Meta-analysis suggests the microbiome responds to Evolve and Resequence experiments in *Drosophila melanogaster*

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ABSTRACT

Background: 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. However, the microbial contribution to host evolution remains poorly understood. Here, we re-analyzed genomic data to characterize the metagenomes from ten Evolve and Resequence (E&R) experiment in *Drosophila melanogaster* to determine how the microbiome changed in response to host selection.

Results: Bacterial diversity was significantly different in 5/10 studies, primarily in traits associated with metabolism or immunity. Duration of selection did not significantly influence bacterial diversity, highlighting the importance of associations with specific host traits. 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 the *D. melanogaster* microbiome.

Conclusions: Our genomic re-analysis suggests the microbiome often responds to host selection; thus, the microbiome may contribute to the response of *Drosophila* in E&R experiments. We outline important considerations for incorporating the microbiome into E&R experiments. The E&R approach may provide critical insights into host-microbiome interactions and fundamental insight into the genomic basis of adaptation.

Keywords: experimental evolution, Drosophila melanogaster, microbiome

41 ***Background***

42 The microbiome has emerged as a key modulator of many organismal phenotypes [1–3].
43 While many studies show the impact of the microbiome on host phenotypes, the evolutionary
44 implications remain enigmatic [4–6]. The microbiome may contribute to host evolution in unique
45 ways. First, large effective population sizes and rapid generation times may enable microbes to
46 evolve more rapidly than hosts [7]. Second, the microbiome likely encodes distinct genes
47 compared to the host genome, potentially expanding the genomic reservoir to enable adaptation
48 to diverse selective pressures [3, 8, 9]. If hosts can leverage this microbial evolution, then the
49 microbiome may alter host evolution.
50
51 Experimental evolution is a powerful tool to study the basis of adaptation, but remains
52 underutilized in the study of host-microbiome evolution [6, 10, 11]. One particularly well-suited
53 class of these studies is Evolve and Resequence (E&R) experiments [12–14]. E&R experiments
54 build on the long history of using artificial selection in evolutionary biology by incorporating new
55 advances in sequencing technologies to measure the genomic responses to selection. E&R
56 experiments are commonly performed in microbes like *E. coli* or yeast, as well as eukaryotes
57 like *Drosophila* [13]. In general, E&R experiments begin with large outbred populations. The
58 population is reared under a particular selective regime. The selective regime can take many
59 forms, ranging from threshold selection (e.g., egg size) or general survival under some sort of
60 stressor (e.g., low nutrition diets). In parallel, to control for genetic drift, control populations are
61 maintained in a benign (i.e., non-selective) environment. After a number of generations, the
62 control and evolved populations are sequenced to identify regions of the genomes associated
63 with the response to selection. For flies and other eukaryotic hosts, selection is explicitly applied
64 to host populations, but may also act upon the microbiome. When the microbiome influences
65 host phenotypic variation, microbial variation may also affect the response to selection in hosts.
66 Thus, the underappreciated interplay between host and microbial variation has the potential to
67 complicate the interpretation of selection responses based strictly on host genetic variation.
68
69 Microbes may be underappreciated drivers of host phenotypic variation. For example,
70 *Wolbachia* infection can rescue deleterious phenotypes in homozygous mutant *Drosophila* lines
71 [15–17]. Body color in aphids is partially determined by *Rickettsia* secondary symbionts [18].
72 These phenotypic effects are not limited to single microbial species, but also include more
73 complex microbiomes. In cows, the microbiome explained 13% of methane emissions [19] and
74 26-42% of fatty acid composition of milk [20]. The microbiome also explained 33% of weight
75 gain in pigs [21]. For both pigs and cows, the microbiome contributed almost as much to traits
76 as host genetics. These examples suggest that the microbiome in many host taxa is an
77 important determinant in host phenotypes, and, in turn, may shape the selection response for

hosts. E&R experiments may thus be missing a substantial component that shapes the host evolutionary response.

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 [22–24]. 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. Our goal here is to explore these publicly available data and using meta-analysis, characterize patterns in the metagenomes of these experiments, and to highlighting potential future directions to show how E&Rs can identify signatures of selection in host-microbiome evolution.

Results

The 10 E&R experiments analyzed for metagenomes ranged in a variety of selective pressures (see Table 1 for full description)--from life history (accelerated development [25], delayed reproduction [26], increased lifespan [27], egg size [28]) to abiotic pressures (desiccation resistance [29], fluctuating temperature [30], salt and heavy metal resistance [31]) to biotic pressures (starvation resistance [32], parasitoid resistance [33], viral resistance [34]). All experiments had replicated control and evolved populations, although replication varied from as few as three to as many as 18 (Table 1). Given the importance of the diet in shaping microbial variation, we examined the reported characteristics of the diet from each study. Importantly, for 9/10 studies, the reported diets did not differ between control and evolved populations (Table 1). Only for the cadmium and salt resistance study [31] were diets different for the entire lifespan between control and evolved populations. The starvation resistance study [32] exerted starvation on adults for four days, and then flies that survived were returned to a standard diet to propagate the next generation. While the diets may have varied between studies, only 5/10 studies described the diet (Table 1). The lack of consistent dietary reporting is a major challenge for *Drosophila*-microbiome studies [35]. As the majority of these studies do not report specific dietary information, we are unable to explore the effects of diet across E&Rs in this analysis.

Because each experiment has replicated control and evolved populations, we compared microbiomes within each experiment. In the E&R context, control populations represent the standing genetic variation from which selection proceeds. Thus, by comparing control and evolved populations within experiment, the effects of many different factors (e.g., local

laboratory environment, different diets, different fly populations) are controlled for in our analysis. For each experiment, bacterial families were differentially abundant in control and evolved populations (Fig. 1; Supp. Figs 1-10 for individual replicates for each experiment). Control and evolved populations tended to harbor similar bacterial families across replicates, within each experiment, as measured through beta-diversity (Jaccard similarity; Fig. 2, Table 2). Only in two experiments did control and evolved populations differ in community membership--accelerated development time and delayed reproduction. Bacterial alpha-diversity frequently responded to experimental evolution (Fig. 3). Evolved populations often exhibited reduced levels of bacterial diversity (4/10 studies), though in one case (accelerated development time) bacterial diversity increased (Table 3 for statistical summary). Taken together, the microbiome frequently shifts in response to host selection (i.e., differences in alpha-diversity), but does not necessarily gain different microbes (i.e., no difference in beta-diversity).

Because the number of generations varied across E&R experiments (from 5-605 *Drosophila* generations; see Table 1), we also tested if change in microbial diversity was correlated with duration of host selection. One hypothesis is that shorter selection experiments provide less opportunity for the microbiome to change, while longer selection provides more opportunity for increased microbial change. The change in microbial diversity was not correlated with duration of selection after controlling for each study as a random effect (Fig. 4, $r=0.05$, $p=0.649$). The specific nature of the selective pressure appears to be more important in driving changes in the evolving microbiome as experiment explained 76% of variance in our model (Table 4). 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 [22]. For other traits, like egg size, the microbiome did not significantly respond to experimental evolution. This analysis suggests that the effect of selection on microbiome is likely trait specific.

We note that, in seven out of ten studies, flies were infected with *Wolbachia* (Table 1). *Wolbachia* was <2% of reads for three studies, but 48-98% for the other four studies. To better understand the association between *Wolbachia* and the microbial response to selection, we focused on these four studies with high relative abundance (Fig. 5). *Wolbachia* was significantly more abundant for evolved populations in starvation and viral resistance, though also tended to increase for desiccation resistance and fluctuating temperatures (Table 5 for statistical summary). Furthermore, excluding *Wolbachia* reads from our analysis revealed different inferences about the response of the evolved microbiome (Fig. 6, Table 3 for statistical summary). First, in desiccation resistance, excluding *Wolbachia* reads reduced the difference in

diversity between control and evolved populations, but did not change our inference as no significant difference was observed for the full bacterial community. Second, excluding *Wolbachia* reads leads to an increase in environmentally acquired bacterial diversity in the evolved lines under fluctuating temperatures. Third, excluding *Wolbachia* reads shows that environmentally acquired bacteria did not respond to selection in starvation resistance as there was no difference between control and evolved lines. Finally, in viral resistance, excluding *Wolbachia* increased environmentally acquired bacterial diversity in evolved lines, while the whole community showed reduced diversity. For the three studies with low *Wolbachia* abundance, excluding *Wolbachia* reads did not impact estimates of diversity (Table 3). Taken together, importantly, we suggest careful consideration towards considering inclusion or exclusion of *Wolbachia* reads, as it 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 *Drosophila* in E&R experiments [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, 3). 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, suggesting that hosts select for bacteria to enhance metabolism [36–38]. 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. 3). It is not surprising that not all selection pressures shape the microbiome; indeed, in *Drosophila*, traits such as activity level, sleep, and some aspects of nutrition are known to not be influenced by the microbiome [39–42]. Our results suggest that the microbiome changes along with host evolution in the E&R context – although we emphasize that the data we present are a re-analysis of existing genomic data and not derived from new manipulative experiments. Our results here contribute to a growing body of literature suggesting that when the microbiome contributes to host phenotypic variation, the microbiome has the potential impact host evolutionary trajectories [6, 43]. The evolutionary interplay between host and their microbiome may play an important role in driving host evolution (beyond the explicit selective pressures exerted in these experiments), and this level of variation should not be ignored in E&R analyses.

We observed several generalities in the microbial response in E&R experiments. First, the microbiome in both control and evolved populations was composed of similar bacterial

189 families (Fig. 1), suggesting selection did not lead to the complete replacement by different
190 bacterial taxa in evolved populations. In evolved populations, only a few of the bacterial families
191 increased in relative abundance. Furthermore, in all studies, replicate lines from both control
192 and evolved populations show similar community compositions, suggesting consistent effects
193 on the microbiome (Figure 2, Supp. Figs. 1-10). Bacteria that contribute to the host adaptation
194 may be more likely to persist under the selective pressure, increasing in abundance and
195 facilitating local adaptation. Second, the increase in abundance of particular bacterial families
196 also contributed to the frequent reduction in diversity. The reduction in diversity likely reflects
197 local adaptation in the microbiome, but potentially also the loss of genetic diversity in the host.
198 We expect that the rapid nature of E&R experiments, combined with strong selective pressures,
199 results in lower heterozygosity levels across the genome following selection in E&R experiments
200 [13]. Host genetics shapes a significant fraction of the fly microbiome [44], and perhaps the loss
201 of diversity in the host genome also contributed to the reduction in microbial diversity observed
202 here. While evolved populations have reduced heterozygosity, they still maintain substantial
203 heterozygosity across the genome. More research is necessary to understand how host
204 genome-wide diversity affects microbial diversity, or if only certain host loci are the key drivers
205 of changes in microbial diversity. Uncovering the specific host genetic loci that may be
206 associated with microbial changes is beyond the scope of this current study; however this is an
207 important factor to consider in future studies.
208
209 For the evolved microbiomes, bacteria may have evolved different functions that hosts
210 can leverage. For example, the relative abundance of *Acetobacteraceae* is enriched in the
211 evolved populations for accelerated development time (Fig. 1A). *Acetobacter* produces acetic
212 acid that modulates the insulin/insulin-like signalling (IIS) growth factor pathway in flies [36].
213 More so, *Acetobacter* is frequently associated with accelerated development compared to other
214 bacteria [24, 36, 45, 46]. The IIS pathway may also integrate metabolic products from other
215 bacteria in the microbiome to help regulate fly metabolism. *Wolbachia* infection has been shown
216 to increase insulin signaling in *Drosophila* [17]. The increased *Anaplasmataceae* abundance in
217 the evolved populations may better regulate metabolic traits to mitigate selection in the
218 starvation resistance experiment (Fig. 1H). We hypothesize that increased relative abundance
219 for particular bacteria in the evolved populations corresponds to functional changes and is
220 suggestive of fitness benefits for the fly. Subsequently, flies transmit and preferentially associate
221 with the beneficial microbes. However, bacteria may also be increasing in the evolved
222 conditions independently of any host selection. To better understand how microbial evolution
223 interacts with host evolution, longitudinal sampling over the course of the evolutionary trajectory
224 is necessary. Identifying if beneficial adaptations emerge first in the microbiome and then alter

allele frequencies in host populations would provide key insights into how host-microbiome interactions shape eukaryotic evolutionary processes.

