**Title: Experimental Evolution Reveals Limited Adaptation in Microbiota-less *Drosophila melanogaster***

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**Abstract**

In single-generation experiments, microbiome removal often affects multiple host traits. However, relatively little is understood about the long-term implications of living in a microbe-free environment. To address this question, we evolved four replicate laboratory populations of *Drosophila melanogaster* without microbes and compared their traits with corresponding control populations that were reared with microbiota. This comparison was done under two assay environments: with-microbes and microbe-free. Prior single-generation experiments on our populations had revealed that microbiota removal significantly affects multiple traits, which suggested that the flies were under strong selection pressure to adapt to the absence of microbes. However, contrary to our expectations, the selected populations underwent very modest changes even after 54-57 generations of selection. Moreover, the magnitude of change in trait values across the with- and without- microbe environments was less for the selected populations than for the controls. RNA-Seq on one of the evolutionary replicates revealed that compared to the control, some anti-microbial peptides (AMPs) in the selected population were up-regulated, while several heat shock proteins (HSPs) were down-regulated. These results suggest that robust host-microbiome integrations on short timescales can nevertheless be labile on longer timescales. We situate these results in the context of the “evolutionary addiction hypothesis” and the “hygiene hypothesis”.

## Introduction

For millions of years, multicellular organisms have lived on this planet in conjunction with their associated microbial communities. Yet, the evolutionary dynamics of the symbiotic relationships between the hosts and their microbiota remain poorly understood. The hologenome theory of evolution, proposed nearly twenty years ago, suggests that the host and its associated microbiota—collectively termed the holobiont—can be considered a unified entity subject to natural selection (Rosenberg & Zilber-Rosenberg, 2018; Zilber-Rosenberg & Rosenberg, 2008). Since its inception, this theory has generated considerable interest and controversies within the scientific community (Bordenstein & Theis, 2015; Douglas & Werren, 2016; Madhusoodanan, 2019; Moran & Sloan, 2015; Morris, 2018). For example, critics have argued that the identity of the host-associated microbiota is often so dynamic (often changing within the host's lifetime (Bana & Cabreiro, 2019)) that the holobiont cannot be considered a well-defined evolutionary unit. Others have pointed out that the interactions between a host and various components of its microbiota can range from beneficial to antagonistic (and everything in between), which leads to variation in selective pressures among constituents of host-microbiome assemblages (Douglas & Werren, 2016). This makes it difficult to consider the holobiont as a meaningful evolutionary unit. Despite the above-mentioned controversies about the status of the holobiont as a unit of selection/evolution, it is generally accepted that microbiota can play a significant role in a host's evolution in multiple ways (Gilbert et al., 2015; Henry et al., 2021; Kolodny et al., 2020; Macke et al., 2017).

Theoretical studies have shown that under the conditions of strong vertical selection and short generation time, hosts can get a fitness advantage from symbiont microbes, even if the microbes pay costs for such an advantage (Vliet & Doebeli, 2019). It has been argued that the microbiome can alter the mean and the variance of the host's phenotypic distribution, which in turn can enable the latter to explore a novel fitness landscape (Henry et al., 2021). These authors also suggest that the variance contributed by microbes can alter the heritability of the traits, thus affecting the host's response to selection. Empirical studies show that microbes can affect various facets of the biology of their hosts (reviewed in (Rosenberg, 2021)), including physiology (Grenier & Leulier, 2020), life history (Macke et al., 2017), behavior (Vuong et al., 2017), and immunity (Belkaid & Hand, 2014). Consequently, when the impact of the symbiont-mediated modulation is substantial, microbes are expected to alter the host's fitness significantly (Ma et al., 2023; Rosenberg & Zilber-Rosenberg, 2016), thereby modulating their evolution.

Microbiota can confer rapid adaptation to the hosts by expanding the host's niche (Borges, 2017; Hoang et al., 2021; Koide, 2023; Kopac & Klassen, 2016). For example, a study showed that stinkbugs can gain insecticide resistance within a single generation by acquiring the bacterial strain of *Burkholderia* that can degrade the insecticide fenitrothion (Kikuchi et al., 2012). This study also showed that along with insecticidal resistance, stinkbugs gained fitness advantages such as higher survival and larger body size that could potentially help this symbiosis spread in the population. Furthermore, it is known that symbiotic microbes can be an important source of phenotypic plasticity for their host (Alberdi et al., 2016), which can serve as an initial step in the host's adaptation to novel environments.

Given the diverse roles that microbiota can play in the evolution of their hosts and the fact that almost all organisms have been co-habiting with microbes for millions of years, it is natural to ask what will happen to organisms if they are forced to live without microbes for long periods of time. We already know that in some species, including caterpillars of tobacco hornworms (Hammer et al., 2017) and butterflies (Phalnikar et al., 2019), removing the microbiota has little or no effects. However, in the vast majority of species, removing the microbiota leads to prominent physiological and behavioral effects (Rosenberg, 2021). If subjected to prolonged microbiota absence, will such species fail to grow beyond a few generations, or will they adapt? If the latter, will the adaptation be fast or slow? Will their ability to adapt to other stresses be affected by the prolonged absence of the microbes?

While the above questions are of interest to evolutionary biologists, there are more pragmatic anthropocentric reasons for understanding the long-term impact of microbiota on host evolution. In modern human societies, the overuse of antibiotics and a hygienic lifestyle, along with other factors, could lead to the disappearance of beneficial microbes (Blaser & Falkow, 2009; Noverr & Huffnagle, 2004). It has been hypothesized that such loss of ancestral microbes (e.g., *H. pylori*) can increase the risk factors for conditions such as gastroesophageal reflux disease (GERD) and childhood asthma (Blaser, 2008; Blaser & Falkow, 2009). Even the microbiota of many naturally occurring species might have been altered due to increased antibiotic load in the environment (Baquero et al., 2019; Larsson & Flach, 2022) and micro-environmental alterations brought by climate change (Williams et al., 2023). Therefore, understanding the evolutionary implications of altering or

removing the microbiota on the hosts has emerged as a key question in biology (Macke et al. 2017; Henry et al. 2021).

Here, we used experimental evolution on *D. melanogaster* populations to understand how a host evolves in the absence of its microbiota. In this species, removing microbiota for a single generation can lead to developmental delay, lower body size (Shin et al., 2011; Storelli et al., 2011), reduced female fecundity (Elgart et al., 2016; Gnainsky et al., 2021; Suyama et al., 2023), and changes in behavioral traits such as diet-induced mating preference (Sharon et al., 2010), foraging (Wong et al. 2017), aggression (Jia et al., 2021), and locomotor activity (Schretter et al., 2018). Thus, one expects that in *Drosophila*, the removal of the microbiota would affect the hosts' fitness in multiple ways, potentially leading to a strong, multi-directional selection pressure. This makes *Drosophila* a well-suited system for studying this question.

We kept four large (~2400 individuals) outbred laboratory populations of *D. melanogaster* microbe-free using bleach (labeled ‘MBL’ populations, for ‘Microbiota-Less’) for more than 50 generations. This involved bleaching *D. melanogaster* eggs every generation and rearing them in sterile food in a sterile environment. Each of these four selected populations had an ancestry-matched control (labeled ‘MB’ populations) where the microbiome was reconstituted from a common source pool just after the bleach treatment. For this reconstitution, microbes were sampled from the environment of the flies via fecal microbiome transfer (Supplementary Figure S9 shows the composition of the ancestral microbiome that is dominated by *Acetobacter* and *Lactobacillus* spp.). We compared the selected MBL and control MB populations in two assay environments: (a) in the presence of microbes (labeled “with-microbes” environment) and (b) in the absence of microbes (labeled “microbe-free” environment). We hypothesized that the host would face substantial selection pressure in the absence of microbes. Therefore, we expected a rapid evolution of the host to maintain the homeostasis seen in the presence of microbes in our ancestral baseline populations. We found that even after surviving for 54 generations without microbes, contrary to expectations, there were only modest adaptations in the selected populations. Compared to the controls, the trait values of the selected populations were found to be less affected by the absence of microbes in the assay environment. RNA-Seq on one of the evolutionary replicates suggested that a cluster of anti-microbial peptides (AMPs) was up-regulated, and another cluster of heat shock proteins (HSPs) was

169 down-regulated in the MBLs. Finally, we discuss the potential implications of these results
170 in terms of existing theories about host-microbiota evolutionary relationships.
171

172 **Methods**

173 **Details of the experimental evolution lines**

174 The experimental evolution lines were derived from the four ancestral baseline populations
175 named $NDB_{1-4}$ (at their generation 21 in the lab). The details about the $NDB_{1-4}$ are provided
176 in Supplementary Text S1.1. Two populations were derived from each ancestral NDB line:
177 MBL ("Microbiota-Less," selected population that is without the microbiome) and MB
178 (corresponding ancestry-matched control population with microbiome) (Supplementary
179 Figure S1). For example, $NDB_1$ gave rise to $MBL_1$ and $MB_1$, $NDB_2$ to $MBL_2$ and $MB_2$, and
180 so on. Thus, populations with the same subscript (e.g., $MBL_2$ and $MB_2$) share a common
181 ancestry and were always assayed together. Each population in this setup is maintained
182 at a size of ~2400 adult flies in plexiglass cages with an additional microbiome removal
183 protocol in each generation, as described in the next section. $NDB_1$ served as the common
184 source of the microbiome for all the MBs (for the rationale of this step, see microbiome
185 reconstitution section). All populations were maintained at 25°C and constant light
186 conditions.

187

188 **Experimental evolution protocol employed each generation**

189 MBLs were handled aseptically inside a Level II biosafety cabinet (Microfilt, India). Any
190 material that came in contact with MBLs for their maintenance was either autoclaved,
191 filtered through a 0.2-micron filter, or surface-sterilized with 70% ethanol.

192 Every generation, on day 12 after egg collection, each population of MBLs and MBs (a
193 total of eight populations) was provided with a moist paste of autoclaved yeast to boost
194 their egg output. This paste also contained a few drops of acetic acid so that the flies were
195 attracted to yeast. A fresh food plate with an exposed vertical food surface was provided
196 for 12-16 hours to these populations at the end of day 13 to collect eggs for the next
197 generation. On day 14, these eggs were collected using a moist paintbrush and
198 transferred to cell strainers (TCP026, HiMedia) for egg dechorionation (Supplementary
199 Figure S2A). The cell strainer was then placed into one of the wells of a 6-well plate.
200 Based on known protocols to rear microbe-free *Drosophila* eggs (Kietz et al., 2018; Koyle
201 et al., 2016; Ridley et al., 2013), these eggs were washed with 2% bleach for 2 min to
202 remove the chorion layer and any associated microbes (Supplementary Figure S2B). The
203 cell strainer with dechorionated eggs was transferred to another well of the 6-well plate for
204 another quick wash with 2% bleach to kill any residual microbes. Next, the cell strainer

with eggs was transferred back-to-back to two fresh wells of the 6-well plate for two successive washes with autoclaved $dH_2O$ to remove bleach. For the MBLs, ~400 eggs were transferred to translucent plastic milk bottles (Laxbro Inc., Catalog No. FLBT-20) with ~50-60 ml autoclaved solid banana-jaggery media (Supplementary Figure S2C). To make MBs, dechorionated eggs were reconstituted with the native microbiome of $NDB_1$ (see next section) before being transferred to the food bottles (Supplementary Figure S2C).

The presence of microbes in MBs and their absence in MBLs is shown by PCR using 16S rRNA universal bacterial primers in Supplementary Figure S5 as described in Supplementary Text S3.1 and S3.2.

**Microbiome reconstitution**

For long-term experimental evolution, we chose microbiome reconstitution to create flies with microbes. Supplementary Figure S3 illustrates the reconstitution protocol in detail. Microbiome from the ancestral $NDB_1$ was used in reconstitution for all the MBs. This ensured that there were no differences across the four MB populations in terms of the microbiota that they harbored. Several distinct sources of the microbiome (say, every $MB_i$ getting their microbiome from their ancestral $NDB_i$) would have meant a greater chance of differential microbial association, which we sought to avoid.

Eight fly vials with non-autoclaved standard banana-jaggery food were set to house the flies for 24 hrs. These flies were collected on day 13 after egg collection. 50 flies with an equal male-to-female ratio were transferred into each vial using mild $CO_2$ anesthesia. After 24 hours, the flies were discarded, and 1 ml of autoclaved water was added to each vial to extract the microbiome. Vials were swirled with water till no visible fecal spots were seen on the walls. This method of sampling of microbes from the frass of flies housed in vials has been used earlier in the literature (Chandler et al., 2022). The microbe-free eggs, after the bleach treatment, were washed with autoclaved water twice and then dipped twice into this microbiome slurry to seed the source microbiome on the eggs. These eggs were then gently transferred to *Drosophila* vials or bottles with autoclaved food to generate flies with reconstituted microbiomes derived from $NDB_1$.

**Common-garden assay environments for the phenotypic assessment**

We explain the assay environments using the $MB_1$-$MBL_1$ (Figure 1) as an example. However, the same is true for the rest of the three groups of MB-MBL as well. $MB_1$ received microbiome from $NDB_1$ every generation.

$MBL_1$ is maintained without any microbes. If we directly compare the phenotypes of $MB_1$-$MBL_1$ in their experimental evolution conditions (i.e., MB with microbes and MBL without microbes) (Figure 1A), any observed differences between MB/MBL lines can be due to their evolutionary history (with/without microbes) or the assay environment (with/without microbes) or both. Therefore, we needed to provide a common assay condition for the phenotypic comparison of $MB_1$-$MBL_1$. We performed a phenotypic comparison between $MB_1$-$MBL_1$ both in the native environment of $MB_1$, which is with-microbes environment, as well as in the native environment of $MBL_1$, which is microbe-free (Figure 1B). For the with-microbe environment, $MB_1$ as well as $MBL_1$ were maintained in this common-garden environment in the presence of microbes for a generation. Assays were then performed on F1 flies (F1 flies generated from the selected and control flies that underwent 17-20 or 54-57 generations of selection) from the with-microbe environment that were raised as described in Supplementary Text S2 and Supplementary Figure S4. For a microbe-free environment, $MB_1$ and $MBL_1$ were maintained in this common-garden environment in the absence of microbes for a generation. Microbe-free F1 flies on which we performed the assays were raised as described in Supplementary text S2 and Supplementary Figure S4. Matching of environments via common-garden rearing for a generation minimizes non-genetic effects and allows us to attribute any observed differences in phenotype to the evolutionary effects (instead of effects due to short-term plastic changes) in which we are primarily interested. The assay design aims to ensure that only evolutionary effects contribute to the selected vs. control comparison.

The detailed validation of these assay conditions is reported in Supplementary Text S5.1-S5.2.

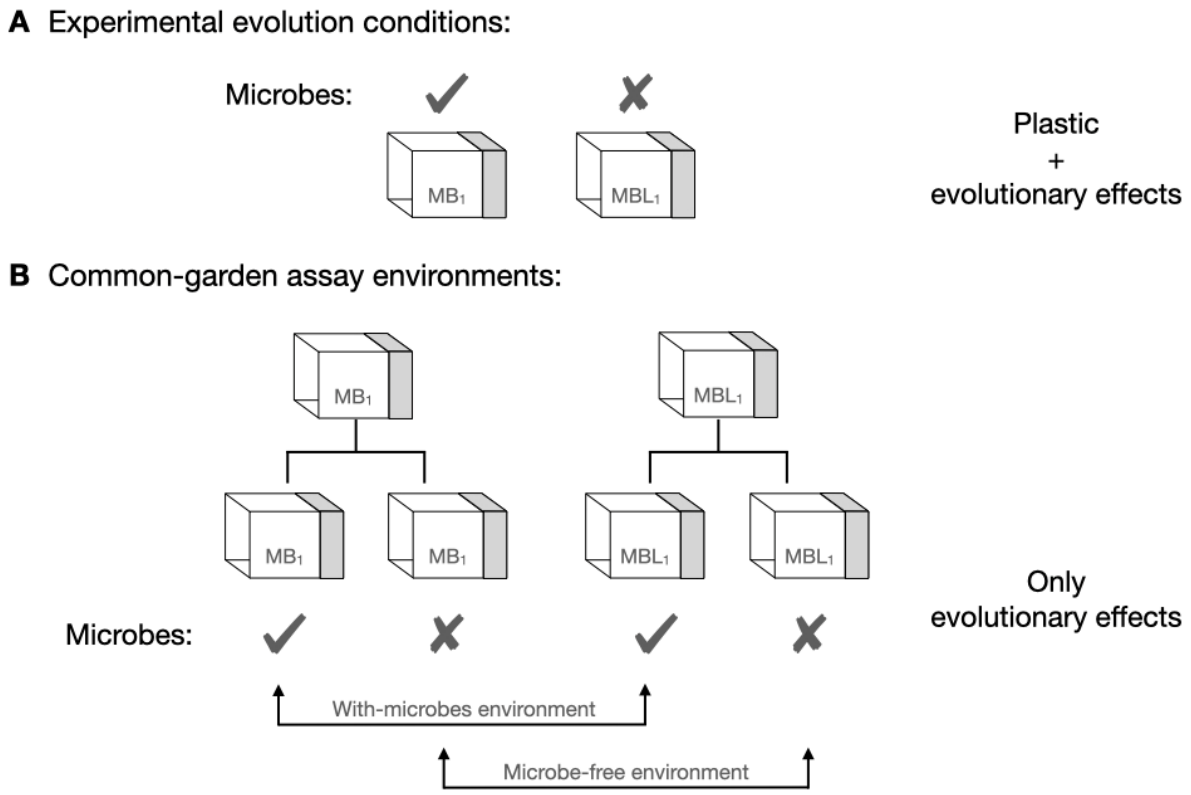


**Figure 1. Illustration of environments in which experimental evolution was carried out and the environments in which phenotypic assays are carried out.**

263

264 **Assays**

265 We measured various traits in the selected and control populations twice, first in
266 generations 17-20 and then in generations 54-57 (the experimental design details of these
267 two assessments are given in Supplementary Text S4.1 and S4.2, respectively).

268

269 For all the following assays, eggs were dechorionated as explained in a previous section
270 (Supplementary Figure S2) and transferred into plastic vials with ~ 6 ml of food at a pre-
271 determined density (~30 eggs per vial unless stated otherwise). All the assays were
272 carried out in autoclaved banana-jaggery food.

273

274 *Egg-to-adult developmental time and Percentage survival to adulthood*

275 A total of ten replicate vials were used per treatment. For each vial, we counted precisely
276 30 eggs under a microscope using a moist paintbrush and transferred them to a thin strip

of solidified 1.3% non-nutritive agar. This piece of agar with exactly 30 eggs was then transferred to the food vial with autoclaved banana-jaggery food. The vials were kept in an incubator at 25°C under constant light conditions. When the flies were about to eclose, the vials were monitored at regular intervals of 2 hours (generations 17-20) or 6 hours (generations 54-57). Every two hours, the males and females were sex separated under mild $CO_2$ anesthesia, and their numbers were noted. When there were no further eclosions in a vial, we counted the total number of eclosed flies and divided that by 30 (i.e., the number of eggs put in the vial) to compute the egg-to-adult survivorship.

