1   **_Wolbachia_ is a nutritional symbiont**

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ABSTRACT

Approximately a quarter of eukaryotes are infected with the bacterium *Wolbachia*. Its broad success as a vertically transmitted infection has been historically difficult to explain given the myriad of parasitic impacts characterized across *Wolbachia*'s host range. Using the *Drosophila* model and their natively associated *Wolbachia,* 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 insect 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 insect development and increase host fitness: a selective advantage that could contribute to its high frequency in nature.

SIGNIFICANCE STATEMENT

Wolbachia is a bacterial symbiont of ~1/4 of all eukaryotic species on earth, often deemed a "parasite" due to selfish manipulations of arthropod reproduction. However, many have theorized there must be more to this symbiosis: parasitic and reproductive impacts alone cannot explain the success and ubiquity of this bacterium. Here, we use *Drosophila* 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: a biosynthetic pathway that all *Wolbachia* lineages encode for. These developmental advantages might help explain the ubiquity of *Wolbachia* infections.

53 **INTRODUCTION**

54 Maternally transmitted microbes have evolved numerous ways to manipulate host physiology

55 ultimately facilitating their own success. Strategies include supplementation of the host's diet via

56 nutrient provisioning, protection against parasites and infections, and even direct manipulation of

57 the host's reproduction (1). A high percentage of arthropod species are infected with bacteria in

58 the genus *Wolbachia* (Alphaproteobacteria: Rickettsiales), a maternally transmitted infection long

59 considered to be a "reproductive parasite" (2-8). This classification was due to the seemingly

60 selfish ways in which *Wolbachia* alters host reproductive outcomes ultimately facilitating its own

61 spread through the population (1, 9, 10). For example, the conversion to asexual reproduction or

62 the induction of sperm-egg incompatibilities (so-called "cytoplasmic incompatibility") by *Wolbachia*

63 can result in a rapid sweep of *Wolbachia*-infected matrilines across a population (11, 12).

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65 However, the line between "parasite" and "mutualist" is becoming increasingly blurred as

66 *Wolbachia*-mediated benefits for the host are uncovered (13). For example, certain strains of

67 *Wolbachia* are clear nutritional mutualists, such as the bed bug-infecting *Wolbachia* that produce

68 B vitamins to support these obligately hematophagous insects (14). Many other *Wolbachia* strains

69 have been characterized for their ability to protect host insects against secondary infections,

70 especially viruses (15). Indeed, evolutionary theory predicts that vertical transmission will select

71 for mutualism, a process that we have been fortunate to watch happen in real time in some

72 *Wolbachia*-host associations (16). Additionally, there are data indicating that *Wolbachia* can

73 spread through a population even in the absence of so-called "parasitic" reproductive

74 manipulations (17).

75

76 We previously identified that *Wolbachia* infection results in a suite of changes to the expression

77 of fly nucleotide metabolism pathways (18). Additionally, comparative genomic data indicate that

78 the biosynthetic pathways for the *de novo* synthesis of purines and pyrimidines have been

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79 conserved across *Wolbachia*, pointing towards their broad importance (19). Furthermore,

80 *Wolbachia* encode for an abundance of amino acid importers, which would provide necessary

81 precursors for *Wolbachia*'s *de novo* synthesis of nucleotides (3, 19). We hypothesized that

82 *Wolbachia* plays a supporting role in host nutrition. We show that *Wolbachia* infection is beneficial

83 for flies, especially under nutrient limited conditions, and that *Wolbachia* is responsive to changes

84 in the expression of fly metabolic pathways. These data support the idea that *Wolbachia* may

85 broadly act as a nutritional symbiont, potentially underlying its evolutionary success as a common

86 infection in the most abundant group of eukaryotes on earth.

87

88 **METHODS**

89 <u>Fly husbandry</u>

90 Fly stocks were maintained on standard Bloomington cornmeal-agar medium at 25 °C on a 24-

91 hour, 12:12 light:dark cycle under density-controlled conditions and 50% relative humidity.

