Title: Experimental evolution of *Drosophila* without its microbiome

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Abstract

The ability of the gut microbiome to contribute to diverse host phenotypes and, in turn, alter host fitness, raises the possibility that the presence of such microbes can influence the host's evolution. However, empirical studies on the evolutionary aspects of host-microbe relationships are rare. Here, we used experimental evolution on laboratory populations of *D. melanogaster* to understand how a host can evolve without its microbiome. For this, we compared replicate *Drosophila* populations reared without the microbes (labeled as MBL_{1-4}, for "Microbiota-less") for 54 host generations with their corresponding controls (labeled as MB_{1-4}), whose microbiome was reconstituted every generation. This comparison was done in two common-garden assay environments: with microbes and microbe-free. We found that contrary to what we expected, only modest adaptations were seen in the MBL populations. When looking at the phenotypic plasticity of MB-MBLs across with-microbe and microbe-free environments, we saw that MBLs had lesser plasticity than MBs. This might hint at MBLs reducing their dependence on microbes. We performed RNA-Seq on MBL_1 and MB_1 populations to see if there were changes in gene expression patterns. We found that a cluster of anti-microbial peptides (AMPs) was up-regulated, and another cluster of heat shock proteins (HSPs) was down-regulated in MBL_1 compared to MB_1. These results show that while the host's homeostasis is perturbed by microbiome manipulation on short timescales, the host-microbiome integration seems more labile on longer timescales, with hosts showing potential for adaptation without the microbes.

Introduction

Host-associated microbes are shown to modulate host biology (Cryan and Dinan 2012; McFall-Ngai et al. 2013; Sommer and Bäckhed 2013; Lynch and Hsiao 2019; McFall-Ngai 2024). These symbiont microbes can enhance the host's nutritional capacity (Douglas 1998; Ridley et al. 2012; Sun et al. 2022), offer protection from pathogens through colonization resistance (Caballero-Flores et al. 2023; Spragge et al. 2023), and shape the host's physiology (Sommer and Bäckhed 2013; Grenier and Leulier 2020), life history (Shin et al. 2011; Gould et al. 2018; Storelli et al. 2018; Lee et al. 2019; Gnainsky et al. 2021; Arias-Rojas and Iatsenko 2022; Suyama et al. 2023), behavior (Cryan and Dinan 2012; Vuong et al. 2017; Wong et al. 2017; Schretter et al. 2018), and immunity (Chervonsky 2013; Lee et al. 2013; Belkaid and Hand 2014; Selosse et al. 2014; Ansaldo et al. 2021; Jordan and Clarke 2024). In hosts where the impact of this symbiont-mediated modulation is substantial, microbes can alter the host's fitness (Rosenberg and Zilber-Rosenberg 2016; Ma et al. 2023). The ability of symbionts to contribute to diverse host phenotypes and, in turn, influence host fitness raises the possibility that the presence of such microbes can influence the host's evolution (Gilbert et al. 2015; Macke et al. 2017; Kolodny et al. 2020; Henry et al. 2021).

As microbiome acquisition can happen on faster timescales than the host's generation time, the microbiome can confer rapid adaptation to the hosts (Zilber-Rosenberg and Rosenberg 2008; Lau and Lennon 2012; Alberdi et al. 2016; Voolstra and Ziegler 2020). These adaptations can begin with the hosts expanding their niche with the help of microbes (Kopac and Klassen 2016; Borges 2017; Kolodny and Schulenburg 2020; Hoang et al. 2021; Koide 2023). 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 the insecticidal resistance, the stinkbugs gained a fitness advantage which could help this symbiosis to spread in the population.

Theoretical studies have shown that under certain conditions (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 and 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.

Another way in which the microbiota can affect the host's evolutionary potential is through phenotypic plasticity. 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 (Hoang et al. 2021).

In light of the above discussion, it comes as no surprise that the potential impacts of microbiota on the evolution of their host has become a major topic of investigation in the field (Macke et al. 2017; Kolodny et al. 2020; Henry et al. 2021). However, 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 (Noverr and Huffnagle 2004; Blaser and Falkow 2009). The recent COVID-19 pandemic-induced lifestyle changes, such as the overuse of soaps and sanitizers, can add to this selection pressure on certain symbiotic microbes. It has been hypothesized that such loss of ancestral microbes (e.g., *H. Pyroli*) can increase the risk factors for conditions such as gastroesophageal reflux disease (GERD) and childhood asthma (Blaser 2008; Blaser and 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 and Flach 2022) and microenvironmental 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 evolutionary biology (Macke et al. 2017; Henry et al. 2021).

Here, we used experimental evolution using *D. melanogaster* as a model system to understand how a host can evolve in the absence of its microbiome. For this, we kept four large (~2400 individuals) outbred populations of *D. melanogaster* microbe-free using bleach (labeled 'MBL' populations) 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 just after the bleach treatment from a common source pool. For this reconstitution, microbes were sampled from the environment of the flies via fecal microbiome transfer. We compared the selected MBL and control MB populations in two assay environments: (a) in the presence of

145 microbes (labeled “with-microbes” environment) and (b) in the absence of microbes
146 (labeled “microbe-free” environment). Previous observations from the lab showed that the
147 symbiotic microbiome associated with our outbred baseline lab population affects diverse
148 host traits and host fitness (Malwade et al., manuscript under prep). This observation
149 underscored the important role that the microbiome plays in maintaining the host’s
150 homeostasis. Based on these results and the available literature, we hypothesized that the
151 host would face substantial selection pressure in the absence of microbes. Therefore, we
152 expected a rapid evolution of the host to maintain the homeostasis seen in the presence of
153 microbes in our ancestral baseline populations.

154 We assayed these populations twice during selection: generations 17-20 and generations
155 54-57. We found that even after 54 generations without microbes, contrary to
156 expectations, there were only modest adaptations in MBL populations. The percent
157 survival from egg to adulthood was higher for MBLs when tested under the microbe-free
158 assay environment. We also found that in the with-microbe assay environment, MBL
159 males had lower desiccation resistance. The increase in survival might be an adaptation,
160 and the reduction of male desiccation resistance might be a cost paid by MBLs. We also
161 saw that the effect sizes over evolutionary differences between MB-MBLs shifted from
162 “small to medium” effects in Gen 17-20 towards “medium to large” effects in Gen 54-57.
163 When looking at the phenotypic plasticity of MB-MBLs across with-microbe and microbe-
164 free environments, we saw that MBLs had lesser plasticity than MBs. This might hint at
165 MBLs reducing their dependence on microbes. To see if there are changes in gene
166 expression patterns, we performed RNA-Seq on one population each of MB and MBL. We
167 found that a cluster of anti-microbial peptides (AMPs) was up-regulated, and another
168 cluster of heat shock proteins (HSPs) was down-regulated in MBL as compared to MBs.
169 These RNA-Seq results also hinted that the peritrophic matrix could be a site of interest for
170 carrying out further investigations related to these populations.

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174
175

176 **Methods**

177 **Details of the experimental evolution lines**

178 The experimental evolution lines were derived from the four ancestral baseline populations
179 named NDB_{1-4} (at their generation 21 in the lab). The details about the NDB_{1-4} are provided
180 in Supplementary Text S1.1. Each ancestral NDB line gave rise to two populations: MBL
181 ("Microbiome-Less," selected population that is without the microbiome) and MB
182 (corresponding ancestry-matched control population with microbiome) (Supplementary
183 Figure S1). For example, NDB_1 gave rise to MBL_1 and MB_1, NDB_2 gave rise to MBL_2 and
184 MB_2, and so on. Thus, populations with the same numerical index (e.g., index 2 for MBL_2
185 and MB_2) share a common ancestry and were always assayed together and treated as
186 blocks in statistical analysis. This design means a total of four replicate selected
187 populations (MBL_{1-4}) were compared against four replicate control populations (MB_{1-4})
188 (Supplementary Figure S1). Each population in this setup is maintained at a population
189 size of ~2400 in the plexiglass cages with the added implementation of a selection
190 protocol and selection conditions described in the next section. NDB_1 served as the
191 common source of the microbiome for all the MBs (for the rationale of this step, see
192 microbiome reconstitution section).

193

194 **Experimental evolution protocol employed each generation**

195 MBLs were handled aseptically inside a Level II biosafety cabinet (make: Microfilt, India).
196 All things that came in contact with MBLs for their maintenance were either autoclaved,
197 filtered through a 0.2-micron filter, or surface-sterilized with 70% ethanol.

198 Every generation, on day 12 after egg deposition, each experimental evolution population
199 of MBLs and MBs (total 8 populations) was provided with autoclaved yeast in the form of
200 moist paste to boost their egg output. This paste also contained a few drops of acetic acid
201 so that the flies are attracted to yeast. A fresh food plate with an exposed vertical food
202 surface was provided for 12-16 hrs to these populations at the end of day 13 to collect
203 eggs for the next generation. On day 14, these eggs were collected using a moist
204 paintbrush and transferred to cell strainers (TCP026, HiMedia) for egg dechorionation
205 (Supplementary Figure S2A). The cell strainer was then placed into one of the wells of a 6-
206 well plate. These eggs were washed with 2% bleach for 2 min to remove the chorion layer
207 and any associated microbes (Supplementary Figure S2B). The cell strainer with

208 dechorionated eggs was transferred to another well of the 6-well plate for another quick
209 wash with 2% bleach to kill any residual microbes. Next, the cell strainer with eggs was
210 transferred back to back to two fresh wells of the 6-well plate for two successive washes
211 with autoclaved dH_2O to remove bleach. To create MBLs, ~400 eggs were transferred to
212 translucent, plastic *Drosophila* milk bottles (Laxbro Inc., Catalog No. FLBT-20) that had
213 ~50-60ml autoclaved banana-jaggery media at the bottom (Supplementary Figure S2C).
214 To make MBs, the native microbiome of NDB_1 was reconstituted (see next section) on the
215 dechorionated eggs before they were transferred to the food bottles (Figure 4.2C).

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

219

220 **The procedure of microbiome reconstitution**

221 We chose passive microbiome reconstitution to create flies with microbes for long-term
222 experimental evolution. Supplementary Figure S3 illustrates the reconstitution protocol in
223 detail. The microbiome was reconstituted from the ancestral NDB_1 population for all the
224 MBs (i.e., MB_{1-4}). This ensured that there were no differences across the four MB
225 populations in terms of the microbiota that they were harboring. Several different sources
226 of the microbiome (say, every MB_i getting their microbiome from their ancestral NDB_i)
227 would mean there is a greater chance of differential microbial association.

228 We describe the microbiome reconstitution protocol in detail in Supplementary Figure S3.
229 The source population for the microbiome reconstitution is NDB_1 unless stated otherwise.
230 A total of eight fly vials with non-autoclaved normal banana-jaggery food were set to keep
231 the flies for 24 hrs. These flies were collected on day 13 after egg collection. In each vial,
232 50 flies with an equal male-to-female ratio were transferred using mild CO_2 anesthesia.
233 After 24 hrs, flies were discarded, and 1 ml of autoclaved water was added to each vial to
234 extract their microbiome. Vials were swirled with water till no visible fecal spots were seen
235 on the vial walls. This method of sampling of microbes from the frass of flies housed in
236 vials has been used earlier in the literature (Chandler et al. 2022). The microbe-free eggs,
237 after the bleach treatment, were washed with autoclaved water twice and then dipped
238 twice into this microbiome slurry to seed the source microbiome on the eggs. These eggs

239 were then gently transferred to *Drosophila* vials or bottles with autoclaved food to generate
240 flies with reconstituted microbiomes derived from NDB_1.

241

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

243 We explain the assay environments using the MB_1-MBL_1 block (Figure 1). However, the
244 same is true for the rest of the three blocks of MB-MBL as well. MB_1 received microbiome
245 from NDB1 every generation. MBL_1 is maintained without any microbes. If we directly
246 compare the phenotypes of MB_1-MBL_1 in their experimental evolution conditions (i.e., MB
247 with microbes and MBL without microbes) (Figure 1A), any observed differences between
248 MB/MBL lines can be due to their evolutionary history (with/without microbes) or the assay
249 environment (with/without microbes) or both. Therefore, we needed to provide a common
250 assay condition for the phenotypic comparison of MB_1-MBL_1. We performed a phenotypic
251 comparison between MB_1-MBL_1 in the native environment of MB_1, which is with-microbes
252 environment, and also in the native environment of MBL_1, which is microbe-free (Figure
253 1B). For the with-microbe environment, MB_1 as well as MBL_1 were maintained in this
254 common-garden environment in the presence of microbes for a generation. F1 flies with-
255 microbes on which we performed the assays were raised as described in Supplementary
256 Text S2 and Supplementary Figure S4. For a microbe-free environment, MB_1 and MBL_1
257 were maintained in this common-garden environment in the absence of microbes for a
258 generation. Microbe-free F1 flies on which we performed the assays were raised as
259 described in Supplementary text Figure S4. Matching of environments via common-garden
260 rearing for a generation minimizes the non-genetic effects, giving us the evolutionary
261 effects in which we are primarily interested.

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

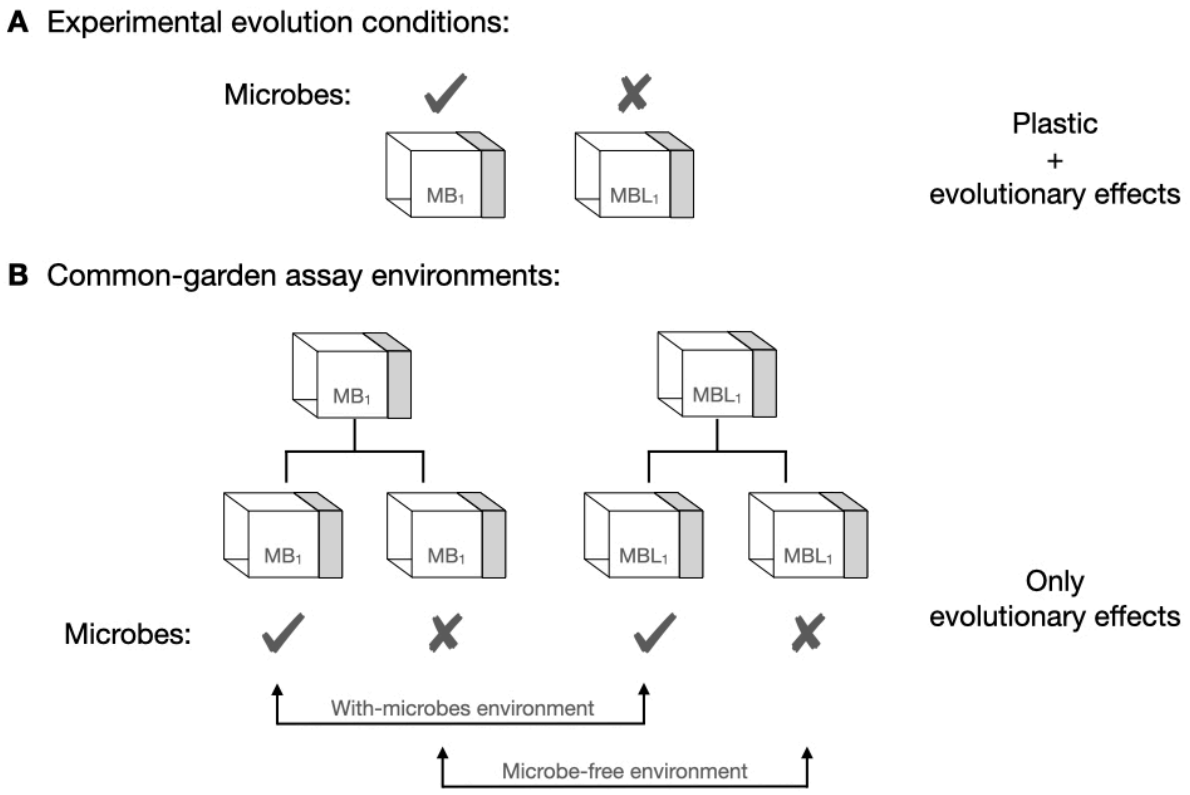

Figure 1. Illustration of environments in which experimental evolution was carried out and the environments in which phenotyoic assays are carried out.

