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Interactions between the microbiome and mating influence the female's transcriptional profile in *Drosophila melanogaster*

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Drosophila melanogaster females undergo a variety of post-mating changes that influence their activity, feeding behavior, metabolism, egg production and gene expression. These changes are induced either by mating itself or by sperm or seminal fluid proteins. In addition, studies have shown that axenic females—those lacking a microbiome—have altered fecundity compared to females with a microbiome, and that the microbiome of the female's mate can influence reproductive success. However, the extent to which post-mating changes in transcript abundance are affected by microbiome state is not well-characterized. Here we investigated fecundity and the post-mating transcript abundance profile of axenic or control females after mating with either axenic or control males. We observed interactions between the female's microbiome and her mating status: transcripts of genes involved in reproduction and genes with neuronal functions were differentially abundant depending on the females' microbiome status, but only in mated females. In addition, immunity genes showed varied responses to either the microbiome, mating, or a combination of those two factors. We further observed that the male's microbiome status influences the fecundity of both control and axenic females, while only influencing the transcriptional profile of axenic females. Our results indicate that the microbiome plays a vital role in the post-mating switch of the female's transcriptome.

Reproductive success is determined by the cumulative effects of behavioral and physiological changes that a female undergoes after mating. In *Drosophila*, these post-mating responses include sperm storage, increased oocyte production and ovulation, a decrease in sleep and the female's propensity to remate, alterations to the female's immune system, and changes in feeding frequency, gut morphology and physiology (reviewed in¹). These phenotypic changes are accompanied by extensive transcriptome changes across several female tissues^{2–11}. These transcriptome changes typically reach their highest magnitude at around 6 h after mating^{5,10}, and often include genes that encode proteolytic/metabolic enzymes and immune response genes^{3,9–11}. Furthermore, these female post-mating responses are influenced by an interplay between the genotypes of the female and her mate^{12–16}, and are induced in part by male ejaculate components that are transferred to the female during mating^{1,4–6,17,18}. The post-mating changes in metabolism and food uptake are thought to be required to meet the high energetic demands of oocyte production^{19,20}, and can potentially be influenced by transient factors such as the host microbiome.

In recent years, *Drosophila melanogaster* has emerged as a valuable model to study fundamental principles of host-microbiome interactions, owing to the availability of genetic resources and a well-characterized and easily-manipulated gut microbiome²¹. Removing the microbiome (bacteria and yeast) from *D. melanogaster* affects a wide range of traits, including the gut transcriptome, which highlights the regulatory effects of the microbiome on tissue homeostasis, carbohydrate and lipid metabolism, proteolysis and immunity^{22–26}. In addition, microbiome-induced transcriptome changes underlie a range of phenotypes such as larval development time^{27–30}, metabolite levels^{30,31}, intestinal stem cell proliferation^{32,33}, behavior^{34,35}, longevity^{28,33,36} and reproductive capacity^{28,37–39}.

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Across microbiome studies of *D. melanogaster*, some effects are consistently observed (e.g. transcriptome changes or changes in metabolite content), while others yield variable results, which likely depend on the experimental design and/or environmental conditions²¹. For example, Schretter et al.³⁵ observed a significant increase in the locomotor activity of axenic flies—those that lack a microbiome—relative to flies with a microbiome, but limited difference in activity was seen by Selkrig et al.⁴⁰. Furthermore, microbiome-induced changes in courtship were not observed by Selkrig et al.⁴⁰ and Leftwich et al.⁴¹, while changes in courtship were observed by Qiao et al.³⁹ and Sharon et al.⁴². Even egg laying, which was consistently observed to be lower in axenic females compared to females with a microbiome in multiple studies^{28,37–40}, was not observed to be lower in axenic females by Ridley et al.³⁰.

The varied results obtained in microbiome studies using *D. melanogaster* could be attributed to variability in nutrients^{43–45}, species and strains of microbiota and host, host age^{46–48}, or the requirement for frequent bacterial replenishment to maintain a stable microbiome^{49,50}. However, another variable that can play a role is female mating status. For example, several studies measured differences in the transcriptomes of female *D. melanogaster* with or without a microbiome, but if or when females mated was not explicitly controlled^(22–24,26). Thus, it is unclear how the interaction between microbiome and mating status influences whole-body transcript abundance in *D. melanogaster*.

Here we use short-read RNA sequencing (RNA-seq) to explore how the interaction between the female's mating status (virgin or mated) and her microbiome state (control or axenic) influence her transcriptome. We further evaluate how the female transcriptome is influenced by the microbiome of the female's mate, because previous work has shown that male mating success is impacted by his microbiome state³⁸. Of particular interest is the post-mating up-regulation of immune response genes which occurs in females of a variety of species⁵¹, including *D. melanogaster*. The post-mating up-regulation of some immunity genes was shown to be elicited by sperm and male seminal fluid proteins^{4–6,17}, but it is unclear whether microbiome state influences post-mating immune gene up-regulation, even though activation of the innate immune system might seem particularly sensitive to prior microbial exposure.

Results

We investigated whether the female's transcriptome is influenced by interactions between the female's microbiome and her mating status. In addition, we asked whether the microbiome status of her mate influences her fecundity and post-mating transcriptional response. To this end, we mated wildtype Canton-S control females (containing a conventional microbiome found in our lab's Canton-S stock) and axenic females (lacking a microbiome) to axenic or control Canton-S males. For these treatments, we measured fecundity across 54 h post-mating and transcript abundance at 6 h post-mating.

Both female and male microbiome status influence egg laying. Over the course of 54 h, we observed a significant interaction between female microbiome status and male microbiome status ($p = 0.035$). Control females mated to control males (CC) produced more eggs than axenic females, with an average of 77 eggs in CC crosses (± 4 ; sample size $n = 50$) versus 48 eggs in axenic females mated to axenic males (AA) (± 5 ; $n = 49$; $p < 0.0001$) and 53 eggs in axenic females mated to control males (AC) (± 6 ; $n = 47$; $p = 0.0004$) (Fig. 1A). Interestingly, the CC cross produced significantly more eggs than the CA cross (control females mated to axenic males), which produced on average 51 eggs (± 4 ; $n = 46$; $p = 0.003$; Fig. 1A). These results suggest that presence of a microbiome in the male has a positive impact on female fecundity.

Many transcripts differ in abundance between axenic and control virgin females, and many more are altered after mating. We investigated whether microbiome state influences a female's transcriptome by directly comparing the transcriptomes of axenic and control females. First, we examined sample clustering using multidimensional scaling and found that axenic and control samples are clearly separated across the first dimension, while virgin and mated samples are separated across the second dimension (Fig. 1B). Next, we compared transcript abundance between axenic and control virgin females and found 181 transcripts that differ in abundance (167 up-regulated and 14 down-regulated) (Fig. 1C,E, Table S1). Finally, we compared transcript abundance between axenic and control mated females, irrespective of their mate's microbiome status, and identified 371 transcripts that are differentially abundant (271 up-regulated and 100 down-regulated) (Fig. 1D,E, Table S1). These two contrasts have 124 genes in common, suggesting that these genes constitute a “core” set that is influenced by the microbiome regardless of mating status (Fig. 1E). In addition, 57 transcripts (51 up-regulated and 6 down-regulated) were affected by the microbiome specifically in virgin females, while 247 (155 up-regulated and 92 down-regulated) were affected by the microbiome specifically in mated females (Fig. 1E).

