

1 **Title: Identification of Microbiota-Induced Gene Expression Changes in the**
2 ***Drosophila melanogaster* Head**

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47 **ABSTRACT**

48 Symbiotic microorganisms exert multifaceted impacts on the physiology of their animal
49 hosts. Recent discoveries have shown the gut microbiota influence host brain function
50 and behavior, but the host and microbial molecular factors required to actuate these
51 effects are largely unknown. To uncover molecular mechanisms that underlie the gut-
52 microbiota-brain axis, we used *Drosophila melanogaster* and its bacterial microbiota as a
53 model to identify microbiota-dependent gene expression changes in the host brain and
54 head. Specifically, we employed RNA-seq and nanoString nCounter technology to identify
55 *Drosophila* genes that exhibit altered transcript levels in fly heads upon elimination of the
56 microbiota. The identified genes, some of which exhibited sex-specific differences, have
57 demonstrated or inferred functional roles in the immune response, metabolism, neuronal
58 activity, and stress resistance. Overall, this study reveals microbiota-responsive genes in
59 the fly head, an anatomical structure not previously investigated in this context. Our
60 results serve as a foundation for future investigations of how microbe-driven gene
61 expression changes impact *Drosophila* biology.

62 INTRODUCTION

63 Throughout their lifespan, animals engage in complex and dynamic interactions
64 with microbial communities that reside both in and on their bodies, and in their
65 environment. Association with a microbiota impacts many physiological and life history
66 traits of the animal host, and in certain environmental contexts is vitally important to
67 supporting host health and homeostasis (McFall-Ngai *et al.* 2013). The microbial
68 communities occupying the mammalian gut promote host immune development (Atarashi
69 *et al.* 2011; Rosser and Mauri 2016), aid food metabolism (Cockburn and Koropatkin
70 2016), and alter neural function and behaviors (Hsiao *et al.* 2013; Sampson and
71 Mazmanian 2015; Sharon *et al.* 2016). In these and other ways, symbiotic
72 microorganisms exert their influence on animal health at every hierarchical level of
73 biological organization, ranging from the molecular to the ecological (Kohl and Carey
74 2016). However, despite the wealth of evidence demonstrating the importance of animal-
75 microbiota interactions, the underlying molecular and mechanistic principles of these
76 interactions are only beginning to be uncovered.

77 *Drosophila melanogaster* and its bacterial gut symbionts provide a
78 microbiologically and genetically controllable system with which to interrogate the
79 molecular basis of microbiota-dependent host traits. In the lab and in the wild, *Drosophila*
80 continuously encounter microbe-rich food substrates (Broderick and Lemaitre 2012;
81 Wong *et al.* 2015; Bost *et al.* 2017, 2018; Adair *et al.* 2018). Unlike mammals and other
82 higher order metazoans, the bacterial communities associated with *Drosophila* are low
83 diversity, and are dominated by culturable taxa, predominantly *Lactobacillus* and
84 *Acetobacter* species (Brummel *et al.* 2004; Wong *et al.* 2011, 2013, 2015; Blum *et al.*

85 2013; Broderick *et al.* 2014; Elya *et al.* 2016; Adair *et al.* 2018). Interactions between
86 *Drosophila* and its external symbionts are also readily studied by generating sterile, germ-
87 free (GF) flies, and gnotobiotic (GNO) flies mono- or polyassociated with defined bacterial
88 isolates (Koyle *et al.* 2016). The microbial tractability of its gut consortium complements
89 the abundant genetic tools that enable thorough investigation of host gene function in
90 *Drosophila*. Thus, the ability to manipulate host genetics and its microbiota makes
91 *Drosophila* an excellent model to study the molecular mechanisms that underlie microbial
92 modulation of host biology.

93 The fly microbiota strongly impact a variety of physiological traits displayed by
94 laboratory *Drosophila*. Both lactic acid and acetic acid bacteria alter the nutritional
95 availability of particular diet substrates that enables or accelerates larval development,
96 and shapes the metabolic profile of adults (Shin *et al.* 2011; Chaston *et al.* 2014, 2016;
97 Huang and Douglas 2015; Matos *et al.* 2017; Storelli *et al.* 2017; Téfit and Leulier 2017;
98 Sannino *et al.* 2018). Microbes in the gut lumen affect local gut biology by stimulating the
99 proliferation of gut epithelial cells (Buchon *et al.* 2009; Jones *et al.* 2013, 2015; Li *et al.*
100 2016; Petkau *et al.* 2017), driving reactive oxygen species production (Jones *et al.* 2013,
101 2015; Guo *et al.* 2014), modulating innate immune activity (Ryu *et al.* 2008; Lee *et al.*
102 2013; Broderick *et al.* 2014; Combe *et al.* 2014; Sansone *et al.* 2015), and protecting
103 against stressors like pathogens and oxidative injury (Ryu *et al.* 2008; Blum *et al.* 2013;
104 Jones *et al.* 2013, 2015; Sansone *et al.* 2015). Newly emerging evidence is connecting
105 the bacterial microbiota with fly behavior and neural function. Flies demonstrate modified,
106 olfactory-mediated feeding and egg-laying preferences in response to individual bacteria,
107 mixed microbial communities, and microbial fermentation products (Farine *et al.* 2017;

108 Fischer *et al.* 2017; Kim *et al.* 2017; Leitão-Gonçalves *et al.* 2017; Liu *et al.* 2017; Wong
109 *et al.* 2017). Adult GF *Drosophila* exhibit increased locomotor activity, which can be
110 suppressed by a *Lactobacillus brevis*-derived, secreted metabolic enzyme, xylose
111 isomerase (Schretter *et al.* 2018). The ability of both *Acetobacter pomorum* and
112 *Lactobacillus plantarum* to promote larval development on nutrient-limited diets requires
113 induction of *Drosophila* insulin-like peptides (Shin *et al.* 2011; Storelli *et al.* 2011), which
114 are synthesized by neuroendocrine insulin-producing cells (IPCs) in the brain and
115 released into circulation to induce systemic developmental and metabolic phenotypes
116 (Géminard *et al.* 2009; Nässel and Broeck 2016). Reciprocally, genetic induction of
117 tumors in the optic lobes of the larval brain and eye-antennal discs was sufficient to
118 perturb the structure and diversity of bacterial communities in the larval gut (Jacqueline
119 *et al.* 2017). These discoveries suggest that modulation of neuronal function by
120 *Drosophila*-associated gut microbes can impact a range of physiological, behavioral, and
121 life-history traits in the insect. However, the host molecules and pathways induced by
122 interactions with the microbiota that result in these microbe-dependent host traits remain
123 largely undiscovered.

124 Several studies have profiled the microbiota's impact on gene expression in the
125 adult *Drosophila* gut (Broderick *et al.* 2014; Guo *et al.* 2014; Elya *et al.* 2016; Petkau *et*
126 *al.* 2017), embryo (Elgart *et al.* 2016), larvae (Erkosar *et al.* 2017), and whole animals
127 (Combe *et al.* 2014; Dobson *et al.* 2016; Bost *et al.* 2017). These investigations found
128 generally similar functional categories of genes differentially expressed under GF
129 conditions across the indicated tissue- and sample-types, namely genes involved in
130 innate immunity, digestion, metabolism, and cellular homeostatic pathways. Notably, all

131 but one of these reports (Bost *et al.* 2017) examined the transcriptomic effects of
132 microbiota elimination solely in female flies. The extent to which microbial effects on
133 global gene expression differ between sexes therefore remains minimally explored.

134 In this study, we sought to identify molecular factors underlying the microbial
135 impacts on *Drosophila* neural function, and behavioral and physiological traits. To
136 accomplish our objective, we screened for microbiota-induced transcriptional changes in
137 the adult *Drosophila* head. The fly head predominantly comprises the brain, eyes,
138 antennae, and head fat body, a major metabolic/endocrine/immune tissue. We
139 hypothesized that the brain and fat body in particular play key, undiscovered roles in
140 mediating microbial impacts on fly behavior and metabolism. Moreover, the head is an
141 anatomical structure that has not been investigated previously in the context of
142 microbiota-dependent global gene expression. We therefore conducted a two-step
143 screen for microbiota-induced transcriptional changes in the head using RNA-seq
144 followed by nanoString technology, and identified both sex-general and sex-specific
145 microbe-sensitive genes. These genes are broadly classifiable by shared functional
146 categories, including innate immunity, neural activity, oxidative stress responses, and
147 metabolism. At the cellular and physiological levels, the microbiota's influence on some
148 of these biological processes in the host insect is well established in the literature. Thus,
149 some of the genes identified in this study are promising candidates that may connect
150 specific known aspects of host function to the microbiota. In other cases, we predict that
151 the genes identified here will lead to the discovery of new microbe-dependent facets of
152 host biology.

153 **MATERIALS AND METHODS**

154 **Fly stocks and general rearing**

155 The primary stock used in this study was the Top Banana *Drosophila melanogaster* strain,
156 generously provided by Michael Dickinson's lab at the California Institute of Technology.
157 This population was caught at the Top Banana fruit stand in Seattle, Washington USA
158 (coordinates 47°40'37.0"N 122°22'37.9"W) in September 2013. Stock cultures were
159 reared on yeast-cornmeal-molasses food of the following recipe: vol/vol or wt/vol; 8.5%
160 molasses, 7% cornmeal, 1.1% brewer's yeast, 0.86% agar (MoorAgar), supplemented
161 with 0.27% propionic acid (Sigma) and 13.5 mL/L of 20% methylparaben (Sigma)
162 dissolved in 95-100% ethanol at room temperature, ~25°C. All experimental cultures,
163 derived as described in the following section, were reared in autoclaved bottles and vials
164 of the above diet with ~0.25g autoclaved yeast granules added topically, and maintained
165 in a light-, temperature-, and humidity-controlled incubator on a 12:12 hour light:dark cycle
166 (lights on 8:00am, lights off 8:00pm) at 22-23°C, 70% humidity.

167 RNA-seq and nanoString experiments were conducted with Top Banana flies harboring
168 a *Wolbachia* infection derived from the wild-caught population; RT-qPCR experiments
169 were conducted with Top Banana cultures cleared of *Wolbachia* by rearing for two
170 generations on indicated diet with 0.25 g/L tetracycline (Sigma), followed by >10
171 generations on regular diet. The w^{1118} stock used in this study is *Wolbachia*-free. The
172 *Wolbachia* status of stocks was determined by PCR amplification of the *Wolbachia*
173 surface protein (*wsp*) gene from DNA extracts prepared from 5-10 adult flies (Table S2).

