

## Correction

### EVOLUTION

Correction for “Gut microbiomes and reproductive isolation in *Drosophila*,” by Philip T. Leftwich, Naomi V. E. Clarke, Matthew I. Hutchings, and Tracey Chapman, which was first published November 6, 2017; 10.1073/pnas.1708345114 (*Proc Natl Acad Sci USA* 114:12767–12772).

The authors note that on page 12768, left column, first full paragraph, line 19, “4% propionic acid” should instead appear as “0.4% propionic acid.”

The authors also note that on page 12768, in the final paragraph beginning in the left column, lines 5–6, “Mantel–Haenszel (MH) test statistics  $\chi^2_1 = 1.35$ ,  $P = 0.24$ ; Dahomey: MH  $\chi^2_1 = 0.35$ ,  $P = 0.55$ ” should instead appear as “Mantel–Haenszel (MH) test statistics  $\chi^2_1 = 0.2$ ,  $P = 0.66$ ; Dahomey: MH  $\chi^2_1 < 0.001$ ,  $P = 0.98$ ”; and, in the same paragraph, line 8, “MH  $\chi^2_1 = 18.15$ ,  $P < 0.001$ ” should instead appear as “MH  $\chi^2_1 = 4.17$ ,  $P = 0.041$ .”

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# Gut microbiomes and reproductive isolation in *Drosophila*

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Experimental studies of the evolution of reproductive isolation (RI) in real time are a powerful way in which to reveal fundamental, early processes that initiate divergence. In a classic speciation experiment, populations of *Drosophila pseudoobscura* were subjected to divergent dietary selection and evolved significant positive assortative mating by diet. More recently, a direct role for the gut microbiome in determining this type of RI in *Drosophila melanogaster* has been proposed. Manipulation of the diet, and hence the gut microbiome, was reported to result in immediate assortative mating by diet, which could be eliminated by reducing gut microbes using antibiotics and recreated by adding back *Lactobacillus plantarum*. We suggest that the evolutionary significance of this result is unclear. For example, in *D. melanogaster*, the microbiome is reported as flexible and largely environmentally determined. Therefore, microbiome-mediated RI would be transient and would break down under dietary variation. In the absence of evolutionary coassociation or recurrent exposure between host and microbiome, there are no advantages for the gut bacteria or host in effecting RI. To explore these puzzling effects and their mechanisms further, we repeated the tests for RI associated with diet-specific gut microbiomes in *D. melanogaster*. Despite observing replicable differences in the gut microbiomes of flies maintained on different diets, we found no evidence for diet-associated RI, for any role of gut bacteria, or for *L. plantarum* specifically. The results suggest that there is no general role for gut bacteria in driving the evolution of RI in this species and resolve an evolutionary riddle.

ecological adaptation | diet | holobiome | gut microflora | assortative mating

The experimental study of key elements of incipient reproductive isolation (RI) in the laboratory has provided important insights into the underlying evolutionary processes involved (1, 2). Such data show that key components of the initiation of reproductive divergence can be observed and studied in real time (3–9). A classic study of the evolution of incipient RI, arising as a side effect of natural selection to different diets, is Dodd's experiment on replicated populations of *Drosophila pseudoobscura* (10). In this, four populations were each placed onto maltose- or starch-based diets and maintained for a period of ~1 y. Mating tests were then conducted within and between replicates maintained on each of the regimes, and significant assortative mating by diet was observed. This has become a textbook example of a "speciation experiment" (1), relevant to understanding speciation by host shifts (11–13).

Many aspects of the mechanisms underlying divergence associated with ecological adaptation or host shifts remain unknown (14, 15). Hence, recent studies that have described mechanistic insights into our understanding of how mate choice is associated with dietary divergence have had a wide impact. For example, there has been much interest prompted by a study that suggested a role for gut bacteria in driving assortative mating in *Drosophila melanogaster* (16, 17). Flies placed on different diets were reported to show instant assortative mating by diet. This was abolished following antibiotic treatment of the adults and was

reestablished by bacterial replacement experiments, specifically by add-back of *Lactobacillus plantarum*. The proposed mechanism was via differential effects of gut bacteria on cuticular hydrocarbons that affect attractiveness (16, 18–20).