The “core” set of microbiome response genes in females are involved in metabolic and immune processes and have an expression bias in the midgut. Among the 124 transcripts that are influenced by the microbiome state regardless of the females' mating status, we found significant enrichment of Biological Process Gene Ontology (GO) terms related to the immune response and carbohydrate, nucleoside, lipid and amino acid metabolism (Fig. S4A). Similarly, enriched Molecular Function GO terms included hydrolase, glucosidase, peptidase and lipase and sterol binding activity (Fig. S4B). The majority of these transcripts (116/124) were up-regulated in control females relative to axenic females. These observations are in accordance with studies that showed that axenic flies have altered levels of glucose, trehalose, triglycerides, proteins and insulin-like signaling^{30,31,54–57}. The core set of microbiome-responsive genes also have a clear expression bias in the female midgut (Fig. 2A). This result is similar to that of²³, who found major transcriptome changes in the gut of axenic females, but detected few changes in non-gut tissues. Only eight transcripts had higher abundance in axenic

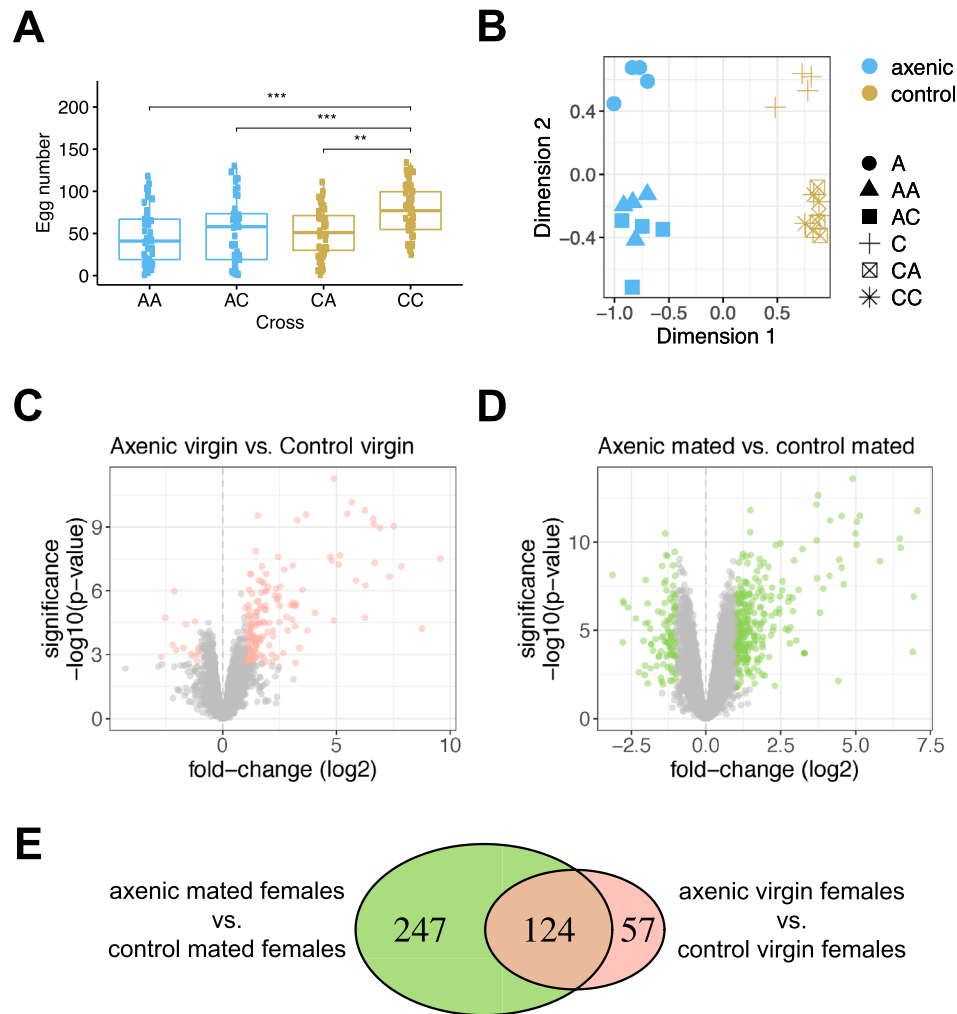


Figure 1. Fecundity and transcript abundance differences between axenic and control females. **(A)** Fecundity of axenic and control females after a single mating to axenic or control males. Each point represents eggs laid by one female over the course of 54 h. The first letter refers to the female's microbiome status, the second letter refers to the male's microbiome status (A = axenic, C = Control). Sample sizes: $n = 49$ for AA, $n = 47$ for AC, $n = 46$ for CA and $n = 50$ for CC. Groups were compared using a generalized linear mixed model with a Poisson response distribution. $***p < 0.001$; $**p < 0.01$ **(B)** Multidimensional scaling plot of replicates and samples used in the study. Microbiome status is represented by colour, and sample origin represented by shape. **(C)** Volcano plot showing the results of a differential expression analysis of control virgin females relative to axenic virgin females. Significant genes (FDR < 0.05 , $>$ two-fold) are shown in pink. **(D)** Volcano plot showing the results of a differential expression analysis of control mated females relative to axenic mated females, averaged across the two male microbiome states. Significant genes (FDR < 0.05 , $>$ two-fold) are shown in green **(E)** Overlap of genes that are influenced by the microbiome in virgin and mated females. Figures were produced using R⁵².

females relative to control females. Of these, *Pka-R1* is noteworthy because it regulates feeding behavior⁵⁸ and PKA signaling acts downstream of dopamine signals to promote ovarian dormancy⁵⁹.

In virgin females specifically, only 57 transcripts were affected by microbiome status (Fig. 1C,E). Most of these (51/57) were up-regulated in control virgin females relative to axenic virgin females. These up-regulated transcripts were significantly enriched for peptidases and carbohydrate transmembrane transporters (Fig. S4C). In addition, we identified 10 genes involved in the immune response and two genes involved in reproduction (*tj*, *Cp36*). Only six transcripts were down-regulated in control relative to axenic virgin females. One of these is *takeout*, which is associated with circadian rhythm, starvation and food intake⁶⁰. We tested the transcript levels of *to* using qRT-PCR on independently collected samples and verified that it was downregulated in control virgin females relative to axenic virgin females ($p = 0.005$, Fig. S6). We also performed a qRT-PCR on *Mtk* (an antimicrobial peptide) and *Tobi* ("target of brain insulin";⁶¹). For both genes, we were able to validate a significant upregulation in control mated females relative to axenic mated females (Fig. S6; *Mtk* $p = 0.04$; *Tobi* $p = 0.007$). *Mtk* and *Tobi* expression was not significantly higher in control virgin females relative to axenic virgin females in our qRT-PCR results.

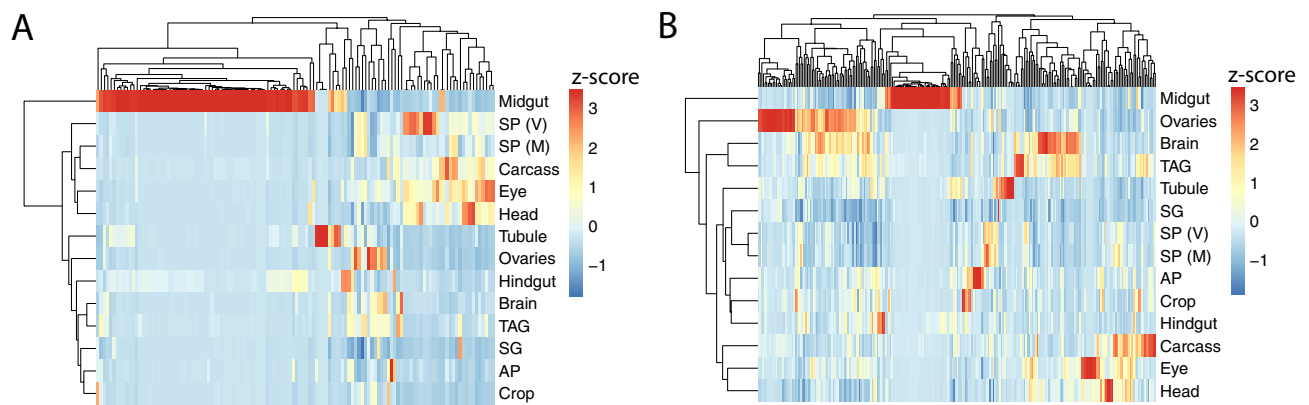


Figure 2. Tissue enrichment scores for differentially abundant transcripts across 14 female tissues. (A) Heatmap for 124 “core” genes whose RNA levels are influenced by the microbiome in both mated and virgin females. (B) Heatmap for 247 genes whose RNA levels are influenced by the microbiome in mated females only. Enrichment scores were calculated using TPM values from FlyAtlas2⁵³. Both heatmaps were generated with the R package pheatmap (v. 1.0.12). (AP = anal plate; M = mated; SG = salivary gland; SP = spermathecae; TAG = Thoracico-abdominal ganglion; V = virgin). Figures were produced using R⁵².

Many of the 124 “core” genes influenced by the microbiome in this study were influenced by the microbiome in previously published studies. The study design we employed is different from the designs used by most published microbiome studies. Specifically, we created “control” flies with a conventional microbiome by adding homogenate of untreated flies onto the fly media, while most studies generate gnotobiotic flies which carry a limited, curated set of bacterial species that are usually found in the fly gut²¹. We created control flies to assess the effects of presence/absence of the microbiome, rather than the effects of specific bacteria. In addition, we did not want to omit potential effects of bacteria present in the reproductive tract, which have been shown to influence reproduction in several other species⁶², but have not been characterized in *D. melanogaster*.

