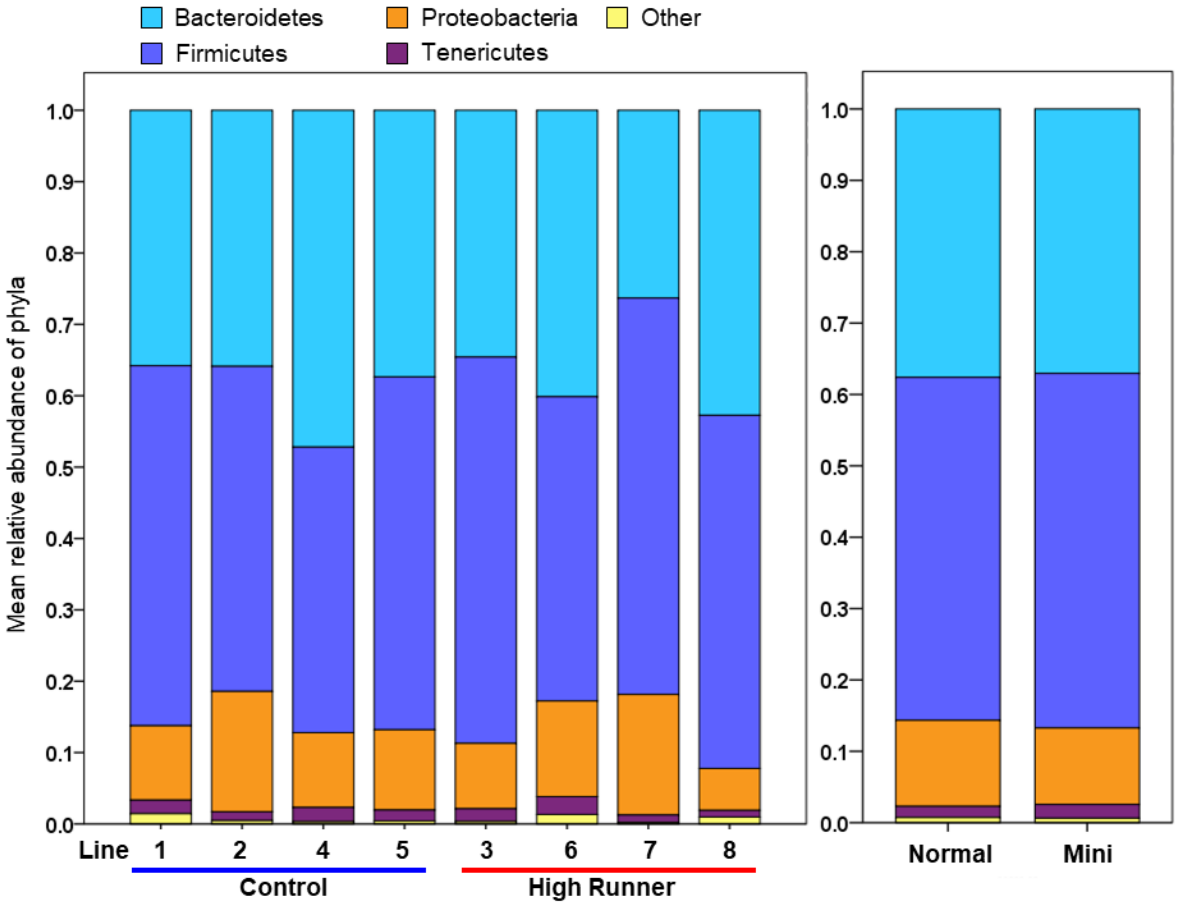

Fig. S1. Community composition of the weanling gut microbiome for all experimental mice (N=95) was dominated by Firmicutes (48.4 ± 14%) (mean ± S.D.) and Bacteroidetes (37.5 ± 17.1%), with additional phyla being much less abundant: Proteobacteria (11.8 ± 8.7%), Tenericutes (1.6 ± 2%), Cyanobacteria (0.28 ± 0.53%), Verrucomicrobia (0.24 ± 1.1%), Actinobacteria (0.09 ± 0.16%), Deferribacteres (0.08 ± 0.24%), Fusobacteria (0.0002 ± 0006%), and TM7 (0.0001 ± 0.0004%).