These results stimulated intense interest in the wider role of the gut microbiome in mate choice and, potentially, speciation (21–24). They also provided a keystone for the upsurge of interest in the "holobiome" concept (e.g., ref. 25), in which the unit of selection is seen as the sum total of the host plus its microbiome. However, the recent interest in gut microbiomes and their potential role in speciation in fruit flies presents a significant evolutionary puzzle. Selection at the level of the holobiome, or a causal role for microbiomes in host speciation, requires coevolutionary associations, microbiome stability, or recurrent exposure between hosts and microbiomes (26). In many situations in which the holobiome is thought to be important, these conditions may not exist. For example, natural populations of *D. melanogaster* are reported to exhibit fairly flexible, environmentally acquired gut microbiomes (e.g., refs. 27–33). Hence the composition of the gut bacterial community seems to depend largely on the ingested diet (32). Strong, and potentially coassociated, evolutionary relationships between *D. melanogaster*

## Significance

The evolutionary significance of assortative mating by diet, mediated by gut bacteria is a puzzle, but it has had a huge impact and has provided a keystone to support increasing interest in the "holobiome." However, in species such as *Drosophila melanogaster* that have flexible gut microbiomes, any reproductive isolation mediated by gut bacteria specific to host diets can only be transient. Here, we replicated and extended tests of this idea. Despite differences in gut microbiomes, we failed to recover previously observed patterns of nonrandom mating and found no evidence that mating preferences were associated with diet or gut bacteria. This suggests that the evolutionary importance of gut microbiomes in host divergence needs careful consideration on a case-by-case basis.

Author contributions: P.T.L. and T.C. designed research; P.T.L., N.V.E.C., and T.C. performed research; P.T.L. analyzed data; and P.T.L., N.V.E.C., M.I.H., and T.C. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: All chimera-checked 16S rRNA gene sequences of representative OTUs, the dereplicated, quality filtered Illumina MiSeq data set file, metadata mapping file, and the final OTU distribution table, along with raw sanger sequencing reads of *L. plantarum* have been deposited to Figshare, available at <https://dx.doi.org/10.6084/m9.figshare.5469316>. The individual raw 16S sequences are available at NCBI Sequence Read Archive (BioProject PRJNA415376). The chimera-checked 16S consensus sequence for *L. plantarum* used for bacterial add-back has been deposited in the NCBI GenBank database (accession no. MG066537).

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hosts and their gut bacteria have not been reported. Hence, a general role for gut bacteria in the maintenance of RI seems unlikely, given the degree of dietary flexibility exhibited by this species. In addition, it is not clear that there can be any benefit to either host or gut bacteria in the absence of any recurrent, potentially coevolved association. Hence the evolutionary significance of this type of association between gut bacteria and host is unclear (26, 34, 35).

These reasons may explain the lack of consistency in tests that have investigated a general role for gut bacteria in mating associations and mate choice in *D. melanogaster* (16, 18, 36–38). To try to resolve these differences, and to investigate the potential mechanisms underlying the role of gut microbes in assortative mating, we repeated the experiments of Sharon et al. (16) (Table S1). We used two independent wild-type strains of *D. melanogaster* (including two strains of Oregon R, the original background tested) for three test populations in total. We first described the gut microbiomes, on the basis that a precondition for assortative mating mediated by diet and/or gut microbiota is that the microbiomes should be at least partially distinct between flies maintained on different diets. Conversely, if microbiomes are distinct, but assortative mating by diet is absent, then a role for gut bacteria would not be supported. We then conducted mate-choice trials following 5, 30, and 35 generations of maintenance on “CMY” (0.65% agar, 7.6% cornmeal, 7.6% molasses, 5% inactivated brewer’s yeast, 0.1% methyl-4-hydroxybenzoate, 0.76% ethanol and 4% propionic acid) or “starch” (3% starch, 5% inactivated brewer’s yeast, 1% agar, 0.5% propionic acid) diets and manipulated gut microbiome composition by using antibiotic and *L. plantarum* add-back treatments. The results revealed that, although there were replicated differences in the gut microbiomes in flies maintained on the different diets, there was no evidence for assortative mating associated with diet, with gut bacteria, or with *L. plantarum* in particular.

## Results and Discussion

**Composition of the Gut Microbiomes of CMY and Starch Flies.** A principal coordinate analysis (PCoA) showed that the bacterial gut microbiomes of the three populations of flies maintained on the same CMY and starch media as in ref. 16 for 30 generations exhibited significant, tight clustering according to CMY or starch diet ( $F_{1,11} = 1.52$ ,  $P < 0.001$ ; Table 1 and Fig. 1A). Independent biological replicates were generally consistent, but more variable among lines on starch (Fig. 1A). Acetobacteriaceae comprised over 50% of the microbiome across all populations reared on the CMY diet, with the next most abundant group being the Lactobacillaceae (Fig. 1B). Flies reared on CMY showed a stable abundance of these core microbes across groups and independent biological replicates. There was a log-fold reduction in the abundance of these same groups of bacteria maintained on starch (Table S2). Instead, species of Rickettsiaceae were found in much greater abundance, particularly in both replicates of the OR2376 line and one replicate of OR25211 (Fig. 1B). This may reflect a reduction in the acquisition of environmental microbes in flies reared on starch (16, 32). The identity and relative abundances of gut microbes from the guts of flies maintained on the different diets were consistent with previous descriptions. Notably, species in the family Enterobacteriaceae were largely absent, and, as reported previously, this absence was associated with a high frequency of Acetobacteriaceae (27–29). Overall, the results showed replicated, significant differences in the gut microbiomes of the flies maintained on different diets.

