Wolbachia is a nutritional symbiont in *Drosophila melanogaster*

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ABSTRACT

The intracellular bacterium *Wolbachia* is a common symbiont of many arthropods and nematodes, well studied for its impacts on host reproductive biology. However, its broad success as a vertically transmitted infection cannot be attributed to manipulations of host reproduction alone. Using the *Drosophila melanogaster* model and their natively associated *Wolbachia* strain "*w*Mel", we show that *Wolbachia* infection supports fly development and buffers against nutritional stress. *Wolbachia* infection across several fly genotypes and a range of nutrient conditions resulted in reduced pupal mortality, increased adult emergence, and larger size. We determined that the exogenous supplementation of pyrimidines rescued these phenotypes in the *Wolbachia*-free, flies suggesting that *Wolbachia* plays a role in providing this metabolite that is normally limiting for fly growth. Additionally, *Wolbachia* was sensitive to host pyrimidine metabolism: *Wolbachia* titers increased upon transgenic knockdown of the *Drosophila de novo* pyrimidine synthesis pathway but not knockdown of the *de novo* purine synthesis pathway. We propose that *Wolbachia* acts as a nutritional symbiont to supplement fly development and enhance host fitness.

SIGNIFICANCE STATEMENT

Wolbachia is a bacterial symbiont of arthropods and nematodes, well described for its manipulations of arthropod reproduction. However, many have theorized there must be more to this symbiosis, even in well-studied *Wolbachia*-host relationships such with *Drosophila*. Reproductive impacts alone cannot explain the success and ubiquity of this bacterium. Here, we use *Drosophila melanogaster* and their native *Wolbachia* infections to show that *Wolbachia* supports fly development and significantly buffers flies against nutritional stress. Additionally, we show that this advantage is likely mediated by pyrimidines. These developmental advantages might help explain the ubiquity of *Wolbachia* infections.

INTRODUCTION

Maternally transmitted microbes have evolved numerous ways to alter host biology, ultimately facilitating their own transmission. Strategies include supplementation of the host's diet via nutrient provisioning, protection against parasites and infections, and even direct manipulation of host reproduction (1). Many insects, other terrestrial arthropods, and some nematode species are infected with bacteria in the genus *Wolbachia* (Alphaproteobacteria: Rickettsiales), a maternally transmitted infection long appreciated for its direct impacts on host reproduction and sex ratios (1-10). Importantly, *Wolbachia* can spread through populations in the absence of such reproductive manipulations (11). And, increasingly, other *Wolbachia*-mediated benefits to the host are being uncovered (12). For example, a suite of *Wolbachia* strains have been characterized for their ability to protect host insects against secondary infections, especially viruses (13). Certain strains of *Wolbachia* are obligate beneficial nutritional symbionts, such as the bed bug-infecting *Wolbachia* that produce B-vitamins to support these hematophagous insects (14). In other insects, facultative associations with *Wolbachia* have been implicated in the balance of cholesterol, iron, or B-vitamins (15-18).

We previously identified that *Wolbachia* infection results in a suite of changes to the expression of *Drosophila melanogaster* nucleotide metabolism pathways (19). Additionally, comparative genomic data indicate that the biosynthetic pathways for the *de novo* synthesis of purines and pyrimidines have been conserved across *Wolbachia*, pointing towards their broad importance (20). Furthermore, *Wolbachia* encode for an abundance of amino acid importers, which would provide necessary precursors for *Wolbachia*'s *de novo* synthesis of nucleotides (3, 20). The conservation of these metabolic pathways might simply represent essential functions for *Wolbachia*'s own metabolism, or they might also be important in the context of interaction with the host. We hypothesized that *Wolbachia* plays a supporting role in host nutrition, and this might be mediated by nucleotides. We show that *Wolbachia* infection is beneficial for flies, especially

79 under nutrient limited conditions, and that *Wolbachia* is responsive to changes in the expression
80 of fly metabolic pathways.
81
82 **METHODS**
83 Fly husbandry
84 *Drosophila melanogaster* stocks with or without their native *Wolbachia* strain "*w*Mel" were
85 maintained on standard Bloomington cornmeal-agar medium at 25 °C on a 24-hour, 12:12
86 light:dark cycle under density-controlled conditions and 50% relative humidity. *Wolbachia*
87 colonization status was confirmed with PCR assays using *Wolbachia*-specific 16S primers
88 WspecF and WspecR (21). Genotypes used in nutritional assays (below) included: DGRP-320
89 (RRID:BDSC_29654), a *Wolbachia*-infected isogenic wild-type strain with genome sequence
90 available which we refer to as "wild type" below (22), and a *Wolbachia*-infected white line, w^{145}
91 (RRID:BDSC_145). *Wolbachia*-cleared counterpart stocks were generated with antibiotics via
92 three generations of tetracycline treatment (20 µg/mL in the fly food for three generations),
93 followed by re-inoculation of the gut microbiome by transfer to bottles that previously harbored
94 male flies from the original stock that had fed and defecated on the media for one week (23).
95 Stocks were allowed to recover from any potential transgenerational effects of the antibiotic
96 treatment for at least an additional ten generations prior to use in any experiment and re-screened
97 for *Wolbachia* infection immediately prior to all experiments. All other fly stocks (e.g., RNAi lines),
98 are detailed in Supplementary Table S1.
99
100 Nutritional stress experiments
101 To generate poor media, Nutri-fly® Bloomington Formulation (Genesee Scientific 66-121) was
102 prepared according to manufacturer's instructions and diluted to indicated percentages while
103 maintaining the full concentrations of agar (5.3 g/L) and propionic acid (4.8 ml/L). Five ml of media
104 was aliquoted into each vial. Flies were placed in mating cages on grape agar plates streaked

105 with yeast paste and acclimated for 24 hours, prior to initiating 4-hour egg lays. Upon hatching, <
106 4-hour old L1 larvae were transferred to media vials (n=20 per vial), and development was scored
107 every 24 hours. Pupation was defined by eversion of the anterior spiracles (24). Each vial was
108 tracked until all pupae eclosed, or until three consecutive days without additional adult
109 emergence.
110
111 Gnotobiotic assays
112 To test if the gut microbiome was playing a role in *Wolbachia*-mediated developmental
113 phenotypes, we reared wild type flies with and without *Wolbachia* on 100% and 25% strength
114 media under gnotobiotic conditions (i.e., without the gut or food microbiome). 10 mL of media
115 (prepared according to protocols in "nutritional stress experiments") was aliquoted into
116 polypropylene wide vials, capped with a Cellulose Acetate Flug® (Flystuff 49-101), and
117 autoclaved on liquid setting for 20 minutes to ensure sterility. Embryos were collected on grape
118 agar with yeast paste as described previously and transferred into 70-micron mesh cell strainers
119 (Falcon 352350). Embryos in the cell strainers were washed with *Drosophila* embryo wash
120 solution (7% NaCl, 0.5% Triton X-100) to remove food debris. After an initial wash, embryos were
121 immersed in a 10% bleach solution for 3 minutes, with gentle mixing every 30 seconds. The
122 embryos were then washed with sterile *Drosophila* embryo wash solution to remove excess
123 bleach. Embryos were subjected to a final rinse with sterile phosphate buffered saline (PBS) and
124 transferred to a sterile agar culture plate (2% agar in deionized water) stained with blue gel food
125 coloring to facilitate counting. A flame-sterilized probe was used to transfer 20 embryos into each
126 vial of sterile media.
127
128 Nutritional supplementation experiments
129 To test the impact of specific nutrients, we prepared 12.5% strength media as above, and
130 selectively added in protein or nucleotides: the precursors and end points of *de novo* nucleotide