174 **Generation of conventional and germ-free *Drosophila* cultures**

175 Synchronous cultures were prepared by collecting embryos laid on apple juice agar plates
176 within a 4-hour time window. Embryos were then collected in embryo wash buffer (1X

177 solution, vol/vol or wt/vol in Milli-Q H₂O: 2% Triton X-100, 7% NaCl). To generate
178 conventional cultures, approximately 150-200 embryos were transferred directly to food
179 bottles by pipette. To generate germ-free and gnotobiotic cultures, embryos were treated
180 for 2 minutes with 50% sodium hypochlorite, rinsed twice in 70% ethanol and twice in
181 sterile Milli-Q H₂O. Approximately 150-200 embryos were then transferred by pipette to
182 either: i) sterile food bottles, for germ-free cultures, or ii) sterile food bottles inoculated
183 with $\sim 10^7$ CFU bacterial liquid culture for gnotobiotic fly cultures (see below). All
184 manipulations were performed inside a sterile laminar flow cabinet. Bottle cultures were
185 then incubated under conditions described above and developed to adulthood. Adult flies
186 were collected under light CO₂ anesthesia on a sterilized pad 0-24 hours post-eclosion
187 and transferred to sterile food vials with ~ 0.05 g autoclaved yeast granules added, 10-20
188 flies of the same sex per vial. For gnotobiotic flies, vials were inoculated with $\sim 10^7$ CFU
189 bacterial liquid culture prior to collecting adult flies (see below). Microbial status of
190 gnotobiotic cultures and sterility of germ-free cultures were routinely checked by
191 homogenizing 5-10 flies in phosphate buffer saline (PBS) and plating on bacterial growth
192 media (see below). Flies were aged in vials to 5-6 days post-eclosion for use in all
193 experiments reported here.

194 **Isolation and identification of gut bacteria from conventional Top Banana flies**

195 Bacteria were collected from either surface-sterilized whole flies or dissected guts from
196 conventional Top Banana male and female flies. For surface sterilization, flies were
197 washed once in 1 mL 10% sodium hypochlorite, once in 1 mL 70% ethanol, and three
198 times in PBS. Guts (proventriculus to hindgut, excluding crop and Malpighian tubules)
199 from 10-20 individual animals were dissected in PBS. Surface sterilized animals or

200 dissected guts were then homogenized in 100 μ L PBS and five 10-fold serial dilutions of
201 homogenate were prepared in PBS. Dilutions were plated on MRS (Difco) and Ace (wt/vol
202 or vol/vol: 0.8% yeast extract, 1.5% peptone, 1% dextrose, 1.5% agar, 0.3% acetic acid,
203 0.5% ethanol) agar plates. MRS plates were sealed with parafilm and incubated at 37°
204 for ~48 hours, and Ace plates were incubated at 30° for ~72 hours. Individual colonies of
205 characteristic morphology were then streaked for isolation on the relevant medium. For
206 taxon identification, the 8F and 1492R primers were used to amplify sequence from the
207 16S rRNA gene of each isolate (Eden *et al.* 1991; Table S2). Amplicon DNA was purified
208 and Sanger sequenced with both the forward and reverse primers. Sequencing results
209 were then searched for highly similar sequences in both the SILVA database using the
210 SINA alignment service (Pruesse *et al.* 2012) and the NCBI nr/nt database via blastn
211 (Altschul *et al.* 1990; Morgulis *et al.* 2008; Camacho *et al.* 2009). Bacterial taxonomies
212 were assigned based on >97% sequence homology (see Table S1). The 16S rRNA
213 sequence for isolate A22 bore >97% similarity with multiple *Acetobacter* strains of
214 different species.

215 **Generation of gnotobiotic *Drosophila* cultures**

216 Overnight cultures of *L. plantarum* L32 and *L. brevis* L28 were grown in MRS broth
217 statically at 37°C, and *A. pasteurianus* A40, and *Acetobacter sp.* A22 were grown in MRS
218 broth at 30°C with shaking. Cells were pelleted, washed twice with PBS, and resuspended
219 in 500 μ L PBS. Cells were then diluted to a suspension of OD₆₀₀=1 and five serial 10-fold
220 dilutions were prepared. Defined volumes of each dilution were then plated on MRS or
221 Ace agar and incubated at 37°C or 30°C for 48 hours. Colonies were then counted

222 manually and CFU/mL constants for $OD_{600}=1$ cell suspensions were calculated for each
223 bacterial isolate (Table S1).

224 To inoculate sterile food bottles with bacterial cell suspensions for gnotobiotic fly culture
225 generation, overnight cultures were grown and washed in PBS as described. Cell
226 suspensions were then diluted to a concentration of $\sim 10^7$ cells in 150 μL volume
227 (calculations based on obtained CFU/mL constants). The entire 150 μL was then pipetted
228 directly onto the surface of sterile food bottles ~ 4 -5 hours prior to addition of
229 dechorionated, sterilized fly embryos (as described above). A cell suspension volume of
230 50 μL was used to inoculate vials with $\sim 10^7$ CFU of each bacteria prior to transfer of
231 newly-eclosed adult flies, as described above.

232 **RNA extraction and sequencing**

233 For each biological replicate, heads from 50 adult male flies were dissected in PBS and
234 transferred to tubes containing 400 μL Trizol Reagent (Invitrogen) and ~ 50 μL zirconia
235 beads (1.0 mm). Head tissues were then immediately homogenized with a mini-
236 beadbeater (Biospec Products) for three 30 second pulses, with 10-15 second pauses in
237 between. RNA was then extracted using the PureLink RNA Mini Kit (Life Technologies)
238 exactly following the kit protocol. Paired-end sequencing was conducted at the University
239 of Southern California Molecular Genomics Core facility on an Illumina NextSeq
240 instrument. The following read-pair counts for each sample were obtained: CV.1
241 53638762 read pairs; CV.2 44963314 read pairs; CV.3 45583437 read pairs; GF.1
242 42930013 read pairs; GF.2 46242287 read pairs; GF.3 51418597 read pairs. Raw read
243 data were provided to C. Kingsford's group at Carnegie Mellon for analyses described
244 below.

245 **RNA-Seq data analysis**

246 Transcript expression quantification estimates were generated using Salmon (Patro *et al.*
247 2017) version 0.6.0 using Release-84 of Ensembl's *Drosophila melanogaster* cDNA
248 library as a reference index. Each paired end file set was processed individually using the
249 variational Bayesian EM algorithm using 30 bootstrapped samples to compute
250 abundance estimates. Transcript-level expression values were converted to gene-level
251 expression using the biomaRt package of the Bioconductor software project (Durinck *et*
252 *al.* 2009). Differential expression was then processed on the gene level using DESeq2
253 (Love *et al.* 2014) on both the full dataset and for each leave-one-out analysis. A gene
254 was defined as “differentially expressed” if it exhibited nonzero expression in both sample
255 sets and had a Benjamini-Hochberg adjusted p-value of <0.1 (see “Results”). Tissue
256 enrichment and Gene Ontology (GO) term enrichment analyses were conducted using
257 the FlyMine integrated database (Lyne *et al.* 2007).

258 **NanoString nCounter analysis**

259 Custom barcoded nanoString probes for genes of interest and housekeeping genes were
260 designed by nanoString Technologies (File S6, sheet “Codeset”). For each biological
261 replicate, heads from 5-10 adult flies (males and virgin females) were removed in PBS
262 and immediately homogenized in Trizol by bead beating, as described in “RNA extraction
263 and sequencing”. RNA was then extracted using the Direct-zol RNA MiniPrep Kit (Zymo
264 Research) exactly following the kit protocol. Hybridization with probe set on nCounter
265 chips was performed following manufacturer’s protocol with 70-100ng RNA per sample.
266 Quality assessment of raw data and normalization were performed using nSolver Analysis
267 Software 3.0. Of note, one sample each of the following conditions were flagged by

268 nSolver QC analysis due to positive control failure: CV males, GF females, GNO females
269 (File S6, sheet: “Raw mRNA counts”). Data from these samples were therefore excluded
270 from subsequent analysis. For genes of interest, mRNA counts were normalized to the
271 geometric mean of count values from each sample for the following four housekeeping
272 genes: *14-3-3 ϵ* , *Su(Tpl)*, *cyp1*, *cyp33*. As our aim was to identify strongly microbiota-
273 responsive genes in either male or female *Drosophila* heads, and not to examine the
274 impact of sex on expression of our genes of interest, we examined expression data across
275 CV, GF, and GNO conditions independently for each sex. Additionally, we refined this
276 analysis by examining the genes with the highest magnitude average expression
277 difference, arbitrarily defined as genes with $|2fc| > 0.4$ or $|2fc| < -0.4$ in either the GF vs. CV
278 or GF vs. GNO comparisons. For each gene examined, we first conducted Levene’s test
279 for equal variance and the Shapiro-Wilk normality test. Data that met the parametric
280 assumptions according to results of these tests ($p < 0.05$) were analyzed via one-way
281 analysis of variance (ANOVA), followed by the Tukey HSD *post hoc* test to compare
282 means between groups. Data that did not meet assumptions of equal variance and a
283 normal distribution were analyzed via the Kruskal-Wallis test with Dunn’s multiple
284 comparisons test.

285 **RT-qPCR analysis**

286 For RT-qPCR experiments, heads were dissected and RNA extracted exactly as
287 described in “NanoString nCounter Analysis”. Each biological replicate represented RNA
288 extracted from heads of 10 individual animals. Pure quality RNA ($A_{260nm/280nm} \sim 2.1$, 400-
289 500ng) was used as template for cDNA synthesis using the qScript cDNA synthesis kit
290 (QuantaBio). Product from cDNA synthesis reactions was used for qPCR with the

291 PerfeCTa SYBR Green Supermix (QuantaBio) in an Applied Biosystems 7300 Real Time
292 PCR System instrument. Primer efficiencies were calculated using LinregPCR software
293 (Ramakers *et al.* 2003; Table S2). Data were normalized to either *Rpl32* or the average
294 of *Rpl32* and *14-3-3ε* housekeeping genes, as indicated, and expression fold changes
295 relative to GF were calculated using the $2^{-\Delta\Delta Ct}$ method. Normalized data were analyzed
296 via one-way analysis of variance (ANOVA), followed by the Tukey HSD *post hoc* test to
297 compare means between groups. Sequences for all primers used are listed in Table S2.

298 **Statistical analysis**

299 The statistical methods and tests employed for each set of experiments are indicated in
300 the relevant Methods sections and are reported in the figure legends. Analyses and figure
301 generation were performed with R version 3.4.0 and GraphPad Prism 7 software.