264

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

266 A subset of flies from the phenotypic assays (at generation 54-57) were flash-frozen in
267 liquid nitrogen and stored at -80^{0}C. These flies from MB_1 and MBL_1 populations were sent
268 to a commercial vendor (miBiome Therapeutics LLP, Mumbai, India) for RNA-Seq, starting
269 with total RNA isolation. RNA-Seq was done only on female flies.

270

271 *RNA isolation*

272 Total RNA was extracted using miRNeasy Mini kit (Qiagen, Catalog No. 1038703)
273 following the manufacturer's protocol. About 30 females were homogenized and lysed in
274 Trizol reagent, followed by chloroform extraction. The aqueous phase was mixed with 1.5x
275 chilled ethanol, and the mix was loaded on the columns provided in the kit. RNA was
276 eluted in 30 μl RNase-free water. The RNA samples were quantitated on a Qubit

277 fluorometer. Appropriate dilutions were loaded on a high-sensitivity RNA screen tape to
278 determine the RNA integrity number (RIN).

279

280 *Library preparation and sequencing*

281 1.5 μg of RNA per sample was depleted of fly rRNA using QIAseq FastSelect - rRNA Fly
282 Kit (Qiagen, Catalog No. 333262). The NEBNext Ultra II directional RNA library prep kit
283 (Illumina, Catalog No. E7760L) was used to construct double-stranded cDNA libraries from
284 the rRNA-depleted RNA. The cleaned libraries were quantitated on a Qubit fluorometer,
285 and appropriate dilutions were loaded on high-sensitivity D1000 screen tape to determine
286 the library size.

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

289

290 *RNA-seq analysis*

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

299

300 **Details of the phenotypic assays**

301 For all the following assays, eggs were processed for dechorionation using bleach as per
302 the protocol illustrated in Supplementary Figure S2. These dechorionated eggs were
303 transferred to the *Drosophila* vials with ~ 6 ml of food at a specified density (30 eggs per
304 vial unless stated otherwise). All the assays were carried out in autoclaved banana-jaggery
305 food.

306 We assayed the following phenotypes in MB_{1-4} vs. MBL_{1-4}.

Percentage survival to adulthood

For this assay, the eggs were collected via synchronized egg collection within 2 hr window. These eggs were then put into food vials after being counted precisely under a microscope using a paintbrush to the specified egg density (here, 30 eggs per vial). In this procedure, eggs were transferred over a thin strip of solidified 1.3% non-nutritive agar with a moist paintbrush. These eggs were spread over the agar strip such that a single egg could be counted and moved into a stack. This egg stack was cut away along with the agar layer using a scalpel that was pre-cleaned with cotton soaked in 70% ethanol. This piece of agar with exactly 30 eggs was transferred to the food vials with autoclaved banana-jaggery food. Males and females were sex separated under mild CO_2 anesthesia upon eclosion, and their numbers were noted. The eclosion of the flies was scored at regular intervals of 2 hours. A total of ten vial replicates were used per treatment for this assay.

Dry body weight

On day 12 after egg deposition, the adult flies were sorted by sex in batches of 20 individuals per sex into 1.5ml micro-centrifuge tubes using CO_2 anesthesia. These flies were then flash-frozen using liquid nitrogen and, if required, stored at -80^{0}C till weighing. The flies were then dried at 60^{0}C for 72 hrs and weighed on an analytical weighing balance (ME104, Mettler Toledo, least count = 0.1mg). 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.

Egg-to-adult developmental time

This assay was conducted similarly to the percent survival to adulthood assay, with the only difference being that eclosion time was noted for emerging flies in the given vial for male and female flies separately. Males and females were sex separated under mild CO_2 anesthesia upon eclosion. The eclosion of the flies was scored at regular intervals of 2

339 hours in the case of assays in generations 17-20 and at regular intervals of 6 hours in the
340 case of assays in generations 54-57.

341

342 *Female fecundity*

343 On day 12 after egg deposition, flies were transferred to fresh food vials. On day 13, a
344 male-female pair was introduced into an aerated 50 ml falcon tube containing a food cup
345 using aspirators, thus completely avoiding CO_2 anesthesia. The food cup provided a
346 surface for egg-laying. The falcon tubes were kept for 12 hrs in an incubator maintained at
347 25^0C. After 12 hours, the total number of eggs laid was counted under a microscope. A
348 total of 50 replicates per treatment were set for this assay.

349

350 *Desiccation resistance*

351 Ten adult flies of the given sex were introduced into an empty plastic vial (Height: 3.5
352 inches x Diameter: 1 inch). The flies had no access to food or water. The vials were
353 plugged with fresh cotton plugs. These vials were checked every two hours for any deaths,
354 till al the flies were dead. The datasheet kept track of flies alive (total flies to start with -
355 count of total dead flies) at a given time point. The average time to death due to
356 desiccation was calculated for the vial. A total of ten such vial replicates were set per
357 treatment per sex.

358

359 *Locomotor activity*

360 The flies' locomotor activity was measured using the *Drosophila* Activity Monitoring (DAM)
361 system (Trikinetics Inc). Using an aspirator, a single fly was introduced into a glass DAM
362 tube (Length: 6.5 cm, Diameter: 4 mm). The locomotor activity for a given fly is the
363 average number of times the fly crosses the infrared beam running through the middle of
364 the glass DAM tube per hour, recorded over six hours. Adult flies (day 12 after egg
365 deposition) were used for this assay. A total of about 32 flies were used per treatment per
366 sex. The dead flies during the six hours were excluded from the analysis. The first 15
367 minutes of activity were excluded from the analysis as this was taken as the time taken for
368 flies to get acclimatized to the glass DAM tubes.

369

370 *Mating latency and mating time*

371 The mating assay was performed on virgin males and females. After the eclosion (typically
372 around day eight after egg deposition), male and female flies were separated under mild
373 CO_2 anesthesia into vials with fly food. The interval of the fly collection was six hours,
374 starting with the very first eclosion, to ensure that the eclosed flies were virgins. Males and
375 females were held separately at the density of ten flies per vial till the mating experiment
376 on day 12 after egg deposition.

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

384

385 **Statistical analysis**

386 The selected populations (MBL_{1-4}) were compared with their corresponding controls (MB_{1-}
387 $_{4}$) in two types of environments (with and without microbiota). The analysis for these two
388 environments was done separately. In each environment, four block means of MB_{1-4} were
389 compared against four block means of MBL_{1-4} using a paired t-test performed in GraphPad
390 Prism (v10.2.1).

391 For interpreting effect sizes using Cohen's d, the following criteria were followed: $d > 0.8$
392 (high); $0.8 > d > 0.5$ (medium); $d < 0.5$ (low) (Cohen 2013). The graphs are plotted in
393 GraphPad Prism (v10.2.1). The figures drawn using BioRender have that specification in
394 their legend description.

395 In the Results section, along with the trends that are statistically significant (i.e., $P < 0.05$),
396 we have also highlighted trends that are not statistically significant (i.e., $P > 0.05$) but have
397 come close to statistical significance ($P \sim 0.1$) <u>with large effect sizes</u> (using Cohen's d).
398 This is highlighted in blue in Supplementary Tables S5-S8, which provide detailed
399 information about the statistical comparisons for the phenotypic assays.

400

401 **Results**

402 **First phenotypic assessment of experimental evolution lines at host generations 17-**
403 **20**

404 We assayed the selected and control populations twice, first in generations 17-20 and then
405 in generations 54-57 (The experimental design details of these two assessments are given
406 in detail in Supplementary Text S4.1 and S4.2, respectively). The results for generations
407 17-20 are provided in Supplementary Text S6. At this point, we observed that only one trait
408 (mating time in the with-microbe environment) showed a difference, and the rest of the
409 traits did not change for the flies kept without the microbiome for 17-20 generations
410 (Supplementary Tables S5 and S6).

411 **Second phenotypic assessment of experimental evolution lines at host generations**
412 **54-57**

413 Supplementary Tables S7 and S8 provide detailed information on the statistical
414 comparison between MB_{1-4} and MBL_{1-4} for the with-microbe assay environment and
415 microbe-free assay environment, respectively. Comparing these tables with the
416 corresponding ones for generation 17 (i.e., Supplementary Tables S5 and S6) shows that
417 there were a larger number of cases in the later generation where the effect sizes were
418 medium or large. We interpret this as a signature of evolutionary divergence.

419

420 *Male traits in host generations 54-57*

421 There was no difference between the body weight of the male MBL_{1-4} and MB_{1-4} flies in the
422 with-microbe assay environment (Paired t-test, $t_3 = 0.32$, $P = 0.7723$, Figure 2A-left). In the
423 microbe-free environment, the body weight of the male MBL_{1-4} was more than their
424 corresponding controls MB_{1-4} with a large effect size even though the P-value was
425 marginally greater than 0.05 (Paired t-test, $t_3 = 2.89$, $P = 0.0628$, Cohen's d = 1.74 (large),
426 Figure 2A-right).

427 The egg-to-adult development time was largely unaffected by the multi-generation
428 absence of microbes, as seen in both assay environments (Supplementary Tables S7 and
429 S8, Figure 2B).

430 MBL_{1-4} had lower desiccation resistance than the MB_{1-4} in the with-microbe environment
431 with a large effect size, although the P-value was marginally greater than 0.05 (Paired t-

432 test, t_3 = 3.16, P = 0.0510, Cohen's d = 1.62, Figure 2C-left). No difference was observed
433 in the microbe-free environment (Paired t-test, t_3 = 0.40, P = 0. 7164, Figure 2C-right).

434 In both environments, MB_{1-4} and MBL_{1-4} showed similar levels of locomotor activity
435 (Supplementary Tables S7 and S8, Figure 2D).

436

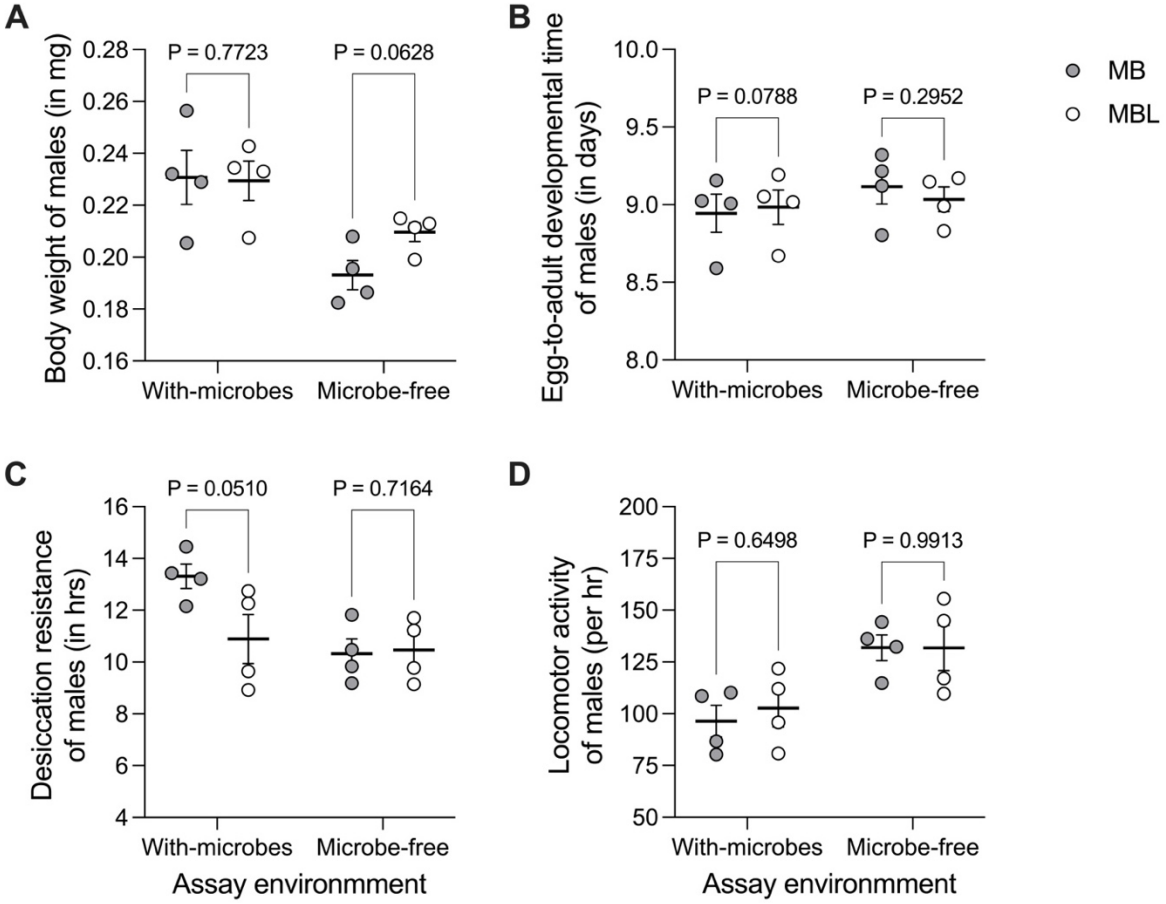

Figure 2. Male traits assessed in Gen 54-57 in with-microbe and microbe-free environments. (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations MB_{1-4} and, similarly, four empty circles are means for each of the four selected populations MBL_{1-4}. The black horizontal line 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.

437

438 *Female traits in host generations 54-57*

439 The body weights of the MB_{1-4} and MBL_{1-4} females were similar in the with-microbes
440 regime (Paired t-test, $t_3 = 0.82$, P = 0.4726, Figure 3A-left) but showed a marginal increase
441 for MBLs in the no-microbe regime which was not statistically significant, although with a
442 large effect size (Paired t-test, $t_3 = 2.34$, P = 0.1015, Cohen's d = 1.35, Figure 3A-right).

443 Female egg-to-adult development time was similar for MB-MBLs after multiple generations
444 without the microbes when assayed in both assay environments (Supplementary Tables
445 S7 and S8, Figure 3B).

446 MBL_{1-4} females had lower desiccation resistance in the with-microbe regime, which was
447 not statistically significant (Paired t-test, $t_3 = 2.45$, P = 0.0916, Cohen's d = 0.89 (large),
448 Figure 3C-left). MBs and MBLs had similar desiccation resistance in the no-microbe
449 regime (Paired t-test, $t_3 = 0.70$, P = 0.5365, Figure 3C-right).

450

451 MB_{1-4} and MBL_{1-4} females had similar locomotor activity levels in the with-microbes
452 environment (Paired t-test, $t_3 = 0.10$, P = 0.9260, Figure 3D-left). In the microbe-free
453 environment, there was a trend of MBL_{1-4} females being more active than the MB_{1-4} that
454 was not statistically significant (Paired t-test, $t_3 = 2.23$, P = 0.1122, Cohen's d = 0.89
455 (large), Figure 3C-left).

