The microbiome responds to Evolve and Resequence experiments in *Drosophila melanogaster*

Lucas P. Henry[1,2], Julien F. Ayroles[1,2]

1. *Dept. of Ecology and Evolutionary Biology, Princeton University*
2. *Lewis Sigler Institute for Integrative Genomics, Princeton University*

email: LPH lhenry@princeton.edu ; JFA jayroles@princeton.edu

Abstract

Experimental evolution has a long history of uncovering fundamental insights into evolutionary processes but has largely neglected one underappreciated component—the microbiome. As eukaryotic hosts evolve, the microbiome may also evolve in response. However, the microbial contribution to host evolution remains poorly understood. Here, we analyzed the metagenomes from 10 E&R experiments in *Drosophila melanogaster* to determine how the microbiome changes in response to host selection. Bacterial diversity was significantly different in 5/10 studies in traits associated with metabolism or immunity. Additionally, we find that excluding reads from a facultative symbiont, *Wolbachia*, in the analysis of bacterial diversity changes the inference, raising important questions for future E&R experiments in *D. melanogaster*. Our results suggest the microbiome often responds to host selection but highlights the need for more work to understand how the microbiome changes the host response to selection.

Introduction

The microbiome has emerged as a key modulator of many organismal phenotypes [1–3]. While many studies show the impact of the microbiome on host phenotypes, the evolutionary implications remain enigmatic [4–6]. The microbiome may contribute to host evolution in unique ways. First, large effective population sizes and rapid generation times may enable microbes to evolve more rapidly than hosts [7]. Second, the microbiome likely encodes distinct genes compared to the host genome, potentially expanding the genomic reservoir to enable adaptation to diverse selective pressures [3,8,9]. If hosts can leverage this microbial evolution, then the microbiome may alter host evolution.

Experimental evolution is a powerful tool to study the basis of adaptation, but remains underutilized in the study of host-microbiome evolution [6,10,11]. One particularly well suited class of studies is Evolve and Resequence (E&R) experiments [12–14]. E&R experiments build on a long history of using artificial selection in evolutionary biology by incorporating new advances in sequencing technologies to measure the genomic responses to selection. E&R experiments are commonly performed in microbes like *E. coli* or yeast, as well as eukaryotes like *Drosophila* [13]. In general, E&R experiments begin with large outbred populations. The population is then reared under a particular selective regime. Selective pressures can take many forms, ranging from threshold selection (e.g., egg size), or general survival under some sort of stressor (e.g., low nutrition diets). In parallel, to control for genetic drift, control populations are maintained in a benign (i.e., non-selective) environment. After a number of generations, the control and evolved populations are sequenced to identify regions of the genomes associated with response to selection. For flies and other eukaryotic hosts, selection is explicitly applied to host populations, but may also act upon the microbiome. When the microbiome influences host phenotypic variation, microbial variation may also affect the response to selection in hosts. Thus, the underappreciated interplay between host and microbial variation has the potential to complicate the interpretation of selection responses based strictly on host genetic variation.

Here, we analyzed the metagenomes from 10 E&R experiments in *Drosophila melanogaster.* Many phenotypes in *D. melanogaster* are responsive to microbial variation, including developmental, metabolic, and immunological traits [15–17]. Furthermore, E&R experiments in *D. melanogaster* capture the evolutionary response to a wide range of different selective pressures, ranging from life history to nutritional to pathogen challenges (Table 1). Thus, E&R experiments in *D. melanogaster* provide a unique opportunity to study how the microbiome responds to host selection.

Methods

We searched the literature for E&R experiments in *D. melanogaster* where replicated selection lines were derived from outbred populations and raw .fastq data were available. We found 10 studies that met these criteria. Our analyses captured a wide range of different selection pressures, from life-history traits to abiotic and pathogen pressures. The diversity of selective pressures and phenotypes under investigation in these studies provides a broad lens to study the microbiome response to host selection. In all cases, the E&R approach sequenced pools of individuals from different selection regimes, but each E&R study had different levels of replication, and number of generations of selection (summarized in Table 1). These were the only data available from published E&R experiments in *D. melanogaster*.