**Assortative Mating by Diet.** We tested the mating preferences of each of the wild-type lines after 5, 30, or 35 generations of maintenance on CMY or starch diets (Fig. 2). There was no significant deviation from random mating across the experiment for two of the lines [OR25211: Mantel–Haenszel (MH) test

statistics  $\chi^2_1 = 1.35$ ,  $P = 0.24$ ; Dahomey: MH  $\chi^2_1 = 0.35$ ,  $P = 0.55$ ]. OR2376 showed a single significant deviation from random mating in one test (MH  $\chi^2_1 = 18.15$ ,  $P < 0.001$ ), but in a diet-disassortative direction. There were no significant differences in the number of homogamic vs. heterogamic matings occurring across all three generations of testing (Fig. S1A and Table S3). The tests for RI showed a weak signal for reproductive outbreeding (preference for mating with flies of the opposite diet type) at generation 5 (Table S3). However, this was not evident at any subsequent time point (Fig. S1B). Overall, the results from the mating tests on the wild-type lines tested following three time points of maintenance on the different diets showed no evidence for significant assortative mating by diet.

**Effect of Antibiotic Treatment and *L. plantarum* Add-Back on Assortative Mating by Diet.** To account for the possibility that differences in the composition of microbiomes between this study and ref. 16 could affect mating responses, we also tested whether the elimination of gut bacteria followed by *L. plantarum* add-back could recreate the proposed pattern of assortative mating (16). We first treated the adults with antibiotics, which effectively eliminated gut microbiomes (Supporting Information), and then retested the flies for mating preferences at three time points, as above. The results showed a pattern of random assortment of matings with respect to diet of origin and no evidence of sexual isolation (Figs. S2 and S3 and Table S3). *L. plantarum* isolated from fly guts of each strain was then fed back to a subset of antibiotic-treated adults from the same strains before testing mating preferences (Fig. S4). No significant mating preferences were generated by *L. plantarum* add-back for any of the three lines tested (MH  $\chi^2_1 = 0.004$ ,  $P = 0.95$ ) (Fig. 3). There were again no differences in the number of homogamic vs. heterogamic matings, and the sexual isolation indices showed no deviation from random mating across any of the three wild-type lines (Fig. S5 and Table S3). Hence there was no evidence that add-back of *L. plantarum* could create a diet-assortative pattern of mating.

**Statistical Power.** An analysis of the statistical power of the experiments presented here revealed that the power of our analyses exceeds that necessary to detect the effect sizes previously reported (refs. 16 and 17; full results are given in Supporting Information). Hence the null results presented are statistically robust and show that the previous published results (16, 17) were not replicated here.

## Conclusions

The compositions of the gut microbiomes of flies held on the different diets were distinct, which is consistent with the observations of a relatively flexible microbiota in this species (27–29, 31–33). However, the mating preferences of the flies were not associated with these microbiome differences. The results showed no evidence for assortative mating by diet or gut microbiome, no excess of homogamic pairings, and no evidence for significant sexual isolation between any of the wild-type strains maintained for the short or long term on different diets that were previously reported to drive significant positive assortative mating (16). The one example of significant sexual isolation was attributable to an excess of disassortative mating by diet (fewer starch-with-starch fly matings than expected) in the OR2376 line at generation 5. The pattern of random mating was not altered by antibiotic treatment, which successfully removed culturable bacteria from the fly guts. The pattern of matings remained random after *L. plantarum* add-back to axenic flies (i.e., there was no excess of matings between the add-back-treated flies). Tested across three populations and over multiple generations of maintenance on the different diets, our results contrast with the results of refs. 16 and 17 and provide no evidence of assortative mating by diet or that mating preference is associated with gut microbiota.

Our results suggest that any effects of gut microbes in mate choice or assortative mating in this species are highly variable and represent proximate effects, or epiphenomena derived from an as-yet unidentified origin. They resolve a puzzle, as they support the assertion that, in this scenario, the different parties (host and microbiome) have limited evolutionary interests in common. Hence, gut bacteria that exhibit flexible and transient associations with their hosts are unlikely to play a general role in host RI. In other species in which there is obligate or recurrent exposure of hosts and their microbiomes or symbionts, such effects can be important (e.g., refs. 39–41).

