GxGxE interactions and adaptive potential of the microbiome in *Drosophila melanogaster*

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Abstract

The microbiome may serve as a reservoir of adaptive potential if hosts can leverage microbial adaptations in stressful environments. However, the facilitation of rapid host adaptation may be limited by interactions between host genotype, microbiome, and environment (G_H x G_M x E). Here, to understand how host x microbiome x environment interactions shape adaptation, we leverage >150 generations of experimental evolution in *Drosophila melanogaster* in a stressful environment, high sugar (HS) diet. The microbiome of HS adapted flies shifted to be dominated by one bacteria, *Acetobacter pasteurianus.* We next performed a fully reciprocal transplant experiment using the dominant control and HS bacteria to measure life-history and metabolic traits in flies. Mismatches between fly evolution and microbiome exerted fitness costs by slowing development and reducing fecundity, especially in the stressful HS diet. The G_H x G_M x E interactions observed suggest that both ecological context and host evolution shape the adaptive potential of the microbiome. We conclude by proposing future directions that highlight the benefit of using experimental evolution to study the contribution of the microbiome to host evolution.

INTRODUCTION

The microbiome has profound effects on many aspects of organismal biology. From modulating physiology [1] to pathogen protection [2] to social interactions [3], the microbiome is frequently implicated as a key regulator of many host phenotypes--but can the microbiome also influence host adaptation? For hosts where microbes are tightly controlled and faithfully transmitted across generations, microbes have clear influence on host evolution [4, 5]. For example, the acquisition of nutrition-provisioning symbionts enables the ecological expansion of sap-feeding insects [6]. For many eukaryotes, though, their microbiomes are environmentally acquired or through other weakly controlled transmission mechanisms [5]. Weak control over microbial transmission disrupts the links between the microbiome and host over generations. Without these links, the microbiome may have limited effects on host evolution [4, 7]. Microbiomes may still have the potential to influence host evolution because environmentally acquired microbes must survive and adapt to external environments. In doing so, microbiomes may acquire beneficial traits that hosts can leverage. Thus, microbiomes have the potential to precede and facilitate rapid host adaptation, but this process is poorly understood.

The environmentally acquired microbiome appears to facilitate rapid host adaptation in several systems. For example, bean beetles acquire pesticide resistance from their soil-dwelling *Burkholderia* symbiont [8]. The gut microbiome of woodrats facilitates survival on toxic plant diets [9]. In plants, drought-adapted soil communities can increase fitness in drought conditions [10]. These studies suggest that microbes can adapt and evolve to stressful conditions in natural environments and buffer the stressor for the host, but much about the evolution of these interactions remains unclear--specifically, we do not know to what extent these microbial effects depend on host genotypes. Host genetic variation may influence the responsiveness to microbial variation such that some combinations of host and microbiome differentially affect host phenotypes within species. The effects on host phenotype will also likely depend on the environmental context, where mismatches between the environment, host genotype, and microbiome may negatively affect host fitness. In other words, host genotype x microbial genotype x environment (G_H x G_M x E) interactions may shape how host-microbiome systems evolve. Such complex G_H x G_M x E interactions are likely to be common, and yet their contribution in shaping host phenotypic variation and adaptation are underexplored. As the study of microbiomes moves beyond descriptive attributes, experimentally assessing the interactions between host and microbial evolution are a key research priority for better understanding the interplay in host-microbiome interactions [11, 12].

The fruit fly, *Drosophila melanogaster*, has emerged as an excellent model to study host-microbe interactions. Flies have relatively simple microbiomes, often composed of fewer than 10 bacterial species from acetic acid and lactic acid bacteria families [13]. The microbiome is largely environmentally acquired through feeding, defecation, and egg smearing during oviposition [14, 15]. After hatching, larvae acquire their microbes through a combination of microbial persistence and colonization and form associations over the lifespan of the fly [16–18]. Thus, the microbiome is mostly environmentally acquired, and these microbes also must persist independently in the habitat. Many fly-associated microbes can be individually cultured and reconstituted in

various combinations to inoculate sterile flies [13, 19]. With the feasibility of microbiome manipulations and rich genetic resources, *Drosophila* is an ideal model to study the interplay between host genetic, microbiome, and environment in shaping host phenotypes.

The microbiome shapes many different aspects of *Drosophila* biology. Axenic flies (without microbiomes) develop slower, display metabolic defects, and dysregulated immune systems compared to those with normal microbiomes [13, 17]. Different components of the microbiome also affect *Drosophila* traits in different ways. Generally, *Acetobacter* species tend to accelerate development while *Lactobacillus* slow development [20, 21]. The variation in microbiomes also affects how flies utilize and store energy, but this depends on fly genetics [20, 22]. For example, lipid storage in only some host genotypes increased in the presence of *Acetobacter tropicalis* [22, 23]. Furthermore, interactions within the microbiome can shift life history tradeoffs between lifespan and fecundity. More complex communities that include multiple species of *Acetobacter* and *Lactobacillus* developed and died faster, but offset fitness costs by increasing fecundity compared to mono-association with the same bacteria [24]. Finally, microbiome composition shaped genomic divergence in field mesocosms during seasonal evolution that matched traits associated with natural *Drosophila* populations [21, 25]. These studies demonstrate the range of effects associated with the microbiome, and importantly, the complex web of host genetic and microbial control that contribute to variation in fly phenotypes.

While the above studies identify key host genotype x microbiome interactions, missing are the environmental effects. Environmental stressors may increase the relative importance of microbial costs or benefits [26–28]. For *Drosophila*, the microbiome may be critical to mitigate dietary stressors, but few studies have tested the role of the microbiome in dietary adaptation. One study found that *Lactobacillus* evolved to promote larval growth and development in mono-association with *Drosophila* on nutrient poor diets [29]. However, nutrient poor diets often limit microbial growth in the fly gut [30]. More so, axenic flies adapted to nutrient poor diets had similar survival and metabolic phenotypes as conventional flies [31], suggesting no benefit of the microbiome in stressful environments. While these studies address what happens to fly or the microbiome, how host evolution and the microbiome together shape host survival in stressful environments remains poorly understood.

Here, we leveraged ~150 generations of fly and microbial adaptation to high sugar (HS) diets. HS diets exert stress on flies, and flies exhibit similar obesity-like phenotypes as humans with elevated triglycerides, insulin resistance, and shortened lifespan [32–34]. To cope with HS stress, flies must convert sugar into lipids [35]. One genus of the microbiome, *Acetobacter* consumes and reduces sugar in the fly gut [36], suggesting *Acetobacter* may contribute to fly adaptation to HS diets. To test this hypothesis, we performed a fully reciprocal host x microbiome x diet transplant experiment using the evolved HS fly genotype and HS *Acetobacter*. If the microbiome facilitates fly adaptation in HS diets, then transferring the HS microbiome into naive hosts may confer adaptive traits. Our results highlight the complexities of environmental context dependency on host-microbiome interactions.

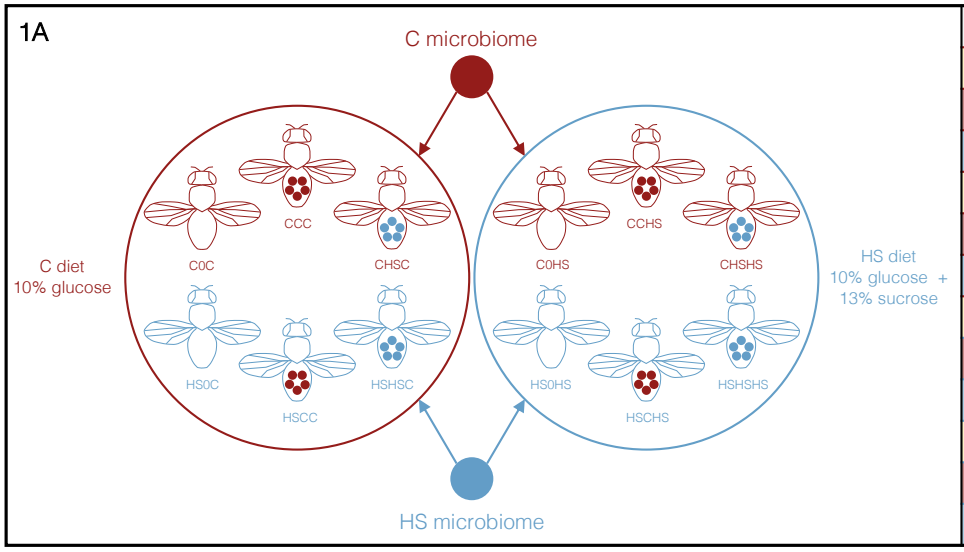
METHODS

Fly populations

All fly populations were maintained at 25ºC with 12 hour light:dark cycles. The base population consisted of an outbred population created by crossing 15 inbred lines initially collected in the Netherland (a subset of the Global Diversity Lines) [37]. Briefly, we crossed each inbred line to another using a round-robin design. The resulting F1s were crossed in a similar pattern to ensure parental representation and that no chromosome would be lost in the first round of crosses. The resulting F2 individuals were placed in large population cages, maintained at large population size (>10,000 flies) and allowed to interbreed freely for ~50 generations before high sugar (HS) selection began. Flies were maintained on the control diet composed of 10% glucose, 10% yeast, 1.2% agar, with 0.04% phosphoric acid and 0.4% propionic acid as preservatives. Three replicate populations for HS selection were derived from the base population and maintained on the same diet, but with an additional 13% sucrose and at lower population size (>5000 flies/cage until generation 100 and ~2000 flies/cage after). The approach used here allowed for natural selection on HS diets; we did not guide or select for particular traits as is often used in experimental evolution in *Drosophila*. In parallel, three replicate populations were reared on control diets as is standard for experimental evolution studies, but these are not the focus of this study. Because we were interested in the microbiome transferring adaptive traits to naive hosts, we defined the outbred base population as our control, non-evolved population. We consider the two populations, C and HS, to be two different genotypes. We note that the flies are not traditional inbred lines and still retain genetic diversity, but for the purposes of this study, we consider their evolutionary history to have generated two distinct genotypes.