Raw sequences were QC filtered and trimmed using Trimmomatic [18] to remove sequencing adapters, remove low quality reads (average quality per base > 15), and drop reads shorter than 20 bases long. Then, bacterial reads were assigned at the family level using Kraken [19]. Relative abundance of bacterial families were determined using Bracken [20]. We removed any low abundance bacterial family that was assigned fewer than 100 reads as potential contaminants.

Microbial diversity was calculated using the Shannon diversity index. We used this metric to test if microbial diversity was different between control and evolved lines. We determined significance using a t-test. We then tested whether two factors were sufficient to explain variation in microbial diversity between control and selected lines: duration of selection (i.e. the number of fly generations) and *Wolbachia*.

First, the duration of selection ranged from 5-605 generations. We reasoned that selection response in the microbiome might be influenced by length of selection (the longer the selection, the more divergent the microbiome between control and evolved lines). To test if the duration of selection was correlated with changes in microbial diversity, we first calculated the average microbial diversity for the control lines. We then subtracted the diversity of each evolved line from the averaged control diversity to calculate change in diversity. Because we had positive and negative changes in diversity, we used the absolute difference. We performed a linear regression between change in diversity and the log10 duration of selection.

Second, given that *Wolbachia* reads often make up the majority of the microbial reads (ranging from 30-99% of reads) we tested whether or not excluding *Wolbachia* reads influenced bacterial diversity. *Wolbachia* is a facultative, intracellular bacteria transmitted exclusively from mother to offspring. *Wolbachia* has no known environmental reservoir and cannot exist outside of the host. These intracellular, maternally transmitted bacteria have the same evolutionary trajectory as the host genome. The shared transmission mode with the host genome may change the response to selection compared to the other portions of the microbiome that are acquired from the environment [5,6]. Furthermore, recent studies suggest that *Wolbachia* may interact with other bacteria in the microbiome [21,22]. Finally, many studies in *D. melanogaster* exclude *Wolbachia* reads when analyzing microbial communities [17,23–26], leaving an open question as to if *Wolbachia* is considered part of the *Drosophila* microbiome. To test the effect of *Wolbachia* on our inference, we removed *Wolbachia* reads from the communities, and then recalculated Shannon diversity. We compared if bacterial communities without *Wolbachia* reads in our analysis significantly differed between control and evolved lines using a t-test.