Figure S2

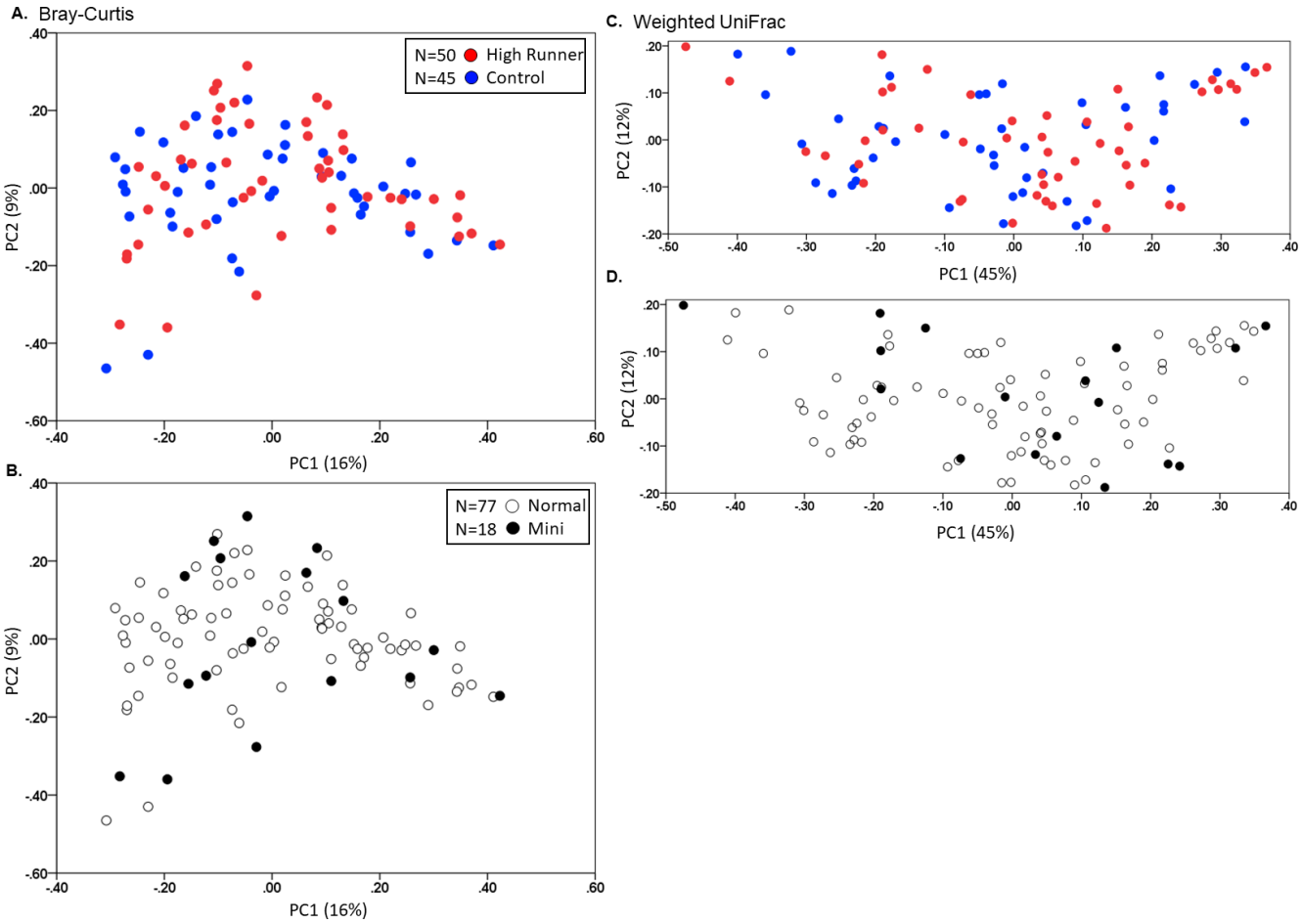

Fig. S2. Beta diversity (among experimental groups) of the weanling fecal microbiome based on 16S rRNA sequence data. A and B are PCoA plots based on Bray-Curtis distances, which consider bacterial OTU sequence relative abundances. C and D are PCoA plots based on weighted UniFrac distances, which consider both bacterial OTU sequence relative abundances and phylogenetic distances. PERMANOVAs based on Bray-Curtis or weighted UniFrac distance matrices indicated no statistically significant separation based on either linetype (Bray-Curtis: R^2=0.0147, P=0.108, weighted UniFrac: R^2=0.0116, P=0.307) or mini-muscle status (Bray-Curtis: R^2=0.009, P=0.638, weighted UniFrac: R^2=0.005, P=0.773).