92 *Wolbachia* colonization status was confirmed with PCR assays using *Wolbachia*-specific 16S

93 primers WspecF and WspecR (20). Genotypes used in nutritional assays (below) included:

94 DGRP-320 (RRID:BDSC_29654), a *Wolbachia*-infected isogenic wild-type strain with genome

95 sequence available which we refer to as "wild type" below (21), and a *Wolbachia*-infected white

96 line, w^{145} (RRID:BDSC_145). *Wolbachia*-cleared counterpart stocks were generated with

97 antibiotics via three generations of tetracycline treatment (20 µg/mL in the fly food for three

98 generations), followed by re-inoculation of the gut microbiome by transfer to bottles that previously

99 harbored male flies from the original stock that had fed and defecated on the media for one week

100 (22). Stocks were allowed to recover from any potential transgenerational effects of the antibiotic

101 treatment for at least an additional ten generations prior to use in any experiment. All other fly

102 stocks (e.g., RNAi lines), are detailed in Supplementary Table S1.

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105　Nutritional stress experiments

106　To generate poor media, Nutri-fly® Bloomington Formulation (Genesee Scientific 66-121) was

107　prepared according to manufacturer's instructions and diluted to indicated percentages while

108　maintaining the full concentrations of agar (5.3 g/L) and propionic acid (4.8 ml/L). Five ml of media

109　was aliquoted into each vial. Flies were placed in mating cages on grape agar plates streaked

110　with yeast paste and acclimated for 24 hours, prior to initiating 4-hour egg lays. Upon hatching, <

111　4-hour old L1 larvae were transferred to media vials (n=20 per vial), and development was scored

112　every 24 hours. Pupation was defined by eversion of the anterior spiracles (23). Each vial was

113　tracked until all pupae eclosed, or until three consecutive days without additional adult

114　emergence.

115

116　Gnotobiotic assays

117　To test if the gut microbiome was playing a role in *Wolbachia*-mediated developmental

118　phenotypes, we reared wild type flies with and without *Wolbachia* on 100% and 25% strength

119　media under gnotobiotic conditions (i.e., without the gut or food microbiome). 10 mL of media

120　(prepared according to protocols in "nutritional stress experiments") was aliquoted into

121　polypropylene wide vials, capped with a Cellulose Acetate Flug® (Flystuff 49-101), and

122　autoclaved on liquid setting for 20 minutes to ensure sterility. Embryos were collected on grape

123　agar with yeast paste as described previously and transferred into 70-micron mesh cell strainers

124　(Falcon 352350). Embryos in the cell strainers were washed with *Drosophila* embryo wash

125　solution (7% NaCl, 0.5% Triton X-100) to remove food debris. After an initial wash, embryos were

126　immersed in a 10% bleach solution for 3 minutes, with gentle mixing every 30 seconds. The

127　embryos were then washed with sterile *Drosophila* embryo wash solution to remove excess

128　bleach. Embryos were subjected to a final rinse with sterile phosphate buffered saline (PBS) and

129　transferred to a sterile agar culture plate (2% agar in deionized water) stained with blue gel food

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130   coloring to facilitate counting. A flame-sterilized probe was used to transfer 20 embryos into each

131   vial of sterile media.

132

133   <u>Nutritional supplementation experiments</u>

134   To test the impact of specific nutrients, we prepared 12.5% strength media as above, and

135   selectively added in protein or nucleotides. Treatments included casein (64.1 g/L; Sigma C7078),

136   inosine (0.74 g/L; Sigma I4125), uridine (0.67 g/L; Sigma U3003), or both inosine and uridine at

137   the aforementioned concentrations. The concentrations of additives were calculated as 87.5% of

138   a previously defined holidic diet (24), to adjust for the 12.5% BDSC media strength.

139   Developmental assays were carried out as described above.

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141   <u>Pupal size measurements</u>

142   After selected developmental assays, pupal casings and dead pupae were removed from vials

143   using a wet paint brush and transferred to glass slides. Pupae were imaged with brightfield

144   microscopy on an ECHO Revolve at 4X. Images were manually annotated in Echo Labs Pro

145   software to measure sizes. Pupal length was defined as the distance between the base of the

146   posterior spiracles to the midway point between the anterior spiracles. Width measurements were

147   taken at the widest part of the pupa. The volume of each pupa was calculated assuming a prolate

148   spheroid shape [$V = (4/3) \pi (width/2)^2 (length/2)$] (Supplemental Figure S1) (25, 26).