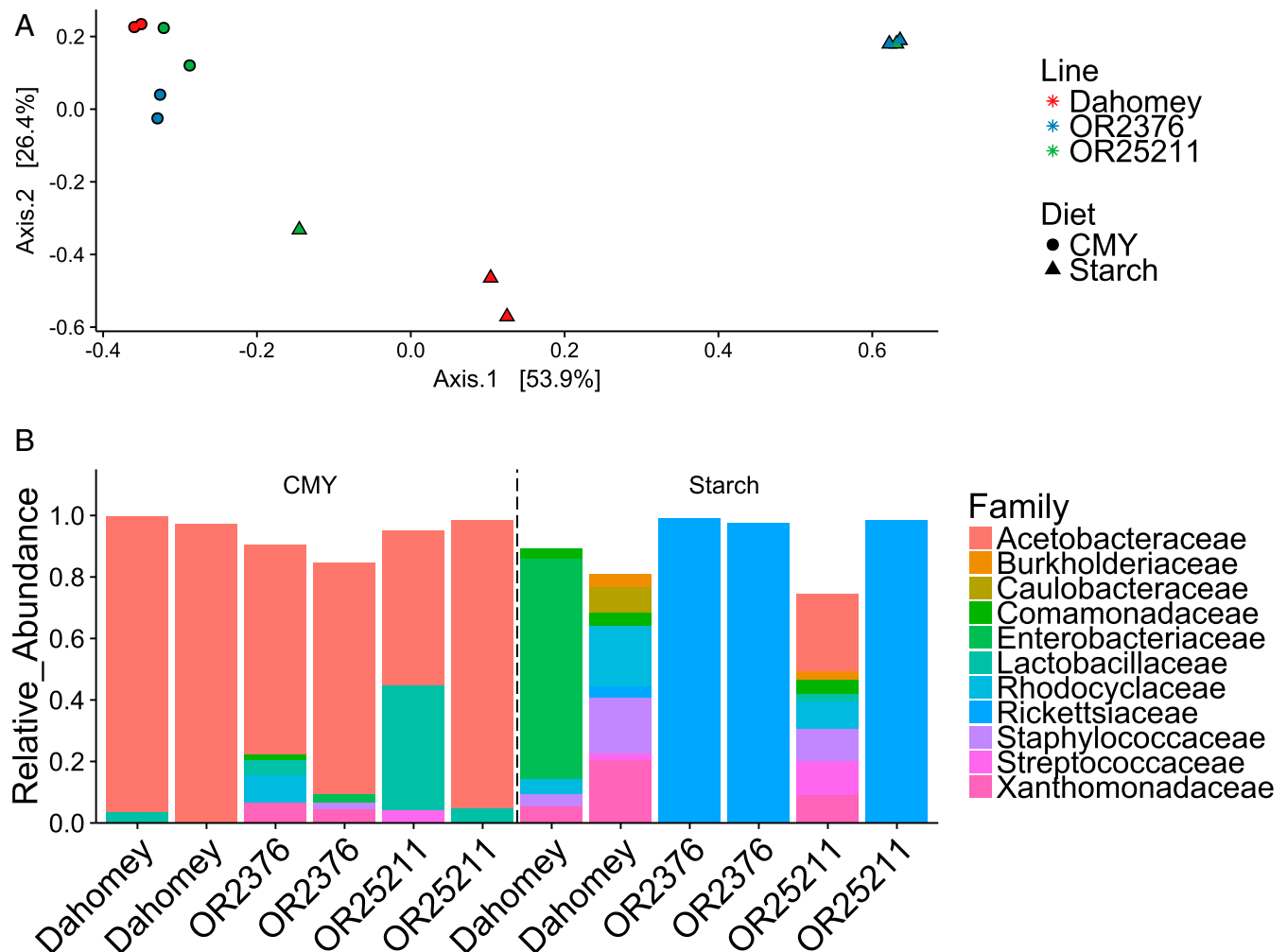
We found no evidence for assortative mating by diet in any of the three lines tested in any of our experiments. The reason for the difference in comparison with the original Dodd study conducted on *D. pseudoobscura* (10) is unclear. The time scale of the maintenance on the different diets is comparable, so the number of generations available for the emergence of assortative mating was similar. It is possible that the strength of selection exerted by the diets on the respective host microbiota differed. In addition, the nature and transmission pattern of the microbiome of *D. pseudoobscura* has not yet been described, and hence a role for gut microbes in mating preferences in this species remains a possibility (e.g., if there were stable, vertical transmission of the

gut microbiome). We suggest that an understanding of the co-association and transmission dynamics of microbiomes within and across hosts is essential to (i) understand the ultimate significance of the effects of gut microbes and (ii) critically evaluate the likely strength of selection at the level of the holobiome. Hence, assessments of the evolutionary importance of the holobiome and the role of gut microbiomes in host adaptation and divergence need careful consideration on a case-by-case basis (26, 35).