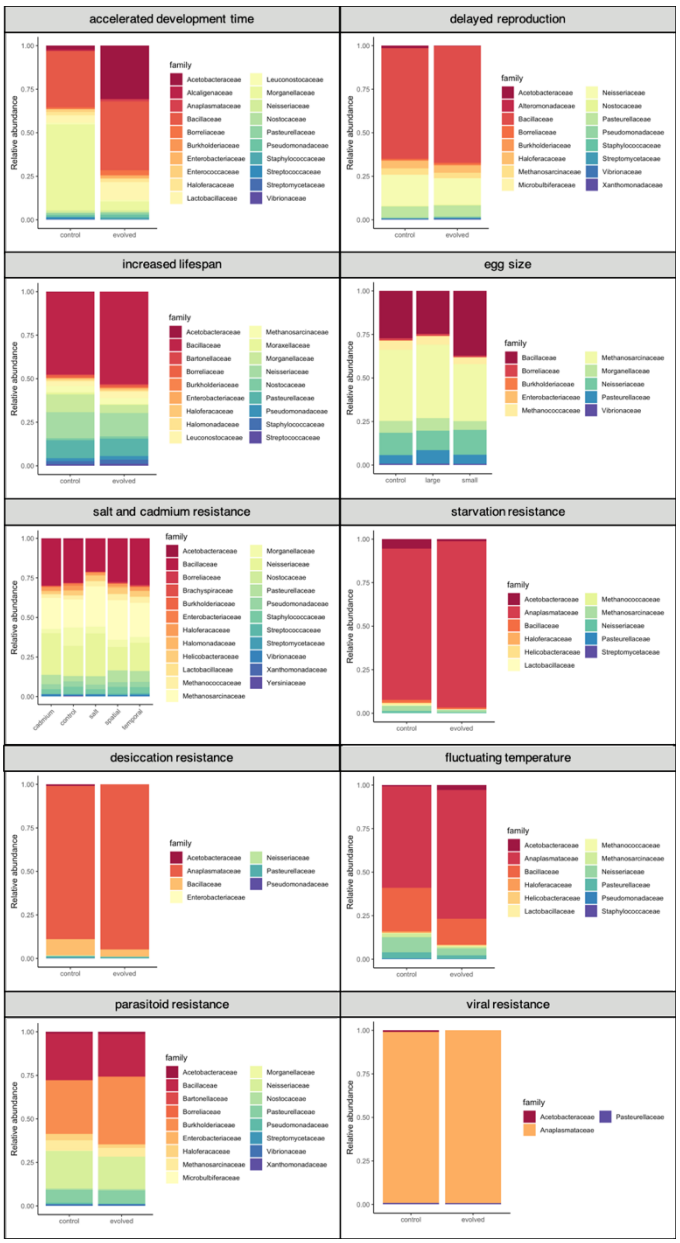

Fig. 1: Relative abundance for bacterial families from the 10 E&R experiments. Each experiment was grouped separately; the colors represent different bacterial families in each.

Results

For each experiment, bacterial families were differentially abundant in control and evolved populations (Fig. 1). Bacterial diversity frequently responded to experimental evolution (Fig. 2). Evolved populations often exhibited reduced levels of bacterial diversity (4/10 studies), though in one case (accelerated development time) bacterial diversity increased (Table 2 for statistical summary). Because the number of generations varied across E&R experiments (from 5-605 *Drosophila* generations), we also tested if change in microbial diversity was correlated with duration of host selection. The change in microbial diversity was only weakly correlated with host generations of

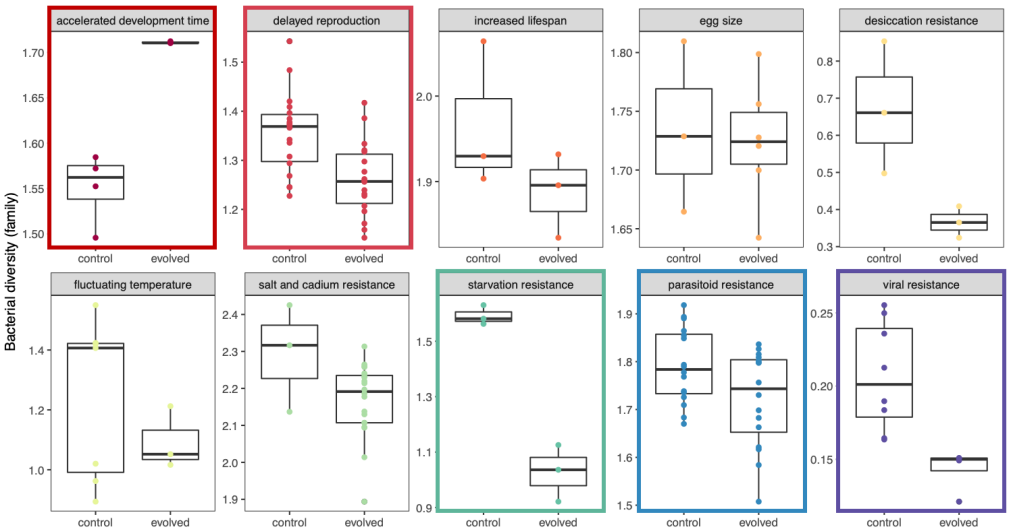

Fig. 2: Bacterial diversity between control and evolve populations in 10 E&R experiments. 5/10 experiments had significantly different bacterial diversity (denoted with the colored outline). Bacterial diversity was calculated at the family level using Shannon diversity metric. Comparisons between control and evolved populations were within each experiment. Each point represents a pool of sequenced flies, and the details of how many flies/experiment is described in Table 1. Color represents each study. Two studies had more than one selection pressure, and the different selection pressure is labelled rather than as "evolved".

selection (Fig. 3, r=0.26, p=0.02). The specific nature of the selective pressure appears to be more important in driving changes in the evolving microbiome. For example, the evolved microbiome in the starvation resistance experiment exhibited the greatest change in bacterial diversity. This may not be surprising given that the *Drosophila* microbiome has been shown to be tightly linked to the regulation of metabolic networks [15]. For other traits, like egg size, the microbiome did not significantly respond to experimental evolution. This analysis suggests that the effect of selection on the microbiome is likely trait specific.