149

150   <u>Knockdown of fly nucleotide metabolism genes</u>

151   *Wolbachia*-infected TRiP stocks were obtained from the Bloomington *Drosophila* Stock Center to

152   knock down expression of genes in the purine and pyrimidine nucleotide biosynthesis pathways

153   (Supplemental File S1). Unmated females carrying a UAS-gene-specific short hairpin were

154   crossed to males with an Act5C-Gal4 driver (RRID:BDSC_3954: $y^1 w^*$; P{w^{+mC}=Act5C-

155   GAL4}17bFO1/TM6B, Tb^1). Unmated, F1 female progeny were sorted as adults into those with

156   the short hairpin and Gal4-driver or those with the short hairpin and the TM6B balancer. At three

157   days old, the sorted flies were flash frozen in liquid nitrogen and then stored at -80 °C for later

158   processing. To determine the effect of the TM6B balancer on *Wolbachia* titer, we crossed

159   *Wolbachia*-infected, Act5C-Gal4 driver females to *Wolbachia*-uninfected UAS-anti-Ppyr\LUC

160   males (RRID:BDSC_31603: y^1 v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF01355}attP2). Unmated, F1 female

161   progeny were sorted and stored as described for the nucleotide biosynthesis gene knockdowns.

162   To determine spatial differences in *Wolbachia* titer, we dissected ovaries from two-day old *r-l*

163   knockdown and sibling control flies. We separated ovaries from living, anesthetized flies in sterile

164   PBS. Ovaries and carcasses from three flies were pooled for each biological replicate and flash

165   frozen, followed by storage at -80 °C for later processing.

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167   <u>Real-time quantitative RT-PCR analyses of target gene expression</u>

168   Flies were homogenized in TRIzol reagent (Invitrogen), and total RNA was extracted following

169   manufacturer's instructions. RNA extractions were DNase treated (RQ1 RNase-free DNase, New

170   England Biolabs) according to the manufacturer's instructions. cDNA was synthesized using

171   MMuLV Reverse Transcriptase (New England Biolabs) with random hexamer primers (Integrated

172   DNA Technologies). Quantitative RT-PCR reactions were performed with SensiFAST SYBR Hi-

173   ROX kit (Bioline) and gene-specific primers (Supplemental File S2). All samples were run in

174   technical duplicate alongside negative controls on an Applied Bioscience StepOnePlus qPCR

175   machine (Life Technologies). Gene expression was normalized to endogenous 18S rRNA

176   expression using the Livak method (27).

177

178   <u>Real-time quantitative PCR analyses of *Wolbachia* titer</u>

179   DNA was extracted from individual flies using the Quick-DNA/RNA Pathogen Miniprep kit (Zymo

180   Research) according to the manufacturer's protocol. *ftsZ* primers were used to quantify *Wolbachia*

181   genome copy numbers, which were normalized to host genome copies via 18S quantification

182   using the Livak method (27). Reactions were performed with SensiFAST SYBR Hi-ROX kit

183   (Bioline), and all samples and negative controls were run in technical duplicate on an Applied

184   Bioscience StepOnePlus qPCR machine (Life Technologies).

185

186   <u>Nucleotide metabolism biosynthesis and gene expression</u>

187   Nucleotide biosynthesis pathway information was downloaded from BioCyc by querying the

188   "*Wolbachia* endosymbiont of *Drosophila melanogaster w*Mel, version 26.5" and "*Drosophila*

189   *melanogaster*, version 26.5" databases (28). Enzymes for each step were cross-referenced with

190   KEGG Pathways, release 105.0 (29). To assess expression of *Wolbachia* metabolic pathways,

191   we analyzed the *Wolbachia* transcriptomes previously extracted from the MODENCODE dataset

192   (30, 31). We leveraged the previously published FPKM values for each *Wolbachia* gene. For

193   simplicity of visualization, mean FPKM is displayed for developmental time points represented by

194   multiple libraries. Fly gene expression for *Wolbachia*-infected and *Wolbachia* uninfected adult

195   females was previously reported (18).