Transplanting microbiomes to test for adaptive significance

To test if the microbiome can transfer adaptive traits, we performed a reciprocal microbiome transplant experiment (Fig. 1). All host genotypes x microbiome x diet interactions were generated in the reciprocal transplant. Both genotypes (C and HS flies) were inoculated with microbiomes (C, HS) or kept sterile and raised in both diets (C and HS) for a total of 12 treatments. This experimental design allowed us to evaluate



ID	treatment	symbol
C0C	C fly + sterile + C diet	
CCC	C fly + C micro + C diet	
CHSC	C fly + HS micro + C diet	
HS0C	HS fly + sterile + C diet	
HSCC	HS fly + C micro + C diet	
HSHSC	HS fly + HS micro + C diet	
C0HS	C fly + sterile + HS diet	
CCHS	C fly + C micro + HS diet	
CHSHS	C fly + HS micro + HS diet	
HS0HS	HS fly + sterile + HS diet	
HSCHS	HS fly + C micro + HS diet	
HSHSHS	HS fly + HS micro + HS diet	

Fig. 1: We leveraged > 150 generations of fly adaptation to HS diets to study the role of the microbiome in adaptation.
A) Experimental design for fully reciprocal host x microbiome x diet transplant to test the role of microbial evolution to fly adaptation. Fly genotypes (C or HS) were inoculated with microbes (none, C, or HS) in two diets (C or HS). The same experimental design was used in two iterations: community transplant or *Acetobacter* transplant. The table shows the abbreviated treatment codes used throughout this study, and it is colored to emphasize the microbiome treatment. The symbols represent the same information visually diagrammed as flies. Red colors represent control, blue colors represent high sugar, and yellow (or empty flies) represent sterile treatments.

how host x microbiome x environment interactions influence the transfer of adaptive phenotypes.

We performed an initial microbiome transplant using fly frass, and we detailed the methods and results in the supplement. In brief, we found that diet strongly structured the microbiome of both donor and recipient flies (Supp. Fig. 1), affected developmental timing and fecundity (Supp. Fig. 2), but the recipient microbiome did not strongly resemble the donor, suggesting the transplant was not faithful. To better understand the host-microbiome interaction, we identified the differentially abundant bacteria between C and HS flies through ANCOM (analysis of composition of microbiomes) implemented in QIIME2 [38]. For ANCOM, analysis was performed on ASVs (amplicon sequence variants). Only ASVs present in >5 fly samples were included. We cultured the most differentially abundant bacteria from HS flies, *A. pasteurianus*, and the significantly differentially abundant bacteria within the *Acetobacter* genus and enriched in C flies, *A. indonesiensis*. We confirmed 16S rRNA sequence identity using Sanger sequencing. Because the 16S rRNA phylogeny is discordant with a multilocus phylogeny in the Acetobacteraceae [39] and for simplicity, we refer to *A. indonesiensis* as C *Acetobacter* and *A. pasteurianus* as HS *Acetobacter*.

To perform the transplant, *Acetobacter* was cultured in liquid MRS media at 25ºC for 2-3 days. Bacterial density was normalized to $OD_{600} = 0.1$, and 50 µl of bacteria (or PBS for sterile treatments) was used to inoculate axenic eggs in sterile diets [19]. For each treatment, 6-10 replicates were maintained. Flies were then reared in a biosafety cabinet at 25ºC and 12:12 light:dark cycle through the lifespan of the recipient flies. At 8-10 days post eclosion, a subset of 4-6 flies from all replicates were homogenized in 100 µl PBS and plated on MRS media. Bacterial growth was visually confirmed to be the original *Acetobacter* isolates and sterility for sterile treatments.

Phenotypic effects of the Acetobacter transplant

To measure developmental time, we counted the number of new pupae every 6 hours during their daytime (i.e., 8am, 2pm, 8pm) until all treatments began eclosing. We used the survival [40] and survminer [41] packages in R to analyze development data. We used Cox proportional hazards to identify how interactions between fly genotype, microbiome, and diet influence development time with the following model:

$$DT = y + \beta_1 G + \beta_2 M + \beta_3 E + \beta_4(G*M) + \beta_5(M*E) + \beta_6(G*E) + \beta_7(G*M*E) + e$$

Where DT = developmental time (pupation), y = grand mean, G = fly genotype (C or HS), M = *Acetobacter* treatment (C, HS, or sterile), E = diet (C or HS), and $e \sim N(0, \sigma_e)$ as residual error.

For fecundity, we counted the number of eggs laid by individual 8-10 day old females (N=644). Females were placed in a 24-well plate with C and HS oviposition media. Oviposition media was similar to the fly diets, but with yeast extract to make the media transparent for ease of egg counting.

We did not find a difference in egg counts between C and HS oviposition media (Supp. Fig. 3, Kruskall-Wallis $X^2 = 2.85$, df=1, p = 0.09), and for the subsequent analyses, pooled together egg counts from both oviposition media. To minimize plate effects, two treatments were assayed on each plate, and treatments were randomly assigned across plates. Females laid eggs for ~4-6 hrs at 25ºC during the late afternoon (2pm-8pm) to capture similar circadian rhythms in egg laying across all treatments. We analyzed fecundity using a zero-inflated Poisson distribution implemented in glmmTMB [42] with the following model:

$$F = y + \beta_1 G + \beta_2 M + \beta_3 E + \beta_4(G*M) + \beta_5(M*E) + \beta_6(G*E) + \beta_7(G*M*E) + d + e$$

Where F = egg count from individual females, y = grand mean, G = fly genotype (C or HS), M = *Acetobacter* treatment (C, HS, or sterile), E = diet (C or HS), where $d \sim N(0, \sigma_o)$ = random effect of oviposition plate (N=29 plates), and $e \sim N(0, \sigma_e)$ as residual error. We tested for significance of fixed effects using Type III ANOVA with Kenward-Roger degrees of freedom using the car package in R [43]. Least square means were used to visualize the interactions with the emmeans package in R [44].

For nutritional indices, we assayed pools (N=95) of three age-matched (8-10 days old) females for protein and total triglycerides. Protein serves as a proxy for size and was measured using a Bradford assay (BioRad 5000205). Total triglycerides represent fat stores for flies and assayed using a colorimetric assay (Sigma TR0100-1KT). Triglycerides were normalized by protein amount [45]. Each assay was performed in triplicate in 96 well plates with all 12 treatments in each plate to minimize batch effects. Triplicate values were averaged; values with standard deviation > 0.02 were removed from analysis. The replicates of treatments were assayed across different assay plates. We visualized the protein and triglyceride data by regressing out the effect of the assay plates to show mean-centered values. We modeled the response as:

$$Y = y + \beta_1 G + \beta_2 M + \beta_3 E + \beta_4(G*M) + \beta_5(M*E) + \beta_6(G*E) + \beta_7(G*M*E) + d + e$$

Where Y= protein or triglyceride amount, y = grand mean, G = fly genotype (C or HS), M = *Acetobacter* treatment (C, HS, or sterile), E = diet (C or HS), where $d \sim N(0, \sigma_o)$ = random effect of assay plate (N=14 assay plates), and $e \sim N(0, \sigma_e)$ as residual error. The three way interaction between fly genotype, *Acetobacter*, and diet was not significant and was removed from the model. We tested for significance of fixed effects using Type III ANOVA with Kenward-Roger degrees of freedom using the car package [43]. Least square means were visualized to show interaction effects with the emmeans package in R [44].

RESULTS

Fly microbiome responds to HS selection

Here, we leveraged ~150 generations of fly and microbial adaptation to HS diets to understand how the microbiome can contribute to host evolution. First, we established that the microbiome changed between C and HS flies (Fig. 2A). HS flies were dominated by *Acetobacter pasteurianus* (86.7% +/- 1.8 SE) and unclassified Acetobacteraceae strain (5.1% +/- 1.8 SE), while C flies were more diverse with *Algoriella xinjiangensis* (37.3% +/- 5.3 SE), *Acetobacter persici* (16.9% +/-2.6 SE), and *Acetobacter indonesiensis* (14.8% +/- 2.1 SE). The most differentially abundant bacteria were *A. pasteurianus* and Acetobacteraceae strain for the HS flies, while *A. xinjiangensis* and *A. indonesiensis* were associated with C flies (Fig. 2B, Supp. Table 1). These bacteria are unique to either C or HS flies, demonstrating that the HS diet selected for different fly microbiomes.

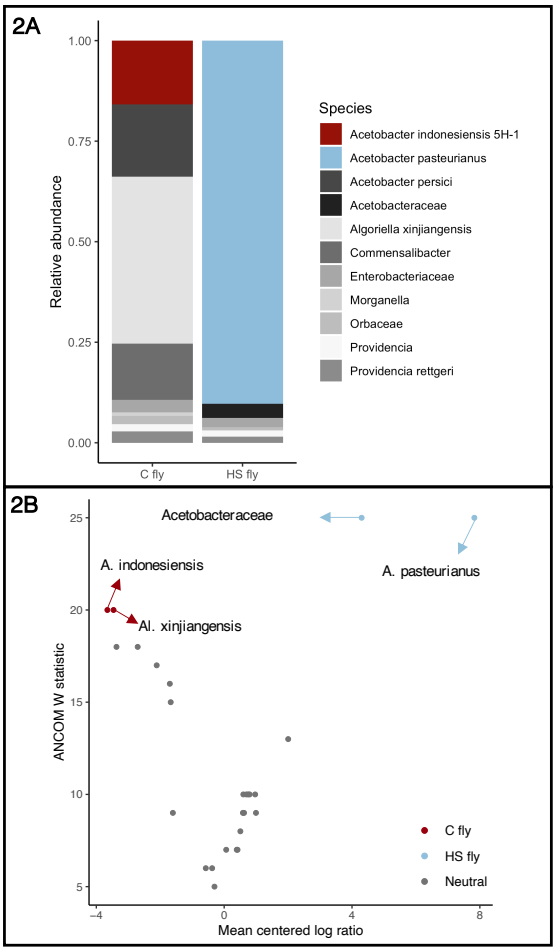


Fig. 2: The microbiome in HS flies is different from C flies. A) Relative abundance of microbiome determined using 16S rRNA sequencing. The C fly microbiome is enriched for *A. indonesiensis*, *A. persici*, and *Algoriella xinjiangensis*. The HS fly microbiome is enriched for *A. pasteurianus*. B) ANCOM results for differentially abundant bacteria between C and HS flies. Colored points represent significantly different ASVs and arrows denote taxonomic classification.