131 biosynthesis. Treatments included casein (for bulk addition of amino acids at 64.1 g/L; Sigma
132 C7078), inosine (0.74 g/L; Sigma I4125), uridine (0.67 g/L; Sigma U3003), or both inosine and
133 uridine at the aforementioned concentrations. The concentrations of additives were calculated as
134 87.5% of a previously defined holidic diet (25), to adjust for the 12.5% BDSC media strength.
135 Developmental assays were carried out as described above.
136
137 Pupal size measurements
138 After selected developmental assays, pupal casings and dead pupae were removed from vials
139 using a wet paint brush and transferred to glass slides. Pupae were imaged with brightfield
140 microscopy on an ECHO Revolve at 4X. Images were manually annotated in Echo Labs Pro
141 software to measure sizes. Pupal length was defined as the distance between the base of the
142 posterior spiracles to the midway point between the anterior spiracles. Width measurements were
143 taken at the widest part of the pupa. The volume of each pupa was calculated assuming a prolate
144 spheroid shape [$V = (4/3)\ \pi\ (\text{width}/2)^2\ (\text{length}/2)$] (Supplemental Figure S1) (26, 27).
145
146 Knockdown of fly nucleotide metabolism genes
147 *Wolbachia*-infected Transgenic RNAi Project ("TRiP") stocks were obtained from the Bloomington
148 *Drosophila* Stock Center to knock down expression of genes in the purine and pyrimidine
149 nucleotide biosynthesis pathways (Supplemental File S1). Unmated females carrying a UAS-
150 gene-specific short hairpin were crossed to males with an Act5C-Gal4 driver (RRID:BDSC_3954:
151 $y^1 w^*$; P{w^{+mC}=Act5C-GAL4}17bFO1/TM6B, Tb^1). Unmated, F1 female progeny were sorted as
152 adults into those with the short hairpin and Gal4-driver or those with the short hairpin and the
153 TM6B balancer. At three days old, the sorted flies were flash frozen in liquid nitrogen and then
154 stored at -80 °C for later processing. To determine the effect of the TM6B balancer on *Wolbachia*
155 titer, we crossed *Wolbachia*-infected, Act5C-Gal4 driver females to *Wolbachia*-uninfected UAS-
156 anti-Ppyr\LUC males (RRID:BDSC_31603: y^1 v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF01355}attP2). Unmated,

157 F1 female progeny were sorted and stored as described for the nucleotide biosynthesis gene
158 knockdowns. To determine spatial differences in *Wolbachia* titer, we dissected ovaries from two-
159 day old *r-l* knockdown and sibling control flies. We separated ovaries from living, anesthetized
160 flies in sterile PBS. Ovaries and carcasses from three flies were pooled for each biological
161 replicate and flash frozen, followed by storage at -80 °C for later processing.
162
163 Real-time quantitative RT-PCR analyses of target gene expression
164 Flies were homogenized in TRIzol reagent (Invitrogen), and total RNA was extracted following
165 manufacturer's instructions. RNA extractions were DNase treated (RQ1 RNase-free DNase, New
166 England Biolabs) according to the manufacturer's instructions. cDNA was synthesized using
167 MMuLV Reverse Transcriptase (New England Biolabs) with random hexamer primers (Integrated
168 DNA Technologies). Quantitative RT-PCR reactions were performed with SensiFAST SYBR Hi-
169 ROX kit (Bioline) and gene-specific primers (Supplemental File S2). All samples were run in
170 technical duplicate alongside negative controls on an Applied Bioscience StepOnePlus qPCR
171 machine (Life Technologies). Gene expression was normalized to endogenous 18S rRNA
172 expression using the Livak method (28).
173
174 Real-time quantitative PCR analyses of *Wolbachia* titer
175 DNA was extracted from individual flies using the Quick-DNA/RNA Pathogen Miniprep kit (Zymo
176 Research) according to the manufacturer's protocol. *ftsZ* primers were used to quantify *Wolbachia*
177 genome copy numbers, which were normalized to host genome copies via 18S quantification
178 using the Livak method (28). Reactions were performed with SensiFAST SYBR Hi-ROX kit
179 (Bioline), and all samples and negative controls were run in technical duplicate on an Applied
180 Bioscience StepOnePlus qPCR machine (Life Technologies).
181
182 Nucleotide metabolism biosynthesis and gene expression

183 Nucleotide biosynthesis pathway information was downloaded from BioCyc by querying the
184 "*Wolbachia* endosymbiont of *Drosophila melanogaster w*Mel, version 26.5" and "*Drosophila*
185 *melanogaster*, version 26.5" databases (29). Enzymes for each step were cross-referenced with
186 KEGG Pathways, release 105.0 (30). To assess expression of *Wolbachia* metabolic pathways,
187 we analyzed the *Wolbachia* transcriptomes previously extracted from the MODENCODE dataset
188 (31, 32). We leveraged the previously published FPKM values for each *Wolbachia* gene. For
189 simplicity of visualization, mean FPKM is displayed for developmental time points represented by
190 multiple libraries. Fly gene expression for *Wolbachia*-infected and *Wolbachia* uninfected adult
191 females was previously reported (19).
192
193 Statistics and Data Visualization
194 Statistics and data visualization were carried out in R version 3.5.0 (33). Significant differences in
195 development were assessed with generalized linear mixed-effects models (package: 'lme4',
196 function 'glmer' (34)) including the proportion of flies that reached a given stage (pupa or adult)
197 as a binomial response, *Wolbachia* presence, day of development, media, and the interaction of
198 the three as fixed effects, and vial as a random effect to account for repeated measures.
199 Significant differences in pupal volume of flies reared on 12.5% media was assessed with a two-
200 way ANOVA (function 'aov') including *Wolbachia*, mortality status, and their interaction as fixed
201 effects. Significant differences in sizes of pupae reared on supplemented media was first
202 assessed with a two-way ANOVA (function 'aov') including *Wolbachia*, media, and their
203 interaction as fixed effects. Paired comparisons between *Wolbachia*-infected and uninfected flies
204 were then assessed with Wilcoxon rank sum tests (function 'wilcox.test'). Gene expression and
205 *Wolbachia* titers from knockdown experiments were assessed with two-way ANOVAs (function
206 'aov') including delta delta Ct as the response, and target locus, genotype (knockdown versus
207 sibling), and their interaction as fixed effects. *Wolbachia* titers in dissected flies were also

208 assessed with ANOVA, here with genotype, tissue, and their interaction as fixed effects. Pairwise
209 comparisons were performed with t-tests.
210
211 **RESULTS**
212 *Wolbachia* is beneficial under nutrient limited conditions
213 To assess the impact of *Wolbachia* infection on fly development, we reared flies on 100% strength
214 and 25% strength media and quantified pupation and adult emergence. We found a significant
215 interaction between *Wolbachia*, media strength, and time that impacted fly pupation ($F_{1,1063}$ =
216 24.7147, $p < 0.0001$) and adult emergence ($F_{1,1063}$ = 22.2897, $p < 0.0001$). Additionally, we found
217 a significant interaction between the presence of *Wolbachia* and the media strength (wild type
218 pupae: $F_{1,1063}$ = 0.6517, p = 0.0063; wild type adults: $F_{1,1063}$ = 0.1746, p = 0.0062; w^{145} pupae: $F_{1,711}$
219 = 8.8129, p = 0.0009; w^{145} adults: $F_{1,711}$ = 19.0998, $p < 0.0001$), along with a significant impact of
220 *Wolbachia* infection alone on fly pupation and adult emergence (wild type pupae: $F_{1,1063}$ = 0.2874,
221 $p < 0.0001$; wild type adults: $F_{1,1063}$ = 0.5375, $p < 0.0001$; w^{145} pupae: $F_{1,711}$ = 10.5483, $p < 0.0001$;
222 w^{145} adults: $F_{1,711}$ = 7.0718, $p < 0.0001$). These effects are driven by the nutrient-limited conditions
223 where *Wolbachia*-infected flies developed faster (1-5 days depending on genotype, Figures 1B
224 and 1E) and a larger percentage of flies reached adulthood. For example, while the wild type flies
225 with and without *Wolbachia* reared on 100% strength media reached adulthood in 82% and 81%
226 of cases, on the 25% strength media these were reduced to 75% and 66% respectively. The
227 *Wolbachia*-mediated advantage was more prominent for a second fly genetic background, w^{145}.
228 Even on 100% strength media, the *Wolbachia*-free w^{145} flies experienced a 5% reduction in adult
229 emergence relative to *Wolbachia*-infected flies (Figure 1D). When reared on 25% strength media,
230 the *Wolbachia*-infected w^{145} flies were more than twice as likely to reach adulthood (70% versus
231 31%, Figure 1E). Across these assays, we found delays in entering pupation are approximately
232 equal to the delay in adult emergence: *i.e.,* the time spent in metamorphosis did not change as a
233 factor of *Wolbachia* infection.