### Materials and Methods

**Stocks and Cultures.** We used two wild-type strains: Dahomey and two lines of Oregon-R (the wild type used in ref. 16) (OR 2376, OR 25211; Bloomington Stock Centre). Dahomey wild-type flies were from a large laboratory population originally collected in the 1970s in Dahomey (Benin) and served as an additional, independently derived wild type to Oregon-R. All flies were originally maintained on a standard sugar-yeast-agar (SYA) medium [50 g sugar, 100 g yeast, 15 g agar, 30 mL Nipagin (Clariant International Ltd.) (10% wt/vol solution), and 3 mL propionic acid per liter].

**Generation and Maintenance of Lines on CMY and Starch Diets.** We placed populations of Dahomey and the two lines of Oregon-R onto the same starch and CMY diets used in ref. 16 (CMY: 0.65% agar, 7.6% cornmeal, 7.6% molasses, 5% inactivated brewer's yeast, 0.1% methyl-4-hydroxybenzoate,



**Fig. 1.** Gut microbiome composition of CMY and starch lines at generation 30. (A) PCoA of the gut bacterial community of the wild-type strains maintained on the CMY or starch diets. Each symbol represents a single biological replicate comprised of a pool of five individuals. There were two independent biological replicates for each treatment. Wild-type strains are indicated by the different colors; circles and triangles indicate the CMY and starch diets, respectively. (B) Stacked bar plot of community composition and distribution of dominant bacterial taxa (>5% abundance, collapsed to Family level) for the gut microbiomes in A.

**Table 1. Results of PERMANOVA analysis of gut microbiome composition between each of the wild-type lines maintained on CMY or starch diets for 30 generations**

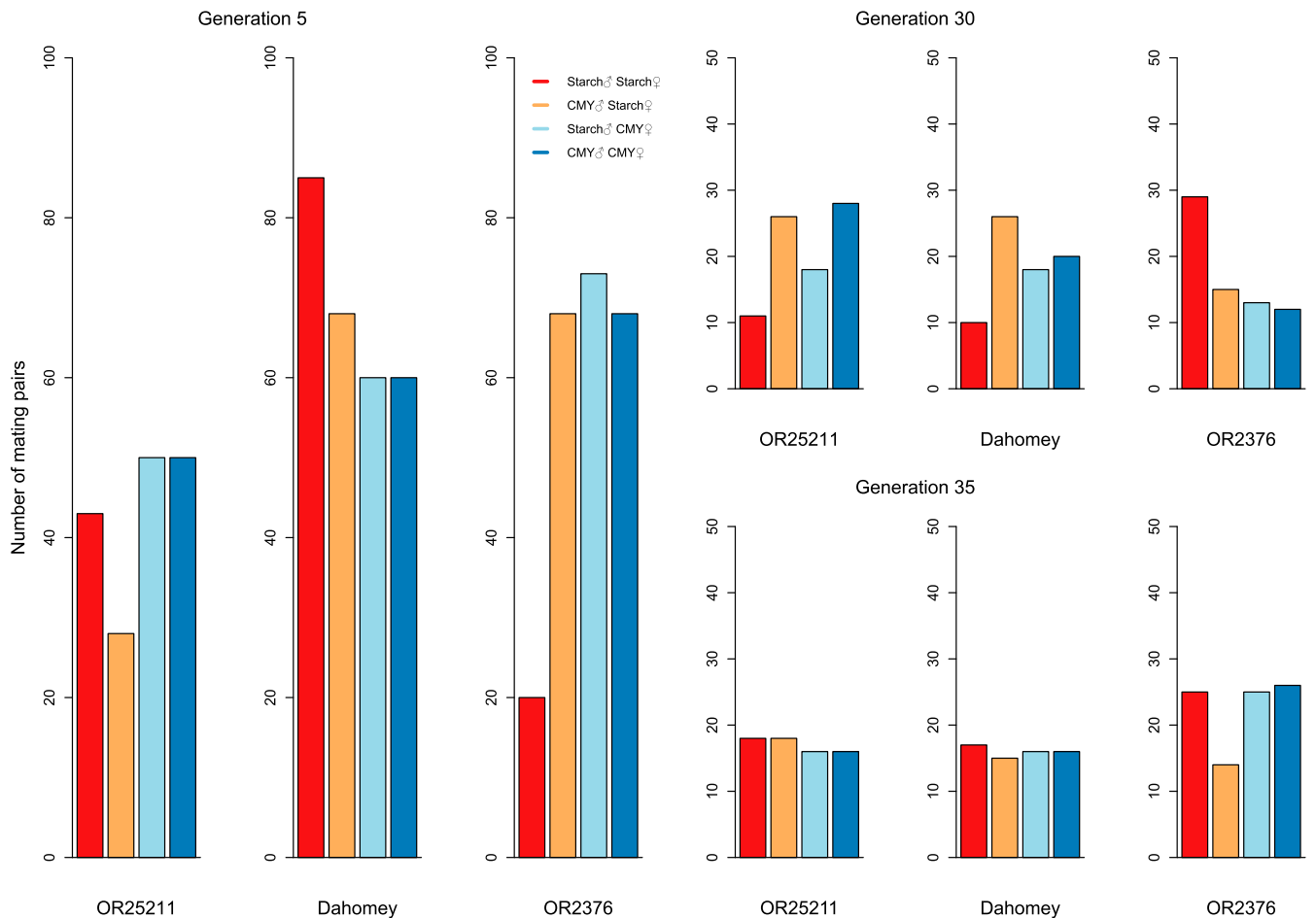
Variable	df	Sum of squares	Mean squares	F	R <sup>2</sup>	P
Line	2	0.36	0.18	1.1	0.102	0.38
Diet	1	1.52	1.52	9.34	0.43	<0.001
Line × diet	2	0.64	0.32	1.98	0.18	0.12
Residuals	6	0.97	0.16		0.28	
Total	11	3.5			1	