Excluding *Wolbachia* reads from our analysis frequently changed our inference about the response of the evolved microbiome (Fig. 4, Table 2 for statistical summary). First, in accelerated development, excluding *Wolbachia* reads did not affect our estimate of bacterial diversity between control and evolved lines. Second, excluding *Wolbachia* reads leads to an increase in the detection of environmentally acquired bacterial diversity in the evolved lines under fluctuating temperatures. Third, excluding *Wolbachia* reads suggests that environmentally acquired bacteria may not be responding to selection in starvation resistance as there was no difference between control and evolved lines. Finally, in viral resistance, excluding *Wolbachia* reveals an increase in environmentally acquired bacterial diversity in evolved lines, while the whole community showed reduced diversity. Taken together, the inclusion or exclusion of *Wolbachia* reads will significantly alter the observed response to selection in the microbiome.

Discussion

To our knowledge, this is the first systematic examination of the microbiome in E&R experiments in *D. melanogaster*. Given the many fundamental insights gained from E&R experiments in *Drosophila* [13], our results here uncover another layer of variation previously unexplored--the microbiome. The microbiome changed under some selective pressures, while it was unaffected by others (Fig. 1, 2).

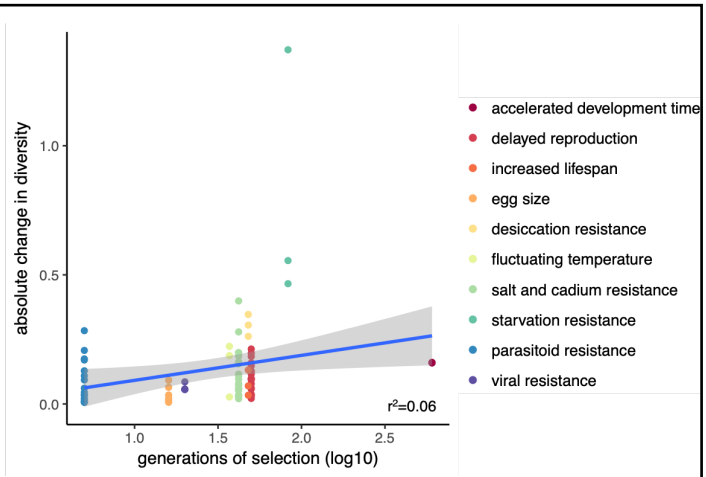

Fig. 3: Evolved bacterial diversity was only weakly correlated with host generations of selection (r = 0.24, p = 0.02). Generations of selection ranged from 5 generations (parasitoid resistance) to > 500 generations (accelerated development). Each point represents the difference between average control diversity and the specific pool of evolved flies for each experiment.