Despite the differences in study design, more than half (52%) of our “core” genes had been reported to be influenced by the microbiome in at least one of three other studies^{22,23,25} (Table S2). Moreover, these 124 genes were enriched for similar functions, i.e., immune response and metabolic processes^{22–25}. Broderick et al.²³ also defined a “core” set of 152 genes whose transcript abundance was influenced by the microbiome in the female gut in flies with distinct genotypes (Oregon-R and Canton-S). We found only 11 genes that overlap between our core set and the core set from²³. This likely reflects the use of gut versus whole fly, or it could be caused by differences in experimental design, such as growth conditions or fly genotypes. Still, these 11 genes fall into several broad functional classes that are affected by the microbiome both in this study and in²³, including immune and stress response genes (*AttA*, *AttB*, *GstD8*), genes affecting gut structure (*Mur29B*, *CG7017*), metabolism (*Npc2e*, *Acbp6*, *Gba1a*, *CG17192*) and gene expression (*CG15533*).

Transcripts involved in reproduction and neuronal function differ in abundance between axenic and control mated females. Next we analyzed the 247 transcripts that differ in abundance specifically between mated axenic and mated control females (Fig. 1D–E). These transcripts have an expression bias to the midgut and the ovary, and to a lesser extent the brain (Fig. 2B). Of the 247 transcripts, 155 transcripts were up-regulated and 93 transcripts were down-regulated in mated control females relative to mated axenic females (Fig. 1D). We did not identify significantly enriched GO terms among the 155 up-regulated genes, but we detected 29 genes associated with “multicellular organism reproduction”. We performed a Gene Set Enrichment Analysis (GSEA) using the fold-changes derived from the contrast between mated axenic females and mated control females, and identified multiple significant Biological Process terms associated with immunity, reproduction and metabolism (Table S3). Notably, for Molecular Function, only hydrolase activity was enriched (Fig. 3A). Genes encoding hydrolases show a strong expression bias in the midgut and are largely composed of maltases and mannosidases (Fig. 3B). Additional up-regulated genes include those involved in the immune response (*Def*, *PGRP-SC1b*, *PGRP-SD*), (dopaminergic) neurotransmission, (*Fer2*, *Catsup*, *Bx*, *Atpal-pha*) (Fig. 3C), and pigment biosynthesis (*yellow-f*, *bw*). Changes in pigment biosynthesis have been described in axenic flies⁶³, and this could reflect a sub-optimal metabolism in the absence of bacteria⁶⁴.

The 93 transcripts with lower abundance in control mated females compared to axenic mated females were significantly enriched for genes involved in sensory perception (Fig. S4D). These included four genes encoding odorant binding proteins (*Obp8a*, *Obp44a*, *Obp56g*, *Obp57c*), genes involved in phototransduction (*Rh5*, *ninaA* and *ninaC*) and a cation channel (*trp*) (Fig. 3C). Furthermore, mRNAs encoding three neuropeptides (*Nplp3*, *Pdf*, *Capa*) and *TpnC4* and *TpnC41C*, which are part of the muscle troponin complex, were down-regulated in control mated females (Fig. 3C).

22 genes respond to mating regardless of female microbiome status. We detected 22 transcripts that were up- or down-regulated in females after mating regardless of microbiome status (Table S4). We detected these transcripts by contrasting transcript abundance in mated females with that of the respective virgin females.

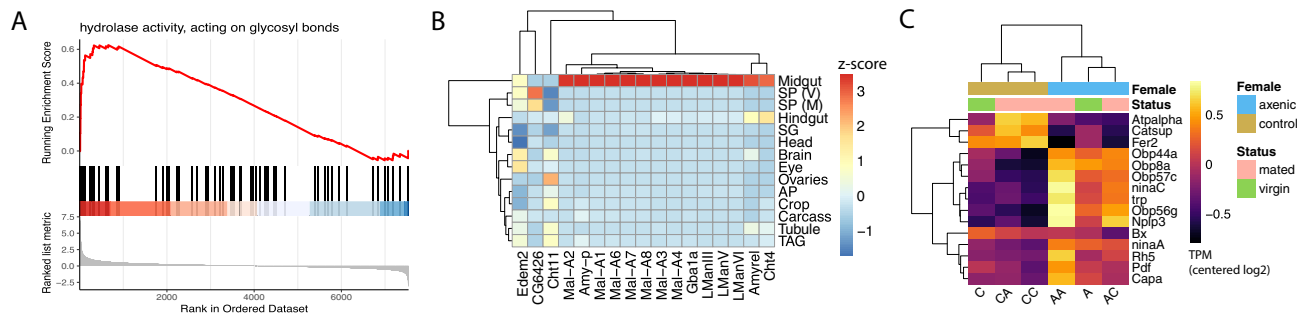


Figure 3. Genes whose mRNA levels are influenced by the microbiome in mated females have roles in metabolism and neuronal functions. **(A)** Gene Set Enrichment Analysis (GSEA) showing that transcripts involved in glycolytic metabolism are generally up-regulated in mated females with a microbiome relative to axenic mated females. The top panel of the GSEA plot shows the running enrichment score for a rank-ordered list of genes that are involved in hydrolase activity (genes are ranked based on the log₂ fold change between mated control and mated axenic females, in decreasing order). Each vertical black line represents a gene that is involved in hydrolase activity. **(B)** Tissue enrichment Z-scores of genes encoding hydrolases, that are differentially expressed between mated axenic and mated control females. These genes show a strong expression bias in the female midgut (abbreviated tissue samples are the same as in Fig. 2). **(C)** Heatmap showing mean centered log₂ TPM for 14 genes with sensory or neuronal functions, whose transcript abundance is altered in mated females depending on whether they have a microbiome or not. (The panel A figure was generated using the gseaplot2 function of the R package enrichplot (v. 1.4.0) and heatmaps in B and C were generated with the R package pheatmap (v. 1.0.12). (A = axenic, C = control). The first letter refers to the female's microbiome status, the second letter refers to the male's microbiome status. Female microbiome and mating status are indicated above the heatmap and in the key. Figures were produced using R⁵².

Mating-responsive genes include three spermathecal serine-type endopeptidases (*Send2*, *CG17239*, *CG17234*); the metallopeptidase *Nep7*; *jhamt*, involved in juvenile hormone synthesis; a maltase, *Mal-B1*; a gene encoding an odorant binding protein *Obp83f*; *wbl*, involved in Toll signaling and dorso-ventral patterning; the antimicrobial peptide *Listericin*; and *CG14191*, which is involved in sarcomere function. Using qRT-PCR on independently collected samples, we confirmed the post-mating upregulation of *jhamt* in both axenic and control females (p values for both contrasts < 0.001; Fig. S6). We further confirmed a downregulation of *Mal-B1* in control females after mating using qRT-PCR ($p = 0.02$; Fig. S6). *Mal-B1* transcript abundance was also lower after mating in axenic females, but that difference was not statistically significant based on the qRT-PCR data.

Male microbiome status does not affect post-mating transcript abundance in control females, but has a major effect on axenic females. Next we examined contrasts that reveal the effect of male microbiome status on the female's post-mating transcriptome. When we directly compared transcript abundance in control females mated to control males with that of control females mated to axenic males, we did not detect any differentially-expressed transcripts (Fig. 4A). This suggests that the male's microbiome does not affect post-mating mRNA levels at six hours in females that have a microbiome. We then compared transcript abundance between axenic females mated to axenic males and axenic females mated to control males and found 136 transcripts that were differentially abundant (Fig. 4A, Table S5). Hierarchical clustering of all samples based on the normalized expression for these 136 genes showed that the transcript abundance of these genes was similar across virgin females and mated control females; mated axenic females formed separate clusters depending on the male they mated with, showing opposite patterns of transcript abundance (Fig. 4B). Only 14 transcripts were detected at a higher level in AC crosses relative to AA crosses. These included three immune effectors (*IM18*, *Dro* and *Listericin*; Fig. 4C). The majority of the transcripts (122/136) were present at a lower level in AC crosses compared to AA crosses. These genes have an expression bias to ovaries and the midgut, brain and thoracico-abdominal ganglion (Fig. S5). Gene Set Enrichment Analysis indicated a significant enrichment of peptidases among the 136 differentially expressed genes. Additional genes of interest identified using GO classification include 17 genes involved in the stress and immune response, 8 genes involved in reproduction (among which are *vas* and *jhamt*, Fig. 4C) and genes with neuronal functions (*Atx2*, *NinaE*, *Bx*, *TBPH*, *Dsk*).

Discussion

This study addressed two main questions: (1) Is a female's transcriptome influenced by interactions between her microbiome and her mating status? and (2) can interactions between the female's microbiome and the male's microbiome influence the female's transcriptome and fecundity? We found evidence for such interactions and discuss their implications for the female's reproduction and metabolism, neuronal functions and immune gene expression. We also note that by using only one host genotype, we cannot rule out that some of these responses, or the magnitude of the responses might be host genotype-specific.