Figure S3

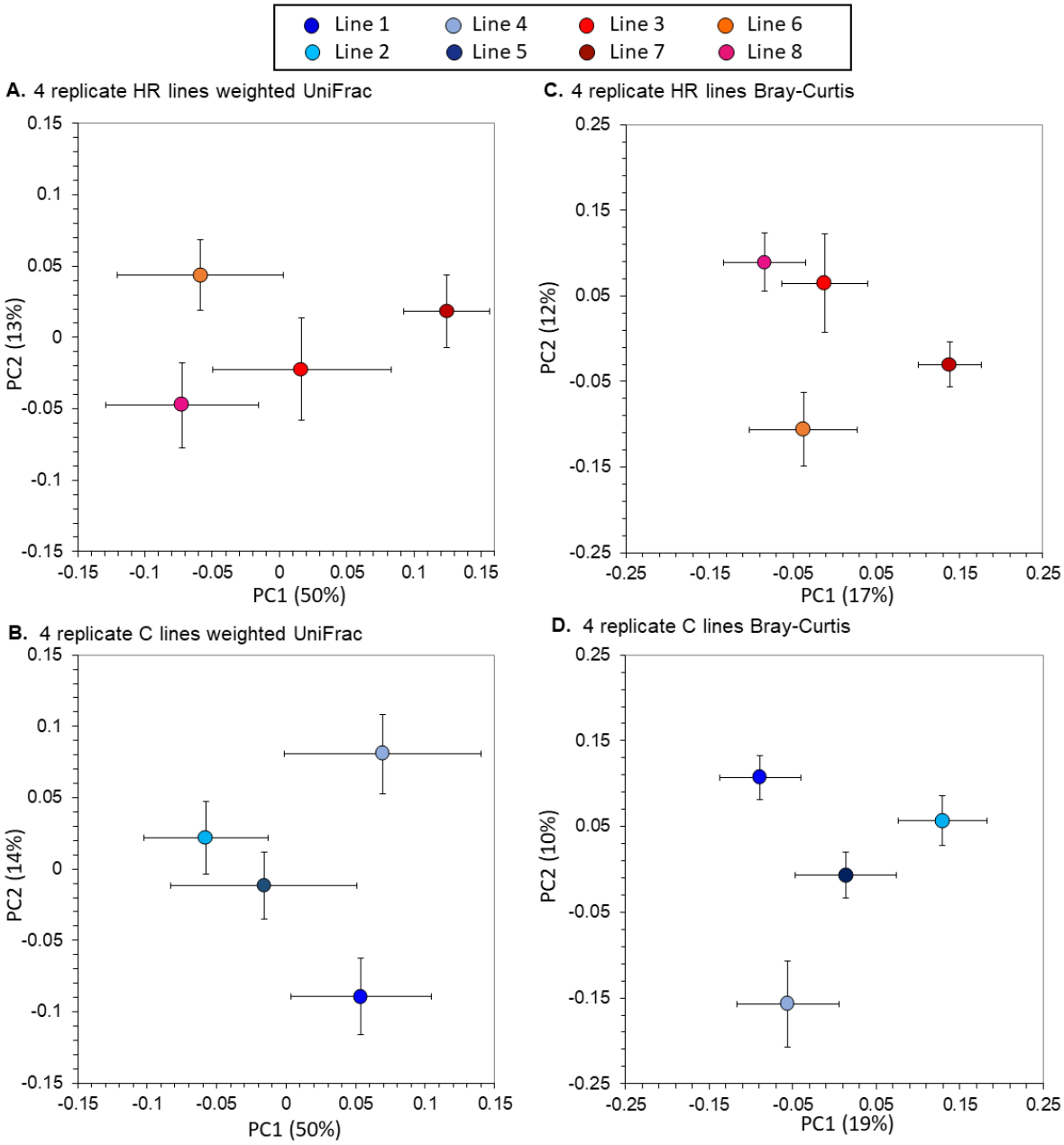

Fig. S3. PCoA plots from separate weighted UniFrac and Bray-Curtis analyses of the 4 HR lines (lab designations 3,6,7,8: A and C) and of the 4 C lines (lab designations 1,2,4,5: B and D). Values are means and standard errors for scores on PCoA axes. Separately, we used PERMANOVAs to test for significant separation among the 4 replicate HR lines and among the 4 non-selected C lines. Differences among the 4 replicate C lines were statistically significant based on Bray-Curtis (R^2=0.051, P=0.005), but not among the 4 replicate HR lines (R^2=0.014, P=0.839). Weighted UniFrac indicated no significant separation among replicate lines for either linetype (HR lines: R^2=0.005, P=0.957; C lines: R^2=0.030, P=0.228).