The temporal aspect of host-microbiome evolution is important, but underexplored and thus poorly understood. Our analysis suggests that time did not significantly affect the difference in diversity between control and evolved populations (Fig. 4). This might be because the microbiome changes rapidly, within a single host generation, but the evidence for rapid change is inconsistent. One study found that the microbiome was significantly different when flies were starved or shifted to a high fat diet [47], while another study found no differences when shifted to low or high sugar diets [48] within their lifespan. Finally, in flies mono-associated with *Lactobacillus* reared in nutrient poor diets, *Lactobacillus* evolved beneficial mutations that promoted fly growth in only 5 fly generations [49]. While most experiments did not manipulate diets in our E&R analysis, the findings from these three studies suggest a range of timescales in which the microbiome may evolve. Conducting longitudinal surveys of the microbiome during experimental evolution is essential to understanding if and how the microbiome shapes host evolutionary trajectories.

Excluding *Wolbachia* reads from the analysis had substantial effects on the inference for the observed response in the microbiome (Fig. 6). This change in inference often resulted from an increase in *Wolbachia* abundance in evolved populations (Fig. 5). The phenotypic effects exerted by *Wolbachia* on their hosts often depend on the degree of increase in abundance. For example, higher abundance provided stronger cytoplasmic incompatibilities [50], increased protection from viruses [51], or greater reductions in lifespan [52]. However, several factors may actually confound the *Wolbachia* results observed in our meta-analysis here. Infection was only assessed from pools of flies – we do not have access to individual level status (Table 1). Relative abundance may reflect the average relative abundance within individuals or heterogeneous infection patterns across individuals. We believe this second scenario of heterogeneous infection across individuals is not likely for the four high *Wolbachia* studies we examined in more detail. In a study that examined how *Wolbachia* infection spreads within outbred populations, *Wolbachia* infection across individuals increased to 80% by 10 generations and nearly 100% by 32 generations [53]. This suggests that for the four high Wolbachia studies, most individuals were infected. However, for the three studies with low *Wolbachia* abundance, it may be that *Wolbachia* infection status is highly heterogeneous across individuals or reflects recent *Wolbachia* infections (or simply contamination). Fly age also affects *Wolbachia* abundance, increasing in older flies [54]. Unfortunately, age of flies was only described in 3/10 studies (Table 1), but was always similar between control and evolved populations. Age might affect our results if control and evolved populations were systematically collected at different

262 ages. We think this unlikely as it would also bias the genomic analysis as survival may differ
263 between control and evolved populations at different ages (e.g., viability selection). Overall,
264 designing experiments that explicitly control for *Wolbachia* infection is necessary to understand
265 its potential influence on host-microbiome evolution [14, 23].
266
267 Importantly, the data presented here cannot answer the question whether *Wolbachia*
268 influences the microbiome during fly evolution--rather, our analysis highlights several
269 complications of *Wolbachia*. There are both practical and biological reasons to exclude
270 *Wolbachia* from most microbiome studies as it is intracellular, low abundance in the gut, has
271 complex effects on host traits, overrepresentation in 16S rRNA profiling--all characteristics
272 distinct from the more common bacteria in the fly microbiome, like *Acetobacter* and
273 *Lactobacillus* [23, 55, 56]. Indeed, many studies in *D. melanogaster* either only use uninfected
274 flies [23, 39, 45, 46] or computationally remove *Wolbachia* reads during 16S rRNA microbiome
275 analysis [24, 57–60]. We emphasize the importance of considering and testing the potential
276 influence of *Wolbachia* on fly adaptation and trait variation. In the studies analyzed here, 7/10
277 studies used flies infected with *Wolbachia*, but only two explicitly mention *Wolbachia* infection
278 status in their flies--fluctuating temperature [30] and viral resistance [34]. Furthermore, only
279 Martins et al. [34] estimated the effects of Wolbachia on host phenotype by clearing *Wolbachia*
280 infections and then re-measuring phenotypes. Notably, no study assessed the change in
281 *Wolbachia* relative abundance. While our approach cannot assess the functional importance,
282 future studies could take a similar approach to Martins et al. by clearing *Wolbachia* infections
283 and re-phenotyping. We acknowledge there is currently ambiguity about including *Wolbachia* in
284 the microbiome as discussed here. We raise these points to highlight unintended potential
285 complications of *Wolbachia* infection, particularly for incorporating the microbiome into E&R
286 studies.
287
288 Furthermore, *Wolbachia* has a variety of effects on fly biology, ranging from reproductive
289 phenotypes to immunity to nutrition [61–63] and may substantially influence *Drosophila*
290 evolution [61, 64]. *Wolbachia* may also affect the microbiome. In a comparison of a single
291 genotype of flies infected and uninfected with *Wolbachia*, uninfected flies had twice as much
292 *Acetobacter* [55]. However, in the same study, a different fly genotype did not display this effect.
293 Yet, another study found that *Wolbachia* infection increased *Acetobacter* abundance [56]. These
294 effects are inconsistent and likely depend on interactions between fly genotype, *Wolbachia*
295 genotype, and environmental conditions. If *Wolbachia* interacts positively or negatively with
296 different bacteria, then *Wolbachia* may also influence how the microbiome shapes host
297 phenotypes and contributes to the host evolutionary trajectory. Taken together, the interactions
298 between *Wolbachia*, host, and microbiome are likely complicated. Many insects are infected

with *Wolbachia* or similar intracellular symbionts, and these microbe-microbe interactions may have important implications for the host [65].

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, as previously mentioned, when flies were monoassociated with *Lactobacillus plantarum* in nutrient poor environments, *L. plantarum* rapidly evolved symbiotic benefits to increase fly fitness [49]. 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 [59]. Both of these studies rely on mono-associations with single microbes, but this likely does not realistically capture host-microbe dynamics. Higher order interactions among bacteria shape phenotypes in *Drosophila* [45, 46]. Interaction among microbes, like cross-feeding of metabolites between *Acetobacter* and *Lactobacillus*, can enable mutually beneficial growth for both bacteria species as well as increases bacterial growth, but critically also alters fly metabolism [66]. Furthermore, even strain-level variation within a bacterial species can have divergent effects on host phenotypes [67, 68]. The technical challenges associated with accurately quantifying genetic variation across complex microbial populations necessitated these mono-association experiments. Fortunately, new emerging methods are enabling the identification of signatures of selection in complex microbiomes [69, 70]. Future experiments with more complex and realistic microbiomes will show how microbe-microbe interactions contribute to host adaptation.

Taken together with our analyses, both the host and microbiome likely evolve in response to selection. More generally, other systems like *Brassica* and *Arabidopsis* have also shown that selection on hosts changes the microbiome as well [71, 72]. 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

336 batch effects during library preparation [69, 73–75]. While we applied an arbitrary cutoff to
337 remove contaminants, it is difficult to know how potential contaminants may affect the observed
338 results. However, contamination would have to differentially affect control and evolved
339 microbiomes to influence our results--which we believe is unlikely. Surveys of microbial diversity
340 in *D. melanogaster* typically use 16S rRNA profiling and find bacteria from the
341 *Acetobacteraceae*, *Firmicutes*, and *Enterobacteriaceae* [22, 24, 76]. Our mapping approach
342 detected these bacteria commonly associated with *D. melanogaster*, but also found abundant
343 methanogens and human commensal microbes (Fig. 1). One discrepancy could arise from our
344 metagenomic approach, which will often lead to different conclusions than 16S rRNA profiling
345 [69]. Mining metagenomes from existing whole genome sequencing is an emerging area of
346 research in the microbiome, and more work is necessary for biological interpretations [5, 69].
347 Finally, none of the flies sequenced in these studies were surface sterilized, and thus, the
348 metagenomes characterized here result from both the external body surface and internal gut
349 microbiome. However, the external microbiome is orders of magnitude less abundant than the
350 internal microbiome across the fly lifespan [77]. While we cannot distinguish between external
351 and internal microbiomes in this analysis, future studies should be clear if the total (external and
352 internal) or gut microbiomes were sequenced. Nevertheless, the consistent differences in the
353 microbiome across experiments shown here highlight how E&R experiments could provide
354 exemplary opportunities to investigate the genetic basis underlying host-microbiome evolution.
355
356 ***Conclusions***
357 For researchers interested in adapting the E&R approach for host-microbiome
358 interactions, we have several key recommendations. As we have suggested above, more
359 intensive temporal sampling to capture both microbial and host evolution is necessary. For
360 *Drosophila*-microbiome E&R experiments, researchers may wish to begin the experimental
361 evolution by standardizing the microbiome between control and evolved populations, like with
362 the 5-species bacterial community commonly used [23, 78] or fly feces to mimic natural, but
363 standardized microbial inoculation [79, 80]. Second, as much of the microbiome is determined
364 by the environment in flies, researchers need to use consistent brands of yeast, preservatives,
365 and other aspects of diet/environment. For example, different preservatives have different
366 effects on the microbiome and behavioral traits [40, 41]. *Drosophila* in different labs in the same
367 building (with the same kitchen for fly food) had different microbiomes [76], suggesting that
368 several aspects of the environment are important in shaping the fly microbiome. Finally, as
369 outlined by Goodrich et al. [81], microbiome research requires careful planning (with both
370 biological and technical controls), extensive documentation, and consistency. Importantly, we
371 are not advocating that every E&R experiment incorporates the microbiome, but note that the
372 microbiome may impact conclusions from E&R experiments. For researchers not explicitly

interested in the microbiome, our primary recommendation is to clear fly lines of *Wolbachia* to avoid potential confounding effects between host genetic and *Wolbachia* evolution as others have suggested [14, 23].

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 effects 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. Our results here suggest that the microbiome might influence host evolution, but do not prove it. To measure how the microbiome affects host evolutionary trajectory, combining several of these techniques will be necessary. Overall, incorporating the microbiome into E&R experiments will provide fundamental insights into host-microbiome evolution.

Methods

We searched the literature for E&R experiments in *D. melanogaster* where replicated control and selection lines were derived from outbred populations and raw .fastq data were publicly 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, enabling generalizations about 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 (summarized in Table 1). We report the diet as described in the publication for each study (Table 1). While most studies did not publish specifics about the diet, we noted the diets that differed between control and evolved populations (only one study [31]); if the publication did not specify, we reasonably assumed diets were the same. These were the only data available from published E&R experiments in *D. melanogaster* at the time of submission.