302 **Data and reagent availability**

303 All fly stocks and bacterial isolates used in this study are available upon request. All
304 supplemental figures and files have been deposited in figshare. Raw RNA-seq data have
305 been deposited in the National Center for Biotechnology Information Sequence Read
306 Archive, BioProject accession number PRJNA514099. File S1 contains gene-mapped
307 RNA-Seq read counts (sheet “Counts”); weighted average of transcript length for each
308 gene based on the number of reads for each transcript (sheet “Transcript length”);
309 abundance representing transcripts per million (TPM; sheet “TPM”); and initial statistical
310 analysis of results, as described in corresponding Results section, with genes ordered by
311 ascending Benjamini-Hochberg adjusted p-values (sheet “Statistical comparison”). File
312 S2 summarizes descriptive results of each leave-out analysis conducted. File S3 lists
313 genes identified as significantly differentially expressed ($p\text{-adj} < 0.1$) in ≥ 2 leave-out

314 analyses, i.e. genes with highest degree of statistical support. File S4 lists the 343 genes
315 identified as significantly differentially expressed ($p\text{-adj}<0.1$) in ≥ 1 leave-out analysis; p -
316 adj values for each analysis conducted are provided for each gene. File S5 contains
317 results from tissue and GO-term enrichment analyses conducted on the genes listed in
318 File S4. File S6 contains the codeset used in nanoString experiments, raw and normalized
319 nCounter mRNA count values, and differential expression calculated as \log_2 fold change
320 values. Table S1 lists the four bacteria isolated from CV Top Banana flies employed for
321 GNO microbial conditions, their closest taxonomic assignment based on 16S rRNA gene
322 sequence data, and the empirically determined CFU/mL constants utilized to equilibrate
323 planktonic bacterial cultures for inoculation of GNO fly cultures (see Materials and
324 Methods). Table S2 lists and provides pertinent information for all primers used in this
325 study.

326 **RESULTS**

327 **Transcriptomic comparison of the heads of microbiota-associated and germ-free** 328 ***Drosophila***

329 To investigate whether and how microbial symbionts that primarily occupy the gut
330 lumen alter gene expression in head tissues, we compared the head transcriptomes of
331 CV adult male *Drosophila* to their sterile GF siblings via RNA-seq. Specifically, we
332 collected and sequenced RNA from the heads of fifty animals per replicate, and examined
333 three, independently reared biological replicates of each microbial condition. We initially
334 analyzed the data by read normalization to transcripts per million (TPM) values, and
335 comparison via the Wald Chi-squared test, with a Benjamini-Hochberg false discovery
336 rate (FDR)-adjusted p -value cutoff of 0.1 serving as our preliminary statistical significance

337 criterion. In this primary analysis, out of 13180 genes with nonzero total read counts, we
338 identified 50 genes as being significantly differentially expressed in the heads of GF vs.
339 CV male *Drosophila* (File S1; Sheet “Statistical comparison”, highlighted cells). These 50
340 genes included 14 genes that showed elevated transcript levels in GF fly heads [\log_2 fold
341 change ($|2fc|>0$], and 36 genes that showed decreased transcript levels relative to CV
342 ($|2fc|<0$). Overall, the magnitude of expression differences we observed were modest: 6
343 of the 14 elevated genes had $|2fc|>0.5$, and 19 of the 36 decreased genes had $|2fc|<-0.5$.
344 Notably, we did not observe any gene expression changes greater than two-fold ($|2fc|>1$
345 or $|2fc|<-1$; Figure 1A, File S1).

346 Closer, visual inspection of the data revealed a considerable degree of gene-to-
347 gene variability in normalized expression levels across replicates for the CV condition,
348 with each replicate displaying a distinct expression pattern (Figure S1) as revealed by
349 principal component analyses (PCA; Figure S2). Moreover, one replicate of the GF
350 condition (designated GF replicate 3; Figure 1B, Figure S1) showed a global expression
351 pattern markedly distinct from the other two replicates, and our PCA supported this
352 replicate as an extreme outlier (Figure S2). The GF outlier immediately suggested the
353 possibility that this this sample was derived from microbe-contaminated fly cultures.
354 However, matched-sample 16S rRNA gene sequence profiling of the dissected guts of all
355 flies used for our RNA-seq samples does not suggest contamination of any GF samples
356 (data available upon request). Similarly, we speculated that the considerable
357 transcriptomic variability among CV flies could be attributable to diversity in the
358 abundance and composition of their un-manipulated microbial communities. This
359 possibility is consistent with the known features of the laboratory *Drosophila* gut

360 microbiota, namely its inconstancy, transiency under certain rearing conditions,
361 dependence on the diet substrate, and high inter-generational and inter-individual
362 compositional variability (Wong *et al.* 2011, 2013, 2015; Blum *et al.* 2013; Broderick *et al.*
363 2014; Chaston *et al.* 2016; Elya *et al.* 2016; Early *et al.* 2017; Jacqueline *et al.* 2017). Our
364 16S rDNA profiling of the gut microbiota of the CV samples did not reveal any substantive
365 differences, but we cannot rule out the possibility that small populations of distinct
366 microbial cohorts influenced the head transcriptional profiles of these samples. Moreover,
367 the Top Banana fly stock utilized in our study harbors a *Wolbachia* infection presumably
368 derived from the original wild-caught population (see Materials and Methods). Variable
369 titers and cohorts of *Wolbachia* endosymbionts may constitute additional sources of
370 variability in the transcriptomic profiles of GF and CV samples.

371 To address the potential for type II error (i.e. false-negatives) in our initial analysis,
372 resulting from the substantial inter-replicate variability of our CV samples and the GF
373 outlier replicate, we conducted a leave-one-out analysis for each replicate in the
374 experiment, resulting in seven total result outputs. Importantly, the number of genes that
375 met our significance criterion (FDR adjusted p-value<0.1) varied dramatically depending
376 on which replicate was omitted from the analysis: for example, omission of GF replicate
377 2 from analysis of the dataset yielded 8 total differentially expressed genes, while
378 omission of CV replicate 1 yielded 177 significantly different genes (File S2, File S3, File
379 S4). The number of significant genes identified in each of the six analyses were generally
380 consistent with the grouping and distribution patterns observed in our PCA results; for
381 example, omission of GF replicate 3, the extreme outlier GF sample, increased the
382 number of significantly different genes to 162, compared to the other analyses.

383 From this approach, we obtained a list of 343 unique candidate genes (Figure 1B;
384 File S4 for FDR-adjusted p-value outputs from each analysis) that were significantly
385 altered in \geq one of our seven analyses (the analysis of the full dataset and each of the six
386 leave-one-out analyses). Notably, most of these genes were significantly differentially
387 expressed in only one out of seven of the analyses; 66 of the 343 candidates were
388 significant in \geq two analyses, and only six genes were differentially expressed in all seven
389 analyses (File S3, File S4). To begin to prioritize individual genes for subsequent analysis,
390 we characterized the functional biological properties of these 343 candidate genes.
391 Examination of tissue-specific expression, based on Affy Calls (“Up” vs. “Down” relative
392 to whole-body expression levels) derived from the FlyAtlas expression database
393 (Chintapalli *et al.* 2007), revealed that of the 343 genes, 176 genes are highly expressed
394 in the head, 107 genes are highly expressed in the fat body (importantly, the FlyAtlas
395 dataset reflects abdominal fat body expression, and not expression in the head-localized
396 fat body), 99 genes are highly expressed in the eye, and 70 genes are highly expressed
397 in the brain (Figure S3, File S5). Gene Ontology (GO) terms enriched (Holm-Bonferroni
398 adjusted p-value $<$ 0.05) in the 248/343 genes down-regulated in GF fly heads primarily
399 encompassed functional categories related to immune function and antimicrobial
400 responses (Figure 1C, File S5). GO terms related to amino acid and fatty acid biosynthetic
401 processes were also enriched within the down-regulated genes (File S5). Enriched GO
402 terms in the 95 genes transcriptionally elevated in GF, compared to CV, heads were
403 dominated by responses to organic chemical stimuli (Figure 1D, File S5).

404 In summary, our RNA-Seq examination of the microbiota’s impact on the head
405 transcriptome of adult male *Drosophila* revealed a relatively small number of low-

406 magnitude gene expression changes resulting from elimination of microbes. The limited
407 number of robustly differentially expressed genes could in part be attributed to a high
408 degree of inter-replicate variability in the expression profiles of each CV fly sample. This
409 explanation is supported by the substantial increase in the number of candidate genes
410 that met our statistical cutoff depending on the exclusion of certain replicates from our
411 analysis. Nevertheless, this initial experiment provided evidence of genes expressed in
412 the adult *Drosophila* head that were transcriptionally responsive to sterile rearing
413 conditions. This list of candidates formed the basis for our subsequent analyses.

414 **Microbial impact on immune, metabolic, and oxidative stress response gene** 415 **expression in *Drosophila* heads identified by secondary nanoString analysis**

416 Our overall goal was to identify specific genes exhibiting microbiota-dependent
417 expression changes in the *Drosophila* head. We reasoned that these gene identities
418 would inform hypotheses about the molecular bases of known and novel microbiota-
419 regulated host physiological traits, and would thereby guide subsequent mechanistic
420 investigations into the roles of these genes within a microbial context. Given this objective,
421 and the modest expression changes and high variance observed in our RNA-Seq results,
422 we conducted a second round of screening on a subset of candidate genes utilizing
423 nanoString nCounter technology. The nCounter system employs a probe-capture
424 barcode platform for direct, digital measurement of mRNA transcripts. These features of
425 the platform enabled us to validate putative gene expression changes via an alternative
426 methodology that obviates the potential bias introduced by the enzymatic processing
427 steps of library preparation. Expression differences consistently observed via both
428 techniques (indirect, relative, genome-wide vs. direct, target-based) would therefore have

429 considerable empirical support as microbiota-affected genes. Additionally, the high-
430 sensitivity of the automated nCounter technology afforded the potential to reveal evidence
431 of type I (“false positive”) errors from our RNA-Seq analyses.

432 For our nCounter study we generated probes for 92 genes chosen from the 343
433 identified candidates (File S5). Our selection criteria included statistical robustness (i.e.
434 the genes with comparatively high support shared by multiple analyses of RNA-seq
435 results, described above) and magnitude of putative differential expression. Further, given
436 our aim of identifying molecular factors involved in microbiota-modulated neural function
437 and behavioral traits, we also prioritized and selected genes implicated in fly behaviors
438 and with known roles in the brain.