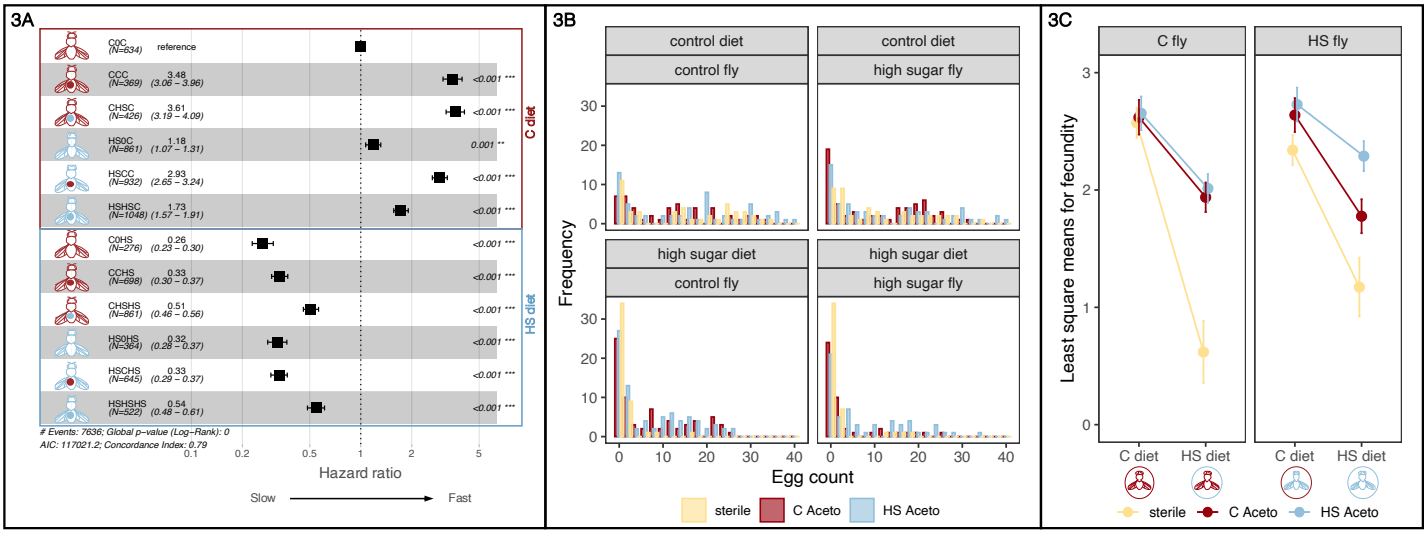


Fig. 3: Life history effects of the *Acetobacter* transplant.
A) Hazard ratio plot for *Acetobacter* effects on development time. Boxplots show hazard ratios and 95% confidence intervals for each treatment relative to C0C. Mismatches between diet and microbiome slowed development. In the C diet, HS flies with C *Acetobacter* developed faster than HS flies with HS *Acetobacter*. However, on the HS diet, C *Acetobacter* slowed, while HS *Acetobacter* accelerated development. The interaction effects on the Cox proportional hazards model between fly genotype x *Acetobacter* x diet best explained variation in development (log-likelihood= -58500, df=7, C^2=465.8, p<0.001). B) Histogram of fecundity from individual females in the *Acetobacter* transplant, faceted by fly genotype and diet. Colors represent the *Acetobacter* treatment. Fecundity is zero skewed, especially for flies on HS diets. C) Least square means (LSM) from fecundity, modeled with a zero-inflated Poisson distribution, show fly genotype x *Acetobacter* x diet interactions shaped fecundity. Colors represent *Acetobacter* treatment. Error bars show standard error for LSMs. For the HS fly genotype, the HS *Acetobacter* increased fecundity relative to the C *Acetobacter*, suggesting a fecundity benefit with the adapted microbiome.

Transplanting Acetobacter influences life history traits

To test if transferring the microbiome could transfer adaptive phenotypes to naive hosts, we performed a fully reciprocal, host genotype x microbiome x diet transplant experiment. We first performed a fecal transplant in flies, but found that the transplant was not faithful (Supp. Fig. 1), as is common for fecal transplants in other taxa [46–48]. We then transplanted the dominant *Acetobacters* from C and HS diets identified above in a fully reciprocal experiment and measured life history and metabolic traits.

Development was shaped by interactions between fly genotype, *Acetobacter*, and diet as was observed in the whole community transplant (Fig. 3A, Supp. Table 2). Importantly, like in the microbiome transplant, all sterile treatments developed slowest. However, flies responded differently to the *Acetobacter* transplant. On the C diet, fly genotype influenced the response to the *Acetobacter* transplant. HS flies with the C *Acetobacter* developed more rapidly (avg. 140.8 hrs +/- 0.54 SE) than with the HS *Acetobacter* (avg. 147.6 hrs +/- 0.60 SE). There was no effect for the C fly. However, on the HS diet, the HS *Acetobacter* accelerated development (HS *Acetobacter* avg. 176.5 hrs +/- .50 SE, C *Acetobacter* avg. 186.8 hrs +/-0.60 SE). The three-way interaction between fly genotype, *Acetobacter*, and diet significantly affected development time (Supp. Table 2, log-likelihood =-58500, X^2 = 47.625, p < 0.0001).

Fecundity responded to interactions between fly genotype, *Acetobacter*, and diet (Fig. 3B, Supp. Table 3). On average, treatments were more fecund on the C diet, while treatments on HS diets laid 54.3% fewer eggs (C avg. 13.0 eggs +/-0.6 SE, HS, avg. 5.9 eggs +/- 0.4 SE). Sterile treatments substantially lowered fecundity, only on the HS diet (+microbes avg. 7.45 eggs +/- 0.5 SE, sterile avg. 1.67 eggs +/- 0.4 SE). Notably, the response to *Acetobacter* treatment depended on fly genotype for the HS diet. On the HS diet, HS flies with HS *Acetobacter* (avg. 8.4 eggs +/-1.4 SE) were more fecund than with C *Acetobacter* (avg. 4.5 eggs +/- 1.0 SE). The interaction between fly genotype, microbiome, and diet significantly affected fecundity (interaction X^2 = 16.4, df = 2, p=0.0003, Fig. 3C, Supp. Table 3).

To gain insight into how these interactions shape host metabolism, we assayed flies for protein and triglyceride (TAG) levels. Protein levels were primarily shaped by interactions between fly genotype and diet (Fig. 4A, Supp. Table 4). On the C diet, HS flies had higher protein levels than C flies, but this relationship was not observed on the HS diet (Fig. 4B, fly genotype x diet interaction, $F_{2,78.1}$ = 18.3, p < 0.0001). *Acetobacter* modified this interaction, but also only on the C diet (*Acetobacter* x diet interaction, $F_{2,78.2}$ = 3.26, p = 0.04). TAG levels were also shaped by interactions between fly genotype and diet (Fig. 4C, Supp. Table 5). On the C diet,

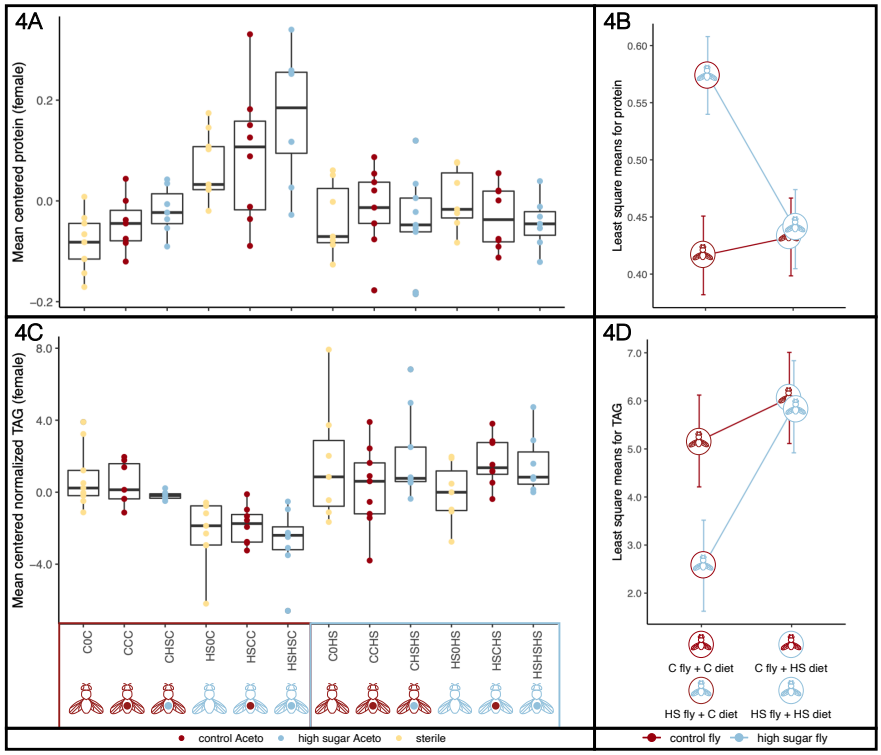


Fig. 4: Metabolic effects of the *Acetobacter* transplant.
A) Boxplot for protein levels. Protein amount is a proxy for size in *Drosophila*. Each point represents a pool of three age-matched female flies. Protein values were mean centered for each batch assayed. Fly genotype, diet, and their interaction significantly affected size ($F_{1,78.1}$ = 18.3, p<0.0001), while *Acetobacter* had minimal effects. B) Least square means (LSM) from the mixed model shows significant interactions between fly genotype and diet. Error bars show standard error for LSMs. For HS flies, protein levels responded to diet, but C flies did not significantly differ. C) Boxplot for triglycerides (TAG) normalized to protein. TAG amount reflects fat storage in *Drosophila*. Each point represents a pool of three age-matched female flies. TAG levels were mean centered to control for each batch assayed. Like for protein levels, interactions between fly genotype and diet shaped TAG levels ($F_{1,78.1}$ = 9.00, p=0.004), but *Acetobacter* had no effect. D) LSMs from the mixed model shows the significant interaction between fly genotype and diet. Error bars show standard error for LSMs. Like for protein, TAG levels responded to diet for HS flies, but not for C flies.