196

197   <u>Statistics and Data Visualization</u>

198   Statistics and data visualization were carried out in R version 3.5.0 (32). Significant differences in

199   development were assessed with generalized linear mixed-effects models (package: 'lme4',

200   function 'glmer' (33)) including the proportion of flies that reached a given stage (pupa or adult)

201   as a binomial response, *Wolbachia* presence, day of development, media, and the interaction of

202   the three as fixed effects, and vial as a random effect to account for repeated measures.

203   Significant differences in pupal volume of flies reared on 12.5% media was assessed with a two-

204   way ANOVA (function 'aov') including *Wolbachia*, mortality status, and their interaction as fixed

205   effects. Significant differences in sizes of pupae reared on supplemented media was first

206   assessed with a two-way ANOVA (function 'aov') including *Wolbachia*, media, and their

207   interaction as fixed effects. Paired comparisons between *Wolbachia*-infected and uninfected flies

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208   were then assessed with Wilcoxon rank sum tests (function 'wilcox.test'). Gene expression and

209   *Wolbachia* titers from knockdown experiments were assessed with two-way ANOVAs (function

210   'aov') including delta delta Ct as the response, and target locus, genotype (knockdown versus

211   sibling), and their interaction as fixed effects. *Wolbachia* titers in dissected flies were also

212   assessed with ANOVA, here with genotype, tissue, and their interaction as fixed effects. Pairwise

213   comparisons were performed with t-tests.

214

215   **RESULTS**

216   <u>*Wolbachia* is beneficial under nutrient limited conditions</u>

217   To assess the impact of *Wolbachia* infection on fly development, we reared flies on 100% strength

218   and 25% strength media and quantified pupation and adult emergence. We found a significant

219   interaction between *Wolbachia*, media strength, and time that impacted fly pupation ($F_{1,1063}$ =

220   24.7147, $p < 0.0001$) and adult emergence ($F_{1,1063}$ = 22.2897, $p < 0.0001$). Additionally, we found

221   a significant interaction between the presence of *Wolbachia* and the media strength (wild type

222   pupae: $F_{1,1063}$ = 0.6517, p = 0.0063; wild type adults: $F_{1,1063}$ = 0.1746, p = 0.0062; w^{145} pupae: $F_{1,711}$

223   = 8.8129, p = 0.0009; w^{145} adults: $F_{1,711}$ = 19.0998, $p < 0.0001$), along with a significant impact of

224   *Wolbachia* infection alone on fly pupation and adult emergence (wild type pupae: $F_{1,1063}$ = 0.2874,

225     $p < 0.0001$; wild type adults: $F_{1,1063}$ = 0.5375, $p < 0.0001$; w^{145} pupae: $F_{1,711}$ = 10.5483, $p < 0.0001$;

226   w^{145} adults: $F_{1,711}$ = 7.0718, $p < 0.0001$). These effects are driven by the nutrient-limited conditions

227   where *Wolbachia*-infected flies developed faster (1-5 days depending on genotype, Figures 1B

228   and 1E) and a larger percentage of flies reached adulthood. For example, while the wild type flies

229   with and without *Wolbachia* reared on 100% strength media reached adulthood in 82% and 81%

230   of cases, on the 25% strength media these were reduced to 75% and 66% respectively. The

231   *Wolbachia*-mediated advantage was more prominent for a second fly genetic background, w^{145}.

232   Even on 100% strength media, the *Wolbachia*-free w^{145} flies experienced a 5% reduction in adult

233   emergence relative to *Wolbachia*-infected flies (Figure 1D). When reared on 25% strength media,

234   the *Wolbachia*-infected w^{145} flies were more than twice as likely to reach adulthood (70% versus

235   31%, Figure 1E). Across these assays, we found delays in entering pupation are approximately

236   equal to the delay in adult emergence: *i.e.,* the time spent in metamorphosis did not change as a