456

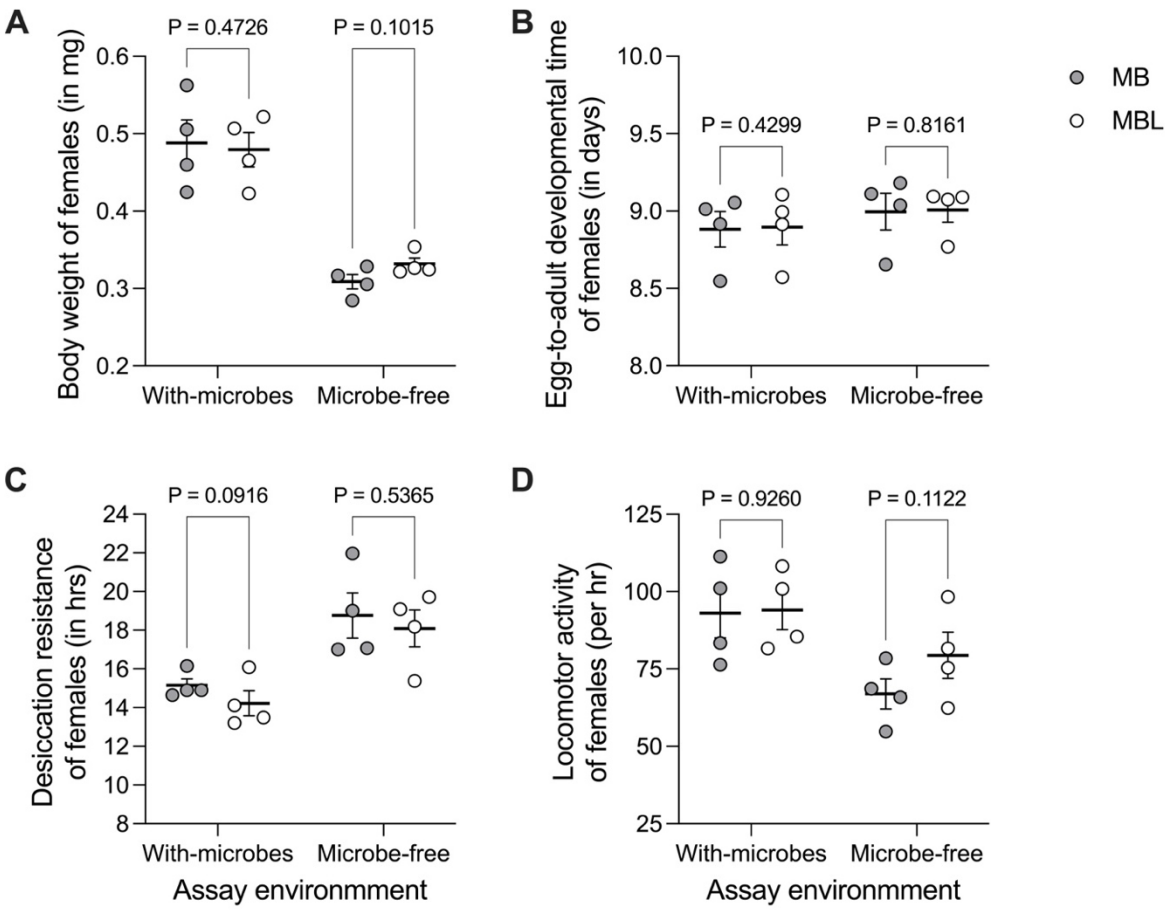

Figure 3. Female traits assessed in Gen 54-57 in with-microbe and microbe-free environments. (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations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

457

458

Pair traits in host generations 54-57

There was a trend of lower survival for MBL_{1-4} than MB_{1-4} in the with-microbe regime, but it is not statistically significant (Paired t-test, $t_3 = 2.31$, P = 0.1044, Cohen's d = 1.81 (large), Figure 4A-left). In the microbe-free environment, MBL_{1-4} had better survival than MB_{1-4} (Paired t-test, $t_3 = 3.72$, P = 0.0388, Cohen's d = 0.83 (large), Figure 4A-right).

There was a trend of MBL_{1-4} laying more eggs than the MB_{1-4} in the with-microbe regime, but this difference was not statistically significant (Paired t-test, $t_3 = 1.75$, P = 0.1791, Cohen's d = 1.67 (large), 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 (Paired t-test, $t_3 = 1.20$, P = 0.3161, Cohen's d = 0.58 (medium), Figure 4B-right).

MBL_{1-4} did not differ from MB_{1-4} in the latency to start the mating, as assessed in both with-microbe and no-microbe regimes (Supplementary Table S7 and S8, Figure 4C).

There was no difference in MB_{1-4} and MBL_{1-4} mating time in the with-microbe regime (Paired t-test, $t_3 = 0.15$, P = 0.8928, Figure 4D-left). In the microbe-free environment, there is a trend of shorter mating times for MBL_{1-4} than MB_{1-4}, but this is not statistically significant (Paired t-test, $t_3 = 2.35$, P = 0.1007, Cohen's d = 1.39 (large), Figure 4D-right).

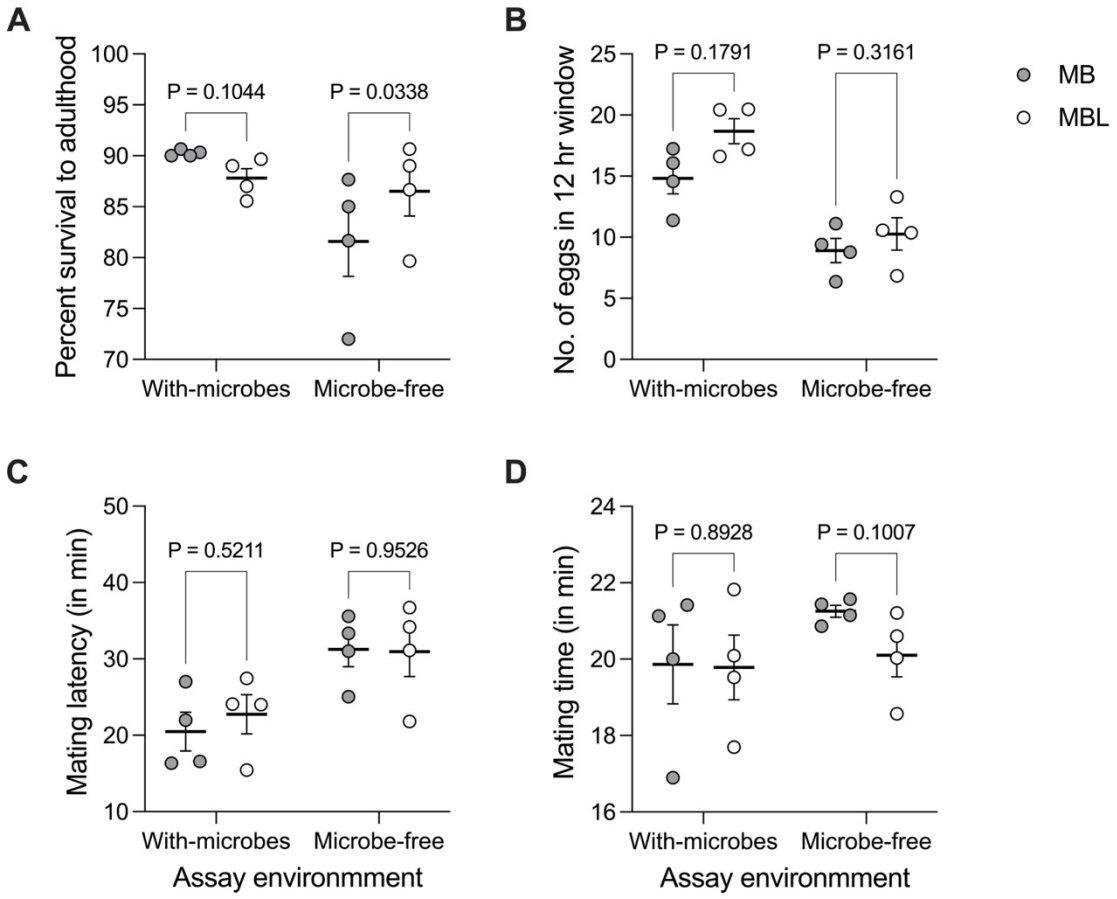

Figure 4. Pair traits assessed in Gen 54-57 in with-microbe and microbe-free environments. (A) Survival to adulthood. (B) Female fecundity. (C) Mating latency. (D) Mating time. In each assay environment, four filled circles are means for each of the four control populations MB_{1-4} and similarly, four 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.

476

477 **MBLs have reduced phenotypic plasticity than the MBs across with-microbe and**
478 **microbe-free environments (Gen 54-57 assays)**

479 Figures 2 to 4 suggested that, compared to the MBs, the trait values of the MBLs changed
480 relatively less across the two assay environments (i.e., with microbes and microbe-free). In
481 other words, the reaction norms for the various traits across these two environments are
482 expected to be flatter for the MBLs than for the MBs. To quantify this observation, we used

483 effect size (Cohen's d) as a proxy for the measure of phenotypic plasticity shown by MB
484 vs. MBL populations *across two environments*. (Note: These across-environment effect
485 sizes are different than those provided in Supplementary Tables S5-S8). Supplementary
486 Text S4.1 and S4.2 discusses the experimental design used to calculate these plastic
487 effect changes and why such analysis could not be done in Gen 17-20.

488 We found that the MBLs, on average, had smaller effect sizes than MBs (Paired t-test, t_{11}
489 = 2.14, P = 0.0554, Cohen's d = 0.49 (small), Figure 5A), possibly hinting at lower
490 phenotypic plasticity than the MBs across two assay environments. Another way of looking
491 at the trait's reaction norm could be through the percentage change that the trait has
492 undergone across environments. The more the percent change, the more the phenotypic
493 plasticity. We found that for the traits that we assessed, MBLs had a lower percentage of
494 change in their trait values across two environments than MBs (Paired t-test, t_{11} = 2.99, P
495 = 0.0123, Cohen's d = 0.39 (small), Figure 5B).

496

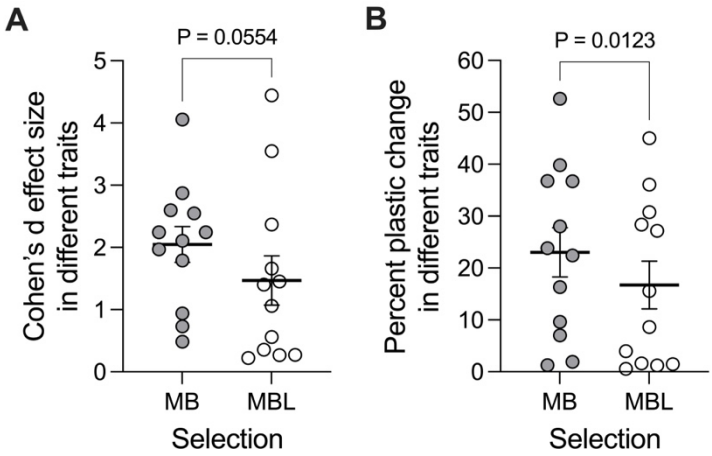

Figure 5. (A) Effect sizes and (B) percentage changes as a proxy for plastic changes across two environments. Means are indicated by black horizontal lines. The error bars represent SEM. The data is analyzed using a paired t-test. For percent change, Wilcoxon matched-pairs signed rank test (a non-parametric test) is also significant (W = -64.0, P = 0.0093).

497

498

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

500 In the previous section, we used effect sizes on phenotype differences across assay
501 environments for MBs as well as MBLs as a proxy *for the phenotypic plasticity* exhibited by
502 these two populations. In this section, we look at how effect sizes have changed *for the*
503 *evolutionary difference between MB-MBLs over time* from the first phenotypic assessment
504 in generations 17-20 to the second assessment in generations 54-57 (Figure 6). These
505 effect sizes are provided in Supplementary Tables S5-S8.

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

513

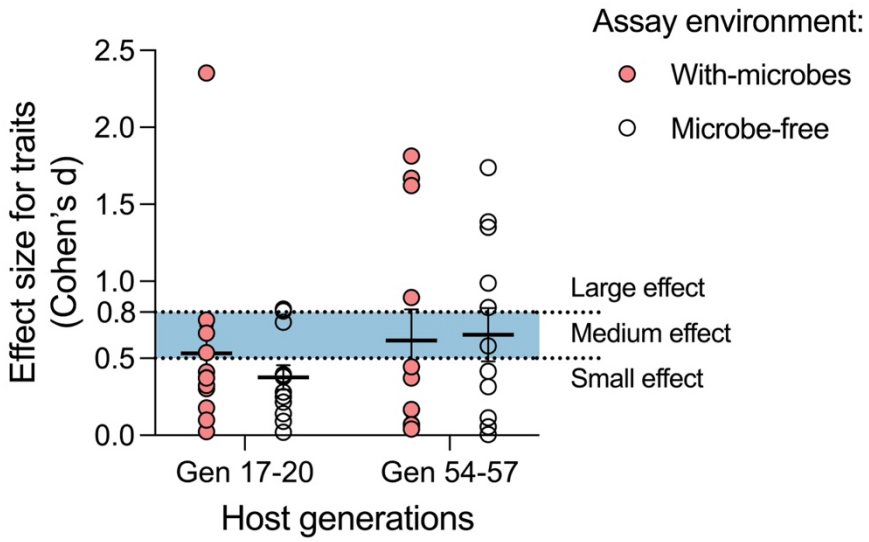

Figure 6. Changes in effect sizes (Cohen's d) over all traits with time. Means are indicated by black horizontal lines. The error bars represent SEM. Coral circles indicate with-microbes environment and empty circles indicate the microbe-free environment.

514

515

516 **RNA-Seq result – Gen 54-57**

517 After investigating the effects of long-term microbiome absence using broad phenotypic
518 assays, we used RNA-Seq to determine if specific transcripts are differentially expressed
519 in MBL_1 vs. MB_1 as a direct consequence of their different evolutionary history. This
520 comparison was done in two assay environments: (a) microbe-free and (b) with-microbe.
521 The protocol for RNA-seq is described in detail in the method section.

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

525 In the with-microbe regime, anti-microbial peptide (AMP) expression is up-regulated in
526 MBL_1 compared to their control MB_1 (Figure 4.20). The full list of up-regulated genes in
527 MBL_1 is given in Supplementary Table S9. We also find that a cluster of heat shock
528 proteins (HSPs) is down-regulated along with several other genes in MBL_1. The full list of
529 down-regulated genes in MBL_1 is given in Supplementary Table S10.

530 Among all the differentially expressed genes, only the significant genes with Log_2(Fold
531 Change) > 1.5 and adjusted P-value < 0.05 are highlighted in Figure 7 and given in
532 Supplementary Tables S9 and S10.

533 Gene ontology analysis of differentially expressed genes is presented in Supplementary
534 Text S7.3.

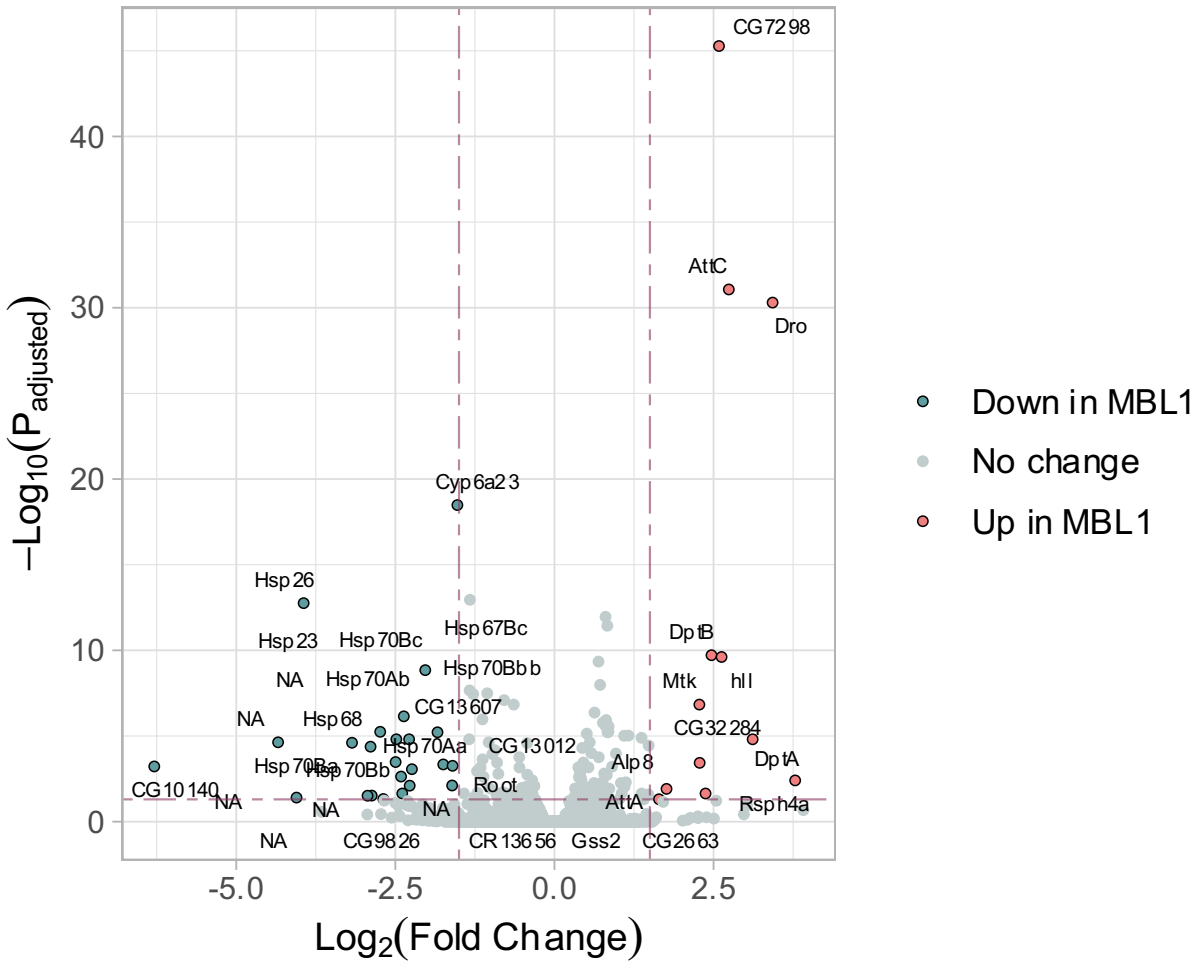

Figure 7. Differentially expressed genes in MBL_1 vs MB_1 in the with-microbes environment using DESeq2. The red dots represents the up-regulated genes, while blue dots represent down-regulated genes in MBL_1. The protocol for the RNA-Seq is described in the method section.