*Dry body weight*

On day 12 after egg collection, the adult flies were anesthetized (using $CO_2$) and sorted by sex into batches of 20 individuals per sex per tube into 1.5 ml micro-centrifuge tubes. These flies were then flash-frozen using liquid nitrogen and, if required, stored at -80$^0$C till weighing. The flies were then air dried at 60$^0$C for 72 hrs and weighed on an analytical weighing balance (ME104, Mettler Toledo, least count = 0.1 mg). First, the total weight of (micro-centrifuge tube + flies) was recorded. Next, flies were removed from the tubes with a dry paintbrush, and the weight of the empty micro-centrifuge tube was recorded. The difference between these two gave us the body weight of 20 flies. This number was divided by 20 to get the average dry body weight per fly. When flies were removed, we counted these flies to double-check if every micro-centrifuge tube had 20 flies. If not, then the total weight was divided by that count. A total of ten micro-centrifuge tubes were weighed per treatment per sex. Thus, 200 flies per treatment per sex were sampled.

*Female fecundity*

On day 12 after egg collection, flies were transferred to fresh food vials. On day 13 (which is the day on which eggs are collected during the selection process), a male-female pair was aspirated (to avoid $CO_2$ anesthesia) into a 50 ml centrifuge tube with several holes in the wall for ventilation. The falcon tube had a food cup stuck to the inner surface of its cap to provide a surface for egg-laying. The centrifuge tubes were kept for 12 hours in an incubator maintained at 25$^0$C and constant light, after which the total number of eggs laid on the food cup was counted under a microscope. A total of 50 replicates per treatment were set for this assay.

*Desiccation resistance*

Ten 12-day old adult flies of a given sex were introduced into an empty plastic vial (Height: 3.5 inches x Diameter: 1 inch) using aspiration to avoid $CO_2$ anesthesia. The flies had no access to food or water. The vials were plugged with fresh cotton plugs and checked manually every two hours for any deaths till all the flies were dead. At each time point, the number of live flies was recorded (total flies to start with - count of total dead flies). The average time to death due to desiccation was calculated for each vial. Ten such replicate vials were set per treatment per sex.

*Locomotor activity*

The flies' locomotor activity was measured using the *Drosophila* Activity Monitoring (DAM) system (Trikinetics Inc). Using an aspirator, a single adult fly (day 12 after egg collection) was introduced into a glass tube (Length: 6.5 cm, Diameter: 5 mm). The locomotor activity for a given fly is the average number of times the fly crosses the infrared beam running through the middle of the glass tube per hour, recorded over six hours. Approximately 32 flies were used per treatment per sex. Data from flies that were seen to be dead at the end of the six-hour recording period were excluded from the analysis. The first 15 minutes of activity were excluded from the analysis as the time taken for the flies to get acclimatized to the glass tubes.

*Mating latency and mating time*

The mating assay was performed on virgin flies. After eclosion (typically around day eight after egg collection), male and female flies were separated under mild $CO_2$ anesthesia into vials with fly food. To ensure that the eclosed flies were virgins, we collected the flies every six hours after the first eclosion (Demerec, 1967). Males and females were held separately at the density of ten flies per vial till the setting up of the mating experiment on day 12 after egg collection.

On the day of the experiment, a female was introduced into a fresh plastic vial using an aspirator, followed by the introduction of a male. The floor of the vials was already covered with solid 1.3% non-nutritive agar to avoid desiccation of the flies. The time at which the male fly was introduced (T1), the time at which mating started (T2), and the time at which mating ended (T3) were noted through live manual observation. The mating latency was calculated as (T2-T1), and the mating time was calculated as (T3-T2). A total of 40 replicates per treatment were used for this assay.

343

344 *Robustness to the presence/absence of microbes*

345 In this study, we looked at the effects of the presence/absence of microbiota across
346 multiple traits. To get an overall picture from this data, we needed to look at a combined
347 measure of the magnitude of the effect of microbiota removal on the trait values. We call
348 this measure the robustness to microbiota removal (or simply, robustness) and
349 conceptualize it as the difference in the average trait value between the with-microbe and
350 the microbe-free conditions. Higher robustness refers to a lower average change in the
351 trait value due to microbiota removal and vice versa. For this, one needs a dimensionless
352 (or a normalized) measure of the trait value, as the various traits measured here (e.g., mg
353 of bodyweight, no. eggs laid, percent survival) have different units. We used two
354 normalized/dimensionless measures that fit the above criteria and then compared the
355 mean robustness for the MB-MBL populations.

356 The first measure was the effect size (Cohen's d) for the MB vs. MBL populations across
357 the two environments: with- and without microbes (Cohen, 2013). In other words, for every
358 trait in each population, we computed the effect sizes of the difference between the trait
359 values in the with-microbe and the microbe-free environments. Thus, for example, for the
360 development time of MBs, we computed the effect size of the difference between the
361 mean development time over the four populations in the with-microbe environment and the
362 microbe-free environment. We reasoned that whichever populations (MBs or MBLs)
363 showed a greater effect size due to microbiome removal for a particular trait exhibited
364 lower robustness for that trait across the with-microbe and microbe-free environments. It
365 should be noted here that these across-environment effect sizes are different from those
366 provided in Tables 1-2 and Supplementary Tables S5-S6. Supplementary Texts S4.1 and
367 S4.2 discuss the experimental design used to calculate these plastic effect changes and
368 why such analysis could not be done in Gen 17-20.

369 The second measure that we used for robustness was the percentage change in the
370 magnitude of the traits across environments.

371

372 **RNA isolation, RNA-Seq, and analysis**

373 A subset of flies from the phenotypic assays (at generation 54-57) were flash-frozen in
374 liquid nitrogen and stored at -80$^0$C. The frozen flies from the $MB_1$ and $MBL_1$ populations
375 were sent to a commercial vendor (miBiome Therapeutics LLP, Mumbai, India) for RNA-

Seq, starting with total RNA isolation. Due to logistical constraints, RNA-Seq was done only on female flies. The details of RNA-Seq are provided in Supplementary Text S1.6.

**Statistical analysis**

The selected populations ($MBL_{1-4}$) were compared with their corresponding controls ($MB_{1-4}$) in two types of environments (with and without microbiota). The analysis for these two environments was done separately. In each environment, means of $MB_{1-4}$ were compared against means of $MBL_{1-4}$ using a paired t-test performed in GraphPad Prism (v10.2.1). We compared the robustness of MBs and MBLs to microbiome removal (i.e., Cohen's *d* and percentage change across environments) using separate paired t-tests.

For interpreting effect sizes using Cohen's d, the following criteria were followed: $d > 0.8$ (high); $0.8 > d > 0.5$ (medium); $d < 0.5$ (low) (Cohen, 2013). The t-tests were performed, and the graphs were plotted in GraphPad Prism (v10.2.1). The figures drawn using BioRender are labeled as such in their description.

In the Results section, along with the trends that are statistically significant (i.e., $P < 0.05$), we have labeled results where $0.05 < P < 0.1$ and Cohen's $d \geq 0.8$ (i.e., large effect size) as trends. This is highlighted in blue in Tables 1-2 and Supplementary Tables S5-S6, which provide detailed information about the statistical comparisons for all the assays.

## Results

### First phenotypic assessment of experimental evolution lines at host generations 17-20

The results for generations 17-20 are provided in Supplementary Text S6. At this point, we observed that barring mating time (which was significantly shorter in MBLs), none of the other traits differed between the MBs and the MBLs (Supplementary Tables S5 and S6).

### Second phenotypic assessment of experimental evolution lines at host generations 54-57

Tables 1 and 2 provide detailed information on the statistical comparison between $MB_{1-4}$ and $MBL_{1-4}$ for the with-microbe and microbe-free assay environments, respectively. Comparing these tables with the corresponding ones for generation 17 (Supplementary Tables S5 and S6) shows that there were a larger number of cases in the later generation where the effect sizes were medium or large. This could be a signature of evolutionary divergence between the MB and MBL populations.

*Male traits in host generations 54-57*

There was no difference between the body weight of the male $MBL_{1-4}$ and $MB_{1-4}$ flies in the with-microbe assay environment (Table 1, Figure 2A-left). In the microbe-free environment, the body weight of the male $MBL_{1-4}$ was more than their corresponding controls $MB_{1-4}$ with a large effect size even though the P-value was marginally greater than 0.05 (Table 2, Figure 2A-right).

The egg-to-adult development time was largely unaffected by the multi-generation deprivation of microbes, as seen in both assay environments (Tables 1 and 2, Figure 2B).

$MBL_{1-4}$ males had lower desiccation resistance than $MB_{1-4}$ in the with-microbe environment with a large effect size, although the P-value was marginally greater than 0.05 (Table 1, Figure 2C-left). However, no difference was observed in the microbe-free environment (Table 2, Figure 2C-right).

Likewise, $MB_{1-4}$ and $MBL_{1-4}$ showed similar levels of locomotor activity in both environments (Tables 1 and 2, Figure 2D).

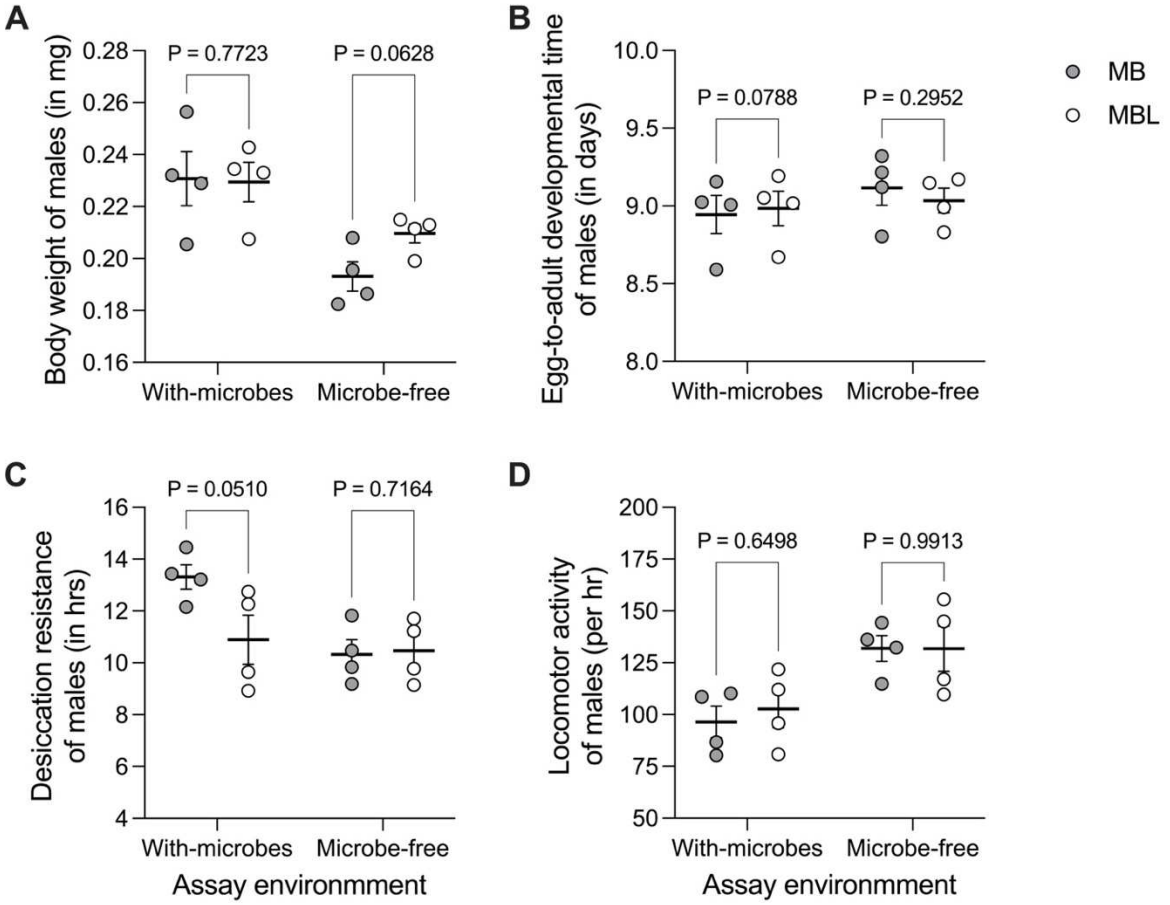


**Figure 2. Male traits assessed in Gen 54-57 in with-microbe and microbe-free environments.** (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations $MB_{1-4}$ and, similarly, four empty circles are means for each of the four selected populations $MBL_{1-4}$. The black horizontal lines represent the grand mean over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over $MB_{1-4}$ was compared against the grand mean over $MBL_{1-4}$ using a paired t-test.

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432 *Female traits in host generations 54-57*

The body weights of the $MB_{1-4}$ and $MBL_{1-4}$ females were similar in the with-microbes regime (Table 1, Figure 3A-left) but showed a marginal increase for MBLs in the no-microbe regime, which was not statistically significant (Table 2), despite a large effect size (d = 1.35, Figure 3A-right).

Female egg-to-adult development time was similar for MB-MBLs after multiple generations without the microbes when assayed in both assay environments (Tables 1 and 2, Figure 3B).

$MBL_{1-4}$ females had lower desiccation resistance in the with-microbe regime, which was not statistically significant (Table 1, Figure 3C-left). MBs and MBLs had similar desiccation resistance in the no-microbe regime (Table 2, Figure 3C-right).

$MB_{1-4}$ and $MBL_{1-4}$ females had similar locomotor activity levels in the with-microbes environment (Table 1, Figure 3D-left). In the microbe-free environment, there was a trend (i.e., large effect size, d = 0.89) towards $MBL_{1-4}$ females being more active than the $MB_{1-4}$ that was not statistically significant (Table 2, Figure 3D-right).

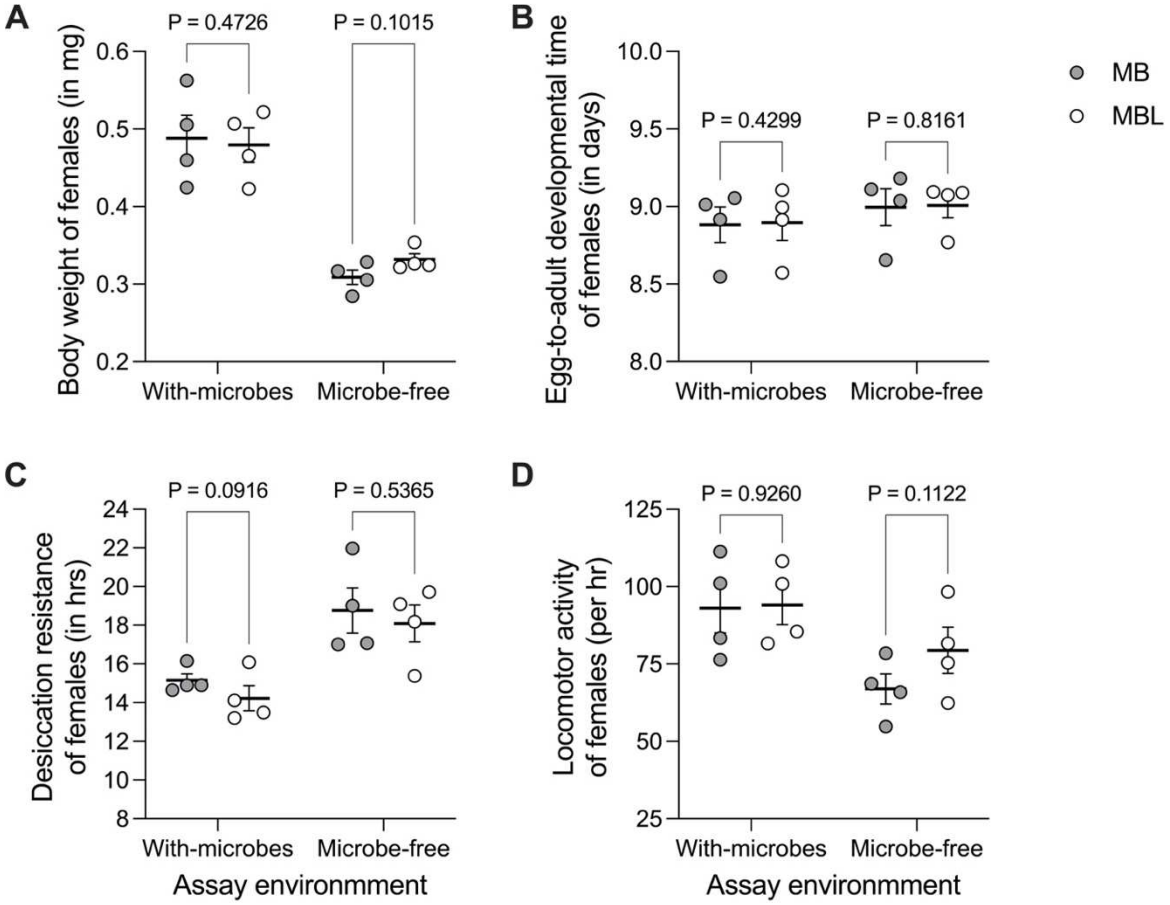


**Figure 3. Female traits assessed in Gen 54-57 in with-microbe and microbe-free environments.** (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations $MB_{1-4}$ and, similarly, four empty circles are means for each of the four selected populations $MBL_{1-4}$. The black horizontal lines represent the grand mean over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over $MB_{1-4}$ was compared against the grand mean over $MBL_{1-4}$ using a paired t-test.

450

*Pair traits in host generations 54-57*

There was a trend (d = 1.81) of lower survival for $MBL_{1-4}$ than $MB_{1-4}$ in the with-microbe regime, but it was not statistically significant (Table 1, Figure 4A-left). In the microbe-free environment, $MBL_{1-4}$ had better survival than $MB_{1-4}$ (Table 2, Figure 4A-right).

There was a trend (d = 1.67) of $MBL_{1-4}$ laying more eggs than the $MB_{1-4}$ in the with-microbe regime, but this difference was not statistically significant (Table 1, Figure 4B-left). In the microbe-free environment, $MBL_{1-4}$ had a trend of slightly higher fecundity, but it was not statistically significant either (Table 2, Figure 4B-right).

$MBL_{1-4}$ did not differ from $MB_{1-4}$ in the latency to start mating, both in the with-microbe and microbe-free regimes (Table 1 and 2, Figure 4C).

There was no difference in $MB_{1-4}$ and $MBL_{1-4}$ mating time in the with-microbe regime (Table 1, Figure 4D-left). In the microbe-free environment, there is a trend (d = 1.39) of shorter mating times for $MBL_{1-4}$ than $MB_{1-4}$, but this is not statistically significant (Table 2, Figure 4D-right).