Using a fecundity assay, we observed that females without a microbiome laid fewer eggs than females with a microbiome. This observation confirms published results^{28,37,39}. Furthermore, we observed lower mRNA abundance of genes involved in egg production in axenic females. This was apparent in axenic virgin females, which

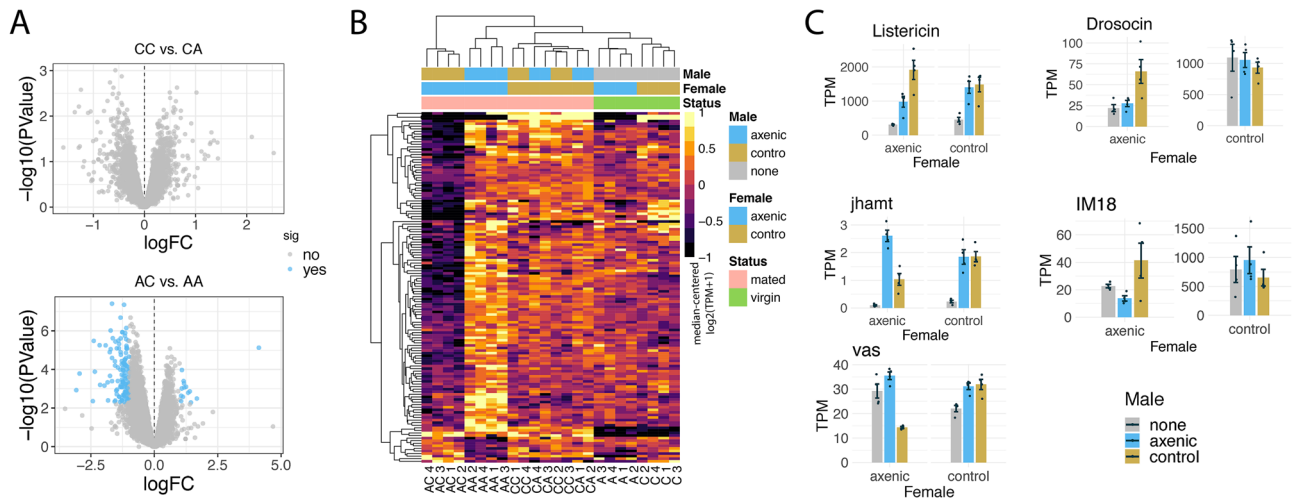


Figure 4. Analysis of female transcriptome changes that are influenced by the male's microbiome status. **(A)** Volcano plot showing the results of a differential expression analysis assessing the effect of male microbiome status on control females (top) and axenic females (bottom). **(B)** Heatmap of normalized, batch-adjusted abundance values (TPM; transcripts per million) for transcripts that are altered in axenic females after mating with an axenic or control male. The heatmap was generated with the R package pheatmap (v. 1.0.12). **(C)** Barplots of TPM values for a subset of genes that are influenced by the male's microbiome in axenic mated females. Error bars represent standard error, and points represent quadruplicate TPM values. Figures were produced using R⁵².

had—compared to control virgin females—lower mRNA levels of *Cp36*, which encodes a chorion protein⁶⁵, and *tj*, which is involved in gonad morphogenesis⁶⁶. After mating, we detected differential abundance of many additional transcripts involved in reproduction. This is likely because mating, and specifically seminal fluid proteins, kickstart egg production¹.

We observed an up-regulation of *jhamt* after mating in all females, whether axenic or control. JHAMT is essential for juvenile hormone (JH) synthesis⁶⁷. JH is an endocrine factor that stimulates the production of vitellogenin and yolk proteins⁶⁸ and JH also suppresses the mated fly's ability to resist infection⁶⁹. JH production is stimulated by the male seminal fluid protein Sex Peptide⁷⁰. The observed up-regulation of *jhamt* mRNA suggests that, in the absence of a microbiome, signals received during mating still elicit an attempt to initiate oogenesis via JH, but somehow oogenesis is curtailed in the absence of a microbiome. One factor that likely contributes is an altered metabolism in axenic vs. control mated females, which was observed both in our study (using transcript abundance) and previously published studies (using transcript abundance or metabolite measurements)^{22–26,30,31}. Our study took these observations further, by showing that the abundance of mRNAs of metabolic genes is lower in the absence of the microbiome both before and after mating. Interestingly, Elgart et al. (2016) showed that *Aldehyde dehydrogenase (Aldh)* expression and activity differed between axenic and control flies and that this influenced oogenesis. In our dataset, the abundance of *Aldh* mRNAs did not differ between axenic and control females, but this could be due to tissue-specific or post-transcriptional regulation. Studies of the post-mating responses in *D. melanogaster* females have shown that mating induces metabolic changes and changes in feeding behavior, likely to accommodate the high energy demands of oogenesis^{19,20,71–74}. Thus, in the absence of a microbiome, a females' ability to manage the metabolic changes needed to sustain egg production might be negatively affected.

Our data further show that the microbiome influences the mRNA abundance of genes with neuronal functions. This class of genes was also reported as influenced by the microbiome by²⁵. Our data show that this phenomenon specifically occurred in mated females and not in virgin females. We observed an up-regulation of genes encoding odorant binding proteins and genes encoding components needed for phototransduction in axenic mated females relative to control mated females. Several studies have shown that fly olfactory behavior changes in the absence of a microbiome or upon changes in microbial composition^{34,39,75,76}, but none reported effects on vision. Transcript abundance of genes involved in olfaction and phototransduction also change after mating in *Drosophila*^{4,5,8,14} and honeybees⁷⁷. In flies, such changes in sensory genes after mating could mediate changes in female receptivity to other males^{78,79} or aid her in finding suitable sites for egg laying^{18,80}. Thus, it is possible that post-mating sensory responses are altered depending on female microbiome state.

We further observed changes in the mRNA levels of two genes with functions in circadian and locomotor behavior, *Bx* and *Pdf*^{81,82}. In addition, two troponins required for muscle contraction had higher mRNA levels in mated axenic females relative to mated control females. *D. melanogaster* female activity levels increase after mating⁸³ and in the absence of a microbiome³⁵. Thus, the transcript changes we observed could reflect those changes in locomotion on a molecular level. In addition, we observed changes in the mRNA levels of genes involved in dopamine signaling (*Fer2*, *Catsup*, *Bx*, *Atpalpha*)^{84,85}. Dopamine has many effects on fly behavior⁸⁶ and the causes and consequences of changes in dopamine signaling cannot be determined based on the current

study. However, these results indicate that interactions between the female's microbiome and her mating status have a significant impact on mRNA levels of neuronal genes.

A common observation in studies of the female post-mating response is that immunity genes are up-regulated after mating^{2,4,10,87}, but the basis for this post-mating induction of immune response genes is not fully understood⁵¹. Here we found that transcripts of immunity genes were up-regulated in control females relative to axenic females, confirming results from other studies^{22–26}. In addition, the transcript levels of some immunity genes were influenced by interactions between the female's microbiome and her mating status. For example, *Def*, *PGRP-SC1b* and *PGRP-SD* had higher mRNA levels in control mated females relative to axenic mated females, while this was not the case when comparing virgin females. This indicates that mating elevates the mRNA levels of these genes only in the presence of a microbiome in the female. On the other hand, transcripts of the antimicrobial peptide *Listericin* were up-regulated by mating in all females, regardless of whether they or their mate had a microbiome. *Listericin* expression has been reported to be regulated by PGRP-LE and JAK-STAT signaling⁸⁸. Our observation is particularly interesting because it suggests that some aspect of mating, without the need for microbiota, can increase the RNA levels of this antimicrobial peptide, perhaps by activating JAK-STAT signaling rather than Toll and imd signaling, the canonical signaling pathways in response to septic threats. This aspect of mating could be copulatory wounding^{89,90}, or exposure to sperm or seminal fluid proteins^{4–7,17,18,91}. If an axenic female mated to a control male, additional immune transcripts were up-regulated relative to when an axenic female mated to an axenic male (e.g. *IM18*, *Dro*, and *Listericin*), indicating that exposure to microbiota during courtship or copulation stimulates an additional up-regulation of these immune gene transcripts. There is increasing attention for the role of reproductive tract microbiota in reproductive success^{62,92}, and a female- and male-specific reproductive tract microbiome has been characterized in *Anopheles* mosquitos, but whether *D. melanogaster* have reproductive-tract specific microbiomes that can influence the post-mating up-regulation of immune transcripts is not yet known.