535

536

537 **Discussion**

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

539 In previous observations from the lab (Malwade et al., manuscript under prep), we saw
540 that almost all the traits we investigated were affected by the removal of the microbiome.
541 This led us to infer that microbiota is crucial for the realization of a large number of traits in
542 *Drosophila melanogaster*, an observation that corroborates the results of countless studies
543 from the literature (Broderick and Lemaitre 2012; Erkosar et al. 2013; Douglas 2018b;
544 Lesperance and Broderick 2020; Ludington and Ja 2020). This led to the hypothesis that
545 the removal of microbiota is likely to be extremely stressful for the flies, as many traits
546 would be affected at once. Hence, we expected the microbiota-less environment to exert
547 very strong selection pressure on the MBL1-4 flies, leading to changes in multiple life-
548 history traits, maybe including some trade-offs, within relatively few generations. However,
549 the results proved to be otherwise.

550 In the first set of phenotypic assays done in host generations 17-20 (Supplementary Text
551 S6), we saw that the mating time of MBLs was shorter than the MBs (Supplementary
552 Figure S13D, with-microbes environment) and female body weight was slightly elevated
553 for MBLs (Supplementary Figure S12A), microbe-free environment). Apart from these two
554 changes, we did not see any evidence of evolutionary divergence between MB_{1-4}-MBL_{1-4} in
555 either of the two assay environments.

556 In the second set of phenotypic assays done in host generations 54-57 (Figures 2 to 4),
557 we saw that two traits showed significant change. In the microbe-free environment, we
558 found that MBL_{1-4} had better egg-to-adult survival than MB_{1-4} (Figure 4A). In the with-
559 microbes environment, MBL_{1-4} males had lower desiccation resistance (Figure 2C). It is
560 possible that this lower desiccation resistance might be a cost incurred by MBLs in this
561 environment for improving their egg-to-adult survival in the selection environment (i.e.,
562 microbe-free environment).

563 When we looked at the effect sizes over all the traits assessed in Gen 17-20 vs. Gen 54-
564 57 (Figure 6), we saw that the effect size distribution shifted from mostly “small and
565 medium” effects to “small, medium, and large” effects. This suggests that even after 54
566 generations, the selected flies are still diverging from the control flies.

567 We assayed the life-history traits both in the presence and the absence of microbes. Thus,
568 any improvement the fitness of the MBLs (compared to the MBs) in the microbe-free

569 environment can be construed as the effect of adaptation in the selection environment. On
570 the other hand, any reduction in the fitness of the MBLs (again compared to the MBs) in
571 either assay environment would signify a cost of adaptation under microbe-free conditions.
572 Our results showed that there were very few adaptations or trade-offs.

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

583 Another possibility is that even though the selection pressure is strong, the flies did not
584 possess sufficient genetic variability to respond to the selection pressure. This might have
585 been a valid concern if, like many studies on *Drosophila*-microbiota systems, the
586 experiments were performed on highly inbred or iso-female strains (e.g., *Canton-S,*
587 *Oregon-R,* and w^{1118}). However, our fly populations are outbred and were reinvigorated
588 through genetic mixing of four outbred populations only one year prior to the initiation of
589 this study (Supplementary Text S1.3). Our regular observation of these flies indicates that
590 they are vigorous in the cage (in terms of activity) and have high fecundity and egg-to-
591 adult survivorship (A Malwade, personal observations). Therefore, we do not have any
592 reason to believe that these flies did not have sufficient genetic variations. More
593 importantly, the fact that egg-to-adult survivorship did change over time indicates that the
594 populations were indeed evolving, which would not have been possible in the absence of
595 genetic variation.

596 A third possibility is that while some divergence has indeed happened, they have not been
597 picked up by our assays. For example, some previous studies have shown that the role of
598 microbiota in affecting host physiology becomes prominent only in nutritionally unbalanced
599 diets (Shin et al. 2011; Storelli et al. 2011). It is entirely possible that we will see more
600 differences between the MBs and the MBLs if we assay them on a different diet. However,
601 previous observations from the lab (Malwade et al., manuscript under prep) have shown

602 that removing the microbiota leads to changes in almost every trait, even under the
603 standard food medium used in our lab. Therefore, it is unlikely that the lack of differences
604 between MBs and MBLs is due to the food on which they were assayed or selected.

605 This brings us to the fourth possibility that although the removal of microbiota can lead to
606 large-scale changes in a number of life-history and behavioral traits in a single generation,
607 this does not translate into strong selection pressure on an evolutionary timescale. Our
608 results would make sense when we consider the possibility that if the selection pressure
609 on the host to evolve in the absence of microbes is low, then the evolutionary response
610 can be slow and mild, as we observed in this study. We hypothesize that the results of
611 long-term experimental evolution can be explained by the so-called "evolutionary addiction
612 hypothesis" (Douglas 2018a; Moran et al. 2019; Hammer 2023). This hypothesis suggests
613 that when the microbiome is perturbed or culled entirely, hosts experience "withdrawal
614 symptoms" as the host and microbes have lived together for an extended period (Hammer
615 2023). Stated differently, the host can take care of itself in the absence of the microbes, it
616 is just that there is an inertia, borne out of very-long coexistence, towards the same. Thus,
617 when we remove the microbiome for the first time, the host goes in an altered state of
618 physiology as the host had evolved some dependence on the microbes that were always
619 present many generations prior to the manipulation. But, the host still had the capacity to
620 perform its function without the microbiome. Once several generations passed without the
621 microbes, the host adjusted itself to the absence of a microbiome without any major
622 evolutionary changes. This idea of evolutionary addiction might explain why this
623 experimental evolution happened under low selection pressure and why we haven't seen
624 major evolutionary changes in our experimental evolution so far.

625

626 **MBL_{1-4} might be reducing their dependence on the microbes as inferred by the**
627 **overall lesser phenotypic plasticity than the MBs**

628 For the phenotypic assays done in generations 54-57, the differential response of MBs and
629 MBLs in two different environments (with microbes vs. microbe-free) represents the
630 phenotypic plasticity of these two populations. We wanted to find out if losing microbial
631 partners for multiple generations has made the MBLs more independent of them. If MBLs
632 have indeed started becoming less dependent on their microbial partners, then we might

633 see that, across the two environments, MBLs might be phenotypically less plastic, as the
634 presence or absence of microbes would affect MBLs less than the MBs.

635 If there is only one trait under consideration, the phenotypic plasticity of two populations
636 can be compared using the slopes of the reaction norms for each population across the
637 environments: the greater the slope, the more the phenotypic plasticity. What we were
638 looking at was phenotypic plasticity *across multiple traits*. To calculate average phenotypic
639 plasticity across different traits, one needs a dimensionless (or a normalized) measure.
640 This is needed because slopes for different traits (e.g., mg of bodyweight, no. eggs laid,
641 percent survival) cannot be added as they have different units. We used two
642 normalized/dimensionless proxies of phenotypic plasticity that fit the above criteria and
643 then compared the mean phenotypic plasticity for the MB-MBL populations. These proxies
644 were (a) effect size using Cohen's d (Figure 5A) and (b) percent change (Figure 5B). The
645 results suggest that MBLs had lower phenotypic plasticity across with-microbes and
646 microbe-free environments. This observation, in turn, might hint that MBLs might be less
647 dependent on the microbes.

648

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

650 To see if MBLs have started showing differences in gene expression compared to MBs,
651 we performed an RNA-seq on one block of the populations, namely MBL_1 and MB_1.

652

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

654 Heat shock proteins are the proteins that are involved when the organism experiences
655 stress (Feder and Hofmann 1999). Even though, as their name suggests, they play an
656 important part primarily in the heat shock response (Lindquist 1986), some of these
657 proteins can be expressed in response to various other stresses such as cold, crowding,
658 anoxia, desiccation, etc., where these proteins can act as chaperones (King and MacRae
659 2015). We find that several HSPs, such as HSP23, HSP26, HSP67, and HSP68, and
660 subunits from HSPs, such as HSP67 and HSP70, are down-regulated in flies that were
661 maintained without the microbiome for multiple generations.

662

663 *RNA-Seq results on MBL_1 agree with the desiccation resistance data over all four replicate*
664 *MBLs*

665 Lower expression of HSPs in MBL_1 might hint at a muted stress response of the MBLs due
666 to the lower availability of HSPs to counter the stress. This correlation of stress resistance
667 with the expression of HSPs is well documented (Feder and Hofmann 1999). When we
668 looked at the desiccation resistance of MBL_{1-4} vs. MB_{1-4} in the with-microbes regime, we
669 found that MBL_{1-4} indeed had a lower desiccation resistance than MB_{1-4} for males (Figure
670 2C). For females, it comes close to statistical significance as well (Figure 3C). Both these
671 effects are large. While we haven't established a causal link in this trend of lower
672 desiccation resistance, this observation correlates the lower expression of HSPs to a
673 phenotypic readout of lower desiccation resistance.

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

681

682 *Antimicrobial peptides (AMPs) upregulated in MBL_1*

683 Antimicrobial peptides (AMPs) are small cationic molecules that interact with bacterial
684 membranes to damage and kill the bacteria (Brogden 2005). They are widespread across
685 the tree of life, with animals and plants harboring various types of AMPs to deal with
686 pathogens (Mookherjee et al. 2020). In *Drosophila* melanogaster, there are seven types of
687 AMPs whose functions are known, and a detailed discussion on them is available in
688 (Lemaitre and Hoffmann 2007; Hanson and Lemaitre 2020). In addition to their classical
689 role of defense against pathogens, these AMPs are also shown to control the host
690 microbiome (Franzenburg et al. 2013; Mergaert 2018; Marra et al. 2021; Maritan et al.
691 2024).

692 A study that looked at the gene expression changes in the *Drosophila* gut using
693 transcriptomics when the host is colonized with the microbes vs. it is kept without microbes
694 found that the presence of a microbiome leads to upregulated expression of several

695 immune response genes, including AMPs such as Attacin, Drosomycin and stress
696 response genes such as HSP23, HSP67Bb, and HSP70Bc (Broderick et al. 2014). While
697 their observation of the up-regulation of the immune response matches our results in
698 MBL_1, the HSP response is down-regulated in MBL_1, in contrast to the observation by
699 Broderick et al., which was done over the host's lifetime (Broderick et al. 2014). The up-
700 regulation of the immune system upon microbiome colonization is also reported in other
701 subsequent single-generational studies that are done over the host's lifetime (Elya et al.
702 2016; Chandler et al. 2022).

703 These gene expression studies discussed above on *Drosophila* within a host's single
704 generation show that the presence of a microbiome leads to AMP up-regulation compared
705 to when a microbiome is absent. In our case, both MBL_1 and MB_1 were given the same
706 native microbiome for a single generation after 54 generations of microbiome absence for
707 MBLs. As both MB and MBL received the same native microbiome from a common pool
708 (shown in Supplementary Text S5.1-S5.2), their different evolutionary history might be the
709 main driving force in the up-regulation of AMPs, not the differential presence of
710 microbiome.

711

712 *A hygiene hypothesis-like phenomenon can explain the overactive humoral immune*
713 *response in MBLs*

714 MBLs have been kept away from any microbes for more than 50 generations. Several
715 studies have shown that microbes are indispensable for the proper calibration and
716 modulation of the immune system, especially early in life (Mazmanian et al. 2005; Abt et
717 al. 2012; Selosse et al. 2014; Gensollen et al. 2016; Knoop et al. 2017; Henrick et al.
718 2021; Hoang and King 2022; Metcalf et al. 2022; Donald and Finlay 2023). This calibration
719 includes proper sorting of self vs. non-self (Lee 2008; Lhocine et al. 2008). There is a
720 possibility that MBLs might have lost the delicate balance that maintains this calibration
721 and, as a result, are over-expressing AMPs compared to MBs to actively regulate the
722 microbiome.

723 In humans, this phenomenon is often discussed in the context of the hygiene hypothesis
724 (or a refined version of the same, called the old friend hypothesis) (Wills-Karp et al. 2001;
725 Bach 2018; Blackwell 2022). This hypothesis states that exposure to certain
726 microorganisms, particularly in early life, can reduce the frequency of atopic diseases and

727 immune-mediated disorders in organisms (Stiemsma et al. 2015). It is possible that the
728 observed increased levels of AMPs signify immune overactivation via immune
729 miscalibration over many generations. It is already known that *Drosophila* can suffer from
730 autoimmune disorders (Mortimer et al. 2021). Therefore, if our conjecture about the
731 hygiene hypothesis is true then one can predict that the MBLs would be suffering from
732 higher instances of auto-immune disorders, something that we would like to investigate in
733 detail in a future study.

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

736

737 **Conclusion**

738 Contrary to our expectation, removing microbes for the extended period of 54 host
739 generations did not result in prominent evolutionary changes in the host. This might be due
740 to weak evolutionary pressure on the host and can be explained using the "evolutionary
741 addiction hypothesis." This might also mean that, in the case of outbred *Drosophila* as the
742 host, the "host + microbe" unit (as a whole) may not be well-integrated on evolutionary
743 timescales as proposed by the hologenome theory of evolution. This has implications for
744 how host-microbe interactions are viewed and modeled. While in a given host, certain
745 traits can be more affected by the microbes (e.g., egg-to-adult survival in our case), certain
746 other traits may not show this functional integration with microbes (e.g., developmental
747 time in our case) and, hence, remain unaffected after microbiome removal. So, instead of
748 focusing on the entire "holobiont" as an independent entity, we think looking at the
749 functional integration between host and microbes trait-by-trait might be of greater value to
750 appreciate the microbiome's role in host evolution.

751 ---

752

753

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755

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Supplementary information

Part 1 – Figures (S1 to S3) – Total 3
Part 2 – Text (S1 to S8) – Total 8

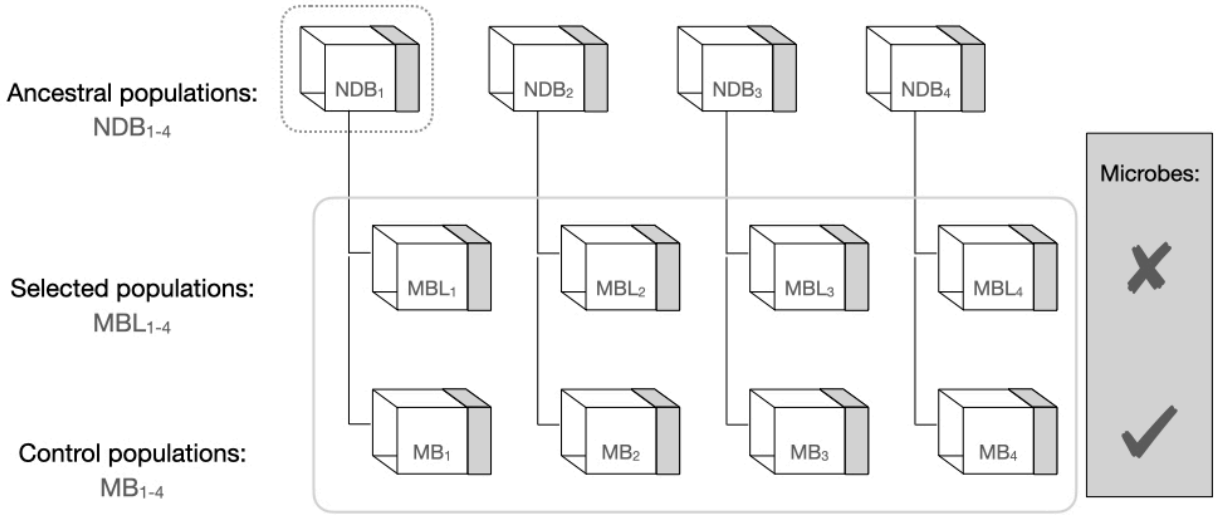

Figure S1. Details of the ancestral and experimental evolution populations.