234

235 Given that the effect size for the *Wolbachia* advantage was genotype dependent and given the
236 more subtle impact of *Wolbachia* in the wild-type flies, we wondered if an even weaker media
237 would result in a stronger advantage for the *Wolbachia*-infected flies. Conversely, *Wolbachia*
238 could be a burden under more significant nutritional stress. Indeed, when flies were raised on
239 12.5% strength media, we again saw significant effects of the interaction between *Wolbachia* and
240 time on the rate and success of pupation and adult emergence (pupae: $F_{1,347} = 77.135$, $p < 0.0001$;
241 adults: $F_{1,347} = 58.9296$, $p < 0.0001$). However, *Wolbachia*-infected flies now developed slower
242 than their uninfected counterparts (6-7 days delayed), but they pupated at higher rates (68%
243 versus 61%) and were more likely to reach adulthood (54% versus 40%, Figure 1C). Furthermore,
244 although *Wolbachia*-infected fly development was slower, it resulted in them attaining, on
245 average, a 23% larger size at pupation ($F_{1,191} = 32.076$, $p < 0.0001$, Figure 1F). Additionally,
246 *Wolbachia*-infected flies experienced lower levels of pupal mortality (35% versus 19%), perhaps
247 related to the finding that flies that died during pupation were significantly smaller ($F_{1,191} = 4.625$,
248 $p = 0.0328$, Figure 1F).

249

250 To determine if the developmental advantage was due to direct effects of *Wolbachia* or indirect
251 effects via *Wolbachia*-mediated impacts on the gut microbiome, we performed the same
252 developmental assay under gnotobiotic conditions (i.e., without a gut or food microbiome). Under
253 gnotobiotic conditions, we saw a strong interactive effect of *Wolbachia* and time that resulted in
254 significantly more, and faster, pupation and adult emergence for infected flies (Figure 1G; pupae:
255 $F_{1,1920} = 153.3796$, $p < 0.0001$; adults: $F_{1,1920} = 129.6080$, $p < 0.0001$). Even on the 100% strength
256 media, only 43% of *Wolbachia*-free flies pupated, compared to 61% of *Wolbachia*-infected flies.
257 Again, *Wolbachia*-free flies had higher levels of pupal mortality: 21% of those that pupated did
258 not emerge as adults, as compared to 13% of the *Wolbachia*-infected pupae. While removing the
259 microbiota negatively impacted fly developmental timing as expected, this effect was exacerbated

260 in the *Wolbachia*-free flies (Figures 1A and 1G). As compared to the conventionally-reared flies
261 on 100% strength media (Figure 1A), gnotobiotic *Wolbachia*-infected flies experienced a two-day
262 developmental delay, and *Wolbachia*-free flies were delayed three days (Figure 1G). The
263 combination of gnotobiotic conditions and 25% strength media resulted in especially impaired fly
264 development: less than 20% of flies reached adulthood (Figure 1H). While there were no
265 significant differences in the proportion of flies that pupated or emerged as adults between
266 *Wolbachia*-infected and uninfected flies in these high-mortality conditions (pupae: $p = 0.9547$;
267 adults: $p = 0.6210$), it is notable that *Wolbachia*-infected flies on average started emerging five
268 days ahead of the *Wolbachia*-free flies (Figure 1H). Finally, we measured the puparia of the
269 gnotobiotic flies reared on 100% strength media (Figure 1I) and found that *Wolbachia* infection
270 resulted in, on average, 10% larger pupal volumes ($F_{1,200} = 11.69$, $p = 0.0008$, Figure 1I), again
271 indicating a significant developmental advantage.

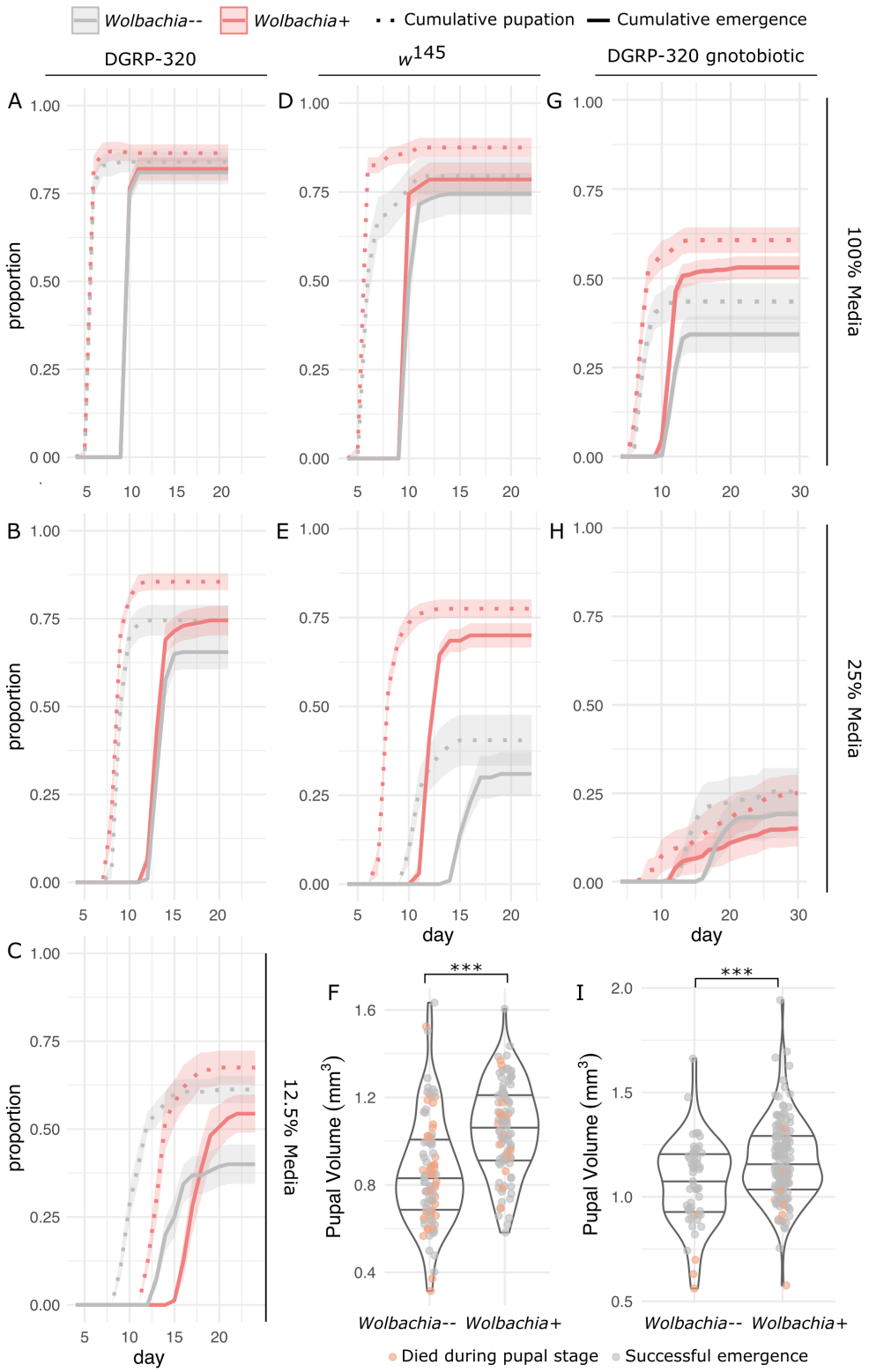


Figure 1. *Wolbachia* infection is beneficial under nutrient limited conditions. Flies (wild type and w^{145}, with and without *Wolbachia*) were reared on different concentrations of media to test