There was a highly significant difference in gut microbiome composition in CMY versus starch diets. Number of permutations was 999, with terms added sequentially (first to last). R<sup>2</sup>, coefficient of determination.

0.76% ethanol, and 4% propionic acid; starch: 3% starch, 5% inactivated brewer's yeast, 1% agar, 0.5% propionic acid). We then tested for assortative mating by diet after 5, 30, and 35 generations of rearing on these diets, with the lines maintained in bottle culture with discrete generations. All experiments and culturing were conducted at 25 °C, 50% relative humidity on a 12-h:12-h light:dark photoperiod. At the emergence for each new generation, a group of 200 females and 200 males were placed into a new bottle containing 70 mL of the appropriate diet. Adults were allowed to lay eggs for 48–72 h before being removed to maintain discrete generations. Each of the CMY and starch lines was maintained in two independent lines of bottle culture.

**Composition of the Gut Microbiomes of CMY and Starch Flies, Using 16S rDNA Sequencing.** Using 16S rDNA sequencing, we examined whether the composition of the microbiomes of the starch and CMY flies differed. We compared samples at generation 30 from each of the three lines of *Drosophila* on both CMY and starch media by Illumina sequencing of 16S rRNA genes. We first extracted the DNA by collecting five adults per sample, followed by surface sterilization. The extracted gut tissue was homogenized by grinding with plastic pestles inside 2-mL microcentrifuge tubes and using three freeze/thaw cycles in liquid nitrogen. Samples were then incubated with 180 µL lysis buffer [20 mM Tris-HCl (pH 8.0), 2 mM sodium EDTA, 1.2% Triton-X 100, 20 mg/mL lysozyme] and incubated at 37 °C for 90 min, with brief bead beating at 45 min in a bead beater with 0.1-mm glass beads (Fisher) for 3 min. Twenty microliters of extraction buffer [2 M Tris-HCl (pH 8.5), 2.5 M NaCl, 0.25 M EDTA, 5% wt/vol SDS] and 15 µL of Proteinase K (20 mg/mL) were added, and samples were incubated overnight at 55 °C. After this lysis, 30 µL of 3 M sodium acetate was added, and the samples were allowed to sit for 30 min; tubes were inverted every 10 min for mixing. The samples were then centrifuged at 11,000 × g for 10 min. Three hundred microliters of 100% ice-cold isopropanol were added to each sample and incubated at room temperature for 30 min, followed by centrifuging at 18,000 × g for 30 min. The supernatant was discarded, and the pellet was washed in 70% ice-cold EtOH, air dried, and resuspended in 20 µL 10 mM Tris-Cl, pH 8.5.

Approximately 100 ng of DNA per sample was used as the template for amplification of the 16s rDNA gene. Bacterial universal primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3) were used to amplify a 291-bp fragment. The reverse PCR primer was bar-coded with a 12-base error-correcting Golay code to facilitate multiplexing (42). PCR conditions were as follows: initial denaturation at 98 °C for 3 min, 35 cycles at 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, with a final



**Fig. 2.** Number of matings between wild-type lines maintained on CMY or starch diets. Bar plots represent the number of mating pairs formed in quartet mating tests between CMY and starch diet lines derived from each wild-type population. Matings were scored at generation 5, 30, and 35 of selection of the lines on the two diets. Before mating tests, all flies were reared for one generation on the CMY diet (as in ref. 16).