HS flies had lower TAG levels than C flies, but this relationship was not observed on the HS diet (Fig. 4D, fly genotype x diet interaction, $F_{1,78.0}$ = 9.00, p = 0.004). *Acetobacter*, alone or through interactions, did not influence TAG levels. Thus, fly metabolism was primarily shaped by host genetics and diet, but not by differences between *Acetobacters*.

DISCUSSION

Here, we explored how the microbiome influences host fitness in stressful environments. Through reciprocal host x microbiome x diet transplant experiments, we show that G_H x G_M x E interactions shape host fitness by modifying developmental timing and fecundity. Our results highlight the complex interactions generated by the microbiome, and we discuss important future directions to further dissect these relationships.

Acetobacter dominates HS microbiome

Acetobacter was the most abundant genus in the HS flies (Fig. 2). *Acetobacter* are common in flies, both in laboratory settings and wild environments [17, 49–52]. Furthermore, *Acetobacter* are sugar specialists and tolerate highly acidic environments, like during vinegar fermentation [53]. In the context of *Drosophila* biology, some strains of *Acetobacter* reduce glucose levels and enhance oxidation of ethanol, which promotes larval development [54, 55]. Additionally, reducing glucose levels is critical for flies to survive HS diets [35]. Over the course of experimental evolution, both fly and microbiome were exposed to HS diets, and in turn, the HS microbiome was dominated by *A. pasteurianus* (Fig. 2B). Taken together, these results indicate that HS selection also changed the fly microbiome, selecting for bacteria that could survive HS stress.

In our attempt to transplant the microbiome, we expected that the HS diet would promote differentiation between C and HS microbiome recipients. However, several lines of evidence suggest that the microbiome transplant was not faithful as recipient microbiomes did not resemble donor microbiomes. First, both the C and HS microbiomes were distinct in bacterial composition and variance across donors, frass, and recipients (Supp. Fig. 4). Only a few species were common in both fly and frass, but were in different relative abundances, much like is found in mammals and their fecal microbiomes [56, 57]. More so, the amount of variation in microbial community composition explained by the microbiome transplant was much lower on the HS diet (HS:

r^2=0.04, C: r^2=0.43, Supp. Fig. 1). While we did not detect contamination in the sterile treatments (Supp. Fig. 5), we are unsure why the microbiome transplant was unfaithful. This appears to be a common issue with fecal transplants in mammals as well [46–48]. We did note that the abundance of microbes differed between C and HS frass in the transplant (Supp. Fig. 6). This suggests that the HS diet also suppresses microbial abundance, in addition to microbial diversity. Much remains unknown about how ecological and evolutionary conditions can promote or impede microbiome transplants [46–48]. Host and environmental factors that support microbial diversity may increase the success of microbiome transplant [58, 59], and those factors that limit microbial growth impede transplants, much like the HS diet that limited both microbial diversity and growth. Because of the difficulty in interpreting the low fidelity of the microbiome transplant and the dominance in the HS microbiome by a single *Acetobacter* species, we subsequently focused on the phenotypic effects of the two dominant *Acetobacters* in the C and HS microbiomes.

Host x microbiome x environment interactions shape life history traits

In *Drosophila*, as well as other holometabolous insects, life history traits represent a classic tradeoff between survival and reproduction [60]. Faster development increases fitness because it maximizes reproductive events before death. Furthermore, *Drosophila* evolved in patchy, ephemeral habitats that enhance competition among larvae and predation risk [61, 62]; faster development can reduce these risks. Delayed development imposes fitness costs unless fecundity later in the host lifespan compensates for that delay. The microbiome has emerged as a novel factor that frequently shapes life history traits of developmental time and fecundity in *Drosophila* [20, 24, 54, 63]--though all of these studies were in benign environments. Our results advance these findings by investigating the fly response in a stressful environment and the role of host evolution in mediating this relationship.

For both developmental time and fecundity, mismatches between host evolution, microbiome, and diet led to fitness costs. For developmental timing, HS flies with HS *Acetobacter* delayed development on the C diet, but for both fly genotypes, the HS *Acetobacter* accelerated development on the HS diet (Fig. 3A). The development effects suggest fitness benefits for the HS *Acetobacter* only on the HS diet, but costs for the C *Acetobacter*. Fecundity also suffered from mismatches with the environment, though predominantly for HS flies on the HS diet (Fig. 3C). HS flies experienced relative increases in fecundity with the HS *Acetobacter*, but decreased with the C *Acetobacter*. C flies did not respond to differences between the C and HS *Acetobacter* in either diet. The G_H x G_M x E interactions for both development and fecundity suggest that the C and HS flies evolved to respond differently to microbial variation, especially in the stressful HS diet.

Our results highlight the surprising responsiveness of developmental timing to microbial variation in *Drosophila*. More so, recent studies have suggested that microbial variation often modulates developmental timing in many different kinds of organisms, like killifish [64], dung beetles [65], and *Arabidopsis* [66]. One proposed mechanism is that the microbiome triggers development that matches host phenotypes to maximize fitness in the local environment [67]. If so, the microbes must provide a more reliable signal than the host can detect alone, but the host must also have a mechanism to detect the signal.

Our results suggest that HS *Acetobacter* may provide a signal to shift development on the HS diet, potentially through promoting fat storage in larvae as found in other *Acetobacter* species [54, 55]. For signal detection, the responsiveness of the HS fly to *Acetobacter* variation suggests the evolution of a novel mechanism to increase fecundity through the insulin/insulin-like signaling (IIS) pathway. IIS modulates endogenous developmental timing and reproduction, but is also affected by the microbiome [54, 68, 69]. IIS is also directly involved in mitigating HS stress as flies with a deficient IIS pathway exhibit slower development and shortened lifespan on HS diets [32]. Thus, the IIS pathway may integrate signals from the microbiome. While we did not detect *Acetobacter* effects on two metabolic indices (Fig. 4), additional phenotyping for other indices, like glucose or trehalose, and in the larvae may provide additional insight into the mechanistic basis underlying the life history phenotypes. Identifying the molecular variation and gene expression patterns that differ between C and HS flies, like the IIS pathway, will demonstrate how the microbiome altered the response to selection in hosts during adaptation.

Adaptive potential of the microbiome depends on evolutionary and ecological context

Many have predicted that the microbiome could accelerate local adaptation in the host, facilitating phenotypic changes to buffer local stressors [6, 70–72]. If so, transplanting the locally adaptive microbiomes should confer adaptive phenotypes to naive individuals. However, the benefit may depend on interactions between host genotype and environment. G_H x G_M x E interactions have the potential to facilitate or impede evolution in challenging environments [11, 12]. Through combining experimental evolution with reciprocal transplants, our data allowed us to test if and how ecological context and host evolution shape the adaptive potential of the microbiome.

To shape adaptive potential, the microbiome must contribute to host phenotype, not unlike traditional (host) additive genetic variance [72]. For development, the microbiome conferred an adaptive phenotype--HS *Acetobacter*

accelerated development similarly for both the naive C and adapted HS fly in the HS diet (Fig. 3A). The G_H x G_M x E interaction for development illustrates how diet, the ecological context, may increase the fitness benefit of locally adaptive microbiomes. However, the G_H x G_M x E interaction that shaped fecundity is suggestive of how genetic interactions may impede the transfer of adaptive potential. The naive host, C flies, did not receive a fecundity increase from the HS *Acetobacter* in the HS diet, but HS flies did (Fig. 3C). The presence of G_H x G_M x E interactions does not imply that the microbiome was unimportant to fly evolution. Rather, the responsiveness of the HS fly to microbial variation in both diets suggests that HS flies evolved a specialized association with their microbiome. Thus, the HS fly evolved to select for beneficial *Acetobacter* traits, which may lead to the dominant HS *Acetobacter*. Taken together, our results demonstrate how both the ecological context and host evolution shape the adaptive potential of the microbiome.

More broadly, the G_H x G_M x E interactions observed here provide several insights into host-microbiome evolution. First, our work contributes to the growing body of literature that environmentally acquired microbes are important in stressful environments. Stressful environments are predicted to increase the relative importance of mutualisms [26–28], though how often the microbiome behaves evolutionarily like a traditional mutualism remains contentious [4, 5, 72]. In plants, stress-adapted soil microbiomes improve germination and seedling survival in stressful environments [73, 74], highlighting the importance of early life traits like we also observed for development. Second, host genotypes may vary in their ability to leverage beneficial microbes in stressful environments. Genetic interactions may limit the benefits of locally adaptive microbiomes, like we observed for fecundity. Understanding these G_H x G_M interactions will be especially important in using evolution to engineer beneficial microbiomes to solve pressing problems in public health and agriculture [11, 12, 75]. Our results suggest how the microbiome may shape host adaptation, but we focused only on the endpoints of fly adaptation. Longitudinal sampling over the evolutionary path will provide key insights into how host genetics and environment shape the adaptive potential of the microbiome.