275 the impact of *Wolbachia* infection. Biological replicates included 20 larvae per each of 10 vials.
276 Dotted and solid lines indicate cumulative pupation and adult emergence, respectively, with
277 shaded regions defining standard error. All *Wolbachia*-infected treatments are in red, and
278 *Wolbachia*-uninfected in grey. **(A)** Conventionally reared wild type flies on 100%, **(B)** 25%, and
279 **(C)** 12.5% strength media. **(D)** Conventionally reared w^{145} flies on 100% and **(E)** 25% strength
280 media. **(F)** Pupal volumes of wild type flies with and without *Wolbachia*, derived from the 12.5%
281 media-reared flies in (C). **(G)** Gnotobiotic wild type flies on 100% and **(H)** 25% strength media. **(I)**
282 Pupal volumes of the gnotobiotic-reared flies on 100% strength media derived from (G). Orange
283 datapoints (H-I) indicate pupae that did not eclose into adults. *** $p < 0.001$
284
285 <u>Dynamics of the redundant *de novo* nucleotide biosynthesis pathways</u>
286 We next considered the role that *Wolbachia* may be playing in supporting fly development during
287 these nutritionally limited conditions. We previously found that nucleotide metabolism is a
288 significant point of interaction for flies and *Wolbachia* (19), pathways known to significantly impact
289 fly growth and development (35). Importantly, the pathways for the *de novo* synthesis of purines
290 and pyrimidines are conserved across all *Wolbachia*, and they are redundantly encoded by the
291 fly (Figure 2A-B). Additionally, we previously showed that *Wolbachia* infection results in
292 downregulation of the fly's pyrimidine synthesis enzymes *rudimentary* (-0.273 Fold Change (FC)
293 compared to uninfected flies), *Dhod* (-0.164 FC), and *rudimentary-like* (-0.301 FC) (19). Strikingly,
294 the repressor *su(r)* is upregulated (+0.505 FC)(Figure 2C). In contrast, purine metabolism overall
295 was not obviously distinct between *Wolbachia*-infected and uninfected flies: some enzymes were
296 up- or down- regulated but not the entire pathway (Figure 2C).
297
298 Given the developmental effects of *Wolbachia* infection, we hypothesized that *Wolbachia*
299 nucleotide metabolic pathways would be more highly expressed during growth stages (*i.e.,* pre-
300 metamorphosis). Indeed, from embryogenesis through the third larval instar (L3), most of

Wolbachia's nucleotide metabolism genes were upregulated. At the onset of pupation (the white pre-pupae "WPP" stage), *Wolbachia*'s metabolic gene expression was sharply and broadly downregulated (Figure 2D). These data indicate that *Wolbachia* may be supplementing host nutrition during critical growth stages, and either limiting negative impacts of infection during metamorphosis and reproductive stages or shifting roles to better interface with host reproduction and ensure transmission.

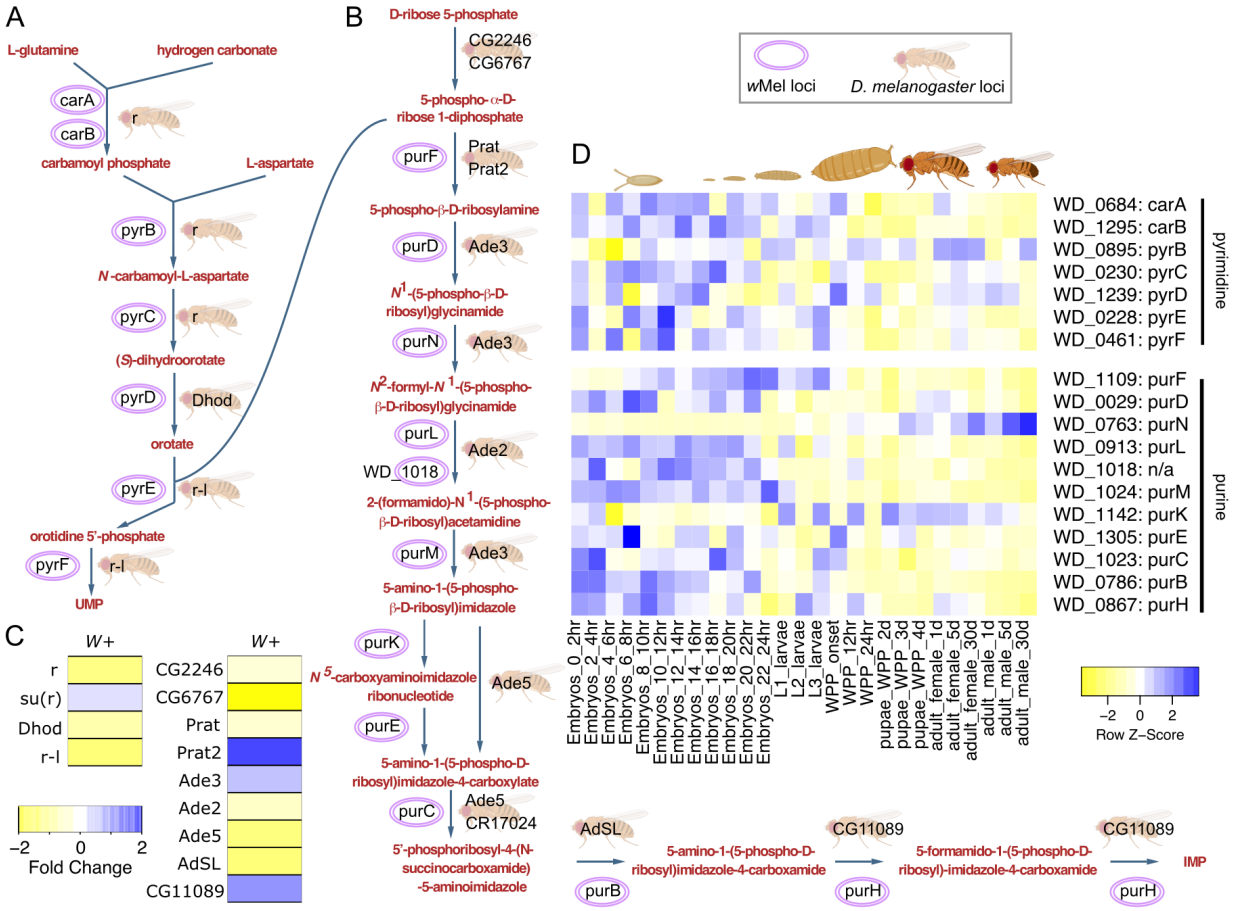


Figure 2. Flies and *Wolbachia* redundantly encode for *de novo* nucleotide biosynthesis. **(A)** Metabolic pathway for *de novo* pyrimidine biosynthesis in *Wolbachia* (left side, purple bacterial schematic) and *Drosophila melanogaster* (right side, fly schematic). **(B)** Metabolic pathway for *de novo* purine biosynthesis in *Wolbachia* (left side or bottom, purple bacterial schematic) and *Drosophila melanogaster* (right side or top, fly schematic). **(C)** Expression of *Drosophila melanogaster* nucleotide biosynthesis loci in *Wolbachia*-infected female flies relative to

314 *Wolbachia*-uninfected controls, derived from published expression data (19). **(D)** Expression of
315 *Wolbachia* nucleotide biosynthesis loci across fly development. *Wolbachia* transcriptomes were
316 generated as part of the MODENCODE project (31, 32). Data are row normalized for inference of
317 per-gene transcriptional changes across development. Fly images are from BioRender.com.
318 Abbreviations: UMP: uridine monophosphate; IMP: inosine monophosphate. Fly gene alternate
319 names: Ade2: Pfas; Ade3: Gart; Ade5: Paics.
320
321 Pyrimidines contribute to the *Wolbachia* infection advantage
322 We reasoned that deficiencies in the diet supplemented by *Wolbachia* could be supplemented by
323 exogenous nutrient addition to the fly food. Therefore, flies with and without *Wolbachia* would
324 have the same growth parameters if this important nutrient was added. Specifically, we focused
325 on the precursors (amino acids) and end-products (uridine and inosine) of the *de novo* nucleotide
326 synthesis pathways, which we supplemented back into the 12.5% media. The developmental
327 assays on supplemented media were repeated with the wild type stocks, avoiding w^{145} because
328 the *white* transporter is known to affect nucleotide metabolism (36). We again saw the *Wolbachia*-
329 mediated developmental advantage in the 12.5% strength media (pupae: $F_{1,235} = 13.9505$, $p =$
330 0.0002; adults: $F_{1,235} = 12.7568$, $p = 0.0004$). Additionally, we saw a subtle but significant positive
331 impact of *Wolbachia* in the 100% strength media as measure by adult emergence (pupae: $F_{1,235}$
332 $= 1.3002$, $p = 0.2538$; adults: $F_{1,235} = 28.0455$, $p < 0.0001$), due to 6% pupal mortality in the
333 *Wolbachia*-free flies that the *Wolbachia*-infected flies did not experience (Figure 3A). While the
334 addition of casein benefited both *Wolbachia*-infected and uninfected flies, *Wolbachia*-free flies
335 pupated at significantly increased rates relative to *Wolbachia*-infected flies ($F_{1,235} = 9.8699$, $p =$
336 0.0017). Again, pupal mortality was quite high in the *Wolbachia*-free flies (36% as compared to
337 9% with *Wolbachia*), resulting in significantly fewer numbers of adults ($F_{1,235} = 6.9988$, $p = 0.0082$).
338 The addition of uridine (product of *de novo* pyrimidine synthesis) specifically benefited *Wolbachia*-
339 free flies, but not the flies that had *Wolbachia* infections. Indeed, uridine supplementation resulted