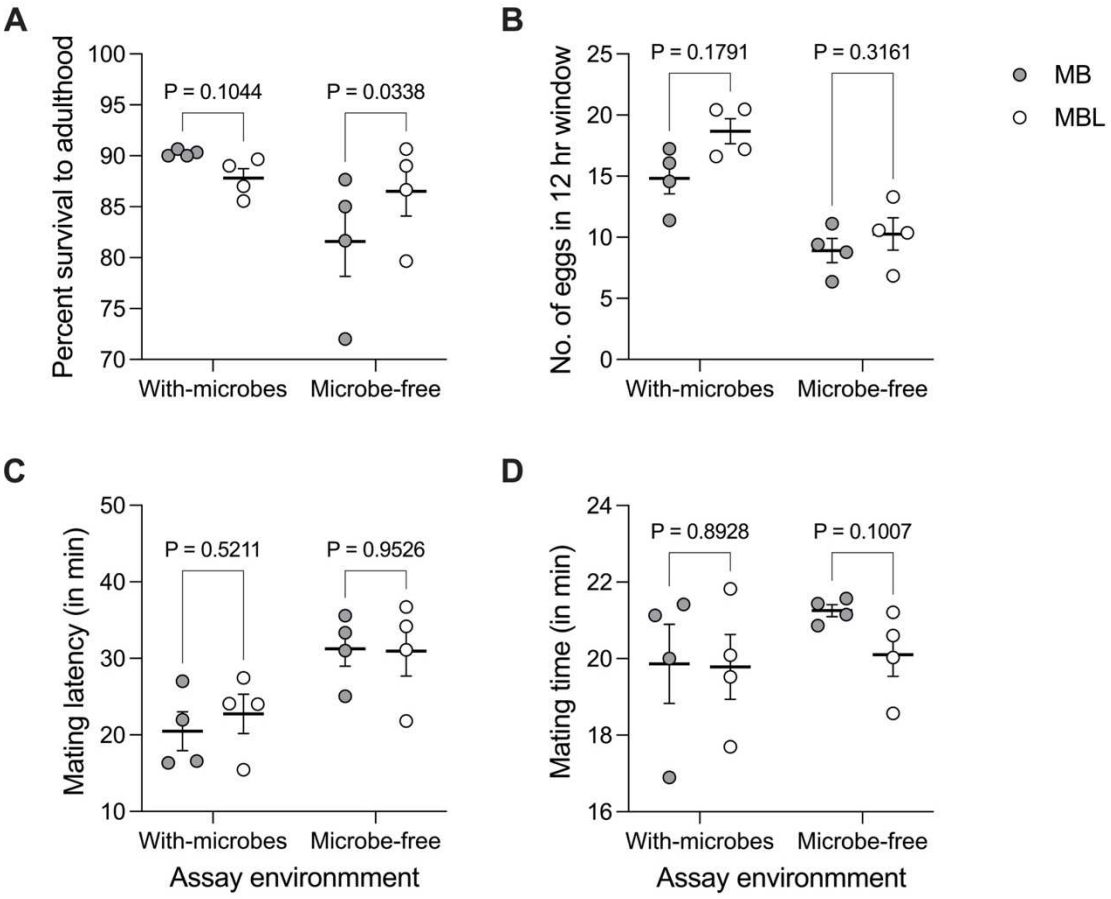


**Figure 4. Pair traits assessed in Gen 54-57 in with-microbe and microbe-free environments.** (A) Survival to adulthood. (B) Female fecundity. (C) Mating latency. (D) Mating time. In each assay environment, four filled circles are means for each of the four control populations $MB_{1-4}$ and similarly, four empty circles are means for each of the four selected populations $MBL_{1-4}$. The black horizontal lines represent the grand means over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over $MB_{1-4}$ was compared against the grand mean over $MBL_{1-4}$ using a paired t-test.

467

468

**Table 1.** Summary of all assays in the with-microbes environment (Gen 54-57). T refers to the grand mean of the MBLs, while C refers to the grand mean of the MBs.

| | Assay | Test statistic | P-value | % change (T - C)/C | Cohen's d | Effect size* | Sample size$ |
|---|---|---|---|---|---|---|---|
| 1 | Dry body weight (Male) | $t_3 = 0.32$ | 0.7723 | -0.6 | 0.07 | Small | 10 |
| 2 | Dry body weight (Female) | $t_3 = 0.82$ | 0.4726 | -1.8 | 0.17 | Small | 10 |
| 3 | Development time (Males) | $t_3 = 2.62$ | 0.0788 | 0.4 | 0.17 | Small | 9 to 10 |
| 4 | Development time (Females) | $t_3 = 0.91$ | 0.4299 | 0.2 | 0.06 | Small | 10 |
| 5 | Survival to adulthood | $t_3 = 2.31$ | 0.1044 | -2.7 | 1.81 | Large | 9 to 10 |
| 6 | Fecundity (Females) | $t_3 = 1.75$ | 0.1791 | 26.0 | 1.67 | Large | 50 |
| 7 | Desiccation resistance (Males) | $t_3 = 3.16$ | 0.0510 | -18.2 | 1.62 | Large | 10 |
| 8 | Desiccation resistance (Females) | $t_3 = 2.45$ | 0.0916 | -6.1 | 0.89 | Large | 10 |
| 9 | Locomotor activity (Males) | $t_3 = 0.50$ | 0.6498 | 6.4 | 0.37 | Small | 30 to 32 |
| 10 | Locomotor activity (Females) | $t_3 = 0.10$ | 0.9260 | 1.1 | 0.07 | Small | 31 to 32 |
| 11 | Mating Latency | $t_3 = 0.72$ | 0.5211 | 11.1 | 0.44 | Small | 34 to 40 |
| 12 | Mating time | $t_3 = 0.15$ | 0.8928 | -0.4 | 0.04 | Small | 34 to 40 |

**Table 2.** Summary of all assays in the microbe-free environment (Gen 54-57). T refers to the grand mean of the MBLs, while C refers to the grand mean of the MBs.

| | Assay | Test statistic | P-value | % change (T - C)/C | Cohen's d | Effect size* | Sample size$ |
|---|---|---|---|---|---|---|---|
| 1 | Dry body weight (Male) | $t_3 = 2.89$ | 0.0628 | 8.5 | 1.74 | Large | 10 |
| 2 | Dry body weight (Female) | $t_3 = 2.34$ | 0.1015 | 7.4 | 1.35 | Large | 10 |
| 3 | Development time (Males) | $t_3 = 1.27$ | 0.2952 | -0.9 | 0.42 | Small | 10 |
| 4 | Development time (Females) | $t_3 = 0.25$ | 0.8161 | 0.1 | 0.05 | Small | 10 |
| 5 | Survival to adulthood | $t_3 = 3.72$ | 0.0338 | 6.0 | 0.83 | Large | 10 |
| 6 | Fecundity (Females) | $t_3 = 1.20$ | 0.3161 | 15.2 | 0.58 | Medium | 50 |
| 7 | Desiccation resistance (Males) | $t_3 = 0.40$ | 0.7164 | 1.3 | 0.11 | Small | 10 |
| 8 | Desiccation resistance (Females) | $t_3 = 0.70$ | 0.5365 | -3.6 | 0.32 | Small | 10 |
| 9 | Locomotor activity (Males) | $t_3 = 0.01$ | 0.9913 | -0.1 | 0.01 | Small | 27 to 32 |
| 10 | Locomotor activity (Females) | $t_3 = 2.23$ | 0.1122 | 18.6 | 0.99 | Large | 32 |
| 11 | Mating Latency | $t_3 = 0.06$ | 0.9526 | -1.0 | 0.05 | Small | 32 to 38 |
| 12 | Mating time | $t_3 = 2.35$ | 0.1007 | -5.4 | 1.39 | Large | 32 to 38 |

*Interpretation for effect sizes using Cohen's d: d > 0.8 (Large); 0.8 > d > 0.5 (Medium); < 0.5 (Small)

[$]Note: See methods for detailed information about sample sizes. For example, the sample size of ten per treatment for body weight indicates ten micro-centrifuge tubes with 20 flies each. In other words, each circle in the plots is an average over this sample size.

**MBLs have greater robustness than the MBs across with-microbe and microbe-free environments (Gen 54-57 assays)**

Figures 2 to 4 suggested that, compared to the MBs, the trait values of the MBLs changed relatively less across the two assay environments (i.e., with-microbes and microbe-free). In other words, the reaction norms for the various traits across these two environments are expected to be flatter for the MBLs than for the MBs. True to this observation, we found that the MBLs, on average, had smaller effect sizes than MBs (Paired t-test, $t_{11} = 2.14$, P = 0.0554, Cohen's d = 0.49 (small), Figure 5A), hinting at lower magnitude of change (and hence greater robustness to microbiome removal) than the MBs across two assay environments. Moreover, MBLs had a lower percentage change in their trait values across two environments than MBs (Paired t-test, $t_{11} = 2.99$, P = 0.0123, Cohen's d = 0.39 (small), Figure 5B).

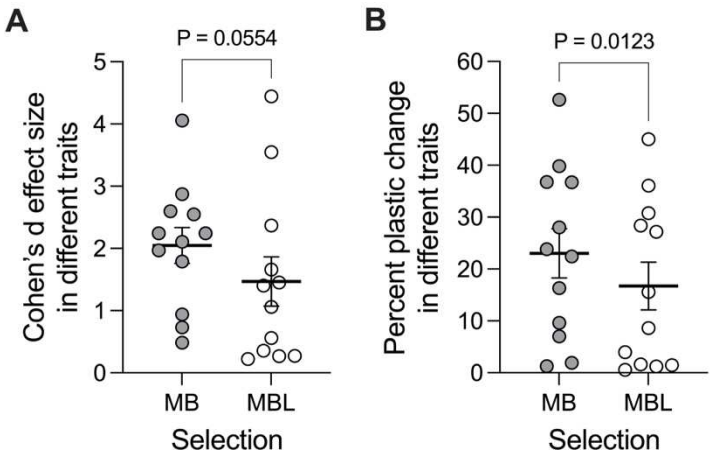


**Figure 5. Robustness to microbiota removal.** (A) Effect sizes and (B) Percentage changes. Means are indicated by black horizontal lines. The error bars represent SEM. The data is analyzed using a paired t-test. For percent change, Wilcoxon matched-pairs signed rank test (a non-parametric test) is also significant (W = -64.0, P = 0.0093).

Taken together, the MBLs had greater robustness (i.e., lesser effect of microbiota removal) than the MBs, even though the effect size was small for both the measures we employed.

**The big picture across both assessments: divergence in MB-MBLs with time?**

In the previous section, we used effect sizes on phenotype differences across assay environments for MBs as well as MBLs to measure the robustness exhibited by these two populations. In this section, we look at how effect sizes have changed *for the evolutionary difference between MB-MBLs over time* from the first phenotypic assessment in generations 17-20 to the second assessment in generations 54-57 (Figure 6). These effect sizes are provided in Supplementary Tables S5-S6 for generations 17-20 and Tables 1-2 for generations 54-57.

The effect sizes (using Cohen's d) for the phenotypic comparison between MB-MBLs in generations 17-20 were mostly small, with some effects being medium or large (Figure 6-left). In generations 54-57, the distribution of effect sizes shifted in the direction of large effects (Figure 6-right). This pattern is seen in both assay environments: with-microbes (circles with a coral fill) and microbe-free (circles without any fill). This suggests that the MBLs are still diverging from MBs even after 54-57 generations of experimental evolution.

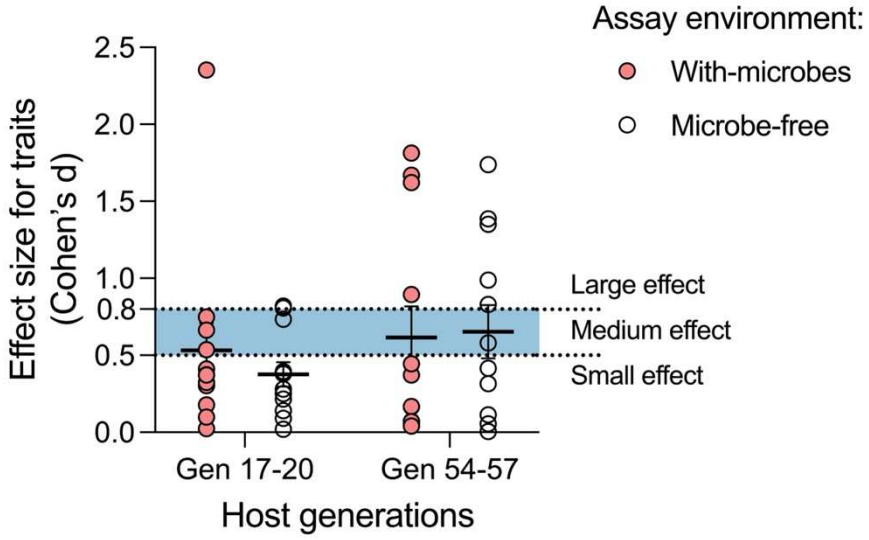


**Figure 6. Changes in effect sizes (Cohen's d) over all traits with time.** Means are indicated by black horizontal lines. The error bars represent SEM. Coral circles indicate with-microbes environment and empty circles indicate the microbe-free environment.

**RNA-Seq results – Gen 54-57**

522 After investigating the effects of long-term microbiome absence using a broad range of
523 phenotypic assays, we used RNA-Seq to determine if specific genes are expressed
524 differentially in $MBL_1$ vs. $MB_1$ as a direct consequence of their different evolutionary
525 history.

526 In the microbe-free regime, where both selected and control populations were assayed
527 without their microbiome, very few transcripts showed differential expression when
528 analyzed using DESeq2 (Supplementary Text S7.1).

529 In the with-microbe regime, anti-microbial peptide (AMP) expression was up-regulated in
530 $MBL_1$ compared to their control $MB_1$ (Figure 7). The complete list of up-regulated genes in
531 $MBL_1$ is given in Supplementary Table S7. We also find that a cluster of heat shock
532 proteins (HSPs) and several other genes in $MBL_1$ are down-regulated. The complete list of
533 down-regulated genes in $MBL_1$ is given in Supplementary Table S8. The gene ontology
534 analysis for differentially expressed genes is presented in Supplementary Text S7.3.

535 Among all the differentially expressed genes, only those genes with $Log_2$(Fold Change) >
536 1.5 and adjusted P-value < 0.05 are highlighted in Figure 7 and given in Supplementary
537 Tables S7 and S8.

538

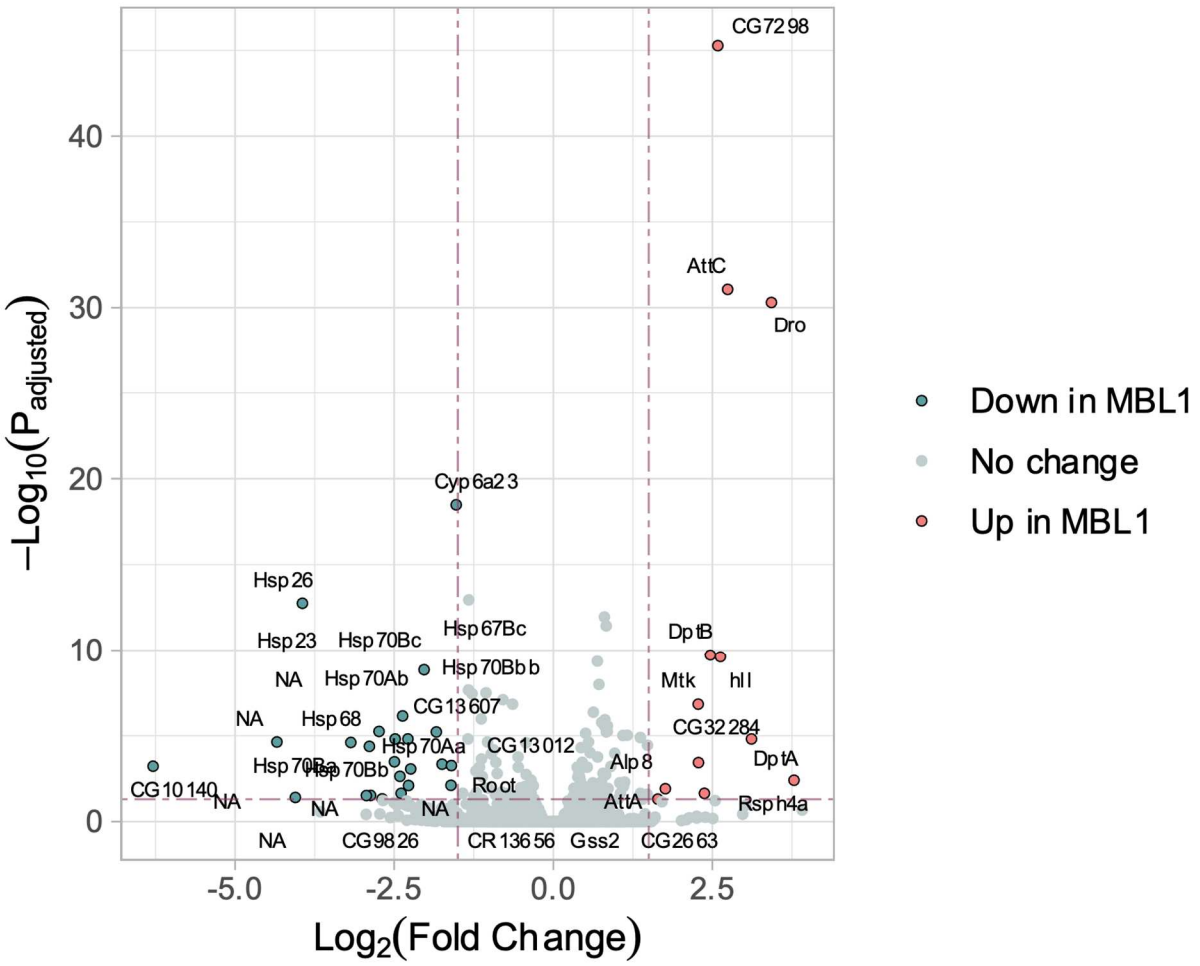


**Figure 7. Differentially expressed genes in $MBL_1$ vs $MB_1$ in the with-microbes environment using DESeq2.** The red dots represent the up-regulated genes, while blue dots represent down-regulated genes in $MBL_1$. The protocol for the RNA-Seq is described in the method section.

539

540

541 **Discussion**

542 **$MBL_{1-4}$ show few changes even after 54 generations without microbes**

543 In a previous study using the ancestral outbred $NDB_{1-4}$ flies (Malwade, 2024), it had been
544 found that upon removal of microbiota, the female flies had lower fecundity, higher
545 desiccation resistance, and lower locomotor activity, while for the males, locomotor activity
546 increased. For both sexes, the other traits, such as body weight, egg-to-adult survival,
547 mating latency, and mating duration, remained unchanged.

548 This led us to infer that microbiota plays an important role in the realization of several traits
549 in *Drosophila melanogaster*, an observation that corroborates the results of numerous
550 studies from the literature (Broderick & Lemaitre, 2012; Douglas, 2018b; Erkosar et al.,
551 2013; Lesperance & Broderick, 2020; Ludington & Ja, 2020). This led to the hypothesis
552 that the removal of microbiota is likely to be extremely stressful for the flies, as many traits
553 would be affected at once. Hence, we expected the microbiota-less environment to exert
554 very substantial selection pressure on the $MBL_{1-4}$ flies, potentially leading to changes in
555 multiple life-history traits, possibly including some trade-offs, within relatively few
556 generations. However, the results of this study indicate otherwise.