Using RNA-seq data, we did not observe effects of male microbiome status on the transcriptome of control females, but we observed significant effects on the transcriptome of axenic females. We wondered whether exposure to bacteria on the male's cuticle during courtship or mating, or exposure to male excreta, could make the mRNA levels of an axenic female more similar to those of a control female. However, at 6 h after mating, that does not appear to be the case. The genes whose mRNA levels were influenced by the male's microbiome had various functions (including egg production) and were mostly down-regulated after mating with a control male. Perhaps a sudden exposure to bacteria during mating does not make axenic female mRNA levels more similar to those of control females because resources are used to initiate an immune response rather than oogenesis. Additional experiments at multiple time points would be necessary to resolve this hypothesis.

Aside from transcript abundance, female fecundity was also influenced by the male's microbiome. Fecundity was lower not only in axenic females, but also in control females that had mated with an axenic male. This indicates that the absence of a microbiome impacts a male's reproductive success. Interactions between a male's reproductive success and his microbiome were also observed by³⁸. For example, Morimoto et al. (2017) observed that gnotobiotic males carrying only *Lactobacillus plantarum* had a longer copulation duration and induced higher short-term egg laying in their mates. Axenic males could differ from control males in pheromone production, or in the production, transfer or quality of seminal fluid proteins or sperm. The reduced fecundity in control females mated to axenic males was not accompanied by transcript level changes in our dataset, possibly due to the time point measured. It is also possible that egg production is unaffected in control females mated to axenic males, but that they differ from control females mated to control males in their frequency of egg deposition.

To conclude, we have shown that a *D. melanogaster* female's transcriptome is influenced by interactions between her microbiome and her mating status, and that both transcript abundance and fecundity are influenced by interactions between the female's microbiome and that of her mate. Our results demonstrate the importance of considering a females' mating status to better understand and interpret the host microbiome's impact on overall fitness.

Materials and methods

Fly stocks, rearing and the generation of axenic and control flies. Canton-S flies were maintained at 25°C on yeast-sucrose-cornmeal food (7 g agar; 12 g yeast; 12 g cornmeal; 40 g sucrose; 1000 ml water, 26.5 ml Tegosept; 12 ml acid mixture) in a 12 h light/dark cycle. To generate axenic and control flies, we followed the protocol described by⁹³. Briefly, population cages of Canton-S flies were set up and females were allowed to oviposit on grape juice agar plates for 2–3 days until robust egg-laying began. On the third day, embryos were collected and treated twice (2.5 min each time) with a 0.6% sodium hypochlorite solution and triple-rinsed in autoclaved distilled water in a laminar flow hood. Axenic embryos were allowed to hatch in 50 ml sterile vials containing yeasted autoclaved food with 40 ul of 1X PBS added to the food surface. To generate controls, the same embryo dechoriation procedure was followed, but the tubes for the control samples received 40 ul of Canton-S adult fly homogenate (prepared in aliquots of 200 ul, at a concentration of 50 flies in 200 ul 1X PBS) on the food surface to add the full set of bacteria found in our lab's Canton-S stock. After 7 days, no differences in developmental rate were apparent, and pupae from axenic and control tubes were collected and twice treated with 0.6% sodium hypochlorite for 30 sec and subsequently rinsed three times in autoclaved distilled water. Pupae were individually placed into a vial with sterile food. Each vial with a 7-day old control pupa received 20 ul of Canton-S fly homogenate on the food surface (on average 2.5 flies ground up for each vial). Each vial with an axenic pupa received 20 ul of sterile 1X PBS. Since only control flies received fly homogenate, we measured the dry weight of control and axenic virgin females to ensure that the addition of fly homogenate did not result in an increased body mass of control relative to axenic flies. We desiccated the abdomen and thorax of 15 axenic virgin females and 14 control virgin females in a drying oven overnight at 60°C. Dry weight was measured using

a Sartorius CP2P microbalance. We only weighed the abdomen and thorax because appendages and heads were prone to dislodge after storage at -80°C . A Wilcoxon test indicated no significant difference in dry mass between control and axenic virgin females ($p = 0.57$; Fig. S1A).

Confirmation of microbiome status. We performed two assays to ensure axenic flies were germ-free and to ensure the presence of a microbiome in control flies: 1) Individual flies were homogenized in De Man, Rogosa and Sharpe (MRS) broth and plated on MRS plates as in⁹³, which were then incubated at 29°C for 2–3 days and checked for colonies, and 2) a PCR assay was performed according to the methods in³⁰. Briefly, genomic DNA was extracted from 3 to 10 pooled axenic or control larvae, pupae or adult flies. PCR was run using primers designed by³⁰ for a conserved region of the bacterial 16S rRNA gene. PCR products were run on a 1% agarose gel to confirm the absence of bacteria in axenic individuals and the presence of bacteria in controls (Fig. S1B). Ethidium bromide-stained gels were imaged using Gel Doc EQ (Biorad, CA). An image with modified contrast is shown in Fig. S1B and the original gel image is shown in Fig. S1C. The contrast modification was performed on Adobe Photoshop (release 20.0.10) by applying a brightness and contrast adjustment of -108 and 55 , respectively. We also compared levels of bacteria in 9–12 pooled adults of our control flies with levels of bacteria in 9–12 pooled untreated (not dechorionated) adult Canton-S flies and found an enrichment of bacteria in our control flies (Fig. S1B). Absence of *Wolbachia pipientis* in our lab's Canton-S stock was confirmed using a PCR assay described by⁹⁴.

Mating assay and sample collection for RNA-seq and qRT-PCR. Five day old virgin flies were used for the mating assays. Axenic and control females and males were singly mated in a 2x2 full factorial design: control females \times control males (CC), control females \times axenic males (CA), axenic females \times axenic males (AA) and axenic females \times control males (AC). All matings took place between 8 a.m. and 12 p.m. Matings were observed and males were removed from the vial after copulation ended. The end time of copulation was recorded and females were flash-frozen 6 h after mating, at which time virgin axenic and control females from the same cohort were also flash-frozen. For RNA-seq, we collected four replicates for each of the six treatments on the same day. Around ten females were pooled per replicate. To carry out qRT-PCR confirmations using samples independent from those used for the RNA-seq, we used flies produced from eggs that were dechorionated on a different day from the ones used for the RNA-seq samples. Females were mated to males of the same microbiome status as themselves and were flash frozen 6 h after mating, at which time virgin females were also frozen. For each treatment for the qRT-PCR, three replicates of 10 pooled females were collected.

RNA extraction, RNA-seq library preparation and qRT-PCR methods. To extract whole RNA, a pool of ~ 10 frozen females from each sample was homogenized in TRIzol following manufacturer's guidelines (Thermo Fisher Scientific Inc., MA). Following liquid phase separation, the RNA-containing upper layer was subjected to column purification and DNase treatment using the RNeasy Mini Kit (Qiagen inc., MD). Purified RNA was quantified and saved at -80°C for library preparation. RNA-seq libraries were made using the Lexogen 3' FWD kit following the manufacturer's protocol (Lexogen, NH). Libraries were quantified on an Agilent 2100 Bioanalyzer before pooling and cluster generation/sequencing on an Illumina NextSeq platform.

For qRT-PCR, RNA was extracted as above. RNA was DNase treated using RQ1 RNase-Free DNase (Promega, WI) and cDNA was synthesized using SMARTScribeTM Reverse Transcriptase (Clontech, CA). qRT-PCR reactions were run on three biological replicates, each with three technical replicates, on a Roche LightCycler 480 Instrument II using LightCycler 480 SYBR GreenI Master (Roche, NJ). Primers were designed using Primer Blast, except for the gene *jhamt*, for which we used primers designed by⁶⁹ and we verified that primer efficiency was above 80%. Primer sequences can be found in Table S6. *Rp49* or *Nervana* were used as control genes. We verified that these genes were not among the differentially expressed genes for the contrasts of interest. Ct values were analyzed using linear models in R. For each of the five genes tested, we set up an independent linear model using Ct value of the gene of interest as response variable, and using "sample" (A, C, AA or CC) and Ct value of the resp. housekeeping gene as explanatory variables (both fixed effects). The linear models were run on three biological replicates for each gene tested. Each biological replicate was the average of three technical replicates. After fitting the models, we calculated estimated marginal means (EMM), standard error of the EMM and pairwise contrasts between samples using the R package emmeans⁹⁵. p values of pairwise contrasts were corrected for multiple testing using the Benjamini-Hochberg method⁹⁶. We used a Shapiro test to ensure that residuals of the fitted models followed a normal distribution and used a Levene's test to ensure homogeneity of variance of the Ct values.