Raw sequences were cleaned using Trimmomatic [82] to remove sequencing adapters, remove low quality reads (average quality per base > 15), and drop reads shorter than 20 bases

410 long. Then, bacterial reads were assigned at the family level using Kraken [83]. Relative
411 abundance of bacterial families were determined using Bracken [84]. We removed any low
412 abundance bacterial family that was assigned fewer than 100 reads as potential contaminants.
413
414 Bacterial data was analyzed using the phyloseq package [85]. To assess if bacterial
415 communities were fully sampled, rarefaction was performed (step size =1000) using ggrare [86].
416 Rarefaction curves indicate communities were fully sampled in all experiments (Supp. Figs 1-
417 10). Beta-diversity to test differences in community composition between control and evolved
418 populations was performed using PERMANOVA on Jaccard similarity. Bacterial alpha-diversity
419 was calculated using the Shannon diversity index. For each experiment, each line was
420 subsampled with replacement to the minimum number of reads in the experiment. Diversity was
421 calculated on this rarefied library. The subsampling was performed 100 times to minimize
422 stochasticity and artificial inflation of diversity associated with rarefaction [87]. Diversity was
423 then averaged across the 100 subsampling efforts and compared between control and evolved
424 lines. We determined significance using Welch's t-test. We then tested whether two factors
425 were sufficient to explain variation in microbial diversity between control and evolved lines:
426 duration of selection (i.e., the number of fly generations) and *Wolbachia* infection.
427
428 First, the duration of selection ranged from 5-605 generations. We reasoned that
429 selection response in the microbiome might be influenced by length of selection (the longer the
430 selection, the more divergent the microbiome between control and evolved lines). To test if the
431 duration of selection was correlated with changes in microbial diversity, we first calculated the
432 average microbial diversity for the control lines. We then subtracted the diversity of each
433 evolved line from the averaged control diversity to calculate change in diversity. Because we
434 had positive and negative changes in diversity, we used the absolute difference. We performed
435 a linear regression between change in diversity and the log10 duration of selection, modeled as
436 $Y = a + b + e$, where Y= change in diversity, a = log10 duration of selection, b = random effect of
437 experiment, and e = residual error. Lme4 was used to perform the regression in R [88].
438
439 Given that *Wolbachia* reads frequently make up the majority of the microbial reads
440 (Supp. Table 1), we examined if *Wolbachia* relative abundance differed between control and
441 evolved populations. We focused on only the four studies with *Wolbachia* relative abundance
442 >2%. Statistical significance was assessed with a Kruskal-Wallis test on Wolbachia relative
443 abundance. Second, we tested whether or not excluding *Wolbachia* reads influenced bacterial
444 diversity. *Wolbachia* is a facultative, intracellular bacteria transmitted exclusively from mother to
445 offspring. *Wolbachia* has no known environmental reservoir and cannot exist outside of the host.
446 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 [55, 56]. Finally, many studies in *D. melanogaster* exclude *Wolbachia* reads when analyzing microbial communities [24, 57–60], 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 the Anaplasmataceae family (all of which are *Wolbachia* in flies) reads from the communities, and then recalculated Shannon diversity through the same procedure as above. Libraries were rarified to the new minimum size following *Wolbachia* removal. We compared if bacterial communities without *Wolbachia* reads significantly differed between control and evolved lines using a t-test.

List of abbreviations: E&R: Evolve and Resequence; IIS: insulin/insulin-like signalling

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The raw .fastq data are available in the NCBI Sequence Read Archive, and accession numbers can be found in Supp. Table 2. The code used to analyze data can be found https://github.com/lphenry/extgeno_e-r.

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Authors' contributions: LPH and JFA designed research. LPH collected, analyzed data and wrote the manuscript with JFA contributing to revisions. All authors read and approved the manuscript.

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Supplementary information

Supplementary information accompanies this manuscript.

Additional file 1: Supp. Table 1: Relative abundance of Wolbachia in each of the 10 E&R experiments.

Additional file 2: Supp. Table 2: List of accession numbers for raw genomic data from the 10 E&R experiments.

Additional file 3: Supp. Figures 1-10: Distribution of sequencing depth, rarefaction curves, and relative abundance of bacteria (family-level) for each of the 10 E&R experiments.

Additional file 4: Supp. Figure 11: Estimates of random effects (i.e., E&R experiment) from linear model to test the correlation between microbial change and duration of selection.

716 **Table 1: Evolve & Resequence studies analyzed**

Pressure	Evolved Phenotype	Duration of Selection (generations)	*Wolbachia* (% reads, min-max)	Experimental design (D: diet, S: sequencing)
Accelerated development[25]	Flies developed from egg to adult 20% faster than control	605	Infected (0.01-1.40%)	D: Banana, corn syrup, agar. S: 25 females (age not reported) pooled from each line; 4 control and 4 evolved.
Delayed reproduction[26]	Age of reproduction increased from 28 to 40 days	50	Uninfected (0%)	D: Not specified. S: 100 females (age not reported) pooled from each line; 18 control and 18 evolved.
Increased lifespan[27]	Median lifespan was increased from 4 weeks to 7-8 weeks	48	Uninfected (0%)	D: Agar, yeast, sugar, oatmeal. S: 250 males + 250 females (age not reported) pooled from each line; 3 control and 3 evolved.
Egg size[28]	Egg size was selected ~20% larger and smaller eggs	16	Infected (0.02-0.11%)	D: Not specified. S: 100 females (age not reported) pooled from each line; 3 control, 3 small, 3 large.
Desiccation resistance[29]	Desiccation resistance (hrs until 80% mortality) increased 70-80%	48	Infected (70.2-89.7%)	D: Yeast, cornmeal, agar. S: 100 females (age not reported) pooled from each line; 3 control and 3 evolved.
Fluctuating temperature[30]	Survival under fluctuating temps 18-28ºC daily	37	Infected (48.7-75.1%)	D: Standard media. S: 500 females (~7 days old) 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[31]	Survival in constant, spatially, temporally varying salt and/or cadmium	42	Infected (0.01-0.03%)	D: Yeast, cornmeal, sugar agar. Diet differed between control and evolved. S: 70 females (age not reported) pooled from each line; 3 control lines and 5 lines for each selection pressure.
Starvation resistance[32]	Starvation resistance (hrs to death w/o food) increased ~25%	83	Infected (53.0-78.4%)	D: Not specified. S: 100 females (4 days old) pooled for each line; 3 control and 3 evolved lines.
Parasitoid resistance[33]	Resistance to parasitoid increased from 20% to 50%	5	Uninfected (0%)	D: Not specified. S: 50 females (~5 hrs old) pooled from each line; 16 control and 16 evolved lines.
Viral resistance[34]	Resistance to Drosophila C virus increased from 25% to 75%	20	Infected (95.1-98.1%)	D: Standard cornmeal-agar. S: 200 individuals (age not reported) pooled from each line; 4 control, 4 procedure control, 4 evolved.

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Table 2: Summary statistics for beta-diversity (Jaccard similarity)

Accelerated development time						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
group	1	0.30394	0.303937	40.862	0.89098	0.022*
Residuals	5	0.03719	0.007438		0.10902	
Total	6	0.34113			1	
Delayed reproduction						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
group	1	0.09902	0.099022	3.9017	0.10294	0.008**
Residuals	34	0.86288	0.025379		0.89706	
Total	35	0.9619			1	
Increased lifespan						
	Df	Sum Sq	Mean Sq	F	N.Perm	Pr(>F)
Groups	1	0.0031624	0.0031624	1.846	719	0.3014
Residuals	4	0.0068525	0.0017131			
Egg size						
	Df	Sum Sq	Mean Sq	F	N.Perm	Pr(>F)
Groups	1	0.0020672	0.0020672	0.7579	999	0.434
Residuals	7	0.0190935	0.0027276			
Desiccation resistance						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
group	1	0.044141	0.044141	1.8976	0.32176	0.2
Residuals	4	0.093047	0.023262		0.67824	
Total	5	0.137188			1	
Fluctuating temperature						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
group	1	0.11007	0.11008	1.9823	0.19858	0.203
Residuals	8	0.44424	0.05553		0.80142	
Total	9	0.55432			1	
Salt + cadmium resistance						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
group	1	0.05652	0.056519	2.1164	0.09155	0.085
Residuals	21	0.56081	0.026705		0.90845	
Total	22	0.61733			1	
Starvation resistance						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
group	1	0.019872	0.019872	2.0937	0.34359	0.2
Residuals	4	0.037964	0.009491		0.65641	
Total	5	0.057836			1	
Parasitoid resistance						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
group	1	0.04295	0.04295	0.64716	0.02112	0.533
Residuals	30	1.99099	0.066366		0.97888	
Total	31	2.03394			1	
Viral resistance						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
group	1	0.0384	0.0384	2.673	0.21092	0.088
Residuals	10	0.14366	0.014366		0.78908	
Total	11	0.18206			1	

722 Table 3: Statistical differences between control and evolved microbiomes

Pressure	**test stat**	**df**	**significance**
accelerated development	t = -8.116	df = 3.009	p-value = 0.004
accelerated development without *Wolbachia*	t = -5.511	df = 3.052	p-value = 0.011
delayed reproduction	t = 3.558	df = 33.652	p-value = 0.001
increased lifespan	t = 1.364	df = 3.172	p-value = 0.261
egg size	t = 0.213	df = 3.105	p-value = 0.845
egg size without *Wolbachia*	t = 0.250	df = 3.077	p-value = 0.818
desiccation resistance	t = 2.883	df = 2.224	p-value = 0.090
desiccation resistance without *Wolbachia*	t = 0.394	df = 3.985	p-value = 0.714
fluctuating temperature	t = 1.238	df = 7.996	p-value = 0.251
fluctuating temperature without *Wolbachia*	t = -4.135	df = 6.886	p-value = 0.005
salt and cadmium resistance	t = 1.431	df = 2.287	p-value = 0.274
salt and cadmium resistance without W*olbachia*	t = 1.431	df = 2.287	p-value = 0.274
starvation resistance	t = 10.448	df = 2.065	p-value = 0.008
starvation resistance without *Wolbachia*	t = -1.413	df = 2.903	p-value = 0.255
parasitoid resistance	t = 2.179	df = 28.394	p-value = 0.038
viral resistance	t = 4.265	df = 9.829	p-value = 0.002
viral resistance without *Wolbachia*	t = -2.851	df = 7.193	p-value = 0.024

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724 Table 4: Summary statistics of mixed model to assess the relationship between duration of
725 selection and change in diversity. Random effect was modeled as experiment (i.e., accelerated
726 development time, starvation resistance, etc.).

Fixed effects			
	Estimate (std. error)	T-value	Pr (> | t |)
Intercept	-0.185 (0.162)	-1.137	0.291
log10.length	0.046 (0.097)	0.473	0.649
Random effects			
	Variance	Std. deviation	
Experiment	0.0225	0.1500	
Residual	0.007	0.084	
Summary			
Observations		79	
Log Likelihood		66.128	
Akaike Inf. Crit.		-124.256	
Bayesian Inf. Crit.		-114.779	

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729 Table 5: Statistical differences in *Wolbachia* relative abundance between control (C) and
730 evolved (E) populations.

Experiment	C min	C max	C avg	E min	E max	E avg
Desiccation resistance						
Relative abundance	0.775	0.906	0.849	0.926	0.936	0.932
Kruskal-Wallis X^2 = 3.8571, df = 1, p-value = 0.04953						
Fluctuating temperature						
Relative abundance	0.488	0.757	0.628	0.685	0.752	0.723
Kruskal-Wallis X^2 = 1.0519, df = 1, p-value = 0.3051						
Starvation resistance						
Relative abundance	0.837	0.854	0.844	0.918	0.943	0.932
Kruskal-Wallis X^2 = 3.8571, df = 1, p-value = 0.04953						
Viral resistance						
Relative abundance	0.955	0.977	0.968	0.98	0.984	0.981
Kruskal-Wallis X^2 = 7.3846, df = 1, p-value = 0.006578						

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746 Fig 1: Relative abundance for bacterial families from the 10 E&R experiments. Each experiment
747 was grouped separately; the colors represent different bacterial families in each. Only bacteria
748 that comprised >1% of total reads were visualized.
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750 Fig. 2: PCoA plots for beta-diversity using Jaccard similarity for the 10 E&R experiments show
751 the majority of control and evolved populations harbor similar bacterial families. Each
752 experiment was grouped separately; grey color represents control populations, and the colored
753 points represent evolved populations. Each sequenced pool is shown as a point with lines
754 connecting to the centroid based on PCoA clustering. The two studies (accelerated
755 development time and delayed reproduction) with significantly divergent microbiomes between
756 control and evolved populations are outlined in colored boxes. P-values are shown for all
757 studies.
758
759 Fig. 3: Bacterial diversity between control and evolve populations in 10 E&R experiments. 5/10
760 experiments had significantly different bacterial diversity (denoted with the colored outline and
761 asterisk). Bacterial diversity was calculated at the family level using Shannon diversity metric.
762 Comparisons between control and evolved populations were within each experiment. Each point
763 represents a pool of sequenced flies showing replication within control and evolved groups, and
764 the details of how many flies/experiments are described in Table 1. Color represents each
765 experiment.
766
767 Fig. 4: Evolved bacterial diversity was not significantly correlated with duration of selection after
768 controlling for differences between experiments as random effects (r = 0.05, p=0.649). Random
769 effects (experiment) explained 76% of variation (see Supp. Fig. 11). Generations of selection
770 range from 5 generations (parasitoid resistance) to > 500 generations (accelerated

development). Each point represents the difference between average control diversity and each pool of evolved flies for each experiment. Points are colored by experiment.