557

558 In the first set of phenotypic assays done between host generations 17-20 (Supplementary
559 Text S6), we saw that the mating time of MBLs was shorter than the MBs (Supplementary
560 Figure S13D, with-microbes environment), and female body weight was slightly elevated
561 for MBLs (Supplementary Figure S12A), microbe-free environment). Apart from these two
562 changes, we did not see any evidence of evolutionary divergence between $MB_{1-4}$-$MBL_{1-4}$ in
563 either of the two assay environments. This lack of divergence could be potentially
564 attributed to the relatively short duration of the selection regime at that point.

565

566 In the second set of phenotypic assays done between host generations 54-57 (Figures 2
567 to 4), we saw that two traits showed significant change. In the microbe-free environment,
568 we found that $MBL_{1-4}$ had better egg-to-adult survival than $MB_{1-4}$ (Figure 4A). In the with-
569 microbes environment, $MBL_{1-4}$ males had lower desiccation resistance (Figure 2C). It is
570 possible that this lower desiccation resistance might be a cost incurred by MBLs in this
571 environment.

572 When we looked at the effect sizes over all the traits assessed in Gen 17-20 vs. Gen 54-
573 57 (Figure 6), we saw that the effect size distribution shifted from mostly “small and

medium" effects to "small, medium, and large" effects. This suggests that even after 54 generations, the selected flies might still be diverging from the control flies.

We assayed the life-history traits both in the presence and the absence of microbes. Thus, any improvement in the fitness of the MBLs (compared to the MBs) in the microbe-free environment can be construed as the effect of adaptation in the selection environment. On the other hand, any reduction in the fitness of the MBLs (again compared to the MBs) in either assay environment would signify a cost of adaptation under microbe-free conditions. Our results showed very few adaptations or costs for the MBLs.

There are several potential ways, not all mutually exclusive, to interpret these observations. One possibility is that the selection pressure on the MBLs was so strong that they responded extremely fast, and therefore, by the time we assayed them first (i.e., by 17-20 generations), they had already responded maximally to the selection pressure and were back to the MB levels across all traits. However, if this were to be the case, in the microbe-free assay environments, we would have expected the MBLs to have generally greater fitness than the MBs. This is because the microbe-free assay environment is the "selection" environment for the MBLs but a novel environment for the MBs. Since the MBLs did not do better than the MBs in this assay environment, we conclude that the former had not adapted too well in the selection environment.

Another possibility is that even though the selection pressure is strong, our fly populations did not carry sufficient genetic variability to respond to the selection pressure. This might have been a valid concern if, like many studies on *Drosophila*-microbiota systems (e.g., (Ridley et al., 2013; Shin et al., 2011)), the experiments were performed on highly inbred or iso-female strains (e.g., *Canton-S, Oregon-R,* and $w^{1118}$). However, our fly populations are outbred, are vigorously active in the cage, and have high fecundity and egg-to-adult survivorship (personal observations). Therefore, we have no reason to believe these populations lacked genetic variation. More importantly, the fact that egg-to-adult survivorship did change over time indicates that the populations were indeed evolving, which would not have been possible in the absence of genetic variation.

A third possibility is that while some divergence has indeed happened, they have not yet been picked up by our assays. For example, some previous studies have shown that the role of microbiota in affecting host physiology becomes prominent only in nutritionally unbalanced diets (Shin et al., 2011; Storelli et al., 2011). It is entirely possible that we will see more differences between the MBs and the MBLs if we assay them on a different diet. However, previous observations on these populations raised on standard food medium have shown that removing the microbiota leads to changes in multiple traits, even within a single generation (Malwade, 2024). Therefore, it is unlikely that the lack of differences between MBs and MBLs is due to the medium on which they were assayed or selected.

This brings us to the fourth possibility that although the removal of microbiota can lead to large-scale changes in a number of life-history and behavioral traits in a single generation, this does not translate into strong selection pressure on an evolutionary timescale. Our results would make sense when we consider the possibility that if the selection pressure on the host to evolve in the absence of microbes is low, then the evolutionary response can be slow and mild, as we observed in this study. We suggest that the results of our long-term experimental evolution are consistent with the so-called “evolutionary addiction hypothesis” (Douglas, 2018a; Hammer, 2024; Moran et al., 2019). This hypothesis suggests that when the microbiome is perturbed or culled entirely, hosts experience “withdrawal symptoms” as the host and microbes have lived together for an extended period (Hammer, 2024). Stated differently, although the host can function in the absence of the microbes, there is an inertia borne out of very long coexistence that temporarily affects the host. Thus, when we remove the microbiome for the first time, the host enters an altered state of physiology as it has already evolved some dependence on the previously ever-present microbes. Nevertheless, the host still has the capacity to perform its function without the microbiome, and within a few generations of microbe-free existence, it adjusts to the absence without any major phenotypic changes. This idea of evolutionary addiction might offer one potential explanation for the apparently low/weak selection pressure in our experimental evolution regime, and why we have not seen major evolutionary changes in our experimental evolution so far.

We speculate that the idea of evolutionary addiction is also consistent with the increased robustness seen in the MBLs (Fig 5). If growing in the absence of the microbes were simply helping the MBLs to come out of their “evolutionary addiction,” then one would

640 expect that the microbiota would play a relatively lesser role in shaping their traits (relative
641 to the MBs). In other words, the MBLs are expected to become more independent of their
642 microbiota, increasing their robustness across the with- and without-microbes
643 environments (by decreasing the sensitivity to microbiome presence/absence). Both
644 proxies of robustness, Cohen's d (Fig 5A) and percentage change (Fig 5B), suggested that
645 this is the case. However, the effect size in both cases was small, which suggests that the
646 MBLs still have some distance to go to "kick their addiction," to function fully independent
647 of the microbiome, and the phenomenon is perhaps incipient at this stage.

648 We note here that our conceptualization of robustness is close to phenotypic plasticity.
649 However, phenotypic plasticity is typically defined on genotypes (Agrawal, 2001; Sommer,
650 2020), whereas our traits were measured at the population level, which likely consisted of
651 multiple genotypes. That is why we refrained from calling our MBL populations less plastic.
652 However, our observations on robustness do raise the possibility that the plasticity of the
653 MBL populations might have been altered due to selection. This conjecture needs to be
654 examined in detail in future studies.

655

656 **$MBL_1$ and $MB_1$ have at least three sets of differentially expressed gene clusters**

657 To see if MBLs have started showing differences in gene expression compared to MBs,
658 we performed an RNA-seq on one group of populations, namely $MBL_1$ and $MB_1$. We here
659 note that these findings, on only one of the four evolutionary replicates, are preliminary.

660

661 *A cluster of heat shock proteins (HSPs) is downregulated in $MBL_1$*

662 Heat shock proteins are the proteins that are involved when the organism experiences
663 stress (Feder & Hofmann, 1999). Even though, as their name suggests, they play an
664 important role primarily in the heat shock response (Lindquist, 1986), some of these
665 proteins can be expressed in response to various other stresses such as cold, crowding,
666 anoxia, desiccation, etc., where these proteins can act as chaperones (King & MacRae,
667 2015). We find that HSPs (*HSP23, HSP26, HSP67, and HSP68*) and HSP subunits
668 (*HSP67 and HSP70*) are down-regulated in $MBL_1$ flies that were maintained without the
669 microbiome for multiple generations.

670

*RNA-Seq results on $MBL_1$ might be consistent with the desiccation resistance trends over all four replicate MBLs*

Lower expression of HSPs in $MBL_1$ suggests a potentially muted stress response of the MBLs due to the lower availability of HSPs to counter the stress. This correlation of stress resistance with the expression of HSPs is well-documented (Feder & Hofmann, 1999). When we looked at the desiccation resistance of $MBL_{1-4}$ vs. $MB_{1-4}$ in the with-microbes regime, we found that $MBL_{1-4}$ had a trend of lower desiccation resistance than $MB_{1-4}$ for males (Figure 2C) but not for females (Figure 3C). Both these effects were large in size. While we have not established a causal link in this trend of lower desiccation resistance, this observation could be a potential link between the lower expression of HSPs and lower desiccation resistance.

Looking at this desiccation-HSP expression correlation, one can speculate that the thermal stress tolerance of $MBL_{1-4}$ will be lower than that of $MB_{1-4}$ due to lower expression and, hence, lower levels of HSPs. But that need not be necessarily true. A study of land snails has shown that desiccation-sensitive species maintain more HSPs than desiccation-resistant species (Mizrahi et al., 2010). If the same logic holds for thermal stress tolerance in *Drosophila*, then the lower expression of HSP might hint at a better ability of MBLs to resist heat stress. Thus, from this data, we cannot predict the ability of the MBLs to withstand thermal stress.

*Antimicrobial peptides (AMPs) are upregulated in $MBL_1$*

Antimicrobial peptides (AMPs) are small cationic molecules that interact with bacterial membranes to damage and kill the bacteria (Brogden, 2005). They are widespread across the tree of life, with animals and plants harboring various types of AMPs to deal with pathogens (Mookherjee et al., 2020). In *Drosophila melanogaster*, there are seven types of AMPs whose functions are known, and a detailed discussion on them is available elsewhere (Hanson & Lemaitre, 2020; Lemaitre & Hoffmann, 2007). In addition to their classical role of defense against pathogens, these AMPs are also shown to control the host microbiome (Franzenburg et al., 2013; Maritan et al., 2024; Marra et al., 2021; Mergaert, 2018).

A study that looked at the gene expression changes in the *Drosophila* gut using transcriptomics when the host is colonized with microbes vs. when it is kept without

704 microbes found that the presence of a microbiome leads to upregulated expression of
705 several immune response genes, including AMPs such as *Attacin*, *Drosomycin* and stress
706 response genes such as *HSP23*, *HSP67Bb*, and *HSP70Bc* (Broderick et al., 2014). While
707 their observation of the up-regulation of the immune response matches our results in
708 $MBL_1$, the HSP response is down-regulated in $MBL_1$, in contrast to the observation by
709 Broderick et al., which was done over the host's lifetime (Broderick et al., 2014). The up-
710 regulation of the immune system upon microbiome colonization is also reported in other
711 subsequent single-generational studies that are done over the host's lifetime (Chandler et
712 al., 2022; Elya et al., 2016).

713 The above single-generation studies on *Drosophila* indicate that the presence of a
714 microbiome leads to AMP up-regulation compared to when the microbiome is removed. In
715 our study, as both MB and MBL received the same native microbiome from a common
716 pool (shown in Supplementary Text S5.1-S5.2), their different evolutionary history should
717 be the driving force in the up-regulation of AMPs, not the differential presence of
718 microbiome.

719

720 *A hygiene hypothesis-like phenomenon can potentially explain the overactive humoral*
721 *immune response in MBLs*

722 MBLs have been kept away from any microbes for more than 50 generations. Several
723 studies have shown that microbes are indispensable for the proper calibration and
724 modulation of the immune system, especially early in life (Abt et al., 2012; Donald &
725 Finlay, 2023; Gensollen et al., 2016; Henrick et al., 2021; Hoang & King, 2022; Knoop et
726 al., 2017; Mazmanian et al., 2005; Metcalf et al., 2022; Selosse et al., 2014). This
727 calibration includes proper sorting of self vs. non-self (W.-J. Lee, 2008; Lhocine et al.,
728 2008). There is a possibility that MBLs might have lost the delicate balance that maintains
729 this calibration and, as a result, are over-expressing AMPs compared to MBs to actively
730 regulate the microbiome. This possibility can be validated in future experiments.

731 In humans, this phenomenon is often discussed in the context of the hygiene hypothesis
732 (or a refined version of the same, called the old friend hypothesis) (Bach, 2018; Blackwell,
733 2022; Wills-Karp et al., 2001). This hypothesis states that exposure to certain
734 microorganisms, particularly in early life, can reduce the frequency of atopic diseases and
735 immune-mediated disorders in organisms (Stiemsma et al., 2015). It is possible that the
736 observed increased levels of AMPs signify immune overactivation via immune
737 miscalibration over many generations. It is already known that *Drosophila* can suffer from

autoimmune disorders (Mortimer et al., 2021). Therefore, if our conjecture about the hygiene hypothesis is true, then one might predict that the MBLs would suffer from higher instances of autoimmune disorders, which we would like to investigate in detail in a future study using all four evolutionary replicates.

Apart from the HSPs and AMPs, another smaller set of chitin-binding genes is also differentially expressed in $MBL_1$. We have discussed this in Supplementary Text S8.

We have discussed multiple speculative insights in the latter part of the discussion, even though they are not substantially supported by our current data, as experimental microbiome manipulation studies on host-microbiome long-term interactions are rare and demanding, and at least some of these insights that we discussed might help generate further falsifiable hypotheses in *Drosophila* and in other hosts.

## Conclusion

Our study involved *Drosophila melanogaster,* where the short-term removal of microbiota is known to have major effects across multiple traits (Gnainsky et al., 2021; Jia et al., 2021; Schretter et al., 2018; Shin et al., 2011; Storelli et al., 2011; Suyama et al., 2023). Therefore, we expected that the removal of the microbiota for an extended period would force the populations to evolve along multiple trait axes, potentially with associated life-history costs. Contrary to our expectations, even after 54 generations, there were only modest evolutionary changes in the host. This might be due to weak evolutionary pressure on the host and may be situated in the framework of the “evolutionary addiction hypothesis.” This might also mean that, for the outbred *Drosophila* system, the “host + microbe” unit (i.e., the holobiont) might represent a somewhat weak integration. Based on our results, we cannot claim that the cost of evolution in the absence of the microbiome will be negligible in other systems. However, our results suggest that if the host pays large costs of microbiota removal across multiple traits in a single generation, then that does not necessarily mean that the host is under strong selection pressure and will evolve to counter this pressure in the long run. In fact, very little can be predicted about the long-term effects of evolution under a no-microbe condition based on insights from the existing single-generation studies. Previous theoretical (Henry et al., 2021; Vliet & Doebeli, 2019) and empirical (reviewed in (Rosenberg, 2021)) studies have suggested that microbiota can affect the evolutionary trajectories of their hosts. Our study suggests a more nuanced picture. Taken together, our findings indicate that the role of microbiota in determining long-term evolutionary trajectories deserves further critical examination.

775 **References**

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**Supplementary information**

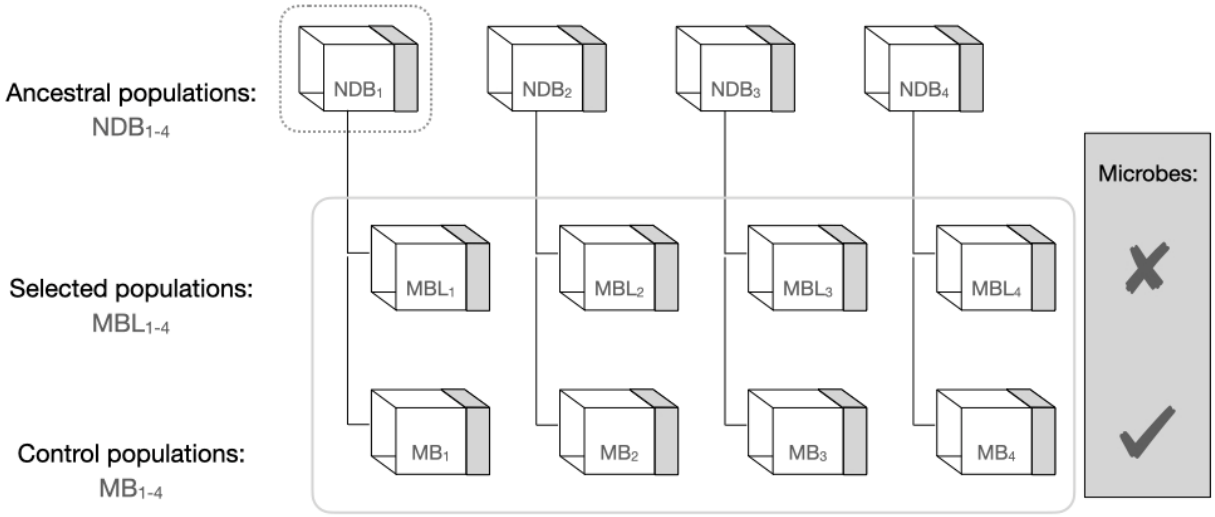


**Figure S1. Schematic showing the design of the study.**

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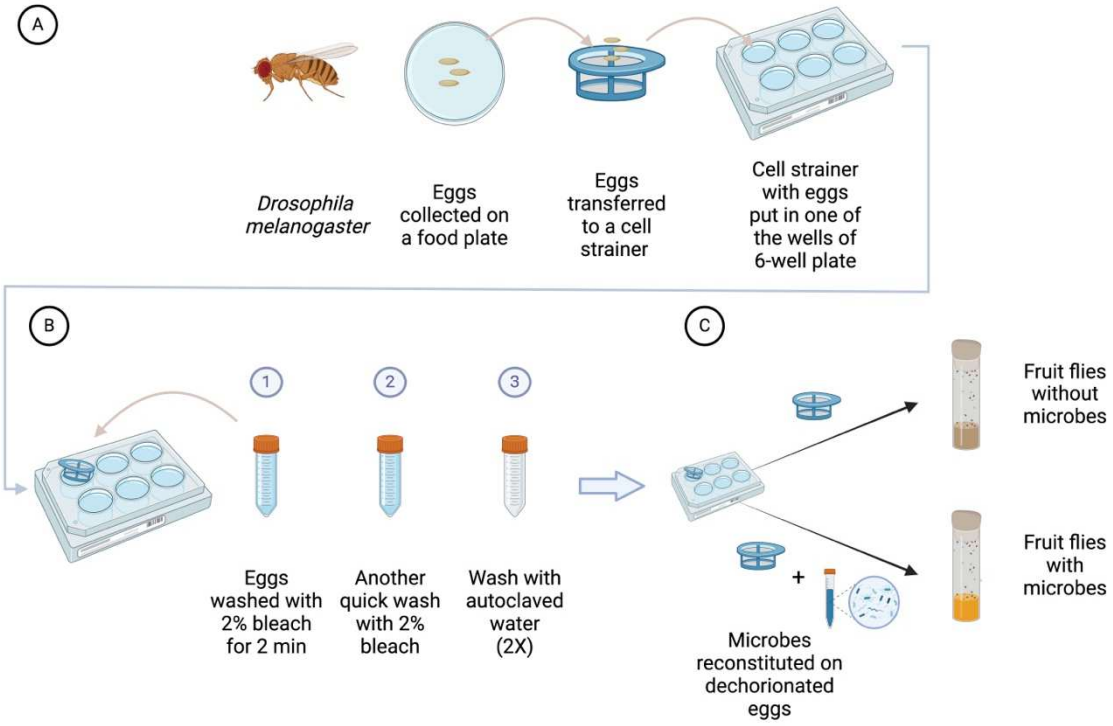


**Figure S2. Details of the egg dechorionation protocol for making $MBL_{1-4}$ and $MB_{1-4}$ populations.** Created in BioRender.com.