991

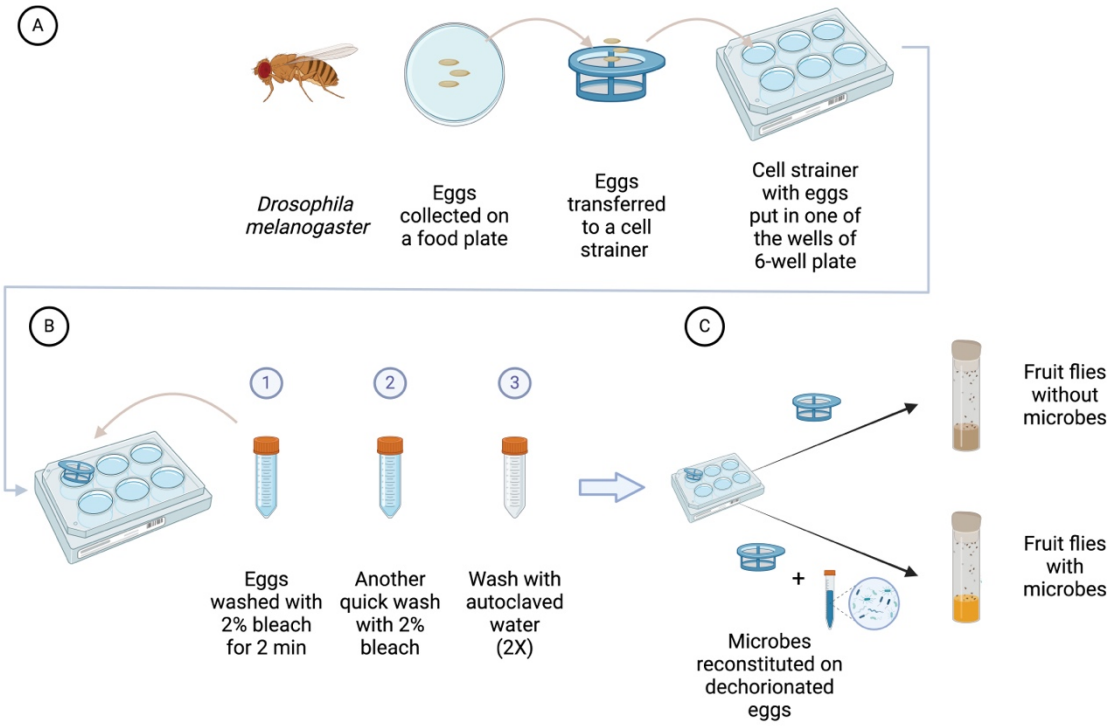

Figure S2. Egg dechorionation protocol using bleach to make MBL_{1-4} and MB_{1-4} populations. Created in BioRender.com.

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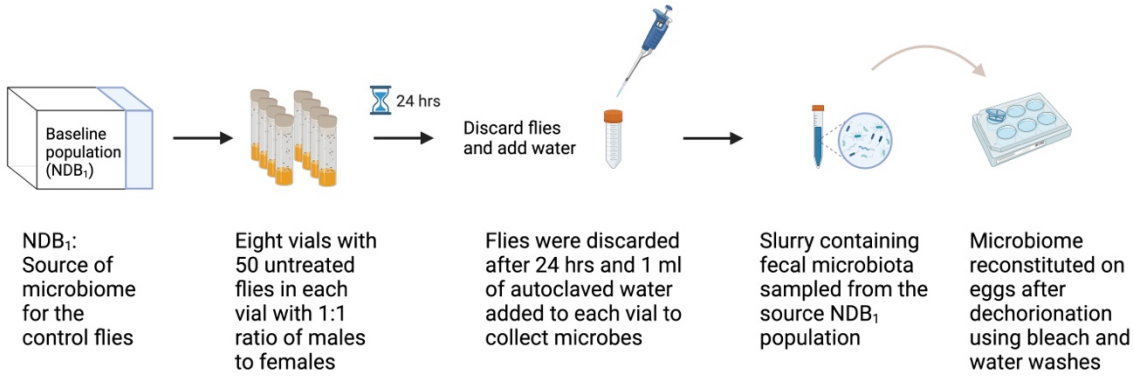

Figure S3. Detailed protocol of microbiome reconstitution from NDB_1. Created in BioRender.com.

1002 **Text S1 Details of the baseline populations and amplicon sequencing**
1003

1004 **S1.1 *Drosophila* NDB stocks and rearing**

1005 Wolbachia-free *Drosophila* melanogaster used in the single-generation study were derived from
1006 four large, outbred laboratory populations named New Dey Baselines (NDB_{1-4}). These fly lines
1007 were maintained on the standard banana-jaggery food with a 14-day discrete generation cycle at
1008 250C under constant light conditions with a breeding population of ~2400 individuals. NDBs trace
1009 their ancestry to the DB populations (see Supplementary Text S1.3 for the details), which in turn
1010 trace back to the wild-caught IV lines (Ives 1970; Rose 1984) via the JB_{1-4} populations (Sheeba et
1011 al. 1998).

1012

1013 **S1.2 Maintenance regime of baseline *Drosophila* populations (NDB_{1-4})**

1014 In the lab, adult *Drosophila* of four baseline populations (NDB_{1-4}) are maintained in plexiglass
1015 cages (25 cm x 21 cm x 16 cm) at a large population size of ~2400 per population. On day 12
1016 (after egg deposition), the fly population is provided with a moist yeast paste on top of the banana-
1017 jaggery food to boost egg output. This yeast paste also contains a few drops of glacial acetic acid
1018 to attract the flies to the paste. At the end of day 13, a food plate, with vertical edges of food
1019 exposed for oviposition, is provided for 12-16 hrs. On day 14, deposited eggs are collected by
1020 cutting thin food strips from the exposed surface, under a binocular stereo microscope. The size of
1021 the strips is adjusted under the microscope using a clean scalpel such that each strip contains
1022 about 400 eggs. These strips are then transferred to plastic and translucent *Drosophila* milk bottles
1023 containing ~50-60ml of banana-jaggery food at the bottom. This marks the new generation's 0th
1024 day after egg deposition. Adult flies arising from six such bottles are transferred to fresh plexiglass
1025 cages on the 12th day (post egg collection). This gives rise to a new generation with a population
1026 size of ~2400 individuals, which do not coexist with the adults of their parental generation at any
1027 stage.

1028

1029 **S1.3 *Drosophila* DB stocks and rearing**

1030 NDB_{1-4} were derived from their most recent ancestors in our lab, Dey Baselines (DB_{1-4}) (Sah et al.
1031 2013). These four DBs were maintained in our lab for 220 generations and were used as baselines
1032 for this period. We pooled an equal proportion of eggs from each DB population and created four
1033 independent NDBs. We maintained NDBs separately for ~10 generations without any assays for
1034 them to stabilize after this intermixing. The maintenance of DBs was similar to NDBs except for two
1035 differences: (a) DBs were maintained in Drosophila 8-dram vials at an egg density of ~ 60 per vial
1036 with ~ 6ml of food as opposed to NDBs that are reared in milk bottles (b) DBs were yeasted on
1037 18th day, provided with a cut plate on 20th day, and their eggs were collected for next generation
1038 on day 21. Thus, DBs followed a 21-day discrete generation cycle as opposed to a 14-day discrete
1039 generation cycle followed by NDBs.

1040

1041 **S1.4 Population-level 16S rRNA amplicon sequencing**

1042 To know the relative microbiota composition in *Drosophila* populations, DNA was extracted as
1043 described in section S3.2.2. DNA was run on 1% agarose gel to see the extraction quality. After
1044 the integrity check on agarose gel, DNA was sent for 16S rRNA V3-V4 region amplicon
1045 sequencing to a commercial vendor (1st BASE Laboratories, Malaysia). Sample quality was again
1046 confirmed by the vendor using agarose gel, spectrophotometer, and fluorometric method. All
1047 samples passed DNA quality check and were subjected to amplicon PCR followed by a quality
1048 check. These samples were then processed to prepare amplicon libraries using 2-step PCR
1049 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. Then, the forward and reverse reads were merged using QIIME. DADA2 pipeline (https://benjjneb.github.io/dada2/) 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 (https://scikit-learn.org/stable/) 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).

1063 **Text S2 - Note on the use of F1 flies for the assays**

1064 In our flies, we removed microbes via dechorionation at the egg stage. Even if we have taken care
1065 of the confounding effect of bleach by using proper control, we wanted to keep the effect of the
1066 bleach on flies to a bare minimum (ideally none). To that end, we employed an additional measure
1067 of using F1 flies for the assays, as explained below (Supplementary Figure S4).

1068 In this approach, to generate flies without the microbes, we followed a protocol employed by
1069 several other studies where F1 flies (progeny of the flies that experienced the bleach at the egg
1070 stage) were used in the assays (Pais et al. 2018; Selkrig et al. 2018; Lee et al. 2019). This ensured
1071 that the F1 assay flies of both types, microbiome-reconstituted (M+) and microbe-free (M-), did not
1072 experience bleach in their lifetime (their respective parent population did). To generate F1 M-
1073 assay flies, F1 eggs were collected from the flies without microbes (i.e., F0 M- flies, Supplementary
1074 Figure S4) and were raised in aseptic conditions, ensuring the absence of microbes. To generate
1075 control F1 M+ assay flies, F1 eggs were collected without any manipulation from the flies whose
1076 microbiome was reconstituted (i.e., F0 M+ flies, Supplementary Figure S4).

1077 In our conventional/untreated baseline lab populations, about 86 percent of the flies survive till
1078 adulthood. With the approach of using the F1 flies we described, control F1 assay flies whose
1079 microbiome was reconstituted (i.e., M+ flies) showed just an additional ~5 percent mortality,
1080 showing that the overall mortality is kept to a minimum.

1081

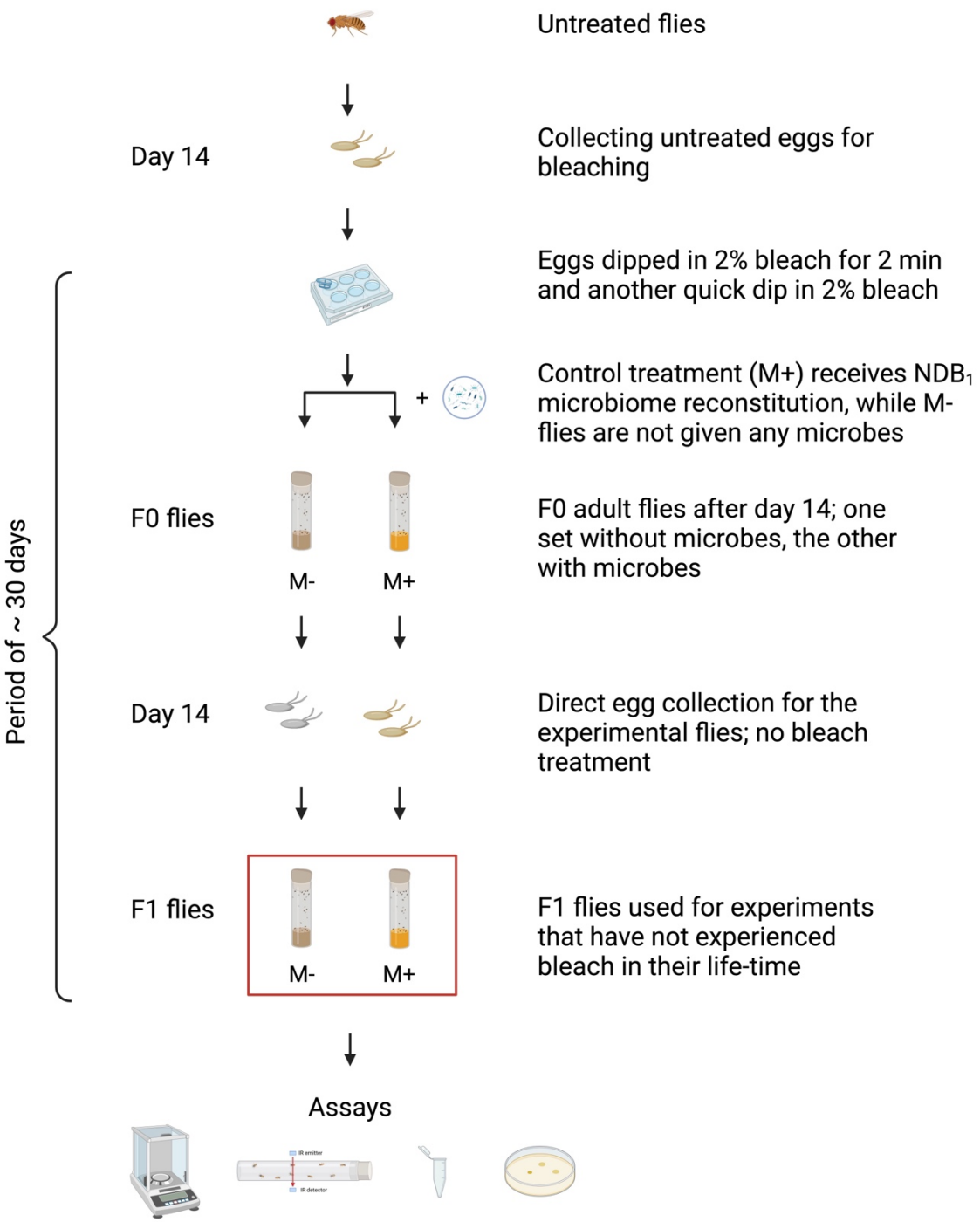

Figure S4. Illustration of the full experimental design to study microbiomes' contribution to host phenotypes. To create two types of flies (with the minimal effect of bleach), flies with microbes (M+) and without them (M-), we combined two procedures. First, we chose microbiome-reconstituted flies as 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.