439 The multiplexed nature of the nCounter technology also allowed us to test the
440 additional variables of a GNO microbial condition and host sex. Which bacterial taxa or
441 cohorts of taxa, and what functional attributes of microbial populations contribute to a
442 microbiota-dependent host trait, are crucial questions inherent to all studies of the
443 mechanistic basis of host-microbe symbioses. Moreover, as indicated above, the
444 microbiota of CV-reared laboratory *Drosophila* is inconstant and compositionally variable
445 within and across fly populations. We generated flies with a standardized, GNO
446 microbiota for examination in our nanoString experiment, to: (i) begin to identify the
447 specific bacteria responsible for observed gene expression changes, facilitating future
448 explorations of microbial mechanisms responsible for identified host traits, and (ii) reduce
449 inter-sample variability in microbiota composition as a potential factor contributing to
450 variable gene expression. Our GNO flies were reared from embryo to 5-6-day adulthood
451 in continuous polyassociation with four bacterial strains isolated from CV Top Banana

452 cultures: *Lactobacillus brevis*, *Lactobacillus plantarum*, *Acetobacter sp.* (unresolved
453 species/strain-level taxonomic assignment; see Materials and Methods), and *Acetobacter*
454 *pasteurianus* (Table S1). These strains represent the bacterial taxa most commonly
455 associated with laboratory *Drosophila* cultures (Broderick and Lemaitre 2012; Douglas
456 2018). In addition, as noted above, reports surveying microbiota effects on *Drosophila*
457 gene expression are dominated by studies focused solely on female flies. Our RNA-seq
458 experiment used only male heads, and we speculated that some putative expression
459 changes might be male-specific, or exhibit a different expression pattern in females. We
460 therefore included both male and female flies in our nanoString study to determine
461 whether any tested candidate genes responded differently to host microbial condition as
462 a function of the animal's sex. In all, our final experimental matrix for nanoString analysis
463 included heads from male and female CV, GF, and GNO flies.

464 As with our RNA-seq results, most gene expression changes observed in the
465 nanoString study were modest. More specifically, we observed relatively few genes with
466 high-magnitude expression differences in both GF vs. CV and GF vs. GNO comparisons
467 (Figure 2A, 2B). For both males and females, more genes were strongly down-regulated
468 in the heads of GF compared to microbe-associated flies than were upregulated in GF
469 heads (Figure 2A, 2B, File S6). Notably, genes down-regulated in GF male heads were
470 generally more strongly repressed relative to CV flies than relative to GNO flies (Figure
471 2A; the magnitude of the blue bars exceeds that of the gray bars). Conversely, in females
472 down-regulated genes were suppressed to a greater magnitude in relation to the GNO
473 condition than vs. CV animals (Figure 2B; the magnitude of the gray bars exceeds that of
474 the blue bars). These trends, in addition to specific gene expression differences (see

475 below), suggest that host sex impacts head-localized transcriptional responses to the
476 microbial environment, consistent with previous observations in the gut (Bost *et al.* 2017).

477 The fundamental objective of our secondary nanoString screen was to identify
478 individual genes with robust, microbiota-affected expression in either male or female
479 *Drosophila* heads. We therefore examined the genes with the greatest magnitude of
480 change in either the GF/CV or GF/GNO comparison (arbitrarily defined as $|2fc| > 0.4$ or
481 $|2fc| < -0.4$; 32 genes for males, 40 genes for females: File S6, sheet “Log2FoldChange”),
482 and statistically compared the effect of the three microbial conditions on these genes
483 separately for each sex (see Materials and Methods; File S6, sheet “Log2FoldChange”).
484 Using this approach, we identified 19 genes that were differentially expressed ($p < 0.05$) in
485 \geq one of the four comparisons: (i) male GF vs. CV, (ii) male GF vs. GNO, (iii) female GF
486 vs. CV, (iv) female GF vs. GNO. For both males and females, a greater number of
487 significant expression changes were observed in the GF to GNO comparison (11 genes
488 for males, 13 genes for females) than in the GF to CV comparison (4 genes for males, 1
489 gene for females). Six of the 19 genes were differentially expressed in both males and
490 females, and for all six the directions of change between microbial conditions were the
491 same in both sexes. Notably, while all 92 of the genes in our codeset were selected from
492 our leave-out analyses-derived 343 RNA-seq candidates, most of the genes (11/19) with
493 significant expression differences in our nanoString results were significantly different in
494 only one leave-out analysis out of our seven total comparisons (File S6, sheet “RNAseq
495 comparison”).

496 The genes down-regulated in the heads of both male and female GF flies relative
497 to microbe-associated flies were dominated by immune genes (Figure 3), specifically, two

498 extracellular peptidoglycan recognition proteins (PGRPs), *PGRP-SB1* and *PGRP-SD*,
499 and eight antimicrobial peptides (AMPS). As previously indicated, more significant
500 expression differences were observed in the GF vs. GNO comparison than in the GF vs.
501 CV comparison. The PGRP genes were significantly reduced exclusively in GF female
502 compared to GNO female heads (Figure 3A, B). Four AMPs were significantly reduced
503 only in male GF vs. male GNO heads (*DptA*, *DptB*, *Dro*, and *edin*; Figure 3F, G, H, I), and
504 the remaining four were reduced in both male and female GF vs. GNO and/or CV heads
505 (*AttA*, *AttB*, *AttC*, and *Mtk*; Figure 3C, D, E, J). While only these ten immune genes
506 achieved statistical significance in \geq one comparison, the observable trend of elevated
507 transcript levels in microbe-associated flies compared to sterile flies was consistent
508 between sexes for all immune genes assayed (Figure 3A-J; File S6), including those with
509 no statistically significant differences in any comparison (Figure S4). As noted in our RNA-
510 seq results, the failure to achieve statistical significance likely reflects substantial
511 transcript count variability among the microbe-associated conditions, as compared to the
512 more consistent, low transcript levels detected in GF samples.

513 Seven additional genes that exhibited statistically significant responses to host
514 microbial condition in \geq one comparison had known or predicted functions in aging,
515 oxidative stress resistance and general detoxification, and metabolism. Interestingly,
516 each of these genes responded to host microbial condition in a sex specific manner. The
517 cytochrome P450 gene *cyp6a17* was elevated in GF male, but not female heads (Figure
518 4A), while the small mitochondrial heat shock protein *Hsp22* and the serine
519 hydroxymethyl transferase *Shmt/CG3011* were upregulated exclusively in GF females
520 (Figure 4B, C). Conversely, four experimentally uncharacterized genes with predicted

521 metabolic functions (inferred from sequence homology and predicted domain
522 architecture) were all down-regulated in GF vs. microbe-associated heads, again with
523 gene-by-gene sex differences. *CG10960* (functionally annotated as involved in sugar
524 transport), *CG4757* (inferred carboxylesterase activity), and *CG3036* (a putative anion
525 transporter) were all suppressed in GF females, but not males (Figure 4D, E, F). Another
526 uncharacterized gene, *CG10512*, with predicted malate dehydrogenase activity, was
527 more highly expressed in both male and female GNO, but not CV, heads relative to GF
528 heads (Figure 4G). An uncharacterized gene with no domain-based annotation and no
529 experimentally determined function, *CG7296*, was also elevated in male GNO vs. GF
530 heads (Figure 4H).

531 **Individual bacteria alter *Arc1* expression in the adult *Drosophila* head**

532 *Drosophila* activity-regulated cytoskeleton associated protein 1 (*Arc1*) also
533 responded strongly to microbial condition in Top Banana heads. Specifically, we found
534 *Arc1* was elevated approximately twofold in GF male heads compared to both CV and
535 GNO males, and more moderately elevated in GF female compared to GNO female
536 heads (Figure 5A). *Arc1* encodes an ~29 kDa protein evolutionarily related to
537 retrotransposon Group-specific antigen (Gag)-like proteins. *Arc1* proteins multimerize to
538 form capsid-like structures that facilitate intercellular mRNA transfer via exosomes at the
539 larval neuromuscular junction (Ashley *et al.* 2018), and this mechanism is conserved in
540 mammalian *Arc/Arg3.1* (Pastuzyn *et al.* 2018). Moreover, *Drosophila Arc1* function in a
541 particular subset of neurons in the larval brain promotes systemic metabolic homeostasis
542 and prevents hyperlipidemia (Mosher *et al.* 2015). These metabolic consequences of
543 *Arc1* genetic perturbation resemble those observed in flies grown under sterile conditions

544 (Shin *et al.* 2011; Storelli *et al.* 2011; Ridley *et al.* 2012; Wong *et al.* 2014; Newell and
545 Douglas 2014; Dobson *et al.* 2015; Huang and Douglas 2015; Chaston *et al.* 2016; Kim
546 *et al.* 2017; Judd *et al.* 2018; Kamareddine *et al.* 2018).

547 Numerous published reports have demonstrated that association with individual
548 bacterial species and strains is sufficient to recapitulate CV- and/or polyassociated GNO-
549 like host molecular and physiological traits. In some examples, a mixed microbial
550 community's impacts on the host are attributable to the activities of a specific, single
551 community member, while in other examples monoassociation with any bacterial
552 commensal is sufficient to recapitulate the effects of the polymicrobial community (Shin
553 *et al.* 2011; Storelli *et al.* 2011; Newell and Douglas 2014; Elya *et al.* 2016; Daisley *et al.*
554 2017; Téfit and Leulier 2017; Fischer *et al.* 2017; Kim *et al.* 2017; Leitão-Gonçalves *et al.*
555 2017; Matos *et al.* 2017; Obadia *et al.* 2017; Wong *et al.* 2017; Judd *et al.* 2018; Sannino
556 *et al.* 2018). We asked whether suppression of *Arc1* expression in the heads of CV and
557 GNO relative to GF flies is due to association with specific bacterial taxa, or represents a
558 generalized response to the presence of microbes. Given that our standardized,
559 simplified, four bacteria GNO microbial community was sufficient to restore CV level
560 expression to male flies (Figure 5A), we tested this hypothesis by focusing on these four
561 species. Specifically, we reared Top Banana *Drosophila* cultures in monoassociation with
562 each of the four bacteria comprising our GNO condition, and measured relative *Arc1*
563 expression in the heads of adult male flies via RT-qPCR. Consistent with our nanoString
564 results (Figure 5A), polyassociation with the four-species GNO community resulted in
565 significantly decreased *Arc1* expression relative to GF heads, and monoassociation with
566 *Acetobacter sp.*, *A. pasteurianus*, or *L. brevis* was sufficient to downregulate *Arc1* to the

567 same extent (Figure 5B). *Arc1* expression was also reduced in *L. plantarum*
568 monoassociated heads relative to GF, but the difference was not significant (Figure 5B).
569 These results suggest that the transcriptional downregulation of *Arc1* in the heads of GNO
570 flies is primarily attributable to interactions with the two *Acetobacter* isolates and *L. brevis*,
571 but not to the presence of *L. plantarum*. As each of these bacteria alone recapitulates the
572 transcriptional difference induced by the polymicrobial community, this microbiota-
573 dependent *Arc1* suppression may reflect a common host response to the presence of
574 certain prokaryotic organisms.

575 **Microbiota-dependent gene expression changes in the *Drosophila* head are** 576 **sensitive to host genetic background**

577 Host genotype substantially affects microbial impacts on certain host traits in
578 *Drosophila* (Brummel *et al.* 2004; Broderick *et al.* 2014; Dobson *et al.* 2015; Chaston *et*
579 *al.* 2016; Obata *et al.* 2018). Because our gene expression screen employed the recently
580 established wild-type *Drosophila* stock Top Banana, we asked whether select microbiota-
581 dependent gene expression changes are unique to this genetic background, or represent
582 more common microbial effects. To address this, we used RT-qPCR to measure relative
583 transcript levels of three strongly microbiota-responsive genes from our screen (*Arc1*,
584 *Hsp22*, and *DptA*) in the heads of CV-, GF-, and GNO-reared w^{1118} flies, a widely-utilized
585 laboratory *Drosophila* wild-type stock.