Finally, we acknowledge that despite the ~150 generations of adaptation to the HS diet, the HS diet still comes at a fitness cost to the HS flies. For example, developmental time was still slower on the HS diet compared to the C diet (Fig. 3A). HS flies were less fecund on the HS diet than on the C diet (Fig. 3C). For metabolic indices, genotypic differences were only observed on the C diet (Fig 4). One possibility for these unexpected results is the use of dechorionation to manipulate the microbiome. Dechorionation, which removes the chorion that protects eggs, is a common tool for the *Drosophila* microbiome [13, 19]. Dechorionation has conflicting effects on different aspects of fly physiology. One study found no significant effect on metabolic indices, like protein and TAG levels, compared to conventionally reared flies [76]. However, another study found that dechorionation increased overall mortality, though improved starvation resistance [77]. It is possible that dechorionation affected our results, but we did not test for this effect. We raise this point as one challenging aspect of using experimentally evolved hosts for microbiome manipulations, as adaptation to novel environments may change host biology in unexpected ways.

Conclusions and research priorities

Here, we leveraged host and microbial evolution from ~150 generations of adaptation to a stressful diet. The fly populations used in this experiment were originally conceived as an Evolve & Resequence (E&R) experiment. The E&R approach in *Drosophila* uses outbred populations to identify signatures of selection across the genome and has illuminated many important findings about the role of demography, population genetics, and genetic architecture in shaping adaptive traits [78, 79]. However, missing from this framework is the role of the microbiome. In a survey of ten E&R experiments, the microbiome also frequently responded to experimental evolution, particularly in traits associated with metabolism [80]. Taken together, our data here also highlight how the microbiome may contribute to host adaptation, previously unseen by focusing only on the *Drosophila* genome. Our study provides the first experimental dissection of the microbial response and influence on host traits in the E&R context. Through reciprocal transplants, we identified how interactions between fly genotype, microbiome, and diet shape fitness related traits.

The data here suggest that the microbiome can contribute to host fitness, but many questions remain about its evolutionary impact. We focused on the endpoints of evolution between host and microbiome, but the evolutionary trajectory of the interaction is unknown. Longitudinal studies that characterize microbial change as well as phenotypic effects would demonstrate if microbial evolution precedes host evolution and whether this shapes the host evolutionary trajectory. To better understand the evolutionary trajectory, reciprocal transplants over the course of adaptation will also show the relative importance of the microbiome compared to host evolution. Experimental evolution is a powerful, but underutilized tool in the study of host-microbiome evolution [11, 72, 81]. Ultimately, the evolutionary benefits of the microbiome will depend on both the environmental and host genetic context, as suggested by the complex interactions observed here. Future experiments that incorporate both host and microbial evolution will identify how genetic interactions shape adaptive responses in stressful environments.

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TABLES

Table 1: ANCOM results for differentially abundant ASVs from C and HS fly microbiomes. Taxonomy was characterized to the lowest level an assignment could be made, and most ASVs were classified to species level.

ASV ID	clr	W	sig	taxonomy
d84f3e1a7dbb97bc4695b954c8a2d2b2	4.30129864	25	HS fly	Acetobacteraceae
fb59adcbdd838dc7853f7388afb62b5a	7.82830283	25	HS fly	Acetobacter pasteurianus
9329896cd08f88a88296d921369cd8ea	-3.4609509	20	C fly	Algoriella xinjiangensis
f0d11f9f8ba452d5485a79e0b31b538f	-3.6539534	20	C fly	Acetobacter indonesiensis
3f2be3d57ac38f60b0b9c56fa5857f63	-2.7057494	18	Neutral	Algoriella xinjiangensis
70e118808cdc81b5b2670837c7833d8d	-3.3688051	18	Neutral	Acetobacter persici
dd4826376c1de49b8687c4f882d3bcf3	-2.1106948	17	Neutral	Enterobacteriaceae
606aa8c7117f801dc5ccc8c2a78c68c3	-1.7000214	16	Neutral	Commensalibacter
1c735b8a08dcbd5ca158987733234114	-1.6719837	15	Neutral	Algoriella xinjiangensis
da74ac9885f0aa2d29ec4338b46aa051	2.00310762	13	Neutral	Orbaceae
4043baca9447b9860efabab56603efc0	0.79635173	10	Neutral	Acetobacter species
61fd19eda2dee9c93613432491556a99	0.69392446	10	Neutral	Alphaproteobacteria
7070daee4850cf801719eab99ce88a82	0.96974134	10	Neutral	Commensalibacter
b3ccbf1d4fc85411f948429591f371cf	0.59968784	10	Neutral	Pseudomonas
ca7279bfdcb977287193c567f96b025c	0.75112887	10	Neutral	Acetobacter indonesiensis
0a04cded1e993516d5cdc851bfe07180	-1.6048832	9	Neutral	Algoriella xinjiangensis
6e4961fe480153e97ebdcf8fca4913fb	0.99515931	9	Neutral	Providencia
8dc187c9810636b7199c030caaaf0694	0.58697562	9	Neutral	Morganella
9f9bb5e1a63c5a0820d1571839534a2f	0.62165362	9	Neutral	Commensalibacter
677d95c5a0bc1c25435861fe262cb487	0.50699766	8	Neutral	Alphaproteobacteria
4af26492bbe6d0d4980bc82c2f08f32d	0.3940931	7	Neutral	Providencia
ad8b60b9428bc76f3b50e428f4f5a29f	0.41862229	7	Neutral	Stenotrophomonas
f72576c61dde7b9b6ad683dea71b105f	0.06411428	7	Neutral	Enterobacteriaceae
3c3c65e801e07d1be83bfa6d962b5627	-0.3759816	6	Neutral	Commensalibacter
681de033774ada405895dd81abc7593f	-0.5750105	6	Neutral	Providencia rettgeri
97b76e52dafa224603888fb56ef0b831	-0.3031253	5	Neutral	Commensalibacter

Table 2A: Analysis of deviance for Cox model with interactions between fly genotype, microbiome, and diet for developmental timing in the *Acetobacter* transplant.

Category	Log-likelihood	X^2	Df	*P* value
Null	-60640			
Fly genotype	-60584	111.642	1	<2.20E-16***
Acetobacter	-60517	134.874	2	<2.20E-16***
Diet	-58732	3568.585	1	<2.20E-16***
Fly x Acetobacter	-58675	115.244	2	<2.20E-16***
Fly x Diet	-58658	34.288	1	4.75E-09***
Acetobacter x Diet	-58523	268.613	2	<2.20E-16***
Fly x Acetobacter x Diet	-58500	47.625	2	4.55E-11***

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Table 2B: Model selection statistics for developmental timing in the *Acetobacter* transplant. Model 1 is without interactions, while Model 2 includes all two and three-way interactions.

Model	Log-likelihood	X^2	X^2 Df	P(>|Chi|)
Model 1	-58732			
Model 2	-58500	465.77	7	<2.20E-16***

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Table 3A: Fixed effects for fecundity in the *Acetobacter* transplant. Significance was evaluated using Type III Wald F tests with Kenward-Roger degrees of freedom.

Category	X^2	Df	*P value*
Intercept	637.8446	1	<2.20E-16***
Fly genotype	4.0333	1	0.0446106*
Acetobacter	39.7839	2	2.30E-09***
Diet	53.9716	1	2.03E-13***
Fly x Acetobacter	7.787	2	0.0203736*
Fly x Diet	8.4003	1	0.0037517**
Acetobacter x Diet	14.4492	2	0.0007285***
Fly x Acetobacter x Diet	16.3387	2	0.0002832***

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Table 3B: Model selection statistics for fecundity in the *Acetobacter* transplant. Model 1 is without interactions, while Model 2 includes all two and three-way interactions.

Model	Df	AIC	BIC	logLik	deviance	X^2	X^2 Df	*P* value
Model 1	7	6333.5	6365	-3159.8	6319.5			
Model 2	14	6296.8	6359.8	-3134.4	6268.8	50.753	7	1.03E-08***

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Table 4A: Fixed effects for protein (as a proxy for size) in the *Acetobacter* transplant. Significance was evaluated using Type III Wald F tests with Kenward-Roger degrees of freedom.

Category	F	Df	Df.res	*P* value
Intercept	229.8272	1	7.004	1.30E-06***
Fly genotype	21.9301	1	78.06	1.17E-05***
Diet	11.3077	1	78.062	0.001198**
Acetobacter	0.9362	2	78.013	0.396492
Fly x Diet	18.3091	1	78.068	5.28E-05***
Fly x Acetobacter	0.3589	2	78.066	0.699579
Diet x Acetobacter	3.2618	2	78.156	0.043595*

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Table 4B: Model selection statistics for protein in the *Acetobacter* transplant. Model 1 is without interactions, while Model 2 includes all two-way interactions. The three-way interaction was not significant and removed from the analysis.

Model	Df	AIC	BIC	logLik	deviance	X^2	X^2 Df	*P* value
Model 1	7	-149.89	-132.01	81.943	-163.89			
Model 2	12	-164.99	-134.34	94.494	-188.99	25.102	5	0.0001332***

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Table 5A: Fixed effects for triglyceride accumulation in the *Acetobacter* transplant. Significance was evaluated using Type III Wald F tests with Kenward-Roger degrees of freedom.