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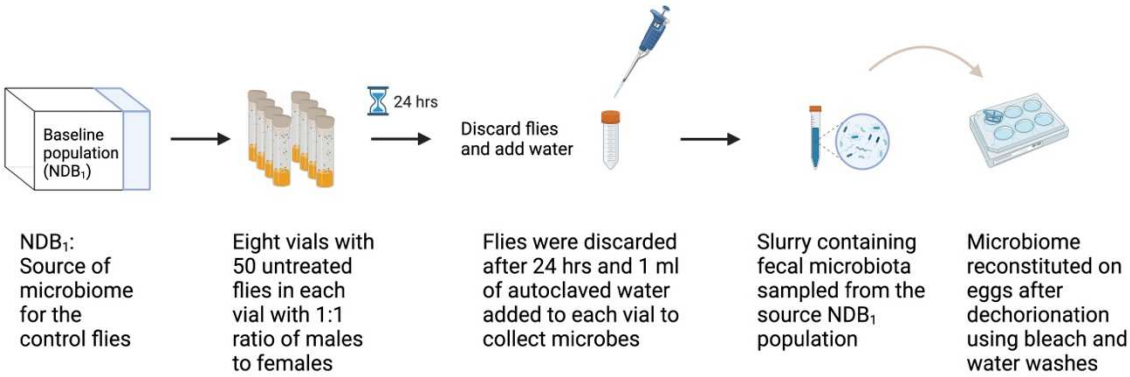


**Figure S3. Schematic of the detailed protocol of microbiome reconstitution from $NDB_1$.** Created in BioRender.com.

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1078 **Supplementary information**
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1080
1081 ## Text S1 Details of the baseline populations and amplicon sequencing
1082

1083 ### S1.1 *Drosophila* NDB stocks and rearing

1084 Wolbachia-free *Drosophila melanogaster* used in the single-generation study were derived from
1085 four large, outbred laboratory populations named New Dey Baselines ($NDB_{1-4}$). These fly lines
1086 were maintained on the standard banana-jaggery food with a 14-day discrete generation cycle at
1087 25°C under constant light conditions with a breeding population of ~2400 individuals. NDBs trace
1088 their ancestry to the DB populations (see Supplementary Text S1.3 for the details), which in turn
1089 trace back to the wild-caught IV lines (Ives, 1970; Rose, 1984) via the $JB_{1-4}$ populations (Sheeba et
1090 al., 1998).

1091 Pairs of MB-MBL populations that have the same subscript are ancestry-matched, and are referred
1092 to as "block" in this document (e.g., $MB_1$ and $MBL_1$ are together called block 1).

1093

1094 ### S1.2 Maintenance regime of baseline *Drosophila* populations ($NDB_{1-4}$)

1095 In the lab, the adults of the four populations ($NDB_{1-4}$) are maintained in plexiglass cages (25 cm x
1096 21 cm x 16 cm) at a large population size of ~2400 per population. On day 12 after egg collection,
1097 the fly population is provided with a moist yeast paste on top of the banana-jaggery food to boost
1098 egg output. This yeast paste also contains a few drops of glacial acetic acid to attract the flies to
1099 the paste. At the end of day 13, a food plate, with vertical edges of food exposed for oviposition, is
1100 provided for 12-16 hrs. On day 14, deposited eggs are collected by cutting thin food strips from the
1101 exposed surface, under a binocular stereo microscope. The size of the strips is adjusted under the
1102 microscope using a clean scalpel such that each strip contains about 400 eggs. These strips are
1103 then transferred to plastic and translucent *Drosophila* milk bottles containing ~50-60 ml of banana-
1104 jaggery food at the bottom. This marks the new generation's 0th day after egg collection. Adult
1105 flies arising from six such bottles are transferred to fresh plexiglass cages on the 12th day (post
1106 egg collection). This gives rise to a new generation with a population size of ~2400 individuals,
1107 which do not coexist with the adults of their parental generation at any stage.

1108

1109 ### S1.3 *Drosophila* DB stocks and rearing

1110 $NDB_{1-4}$ were derived from their most recent ancestors in our lab, Dey Baselines ($DB_{1-4}$) (Sah et al.,
1111 2013). These four DBs were maintained in our lab for 220 generations and were used as baselines
1112 for this period. We pooled an equal proportion of eggs from each DB population and created four
1113 independent NDBs. We maintained NDBs separately for ~10 generations without any assays for
1114 them to stabilize after this intermixing. The maintenance of DBs was similar to NDBs except for two
1115 differences: (a) DBs were maintained in *Drosophila* 8-dram vials at an egg density of ~ 60 per vial
1116 with ~ 6 ml of food as opposed to NDBs that are reared in milk bottles (b) DBs were yeasted on
1117 18th day, provided with a cut plate on 20th day, and their eggs were collected for next generation
1118 on day 21. Thus, DBs followed a 21-day discrete generation cycle as opposed to a 14-day discrete
1119 generation cycle followed by NDBs.

1120

1121 ### S1.4 Population-level 16S rRNA amplicon sequencing

1122 To know the relative composition of bacteria in *Drosophila* populations, DNA was extracted as
1123 described in Supplementary Text S3.2.2. DNA was run on 1% agarose gel to see the extraction
1124 quality. After the integrity check on agarose gel, DNA was sent for 16S rRNA V3-V4 region
1125 amplicon sequencing to a commercial vendor (1st BASE Laboratories, Malaysia). Sample quality
1126 was again confirmed by the vendor using agarose gel, spectrophotometer, and fluorometric
1127 method. All samples passed DNA quality check and were subjected to amplicon PCR followed by a
1128 quality check. These samples were then processed to prepare amplicon libraries using 2-step PCR

according to Illumina's 16S metagenomic library preparation guidelines (Link to the guidelines - https://sapac.support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html). These libraries were processed using Illumina's MiSeq platform using 2x300bp chemistry.

**S1.5 Data analysis of amplicon sequencing**

Sequence adapters and low-quality reads were removed from the paired-end reads before the first 2,00,000 raw reads were extracted using BBTools (https://sourceforge.net/projects/bbmap/). The forward and reverse reads were merged using QIIME (Bolyen et al., 2019). DADA2 pipeline (Callahan et al., 2016) was used to remove low-quality regions and chimeric errors. The resulting data was in the form of amplicon sequence variants (ASVs). The taxonomic classification was done using scikit-learn (Pedregosa et al., 2011) and the Naive Bayes classifier against the SILVA database (release 132). This part of the analysis was performed by the commercial vendor (1st BASE Laboratories, Malaysia).

**S1.6 Details of RNA-seq**

*RNA isolation*

Total RNA was extracted using miRNeasy Mini kit (Qiagen, Catalog No. 1038703) following the manufacturer's protocol. About 30 females were homogenized and lysed in Trizol reagent, followed by chloroform extraction. The aqueous phase was mixed with 1.5x chilled ethanol, and the mix was loaded on the columns provided in the kit. RNA was eluted in 30 µl RNase-free water. The RNA samples were quantitated on a Qubit (Thermo Fisher Scientific) fluorometer. Appropriate dilutions were loaded on a high-sensitivity RNA screen tape to determine the RNA integrity number (RIN).

*Library preparation and sequencing*

1.5 µg of RNA per sample was depleted of fly rRNA using QIAseq FastSelect - rRNA Fly Kit (Qiagen, Catalog No. 333262). The NEBNext Ultra II directional RNA library prep kit (Illumina, Catalog No. E7760L) was used to construct double-stranded cDNA libraries from the rRNA-depleted RNA. The cleaned libraries were quantitated on a Qubit fluorometer, and appropriate dilutions were loaded on a high-sensitivity D1000 screen tape to determine the library size.

These libraries were sequenced on the Illumina NGS platform with 2x150bp chemistry to generate 10 million paired-end reads (3 Gb data per sample).

*RNA-seq analysis*

The quality of the reads was assessed using *FastQC v0.11.9* (Andrews, 2010). These reads were passed through *fastp* to remove adaptors and low-quality bases. The filtered reads were mapped against the *Drosophila melanogaster* BDGP6.46 top-level chromosome (GCA_000001215.4) retrieved from ENSEMBL using *HISAT2* (Kim et al., 2019). The program *featureCounts* (mode = fragment) was used to count mapped reads. The count table from *featureCounts* was given as input to *DESeq2* (Love et al., 2014) to find differentially expressed genes in the populations. Transcripts with counts less than ten were removed from all samples. The false discovery rate (FDR) cut-off was at 0.05, and the log fold change (LFC) threshold was at 1.5.

## Text S2 - Note on the use of F1 flies for the assays

In our flies, we removed microbes via dechorionation at the egg stage. Even if we have taken care of the confounding effect of bleach by using proper control, we wanted to keep the effect of the bleach on flies to a bare minimum (ideally none). To that end, we employed an additional measure of using F1 flies for the assays, as explained below (Supplementary Figure S4).

In this approach, to generate flies without the microbes, we followed a protocol employed by several other studies where F1 flies (progeny of the flies that experienced the bleach at the egg stage) were used in the assays (H.-Y. Lee et al., 2019; Pais et al., 2018; Selkrig et al., 2018). This ensured that the F1 assay flies of both types, microbiome-reconstituted (M+) and microbe-free (M-), did not experience bleach in their lifetime (their respective parent population did). To generate F1 M- assay flies, F1 eggs were collected from the flies without microbes (i.e., F0 M- flies, Supplementary Figure S4) and were raised in aseptic conditions, ensuring the absence of microbes. To generate control F1 M+ assay flies, F1 eggs were collected without any manipulation from the flies whose microbiomes were reconstituted (i.e., F0 M+ flies, Supplementary Figure S4).

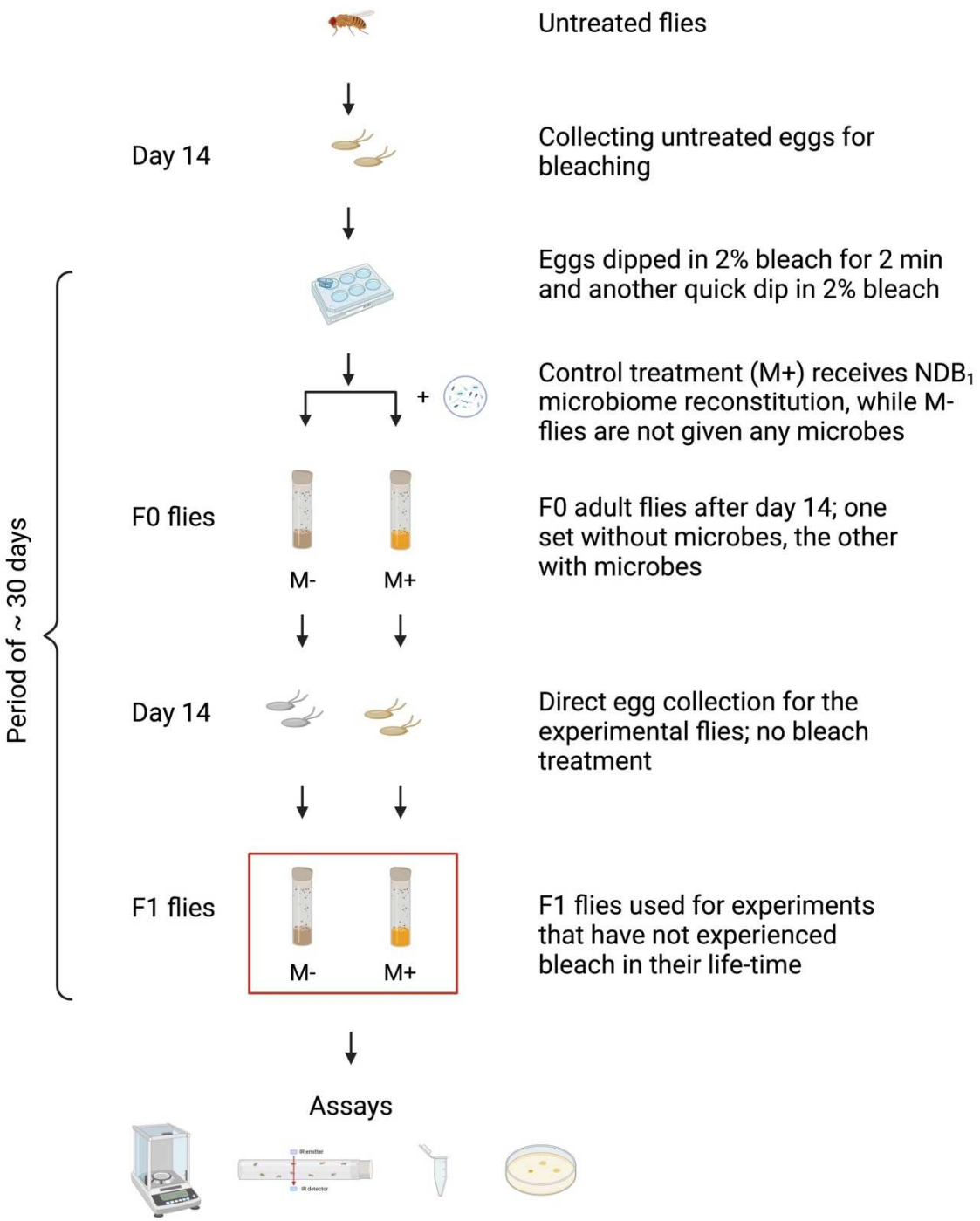


**Figure S4. Schematic of the full experimental design to study microbiomes' contribution to host phenotypes.** To create two types of flies (with the minimal effect of bleach), flies with microbes (M+) and without them (M-), we combined two procedures. First, we chose microbiome-reconstituted flies as a control instead of using untreated flies. The F0 flies of both (M+ and M-) types were generated using bleach at the egg stage. This protocol is illustrated in detail in Figure S2. Second, as discussed in Supplementary Text S2, assays were conducted on progeny of these F0 flies (i.e., F1 flies) that have not experienced bleach in their lifetime to avoid any recency effect of bleach. The eggs collected for F1 experimental flies either had the microbes (M+) or lacked the microbes (M-) depending on the state of their parent's microbiome. Created in BioRender.com.

1187

1188 **Text S3.1 - Assessing the microbiome status of MBs and MBLs during experimental**
1189 **evolution**
1190
1191 We plated adult flies from $MBL_{1-4}$ every generation to see if bacteria are indeed absent in these
1192 populations (data not shown here). Except for generations and blocks specified in Supplementary
1193 Table S1, in all other generations, MBLs did not show the presence of a microbiome. We
1194 sometimes did observe ~1-2 colonies (per 10 flies plated) in some replicates.
1195
1196 **Table S1. Generations in which MBLs showed transient contamination**
1197

| Generation | Affected MBL blocks |
|---|---|
| 37 | Block 1 and 3 |
| 38 | Block 1 |
| 39 | Block 1 |

1198
1199 $MB_{1-4}$ were plated every 2-3 generations and CFUs were counted to make sure they received the
1200 reconstituted microbiome (data not shown here).
1201
1202 To confirm that our protocol indeed removed both culturable and unculturable microbes, we
1203 performed PCR with 16S rRNA universal primers (Supplementary Text S3.2.2) on DNA extracted
1204 from adult $MB_{1-4}$ and $MBL_{1-4}$ females (day 15 after egg collection) in generation 40. In
1205 Supplementary Figure S5, we see that $MB_{1-4}$ show the presence of the reconstituted microbiome,
1206 and the microbiome is absent from $MBL_{1-4}$. This also confirms that, by generation 40, the
1207 contamination that we saw in MBLs in previous generations was completely gone.
1208

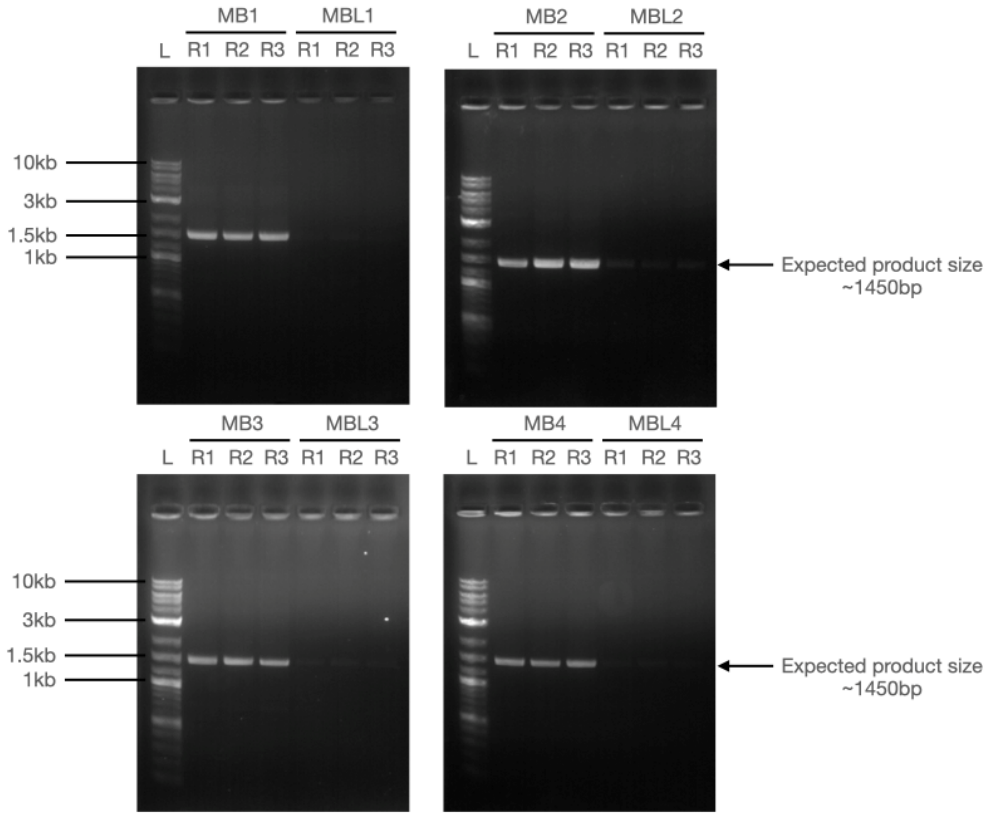


**Figure S5. 16S rRNA PCR to check the status of microbiome in $MB_{1-4}$ and $MBL_{1-4}$.**
1209
1210
1211

1212 **Text S3.2 Fly-associated bacterial load analysis**

1213 **S3.2.1. Culture-dependent method**

1214 Adult flies (day 12 after egg collection, unless stated otherwise) were sorted by sex under mild
1215 $CO_2$ anesthesia. Five male and five female flies under anesthesia were surface sterilized with 70%
1216 ethanol in 1 ml microcentrifuge tubes. These flies were then homogenized in 200 µl of autoclaved
1217 $dH_2O$ and plated on the nutrient agar (HiMedia) or the de Man, Rogosa, and Sharpe (MRS)
1218 medium (HiMedia) and incubated for 72 hrs and 48 hrs, respectively, at $25^0$C under aerobic
1219 conditions. Dilutions with 10-100 colonies were counted, and the counts were multiplied by the
1220 overall dilution factor to estimate colony-forming units (CFUs) per fly.