Fig. 5: *Wolbachia* relative abundance tended to increase in evolved populations for four studies, but was only statistically significant in starvation resistance and viral resistance (denoted with colored box and asterisk). Comparisons were made within each study between control and evolved populations. Each point represents a pool of flies.

Fig. 6: Differences in bacterial diversity when including (full bacterial community) or excluding *Wolbachia* reads in four of the E&R experiments analyzed. Each point represents bacterial diversity for a pool of sequenced flies. Scale bars are different because diversity comparisons are within either the full community or *Wolbachia* removed groupings for each experiment. 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.

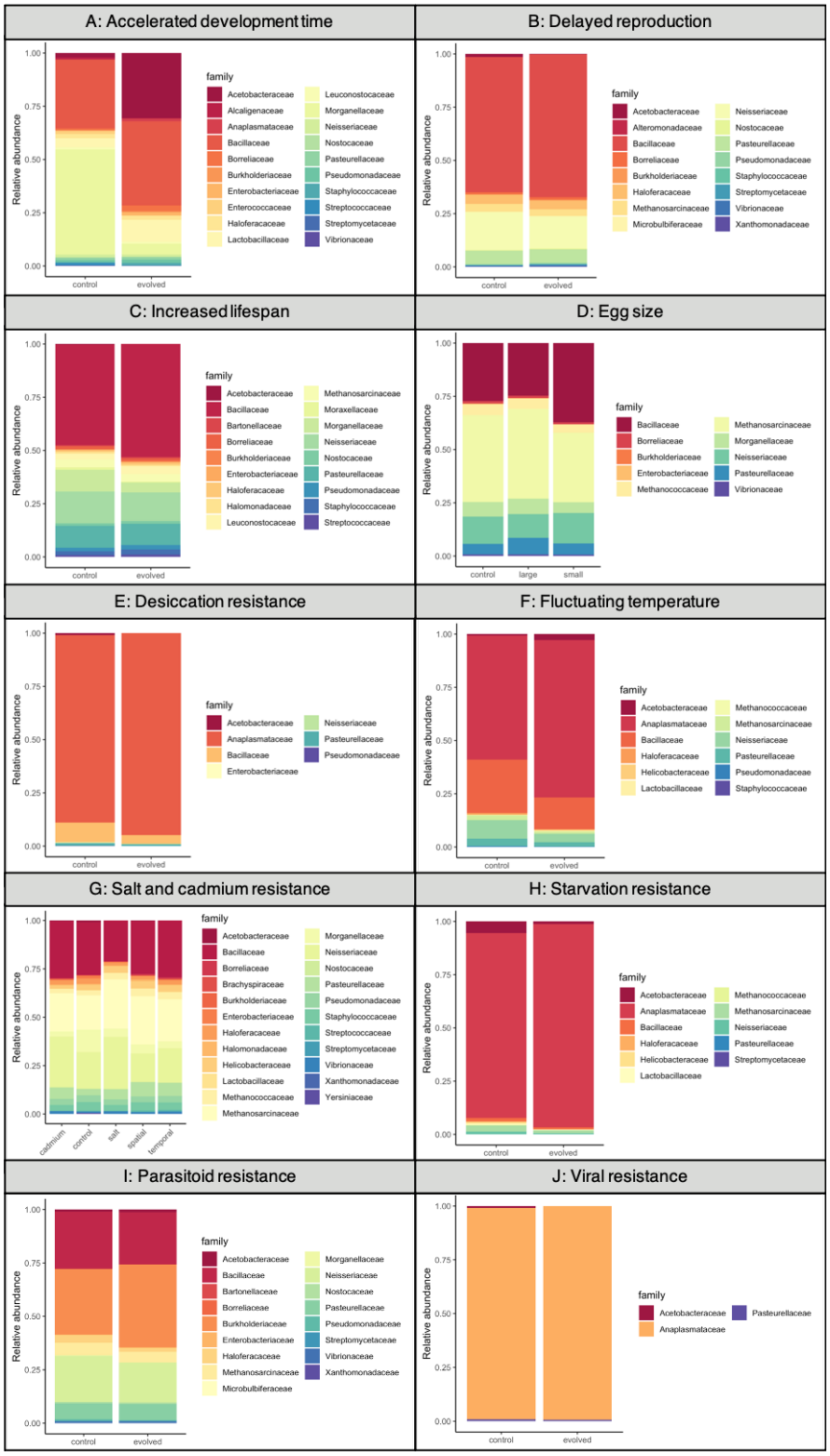


Figure 1: Relative abundance for bacterial families from the 10 E&R experiments. Each experiment was grouped separately; the colors represent different bacterial families in each. Only bacteria that comprised >1% of total reads were visualized.

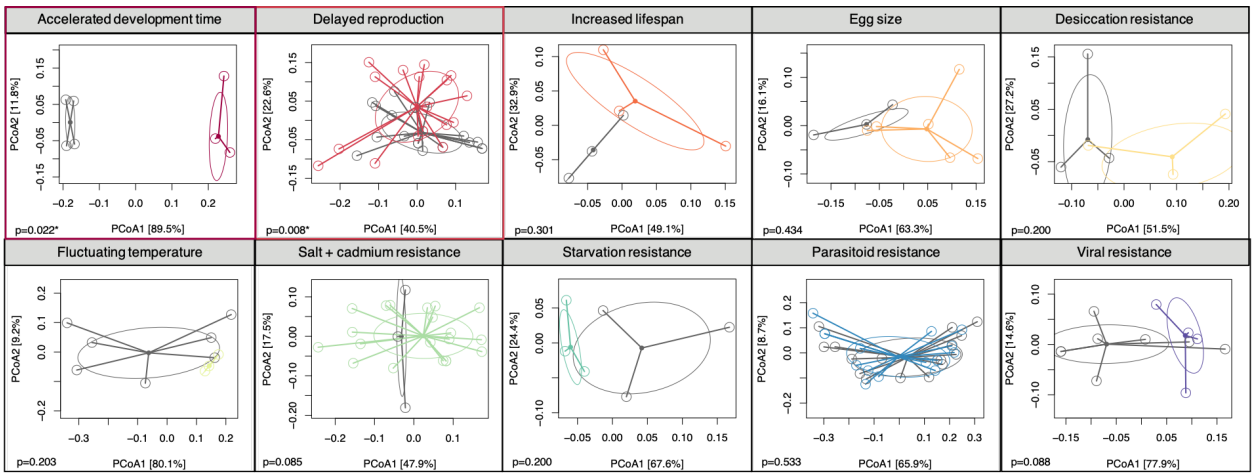


Fig. 2: PCoA plots for beta-diversity using Jaccard similarity for the 10 E&R experiments show the majority of control and evolved populations harbor similar bacterial families. Each experiment was grouped separately; grey color represents control populations, and the colored points represent evolved populations. Each sequenced pool is shown as a point with lines connecting to the centroid based on PCoA clustering. The two studies (accelerated development time and delayed reproduction) with significantly divergent microbiomes between control and evolved populations are outlined in colored boxes. P-values are shown for all studies.

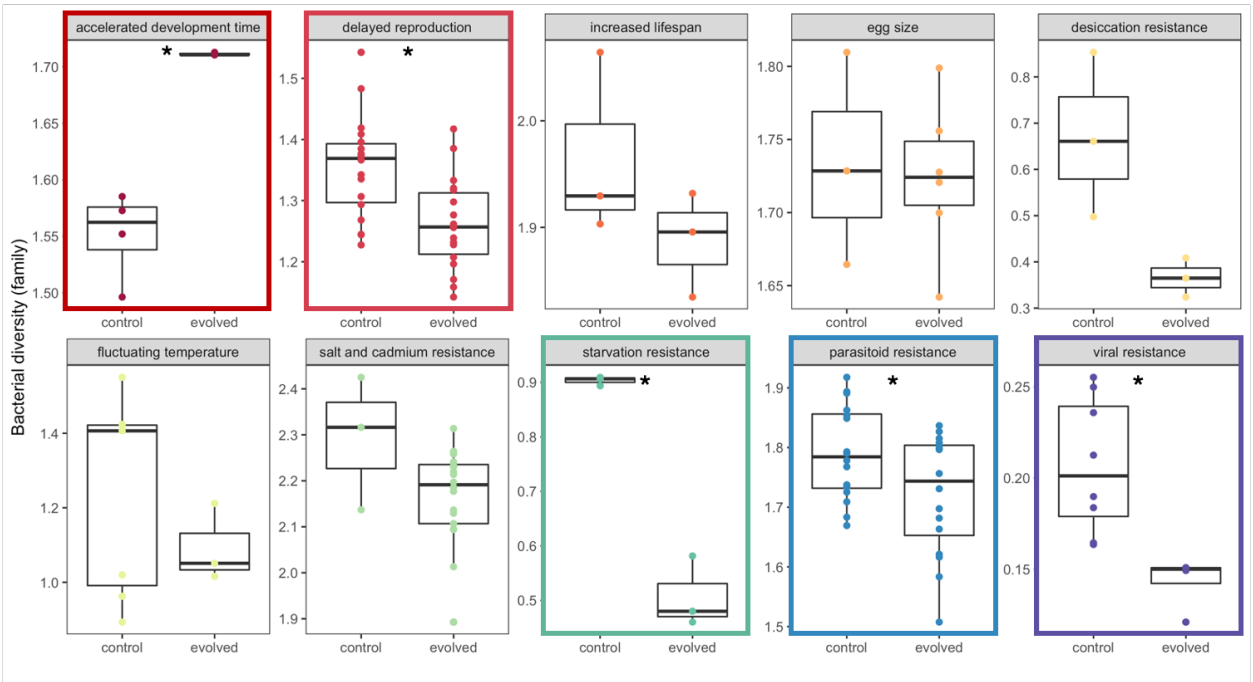


Fig. 3: Bacterial diversity between control and evolve populations in 10 E&R experiments. 5/10 experiments had significantly different bacterial diversity (denoted with the colored outline and asterisk). Bacterial diversity was calculated at the family level using Shannon diversity metric. Comparisons between control and evolved populations were within each experiment. Each point represents a pool of sequenced flies showing replication within control and evolved groups, and the details of how many flies/experiments are described in Table 1. Color represents each experiment.