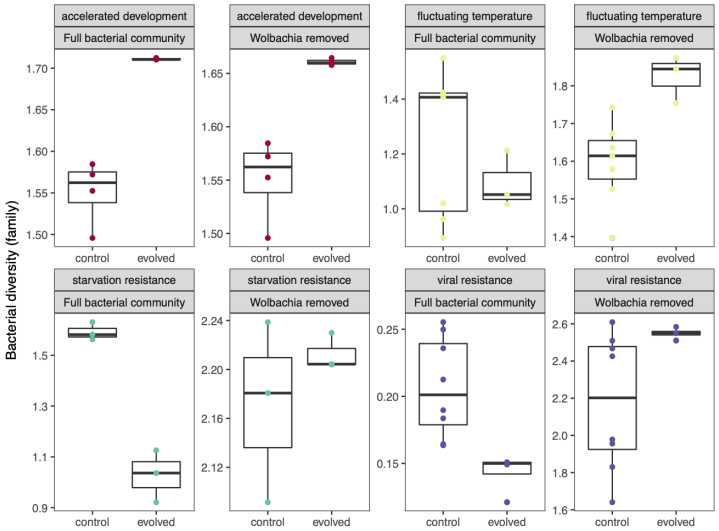

Fig. 4: Differences in bacterial diversity when including (full bacterial community) or excluding *Wolbachia* in 4 of the E&R experiments analysed. Each point represents bacterial diversity for a pool of sequenced flies. The four experiments highlight how the difference between control and evolved microbiomes depends on whether *Wolbachia* is included. For example, for starvation resistance, diversity for the whole community is reduced when *Wolbachia* is included. However, when *Wolbachia* is excluded, there is no response in bacterial diversity between control and evolved populations. Full summary of differences can be found in Table 2.

Pressures closely linked to metabolic processes, like starvation resistance or development time, or immunity affected microbial diversity the most. In *Drosophila*, bacterial genes that increase glucose assimilation and fat storage are necessary for bacterial establishment in the host gut [27–29]. Other pressures, like selection for increased lifespan, egg size, or abiotic stressors (e.g., temperature and heavy metals), did not substantially impact microbial diversity (Fig. 2). It is not surprising that not all selection pressures shape the microbiome; indeed in *Drosophila*, traits like activity, sleep, and some aspects of nutrition are known to not be influenced by the microbiome [30–33]. Our results here contribute to a growing body of literature suggesting that when the microbiome contributes to host phenotypic variation, the microbiome may also impact host evolutionary trajectories [6,34].

We observed several generalities in the microbial response across the E&R experiments. First, the microbiome in both control and evolved populations was composed of similar bacterial families (Fig. 1), suggesting selection did not lead to the complete replacement by different bacterial taxa in evolved populations. In the evolved populations, only a few of the bacterial families increased in relative abundance. Bacteria that contribute to host adaptation may be more likely to persist under selective pressure, increasing in abundance and facilitating local adaptation. Second, the increase in abundance of particular bacterial families also contributed to the frequent reduction in diversity. The reduction in diversity may reflect local adaptation in the microbiome, but also the loss of genetic diversity in the host. We expect that the rapid nature of E&R experiments, combined with strong selective pressures, should lead to a marked reduction in genetic diversity resulting in lower heterozygosity levels across the genome following selection in E&R experiments [13]. Host genetics shapes a significant fraction of the fly microbiome [35], and perhaps the loss of diversity in the host genome also contributed to the reduction in microbial diversity observed here. Correlating host genetic changes with microbial changes is beyond the scope of this current study; however, this is an important factor to consider in future studies.

Excluding *Wolbachia* reads from the analysis had substantial effects on the inference for the observed response in the microbiome (Fig. 4). Importantly, the data presented here do not answer the question of whether or not *Wolbachia* influences the microbiome during fly evolution--rather, our analysis highlight several complications of *Wolbachia*. There are both practical and biological reasons to exclude *Wolbachia* from most microbiome studies as it is intracellular, has low abundance in the gut, has complex effects on host traits, and is overrepresented in 16S rRNA profiling—all characteristics distinct from the more common bacteria in the fly microbiome, like *Acetobacter* and *Lactobacillus* [16,21,22]. Indeed, many studies in *D. melanogaster* either only use uninfected flies [16,30,36,37] or computationally remove *Wolbachia* reads during 16S rRNA microbiome analysis [17,23–26]. We emphasize the importance of considering and testing the potential influence of *Wolbachia* on fly adaptation and host trait variation. In the studies analyzed here, 7/10 studies used flies infected with *Wolbachia*, but only two explicitly mention *Wolbachia* infection status in their flies--fluctuating temperature [38] and viral resistance [39]. Furthermore, only Martins et al. [39] estimated the effects of *Wolbachia* on host phenotype. We acknowledge there is currently ambiguity about including

Wolbachia in the microbiome as discussed here, and we raise these points to highlight unintended potential complications of *Wolbachia* infection, particularly for incorporating the microbiome into E&R studies.