340 in a rescue phenotype where equivalent numbers of *Wolbachia*-infected and uninfected flies
341 emerged as adults ($p = 0.9281$). Importantly, emergence of *Wolbachia*-free flies supplemented
342 with uridine was not significantly different than the *Wolbachia*-infected flies on the standard 12.5%
343 strength media ($p = 0.9097$) (Figure 3A). In fact, *Wolbachia*-free flies supplemented with uridine
344 developed slightly faster than their infected counterparts (pupae: $F_{1,235} = 2.2401$, $p = 0.1348$;
345 adults: $F_{1,235} = 10.5842$, $p = 0.0011$). The addition of inosine (product of *de novo* purine synthesis)
346 was toxic and reduced pupation and emergence for all flies. As such there were no significant
347 differences between *Wolbachia*-infected and uninfected flies in these high mortality (*i.e.*, zero-
348 inflated) conditions (pupae: $F_{1,235} = 0.9217$, $p = 0.335$; adults: $F_{1,235} = 0.3519$, $p = 0.553$).
349 Simultaneously adding uridine and inosine resulted in more intermediate, albeit still quite lethal
350 developmental phenotypes with no significant impact of *Wolbachia* ($F_{1,235} = 1.3471$, $p = 0.2459$;
351 adults: $F_{1,235} = 1.0400$, $p = 0.307$).
352
353 Congruent with the previous developmental assays (Figure 1), *Wolbachia* infection had a
354 significant impact on pupal size, both due to interacting effects with the media ($F_{5,470} = 2.55$, $p =$
355 0.0272), and *Wolbachia* alone ($F_{1,470} = 37.31$, $p < 0.0001$) (Figure 3B). Furthermore, *Wolbachia*-
356 infected flies were significantly larger when reared on 100% ($p = 0.0401$), 12.5% ($p = 0.0316$),
357 and casein-enriched 12.5% media ($p < 0.0001$). However, the addition of uridine to the 12.5%
358 media resulted in similarly sized flies regardless of *Wolbachia* infection ($p = 0.3685$). In summary,
359 *Wolbachia*-free flies were rescued to the same size and level of adult emergence as *Wolbachia*-
360 infected counterparts in uridine (i.e., pyrimidine) supplemented media.

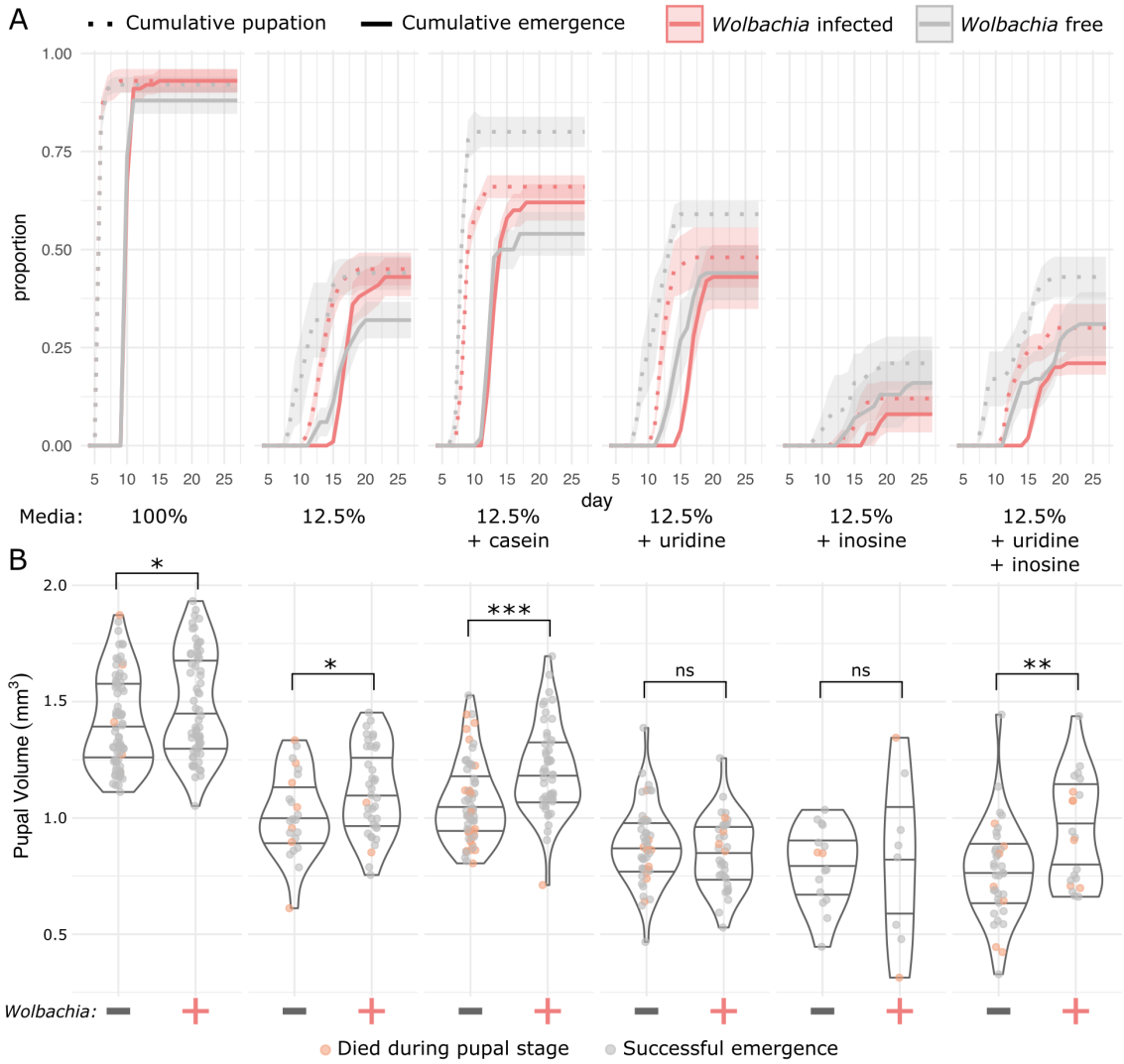
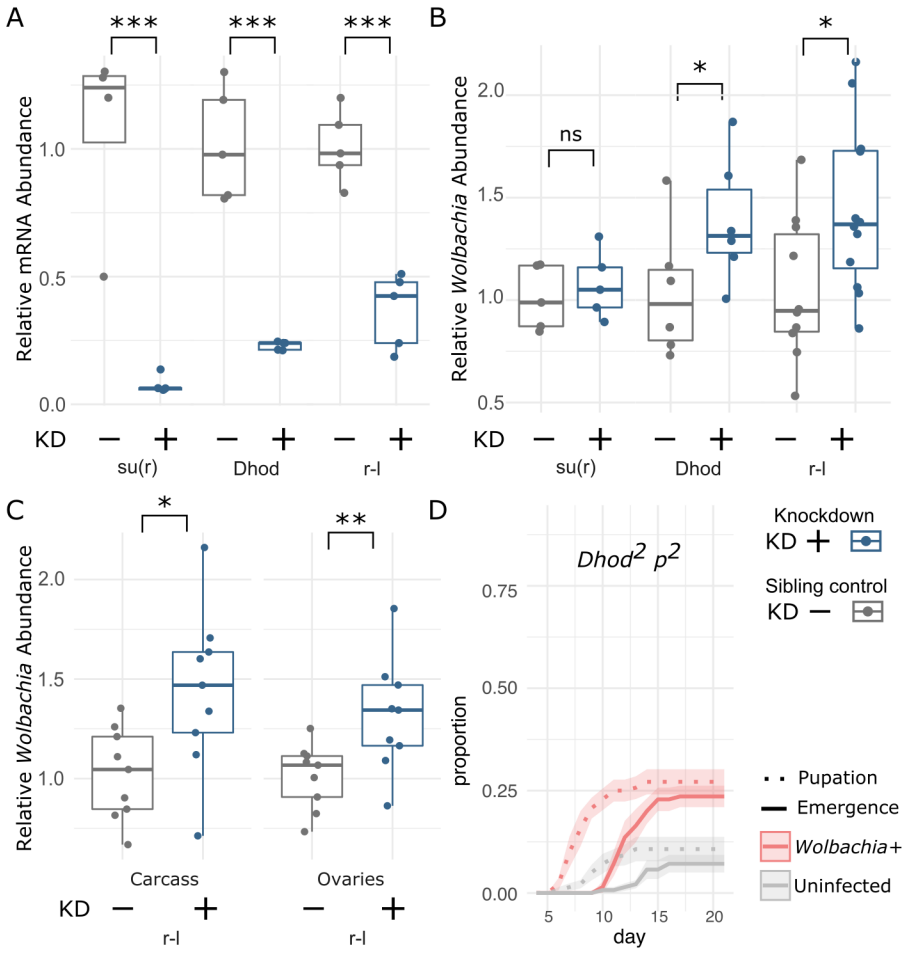