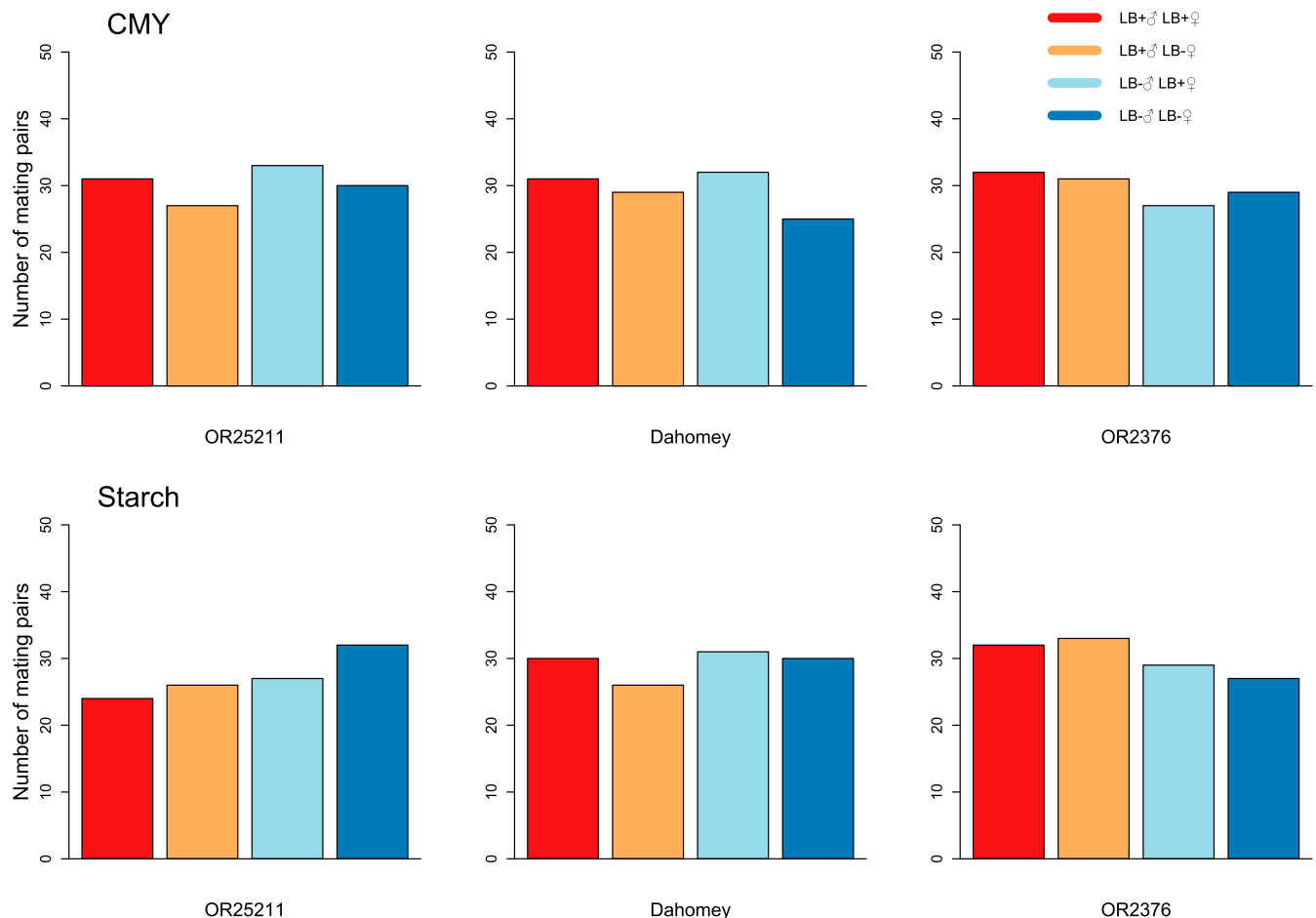
extension for 10 min at 72 °C. Products were pooled at equimolar ratios, and the pool was cleaned with an Agencourt AMPure XP kit (Beckman Coulter). Sequencing was conducted on the Illumina MiSeq 2 × 250 bp platform (provided by the Earlham Institute) according to protocols described in ref. 42.

Sample reads were assembled with mothur v1.32 (43). Chimeric sequences were removed using the USEARCH software based on the UCHIME algorithm (44). Operational taxonomic units (OTUs) were selected using de novo out-picking protocols with a 97% similarity threshold. Taxonomy assignment of OTUs was performed by comparing sequences to the Silva database. Permutational multivariate analysis of variance (PERMANOVA) with 1,000 permutations was used to first identify whether differences in OTU abundances between samples were described more accurately by diet or genotype (45). Linear discriminant analysis coupled with effect size (LEfSe) was performed to identify the bacterial taxa differentially represented in the two diets at Family or higher taxonomic levels (46). Jack-knifed beta diversity of unweighted UniFrac distances was calculated with 10× subsampling, and these distances were visualized by PCoA. The R packages phyloseq and ggplot2 were used for data analysis and visualizing the results, respectively (47, 48).