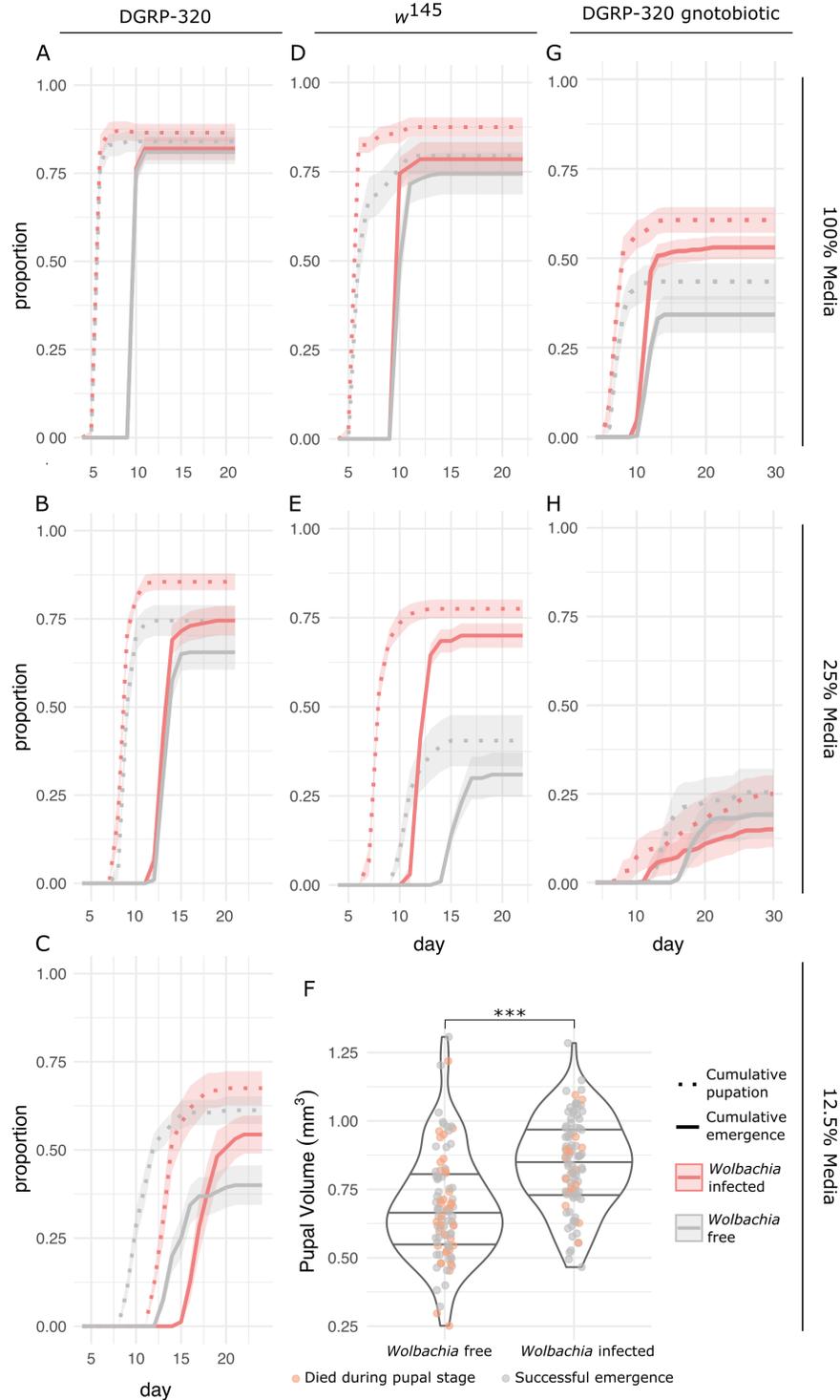237   factor of *Wolbachia* infection.