Figure 3. Uridine rescues *Wolbachia*-free flies. Wild type flies were reared on 100% media, 12.5% media, or 12.5% media containing either casein, uridine, inosine, or both inosine and uridine. The media designations in the middle correspond to the figures above and below. Biological replicates included 20 larvae per each of five vials. **(A)** Dotted and solid lines indicate cumulative pupation and adult emergence, respectively, with shaded regions defining standard error. *Wolbachia*-infected treatments are in red and *Wolbachia*-uninfected are in grey. **(B)** Pupae from (A) were removed post-developmental assay, measured, and their volume was calculated. Orange datapoints indicate pupae that did not eclose into adults. Significance annotations for

370 pupal sizes comparisons are the same regardless of whether flies that died during pupation were
371 included. ***p < 0.001; **p < 0.01; *p < 0.05; ns = not significant.
372
373 <u>*Wolbachia* infection is responsive to fly pyrimidine biosynthesis gene expression</u>
374 To determine whether *Wolbachia* infection was sensitive to changes in host metabolism, we
375 knocked down individual genes within the fly's *de novo* nucleotide synthesis pathways (Figure
376 2A-B) using *Wolbachia*-infected RNAi fly stocks (Figure 4A). Short-hairpin RNA (shRNA)
377 expression was driven in the entire body of the fly by an Act5C-Gal4 driver. We compared gene
378 expression and *Wolbachia* titer in knockdown flies to sibling controls (flies not expressing a gene-
379 targeting shRNA, and instead with the TM6B balancer). We successfully knocked down three
380 genes in the pyrimidine synthesis pathway (*su(r)*, *Dhod*, *rudimentary-like*) and four genes in the
381 purine synthesis pathway (*prat2*, *ade2*, *ade5*, *CG11089*) (Figure 4A, Supplemental Figure S2).
382 Several gene knockdowns were lethal to fly development, including *rudimentary* in the de novo
383 pyrimidine biosynthesis pathway and *CG2246*, *CG6767*, *Prat*, and *AdSL* in the de novo purine
384 biosynthesis pathway. Broad-spectrum knockdown of *de novo* purine synthesis had no significant
385 impacts on *Wolbachia* titer (gene*knockdown: $F_{3,31} = 0.652$, $p = 0.588$; gene: $F_{3,31} = 0.644$, $p =$
386 0.593; knockdown: $F_{1,31} = 0.593$, $p = 0.447$; Supplemental Figure S2). In contrast, knockdown of
387 the *de novo* pyrimidine synthesis pathway resulted in significant increases in *Wolbachia* titer ($F_{1,38}$
388 $= 10.3916$, $p = 0.0026$; Figure 4B). This pattern was specifically driven by the enzymatic loci
389 (*Dhod*: $p = 0.034$ and *rudimentary-like* (*r-l*)*:* $p = 0.013$). In contrast, knockdown of the negative
390 regulator *sur(r)* had no effect on *Wolbachia* titer ($p = 0.530$), perhaps indicating that *Wolbachia*
391 titers are responsive to low fly pyrimidine synthesis activity, but not elevated levels. To account
392 for any effects of the TM6B balancer on measurements of *Wolbachia* abundance, we crossed the
393 Act5C-Gal4 driver to a RNAi line expressing shRNA against a foreign gene: *Photinus pyralis*
394 luciferase (UAS-anti-Ppyr\LUC). We found no significant changes in *Wolbachia* titer, or in
395 nucleotide gene expression that could be driving the results in Figure 4B (Supplemental Figure

S3). We then asked if the change in *Wolbachia* titer was tissue-specific by measuring the relative *Wolbachia* titers in ovaries and the remaining carcasses after knockdown of *r-l* (Figure 4C). There was no significant interaction of knockdown and tissue ($F_{1,32} = 0.20$, $p = 0.658$) or of tissue alone ($F_{1,32} = 0.20$, $p = 0.658$) on *Wolbachia* titers. We again saw a significant effect of *r-l* knockdown on *Wolbachia* titers ($F_{1,32} = 13.07$, $p = 0.001$) both in ovaries ($p = 0.007$) and in carcasses ($p = 0.0127$) (Figure 4C). Finally, given these interactions with pyrimidine synthesis, we used flies with a homozygous hypomorphic allele *Dhod*^{2} (RRID:BDSC_1936, Supplemental Table S1) to assess the importance of *Wolbachia* in a pyrimidine synthesis-impaired genetic background. This stock is naturally infected with *Wolbachia*, but removal of the symbiont lead to significant delays and reductions in pupation and adult emergence (Figure 4D; pupae: $F_{1,247} = 11.0058$, $p = 0.0009$; adults: $F_{1,247} = 12.0550$, $p = 0.0057$).



408 **Figure 4. *Wolbachia* titers are responsive to fly pyrimidine synthesis gene expression.**
409 Expression of fly genes in the *de novo* pyrimidine pathway was constitutively knocked down using
410 standard transgenic approaches. **(A)** *Drosophila* gene knockdown was verified with qRT-PCR
411 leveraging gene specific primers and normalization to 18S expression. **(B)** Relative *Wolbachia*
412 titers in whole adult females and **(C)** dissected ovaries and carcasses were quantified with qPCR
413 using *Wolbachia*-specific primers and normalization to fly genome copy number via 18S
414 amplification. Legend: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns p = not significant. **(D)** Development
415 of conventionally reared *Wolbachia*-infected and -uninfected $Dhod^2$ p^2 flies on 100% strength
416 media. Biological replicates included 20 larvae per each of ten vials. Dotted and solid lines indicate
417 cumulative pupation and adult emergence, respectively, with shaded regions defining standard
418 error. *Wolbachia*-infected flies are in red and *Wolbachia*-uninfected are in grey.
419

420 **DISCUSSION**

421 *Wolbachia* has long been appreciated for its diverse impacts on a range of ecdysozoan hosts.
422 There has been increasing speculation as to the role *Wolbachia* plays in host metabolism more
423 broadly, often times in the context of specific metabolites (12, 19, 20). For example, *Wolbachia*-
424 infection contributes to B-vitamin supplementation in a planthopper (18), bed bugs (14), and likely
425 some species of solitary bees (37). Iron has been the subject of a number of investigations into
426 *Wolbachia*-host interactions (15). In *Drosophila melanogaster, Wolbachia* infection buffers
427 against stresses associated with low or high concentrations of iron in the diet (38). *Wolbachia*
428 infection is additionally associated with changes in host ferritin expression and iron homeostasis
429 in other insects such as *Drosophila simulans* and the parasitoid wasp *Asobara tabida* (16). Given
430 the range of metabolites and physiological processes that have been implicated in *Wolbachia*-
431 host interactions, we focused initial experiments on using a more holistic approach to
432 understanding fly development in the context of diet and *Wolbachia* infection.
433