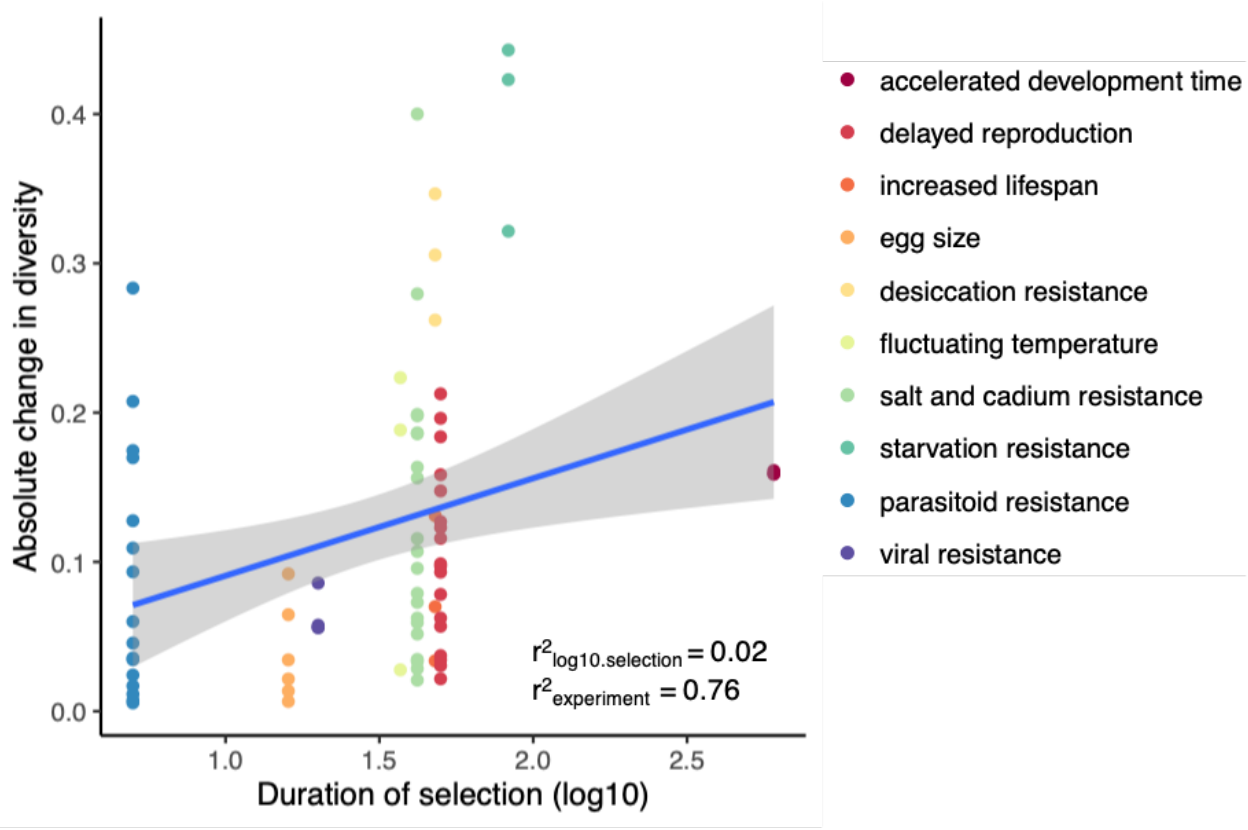


Fig. 4: Evolved bacterial diversity was not significantly correlated with duration of selection after controlling for differences between experiments as random effects (*r* = 0.05, p=0.649). Random effects (experiment) explained 76% of variation (see Supp. Fig. 11). Generations of selection range from 5 generations (parasitoid resistance) to > 500 generations (accelerated development). Each point represents the difference between average control diversity and each pool of evolved flies for each experiment. Points are colored by experiment.

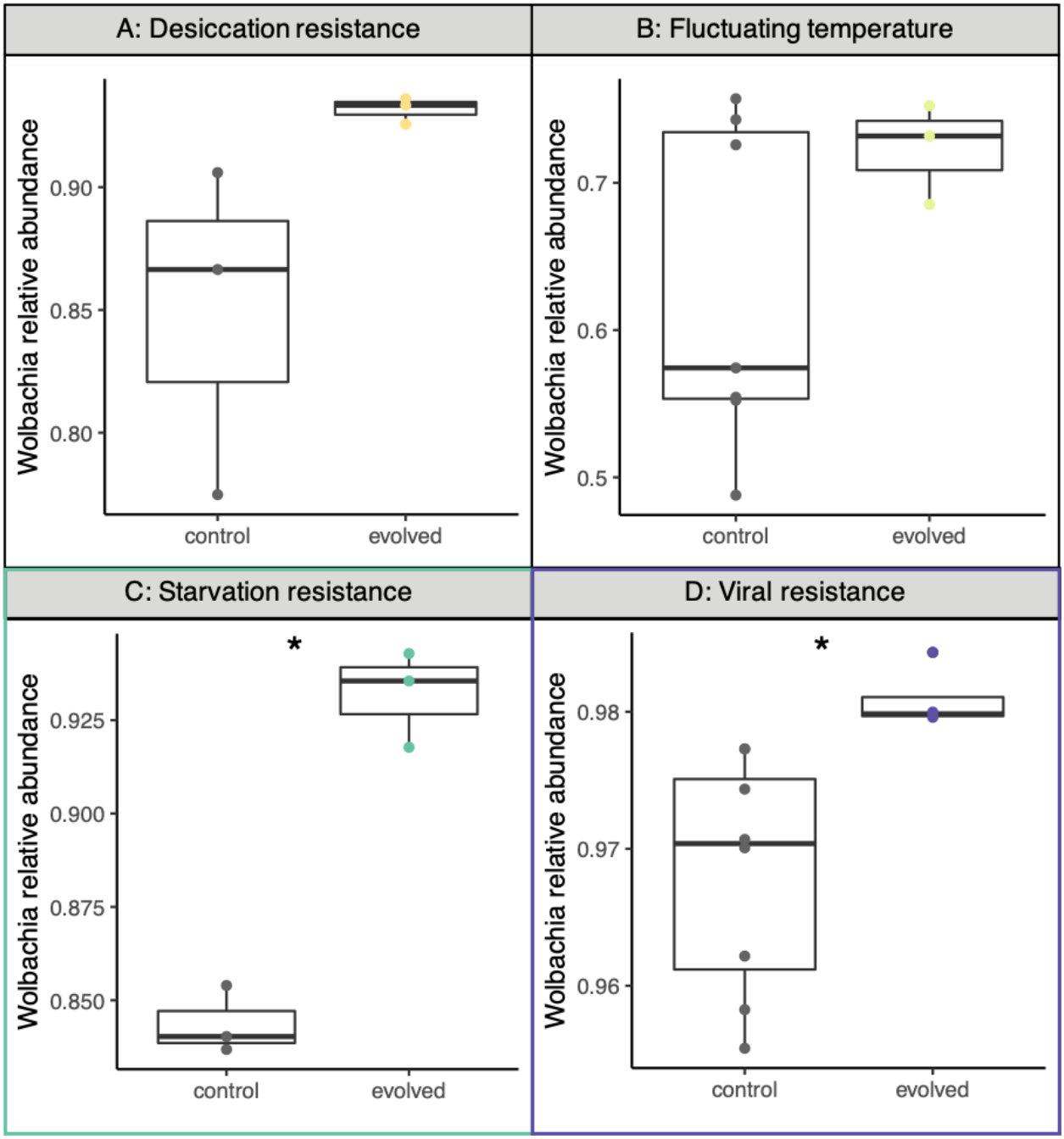


Fig. 5: *Wolbachia* relative abundance tended to increase in evolved populations for four studies, but was only statistically significant in starvation resistance and viral resistance (denoted with colored box and asterisk). Comparisons were made within each study between control and evolved populations. Each point represents a pool of flies.

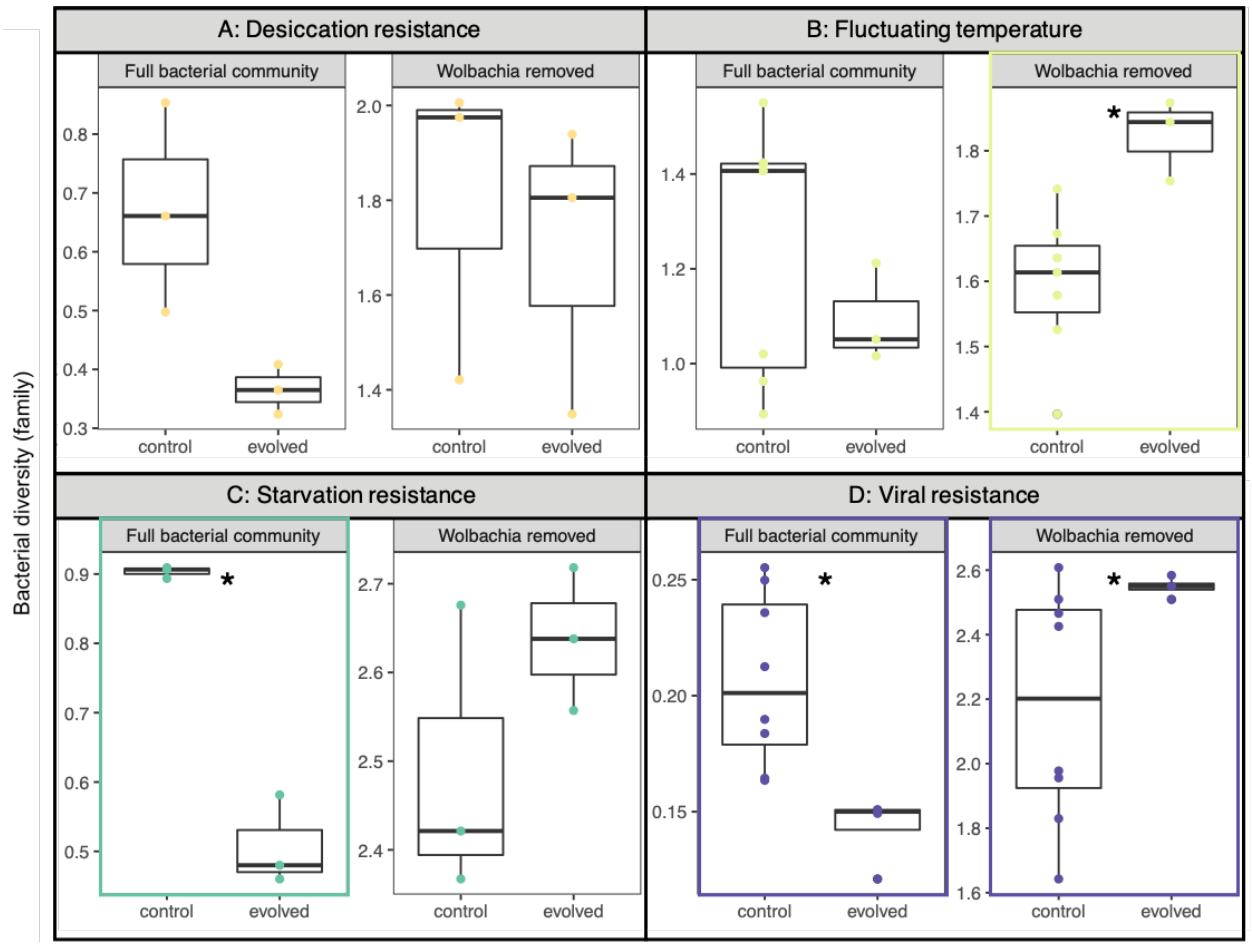


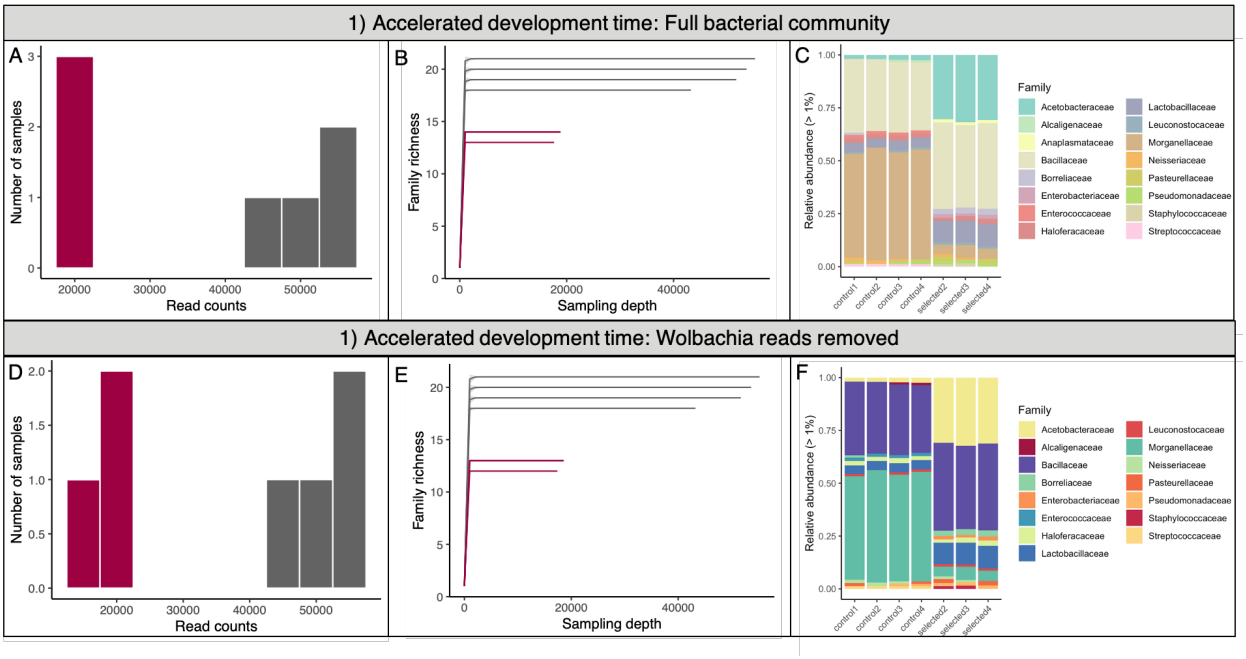
Fig. 6: Differences in bacterial diversity when including (full bacterial community) or excluding *Wolbachia* reads in four of the E&R experiments analyzed. Each point represents bacterial diversity for a pool of sequenced flies. Scale bars are different because diversity comparisons are within either the full community or *Wolbachia* removed groupings for each experiment. 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.