586 As in Top Banana flies (Figure 5A), *Arc1* was expressed at lower levels in the
587 heads of bacteria-associated CV and GNO male w^{1118} *Drosophila* relative to GF males
588 (Figure 6A), however there was no difference in expression across conditions in w^{1118}
589 female heads (Figure 6B). Similarly, *Hsp22*, which exhibited female-specific elevation in

590 the heads of GF Top Banana flies compared to GNO females (Figure 4B) was unaffected
591 in w^{1118} , showing roughly equivalent expression across all conditions (Figure 6C, D). The
592 representative AMP gene *DptA*, showed a high fold expression change in the heads of
593 CV and GNO w^{1118} males and females compared to their sterile siblings like in Top
594 Banana (Figure 3F, 6E,F). However, the fold elevation relative to GF was highest and
595 statistically significant only in GNO females (Figure 6E, F). Nonetheless, this observation
596 was consistent with the robust AMP expression changes observed in our preceding
597 screen results. These data provide additional support for the hypotheses that host sex
598 and genotype are important factors governing *Drosophila*'s transcriptional response to
599 association with the microbiota. More specifically, these data suggest that some gene
600 expression changes identified in our screen may be sensitive to host genomic
601 composition.

602 **DISCUSSION**

603 To date a variety of microbe-dependent physiological and behavioral traits have
604 been described in *Drosophila* (Broderick and Lemaitre 2012; Martino *et al.* 2017; Douglas
605 2018), however the host molecular processes underlying these effects are largely
606 unknown. In this study, we utilized RNA-seq and nanoString technology to uncover
607 microbiota-responsive genes in the adult *Drosophila* head. Our RNA-seq investigation of
608 gene expression changes induced by GF rearing revealed a high degree of inter-replicate
609 variability in global expression profiles, particularly among CV samples. Additionally, all
610 putative microbiota-dependent gene expression changes we identified, in both RNA-seq
611 and nanoString analyses, were of relatively low magnitude (Figure 1A, Figure 2). In
612 contrast, previous studies examining microbiota-induced gene expression identified

613 stronger transcriptional differences (i.e. \geq twofold) in the adult *Drosophila* gut (Broderick
614 *et al.* 2014; Guo *et al.* 2014; Elya *et al.* 2016; Petkau *et al.* 2017), in the embryos of sterile
615 parents (Elgart *et al.* 2016), in third instar larvae (Erkosar *et al.* 2017), and in whole flies
616 (Combe *et al.* 2014; Dobson *et al.* 2016; Bost *et al.* 2017). Excepting the proboscis
617 mouthparts and anterior-most foregut epithelium, the fly head largely consists of tissues
618 that are unlikely to directly interact with microbes. It may therefore follow that expression
619 changes in the head, while potentially no less functionally important, would be modest
620 compared to microbial impacts on gene expression in the gut. Moreover, the head
621 contains multiple highly distinct cell- and tissue-types (i.e. eyes, ocelli, brain, antennae,
622 tracheal tissue, fat body, etc). Large transcriptional changes in small cell numbers could
623 therefore be masked by sampling whole heads. Nevertheless, our study is the first
624 transcriptome-scale assay of microbiota-dependent gene expression in an adult fly
625 anatomical structure distinct from the gut. This study is also noteworthy as only the
626 second to examine microbial effects on gene expression in both male and female flies
627 (Bost *et al.* 2017), and our findings are congruent with that report. Specifically, in both our
628 RNA-seq results and those of Bost *et al.*, chemical response genes, including cytochrome
629 p450-encoding genes, were the dominant functional categories upregulated in GF male
630 animals, while immune genes constituted the major downregulated functional categories
631 in sterile males. Moreover, our nanoString experiment revealed multiple genes that were
632 microbiota-responsive only in one sex, consistent with the sex-specific transcriptome
633 profiles observed by Bost *et al.* These findings emphasize host sex as a critical variable
634 that affects molecular outcomes of host-microbe association in *Drosophila*.

635 Most of the genes revealed by our screen have known or inferred roles in immune
636 function, metabolism, aging, and oxidative stress responses. Intriguingly, numerous
637 published studies have provided evidence that commensal microbes also modulate each
638 of these physiological processes; examples of these connections are described in the
639 following sections. We propose that the genes identified here are promising candidates
640 for future study of the functional molecular basis for known microbe-dependent
641 *Drosophila* traits.

642 **Microbiota-induced immune gene expression in the adult *Drosophila* head**

643 Innate immune genes, namely AMPs and PGRPs, were suppressed in the heads
644 of GF compared to microbiota-associated flies in both our RNA-seq and nanoString
645 experiments (Figure 1A, 1C, Figure 3, Figure S4). Prior transcriptomic studies have also
646 demonstrated microbiota-driven transcriptional induction of IMD pathway genes in the
647 adult gut and in whole animals (Ryu *et al.* 2008; Buchon *et al.* 2009; Broderick *et al.* 2014;
648 Guo *et al.* 2014; Clark *et al.* 2015; Sansone *et al.* 2015; Dobson *et al.* 2016; Petkau *et al.*
649 2017; Bost *et al.* 2017). Some reports suggest that this priming of intestinal immune
650 function by the commensal microbiota is important to maintenance of gut homeostasis
651 and protection against injury in young flies; as the animal ages, over-induction of immune
652 responses contributes to immunosenescence, inflammation, deterioration of gut
653 homeostasis and integrity, and ultimate mortality (Ryu *et al.* 2008; Buchon *et al.* 2009;
654 Blum *et al.* 2013; Guo *et al.* 2014; Clark *et al.* 2015; Sansone *et al.* 2015; Li *et al.* 2016;
655 Lindberg *et al.* 2018). Whether microbiota-induced IMD activity in adult organs and
656 tissues other than the gut also contributes to these phenomena is not known, and our
657 data suggest this question warrants investigation. Importantly, the differences in immune

658 gene activation we observed may reflect transcriptional changes in head cell types that
659 most directly contact microbes, such as the labellum and anterior-most foregut, or the
660 head fat body. While less is known about immune activity in the head fat body, the
661 abdominal fat body is the principle site of humoral immune induction during pathogenic
662 infection (Ferrandon *et al.* 2007). Interestingly, *DptB* (one of the AMPs that emerged from
663 our screen) was recently shown to be highly upregulated in adult male heads following
664 long-term-memory-inducing behavioral training paradigms, and reciprocally its
665 expression in the head fat body was required for the formation of long-term memories
666 (Barajas-azpeleta *et al.* 2018). Further examples in the literature also point to connections
667 between immune pathway activity in head tissues and complex physiological and
668 behavioral phenotypes. AMP transcription in the brain increases with age, and
669 overexpression of IMD-regulated AMPs in the adult brain is sufficient to reduce longevity
670 and geotactic proficiency; conversely, genetic dampening of immune activity in both the
671 CNS and glia is sufficient to extend lifespan and climbing ability (Kounatidis *et al.* 2017).
672 In *Drosophila* models of neurological pathology, such as Fragile X syndrome, glial activity
673 and activation of innate immune function in the brain contributes to neurodegenerative
674 phenotypes and disease symptoms (Cao *et al.* 2013; Petersen *et al.* 2013; O'Connor *et*
675 *al.* 2017). Enteric infection by the pathogen *Pectinobacterium carotovora carotovora* 15
676 also exacerbates the neurological and physiological deterioration of a *Drosophila*
677 Alzheimer's disease model, in a manner involving hemocyte recruitment to the adult brain
678 (Wu *et al.* 2017). Potential contributions of the commensal microbiota to these
679 neuroimmunological processes and their impacts on animal health have not been
680 thoroughly examined. Our observation of microbiota-induced innate immune responses

681 in the heads of adult flies may therefore have important implications for the similar
682 physiological and life-history traits of flies.

683 **Modulation of aging and stress response gene expression by the microbiota**

684 Our screen also revealed microbiota-responsive genes with known roles in the
685 oxidative stress response and in pro-longevity functions. Organisms must respond to
686 reactive oxygen species (ROS)-induced oxidative injury throughout their lifespans. ROS
687 are constantly generated as metabolic byproducts, and their accumulation and the
688 consequent macromolecular damage over time is a major contributor to cellular
689 senescence and normal aging (Johnson *et al.* 1996; Lin and Beal 2003; Ewald 2018).
690 Accordingly, in flies and other model organisms, overexpression of genes that promote
691 proteostasis—such as chaperones, proteasomal subunits, and reducing enzymes—curtails
692 age-related ROS elevation and extends longevity (Lin and Beal 2003; Back *et al.* 2012;
693 Wang *et al.* 2013). Three genes we found to have increased expression in GF fly heads
694 in one or both screen steps, *Hsp22*, *NAD-dependent methylenetetrahydrofolate*
695 *dehydrogenase-methenyltetrahydrofolate cyclohydrolase (Nmdmc)*, and *Ecdysone-*
696 *induced protein 71CD/methionine-S-sulfoxide reductase A (Eip71CD/MsrA)*, all have
697 been shown to enhance oxidative stress resistance and lifespan upon overexpression
698 (Morrow *et al.* 2004; Roesijadi *et al.* 2007; Chung *et al.* 2010; Yu *et al.* 2015). *Hsp22*
699 encodes a small mitochondrial chaperone that functions in the unfolded protein response,
700 and was upregulated specifically in female GF flies in our nanoString study (Figure 4B).
701 While it did not meet our criterion of statistical significance, *Nmdmc* was also highly
702 upregulated, on average, in the heads of GF vs. GNO female flies in our nanoString
703 results (File S6). This gene encodes a mitochondria-localized folate-dependent enzyme

704 involved in purine biosynthesis, and may extend lifespan by increasing mitochondrial DNA
705 copy number through an unknown mechanism (Yu *et al.* 2015). *Eip71CD/MsrA* was
706 among the most statistically robust genes elevated in GF vs. CV male heads in our RNA-
707 seq screen (Figure 1A, File S1, File S3), and was the most highly upregulated (though
708 not significantly different) gene in GF vs. GNO male heads assayed by nanoString (File
709 S6). *MsrA* is a repair enzyme that reduces methionine-S-sulfoxide (generated by
710 oxidative damage to proteins) to methionine, and its pan-neuronal overexpression is
711 sufficient for enhanced longevity (Chung *et al.* 2010). Another gene of interest with
712 putative detoxification and stress response function was *Cyp6a17*, which increased in GF
713 male, but not female, heads compared to both CV and GNO animals (Figure 4A).
714 *Cyp6a17* belongs to the cytochrome P450 gene family, which encodes a broad range of
715 enzymes that oxidize toxic compounds (Bergé *et al.* 1998). In flies, *Cyp6a17* is enriched
716 in the mushroom body of the adult brain where its activity downstream of cAMP-PKA
717 signaling modulates temperature preference behavior via an unknown mechanism (Kang
718 *et al.* 2011).