1082

1083 **Text S3.1 - Assessing the microbiome status of MBs and MBLs during experimental**
1084 **evolution**
1085
1086 We plated adult flies from MBL_{1-4} every generation to see if the microbiome is indeed absent in
1087 these populations (data not shown here). Except for generations and blocks specified in
1088 Supplementary Table S1, in all other generations, MBLs did not show the presence of a
1089 microbiome. We sometimes did observe ~1-2 colonies (per 10 flies plated) in some replicates.
1090
1091 **Table S1. Generations in which MBLs showed transient contamination**
1092

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

1093
1094 MB_{1-4} were plated every 2-3 generations and CFUs were counted to make sure they received the
1095 reconstituted microbiome (data not shown here).
1096
1097 In generation 40 of the selection line, we performed PCR with 16S rRNA universal primers
1098 (Supplementary Text S3.2.2) on DNA extracted from adult MB_{1-4} and MBL_{1-4} females (day 15 after
1099 egg collection). In Supplementary Figure S5, we see that MB_{1-4} show the presence of the
1100 reconstituted microbiome, and the microbiome is absent from MBL_{1-4}. This also confirms that, by
1101 generation 40, the contamination that we saw in MBLs in previous generations was completely
1102 gone.
1103

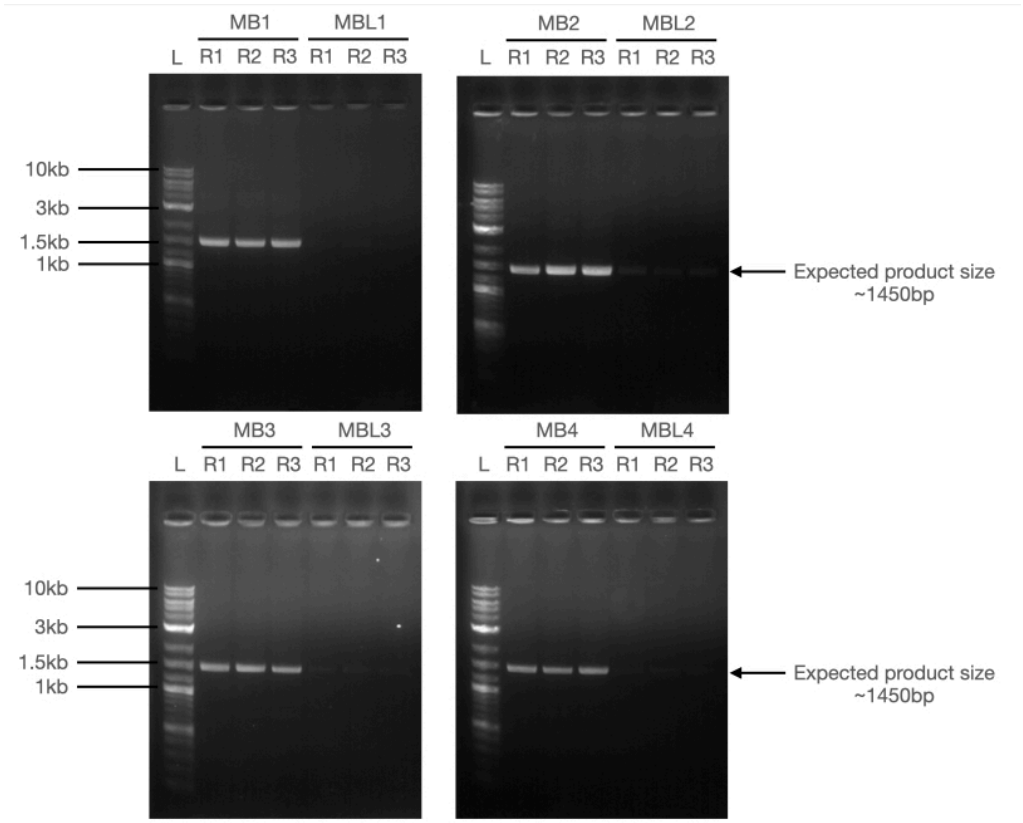

Figure S5. 16S rRNA PCR to check the status of microbiome in MB_{1-4} and MBL_{1-4} .
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1108 **Text S3.2 Fly-associated bacterial load analysis**

1109 **S3.2.1. Culture-dependent method**

1110 Adult flies (day 12 after egg deposition, unless stated otherwise) were sorted by sex under mild
1111 CO_2 anesthesia. Five male and five female flies under anesthesia were surface sterilized with 70%
1112 ethanol in 1 ml microcentrifuge tubes. These flies were then homogenized in 200 μl of autoclaved
1113 dH_2O and plated on the nutrient agar (HiMedia) or the de Man, Rogosa, and Sharpe (MRS)
1114 medium (HiMedia) and incubated for 72 hrs and 48 hrs, respectively, at 25^0C under aerobic
1115 conditions. Dilutions with 10-100 colonies were counted, and the counts were multiplied by the
1116 overall dilution factor to estimate colony-forming units (CFUs) per fly.

1117 **S3.2.2. Culture-independent method**

1118 To test the bacterial load using PCR, total DNA was extracted from eight to ten adult flies using the
1119 QIAamp DNA Mini kit (Qiagen, Catalogue No. 51304) as per the manufacturer's instructions. We
1120 modified three steps of the manufacturer's protocol in line with the protocol (Jehrke et al. 2018).
1121 First, we used mechanical homogenization (with a homemade handheld motorized pestle mixer) to
1122 lyse the tissue in 1.5ml Eppendorf tubes instead of just Proteinase-K treatment. Second, 20 μl of
1123 20 mg/ml RNase A (HiMedia, Catalogue No. DS0003-5ML) was added to each sample after tissue
1124 lysis. Third, the sample was incubated with Buffer AL at 70^0C for 30 min instead of 10 min. We
1125 avoided any vortex step after tissue lysis to minimize the shearing of the DNA. The quality of total
1126 DNA was checked on the agarose gel. This DNA, composed of the fly and microbial genomes, was
1127 used as a template for PCR using 16S bacterial universal primers with a product size of about
1128 1.5kb. The details of the primers are given below in Supplementary Table S2. The primers were
1129 ordered from Sigma-Aldrich, India. The PCR reactions were set up using GoTaq® Flexi DNA
1130 Polymerase (Promega, Catalogue No. M829) with 40 ng gDNA input. The initial denaturation was
1131 at 94^0C for 2 min, followed by 30 cycles of (denaturation at 94^0C for 30s, annealing at $T_m = 60^0$C
1132 for 1 min, extension at 72^0C for 1 min), with final extension 72^0C for 10 min. The PCR products
1133 were visualized on 1% agarose gel with 1kb plus DNA ladder (NEB, Catalogue No. N3200S) as the
1134 molecular weight marker.

1135 **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)

1136
1137

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 in 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 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 plastic effects across two assay environments (with-microbes and microbes-free) on both of these populations were more significant than the effects of the selection (Figures S11-S13). (By plastic effects, we mean differences seen in a phenotype when the environment changes without a change in host genotype). However, as with-microbes and microbe-free flies were generated in separate host generations (see Supplementary Table S3), we couldn't compare these plastic effects quantitatively with this design. We amended our experimental design so that these plastic effects could be compared directly by conducting the assays in the two environments simultaneously. Supplementary Table S4 shows the details of our experimental plan for the set of assays conducted in 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 plastic 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

1215 **Text S5.1 - Validation of microbiome status in the flies raised in two different assay**
1216 **environments**

1217 **S5.1.1 By plating fly homogenates**

1218 In the with-microbe environment, on both MRS and NA media, we see the presence of microbes (
1219 Supplementary Figure S6, Set-B and Set-C). Set-A is the autoclaved water that is used to
1220 homogenize the flies. The serial dilutions with ~10-100 colonies were counted to get the CFUs/fly.
1221 While Supplementary Figure S6A shows the representative picture from block 1, CFUs/fly for all
1222 four blocks of MBs and MBLs are compared using paired t-test on block means in Figure S7.
1223 In the microbe-free environment, both MB_1 (Supplementary Figure S6B) and MBL_1 (Supplementary
1224 Figure S6C) show the absence of the microbiome. While block 1 plates do not have any microbes,
1225 other blocks show a few colonies in some replicates (data not shown here).
1226

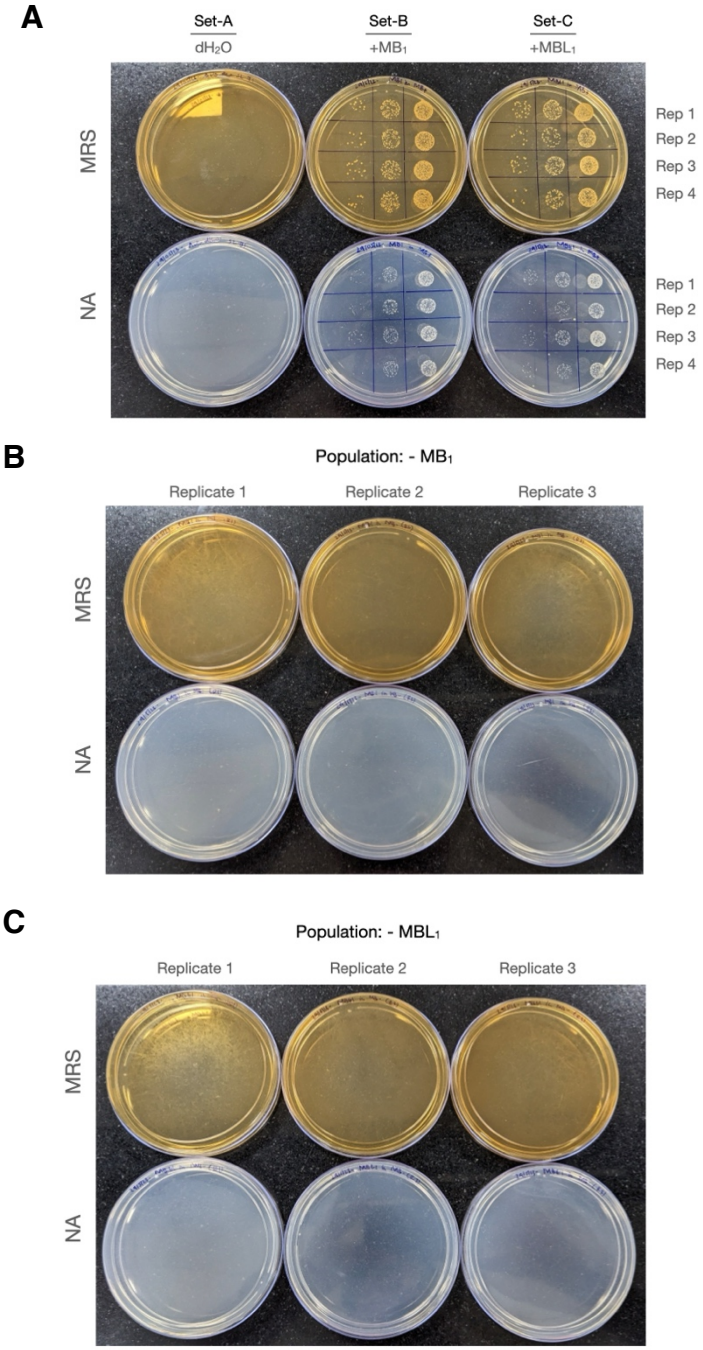

Figure S6. MRS and NA plates showing presence of microbes in with-microbe environment and their absence in microbe-free environment. (A) MB_1 and MBL_1 in with-microbe environment (n = 4). The spots show different serial dilutions used to calculate CFUs/fly.

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).

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1229 **S5.1.2 MBs and MBLs received similar microbial loads in the with-microbe environment**
1230 **(Gen 54-57 assays)**
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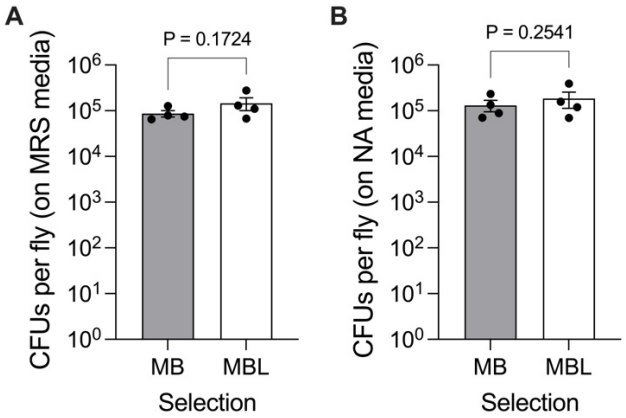

Figure S7. Bacterial load in the MB_{1-4}-MBL_{1-4} flies (with-microbe environment) as determined by plating fly homogenates. CFUs/fly on (A) MRS-agar (B) NA-agar. The bacterial load in MBs vs. MBLs is compared using paired t-test on block means. Block means are indicated by black filled circles. The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

1232
1233
1234 **S5.1.3 PCR showing the presence of microbiome in the with-microbiome environment and**
1235 **depletion of the microbiome in microbe-free environment**
1236
1237 To validate the status of the microbiome in both environments with a culture-independent method,
1238 we extracted DNA from the four types of populations (Main text, Figure 1B). We performed PCR
1239 with 16S rRNA universal primers (details in Supplementary Text S3.2). For PCR, a subset of flies
1240 used for the assay on day 12 were flash-frozen in liquid nitrogen and kept at -80^{0}C for further use.
1241 For the with-microbe environment, 12 female flies from only MB_1 and MBL_1 (block 1) were used for
1242 the DNA extraction. For the microbe-free environment, total DNA was extracted from 12 female
1243 flies (3 flies per block, four such blocks). The PCR was done with 40ng of input DNA with 30
1244 cycles.
1245
1246 In the with-microbe environment, MB_1 and MBL_1 showed the presence of microbes as expected
1247 (Supplementary Figure S8). In the microbe-free environment, while two replicates have no band,
1248 one replicate each from pooled MB_{1-4} and MBL_{1-4} contains a faint band. This faint band might be
1249 indicative of a few colonies that show up on NA and MRS-agar plates, even after aseptic handling
1250 (data not shown). This signal might get picked up due to saturating PCR conditions. As we are not
1251 bleaching assay flies or using any antibiotics to avoid their toxic effects, we are essentially
1252 maintaining flies without any chemicals for about two consecutive generations, i.e., for ~30 days
1253 (Supplementary Figure S4). So, there might be a tradeoff between the efficiency of keeping all the
1254 microbes away without any chemicals and how long that efficiency can be sustained without any
1255 chemical agent. Another possibility is that the signal is from very few non-culturable bacteria.
1256
1257 These results match the observations in the Supplementary Text S5.1.1 obtained after plating flies
1258 on NA and MRS. Together, the evidence showed that both MB-MBL populations in a with-microbe
1259 environment harbor microbiome and in microbe-free environments do not have microbes with
1260 them.
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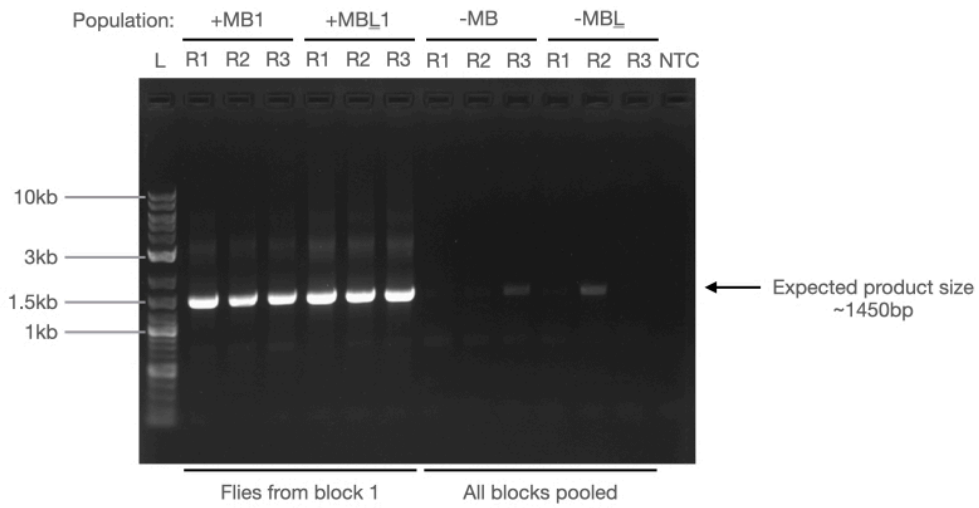

Figure S8. PCR on MB_1 and MBL_{1-4} to confirm the status of microbiome.