Read processing, alignment and differential expression analysis. Raw reads were processed by trimming 10 bases from the 5' end and quality trimming from the 3' end to a minimum quality PHRED score of 20. Processed reads were mapped to the *D. melanogaster* transcriptome (Flybase r6.23) with bowtie2, and read counts and normalized abundances were extracted using eXpress^{97,98}. All differential expression analyses were performed in R using the packages EdgeR⁹⁹ and RUVseq¹⁰⁰. We filtered genes with $\text{cpm} < 1$ in at least 4 samples, leaving 7,649 genes in the dataset. After normalizing counts based on library size, a clear batch effect was visible (Fig. S2A). We used RUVseq to identify $k = 3$ additional variables that were added to the linear model in EdgeR. These variables were estimated by RUVseq based on the residuals from a linear model fitted with the sample variables. Adjusting for three additional unknown variables resulted in improved clustering of samples in a PCA plot (Fig. S2B) and improved Pearson's correlations between replicates of the same sample.

We set up contrasts to 1) identify changes in transcript abundance in the female that depend on her microbiome state, 2) identify mating-responsive transcripts in females, and 3) identify changes in transcript abundance in

females that are influenced by the microbiome state of her mate. We removed differentially expressed transcripts that contained identical counts across multiple genes due to ambiguity in read mapping. Transcripts were considered significantly differentially abundant if the change was >two-fold and had a *p* value adjusted for multiple testing <0.05⁹⁶. The package ClusterProfiler¹⁰¹ was used for Gene Ontology (GO) and Gene Set Enrichment Analysis (GSEA). GSEA was based on fold changes for all 7649 genes in the filtered dataset, using a minimal gene set of 50 genes and a maximal gene set of 500 genes. GO enrichment analysis was performed on the genes with differentially abundant transcripts, using a minimal gene set of 2 genes, and using all 7,649 genes as background. We called a GO category as significantly enriched if it had an adjusted *p* value ≤0.05. DAVID^{102,103} and Flybase¹⁰⁴ were queried for further functional annotation of genes.

Tissue enrichment calculation. To determine if differentially abundant transcripts had an expression bias to particular female tissues, we used a custom analysis of gene expression data from the FlyAtlas (version 2) database⁵³ (<https://github.com/YazBrammah/FlyAtlas2>). We calculated tissue enrichment by dividing the normalized expression value (in transcripts per million, or TPM) of the gene of interest in the tissue of interest by the TPM value for that gene in the whole female body. As described on <http://flyatlas.gla.ac.uk/FlyAtlas2/index.html?page=help#>, when whole body TPM values were <2, we set them to 2 for the enrichment calculation.

Fertility assay. We performed a fertility assay by measuring the number of eggs produced by axenic (A) and control (C) females that were mated to axenic or control males. Matings for the egg laying assay were performed as described above, with sample sizes (female designated first in the cross): *n* = 49 for AA, *n* = 47 for AC, *n* = 46 for CA and *n* = 50 for CC. These sample sizes exclude females that did not survive or escaped during the assay. At the end of copulation, which occurred in vial 0 (V0), males were removed and females were transferred into a new vial (V1), in which they were allowed to lay eggs for 6 h. After 6 h, females were moved to V2 for 24 h, then transferred to V3 for 24 h, after which the females were discarded. Each time females were transferred to a new vial, egg number in the previous vial was recorded. Fly food in V0 was prepared as described above for the RNA-seq assay. V1, V2 and V3 contained the same autoclaved food as described above, but without the addition of yeast or fly homogenate. For each time point, we assessed the presence or absence of bacteria in 2–3 pooled flies using PCR for bacterial 16S rRNA. At each time point, bacteria were absent in axenic flies. Control flies contained bacteria at each time point, but the amount decreased with each transfer onto sterile food that did not contain fly homogenate (Fig. S3; gels were imaged as described above). The total number of eggs produced by each female was analyzed in R using a generalized linear mixed model with an assumed Poisson response distribution (lme4;¹⁰⁵), with fixed effects for female microbiome status, male microbiome status and their interaction, an observation-level random effect to account for overdispersion¹⁰⁶ and a random effect to account for the person counting the eggs. The package emmeans (<https://cran.r-project.org/web/packages/emmeans/index.html>) was used to calculate *p* values for pairwise comparisons between the four treatments (corrected for multiple testing). Count data are available in Table S7.

Data and code availability. The raw Illumina short-read sequences are available through the Sequence Read Archive (SRA) under project accession PRJNA629997. The analysis scripts and code to reproduce analysis results and generate manuscript figures are all available in a GitHub repository (<https://github.com/YazBrammah/Axenic.PM>).

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References

- Avila, F. W., Sirot, L. K., LaFlamme, B. A., Rubinstein, C. D. & Wolfner, M. F. Insect seminal fluid proteins: identification and function. *Annu. Rev. Entomol.* **56**, 21–40 (2011).
- Innocenti, P. & Morrow, E. H. Immunogenic males: a genome-wide analysis of reproduction and the cost of mating in *Drosophila melanogaster* females. *J. Evol. Biol.* **22**, 964–973 (2009).
- Lawniczak, M. K. N. & Begun, D. J. A genome-wide analysis of courting and mating responses in *Drosophila melanogaster* females. *Genome* **47**, 900–910 (2004).
- McGraw, L. A., Gibson, G., Clark, A. G. & Wolfner, M. F. Genes regulated by mating, sperm, or seminal proteins in mated female *Drosophila melanogaster*. *Curr. Biol.* **14**, 1509–1514 (2004).
- McGraw, L. A., Clark, A. G. & Wolfner, M. F. Post-mating gene expression profiles of female *Drosophila melanogaster* in response to time and to four male accessory gland proteins. *Genetics* **179**, 1395–1408 (2008).
- Peng, J., Zipperlen, P. & Kubli, E. *Drosophila* sex-peptide stimulates female innate immune system after mating via the toll and imd pathways. *Curr. Biol.* **15**, 1690–1694 (2005).
- Short, S. M. & Lazzaro, B. P. Reproductive status alters transcriptomic response to infection in female *Drosophila melanogaster*. *G3* **3**, 827–840 (2013).
- Dalton, J. E. *et al.* Dynamic, mating-induced gene expression changes in female head and brain tissues of *Drosophila melanogaster*. *BMC Genom.* **11**, 541 (2010).
- Kapelnikov, A., Rivlin, P. K., Hoy, R. R. & Heifetz, Y. Tissue remodeling: a mating-induced differentiation program for the *Drosophila oviduct*. *BMC Dev. Biol.* **8**, 114 (2008).
- Mack, P. D., Kapelnikov, A., Heifetz, Y. & Bender, M. Mating-responsive genes in reproductive tissues of female *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 10358–10363 (2006).
- Prokupek, A. M., Kachman, S. D., Ladunga, I. & Harshman, L. G. Transcriptional profiling of the sperm storage organs of *Drosophila melanogaster*. *Insect Mol. Biol.* **18**, 465–475 (2009).