1221 **S3.2.2. Culture-independent method**

1222 To test the bacterial load using PCR, total DNA was extracted from eight to ten adult flies using the
1223 QIAamp DNA Mini kit (Qiagen, Catalogue No. 51304) as per the manufacturer's instructions. We
1224 modified three steps of the manufacturer's protocol in line with the protocol from an earlier study
1225 (Jehrke et al., 2018). First, we used mechanical homogenization (with a homemade handheld
1226 motorized pestle mixer) to lyse the tissue in 1.5 ml Eppendorf tubes instead of just Proteinase-K
1227 treatment. Second, 20 µl of 20 mg/ml RNase A (HiMedia, Catalogue No. DS0003-5ML) was added
1228 to each sample after tissue lysis. Third, the sample was incubated with "Buffer AL" at $70^0$C for 30
1229 min instead of 10 min. We avoided any vortex step after tissue lysis to minimize the shearing of the
1230 DNA. The quality of total DNA was checked using agarose gel electrophoresis. This DNA,
1231 composed of the fly and microbial genomes, was used as a template for PCR using 16S bacterial
1232 universal primers with a product size of about 1.5 kb. The details of the primers are given below in
1233 Supplementary Table S2. The primers were ordered from Sigma-Aldrich, India. The PCR reactions
1234 were set up using GoTaq® Flexi DNA Polymerase (Promega, Catalogue No. M829) with 40 ng
1235 gDNA input. The initial denaturation was at $94^0$C for 2 min, followed by 30 cycles of (denaturation
1236 at $94^0$C for 30s, annealing at $T_m = 60^0$C for 1 min, extension at $72^0$C for 1 min), with final extension
1237 $72^0$C for 10 min. The PCR products were visualized on 1% agarose gel with 1kb plus DNA ladder
1238 (NEB, Catalogue No. N3200S) as the molecular weight marker.

1239 **Table S2. 16S bacterial universal primers used for PCR**

| Primer | Sequence | Label | Reference |
|---|---|---|---|
| Forward | AGAGTTTGATCCTGGCTCAG | fD1 | (Weisburg et al., 1991) |
| Reverse | ACGGCTACCTTGTTACGACTT | rP2 | (Weisburg et al., 1991) |

1240
1241

**Text S4.1 – First phenotypic assessment of experimental evolution lines at host generations 17-20**

After 17 generations of selection, we looked at various host traits to see if the experimental evolution without the microbes has led to any divergence in MBLs vis-à-vis the MBs. This assessment was done under two assay environments: (1) with-microbes and (2) microbe-free.

In this design, logistically, it was not possible to assay all four blocks of MB and MBL together. Hence, we assayed them in sets of two blocks, namely 1 - 2 and 3 - 4. Supplementary Table S3 gives details of the generations in which the assays took place. MB and MBL populations in each block were always assayed together in the same generation to know the effect of the selection procedure (e.g., $MB_1$ compared with $MBL_1$). For all assays done in this set, flies for separate environments (i.e., with-microbes and microbe-free) were generated in different host generations (Supplementary Table S3). As the two different environments were not assayed in the same host generation, we do not discuss the effect of the environment on these populations. We only discuss the effect of selection in this first set of assays.

**Table S3. Details of the assay environments and host populations for Gen 17-20 assays**

| No. | Assay environment | Block | Host generation |
|---|---|---|---|
| 1 | With-microbes | 3 and 4 | 17 |
| | | 1 and 2 | 18 |
| 2 | Microbe-free | 1 and 2 | 19 |
| | | 3 and 4 | 20 |

The validation of the microbiome states of flies raised in with-microbes and microbe-free assay environments is reported in Supplementary section S5.1.

**Text S4.2 - Second phenotypic assessment of experimental evolution lines at host generations 54-57**

In the first assessment of $MBL_{1-4}$ vs. $MB_{1-4}$ that happened in generations 17 to 20 (Supplementary Text S4.1), we noticed that the assay environments (with-microbes and microbes-free) had a greater effect in determining the trait-values in both sets of populations than the selection regime (Figures S11-S13). However, since the with-microbes and microbe-free flies were generated in separate host generations (see Supplementary Table S3), we could not compare the relative effects of assay environment vs. selection quantitatively with this design. Therefore, during the next set of assays during generation 54-57, we amended our experimental design by conducting the assays in the two environments simultaneously. This allowed us to directly compare the contributions of selection vs. assay environment. Supplementary Table S4 shows the details of our experimental protocol for the set of assays conducted during generations 54 to 57. This amendment meant, due to logistical reasons, we could only assess one block at a time in the same host generation (in contrast to the Gen 17-20 assays where two blocks were assessed in the same host generation).

This experimental design allows us to make two kinds of comparisons: (1) the effect of experimental evolution in a common-garden assay environment obtained by directly comparing MBs vs. MBLs (Main text, Figures 2 to 4) and (2) the effect of the presence vs. absence of microbes on both MB and MBL populations (Main text, Figure 5)

**Table S4. Details of the assay environments and host populations for Gen 54-57 assays**

| No. | Common-garden environment | Block | Host generation |
|---|---|---|---|
| 1 | With-microbes | 1 | 54 |
| | | 2 | 55* |
| | | 3 | 56 |
| | | 4 | 57 |
| 2 | Microbe-free | 1 | 54 |
| | | 2 | 55* |
| | | 3 | 56 |
| | | 4 | 57 |

*For block 2, body weight and female fecundity were assessed in Gen 59

**Text S5.1 - Validation of microbiome status in the flies raised in two different assay environments**

**S5.1.1 By plating fly homogenates**

In the with-microbe environment, on both MRS and NA media, we see the presence of microbes (Supplementary Figure S6, Set-B and Set-C). Set-A is the autoclaved water that is used to homogenize the flies. The serial dilutions with ~10-100 colonies were counted to get the CFUs/fly. While Supplementary Figure S6A shows a representative picture from block 1, CFUs/fly for all four blocks of MBs and MBLs are compared using paired t-test on block means in Figure S7.
In the microbe-free environment, both $MB_1$ (Supplementary Figure S6B) and $MBL_1$ (Supplementary Figure S6C) show the absence of the microbiome. While block 1 plates do not have any microbes, other blocks show a few colonies in some replicates (data not shown here).

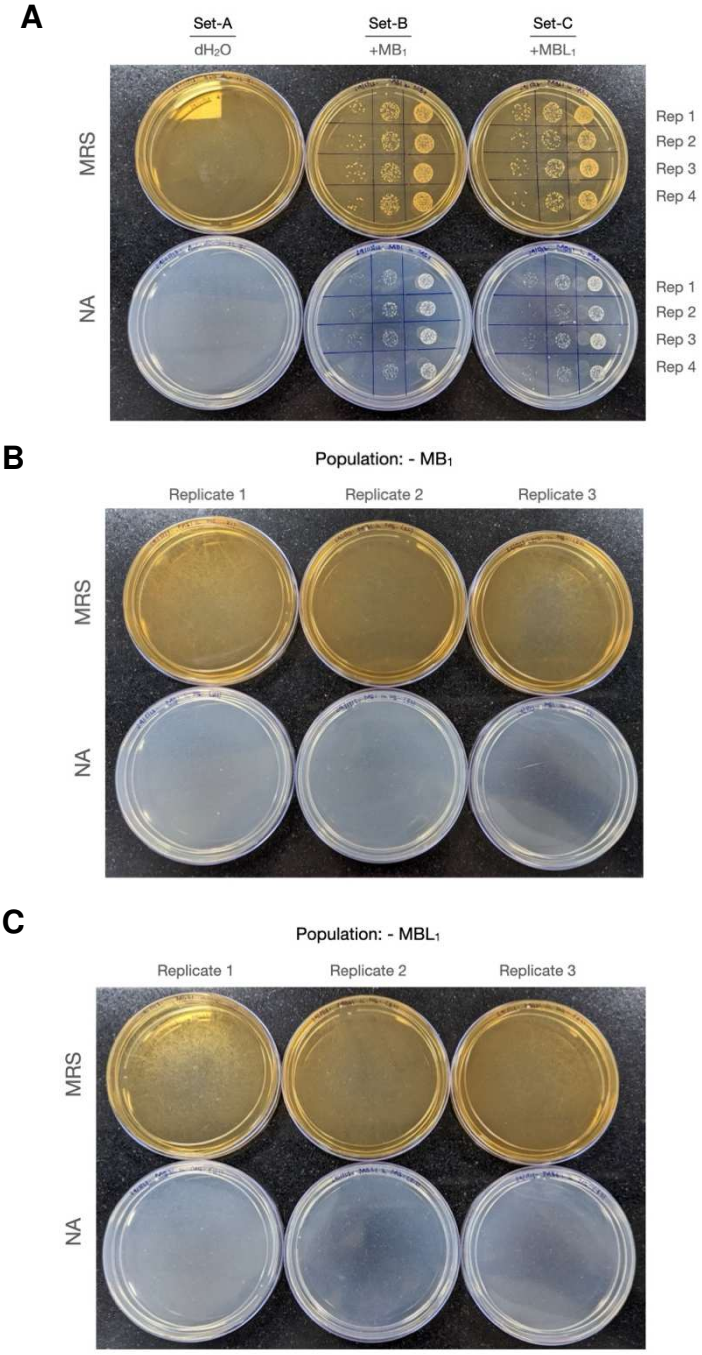


**Figure S6. MRS and NA plates showing presence of microbes in with-microbe environment and their absence in microbe-free environment.** (A) $MB_1$ and $MBL_1$ in with-microbe environment (n = 4). The spots show different serial dilutions used to calculate CFUs/fly.

These CFU/fly for all the replicate populations are shown in Supplementary Figure S7. (B) $MB_1$ microbe-free environment (n = 3). (C) $MBL_1$ microbe-free environment (n = 3).

**S5.1.2 MBs and MBLs received similar microbial loads in the with-microbe environment (Gen 54-57 assays)**

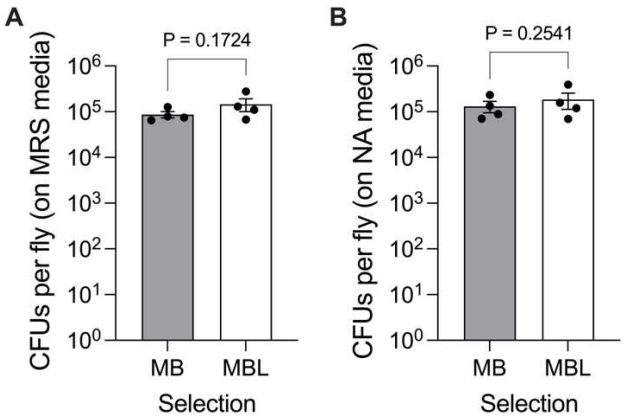


**Figure S7. Bacterial load in the $MB_{1-4}$-$MBL_{1-4}$ flies (with-microbe environment) as determined by plating fly homogenates.** CFUs/fly on (A) MRS-agar (B) NA-agar. The bacterial load in MBs vs. MBLs is compared using paired t-test on block means. Block means are indicated by black filled circles. The error bar represents SEM. We see that the process of microbiome reconstitution leads to MBs and MBLs carrying similar bacterial loads.

**S5.1.3 PCR showing the presence of microbiome in the with-microbiome environment and depletion of the microbiome in microbe-free environment**

To validate the status of the microbiome in both environments with a culture-independent method, we extracted DNA from the four types of populations (Main text, Figure 1B). We performed PCR with 16S rRNA universal primers (details in Supplementary Text S3.2). For PCR, a subset of flies used for the assay on day 12 were flash-frozen in liquid nitrogen and kept at -80$^0$C for further use. For the with-microbe environment, 12 female flies from only $MB_1$ and $MBL_1$ (block 1) were used for the DNA extraction. For the microbe-free environment, total DNA was extracted from 12 female flies (3 flies per block, four such blocks). The PCR was done with 40ng of input DNA with 30 cycles.

In the with-microbe environment, $MB_1$ and $MBL_1$ showed the presence of microbes as expected (Supplementary Figure S8). In the microbe-free environment, while two replicates have no band, one replicate each from pooled $MB_{1-4}$ and $MBL_{1-4}$ contains a faint band. This faint band might be indicative of a few colonies that show up on NA and MRS-agar plates, even after aseptic handling (data not shown). This signal might get picked up due to saturating PCR conditions. As we are not bleaching assay flies or using any antibiotics to avoid their toxic effects, we are essentially maintaining flies without any chemicals for about two consecutive generations, i.e., for ~30 days (Supplementary Figure S4). So, there might be a tradeoff between the efficiency of keeping all the microbes away without any chemicals and how long that efficiency can be sustained without any chemical agent. Another possibility is that the signal is from very few non-culturable bacteria.

These results match the observations in Supplementary Text S5.1.1 obtained after plating flies on NA and MRS. Together, the evidence showed that both MB-MBL populations in a with-microbe environment harbor microbiome and in microbe-free environments have extremely few to no microbes.

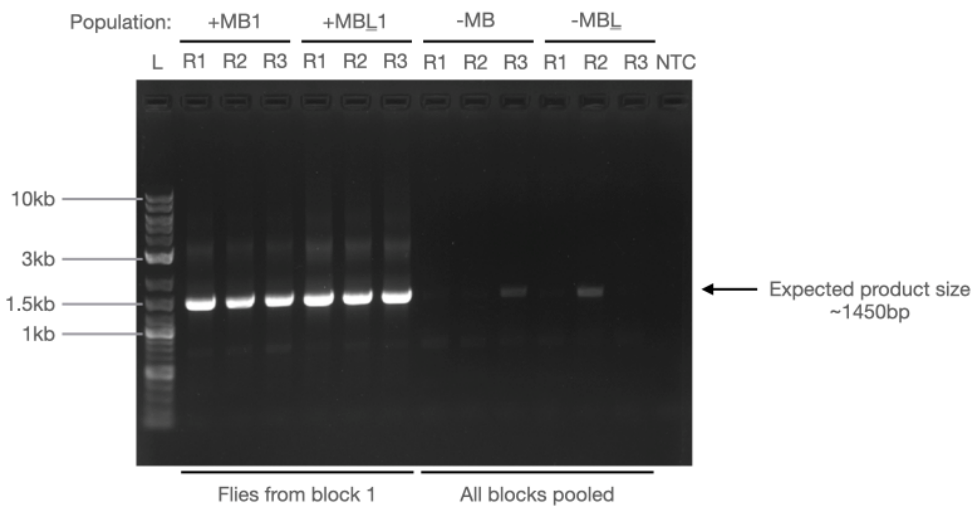


**Figure S8. PCR on $MB_1$ and $MBL_{1-4}$ to confirm the status of microbiome.**

## S5.2 Validation of microbiome composition of flies raised in with-microbes assay environment

### S5.2.1 MB-MBL generation 17-20 assays

In the previous section, through plating fly homogenates and PCR, we showed that flies raised in the with-microbes environment indeed have the microbes with them. However, the PCR only indicates that microbes are present - it does not tell us the composition of the reconstituted microbiome. In Supplementary Figure S7, we have established that the number of microbes received by MB and MBL populations is similar. In this section, we test if the reconstituted microbiome composition is similar for MB-MBLs, and this, in turn, matches the source microbiome composition of $NDB_1$.

To validate that the composition of the reconstituted microbiome of all four blocks ($MB_{1-4}$ and $MBL_{1-4}$) reflects the native $NDB_1$ composition, we performed amplicon sequencing on the V3-V4 region of the 16S rRNA gene (Supplementary Figure S9, method details in Supplementary Text S1.4). Three biological replicates were processed per population, giving us 24 samples (8 populations: 4 MBs + 4 MBLs, 3 replicates each). The native source pool of $NDB_1$ (at Gen 56) was also sequenced. Thus, Supplementary Figure S9 shows a total of 27 samples (24 from $MB_{1-4}$-$MBL_{1-4}$ and 3 from $NDB_1$).

The $MB_{1-4}$-$MBL_{1-4}$ populations also mirror this broad microbiome composition of $NDB_1$ with the dominance of *Acetobacter* spp. but with small fluctuations across MB-MBLs in *Acetobacter* and *Lactobacillus* fractions (Supplementary Figure S9). (In 2020, the old genus *Lactobacillus* was reclassified into 25 new different genera. *Lactiplantibacilus* and *Levilactobacilus* both belong to the old composite *Lactobacillus* genus.)

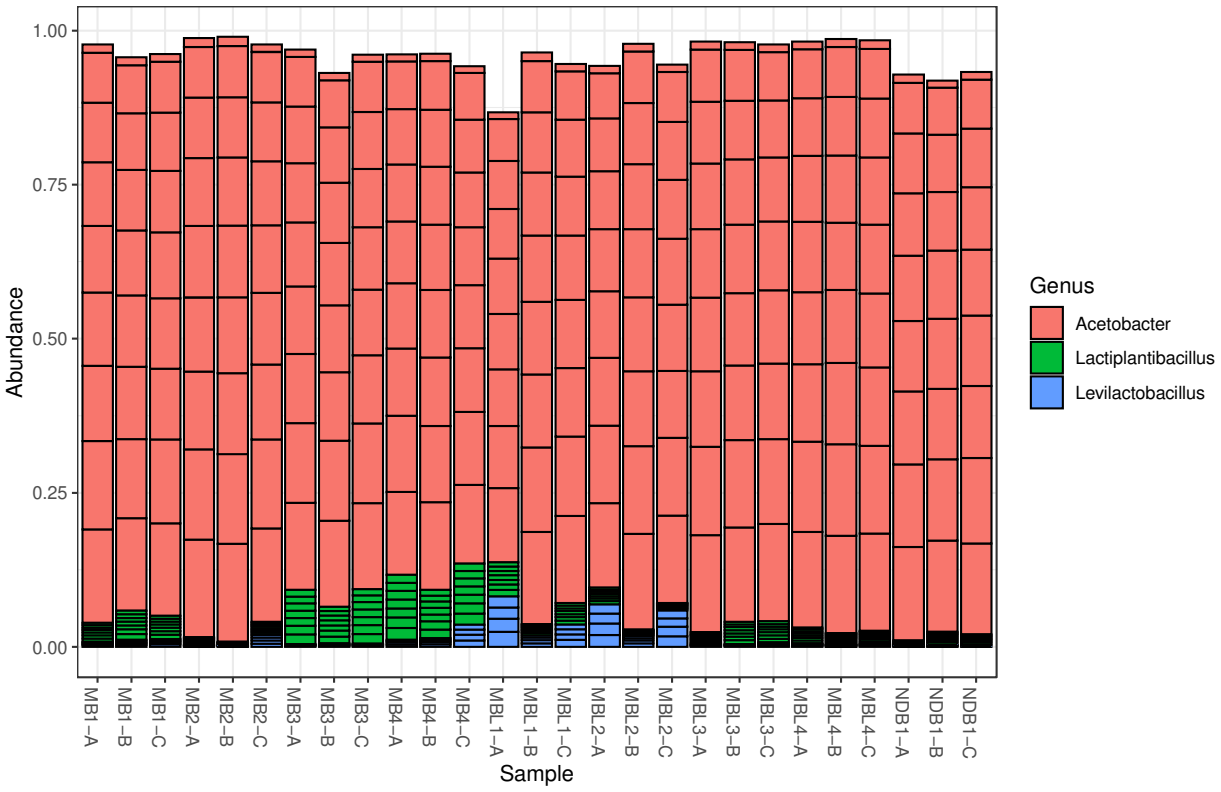


**Figure S9. Validation of microbiome composition after reconstitution in Gen 17-20 assays.** The result of population-level 16S rRNA amplicon sequencing across four replicate populations of $MB_{1-4}$ and four replicate populations of $MBL_{1-4}$. Native microbiome composition of source population $NDB_1$ is also given for the reference (last three columns). Most abundant 20 taxa are shown for each population. For each population, three independent replicates where processed (labelled “A”, “B”, “C”). Each replicate consisted of pooled DNA extracted from surface sterilized full body homogenates of 8-10 females. The sequencing method and sequencing data analysis are described in Supplementary Text S1.4 and S1.5 respectively.