Category	F	Df	Df.res	*P* value
Intercept	30.7704	1	7.002	0.0008616***
Fly genotype	12.0201	1	78.037	0.0008591***
Diet	27.3194	1	78.039	1.40E-06***
Acetobacter	0.028	2	78.008	0.9724439
Fly x Diet	8.9996	1	78.043	0.0036234**
Fly x Acetobacter	1.7817	2	78.041	0.1751283
Diet x Acetobacter	1.771	2	78.098	0.1769287

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

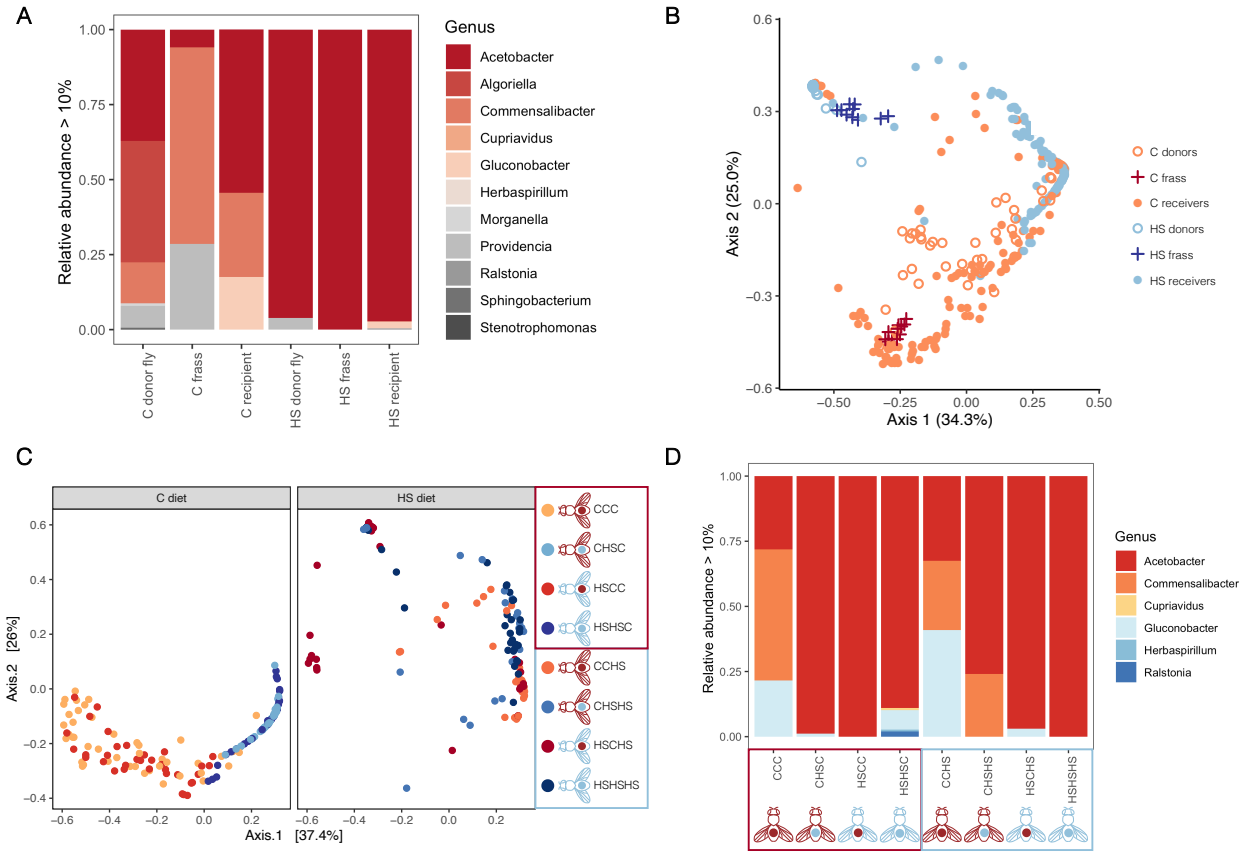
Table 5B: Model selection statistics for triglyceride in the *Acetobacter* transplant. Model 1 is without interactions, while Model 2 includes all two-way interactions. The three-way interaction was not significant and removed from the analysis.

Model	Df	AIC	BIC	logLik	deviance	X^2	X^2 Df	*P* value
Model 1	7	440.31	458.19	-213.16	426.31			
Model 2	12	433.09	463.73	-204.54	409.09	17.226	5	0.00409**

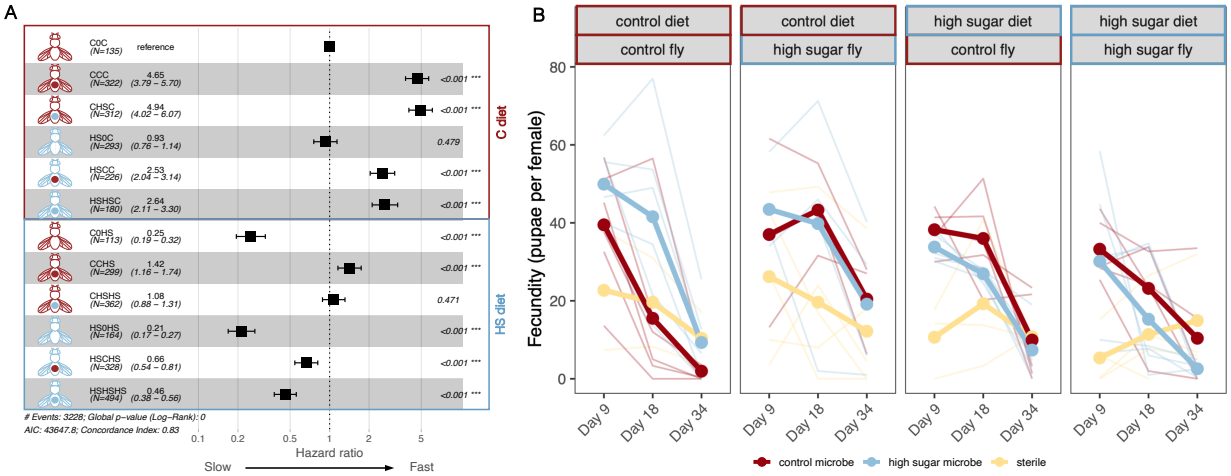
Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

SUPPLEMENTARY FIGURES

Supp. Fig. 1: ***SUPPLEMENTARY FIGURES***

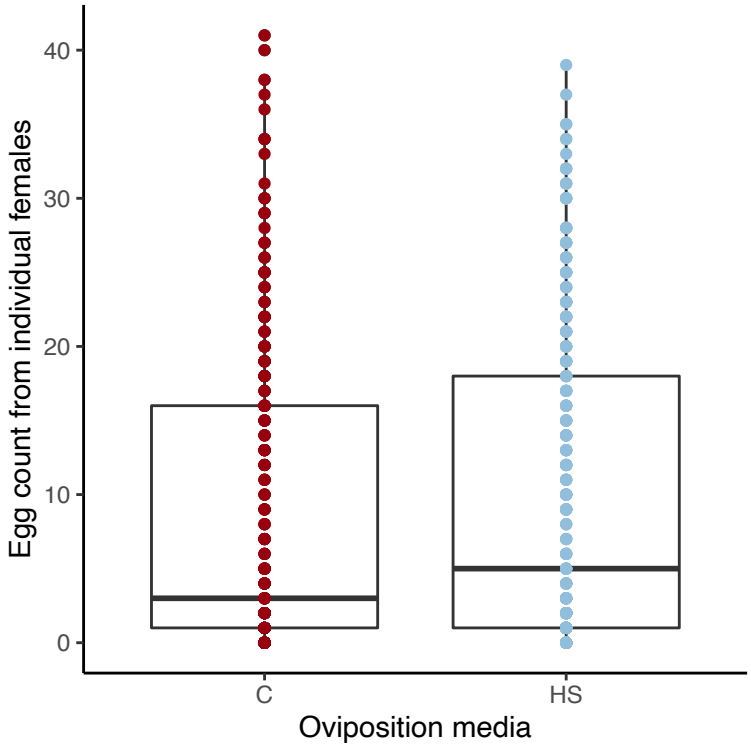


Supp. Fig. 1: 16S rRNA characterization of microbiome transplant.
A) Relative abundance at the genus level for donors, frass, and recipients in microbiome transplant. Each step of the transplant (i.e., donor, frass, recipient) shows overlap in only a few species. B) PCoA plots for Bray-Curtis distance based on relative abundance of ASVs for the microbiome transplant. Points represent individual flies, except for the frass (which was pooled from 250 flies and 6-8 replicates sequenced). Axis 1 mostly separates between frass and flies, while Axis 2 shows some separation between C and HS microbiomes. C) PCoA plots for Bray-Curtis distance for only the recipients, separated by diet. On the C diet, flies that received the C microbiome were distinct from those with the HS microbiome, suggesting fidelity of the microbiome transplant. However, on the HS diet, the microbiome transplant did not reconstitute differences between C and HS microbiomes in the recipient. D) Relative abundance of genera for each treatment in the recipients. The C recipients were composed of primarily Acetobacter and Commensalibacter genera, while only Acetobacter genera in HS recipients, especially on HS diets.