238

239   Given that the effect size for the *Wolbachia* advantage was genotype dependent and given the

240   more subtle impact of *Wolbachia* in the wild-type flies, we wondered if an even weaker media

241   would result in a stronger advantage for the *Wolbachia*-infected flies. Conversely, *Wolbachia*

242   could be a burden under more significant nutritional stress. Indeed, when flies were raised on

243   12.5% strength media, we again saw significant effects of the interaction between *Wolbachia* and

244   time on the rate and success of pupation and adult emergence (pupae: $F_{1,347}$ = 77.135, p < 0.0001;

245   adults: $F_{1,347}$ = 58.9296, p < 0.0001). However, *Wolbachia*-infected flies now developed slower

246   than their uninfected counterparts (6-7 days delayed), but they pupated at higher rates (68%

247   versus 61%) and were more likely to reach adulthood (54% versus 40%, Figure 1C). Furthermore,

248   although *Wolbachia*-infected fly development was slower, it resulted in them attaining, on

249   average, a 23% larger size at pupation ($F_{1,191}$ = 32.076, p < 0.0001, Figure 1F). Additionally,

250   *Wolbachia*-infected flies experienced lower levels of pupal mortality (35% versus 19%), perhaps

251   related to the finding that flies that died during pupation were significantly smaller ($F_{1,191}$ = 4.625,

252   p = 0.0328, Figure 1F).

253

254   To determine if the developmental advantage was due to direct effects of *Wolbachia* or indirect

255   effects via *Wolbachia*-mediated impacts on the gut microbiome, we performed the same

256   developmental assay under gnotobiotic conditions (i.e., without a gut or food microbiome). Under

257   gnotobiotic conditions, we saw a strong interactive effect of *Wolbachia* and time that resulted in

258   significantly more, and faster, pupation and adult emergence for infected flies (Figure 1G; pupae:

259    $F_{1,1920}$ = 153.3796, p < 0.0001; adults: $F_{1,1920}$ = 129.6080, p < 0.0001). Even on the 100% strength

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260   media, only 43% of *Wolbachia*-free flies pupated, compared to 61% of *Wolbachia*-infected flies.

261   Again, *Wolbachia*-free flies had higher levels of pupal mortality: 21% of those that pupated did

262   not emerge as adults, as compared to 13% of the *Wolbachia*-infected pupae. While removing the

263   microbiota negatively impacted fly developmental timing as expected, this effect was exacerbated

264   in the *Wolbachia*-free flies (Figures 1A and 1G). As compared to the conventionally-reared flies

265   on 100% strength media (Figure 1A), gnotobiotic *Wolbachia*-infected flies experienced a two-day

266   developmental delay, and *Wolbachia*-free flies were delayed three days (Figure 1G). The

267   combination of gnotobiotic conditions and 25% strength media resulted in especially impaired fly

268   development: less than 20% of flies reached adulthood (Figure 1H). While there were no

269   significant differences in the proportion of flies that pupated or emerged as adults between

270   *Wolbachia*-infected and uninfected flies in these high-mortality conditions (pupae: p = 0.9547;

271   adults: p = 0.6210), it is notable that *Wolbachia*-infected flies on average started emerging five

272   days ahead of the *Wolbachia*-free flies (Figure 1H).

11

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 the impact of *Wolbachia* infection. Biological replicates included 20 larvae per each of 10 vials.

277   Dotted and solid lines indicate cumulative pupation and adult emergence, respectively, with

278   shaded regions defining standard error. All *Wolbachia*-infected treatments are in red, and

279   *Wolbachia*-uninfected in grey. **(A)** Conventionally reared wild type flies on 100%, **(B)** 25%, and

280   **(C)** 12.5% strength media. **(D)** Conventionally reared w^{145} flies on 100% and **(E)** 25% strength

281   media. **(F)** Pupal volumes of wild type flies with and without *Wolbachia*, derived from the 12.5%

282   media-reared flies in (C). Orange datapoints indicate pupae that did not eclose into adults. *** p

283   < 0.001 **(G)** Gnotobiotic wild type flies on 100% and **(H)** 25% strength media.

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* (18), pathways known to significantly impact

289   fly growth and development (34). 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

293   compared to uninfected flies), *Dhod* (-0.164 FC), and *rudimentary-like* (-0.301 FC) (18). 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

301   *Wolbachia's* nucleotide metabolism genes were upregulated. At the onset of pupation (the white

302   pre-pupae "WPP" stage), *Wolbachia*'s metabolic gene expression was sharply and broadly

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303 downregulated (Figure 2D). These data indicate that *Wolbachia* may be supplementing host

304 nutrition during critical growth stages, and either limiting negative impacts of infection during

305 metamorphosis and reproductive stages or shifting roles to better interface with host reproduction