719 Higher basal expression of these pro-longevity genes in GF *Drosophila* may
720 contribute to the extended lifespan that has been observed for GF animals (Petkau *et al.*
721 2014; Clark *et al.* 2015; Fast *et al.* 2018; Iatsenko *et al.* 2018; Sannino *et al.* 2018).
722 Interestingly, the *Drosophila* microbiota also induce secreted ROS production by
723 enterocytes that limits the gut bacterial population size, preventing dysbiosis and
724 premature aging (Guo *et al.* 2014; Iatsenko *et al.* 2018). Thus, the complete absence of
725 microbes should result in less oxidative damage, and therefore less upregulation of
726 oxidative damage counteracting genes, at least in the gut. Induction of *Nmdmc*, *Eip71CD*,

727 and *Hsp22* in the heads of GF flies must therefore occur via signals independent of
728 microbe-induced ROS.

729 Increased expression of these stress response genes under non-stress conditions
730 may reflect a basal susceptibility to oxidative injury in adults lacking microbial symbionts.
731 Consistent with this, upregulation of *Cyp6a17* (and other cytochrome P450 genes
732 observed in our RNA-seq analysis; File S5) may suggest a greater burden on the animal
733 to detoxify ingested compounds and metabolic byproducts in the absence of microbes
734 that normally serve this function. The baseline stressed condition may be exacerbated
735 upon encountering environmental stressors, suggesting an explanation for the greater
736 susceptibility of GF flies to chemical oxidative challenge (Jones *et al.* 2015; Naudin *et al.*
737 2019). Microbiota-dependent ROS production has not been examined in tissues other
738 than the gut, nor has there been past indication of the sex-specific effects that we
739 observed. This suggests that there is much more to learn about the molecular
740 mechanisms connecting the microbiota to host oxidative stress resistance and lifespan.

741 **Novel microbiota-regulated metabolic genes**

742 Many of the most profound effects of the microbiota on animal physiology occur
743 via their metabolic activities. Bacterial commensals in the mammalian gut utilize host
744 dietary polysaccharides as carbon sources, and many of the resultant secondary
745 metabolites are absorbed by host tissues where they can have systemic effects on
746 multiple organ systems (Cockburn and Koropatkin 2016; Daien *et al.* 2017). Mice and
747 rats raised GF or subjected to aggressive antibiotic regimens exhibit reduced body fat
748 levels and a decreased basal metabolic rate (Smith *et al.* 2007). Removal of the
749 *Drosophila* microbiota also drastically impacts host metabolic function. On nutrient-rich

750 diets, bacteria modulate the glucose content of the diet substrate influencing the
751 metabolic and nutritional profiles of adults (Ridley *et al.* 2012; Newell and Douglas 2014;
752 Newell *et al.* 2014; Wong *et al.* 2014; Dobson *et al.* 2015; Huang and Douglas 2015).
753 Microbial enhancement of dietary nutritional availability, particularly B-vitamins, promotes
754 the fitness of the host—assayed by parameters including developmental rate, adult mass,
755 and fecundity—particularly on protein-limited diets (Storelli *et al.* 2011, 2017; Wong *et al.*
756 2014; Leitão-Gonçalves *et al.* 2017; Matos *et al.* 2017; Bing *et al.* 2018; Sannino *et al.*
757 2018). *Acetobacter*-derived acetic acid induces both insulin signaling and the IMD
758 pathway to reduce hyperlipidemic phenotypes and promote fly development (Shin *et al.*
759 2011; Hang *et al.* 2014; Kamareddine *et al.* 2018). However, host mechanisms activated
760 by microbial depletion that yield these metabolic perturbations are unknown.

761 Second to immune activity, the GO-terms enriched among the genes
762 downregulated in GF flies in our RNA-seq study were dominated by metabolic functions,
763 specifically amino acid and fatty acid biosynthetic processes (File S5). This observation
764 raises the intriguing possibility that microbe-induced expression of certain genes found
765 here enables flies to derive the optimal nutritional benefit from their diet substrate, and
766 potentially from dead microbial cells themselves. We also identified, via nanoString, the
767 serine hydroxyl-methyl transferase *Shmt* and five unstudied genes with domain-based
768 predicted functions in metabolic activity, including sugar transport and enzymatic organic
769 chemical modifiers, which responded in both directions to elimination of the microbiota,
770 more frequently in females than in males (Figure 4). Substantial additional work is
771 required to investigate the uncharacterized, putative metabolic *Drosophila* genes

772 identified here as potential nodes in the complex interplay between laboratory *Drosophila*,
773 its bacterial symbionts, sex, diet, and nutrition.

774 *Arc1* is the *Drosophila* homolog of mammalian activity-regulated cytoskeleton
775 associated protein (ARC), a neuronal protein that is required for strengthening synaptic
776 connections, dendrite maturation, and learning and memory formation in mice
777 (Tzingounis and Nicoll 2006; Shepherd and Bear 2011). Genetic defects in *Arc/Arg3.1*
778 have been connected to neurological disorders, including Alzheimer's disease and Fragile
779 X syndrome (Park *et al.* 2008; Rudinskiy *et al.* 2012). Like its vertebrate homologues,
780 *Drosophila Arc1* is expressed in the larval and adult brain and neuroendocrine cells
781 (Mattaliano *et al.* 2007; Mosher *et al.* 2015). Interestingly, flies null for *Arc1* exhibit
782 metabolic defects, specifically increased fat stores and dysregulation of central carbon
783 metabolism (Mosher *et al.* 2015), as well as enhanced starvation resistance (Mattaliano
784 *et al.* 2007). *Drosophila Arc1* encodes a retroviral GAG-like protein which multimerizes to
785 form capsid-like structures. These structures mediate trans-synaptic, vesicular transfer of
786 *Arc1* mRNA and other mRNAs at the larval neuromuscular junction (Ashley *et al.* 2018),
787 a mechanism conserved in the vertebrate protein (Pastuzyn *et al.* 2018). Connections
788 between the molecular mechanism of *Arc1* activity and its functions in neuronal activity
789 and metabolism are unknown. Interestingly, in addition to our identification of *Arc1* as a
790 microbe-responsive host gene, *Arc1* appears in several published transcriptomic datasets
791 as among the most significantly microbiota-responsive genes. However, while we found
792 a significant elevation of *Arc1* in the heads of GF males and females (Fig. 5A), these
793 studies all observed a decrease in *Arc1* expression in gut or whole GF females (Guo *et*
794 *al.* 2014; Dobson *et al.* 2016; Petkau *et al.* 2017). This discrepancy may further

795 underscore the importance of tissue-specificity of microbial impacts on gene expression.
796 Additionally, these studies were predominantly conducted in the Canton-S wild-type fly
797 stock, and our RT-qPCR experiments conducted on *w¹¹¹⁸ Drosophila* suggest that the
798 *Arc1* expression change may be sensitive to host genotype, sex, and/or an interaction
799 between the two (Figure S5). Nevertheless, the potential connections between the
800 bacterial microbiota, *Arc1* expression and function, and *Drosophila* metabolism represent
801 a promising avenue for future investigation.

802 **Summary and concluding remarks**

803 Together this study has revealed genes that exhibit altered expression in the head
804 of young adult *Drosophila* upon elimination of the microbiota. We hypothesize that some
805 of these genes contribute to the host molecular mechanisms underlying known
806 microbiota-impacted traits, including metabolic function, stress resistance, and aging. In
807 addition, many genes not prioritized in our screening process nevertheless trended with
808 differential expression patterns in our RNA-seq study, and may still represent bona fide,
809 biologically relevant microbiota-regulated genes. Examples include genes with roles in
810 circadian rhythms, visual and odor perception, and cellular responses to hypoxia (File
811 S5). We predict that analysis of these gene expression changes at the tissue and cell
812 type specific level will reveal connections between the gut microbiota and many novel
813 aspects of host physiology and behavior.

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1140 FIGURE LEGENDS

1141 **Figure 1**

1142 Overview of the head transcriptomic response microbiota elimination in adult male Top
1143 Banana *Drosophila*. (A) Volcano plot representation of RNA-seq results plotting all genes
1144 with non-zero read counts according to statistical significance (Benjamini-Hochberg
1145 adjusted p-value) as a function of fold change in GF vs. CV male heads. Genes with \log_2
1146 fold change >0.5 and FDR-adjusted p-value <0.05 are highlighted. (B) Heatmap
1147 representation of 343 candidate genes identified as statistically significant ($p\text{-adj}<0.1$) in
1148 at least one leave-out analysis conducted on the entire RNA-seq dataset (File S4). Each
1149 column represents a biological replicate of the indicated condition (RNA from 50 individual
1150 heads/replicate); colors represent row Z-scores derived from transcripts per million (TPM)
1151 values for each gene. (C) GO-terms enriched (Holm-Bonferroni adjusted p-value <0.001 ,
1152 ≥ 20 genes) in the 248/343 candidate genes downregulated ($\log_2\text{fc}<0$) in the heads of male
1153 GF compared to CV flies. (D) GO-terms enriched (Holm-Bonferroni adjusted p-
1154 value <0.05) in the 95/343 candidate genes upregulated ($\log_2\text{fc}>0$) in the heads of male GF
1155 compared to CV flies.

1156

1157 **Figure 2**

1158 Microbiota-dependent gene expression changes in adult male (A) and female (B) Top
1159 Banana *Drosophila* heads revealed by secondary screening with the nanoString nCounter
1160 platform. Blue bars represent \log_2 fold change values comparing GF and CV heads; gray
1161 bars represent \log_2 fold change values comparing GF heads to GNO flies reared in
1162 polyassociation with a four-species microbial community consisting of *Acetobacter* and
1163 *Lactobacillus* bacteria isolated from CV Top Banana cultures. Data are independently
1164 ordered for each sex based on \log_2 fold change values for the GF vs. GNO comparison,
1165 with GF vs. CV value adjacent for each gene. Fold change values are calculated based
1166 on means of normalized mRNA counts for each condition ($n=5$ replicates each for CV
1167 males, GF females, GNO females, and $n=6$ replicates each for CV females, GF males,
1168 GNO males; 5-10 heads/replicate). The values plotted here are also found in File S6
1169 “Log2FoldChange”.