12. Chow, C. Y., Wolfner, M. F. & Clark, A. G. The genetic basis for male x female interactions underlying variation in reproductive phenotypes of *Drosophila*. *Genetics* **186**, 1355–1365 (2010).
13. Clark, A. G., Begun, D. J. & Prout, T. Female x male interactions in *Drosophila* sperm competition. *Science* **283**, 217–220 (1999).
14. Delbare, S. Y. N., Chow, C. Y., Wolfner, M. F. & Clark, A. G. Roles of female and male genotype in post-mating responses in *Drosophila melanogaster*. *J. Hered.* **108**, 740–753 (2017).
15. Reinhart, M., Carney, T., Clark, A. G. & Fiumera, A. C. Characterizing male-female interactions using natural genetic variation in *Drosophila melanogaster*. *J. Hered.* **106**, 67–79 (2015).
16. Civetta, A. & Ranz, J. M. Genetic factors influencing sperm competition. *Front. Genet.* **10**, 820 (2019).
17. Domanitskaya, E. V., Liu, H., Chen, S. & Kubli, E. The hydroxyproline motif of male sex peptide elicits the innate immune response in *Drosophila females*. *FEBS J.* **274**, 5659–5668 (2007).
18. Gioti, A. *et al.* Sex peptide of *Drosophila melanogaster* males is a global regulator of reproductive processes in females. *Proc. Biol. Sci.* **279**, 4423–4432 (2012).
19. Apper-McGlaughon, J. & Wolfner, M. F. Post-mating change in excretion by mated *Drosophila melanogaster* females is a long-term response that depends on sex peptide and sperm. *J. Insect Physiol.* **59**, 1024–1030 (2013).
20. Reiff, T. *et al.* Endocrine remodelling of the adult intestine sustains reproduction in *Drosophila*. *Elife* **4**, e06930 (2015).
21. Douglas, A. E. Contradictory results in microbiome science exemplified by recent research. *MBio.* **9**(5), e01758-18 (2018).
22. Bost, A. *et al.* Functional variation in the gut microbiome of wild *Drosophila* populations. *Mol. Ecol.* **27**(13), 2834–2845 (2018).
23. Broderick, N. A., Buchon, N. & Lemaitre, B. Microbiota-induced changes in *Drosophila melanogaster* host gene expression and gut morphology. *MBio* **5**, e01117-14 (2014).
24. Combe, B. E. *et al.* *Drosophila* microbiota modulates host metabolic gene expression via IMD/NF- κ B signaling. *PLoS ONE* **9**(4), e94729 (2014).
25. Dobson, A. J., Chaston, J. M. & Douglas, A. E. The *Drosophila* transcriptional network is structured by microbiota. *BMC Genom.* **17**, 975 (2016).
26. Elya, C., Zhang, V., Ludington, W. B. & Eisen, M. B. Stable host gene expression in the gut of adult *Drosophila melanogaster* with different bacterial mono-associations. *PLoS ONE* **11**, e0167357 (2016).
27. Bakula, M. The persistence of a microbial flora during postembryogenesis of *Drosophila melanogaster*. *J. Invertebr. Pathol.* **14**, 365–374 (1969).
28. Gould, A. L. *et al.* Microbiome interactions shape host fitness. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E11951–E11960 (2018).
29. Newell, P. D. & Douglas, A. E. Interspecies interactions determine the impact of the gut microbiota on nutrient allocation in *Drosophila melanogaster*. *Appl. Environ. Microbiol.* **80**(2), 788–796 (2013).
30. Ridley, E. V., Wong, A.C.-N., Westmiller, S. & Douglas, A. E. Impact of the resident microbiota on the nutritional phenotype of *Drosophila melanogaster*. *PLoS ONE* **7**, e36765 (2012).
31. Wong, A.C.-N., Dobson, A. J. & Douglas, A. E. Gut microbiota dictates the metabolic response of *Drosophila* to diet. *J. Exp. Biol.* **217**, 1894–1901 (2014).
32. Buchon, N., Broderick, N. A., Chakrabarti, S. & Lemaitre, B. Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. *Genes Dev.* **23**, 2333–2344 (2009).
33. Fast, D., Duggal, A. & Foley, E. Monoassociation with *Lactobacillus plantarum* disrupts intestinal homeostasis in adult *Drosophila melanogaster*. *MBio.* **9**(4), e01114-18 (2018).
34. Fischer, C. N., Trautman, E. P., Crawford, J. M., Stabb, E. V., Handelsman, J. & Broderick, N. A. Metabolite exchange between microbiome members produces compounds that influence *Drosophila* behavior. *eLife*, **6** (2017).
35. Schretter, C. E. *et al.* A gut microbial factor modulates locomotor behaviour in *Drosophila*. *Nature* **563**, 402–406 (2018).
36. Obata, F., Fons, C. O. & Gould, A. P. Early-life exposure to low-dose oxidants can increase longevity via microbiome remodelling in *Drosophila*. *Nat. Commun.* **9**, 975 (2018).
37. Elgart, M. *et al.* Impact of gut microbiota on the fly's germ line. *Nat. Commun.* **7**, 1–11 (2016).
38. Morimoto, J., Simpson, S. J. & Ponton, F. Direct and trans-generational effects of male and female gut microbiota in *Drosophila melanogaster*. *Biol. Lett.* **13**(7), 20160966 (2017).
39. Qiao, H., Keeseey, I. W., Hansson, B. S. & Knaden, M. Gut microbiota affects development and olfactory behavior in *Drosophila melanogaster*. *J. Exp. Biol.* **222**, jeb192500 (2019).
40. Selkig, J. *et al.* The *Drosophila* microbiome has a limited influence on sleep, activity, and courtship behaviors. *Sci. Rep.* **8**, 10646 (2018).
41. Leftwich, P. T., Clarke, N. V. E., Hutchings, M. I. & Chapman, T. Gut microbiomes and reproductive isolation in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* **114**(48), 12767–12772 (2017).
42. Sharon, G., Segal, D. & Rosenberg, E. Role of bacteria in mating preference in *Drosophila melanogaster*. In *Beneficial Microorganisms in Multicellular Life Forms*, pp. 57–69 (Springer, Berlin, 2012).
43. Chandler, J. A., Eisen, J. A. & Kopp, A. Yeast communities of diverse *Drosophila* species: comparison of two symbiont groups in the same hosts. *Appl. Environ. Microbiol.* **78**(20), 7327–7336 (2012).
44. Wong, C. N. A., Ng, P. & Douglas, A. E. Low-diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. *Environ. Microbiol.* **13**(7), 1889–1900 (2011).
45. Wong, A.C.-N. *et al.* The host as the driver of the microbiota in the gut and external environment of *Drosophila melanogaster*. *Appl. Environ. Microbiol.* **81**, 6232–6240 (2015).
46. Obadia, B. *et al.* Probabilistic invasion underlies natural gut microbiome stability. *Curr. Biol.* **27**, 1999–2006.e8 (2017).
47. Staubach, F., Baines, J. F., Künzel, S., Bik, E. M. & Petrov, D. A. Host species and environmental effects on bacterial communities associated with *Drosophila* in the laboratory and in the natural environment. *PLoS ONE* **8**, e70749 (2013).
48. Wong, A.C.-N., Chaston, J. M. & Douglas, A. E. The inconstant gut microbiota of *Drosophila* species revealed by 16S rRNA gene analysis. *ISME J.* **7**, 1922–1932 (2013).
49. Blum, J. E., Fischer, C. N., Miles, J. & Handelsman, J. Frequent replenishment sustains the beneficial microbiome of *Drosophila melanogaster*. *MBio* **4**, e00860-3 (2013).
50. Pais, I. S., Valente, R. S., Sporniak, M. & Teixeira, L. *Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. *PLoS Biol.* **16**, e2005710 (2018).
51. Lawniczak, M. K. N. *et al.* Mating and immunity in invertebrates. *Trends Ecol. Evol.* **22**, 48–55 (2007).
52. R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
53. Leader, D. P., Krause, S. A., Pandit, A., Davies, S. A. & Dow, J. A. T. FlyAtlas 2: a new version of the *Drosophila melanogaster* expression atlas with RNA-Seq, miRNA-Seq and sex-specific data. *Nucleic Acids Res.* **46**, D809–D815 (2018).
54. Huang, J.-H. & Douglas, A. E. Consumption of dietary sugar by gut bacteria determines *Drosophila* lipid content. *Biol. Lett.* **11**(9), 20150469 (2015).
55. Newell, P. D. *et al.* In vivo function and comparative genomic analyses of the *Drosophila* gut microbiota identify candidate symbiosis factors. *Front. Microbiol.* **5**, 576 (2014).
56. Yamada, R., Deshpande, S. A., Bruce, K. D., Mak, E. M. & Ja, W. W. Microbes promote amino acid harvest to rescue undernutrition in *Drosophila*. *Cell Rep.* **10**, 865–872 (2015).
57. Shin, S. C. *et al.* *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science* **334**, 670–674 (2011).