306 and ensure transmission.
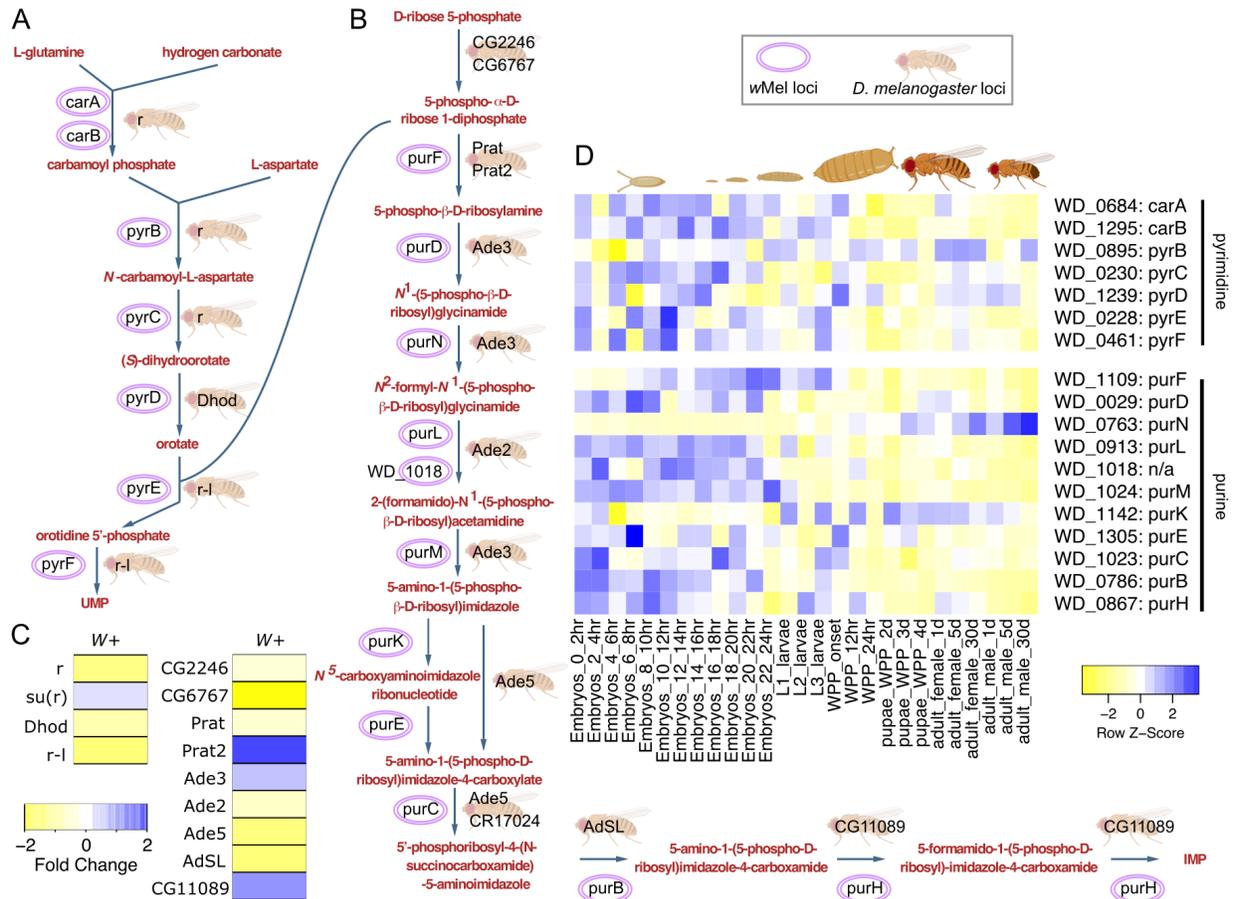
308 **Figure 2. Flies and *Wolbachia* redundantly encode for *de novo* nucleotide biosynthesis.**

309 **(A)** Metabolic pathway for *de novo* pyrimidine biosynthesis. **(B)** Metabolic pathway for *de novo*

310 purine biosynthesis. **(C)** Expression of *Drosophila melanogaster* nucleotide biosynthesis loci in

311 *Wolbachia*-infected female flies relative to *Wolbachia*-uninfected controls, modified from (18). **(D)**

312 Expression of *Wolbachia* nucleotide biosynthesis loci across fly development. *Wolbachia*

313 transcriptomes were generated as part of the MODENCODE project (30, 31). Data are row

314 normalized for inference of per-gene transcriptional changes across development. Fly images are

14

315   from BioRender.com. Abbreviations: UMP: uridine monophosphate; IMP: inosine

316   monophosphate. Fly gene alternate names: Ade2: Pfas; Ade3: Gart; Ade5: Paics.