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1265 **S5.2 Validation of microbiome composition of flies raised in with-microbes assay**
1266 **environment**
1267
1268 **S5.2.1 MB-MBL generation 17-20 assays**
1269
1270 In the previous section, through plating fly homogenates and PCR, we showed that flies raised in
1271 the with-microbes environment indeed have the microbes with them. However, the PCR only
1272 indicates that microbes are present - it does not tell us the composition of the reconstituted
1273 microbiome. In Supplementary Figure S7, we have established that the number of microbes
1274 received by MB and MBL populations is similar. In this section, we test if the reconstituted
1275 microbiome composition is similar for MB-MBLs, and this, in turn, matches the source microbiome
1276 composition of NDB_1.
1277
1278 To validate that the composition of the reconstituted microbiome of all four blocks (MB_{1-4} and MBL_{1-}
1279 $_{4}$) reflects the native NDB_1 composition, we performed amplicon sequencing on the V3-V4 region of
1280 the 16S rRNA gene (Supplementary Figure S9, method details in Supplementary Text S1.4). Three
1281 biological replicates were processed per population, giving us 24 samples (8 populations: 4 MBs +
1282 4 MBLs, 3 replicates each). The native source pool of NDB_1 (at Gen 56) was also sequenced.
1283 Thus, Supplementary Figure S9 shows a total of 27 samples (24 from MB_{1-4}-MBL_{1-4} and 3 from
1284 NDB_1).
1285
1286 The MB_{1-4}-MBL_{1-4} populations also mirror this broad microbiome composition of NDB_1 with the
1287 dominance of *Acetobacter* spp. but with small fluctuations across MB-MBLs in *Acetobacter* and
1288 *Lactobacillus* fractions (Supplementary Figure S9). (In 2020, the old genus *Lactobacillus* was
1289 reclassified into 25 new different genera. *Lactiplantibacilus* and *Levilactobacilus* both belong to the
1290 old composite *Lactobacillus* genus.)
1291

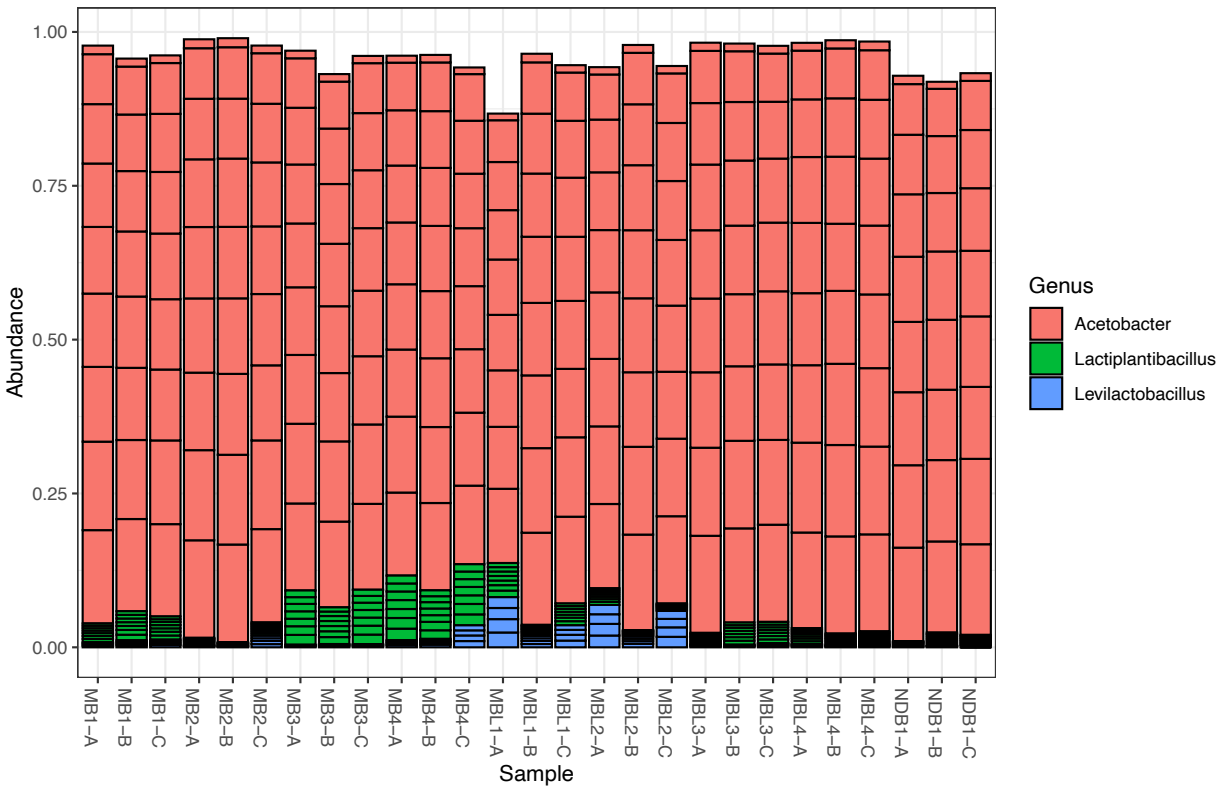

Figure S9. Validation of microbiome composition after reconstitution in Gen 17-20 assays. The result of population-level 16S rRNA amplicon sequencing across four replicate populations of MB_{1-4} and four replicate populations of MBL_{1-4}. Native microbiome composition of source population NDB_1 is 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.

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1294 **S5.2.2 MB-MBL generation 54-57 assays**
1295
1296 In generation 54-57 assays, we sequenced microbiomes from MB_1 and MBL_1 that were raised in
1297 the with-microbes environments (Supplementary Figure S10). We see that MB_1 and MBL_1 have
1298 received microbiomes that are very close in composition. *Acetobacter* spp. dominates the
1299 microbiomes of both populations.
1300
1301 Together, these sequencing results for generations 17-20 assay and for generations 54-57 assay
1302 show that microbiome composition received by MBs and MBLs is broadly similar to each other and
1303 to the source microbiome of NDB_1 (Supplementary Figure S9-last three lanes).
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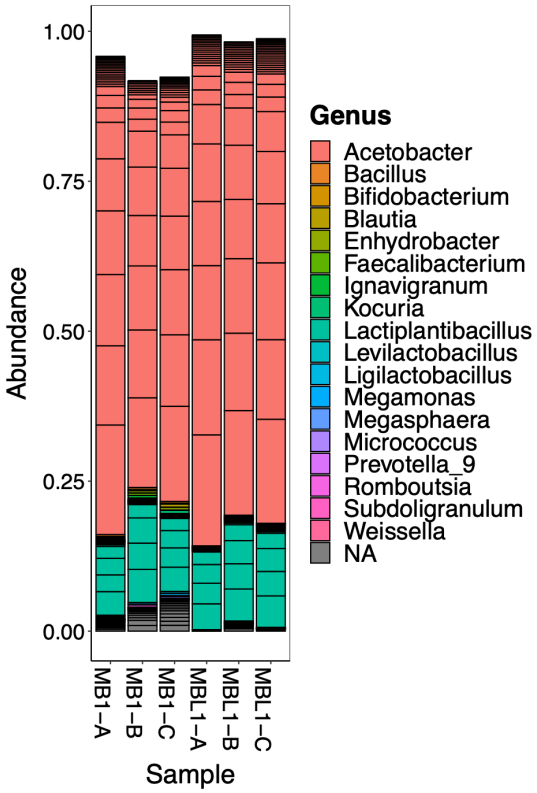

Figure S10. Validation of microbiome composition after reconstitution in Gen 54-57 assays. Most abundant 100 taxa are shown for each population. For each population, three independent replicates where processed (labelled “A”, “B”, “C”). Each replicate consisted of pooled DNA extracted from surface sterilized 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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1309 **Text S6 - First phenotypic assessment of experimental evolution lines at host generations**
1310 **17-20**
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1312 **S6.1 Male traits in host generations 17-20**
1313
1314 None of the four phenotypes tested for males – adult body weight, egg-to-adult development time,
1315 desiccation resistance, and locomotor activity- showed an effect of selection as assessed by the
1316 paired t-test over block means (Supplementary Figure S11A-D). Supplementary Tables S5 and S6
1317 provide details of the statistical comparison for the with-microbe assay environment and microbe-
1318 free assay environment, respectively.
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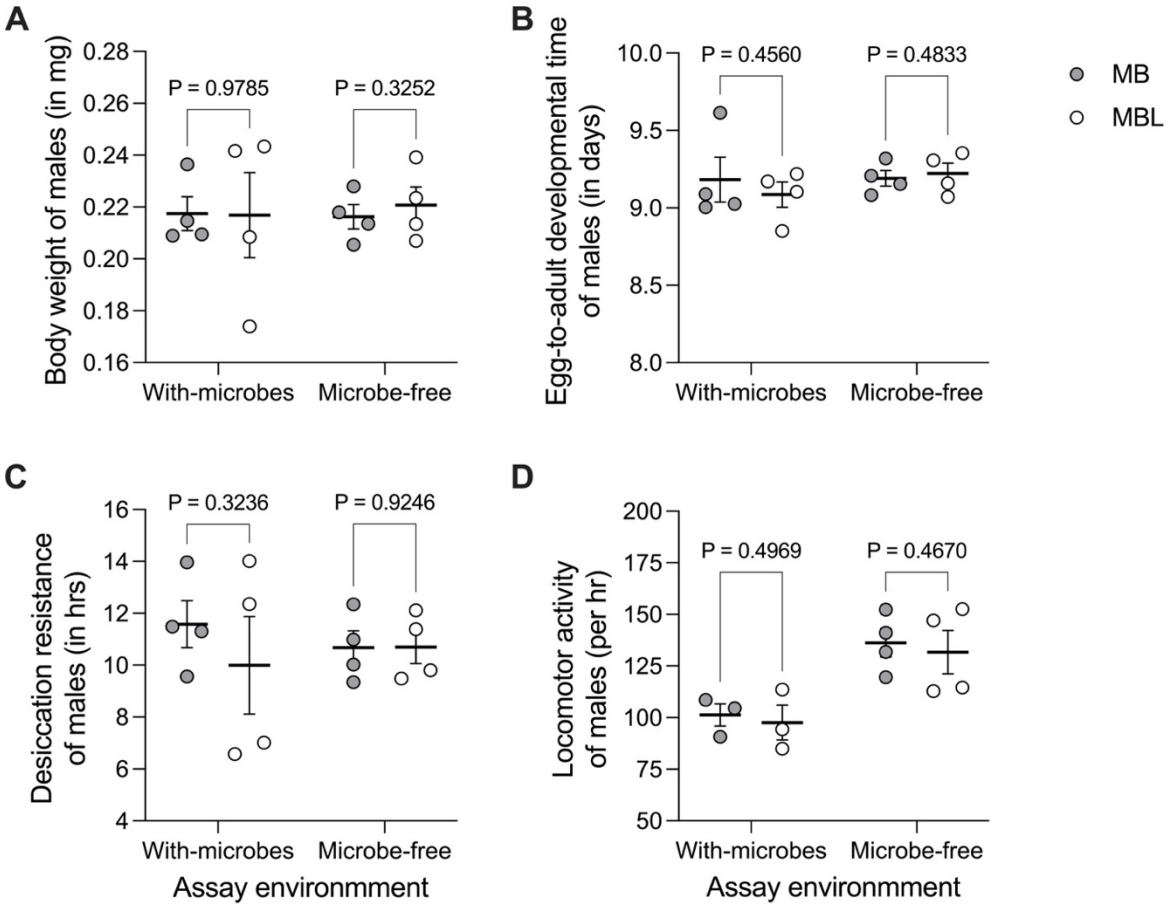

Figure S11. Male traits assessed in Gen 17-20 in with-microbe and microbe-free environments. (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations 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.

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1328 **S6.2 Female traits in host generations 17-20**
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1330 In the case of females, egg-to-adult development time, desiccation resistance, and locomotor
1331 activity didn't differ between MBs and MBLs (Supplementary Figure S12A-D) (Supplementary
1332 Tables S5 and S6). The only trait in females that showed some difference between the MBs and
1333 the MBLs was female body weight in the microbe-free environment (Paired t-test, $t_3 = 3.05$, P =
1334 0.0554, Cohen's d = 0.81 (large), Supplementary Figure S12A). Here, although the p-value is
1335 slightly larger than our pre-determined level of statistical significance (i.e., 0.05), we still interpret
1336 this trait as the effect size was found to be large.
1337

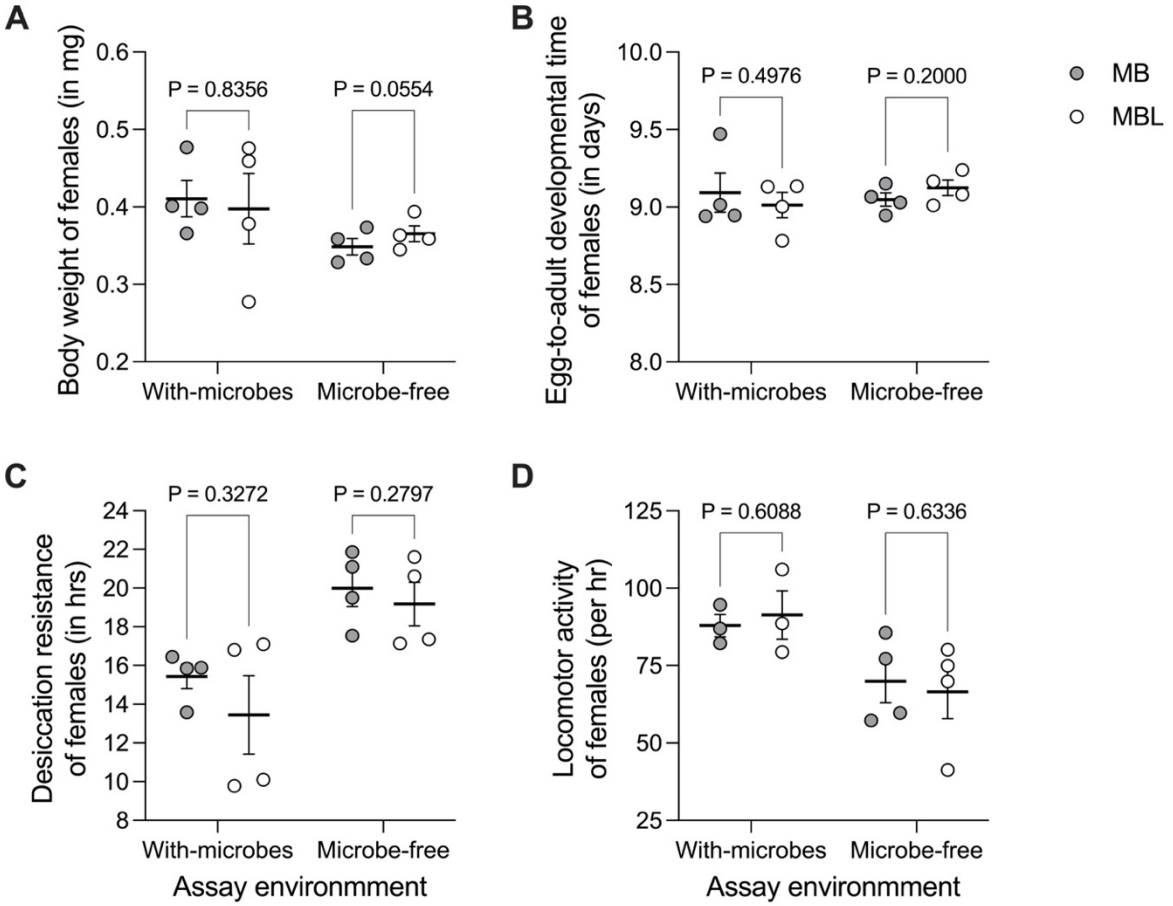

Figure S12. Female traits assessed in Gen 17-20 in with-microbe and microbe-free environments. (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control 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.
1338

1339 **S6.3 Pair traits in host generations 17-20**
1340
1341 In traits where both males and females were involved in the assays - percent survival to adulthood,
1342 female fecundity over 12 hrs (assayed in the presence of a male), and mating latency - did not
1343 show any difference (Supplementary Figure S13A-C, Supplementary Tables S5 and S6). The
1344 mating time for MBL_{1-4} was lower than MB_{1-4} in the with-microbes environment (Paired t-test, t_3 =
1345 3.67, P = 0.0349, Cohen's d = 2.35 (large), Supplementary Figure S13D).
1346