1396
1397
1398 **S5.2.2 MB-MBL generation 54-57 assays**
1399
1400 In generation 54-57 assays, we sequenced microbiomes from $MB_1$ and $MBL_1$ that were raised in
1401 the with-microbes environments (Supplementary Figure S10). Together, the sequencing results for
1402 generations 17-20 and for generations 54-57 show that microbiome compositions of MBs and
1403 MBLs are broadly similar to each other and to the source microbiome of $NDB_1$ (Supplementary
1404 Figure S9-last three lanes).
1405

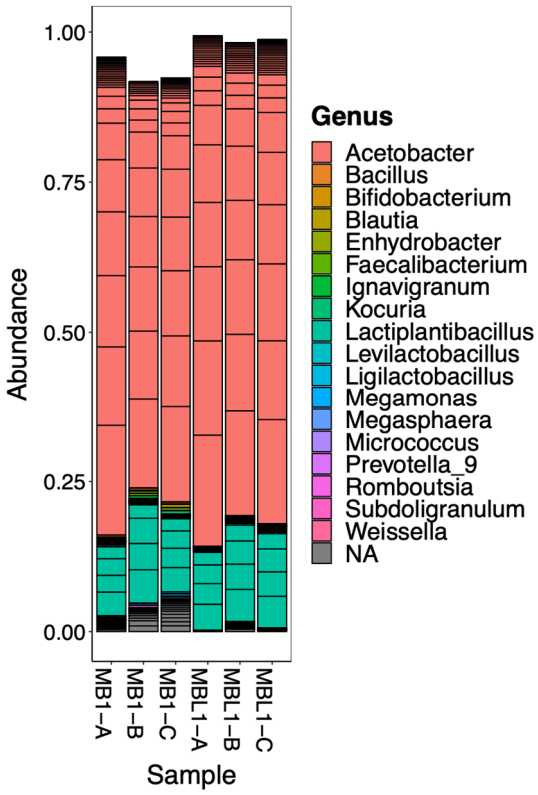


**Figure S10. Validation of microbiome composition after reconstitution in Gen 54-57 assays.** Most abundant 100 taxa are shown for each population. For each population, three independent replicates where processed (labelled “A”, “B”, “C”). Each replicate consisted of pooled DNA extracted from surface sterilized full body homogenates of 8-10 females. The sequencing method and sequencing data analysis are described in Supplementary Text S1.4 and S1.5 respectively.

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1410 **Text S6 - First phenotypic assessment of experimental evolution lines at host generations**
1411 **17-20**
1412
1413 **S6.1 Male traits in host generations 17-20**
1414
1415 None of the four phenotypes tested for males – adult body weight, egg-to-adult development time,
1416 desiccation resistance, and locomotor activity- showed an effect of selection as assessed by the
1417 paired t-test over block means (Supplementary Figure S11A-D). Supplementary Tables S5 and S6
1418 provide details of the statistical comparison for the with-microbe assay environment and microbe-
1419 free assay environment, respectively.
1420

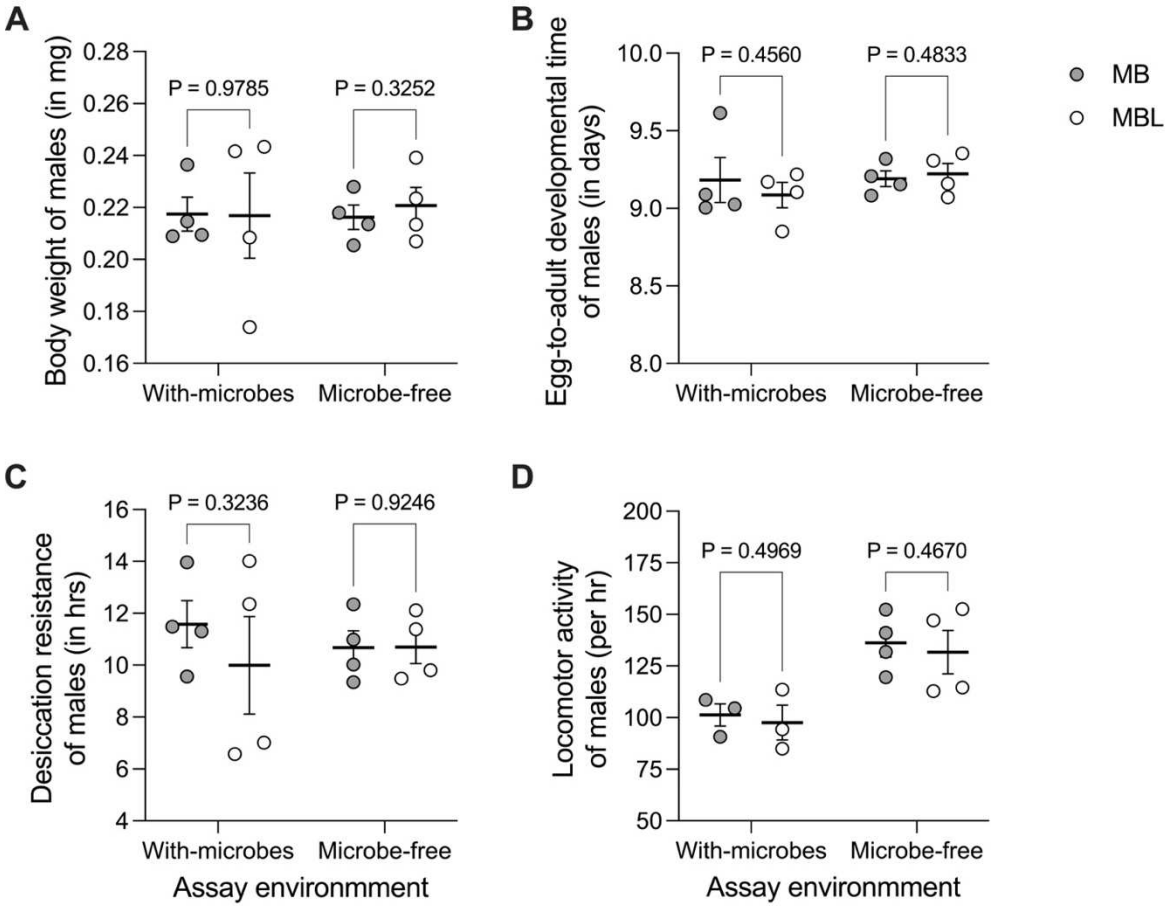


**Figure S11. Male traits assessed in Gen 17-20 in with-microbe and microbe-free environments.** (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations $MB_{1-4}$ and, similarly, four empty circles are means for each of the four selected populations $MBL_{1-4}$. The black horizontal lines represent the grand means over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over $MB_{1-4}$ was compared against the grand mean over $MBL_{1-4}$ using a paired t-test. DAM data for block 4 in with-microbes environment is not included here in the analysis.

1421
1422
1423
1424 **S6.2 Female traits in host generations 17-20**
1425
1426 In the case of females, egg-to-adult development time, desiccation resistance, and locomotor
1427 activity didn't differ between MBs and MBLs (Supplementary Figure S12A-D) (Supplementary
1428 Tables S5 and S6). The only trait in females that showed some difference between the MBs and
1429 the MBLs was female body weight in the microbe-free environment (Paired t-test, $t_3$ = 3.05, P =

0.0554, Cohen's d = 0.81 (large), Supplementary Figure S12A). Here, although the p-value is slightly larger than our pre-determined level of statistical significance (i.e., 0.05), we still interpret this trait as the effect size was found to be large.

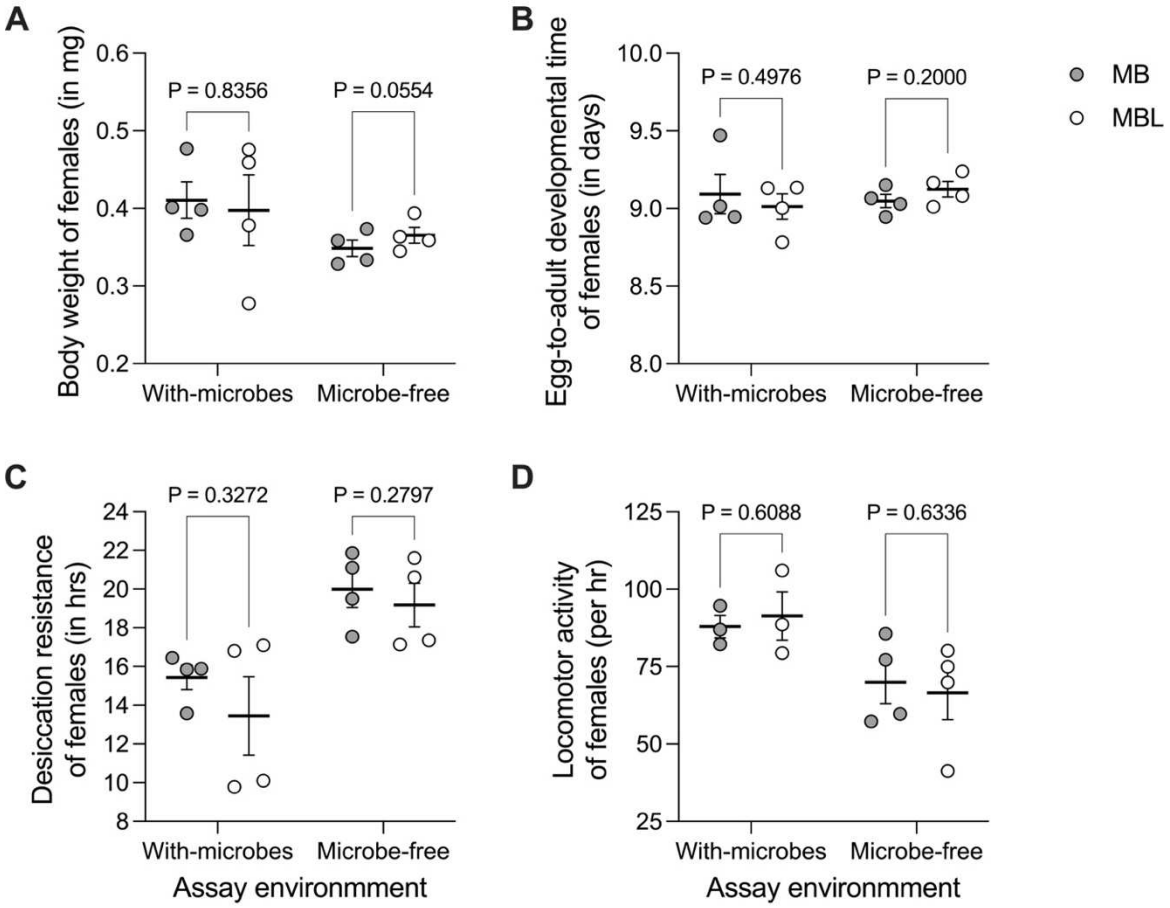


**Figure S12. Female traits assessed in Gen 17-20 in with-microbe and microbe-free environments.** (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations $MB_{1-4}$ and similarly, four empty circles are means for each of the four selected populations $MBL_{1-4}$. The black horizontal lines represent the grand means over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over $MB_{1-4}$ was compared against the grand mean over $MBL_{1-4}$ using a paired t-test. DAM data for block 4 in with-microbes environment is not included here in the analysis.

1435 **S6.3 Pair traits in host generations 17-20**
1436
1437 In traits where both males and females were involved in the assays - percent survival to adulthood,
1438 female fecundity over 12 hrs (assayed in the presence of a male), and mating latency - did not
1439 show any difference (Supplementary Figure S13A-C, Supplementary Tables S5 and S6). The
1440 mating time for $MBL_{1-4}$ was lower than $MB_{1-4}$ in the with-microbes environment (Paired t-test, $t_3$ =
1441 3.67, P = 0.0349, Cohen's d = 2.35 (large), Supplementary Figure S13D).
1442

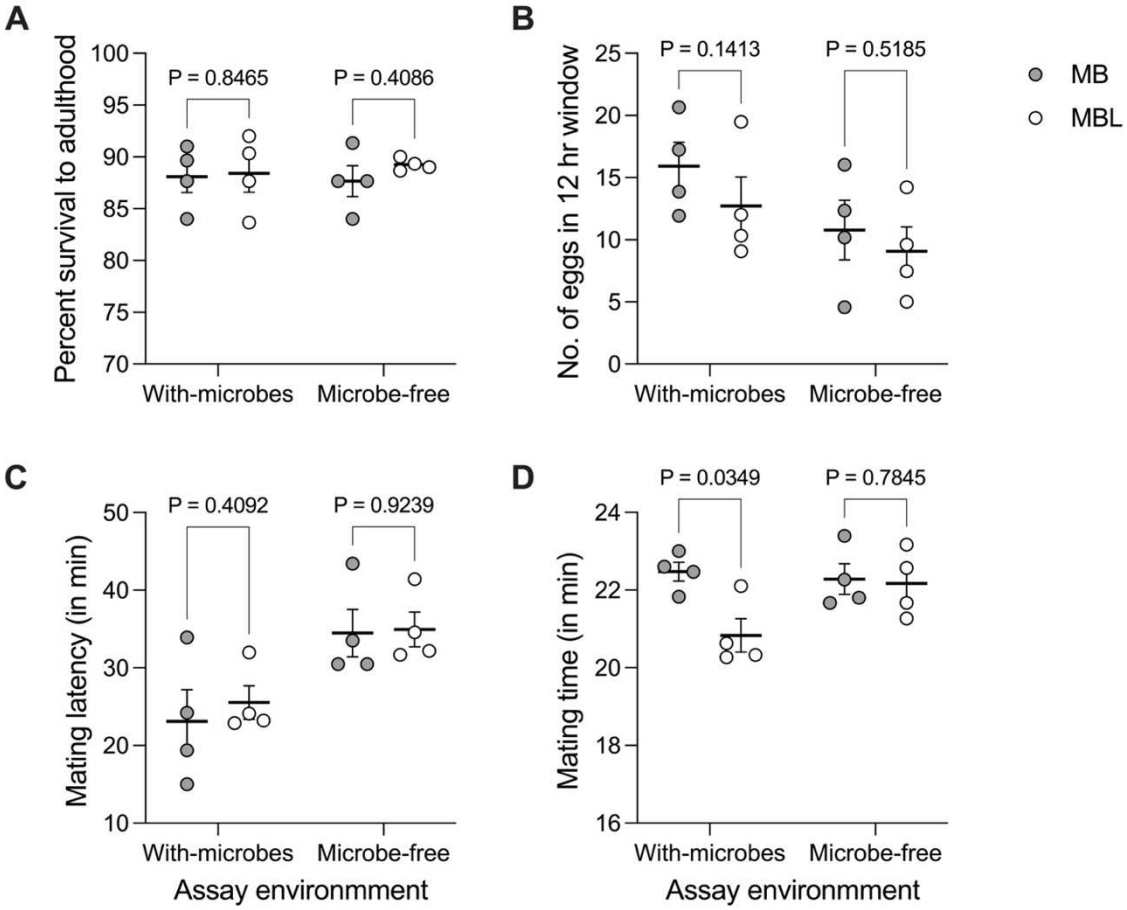


**Figure S13. Pair traits assessed in Gen 17-20 in with-microbe and microbe-free environments.** (A) Survival to adulthood. (B) Female fecundity. (C) Mating latency. (D) Mating time. In each assay environment, four filled circles are means for each of the four control populations $MB_{1-4}$ and similarly, four empty circles are means for each of the four selected populations $MBL_{1-4}$. The black horizontal lines represent the grand means over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over $MB_{1-4}$ was compared against the grand mean over $MBL_{1-4}$ using a paired t-test.

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1446 **Table S5.** Summary of all assays in the with-microbes environment (Gen 17-20). T refers to the
1447 grand mean of the MBLs, while C refers to the grand mean of the MBs.
1448

| | Assay | Test statistic | P-value | % change (T - C)/C | Cohen's d | Effect size* | Sample size$ |
|---|---|---|---|---|---|---|---|
| 1 | Dry body weight (Male) | $t_3 = 0.03$ | 0.9785 | -0.3 | 0.02 | Small | 10 |
| 2 | Dry body weight (Female) | $t_3 = 0.23$ | 0.8356 | -3.1 | 0.18 | Small | 10 |
| 3 | Development time (Males) | $t_3 = 0.85$ | 0.4560 | -1.1 | 0.41 | Small | 10 |
| 4 | Development time (Females) | $t_3 = 0.77$ | 0.4976 | -0.9 | 0.37 | Small | 10 |
| 5 | Survival to adulthood | $t_3 = 0.21$ | 0.8465 | 0.4 | 0.10 | Small | 10 |
| 6 | Fecundity (Females) | $t_3 = 1.96$ | 0.1413 | -20.1 | 0.75 | Medium | 50 |
| 7 | Desiccation resistance (Males) | $t_3 = 1.18$ | 0.3236 | -13.7 | 0.54 | Medium | 10 |
| 8 | Desiccation resistance (Females) | $t_3 = 1.17$ | 0.3272 | -12.9 | 0.66 | Medium | 10 |
| 9 | Locomotor activity (Males) | $t_3 = 0.82$ | 0.4969 | -3.7 | 0.30 | Small | 25 to 32 |
| 10 | Locomotor activity (Females) | $t_3 = 0.60$ | 0.6088 | 3.9 | 0.32 | Small | 31 to 32 |
| 11 | Mating Latency | $t_3 = 0.96$ | 0.4092 | 10.4 | 0.37 | Small | 30 |
| 12 | Mating time | $t_3 = 3.67$ | 0.0349 | -7.3 | 2.35 | Large | 30 |

1449
1450 **Table S6.** Summary of all assays in the microbe-free environment (Gen 17-20). T refers to the
1451 grand mean of the MBLs, while C refers to the grand mean of the MBs.
1452

| | Assay | Test statistic | P-value | % change (T - C)/C | Cohen's d | Effect size* | Sample size$ |
|---|---|---|---|---|---|---|---|
| 1 | Dry body weight (Male) | $t_3 = 1.17$ | 0.3252 | 2.1 | 0.38 | Small | 10 |
| 2 | Dry body weight (Female) | $t_3 = 3.05$ | 0.0554 | 4.8 | 0.81 | Large | 10 |
| 3 | Development time (Males) | $t_3 = 0.80$ | 0.4833 | 0.4 | 0.28 | Small | 10 |
| 4 | Development time (Females) | $t_3 = 1.64$ | 0.2000 | 0.8 | 0.82 | Large | 10 |
| 5 | Survival to adulthood | $t_3 = 0.96$ | 0.4086 | 1.8 | 0.73 | Medium | 9 to 10 |
| 6 | Fecundity (Females) | $t_3 = 0.73$ | 0.5185 | -15.9 | 0.39 | Small | 50 |
| 7 | Desiccation resistance (Males) | $t_3 = 0.10$ | 0.9246 | 0.2 | 0.02 | Small | 10 |
| 8 | Desiccation resistance (Females) | $t_3 = 1.32$ | 0.2797 | -4.1 | 0.39 | Small | 10 |
| 9 | Locomotor activity (Males) | $t_3 = 0.83$ | 0.4670 | -3.3 | 0.25 | Small | 26 to 32 |
| 10 | Locomotor activity (Females) | $t_3 = 0.53$ | 0.6336 | -4.8 | 0.22 | Small | 30 to 32 |
| 11 | Mating Latency | $t_3 = 0.10$ | 0.9239 | 1.4 | 0.09 | Small | 30 |
| 12 | Mating time | $t_3 = 0.30$ | 0.7845 | -0.5 | 0.14 | Small | 30 |

1453

*Interpretation for effect sizes using Cohen's d: d > 0.8 (Large); 0.8 > d > 0.5 (Medium); < 0.5 (Small)
$Note: See methods for detailed information about sample sizes. For example, the sample size of ten per treatment for body weight indicates ten micro-centrifuge tubes with 20 flies each. Also, this is a sample size per block, i.e., each circle in the plots is an average over this sample size.