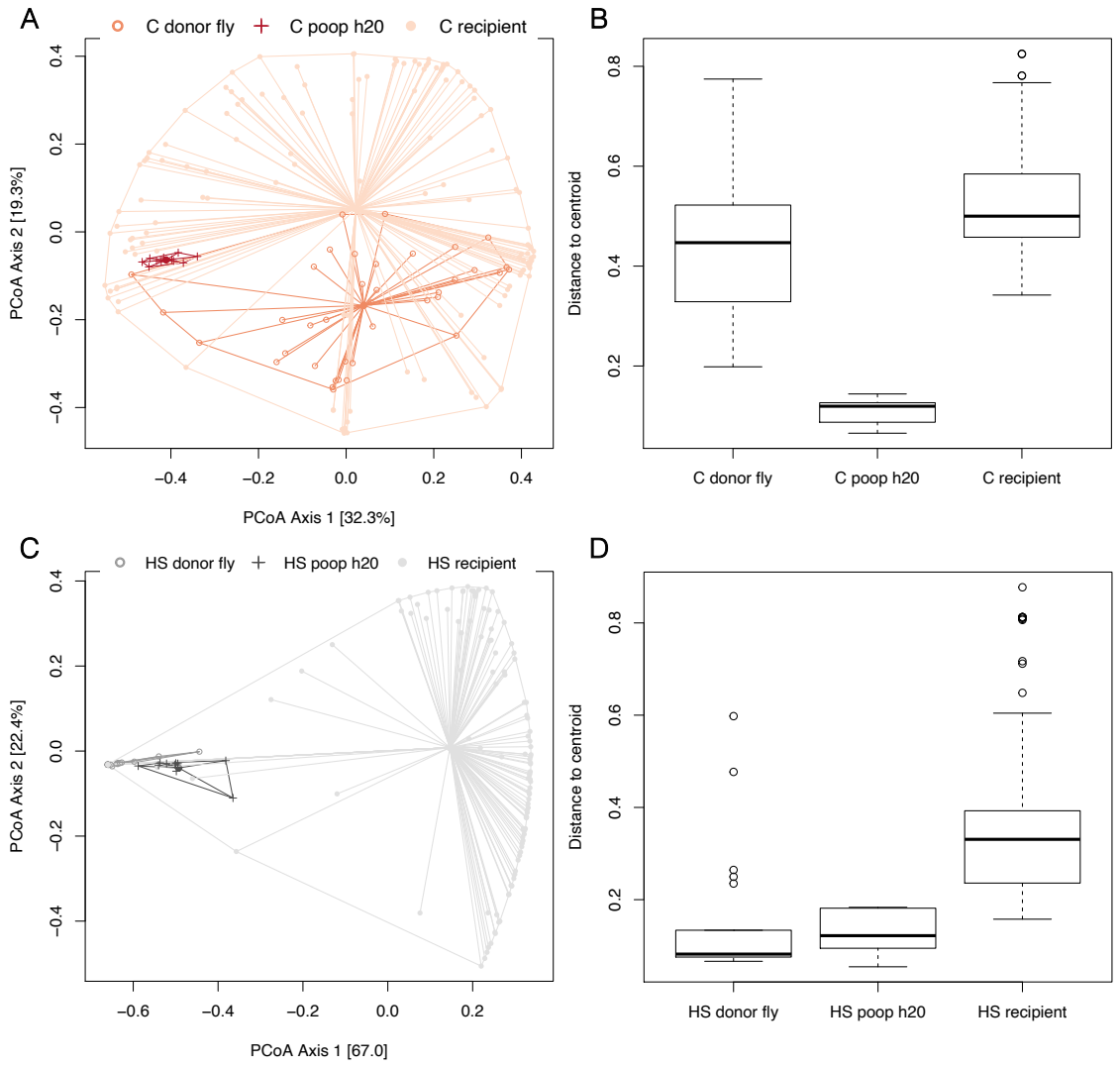


Supp. Fig. 2: Life history effects of the microbiome transplant.

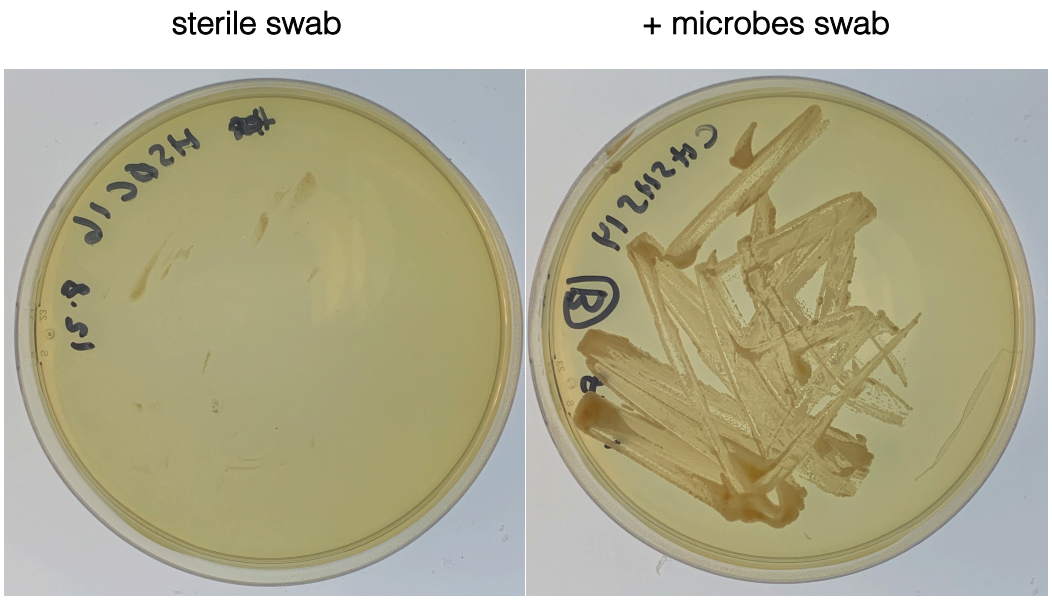
A) Hazard ratio plot for microbiome effects on developmental time. Boxplots show hazard ratios and 95% confidence intervals for each treatment relative to C0C. On the C diet, both fly genotypes with either C or HS microbiomes developed faster than sterile flies. However, on HS diet, and the C microbiome accelerated while HS microbiome slowed development. The interaction effects on the Cox proportional hazards model best explained variation in development (log-likelihood= -21807, df=7, X^2=78.026, p<0.001), suggesting that fly evolution and environment shape phenotypic effects of the microbiome. B) Fecundity effects of the microbiome transplant at three timepoints over 34 days. Plots are faceted by fly genotype and diet. Thick lines with dots represent the average fecundity for the microbiome treatment. Thinner lines represent the replicates. Fecundity declined over time ($F_{2,105.3}$ = 49.4, p<0.0001) and was shaped by both diet ($F_{2,49}$ = 4.35, p=0.02) and microbiome ($F_{1,50}$ = 5.41, p=0.02).



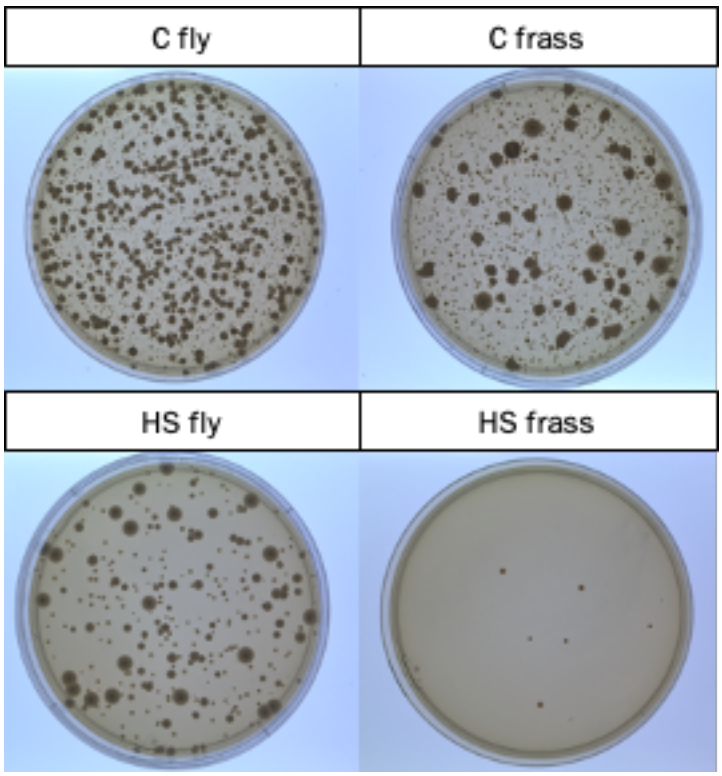
Supp. Fig. 3: Oviposition media differences.
No difference for egg counts between C and HS oviposition media (Kruskall-Wallis X^2 = 2.85, df=1, p = 0.09).



Supp. Fig 4: Differences in betadispersion between donor and recipients in microbiome transplant.
A) The relationship between donor, frass, and recipient on the C diet based on Bray-Curtis dissimilarity. Each point represents a fly, while the lines show the distance to mean value for each. B) Boxplot for centroid dispersion on the C diet. C) The relationship between donor, frass, and recipient on the HS diet based on Bray-Curtis dissimilarity. D) Boxplot for centroid dispersion on the HS diet.



Supp. Fig. 5: Representative images for sterility checks during Acetobacter transplant.
Food was swabbed during development and streaked on MRS plates. Sterile swab shows no growth and only food smudged. Microbes swab shows microbial growth.



Supp. Fig. 6: Differences in bacterial abundance between flies and frass for the C and HS fly genotypes. Figures show cultured bacteria (50 µl of 500 µl homogenate of 5 female flies or 50 µl of frass from ~200 flies) on MRS media incubated for 3 days. C flies harbor higher amounts of bacteria than HS flies. Bacteria in HS frass is especially low abundance.

SUPPLEMENTARY TABLES

Table 6: Comparison of centroid dispersion for donors, frass, and recipients in the microbiome transplant with 999 permutations.

Category	Df	SS	MS	F	*P* value
Microbiome source	5	5.715	1.143	36.282	0.001
Residuals	334	10.522	0.0315		

Table 7: Comparison of microbial community composition for recipients on C diets using PERMANOVA with 999 permutations.

Category	Df	SS	MS	F	R^2	*P* value
Fly genotype	1	0.6954	0.6954	6.129	0.02344	0.004
Microbiome	1	12.6455	12.6455	111.45	0.42627	0.001
Fly genotype x Microbiome	1	0.4395	0.4395	3.874	0.01482	0.01
Residuals	140	15.8849	0.1135	0.5354		
Total	143	29.6653	1			

Table 8: Comparison of microbial community composition for recipients on HS diets using PERMANOVA with 999 permutations.

Category	Df	SS	MS	F	R^2	*P* value
Fly genotype	1	1.8347	1.83472	8.5998	0.06509	0.001
Microbiome	1	1.1485	1.14846	5.3831	0.04074	0.006
Fly genotype x Microbiome	1	1.3113	1.3113	6.1464	0.04652	0.003
Residuals	112	23.8945	0.21334	0.84765		
Total	115	28.189	1			

Table 9: Comparisons of microbial community composition for all recipients using PERMANOVA with 999 permutations.

Category	Df	SS	MS	F	R^2	*P* value
Fly genotype	1	1.189	1.1891	22.026	0.0172	0.001
Microbiome	1	9.302	9.3019	172.301	0.13455	0.001
Diet	1	10.577	10.5766	195.913	0.15299	0.001
Fly genotype x Microbiome	1	0.339	0.3387	6.274	0.0049	0.001
Fly genotype x Diet	1	1.656	1.6562	30.678	0.02396	0.001
Microbiome x Diet	1	4.959	4.9593	91.863	0.07173	0.001
Fly genotype x Microbiome x Diet	1	1.163	1.163	21.543	0.01682	0.001
Tube (random effect)	39	28.451	0.7295	13.513	0.41153	0.001
Residuals	213	11.499	0.054	0.16633		

Total	259	69.135	1

Table 10: Analysis of deviance for Cox model with interactions between fly genotype, microbiome, and diet in the microbiome transplant.