Supp. Table 1: Relative abundance of Wolbachia in each experiment. For each experiment, the minimum, max, and average percentage of Anaplasmataceae reads is presented for both control (C) and evolved (E) lines, as well as total average for the experiment.

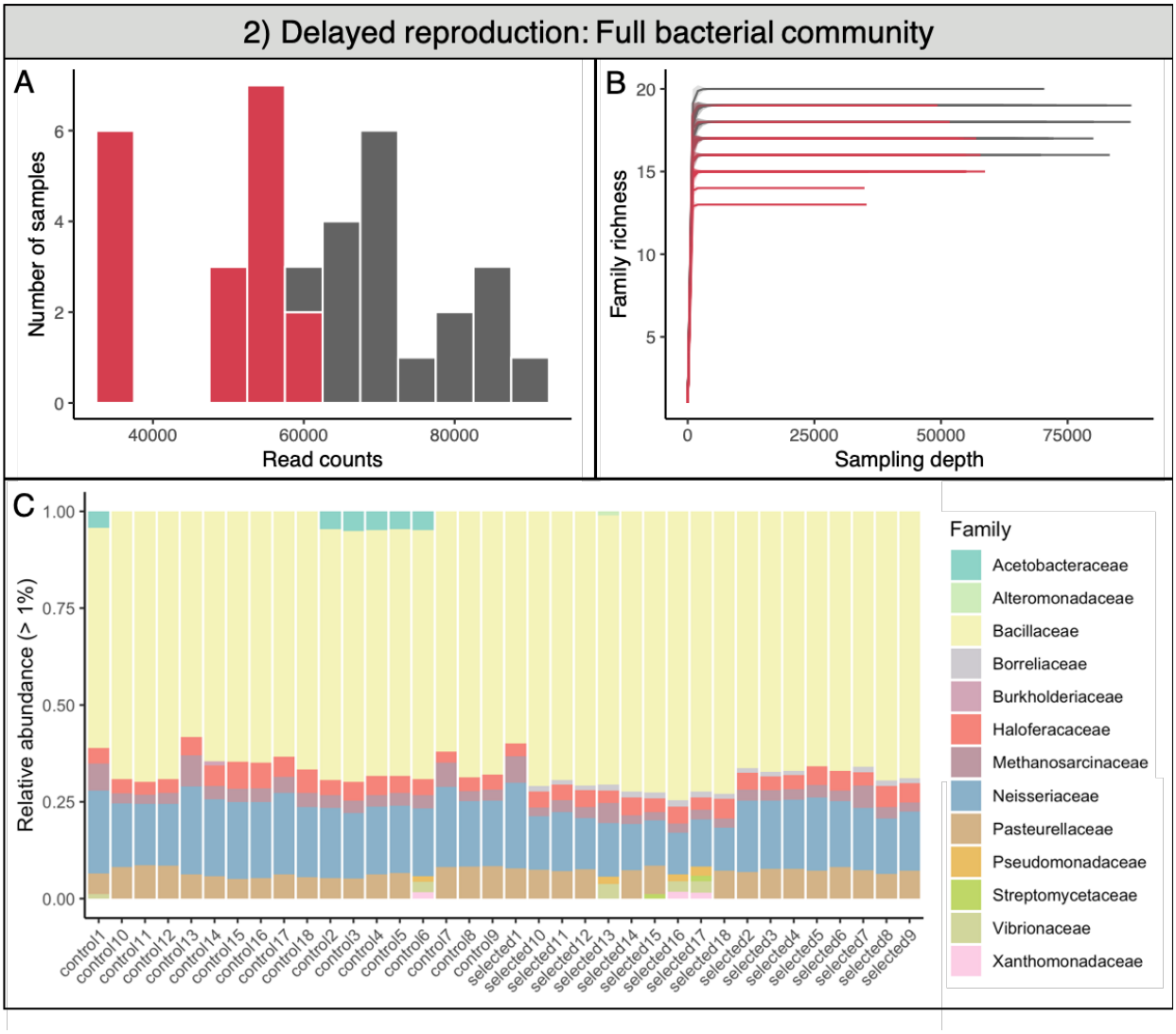
Study	C-Wolb avg	C-Wolb min	C-Wolb max	E-Wolb avg	E-Wolb min	E-Wolb max	Average
Accelerated development	0.02	0.02	0.03	1.32	1.24	1.41	0.67
Delayed reproduction	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Increased lifespan	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Egg size	0.03	0.03	0.05	0.06	0.03	0.12	0.05
Desiccation resistance	76.86	70.21	81.53	88.86	88.43	89.72	82.86
Fluctuating temps	62.51	48.69	75.35	72.22	68.45	75.15	65.42
Salt + cadmium resist	0.01	0.01	0.01	0.01	0.01	0.03	0.01
Starvation resistance	56.21	53.03	58.22	75.48	72.30	78.37	65.85
Parasitoid resistance	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Viral resistance	96.39	95.20	97.31	97.72	97.57	98.09	96.84

Supp. Table 2: Accession numbers for E&R experiments

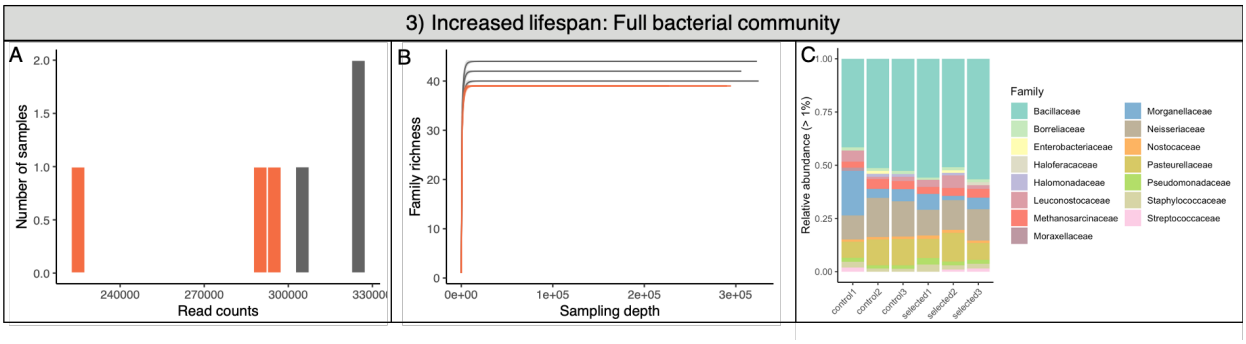
Experiment	*Citation*	*Accession*
Accelerated development time	Burke Nature 2010	SRP002024
Delayed reproduction	Remolina Evolution 2012	SRA038471
Increased lifespan	Michalak BMC Genomics 2017	SRP092765
Egg size	Jha MBE 2015	PRJNA281953
Desiccation resistance	Kang BMC Genomics 2016	SRP066877
Fluctuating temperature	Orozco-terWengel Molecular Ecology 2012	ERP001290
Salt + cadmium resistance	Huang PLOS Genetics 2014	PRJNA257179
Starvation resistance	Hardy MBE 2018	PRJNA315172
Parasitoid resistance	Jalvingh PRSB 2014	PRJNA170455
Viral resistance	Martins PNAS 2014	ERX409654



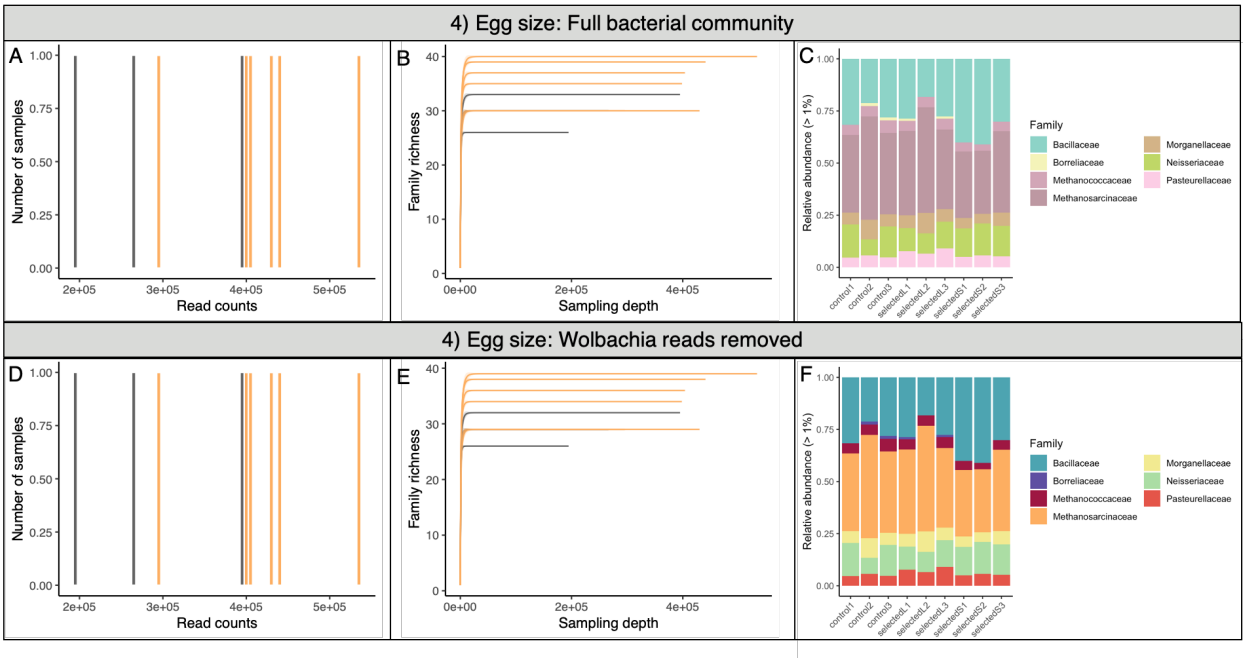
Supp. Fig. 1: Accelerated development time. The control population is in grey, while dark red denotes the evolved populations. A) Histogram of sequencing depth for the seven pools in this study. B) Rarefaction curves suggest that bacterial communities were fully sampled, even though sequencing depth was lower for the evolved populations. C) Relative abundance of each pool shows bacterial taxa with each color. D-F) Histogram of sequencing depth, rarefaction, and relative abundance of bacterial families following removal of *Wolbachia* reads.