Furthermore, *Wolbachia* has a variety of effects on fly biology, ranging from reproductive phenotypes to immunity to nutrition [40–42] and may substantially influence *Drosophila* evolution [40,43]. *Wolbachia* may also directly affect the microbiome. In a comparison based on a single genotype of flies infected and uninfected with *Wolbachia*, uninfected flies had twice as much *Acetobacter* [21]. However, in the same study, a different fly genotype did not display this effect. Yet, another study found that *Wolbachia* increased *Acetobacter* abundance [22]. These effects are inconsistent and likely depend on interactions between fly genotype, *Wolbachia* genotype, and environmental conditions. If *Wolbachia* interacts positively or negatively with different bacteria, then *Wolbachia* may also influence how the microbiome shapes host phenotypes and contributes to the host evolutionary trajectory. Taken together, the interplay between *Wolbachia*, host, and microbiome is likely complicated. Many insects are infected with *Wolbachia* or similar intracellular symbionts, and these microbe-microbe interactions may have important implications for the host [44].

While this is the first examination of the microbiome in the E&R context, other studies have implicated the microbiome in host adaptation in *D. melanogaster*. For example, when flies are monoassociated with *Lactobacillus plantarum* in nutrient poor environments, *L. plantarum* rapidly evolved symbiotic benefits to increase fly fitness [45]. Across replicates, the *de novo* appearance of several SNPs in the acetate kinase gene (*ackA*) in *L. plantarum* promoted larval growth and nutrition, and subsequently, this *L. plantarum* variant increased in frequency across fly generations. In another study, microbiome manipulation shifted allele frequency in seasonally evolving *D. melanogaster* to match latitudinal patterns of fly genetics [25]. Taken together with our analysis, both host and microbiome likely evolve in response to selection. More generally, other systems like *Brassica* or *Arabidopsis* have also shown that selection on hosts changes the microbiome as well [46,47]. In both these studies, transplanting an evolved microbiome into unevolved hosts changed host phenotypes, suggesting that the microbiome has the capacity to transfer adaptive potential. Similar approaches could be applied to *Drosophila* following E&R experiments. Combined with the rich genetic resources and experimental ease in *Drosophila*, microbiome transplants could illuminate key processes underlying host-microbiome evolution.

We note the experiments analyzed here were not designed explicitly to test the role of the microbiome in host adaptation. This may impact our results in several ways. None of these studies were executed with quality control measures that can affect estimates of microbial diversity, such as process blanks during DNA extraction, no template controls during PCR, and batch effects during library preparation [48–51]. While we applied an arbitrary cutoff to remove contaminants, it is difficult to know how potential contaminants may affect the observed results. However, we note that contamination would have to differentially affect control and evolved microbiomes to influence our results--which we believe is unlikely. Surveys of microbial diversity in *D. melanogaster* typically use 16S rRNA profiling and find bacteria from the Acetobacteraceae, Firmicutes, and Enterobacteriaceae [15,17,52]. Our mapping approach detected these bacteria commonly associated with *D. melanogaster*, but also found abundant methanogens and human commensal microbes (Fig. 1). One discrepancy could arise from our metagenomic approach, which will often lead to different conclusions than 16S rRNA profiling [49]. Mining metagenomes from existing whole genome sequencing is an emerging area of research in the microbiome, and more work is necessary for biological interpretations [5,49]. Nevertheless, the consistent differences in the microbiome across experiments shown here highlight how E&R experiments could provide exemplary opportunities to investigate the genetic basis underlying host-microbiome evolution.

In conclusion, the microbiome frequently responded to selection in ten E&R studies in *D. melanogaster.* Our results here associate the microbiome in the host response to some selective pressures, but more work is necessary to partition the relative contribution of host genetics and microbial evolution. We observed large differences in bacterial diversity between control and evolved populations, but a key question remains—if and how the microbiome alters the host response to selection. Combining E&R experiments with approaches from quantitative genetics will be especially fruitful to dissecting the microbial contribution to host evolution [6]. Tracking the rate of microbial evolution over multiple timepoints during fly adaptation will be particularly helpful to elucidate whether the microbiome shapes the host evolutionary trajectory. Partitioning the microbial effects on host phenotype during adaptation may show that microbiome facilitates or impedes host adaptation. Reciprocal transplants over the course of host adaptation will also demonstrate how the microbiome modifies host evolution. Overall, incorporating the microbiome into E&R experiments will provide fundamental insights into host-microbiome evolution.