1170

1171 **Figure 3**

1172 The microbiota induce innate immune gene expression in the heads of adult flies.
1173 nanoString data are represented as individual gene plots for each immunity-related gene
1174 for which microbial condition yielded a statistically significant expression difference in at
1175 least one sex. $n=5$ replicates each for CV males, GF females, GNO females, and $n=6$
1176 replicates each for CV females, GF males, GNO males; 5-10 heads/replicate. Data were
1177 analyzed independently for each sex via one-way ANOVA with Tukey HSD *post-hoc* tests
1178 or Kruskal-Wallis test with Dunn’s multiple comparisons test when assumptions of equal
1179 variance and normal distribution were broken. $p<0.05$ *, $p<0.01$ **, ns=not significant.

1180

1181 **Figure 4**

1182 Sex-specific microbial modulation of expression of genes involved in detoxification,
1183 oxidative stress resistance, and predicted metabolic function in the heads of adult Top
1184 Banana *Drosophila*. nanoString data are represented as plots of individual genes for

1185 which microbial condition yielded a statistically significant expression difference in at least
1186 one sex. n=5 replicates each for CV males, GF females, GNO females, and n=6 replicates
1187 each for CV females, GF males, GNO males; 5-10 heads/replicate. Data were analyzed
1188 independently for each sex via one-way ANOVA with Tukey HSD *post-hoc* tests or
1189 Kruskal-Wallis test with Dunn's multiple comparisons test when assumptions of equal
1190 variance and normal distribution were broken. p<0.05 *, p<0.01 **, ns=not significant.

1191
1192 **Figure 5**
1193 Microbial suppression of *Arc1* expression in adult male Top Banana *Drosophila* heads
1194 can be mediated by interaction with specific, individual bacteria. (A) nanoString data
1195 demonstrating elevation of *Arc1* mRNA in the heads of male and, more moderately,
1196 female flies. n=5 replicates each for CV males, GF females, GNO females, and n=6
1197 replicates each for CV females, GF males, GNO males; 5-10 heads/replicate. (B)
1198 Monoassociation with only three members of the four-species GNO bacterial community–
1199 *Acetobacter sp.*, *L. brevis*, and *A. pasteurianus*, but not *L. plantarum*–is sufficient to
1200 reduce *Arc1* expression in the heads of male Top Banana flies, as measured by RT-
1201 qPCR. n=4 biological replicates of each condition, 10 heads/replicate. Figure represents
1202 fold change relative to GF flies following normalization to the average of housekeeping
1203 genes *Rpl32* and *14-3-3ε* as calculated via $\Delta\Delta C_t$ values. Error bars represent standard
1204 error of the mean. Data from both experiments were analyzed via one-way ANOVA with
1205 Tukey HSD *post-hoc* tests. p<0.05 *, p<0.01 **, p<0.0005 ***, ns=not significant.

1206
1207 **Figure 6**
1208 Candidate microbiota-dependent gene expression effects identified in Top Banana
1209 *Drosophila* are impacted by host genotype. (A) Male, but not (B) female, w^{1118} flies
1210 exhibit microbiota-induced reduction of *Arc1* expression in the head. (C) & (D) *Hsp22*
1211 expression in male and female w^{1118} heads is unaffected by microbial condition, unlike
1212 the female-specific elevation observed in GF Top Banana flies via nanoString. (E) & (F)
1213 *DptA* expression is elevated in the heads of GNO w^{1118} females compared to GF
1214 females; a similar trend that does not achieve statistical significance is observed in male
1215 heads and CV female heads. n=3 biological replicates of each condition, 10
1216 heads/replicate. Figure represents fold change relative to GF flies following
1217 normalization to *Rpl32* $\Delta\Delta C_t$ values. Error bars represent standard error of the mean.
1218 Data were analyzed via one-way ANOVA with Tukey HSD *post-hoc* tests. p<0.0005 ***,
1219 ns=not significant.