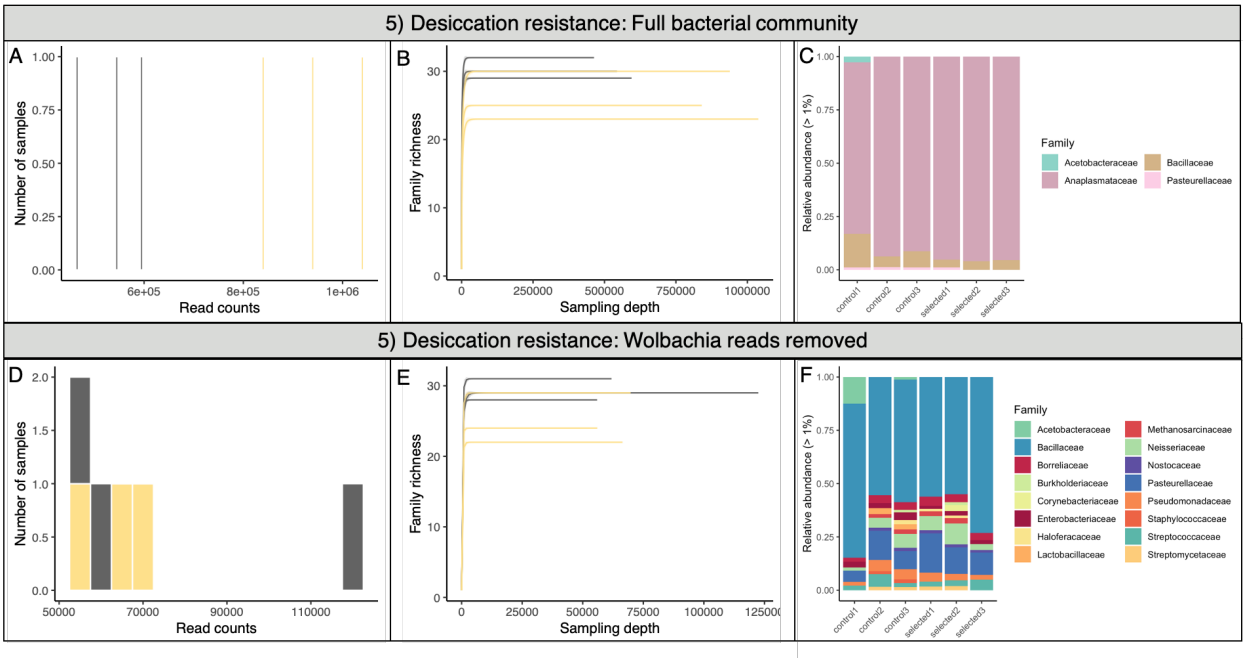
Supp. Fig. 2: Delayed reproduction. The control population is in grey, while red denotes the evolved populations. A) Histogram of sequencing depth for the 36 pools in this study. B) Rarefaction curves suggest that bacterial communities were fully sampled, even though sequencing depth tended to be lower for the evolved populations. C) Relative abundance of each pool shows bacterial taxa with each color. *Wolbachia* was not present in this study.



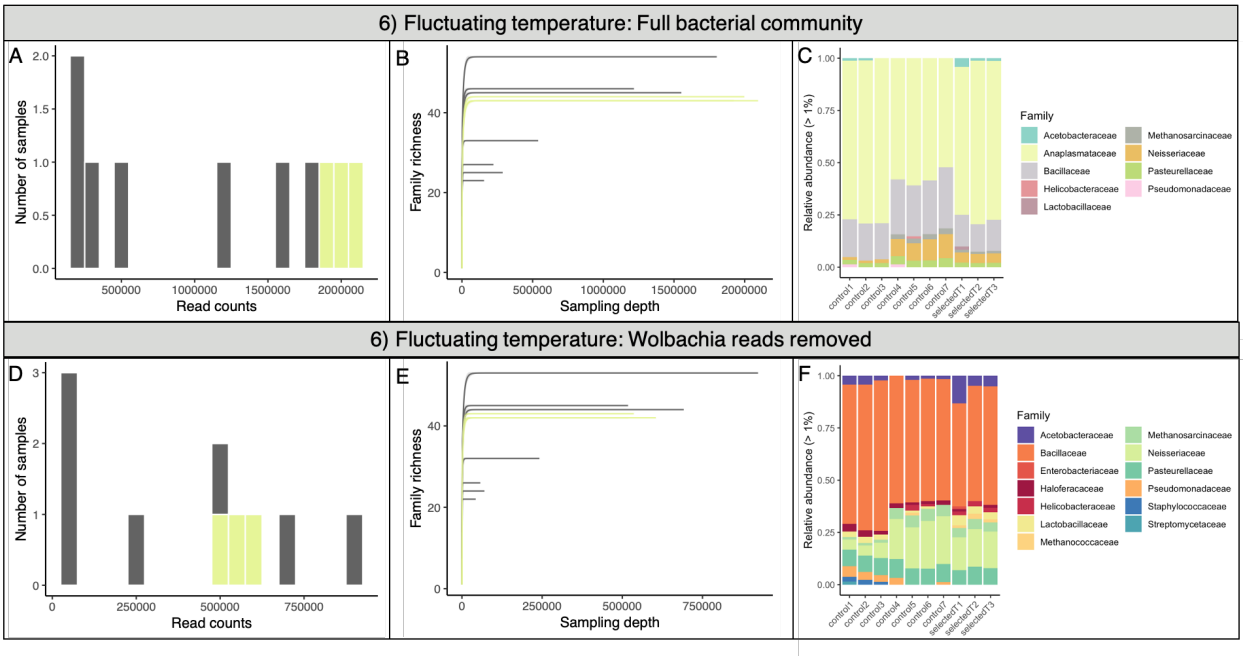
Supp. Fig. 3: Increased lifespan. The control population is in grey, while dark orange denotes the evolved populations. A) Histogram of sequencing depth for the six pools in this study. B) Rarefaction curves suggest that bacterial communities were fully sampled, even though sequencing depth was lower for the evolved populations. C) Relative abundance of each pool shows bacterial taxa with each color. *Wolbachia* was not present in this study.



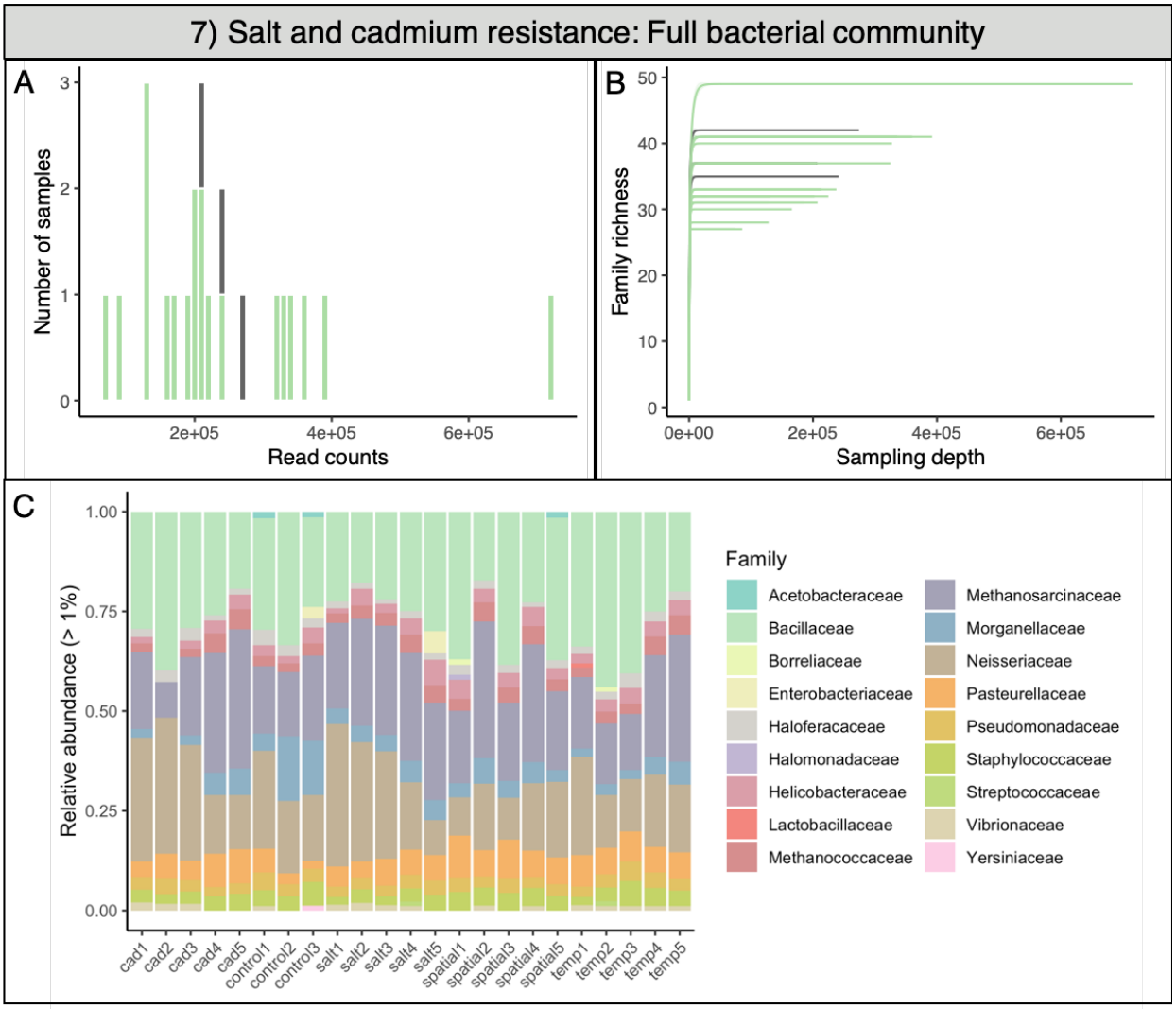
Supp. Fig. 4: Egg size. The control population is in grey, while orange denotes the evolved populations. A) Histogram of sequencing depth for the nine pools in this study. B) Rarefaction curves suggest that bacterial communities were fully sampled, even though sequencing depth was tended to be lower for the control populations. C) Relative abundance of each pool shows bacterial taxa with each color. D-F) Histogram of sequencing depth, rarefaction, and relative abundance of bacterial families following removal of *Wolbachia* reads.