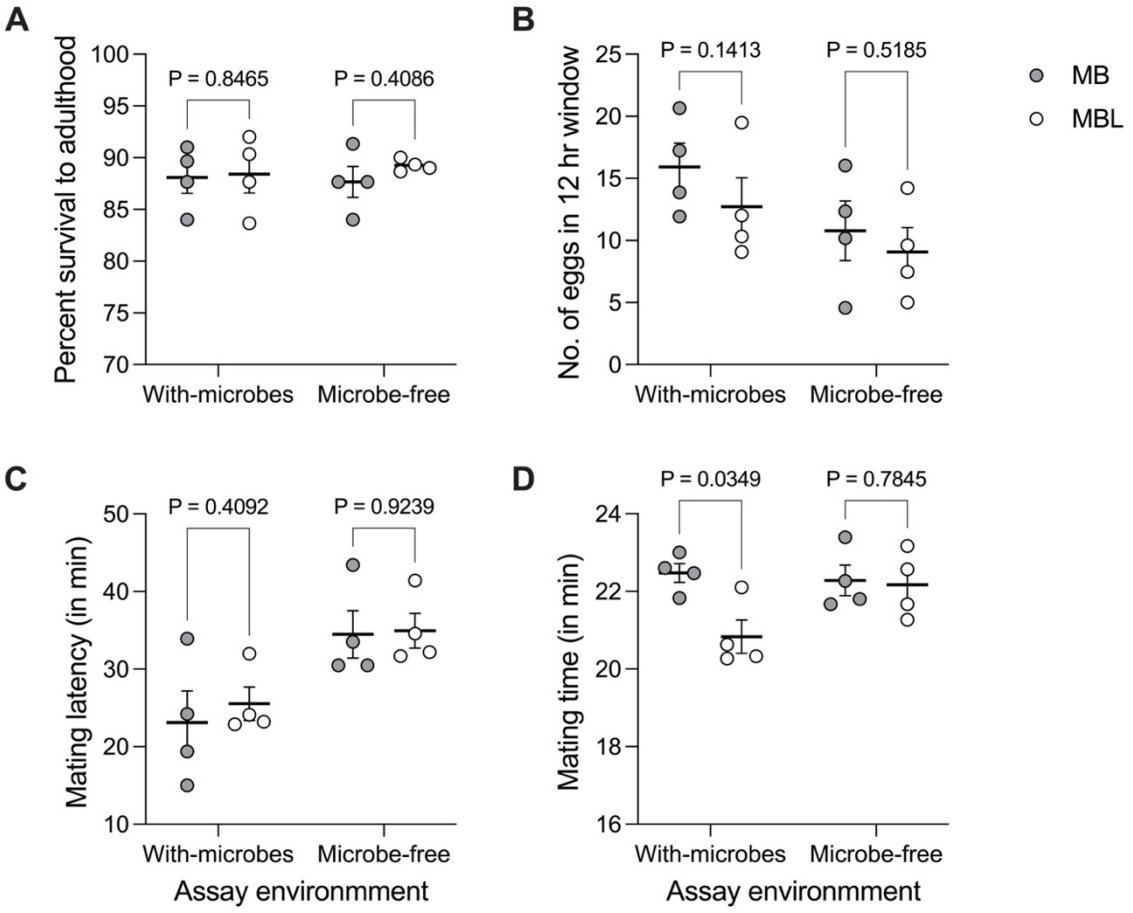

Figure S13. Pair traits assessed in Gen 17-20 in with-microbe and microbe-free environments. (A) Survival to adulthood. (B) Female fecundity. (C) Mating latency. (D) Mating time. In each assay environment, four filled circles are means for each of the four control populations MB_{1-4} and similarly, 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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1350 **Table S5.** Summary of all assays in the with-microbes environment (Gen 17-20). T refers to the
1351 grand mean of the MBLs, while C refers to the grand mean of the MBs.
1352

	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

1353
1354 **Table S6.** Summary of all assays in the microbe-free environment (Gen 17-20). T refers to the
1355 grand mean of the MBLs, while C refers to the grand mean of the MBs.
1356

	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

1357

*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.

1366 **Table S7.** Summary of all assays in the with-microbes environment (Gen 54-57). T refers to the
1367 grand mean of the MBLs while C refers to the grand mean of the MBs.
1368

	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

1369
1370 **Table S8.** Summary of all assays in the microbe-free environment (Gen 54-57). T refers to the
1371 grand mean of the MBLs while C refers to the grand mean of the MBs.
1372
1373

	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. Also, this is a sample size per block, i.e., each circle in the plots is an average over this sample size.

1383 **Text S7 – RNA-Seq result details**
1384
1385 **S7.1 No major changes in the gene expression profile of MB_1 vs MBL_1 in the microbe-free**
1386 **environment**
1387
1388 In the microbe-free regime, where both selected and control populations were assayed without
1389 their microbiome, only a few transcripts showed differential expression when analyzed using
1390 DESeq2 (Supplementary Figure S14). Only the significant genes with Log_2(Fold Change) > 1.5 and
1391 adjusted P-value < 0.05 are highlighted in Figure S14. Except for nAChRbeta2, which is a nicotinic
1392 acetylcholine receptor subunit, and CG13617, which codes for cilium assembly protein DZIP1 (zinc
1393 finger transcription factor), the functions of the other genes (CG14625, CG12693) are not known in
1394 *Drosophila melanogaster*.
1395

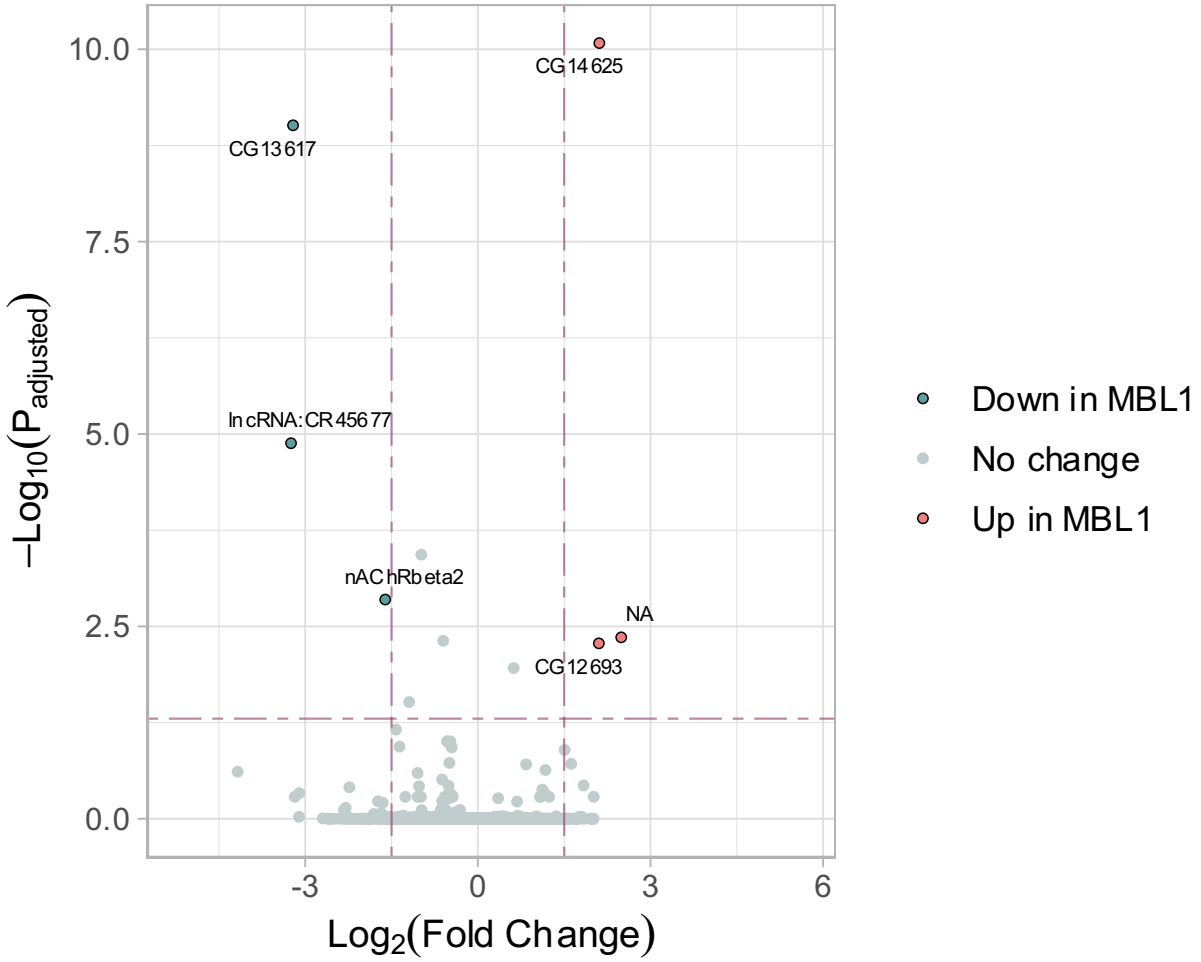

Figure S14. Differentially expressed genes in MBL_1 vs MB_1 in the no-microbe environment using DESeq2. The red dots represents the up-regulated genes, while blue dots represent down-regulated genes in MBL_1. The protocol for the RNA-Seq is described in the method section of the main text.

1396

1397 **S7.2 List of differentially regulated genes in with-microbe assay environment**
1398
1399 **Table S9. 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

1400
1401
1402
1403 **Table S10. List of down-regulated genes in MBL_1 in RNA-Seq (with-microbes environment)**
1404

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	-

S7.3 GO analysis of differentially regulated genes in the with-microbe assay environment

To see the biological processes these genes are involved in, we used the ShinyGo tool (version 0.80) (http://bioinformatics.sdstate.edu/go/) for gene ontology enrichment analysis with 'GO biological process' pathway database. We found that the upregulated genes belong to the pathway involved in the fly's humoral immune response (Figure 4.21), while down-regulated genes belong to the stress response pathway (Figure 4.22).

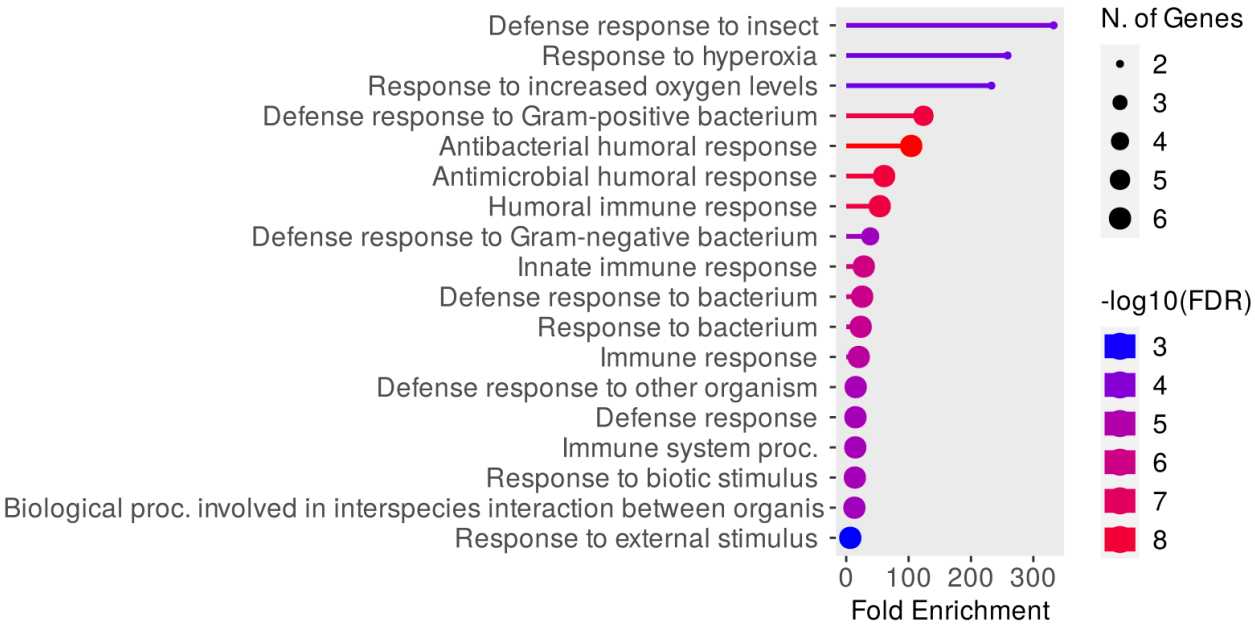

Figure S15. Gene ontology (GO) enrichment analysis for 12 Up-regulated genes.

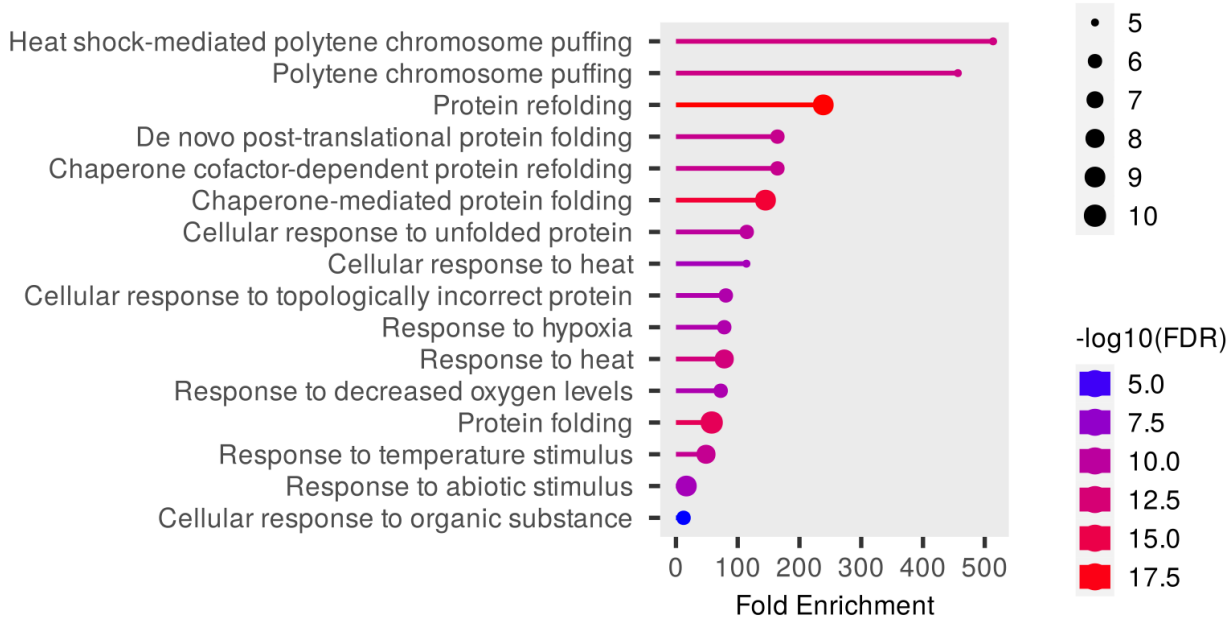

Figure S16. Gene ontology (GO) enrichment analysis for 24 down-regulated genes.

1415 **Text S8 - Another set of differently expressed genes hints at the peritrophic matrix**
1416 **(PM) as a potential region of interest in the MBLs**
1417
1418 Along with HSPs and AMPs, another set of genes that have an altered expression are chitin-
1419 interacting genes. CG7298 and CG32284 are up-regulated chitin-binding enzymes in MBL1, and
1420 down-regulated CG10140 is a probable chitinase. As the RNA-seq is done on the full body of flies,
1421 it is difficult to predict what this means for the fly. But, the annotation of CG7298 in FlyBase offers a
1422 clue. CG7298 is expressed in the proventriculus, which is part of the fly digestive system. A recent
1423 study supports the notion that proventriculus is the site where gut bacteria stably interact with flies,
1424 possibly through interactions with N-acetyl glucosamine, which is a building block of chitin (Dodge
1425 et al. 2023). Moreover, Drosophila melanogaster CG7298 has an ortholog called peritrophin-48 in
1426 related Drosophila species, which is a component of the peritrophic matrix. The peritrophic matrix
1427 is a chitinous layer that separates food bolus from the gut epithelia. This is also implicated as a
1428 physical barrier that keeps gut bacteria away from the gut tissue (Kuraishi et al. 2011; Charroux
1429 and Royet 2012; Rodgers et al. 2017; Erlandson et al. 2019). Taking this all together, we think this
1430 is the region where chitin-interacting proteins are carrying out various processes that might be of
1431 interest. However, the limited available information about these hits prevents us from drawing any
1432 further conclusions at this point in time. In the future, we plan to confirm all the RNA-seq hits and
1433 do additional experiments that can give us more insights.
1434
1435 In a recent study, Kathedar et al. showed that the nicotinic acetylcholine receptor (nAchR)
1436 regulates gut barrier function in Drosophila through the remodeling of the peritrophic matrix
1437 (Katheder et al. 2023). We have seen that in our no-microbe assay regime, nAchRbeta2 gene
1438 expression showed down-regulation in MBL1. This raises the possibility that the processes of
1439 interest happening at the proventriculus that involve the peritrophic matrix might be mediated
1440 through nAch signaling.
1441
1442

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