1462 **Text S7 – RNA-Seq result details**
1463
1464 **S7.1 No major changes in the gene expression profile of $MB_1$ vs $MBL_1$ in the microbe-free**
1465 **environment**
1466
1467 In the microbe-free regime, where both selected and control populations were assayed without
1468 their microbiome, only a few transcripts showed differential expression when analyzed using
1469 *DESeq2* (Supplementary Figure S14). Only the significant genes with $Log_2$(Fold Change) > 1.5 and
1470 adjusted P-value < 0.05 are highlighted in Figure S14. Except for nAChRbeta2, which is a nicotinic
1471 acetylcholine receptor subunit, and CG13617, which codes for cilium assembly protein DZIP1 (zinc
1472 finger transcription factor), the functions of the other genes (CG14625, CG12693) are not
1473 annotated in *Drosophila melanogaster*.
1474

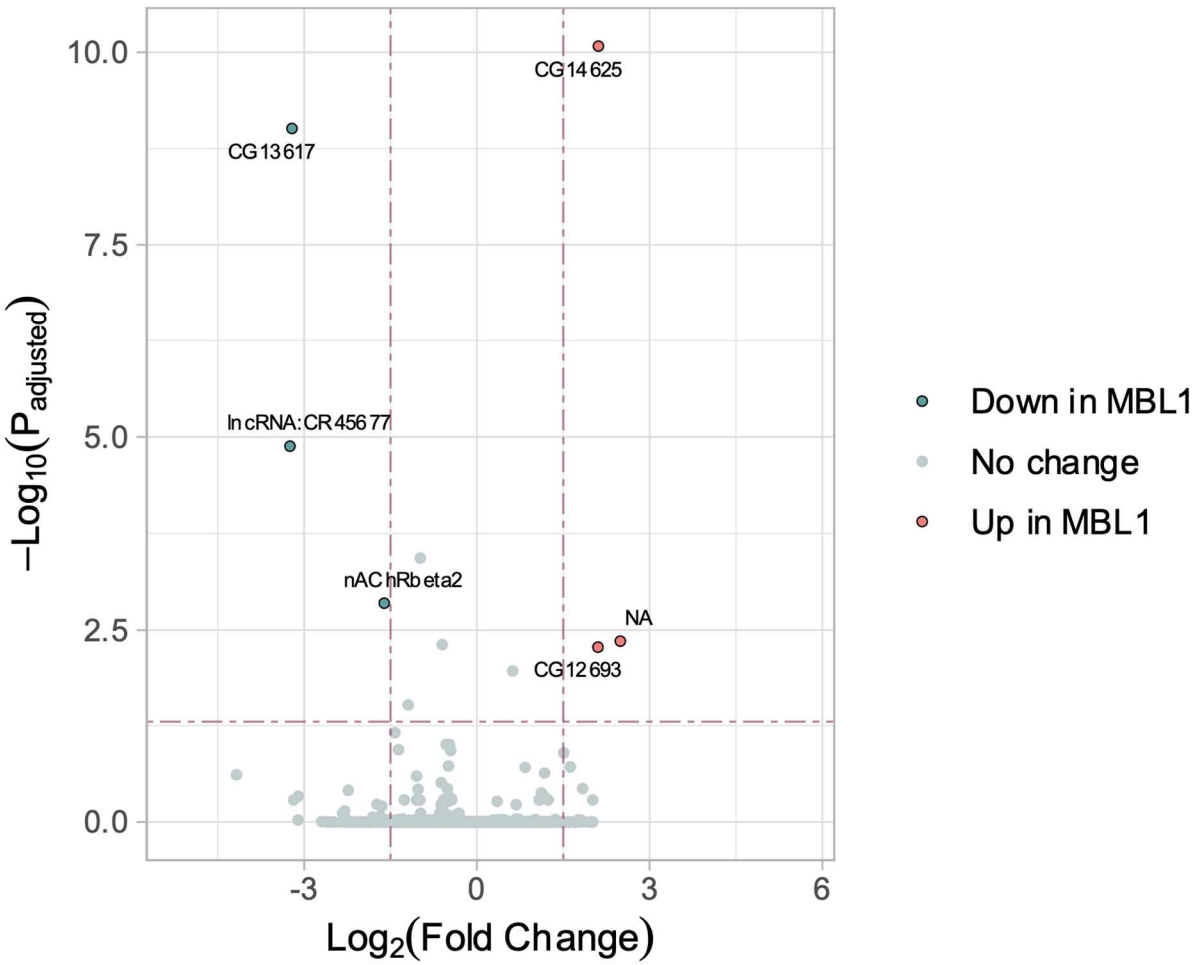


**Figure S14. Differentially expressed genes in $MBL_1$ vs $MB_1$ in the no-microbe environment using DESeq2.** The red dots represent the up-regulated genes, while blue dots represent down-regulated genes in $MBL_1$. The protocol for the RNA-Seq is described in the method section of the main text.
1475

1476 **S7.2 List of differentially regulated genes in with-microbe assay environment**
1477
1478 **Table S7. List of up-regulated genes in $MBL_1$ in RNA-Seq (with-microbes environment)**

| No. | Gene ID | $Log_2$(FC) | Adjusted P-value | Symbol | Chr | Description |
|---|---|---|---|---|---|---|
| 1 | FBgn0036948 | 2.59 | 5.29E-46 | CG7298 | 3L | - |
| 2 | FBgn0041579 | 2.74 | 8.56E-32 | AttC | 2R | Attacin-C |
| 3 | FBgn0010388 | 3.43 | 4.99E-31 | Dro | 2R | Drosocin |
| 4 | FBgn0034407 | 2.47 | 1.91E-10 | DptB | 2R | Diptericin B |
| 5 | FBgn0286723 | 2.63 | 2.44E-10 | hll | 2L | heimdall |
| 6 | FBgn0014865 | 2.28 | 1.46E-07 | Mtk | 2R | Metchnikowin |
| 7 | FBgn0004240 | 3.11 | 1.55E-05 | DptA | 2R | Diptericin A |
| 8 | FBgn0052284 | 2.28 | 0.0004 | CG32284 | 3L | - |
| 9 | FBgn0034957 | 3.78 | 0.0039 | Rsph4a | 2R | Radial spoke head protein 4a |
| 10 | FBgn0034712 | 1.76 | 0.0122 | Alp8 | 2R | Alkaline phosphatase 8 |
| 11 | FBgn0037323 | 2.37 | 0.0227 | CG2663 | 3R | - |
| 12 | FBgn0012042 | 1.64 | 0.0490 | AttA | 2R | Attacin-A |

1479
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1481
1482 **Table S8. List of down-regulated genes in $MBL_1$ in RNA-Seq (with-microbes environment)**
1483

| No. | Gene ID | $Log_2$(FC) | Adjusted P-value | Symbol | Chr | Description |
|---|---|---|---|---|---|---|
| 1 | FBgn0033978 | -1.53 | 3.30E-19 | Cyp6a23 | 2R | Cyp6a23 |
| 2 | FBgn0001225 | -3.94 | 1.75E-13 | Hsp26 | 3L | Heat shock protein 26 |
| 3 | FBgn0001229 | -2.03 | 1.43E-09 | Hsp67Bc | 3L | Heat shock gene 67Bc |
| 4 | FBgn0013279 | -2.37 | 7.09E-07 | Hsp70Bc | 3R | Heat-shock-protein-70Bc |
| 5 | FBgn0001224 | -2.74 | 5.69E-06 | Hsp23 | 3L | Heat shock protein 23 |
| 6 | FBgn0039151 | -1.84 | 6.07E-06 | CG13607 | 3R | - |
| 7 | FBgn0013276 | -2.48 | 1.55E-05 | Hsp70Ab | 3R | Heat-shock-protein-70Ab |
| 8 | FBgn0051354 | -2.28 | 1.55E-05 | Hsp70Bbb | 3R | Hsp70Bbb |
| 9 | FBti0059721 | -4.34 | 2.33E-05 | NA | 3R | - |
| 10 | FBgn0001230 | -3.18 | 2.48E-05 | Hsp68 | 3R | Heat shock protein 68 |
| 11 | FBti0019359 | -2.89 | 4.19E-05 | NA | 3R | - |
| 12 | FBgn0013278 | -2.50 | 0.0003 | Hsp70Bb | 3R | Heat-shock-protein-70Bb |
| 13 | FBgn0030769 | -1.75 | 0.0005 | CG13012 | X | - |
| 14 | FBgn0039152 | -1.60 | 0.0005 | Root | 3R | Rootletin |
| 15 | FBgn0036363 | -6.29 | 0.0006 | CG10140 | 3L | - |
| 16 | FBgn0013275 | -2.24 | 0.0009 | Hsp70Aa | 3R | Heat-shock-protein-70Aa |
| 17 | FBgn0013277 | -2.41 | 0.0023 | Hsp70Ba | 3R | Heat-shock-protein-70Ba |
| 18 | FBgn0052495 | -1.61 | 0.0078 | Gss2 | X | Glutathione synthetase 2 |
| 19 | FBgn0039307 | -2.28 | 0.0079 | CR13656 | 3R | - |
| 20 | FBti0020330 | -2.39 | 0.0227 | NA | 3R | - |
| 21 | RR43222_transposable_element | -2.87 | 0.0300 | NA | X | - |
| 22 | FBti0059720 | -2.94 | 0.0306 | NA | 3R | - |
| 23 | FBti0019362 | -4.05 | 0.0393 | NA | 3R | - |
| 24 | FBgn0034784 | -2.69 | 0.0476 | CG9826 | 2R | - |

1484 **S7.3 GO analysis of differentially regulated genes in the with-microbe assay environment**
1485
1486 To see the biological processes in which these genes are involved, we used the ShinyGo tool
1487 (version 0.80) (http://bioinformatics.sdstate.edu/go/) (Ge et al., 2020) for gene ontology enrichment
1488 analysis with 'GO biological process' pathway database. We found that the upregulated genes
1489 belong to the pathway involved in the fly's humoral immune response (Figure S15), while down-
1490 regulated genes belong to the stress response pathway (Figure S16).
1491

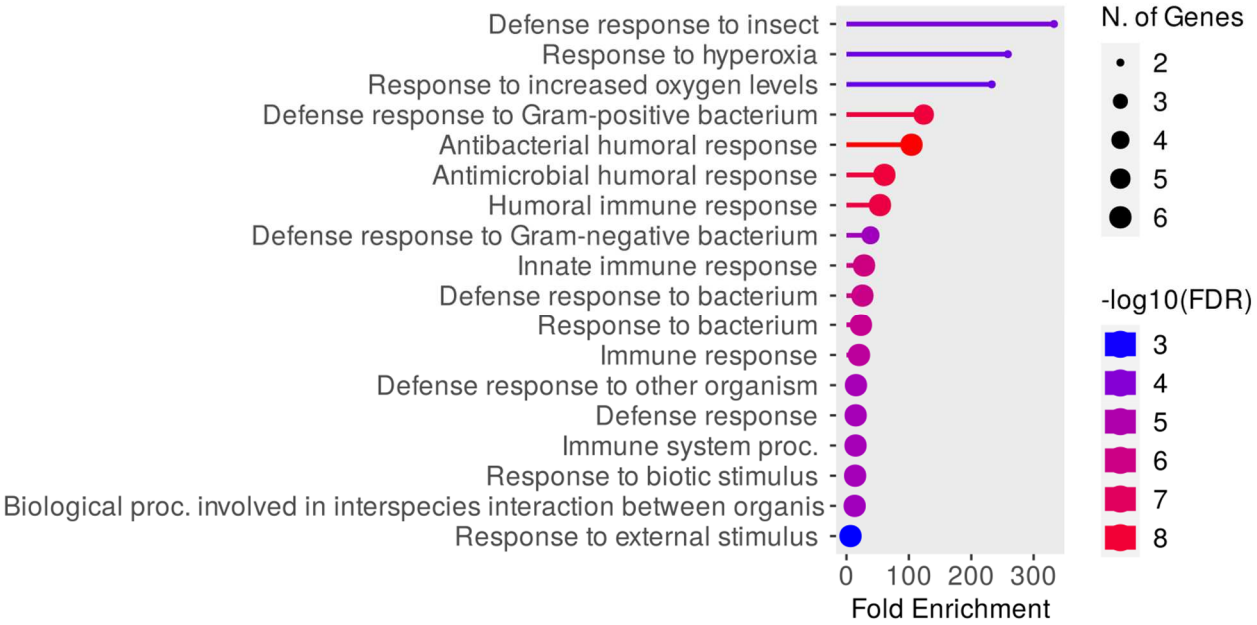


**Figure S15. Gene ontology (GO) enrichment analysis for 12 Up-regulated genes.**
1492

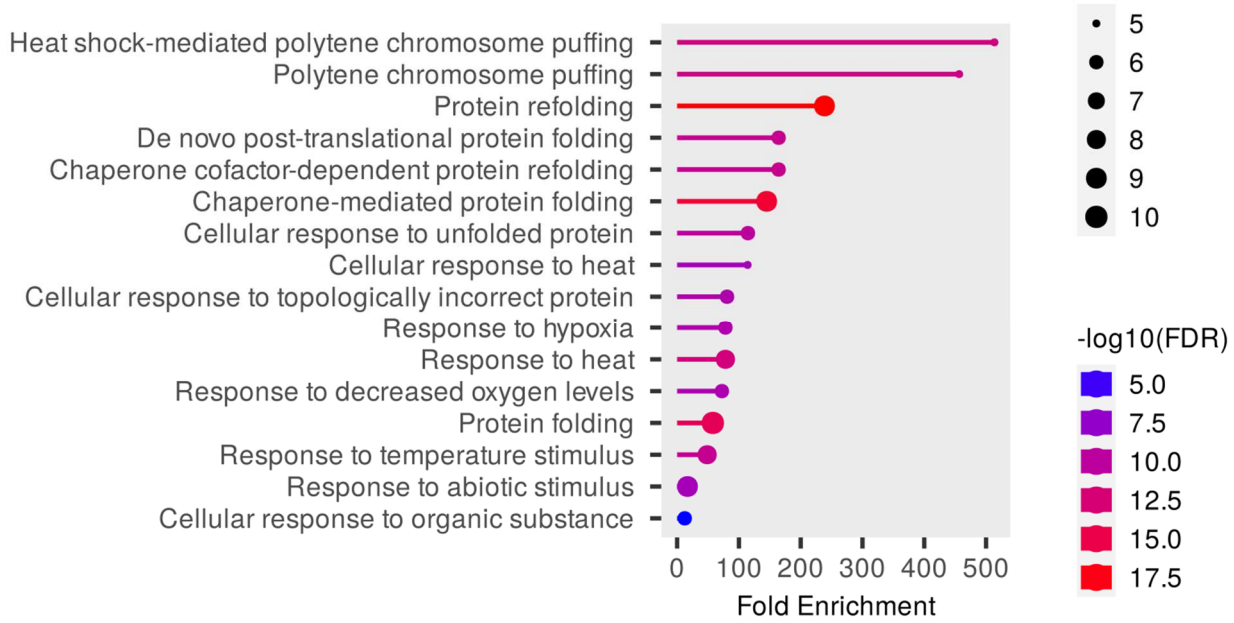


**Figure S16. Gene ontology (GO) enrichment analysis for 24 down-regulated genes.**
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## Text S8 - Another set of differently expressed genes hints at the peritrophic matrix (PM) as a potential region of interest in the MBLs

Along with HSPs and AMPs, another set of genes that have an altered expression are chitin-interacting genes. CG7298 and CG32284 are up-regulated chitin-binding enzymes in $MBL_1$, and down-regulated CG10140 is a probable chitinase. As the RNA-seq is done on the full body of flies, it is difficult to predict what this means for the fly. But, the annotation of CG7298 in FlyBase offers a clue. CG7298 is expressed in the proventriculus, which is part of the fly digestive system. A recent study supports the notion that proventriculus is the site where gut bacteria stably interact with flies, possibly through interactions with N-acetyl glucosamine, which is a building block of chitin (Dodge et al., 2023). Moreover, *Drosophila melanogaster* CG7298 has an ortholog called peritrophin-48 in related *Drosophila* species, which is a component of the peritrophic matrix. The peritrophic matrix is a chitinous layer that separates food bolus from the gut epithelia. This is also implicated as a physical barrier that keeps gut bacteria away from the gut tissue (Charroux & Royet, 2012; Erlandson et al., 2019; Kuraishi et al., 2011; Rodgers et al., 2017). Taken together, we think this is the region where chitin-interacting proteins are carrying out various processes that might be of interest. However, the limited available information about these hits prevents us from drawing any further conclusions at this point in time. In the future, we plan to confirm all the RNA-seq hits and do additional experiments that can give us more insights.

In a recent study, Kathedar et al. showed that the nicotinic acetylcholine receptor (nAchR) regulates gut barrier function in *Drosophila* through the remodeling of the peritrophic matrix (Katheder et al., 2023). We have seen that in our no-microbe assay regime, nAchRbeta2 gene expression showed down-regulation in $MBL_1$. This raises the possibility that the processes of interest happening at the proventriculus that involve the peritrophic matrix might be mediated through nAch signaling.

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1523

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