FIGURE 1

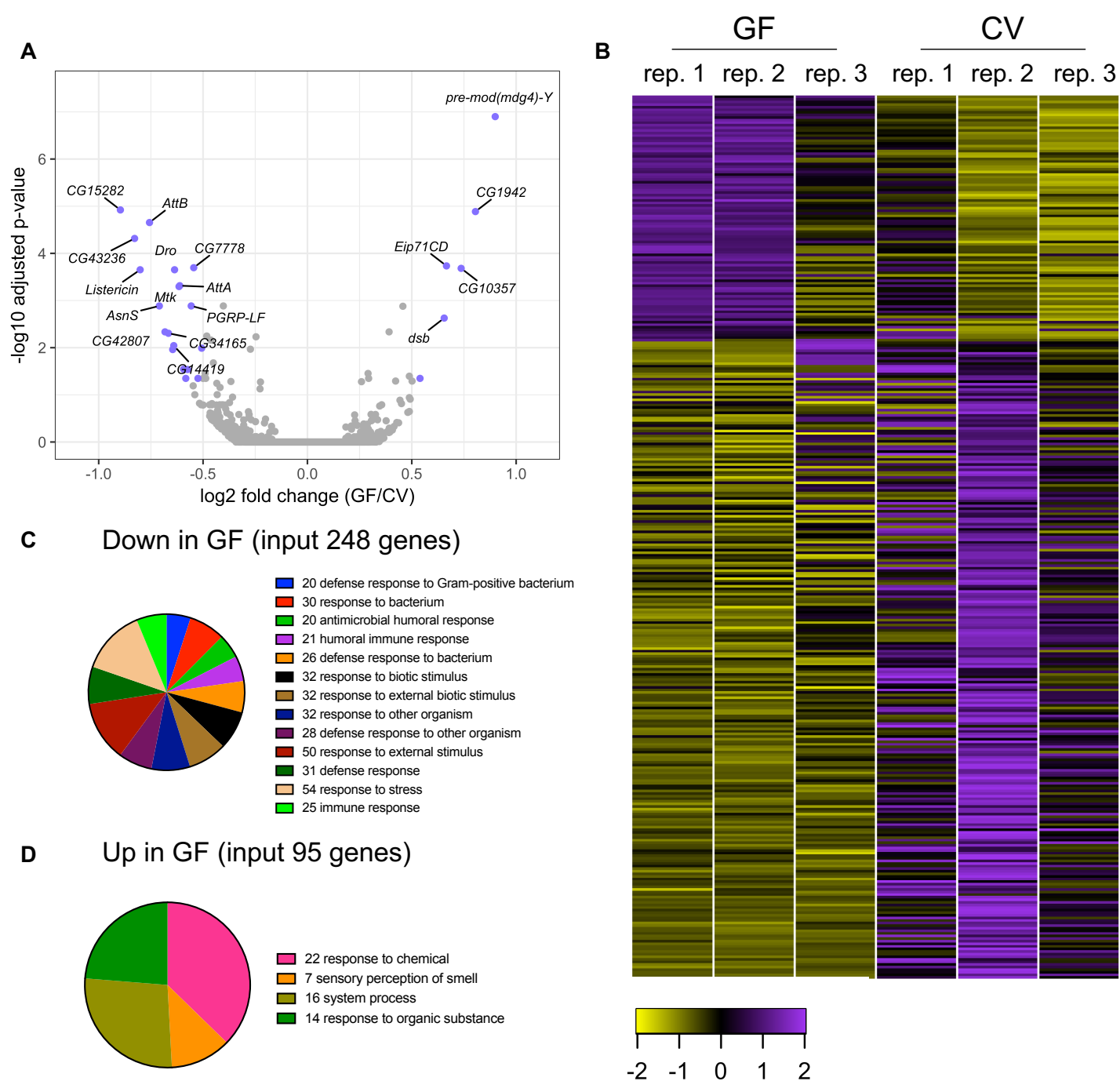


FIGURE 2

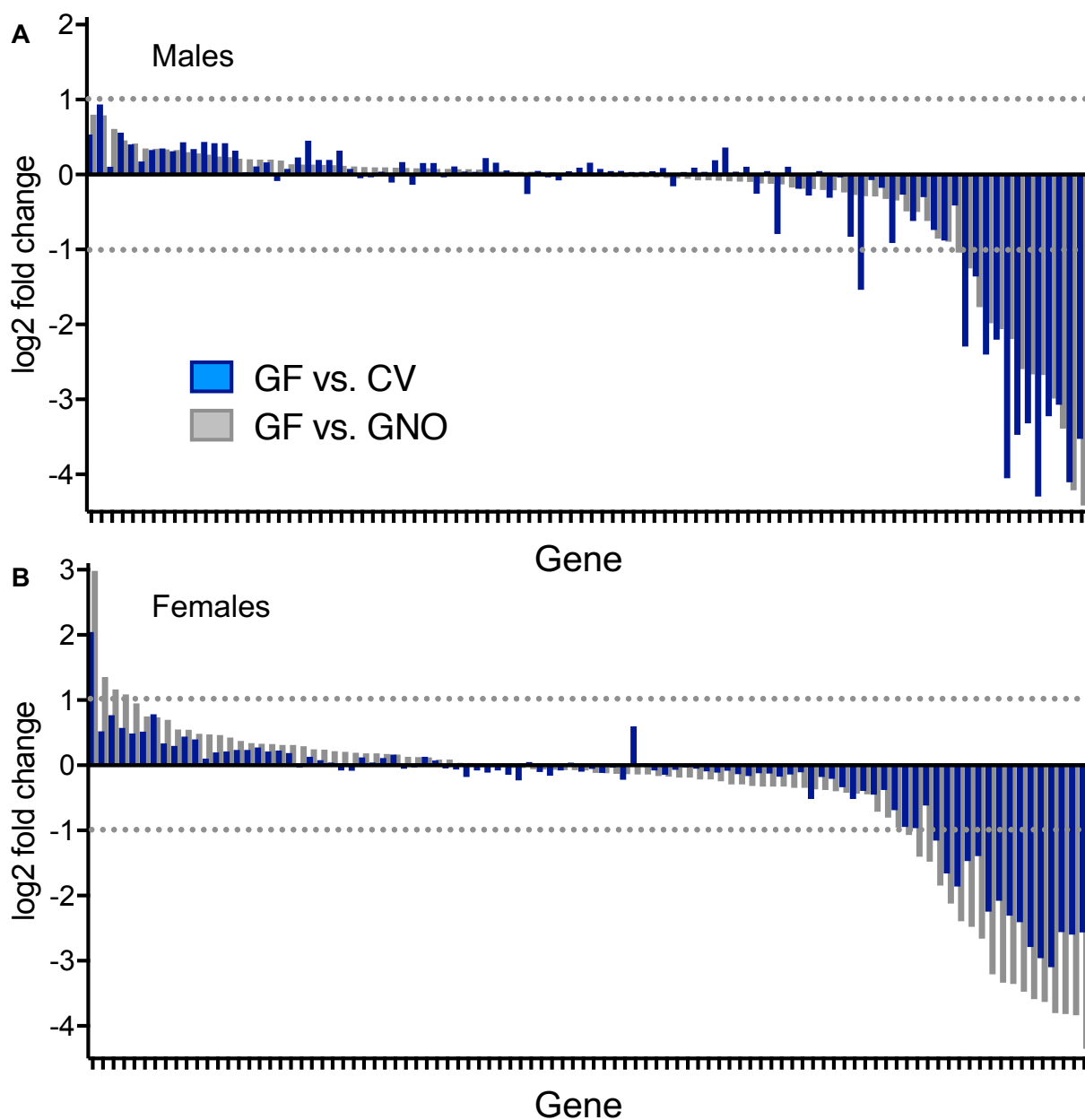


FIGURE 3

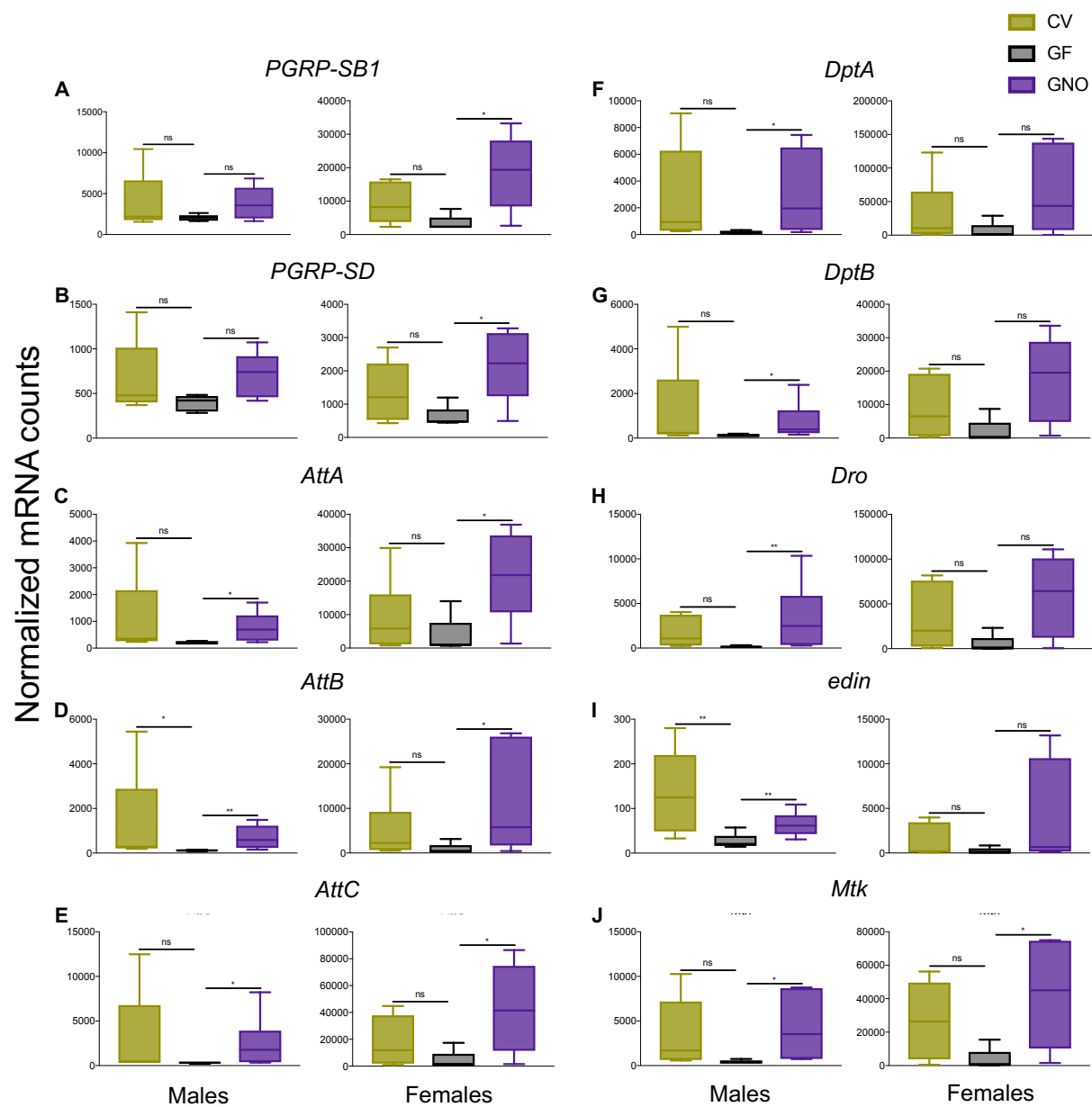


FIGURE 4

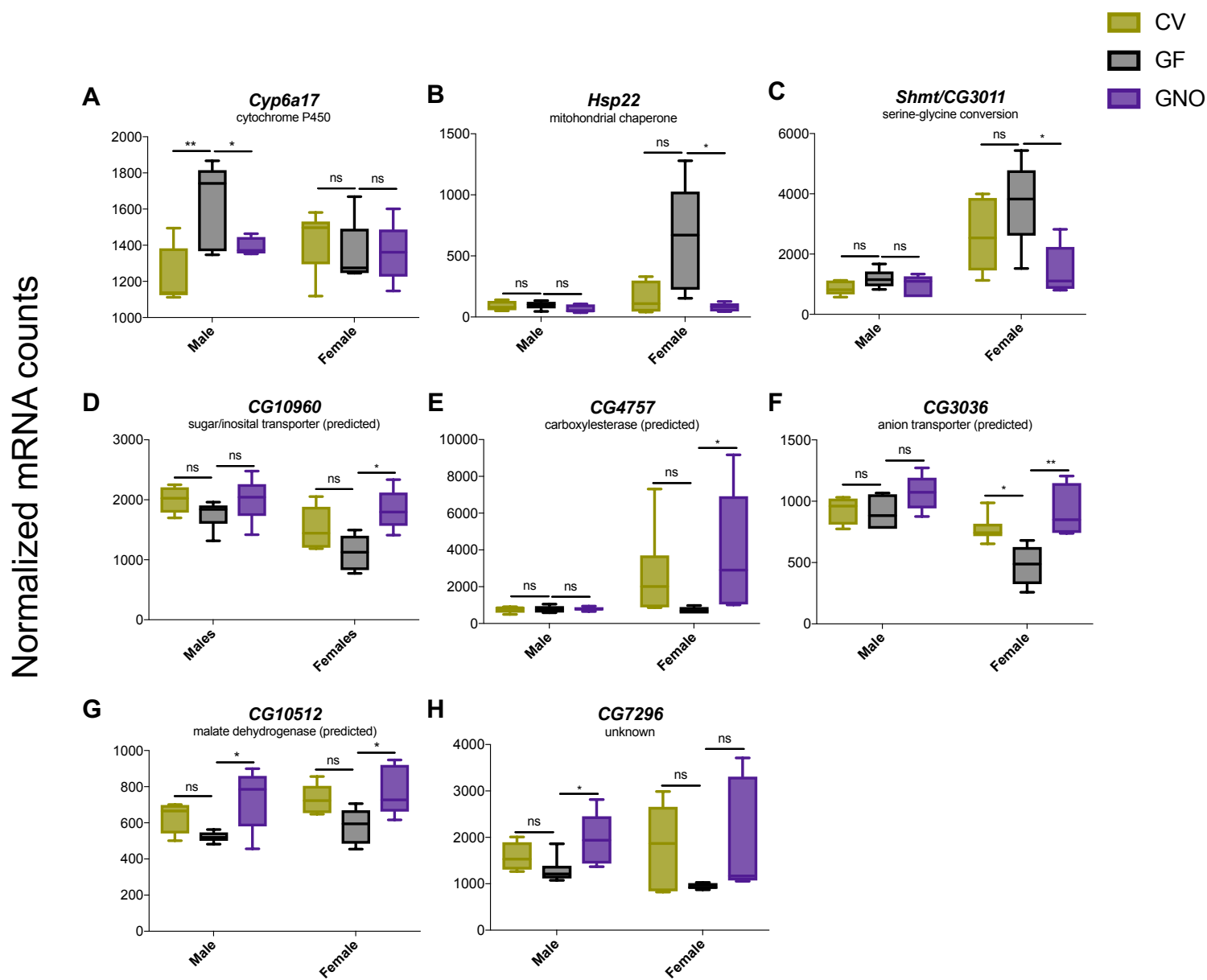


FIGURE 5

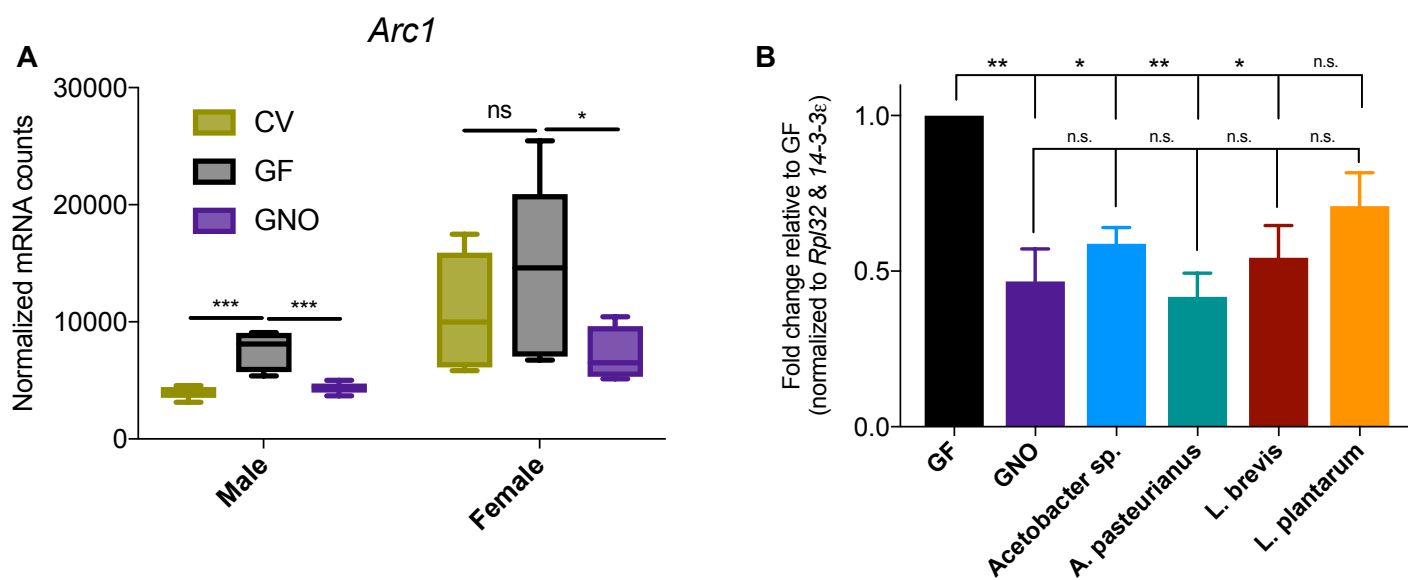


FIGURE 6