Category	Log-likelihood	X^2	Df	*P* value
Null	-22850			
Fly genotype	-22716	268.8227	1	<2.20E-16
Microbiome	-22475	480.3245	2	<2.20E-16
Diet	-21846	1258.5088	1	<2.20E-16
Fly genotype x Microbiome	-21819	53.3452	2	2.61E-12
Fly genotype x Diet	-21817	4.2178	1	0.04
Microbiome x Diet	-21807	20.0385	2	4.45E-05
Fly genotype x Microbiome x Diet	-21807	0.4244	2	0.8088

Table 11: Fixed effects from the mixed model of lifetime fecundity in the microbiome transplant. Significance was evaluated using Type III Wald F tests with Kenward-Roger degrees of freedom.

Category	F	Df	Df.res	*P* value
Intercept	46.6596	1	60.701	4.62E-09
Fecundity time point	49.4043	2	105.269	7.38E-16
Fly genotype	0.0266	1	48.831	0.87103
Microbiome	4.3529	2	49.038	0.01819
Diet	5.4115	1	48.952	0.02419

SUPPLEMENTARY METHODS

Microbiome transplant methods

To test if the microbiome can transfer adaptive traits, we performed a reciprocal microbiome transplant experiment (Fig. 1). All host x microbiome x diet interactions were generated in the reciprocal transplant. Both genotypes (C and HS flies) were inoculated with microbiomes (sterile, C, HS) in both diets (C and HS) for a total of 12 treatments. This allowed us to investigate how host x microbiome x environment interactions influence the transfer of adaptive phenotypes.

To transplant the microbiome, we collected fly frass from 300 C and 300 HS age-matched flies (7-10 days old). Donor flies were placed in sterile 50 ml tubes lined with a plastic sheet and were allowed to defecate for ~ 5hrs. After 5 hrs, flies were immobilized at 4ºC and the plastic sheet removed. Fly frass was washed off the plastic using sterile water to make a fecal solution. Because of large differences in the abundance of bacteria between C and HS flies (Supp. Fig. 7), C frass was resuspended in 6 ml, while HS frass was resuspended in 3 ml. The fecal solution was passed through a fine mesh to remove any fly eggs and other miscellaneous fly material. 50 µl of fecal solution (or sterile water for the sterile treatments) was applied to axenic C and HS fly eggs onto sterile C and HS diets (7.5 ml food in 50 ml tube). Axenic eggs were prepared following standard procedures [1]. Sterile diets were prepared like the normal maintenance diets, but without acid and autoclaved. Flies were then reared in a biosafety cabinet at 25ºC and 12:12 light:dark cycle through the lifespan of the recipient flies. 5-6 replicates of each treatment were maintained and assessed for microbiome and phenotypic effects.

Assessing fidelity of the microbiome transplant

To determine the fidelity of the microbiome transplant, we performed 16S rRNA sequencing on the donor flies, frass, and adult recipient flies. All flies were age matched 7-10 days. Individual flies were sequenced. In brief, the 16S V1-V2 region was amplified, pooled, and digested with BstZ17l to deplete Wolbachia reads [2], and libraries were sequenced using 300 bp paired-end reads using the Illumina MiSeq platform at the Princeton University Genomics Core. We used QIIME2 v2019.4 to cluster into amplicon sequence variants (ASVs) with DADA2 and assign taxonomy with Greengenes classifier trimmed to the 16S rRNA V1-V2 region. Data was imported into phyloseq for visualization and statistical analyses [3].

Flies with fewer than 500 reads were discarded from the analysis. Relative abundance of genera was compared across levels of the transplant (i.e., donor vs. frass vs. recipient). To assess how community structure varied in response to the microbiome treatment, beta-diversity measures were calculated with subsampling down to 500 reads/fly. Microbiomes were visualized with principal coordinate analysis (PCoA) using Bray-Curtis dissimilarity from ASVs. We tested if dispersion differed between the levels of transplant using a permutation test for homogeneity of multivariate dispersion. Because of significant differences in variance between the transplant levels, we focused on differences between groups within the recipients. We performed an ADONIS test to assess how interactions between fly genotype, microbiome, and diet influence the microbiome of recipients.

Effects of the microbiome transplant on fly phenotypes

First, we measured development time in the recipients. We counted the number of new pupae every 6 hours during their day time (i.e., 8am, 2pm, 8pm) until all treatments began eclosing. We used the survival [4] and survminer [5] packages in R to analyse development data. We used Cox proportional hazards to identify how interactions between fly genotype, microbiome, and diet influence development time.

Second, we measured fecundity over the lifespan of recipients. Five females and five males from each replicate were transferred to new tubes. Flies were allowed to lay eggs for 24 hours and then flipped onto new food. Fecundity was measured at three timepoints over 34 days. Flies were flipped every 3-4 days in between fecundity measurements to avoid crowding and competition with developing larvae. Pupae were counted as a measure for fecundity and then divided by the number of females that survived to each fecundity measurement. We then analysed the effects of time, fly genotype, microbiome, and diet on fecundity with tube as a random effect using lme4 [6]. We tested for significance of fixed effects using Type III ANOVA with Kenward-Roger degrees of freedom using the car package in R [7]. We tested if including interactions significantly improved the model fit by using a likelihood ratio test.

SUPPLEMENTARY RESULTS

Microbiome transplant results

To test if transferring the microbiome could transfer adaptive phenotypes to non-evolved hosts, we performed a fully reciprocal, host genotype x microbiome x diet transplant experiment. We assessed the fidelity of the microbiome transplant using 16S rRNA sequencing of the donors, frass, and recipients. For both C and HS flies, the frass (which inoculated the diet of the recipients) was composed of fewer bacterial genera than found in donors (Supp. Fig. 1). The HS donor, frass, and recipient were dominated by Acetobacter species.

To compare how bacterial communities were structured across donor, frass, and recipient, we performed ordination analysis on Bray-Curtis distance matrix (Supp. Fig. 1B). The different steps of the transplant have different centroid dispersion (Supp. Table 6, N_{perm}=999, $F_{5,334}$=36.862, p<0.001). Broadly, the dispersion was greatest for recipients overall (Supp. Fig. 1C). For C flies, dispersion in frass microbial communities is greatly reduced compared to both donors and recipients. However, on HS flies, dispersion in frass and donors is similar, but lower than for recipients. The results from the dispersion analysis suggest that a subset of the microbiome was transferred through the frass, and for the recipients, differences in the structure of microbiome communities accumulated through their lifespan.

Both fly genotype and diet shaped the fidelity of the microbiome transplant in the recipients. Using Bray-Curtis distance to examine microbiome differences in recipients, we find that both microbiome and diet strongly impact beta-diversity in the recipients (Supp. Fig. 1D). On the C diet, recipients cluster by microbiome received (Supp. Table 7, $F_{1,143}$=111.45, r^2=0.43, p=0.001), suggesting a successful microbiome transplant. However, on the HS diet, the microbiome transplant breaks down and only had weak, though significant effects (Supp. Table 8, $F_{1,115}$ = 4.35, r^2 = 0.04, p=0.003). Overall, diet explained 14% and microbiome explained 15% of variation in microbial communities, while interactions between fly, microbiome, and diet had significant, but weak effects (r^2 < 8%, Supp. Table 9). For example, C flies that receive the C microbiome contained Acetobacter, Commensalibacter, and Gluconobacter, whereas HS flies only contained Acetobacter (Supp. Fig. 1D). Thus, the HS diet potentially selected for associations between fly and Acetobacter. Overall, despite the deviation from the donor microbiomes (Supp. Fig. 1C), the microbiome transplant led to different microbiomes between C and HS recipients, especially on the C diet.

Microbiome transplant shapes life history traits, but depends on the environment

The microbiome significantly shaped developmental time, but depended on fly genotype and diet (Supp. Fig. 2A, concordance = 0.825, log-rank test $p < 0.0001$). On the C diet, both fly genotypes responded to the microbiome in a similar way: sterility slowed development (avg. 136.9 +/-0.41 hrs), while C and HS microbiomes accelerated development similarly (C avg. 119.8 +/- 0.49 hrs, HS avg. 121.2 +/- 0.49 hrs). However, on the HS diet, the microbiome shaped development in different ways. The C microbiome (avg. 135.7 +/- 0.59 hrs) accelerated development compared to the HS microbiome (avg. 142.26 +/- 0.52 hrs) and the sterile treatments were the slowest (avg. 167.8 +/- 0.73 hrs). Two-way interactions between fly genotype, microbiome, and diet significantly shaped development time, but the three-way interaction did not (Supp. Table 10). The model with interactions best explained development data compared to one without interactions (log-likelihood = -21807, $X^2 = 78.02$, df =7, $p < 0.0001$). Overall, the results from development suggest that the microbiome and diet contribute to fly development.

To approximate fitness consequences of the microbiome transplant, we measured fecundity over the lifespan of the recipients (Supp. Fig. 2B). Fecundity declined over time ($F_{2,101.4} = 46.6$, $p < 0.0001$). The decline in fecundity was modified by both the diet and microbiome, but not by fly genotype (Supp. Table 11). Diet reduced fecundity by 26% in the HS diet (C diet avg. 26.3 +/- 2.40, HS diet avg. 19.8 +/- 1.69 pupae/female). The microbiome also affected fecundity, but only with the sterile flies having significantly lower fecundity (sterile avg. 15.4 +/-2.25, C avg. 24.5 +/- 2.42, HS avg. 26.1 +/- 2.56). The interaction between microbiome and time was significant ($F_{4,101.3} = 5.84$, $p = 0.0003$), driven by the slower decline in fecundity for the sterile treatments. The model that included this interaction best explained variation in fecundity (log-likelihood = -644.3, $X^2 = 22.44$, $p = 0.0002$). The results from the fecundity trials suggest that the presence of a microbiome can modify fly fitness on the HS diet, but little difference was observed between the C and HS microbiomes.

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