58. Hong, S.-H. *et al.* Minibrain/Dyrk1a regulates food intake through the Sir2-FOXO-sNPF/NPY pathway in *Drosophila* and mammals. *PLoS Genet.* **8**, e1002857 (2012).
59. Andreatta, G., Kyriacou, C. P., Flatt, T. & Costa, R. Aminergic signaling controls ovarian dormancy in *Drosophila*. *Sci. Rep.* **8**, 2030 (2018).
60. Sarov-Blat, L., VenusSo, W., Liu, L. & Rosbash, M. The *Drosophila* takeout gene is a novel molecular link between circadian rhythms and feeding behavior. *Cell* **101**(6), 647–656 (2000).
61. Buch, S., Melcher, C., Bauer, M., Katzenberger, J. & Pankratz, M. J. Opposing effects of dietary protein and sugar regulate a transcriptional target of *Drosophila* insulin-like peptide signaling. *Cell Metab.* **7**, 321–332 (2008).
62. Rowe, M., Veerus, L., Trosvik, P., Buckling, A. & Pizzari, T. The reproductive microbiome: an emerging driver of sexual selection, sexual conflict, mating systems, and reproductive isolation. *Trends Ecol. Evol.* **35**, 220–234 (2020).
63. Tatum, E. L. Development of eye-colors in *Drosophila*: bacterial synthesis of v+ hormone. *Proc. Natl. Acad. Sci. U.S.A.* **25**, 486–490 (1939).
64. Broderick, N. A. & Lemaitre, B. Gut-associated microbes of *Drosophila melanogaster*. *Gut Microbes* **3**, 307–321 (2012).
65. Cernilogar, F. M., Fabbri, F., Andrenacci, D., Taddei, C. & Gargiulo, G. *Drosophila* vitelline membrane cross-linking requires the fs(1)nasrat, fs(1)polehole and chorion genes activities. *Dev. Genes Evol.* **211**, 573–580 (2001).
66. Li, M. A., Alls, J. D., Avancini, R. M., Koo, K. & Godt, D. The large maf factor traffic jam controls gonad morphogenesis in *Drosophila*. *Nat. Cell Biol.* **5**, 994–1000 (2003).
67. Shinoda, T. & Itoyama, K. Juvenile hormone acid methyltransferase: a key regulatory enzyme for insect metamorphosis. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11986–11991 (2003).
68. Bownes, M. Hormonal and genetic regulation of vitellogenesis in *Drosophila*. *Quart. Rev. Biol.* **57**(3), 247–274 (1982).
69. Schwenke, R. A. & Lazzaro, B. P. Juvenile hormone suppresses resistance to infection in mated female *Drosophila melanogaster*. *Curr. Biol.* **27**, 596–601 (2017).
70. Moshitzky, P. *et al.* Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol.* **32**(3–4), 363–374 (1996).
71. Cognigni, P., Bailey, A. P. & Miguel-Aliaga, I. Enteric neurons and systemic signals couple nutritional and reproductive status with intestinal homeostasis. *Cell Metab.* **13**, 92–104 (2011).
72. Lee, K. P., Kim, J.-S. & Min, K.-J. Sexual dimorphism in nutrient intake and life span is mediated by mating in *Drosophila melanogaster*. *Anim. Behav.* **86**(5), 987–992 (2013).
73. Uchizono, S., Tabuki, Y., Kawaguchi, N., Tanimura, T. & Itoh, T. Q. Mated *Drosophila melanogaster* females consume more amino acids during the dark phase. *PLoS ONE* **12**, e0172886 (2017).
74. McMillan, E. A. *et al.* The protein kinase CK2 substrate Jabba modulates lipid metabolism during *Drosophila oogenesis*. *J. Biol. Chem.* **293**, 2990–3002 (2018).
75. Leitão-Gonçalves, R. *et al.* Commensal bacteria and essential amino acids control food choice behavior and reproduction. *PLoS Biol.* **15**, e2000862 (2017).
76. Wong, A. C.-N. *et al.* Gut microbiota modifies Olfactory-Guided microbial preferences and foraging decisions in *Drosophila*. *Curr. Biol.* **27**, 2397–2404.e4 (2017).
77. Liberti, J. *et al.* Seminal fluid compromises visual perception in honeybee queens reducing their survival during additional mating flights. *Elife* **8**, e45009 (2019).
78. Harada, E., Haba, D., Aigaki, T. & Matsuo, T. Behavioral analyses of mutants for two odorant-binding protein genes, obp57d and obp57e, in *Drosophila melanogaster*. *Genes Genet. Syst.* **83**, 257–264 (2008).
79. Matsuo, T., Sugaya, S., Yasukawa, J., Aigaki, T. & Fuyama, Y. Odorant-binding proteins OBP57d and OBP57e affect taste perception and host-plant preference in *Drosophila sechellia*. *PLoS Biol.* **5**, e118 (2007).
80. Zhu, E. Y., Guntur, A. R., He, R., Stern, U. & Yang, C.-H. Egg-laying demand induces aversion of UV light in *Drosophila* females. *Curr. Biol.* **24**, 2797–2804 (2014).
81. Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C. & Taghert, P. H. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* **99**, 791–802 (1999).
82. Tsai, L. T.-Y., Bainton, R. J., Blau, J. & Heberlein, U. Lmo mutants reveal a novel role for circadian pacemaker neurons in cocaine-induced behaviors. *PLoS Biol.* **2**, e408 (2004).
83. Isaac, R. E., Li, C., Leedale, A. E. & Shirras, A. D. *Drosophila* male sex peptide inhibits siesta sleep and promotes locomotor activity in the post-mated female. *Proc. Biol. Sci.* **277**, 65–70 (2010).
84. Wang, Z. *et al.* Catecholamines up integrates dopamine synthesis and synaptic trafficking. *J. Neurochem.* **119**, 1294–1305 (2011).
85. Tas, D. *et al.* Parallel roles of transcription factors dFOXO and FER2 in the development and maintenance of dopaminergic neurons. *PLoS Genet.* **14**, e1007271 (2018).
86. Yamamoto, S. & Seto, E. S. Dopamine dynamics and signaling in *Drosophila*: an overview of genes, drugs and behavioral paradigms. *Exp. Anim.* **63**(2), 107–119 (2014).
87. Alfonso-Parra, C. *et al.* Mating-induced transcriptome changes in the reproductive tract of female *Aedes aegypti*. *PLOS Negl. Trop. Dis.* **10**(2), e0004451 (2016).
88. Goto, A. *et al.* Cooperative regulation of the induction of the novel antibacterial Listericin by peptidoglycan recognition protein LE and the JAK-STAT pathway. *J. Biol. Chem.* **285**, 15731–15738 (2010).
89. Kamimura, Y. Twin intromittent organs of *Drosophila* for traumatic insemination. *Biol. Lett.* **3**, 401–404 (2007).
90. Mattei, A. L., Riccio, M. L., Avila, F. W. & Wolfner, M. F. Integrated 3D view of postmating responses by the *Drosophila melanogaster* female reproductive tract, obtained by micro-computed tomography scanning. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 8475–8480 (2015).
91. Morrow, E. H. & Innocenti, P. Female postmating immune responses, immune system evolution and immunogenic males. *Biol. Rev. Camb. Philos. Soc.* **87**, 631–638 (2012).
92. Perlmutter, J. I. & Bordenstein, S. R. Microorganisms in the reproductive tissues of arthropods. *Nat. Rev. Microbiol.* **18**, 97–111 (2020).
93. Koyle, M. L. *et al.* Rearing the fruit fly *Drosophila melanogaster* under axenic and gnotobiotic conditions. *J. Vis. Exp.* **113**, 54219 (2016).
94. Schneider, D. I., Klasson, L., Lind, A. E. & Miller, W. J. More than fishing in the dark: PCR of a dispersed sequence produces simple but ultrasensitive wolbachia detection. *BMC Microbiol.* **14**, 121 (2014).
95. Lenth, R. *emmeans: Estimated Marginal Means, aka Least-Squares Means* R package version 1.4.8. (2020)
96. Benjamini, Y. & Hochberg, Y. Multiple hypotheses testing with weights. *Scand. J. Stat.* **24**(3), 407–418 (1997).
97. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with bowtie 2. *Nat. Methods* **9**, 357–359 (2012).
98. Forster, S. C., Finkel, A. M., Gould, J. A. & Hertzog, P. J. RNA-eXpress annotates novel transcript features in RNA-seq data. *Bioinformatics* **29**, 810–812 (2013).
99. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**(1), 139–140 (2010).
100. Risso, D., Ngai, J., Speed, T. P. & Dudoit, S. Normalization of RNA-seq data using factor analysis of control genes or samples. *Nat. Biotechnol.* **32**(9), 896–902 (2014).

101. Yu, G., Wang, L.-G., Han, Y. & He, Q.-Y. clusterprofiler: an R package for comparing biological themes among gene clusters. *OMICS: J. Integ. Biol.* **16**(5), 284–287 (2012).
102. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**(1), 44–57 (2009).
103. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **37**, 1–13 (2009).
104. Gramates, L. S., Marygold, S. J., Santos, G. D., Urbano, J.-M., Antonazzo, G., Matthews, B. B., Rey, A. J., Tabone, C. J., Crosby, M. A., Emmert, D. B., Falls, K., Goodman, J. L., Hu, Y., Ponting, L., Schroeder, A. J., Strelets, V. B., Thurmond, J., Zhou, P. & the FlyBase Consortium. FlyBase at 25: looking to the future. *Nucleic Acids Res.* **45**, D663–D671, (2017).
105. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting linear Mixed-Effects models using lme4. *J. Stat. Softw.* **67**, 1 (2015).
106. Harrison, X. A. A comparison of observation-level random effect and Beta-Binomial models for modelling overdispersion in binomial data in ecology & evolution. *PeerJ* **3**, e1114 (2015).

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Author contributions

S.Y.N.D., Y.H.A.-B., M.F.W. and A.G.C. conceived the experiments. S.Y.N.D. and Y.H.A.-B. conducted the experiments. S.Y.N.D. and Y.H.A.-B. analysed the results. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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