434 Here, we show that *Wolbachia* significantly impacts *Drosophila melanogaster* development,
435 resulting in larger flies more likely to reach adulthood. The strength of this phenotype varied by
436 fly genotype and the overall concentration of nutrition in the food: *Wolbachia* infection was highly
437 beneficial under nutrient limited conditions. Removal of the extracellular microbial community
438 indicated that this advantage was directly mediated by *Wolbachia*. In fact, *Wolbachia* significantly
439 buffered flies against the stresses normally associated with removal of the microbiome (39, 40).
440 Furthermore, *Wolbachia* titers were responsive to changes in the expression of fly pyrimidine
441 biosynthesis, and the supplementation of pyrimidines rescued adult emergence of the *Wolbachia*-
442 uninfected flies. We propose that pyrimidines are a significant component of this developmental
443 advantage provided by *Wolbachia*. Indeed, the patterns of *Wolbachia* gene expression seem to
444 be attuned to fly development which could be a strategy for supporting the exponential growth
445 that occurs during larval stages and mitigating fitness impacts of the infection during reproductive
446 stages. Importantly, while the transcription of *Wolbachia*'s nucleotide metabolism pathways are
447 quite high pre-pupation, the flies have comparatively low *Wolbachia* titers during that time (31).
448 Genomic data suggest that *Wolbachia* imports amino acids from the host: while these may be
449 used as precursors for nucleotide biosynthesis, the depletion of free amino acids could induce
450 host stress responses (13, 41). Changes in *Wolbachia* titer and physiology across development
451 and under different nutritional scenarios likely reflect such balancing acts.
452
453 While *Wolbachia* infections have received considerable attention for their impacts on
454 reproduction, we know much less about how *Wolbachia* interact with their host during juvenile
455 stages. Importantly, many insects spend the majority of their lifetime as juveniles. Juvenile insect
456 metabolism is typically quite distinct from adults, and this time period has profound impacts on
457 metamorphosis, adult physiology, and fitness (42). Critically, all insect growth occurs during the
458 larval period: in *Drosophila melanogaster,* larvae increase in body mass by ~200-fold over the
459 course of 4 days (42). We found that *Wolbachia*-free flies had higher levels of pupal mortality,

460 especially during nutritional stress, suggesting insufficient metabolic reserves. This effect was
461 particularly pronounced when flies were reared on an excess of protein (Figure 3). Importantly,
462 amino acids are a key nutritional cue for determining the timing of pupation (i.e., signaling critical
463 weight) due to a direct and indirect effects on the timing and intensity of ecdysone pulses (43-46).
464 An influx of amino acids without other essential nutrients needed for growth might impact
465 nutritional signaling and thus the timing of critical weight, perhaps triggering flies to pupate early
466 without sufficient reserves for metamorphosis. Given *Wolbachia* likely imports amino acids, this
467 may reduce the concentrations of free amino acids and delay signaling critical weight, allowing
468 flies to accumulate more metabolic reserves before pupation.
469
470 During the ~200-fold increase in larval body mass, there is a concomitant 25-fold increase in DNA
471 and RNA content that creates a huge demand for pyrimidines, well exceeding the fly's
472 endogenous biosynthetic capacity (47). Considering that the rate of *Drosophila* development is
473 highly sensitive to *de novo* pyrimidine synthesis (47), bacterially-derived nucleotides could give
474 the host a significant advantage. Pyrimidines are synthesized by a series of metabolic reactions
475 that use ribose sugar and the amino acids glutamine and aspartate to generate uridine-5'-
476 monophosphate, UMP (48): the precursor for uracil, cytosine, and thymine. This pathway is
477 conserved in both *Drosophila* and *Wolbachia*, suggesting that infected flies generate pyrimidines
478 in multiple compartments: the host cytoplasm/mitochondria and *Wolbachia*. *Wolbachia*'s
479 production of pyrimidines could also represent far more than simply additional biosynthesis, as
480 the enzyme dihydroorotate dehydrogenase (*Dhod*) is located in the inner mitochondrial
481 membrane where it couples pyrimidine synthesis to electron transport (49, 50). As such,
482 provisioning of *Wolbachia*-generated pyrimidines could reduce the biosynthetic burden normally
483 imposed upon mitochondria. Additionally, fly enzymes used for pyrimidine synthesis, CAD and
484 UMP synthase, are negatively regulated by UMP (51). Therefore, *Wolbachia* could simultaneously

impact free amino acid pools, supplement pyrimidines, reduce mitochondrial burdens, and spatially segregate components of a normally inhibitory interaction.

While we found that pyrimidines seemed to be important for the *Drosophila*-*Wolbachia* symbiosis, we did not see any changes in *Wolbachia* titer due to suppression of purine synthesis. In fact, the addition of inosine to media negatively impacted flies (Figure 3). Purine nucleotides act as signaling molecules that impact a range of functions. For example, activation of the adenosine receptor globally suppresses fly metabolism (52), which can cause insecticidal effects (53). Another possibility is misincorporation of inosine into RNAs. In mammalian cell lines, this can happen when inosine is in excess, ultimately impacting translation (54). While altering the expression of fly purine metabolic gene expression did not impact the relative abundance pf *Wolbachia* in our experiments, we cannot not rule out the potential for other changes in *Wolbachia* gene expression or physiology. Indeed, we previously found an interactive effect of *Wolbachia* infection and purine gene expression on virus replication (19), highlighting both the importance of purines to the symbiosis and the complexity of the metabolic landscape.

The role of *Wolbachia* as a nutritional symbiont raises numerous questions about the cell biology of *Wolbachia* and how the relationship with the host is regulated. Is *Wolbachia* specifically providing pyrimidines (*e.g.,* via nucleotide transporters)? Or are flies farming and consuming their *Wolbachia* infections (*e.g.,* via autophagy) and pyrimidines so happen to be abundant in *Wolbachia* cells and also a limiting metabolite for the fly? As is the case for many symbioses, it is not always clear if a change in the symbiont (*e.g.,* gene expression or titer), is due to the microbe detecting and responding to the host's status, or the host releasing control of the microbe and allowing a change in microbial physiology. *Wolbachia* do encode for a number of proteins that would allow them to detect their environment and regulate gene expression accordingly (55).

510 However, each *Wolbachia* is enclosed by a host-derived vesicle (56, 57), so perhaps the host
511 tightly regulates *Wolbachia*'s "experience".
512
513 While beneficial nutritional symbioses in insects are not rare, most of the systems for which this
514 is described are of a much more binary nature. Insects that exclusively feed on unbalanced diets
515 (e.g., blood, plant sap) have obligate microbial symbionts to synthesize metabolites that the host
516 cannot generate *de novo*: the partners complement each other (58, 59). Here, both flies and
517 *Wolbachia* have their own complete pathways for nucleotide biosynthesis, which perhaps has led
518 us to overlook the relevance of this biosynthetic redundancy. Our finding that *Wolbachia*
519 significantly impacts the developmental timing, size, and susceptibility to nutritional stress in a
520 well-studied model such as *Drosophila melanogaster* raises the question of the broader relevance
521 of *Wolbachia* infections to insect metabolism and development.
522
523 **DECLARATIONS**
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532
533 Conflicts of interest
534 The authors declare that they have no competing interests.
535

536 Data Availability

537 Supplemental Table S1. Fly stocks

538 Supplemental Table S2. Primers

539 Supplemental Figure S1. Pupal measurement.

540 Supplemental Figure S2. Purine knockdowns.

541 Supplemental Figure S3. Knockdown controls.

542

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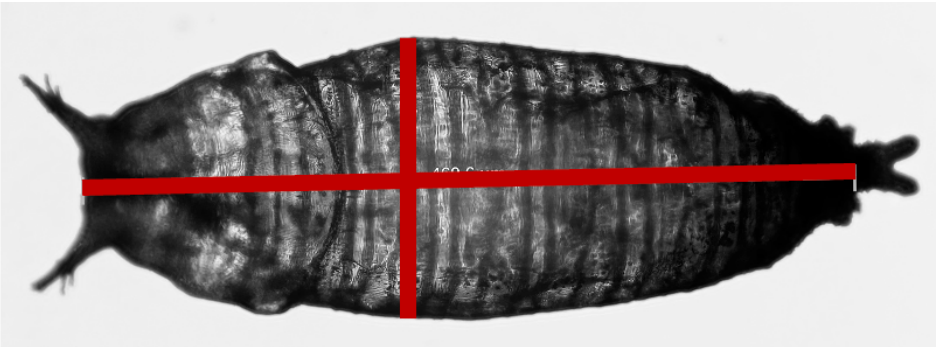
679
680
681 **Supplemental Tables and Figures**
682
683 **Table S1. *Drosophila melanogaster* stocks used in this study.**

BDSC RRID	Complete Genotype	*Wolbachia* infection
41694	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02259}attP2/TM3, Sb[1]	positive
60086	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC05080}attP40	positive

43296	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02669}attP40	positive
51492	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03244}attP2	positive
36686	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01574}attP2	positive
62241	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC05248}attP40	positive
34347	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01336}attP2	positive
53332	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03561}attP40	positive
60019	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC05011}attP40	positive
53339	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03568}attP40	positive
51801	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03359}attP40	positive
55183	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03871}attP40	positive
3954	y[1] w[*]; P{w[+mC]=Act5C-GAL4}17bFO1/TM6B, Tb[1]	positive
31603	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01355}attP2	negative
1936	Dhod[2] p[p]	positive

Table S2. qPCR primers used in this study.