Acknowledgements

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Data Availability

Code used in the analysis is available at https://github.com/lphenry/extgeno. Upon publication, data will be uploaded into public repository.

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Table 1: Evolve & Resequence studies analyzed

Pressure	Evolved Phenotype	Duration of selection (fly generations)	*Wolbachia*	Sequencing
Accelerated development[1]	Flies developed from egg to adult 20% faster than control lines	605	Infected	25 females pooled from each line; 4 control and 4 evolved
Delayed reproduction[2]	Age of reproduction increased from 28 to 40 days	50	Uninfected	100 females pooled from each line; 3 control and 3 evolved
Increased lifespan[3]	Median lifespan was increased from 4 weeks to 7-8 weeks	48	Uninfected	250 males + 250 females pooled from each line; 3 control and 3 evolved
Egg size[4]	Egg size was selected ~20% larger and smaller eggs	16	Infected	100 females pooled from each line; 3 control, 3 small, 3 large
Desiccation resistance[5]	Desiccation resistance (hrs until 80% mortality) increased 70-80%	48	Infected	100 females pooled from each line; 3 control and 3 evolved
Fluctuating temperatures[6]	Survival under fluctuating temps 18-28ºC daily	37	Infected	500 females pooled for each line at different time points; beginning, middle, and end; 3 control and 3 evolved--only compared beginning and end
Salt + cadmium resistance[7]	Survival in constant, spatially, temporally varying salt and/or cadmium	42	Infected	70 females pooled from each line; 3 control lines and 5 lines for each selection pressure
Starvation resistance[8]	Starvation resistance (hrs to death w/o food) increased ~25%	83	Infected	100 females pooled for each line; 3 control and 3 evolved lines
Parasitoid resistance[9]	Resistance to parasitoid increased from 20% to 50%	5	Uninfected	50 females pooled from each line; 16 control and 16 evolved lines
Viral resistance[10]	Resistance to *Drosophila* C virus increased from 25% to 75%	20	Infected	200 individuals pooled from each line; 4 control, 4 procedure control, 4 evolved

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Table 2: Statistical differences for bacterial diversity between control and evolved microbiomes

Pressure	**test stat**	**df**	**significance**
accelerated development	t = -8.134	df = 3.006	p-value = 0.004
accelerated development without *Wolbachia*	t = -5.548	df = 3.060	p-value = 0.011
delayed reproduction	t = 3.567	df = 33.666	p-value = 0.001
increased lifespan	t = 1.369	df = 3.183	p-value = 0.260
egg size	t = 0.213	df = 3.104	p-value = 0.844
egg size without *Wolbachia*	t = 0.251	df = 3.075	p-value = 0.817
desiccation resistance	t = 2.881	df = 2.225	p-value = 0.090
desiccation resistance without *Wolbachia*	t = 0.394	df = 3.985	p-value = 0.714
fluctuating temperature	t = 1.234	df = 7.996	p-value = 0.252
fluctuating temperature without *Wolbachia*	t = -4.122	df = 6.880	p-value = 0.005
salt and cadmium resistance	t = 1.4294	df = 2.285	p-value = 0.274
salt and cadmium resistance without *Wolbachia*	t = 1.4294	df = 2.285	p-value = 0.2743
starvation resistance	t = 9.024	df = 2.460	p-value = 0.006
starvation resistance without *Wolbachia*	t = -0.973	df = 2.161	p-value = 0.426
parasitoid resistance	t = 2.186	df = 28.389	p-value = 0.037
viral resistance	t = 4.262	df = 9.819	p-value = 0.002
viral resistance without *Wolbachia*	t = -2.851	df = 7.187	p-value = 0.024