**Testing for Assortative Mating by Diet.** To test for significant assortative mating by diet, we examined the different wild-type strains following 5, 30, and 35 generations of maintenance on CMY or starch diets. Assortative mating tests were performed as in ref. 16 using quartets of flies comprising one male and one female from the CMY and starch diets. As noted in the correction to the 2010 study (17), only the first mating in any such quartet represents a choice (the second mating being constrained because only one female and male remain). Hence we used the identity of the first pair to mate as the data for tests of assortative mating. For each mating assay experiment, each population was grown for one generation on CMY medium

as in ref. 16, and larvae were raised at a standard density of 100 individuals per vial to both remove any proximate effects of nutrition on mating preference and to minimize environmentally determined differences in body size that might have impacted mating success. At eclosion, flies were collected, and the sexes were separated using light CO<sub>2</sub> anesthesia. Virgin males and females were stored 10 per vial on CMY medium until 1 d before mating. All flies were then anesthetized using light CO<sub>2</sub> anesthesia. Half of the vials from each treatment were selected at random, and the flies within them were given a small wing clip for identification.

For the mating tests, quartets of flies (a single male and female from the CMY treatment and a single male and female from the starch treatment) were aspirated into vials. Wing clipping was used to identify the males and females during the experiment and was rotated in a factorial design (i.e., in half of all tests the CMY males and females were clipped, and in half of all tests the starch males and females were clipped). Hence, the clipping itself was distributed equally across all tests, diet treatments, and sexes so that it could not introduce any systematic confound. The set-up of the mating quartets and the observations of the matings were carried out using a team of researchers who were blind to strain identity. On the day of the mating tests two males were placed in each mating vial (empty vials each containing a moist filter paper strip) followed directly afterward by the two females. The identity of the first pair to mate was then recorded according to the identity of the wing clips of the mating pairs. The clip patterns were decoded after the completion of the mating tests into group/treatment identity. Mating tests were conducted for 5 h from the start of lights on. Pairs were given 2 h to mate, and those that did not mate within this time were discarded. Any vials that contained individuals that died or were immobile during the experiment were discarded. Full sample sizes of initial test numbers and number of matings and nonmatings are detailed in Table S3.



**Fig. 3.** Number of matings between wild-type lines maintained on CMY or starch diets following *L. plantarum* add-back. Bar plots represent the number of mating pairs formed in quartet mating tests between CMY and starch diet lines (generation 38) derived from each wild-type population subjected to an antibiotic mixture to eliminate gut bacteria (as in Fig. S2) and then to *L. plantarum* add-back (LB+) versus non-add-back axenic control (LB-).

**Effect of Microbiome Removal and *L. plantarum* Add-Back on Assortative Mating by Diet.** To rule out the effects of variation in gut microbiome composition, we also tested the effect of gut microbiome removal and *L. plantarum* add-back on assortative mating by diet ([Supporting Information](#)). We treated the adults before the mating tests with a mixture of antibiotics (50 µg/mL tetracycline, 200 µg/mL rifampicin, 100 µg/mL streptomycin) for 48 h to remove their gut bacteria. The effectiveness of this removal was verified as described in [Supporting Information](#). The mating tests on the microbiome-removed flies were then conducted at generations 5, 30, and 35, and *L. plantarum* add-back experiments were performed at generation 38. For the add-back experiment, we isolated *L. plantarum* from each of the three lines (identified to species level by BLAST matching to *L. plantarum*) and tested whether we could generate assortative mating artificially, in the manner proposed in ref. 16, by exposing half of the flies from within the same CMY or starch diet background to  $\pm$  *L. plantarum* and testing for assortative mating as before (for full methods, see [Supporting Information](#)).

**Statistical Analysis of Assortative Mating.** We used the MH test for repeated tests of independence to determine whether repeated observations of mating

pairs showed any deviation from that of random mating. In addition, the number of observed and total possible pairings for each pair type was calculated for each replicate. This was analyzed using JMating v.1.0 (49) to calculate the index of pair sexual isolation (IPSI), a joint isolation index. IPSI varies from  $-1$  to  $+1$ , with  $+1$  being total assortative mating and  $-1$  disassortative mating. Hence, a value of 0 denotes random mating. Following ref. 50, we used IPSI to describe RI at each of the three generational time points. Significance of the coefficient was calculated as the bootstrap probability of rejecting the null hypothesis of random distribution after 10,000 iterations of resampling. All bootstrapping was conducted in JMating; all other statistical analyses were conducted in R v3.3.2 (51). The statistical power of our analyses in comparison with the previous study (16, 17) was then analyzed (for full details, see [Supporting Information](#)).

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