Target	FlyBase ID	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
ftsZ	n/a	TTTTGTTGTCGCAAATACCG	CCATTCCTGCTGTGATGAAA
18S	FBgn0085802	CGAAAGTTAGAGGTTCGAAGGCGA	CCGTGTTGAGTCAAATTAAGCCGC
Prat2	FBgn0041194	AATAGTGACCAGTTTGGGCAAG	CCCAGGTTACCCTTTAACTTCC
ade2	FBgn0000052	CAGCCAGGTTTGTTCCTCTG	TAGCTGCTCGTCAAAGCTCG
ade5	FBgn0020513	CCCGAGGGCTACAGGTTCT	AACTTGGCGGAGACAATTTGC
CG11089	FBgn0039241	CTAGCTTGCGTGGCGCTG	CTGGATAAGATCGAAGCCCTGC
su(r)	FBgn0086450	CATCAAGCACACCACATTGTCG	GTGGGACAGGACTTTTGGCA
Dhod	FBgn0000447	TACGGCATACAAGAACCAGGA	TCATCATGGTACTGTGAAACCG
r-l	FBgn0003257	TGTGATGCAAACCGTATCCGA	GTCCTCGACAATCAGACAGGT

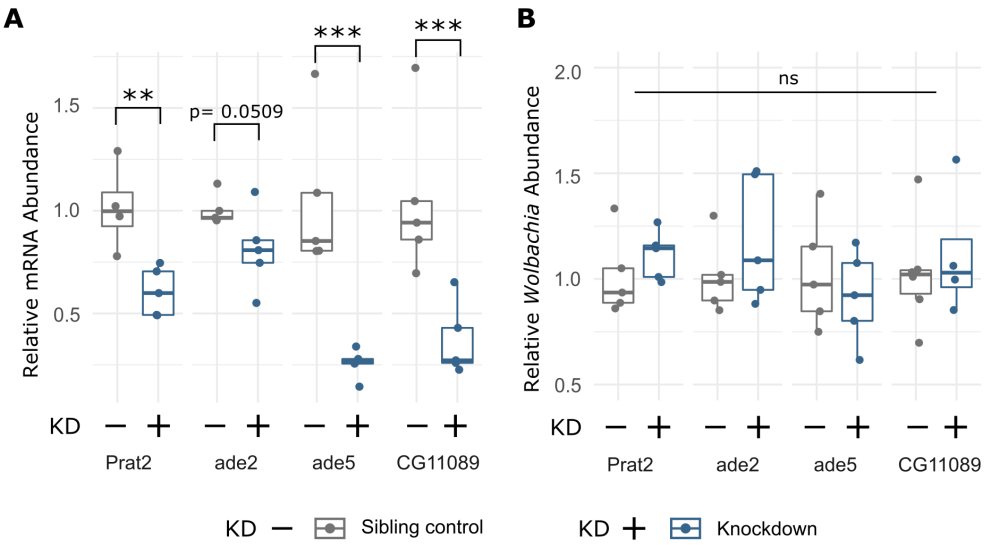


689 **Figure S1. Pupal measurements.** Pupal volume was calculated based on length and width (red

690 lines) and assuming a prolate spheroid shape [$V = (4/3)\ \pi\ (\text{width}/2)^2\ (\text{length}/2)$].

691

692



693

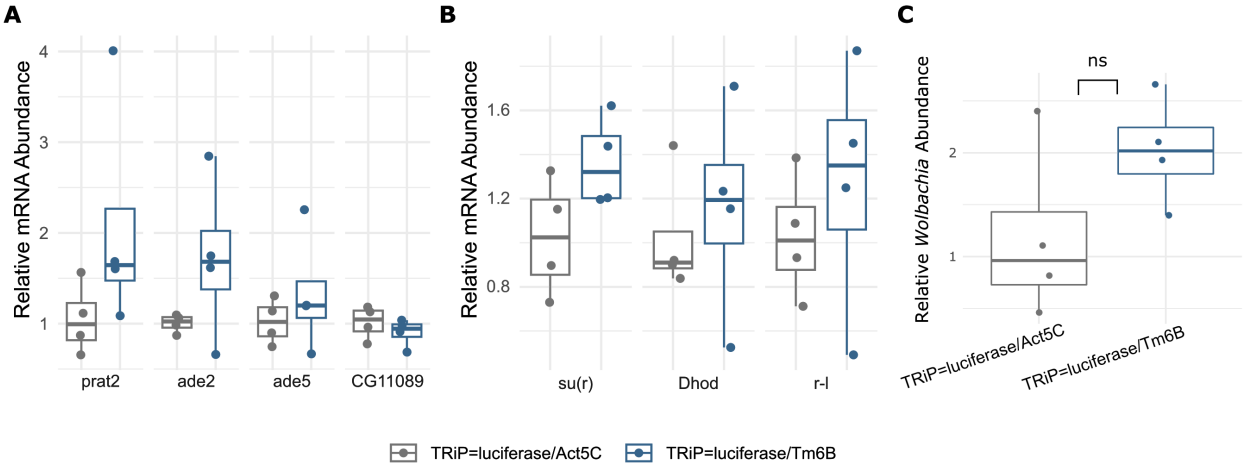
694 **Figure S2. *Wolbachia* titer is not responsive to changes in de novo purine biosynthesis**

695 **gene expression. (A)** qRT-PCR validation of knockdown across four purine loci. Legend: *** p

696 <0.001; ** p < 0.01; * p < 0.05; ns p = not significant. **(B)** Knockdown of purine synthesis had no

697 significant impacts on *Wolbachia* titer (gene*knockdown: $F_{3,31} = 0.652$, $p = 0.588$; gene: $F_{3,31} =$

698 0.644, $p = 0.593$; knockdown: $F_{1,31} = 0.593$, $p = 0.447$).



699

700 **Figure S3. Validation of knockdown controls.** Impact of balancer chromosomes on (A) purine
701 gene expression, (B) pyrimidine gene expression, and (C) *Wolbachia* titer. **(A)** The TM6B
702 balancer had no significant impacts on purine gene expression (gene*balancer: $F_{3,24} = 1.311$, $p =$
703 0.2939; gene: $F_{3,24} = 1.311$, $p = 0.2939$; knockdown: $F_{1,24} = 4.146$, $p = 0.0529$). Importantly, while
704 the mean expression of the purine loci may have been marginally higher in the presence of the
705 balancer chromosome, this means that we would underestimate the effects of knockdown due to
706 an even larger difference between the sibling and knockdown flies (see main text). **(B)** The TM6B
707 balancer had no significant impacts on pyrimidine gene expression (gene*balancer: $F_{2,18} = 0.227$,
708 $p = 0.799$; gene: $F_{2,18} = 0.227$, $p = 0.799$; knockdown: $F_{1,18} = 1.180$, $p = 0.292$). **(C)** There was no
709 significant difference in *Wolbachia* titer due to the balancer chromosome (t-test, $t = 1.8404$, $df =$
710 8, $p = 0.1153$). Again, the trend towards marginally higher *Wolbachia* titer in the presence of the
711 balancer only means that we would underestimate the effects of knockdown.