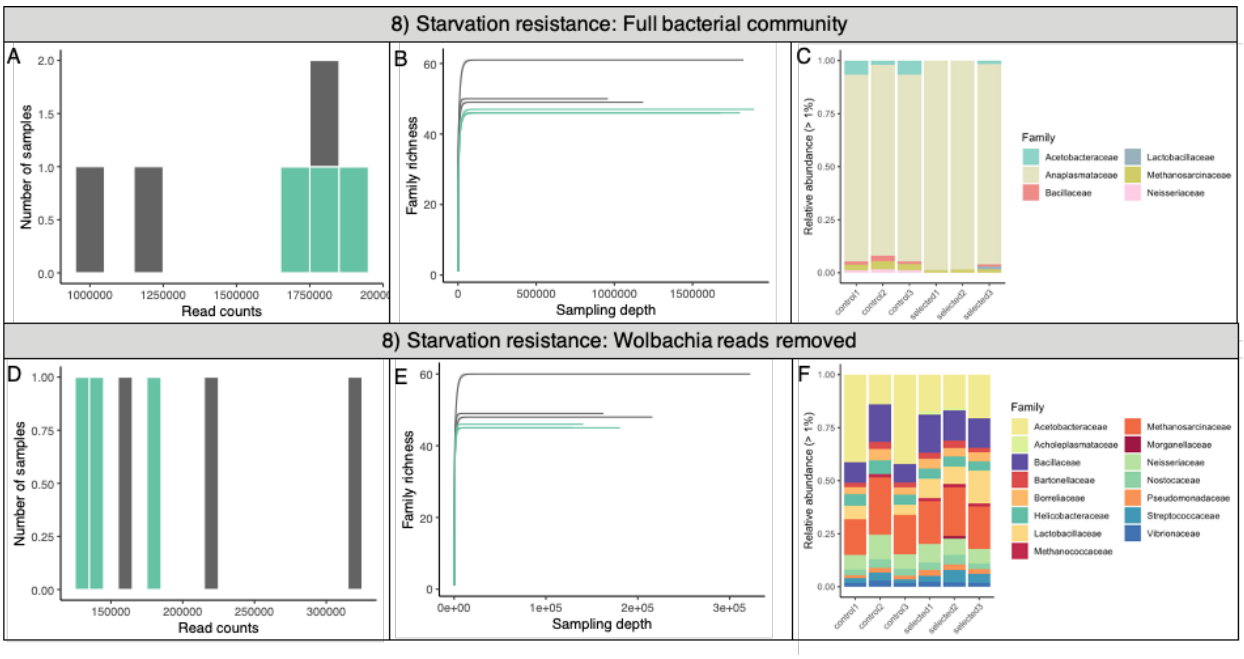
Supp. Fig. 5: Desiccation resistance. The control population is in grey, while dark yellow denotes the evolved populations. A) Histogram of sequencing depth for the six pools in this study. B) Rarefaction curves suggest that bacterial communities were fully sampled. C) Relative abundance of each pool shows bacterial taxa with each color. D-F) Histogram of sequencing depth, rarefaction, and relative abundance of bacterial families following removal of *Wolbachia* reads.



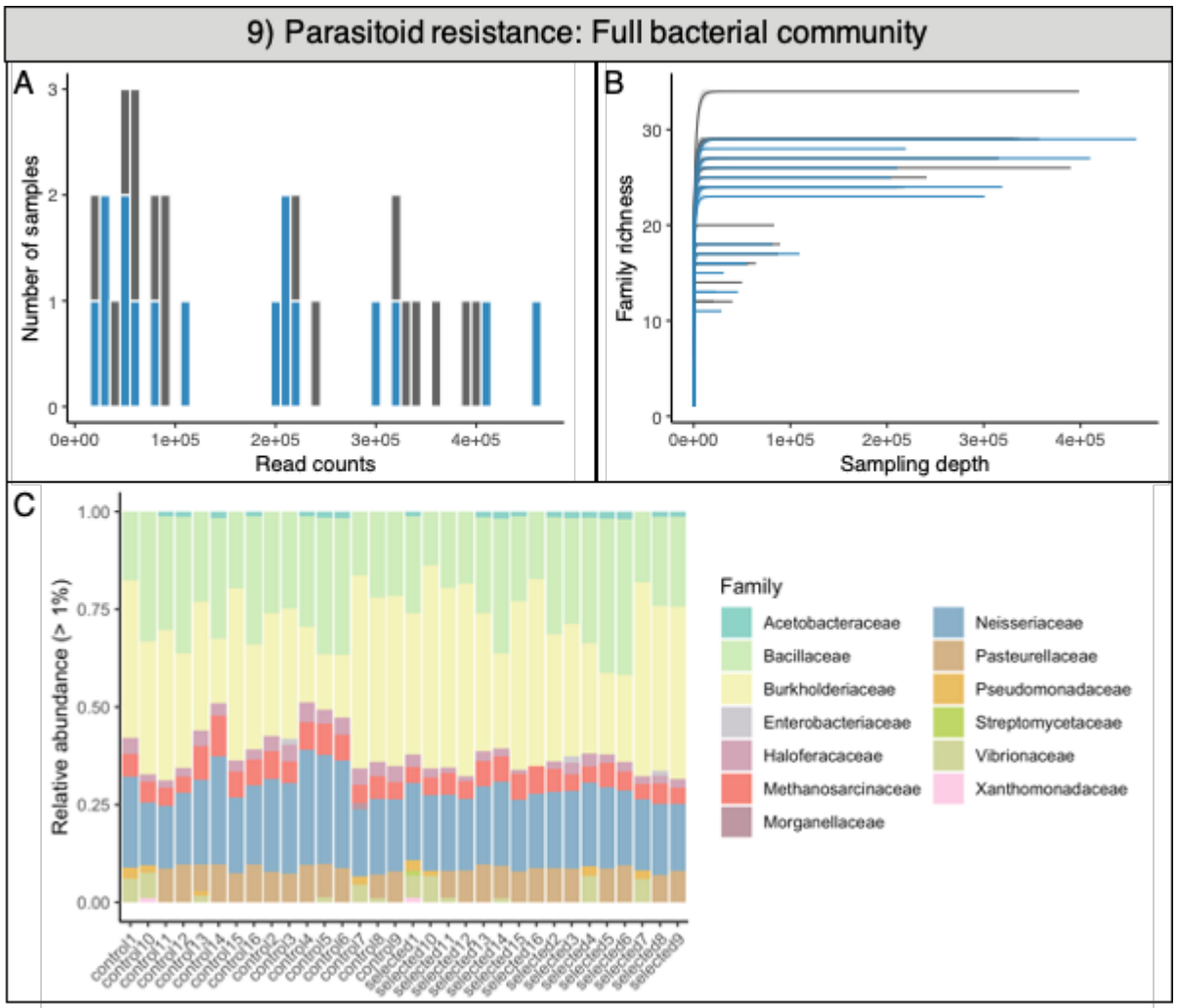
Supp. Fig. 6: Fluctuating temperature. The control population is in grey, while yellow denotes the evolved populations. A) Histogram of sequencing depth for the 10 pools in this study. B) Rarefaction curves suggest that bacterial communities were fully sampled, even though sequencing depth was lower for the control populations. C) Relative abundance of each pool shows bacterial taxa with each color. D-F) Histogram of sequencing depth, rarefaction, and relative abundance of bacterial families following removal of *Wolbachia* reads.



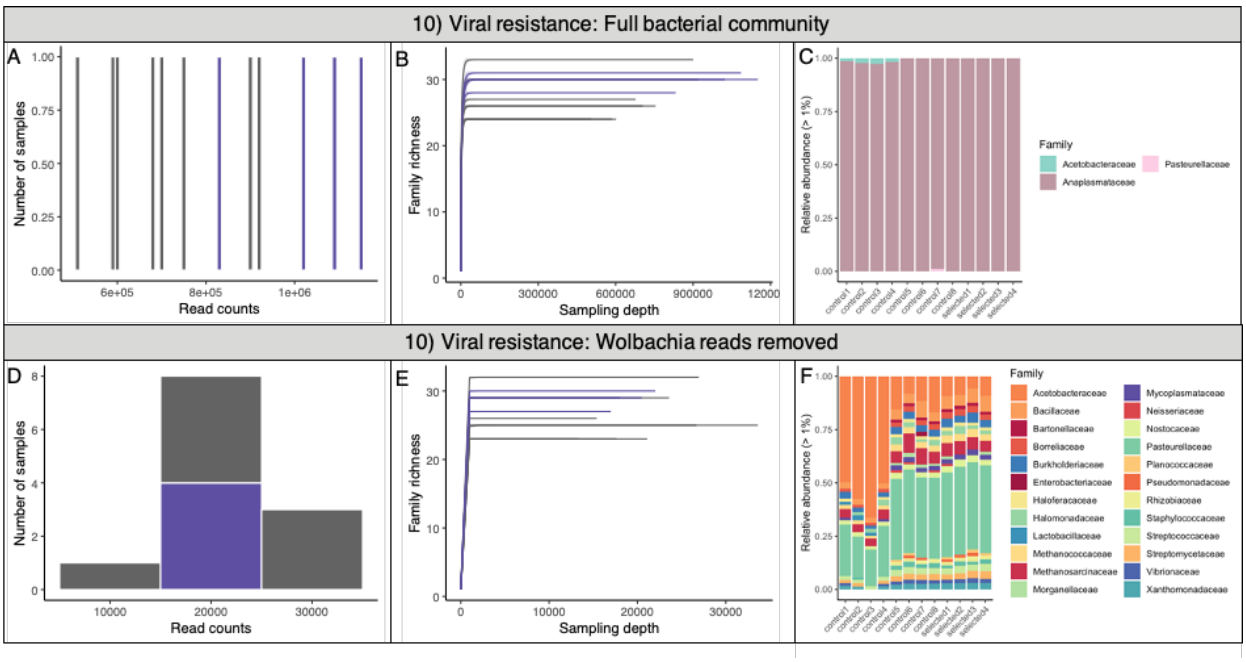
Supp. Fig. 7: Salt and cadmium resistance. The control population is in grey, while green denotes the evolved populations. A) Histogram of sequencing depth for the 23 pools in this study. B) Rarefaction curves suggest that bacterial communities were fully sampled, even though sequencing depth was lower for the evolved populations. C) Relative abundance of each pool shows bacterial taxa with each color. While *Wolbachia* was present in this study, it was below 1% relative abundance.



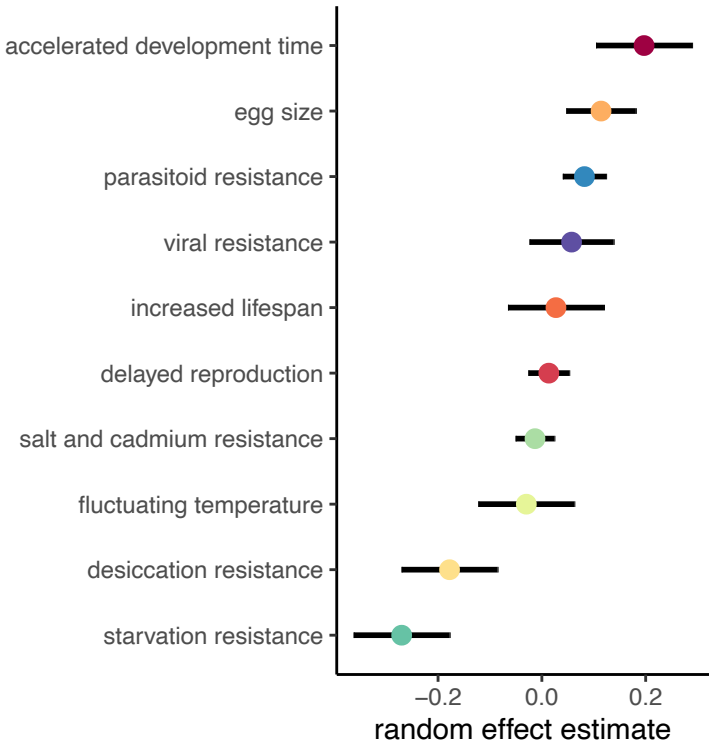
Supp. Fig. 8: Starvation resistance. The control population is in grey, while teal denotes the evolved populations. A) Histogram of sequencing depth for the six pools in this study. B) Rarefaction curves suggest that bacterial communities were fully sampled. C) Relative abundance of each pool shows bacterial taxa with each color. D-F) Histogram of sequencing depth, rarefaction, and relative abundance of bacterial families following removal of *Wolbachia* reads.



Supp. Fig. 9: Parasitoid resistance. The control population is in grey, while blue denotes the evolved populations. A) Histogram of sequencing depth for the 24 pools in this study. B) Rarefaction curves suggest that bacterial communities were fully sampled. C) Relative abundance of each pool shows bacterial taxa with each color. *Wolbachia* was not present in this study.



Supp. Fig. 10: Viral resistance. The control population is in grey, while purple denotes the evolved populations. A) Histogram of sequencing depth for the 12 pools in this study. B) Rarefaction curves suggest that bacterial communities were fully sampled. C) Relative abundance of each pool shows bacterial taxa with each color. D-F) Histogram of sequencing depth, rarefaction, and relative abundance of bacterial families following removal of *Wolbachia* reads.



Supp. Fig. 11: Estimates of random effects for each experiment. Points show conditional means for the random effects and lines show +/- 2 conditional SD.