317

318   <u>Pyrimidines contribute to the *Wolbachia* infection advantage</u>

319   We reasoned that deficiencies in the diet supplemented by *Wolbachia* could be supplemented by

320   exogenous nutrient addition to the fly food. Therefore, flies with and without *Wolbachia* would

321   have the same growth parameters if this important nutrient was added. Specifically, we focused

322   on the precursors and end-products of the *de novo* nucleotide synthesis pathways, which we

323   supplemented back into the 12.5% media. The developmental assays on supplemented media

324   were repeated with the wild type stocks, avoiding w^{145} because the *white* transporter is known to

325   affect nucleotide metabolism (35). We again saw the *Wolbachia*-mediated developmental

326   advantage in the 12.5% strength media (pupae: $F_{1,235}$ = 13.9505, p = 0.0002; adults: $F_{1,235}$ =

327   12.7568, p = 0.0004). Additionally, we saw a subtle but significant positive impact of *Wolbachia*

328   in the 100% strength media as measure by adult emergence (pupae: $F_{1,235}$ = 1.3002, p = 0.2538;

329   adults: $F_{1,235}$ = 28.0455, p < 0.0001), due to 6% pupal mortality in the *Wolbachia*-free flies that the

330   *Wolbachia*-infected flies did not experience (Figure 3A). While the addition of casein benefited

331   both *Wolbachia*-infected and uninfected flies, *Wolbachia*-free flies pupated at significantly

332   increased rates relative to *Wolbachia*-infected flies ($F_{1,235}$ = 9.8699, p = 0.0017). Again, pupal

333   mortality was quite high in the *Wolbachia*-free flies (36% as compared to 9% with *Wolbachia*),

334   resulting in significantly fewer numbers of adults ($F_{1,235}$ = 6.9988, p = 0.0082). The addition of

335   uridine (product of *de novo* pyrimidine synthesis) specifically benefited *Wolbachia*-free flies, but

336   not the flies that had *Wolbachia* infections. Indeed, uridine supplementation resulted in a rescue

337   phenotype where equivalent numbers of *Wolbachia*-infected and uninfected flies emerged as

338   adults (p = 0.9281), almost identical to the rates of adult emergence for *Wolbachia*-infected flies

339   on the standard 12.5% strength media (Figure 3A). In fact, *Wolbachia*-free flies supplemented

340   with uridine developed slightly faster than their infected counterparts (pupae: $F_{1,235}$ = 2.2401, p =

15

341   0.1348; adults: $F_{1,235}$ = 10.5842, p = 0.0011). The addition of inosine (product of *de novo* purine

342   synthesis) was toxic and reduced pupation and emergence for all flies. As such there were no

343   significant differences between *Wolbachia*-infected and uninfected flies in these high mortality

344   (*i.e.*, zero-inflated) conditions (pupae: $F_{1,235}$ = 0.9217, p = 0.335; adults: $F_{1,235}$ = 0.3519, p = 0.553).

345   Simultaneously adding uridine and inosine resulted in more intermediate, albeit still quite lethal

346   developmental phenotypes with no significant impact of *Wolbachia* ($F_{1,235}$ = 1.3471, p = 0.2459;

347   adults: $F_{1,235}$ = 1.0400, p = 0.307).

348

349   Congruent with the previous developmental assays (Figure 1), *Wolbachia* infection had a

350   significant impact on pupal size, both due to interacting effects with the media ($F_{5,470}$ = 2.55, p =

351   0.0272), and *Wolbachia* alone ($F_{1,470}$ = 37.31, p < 0.0001) (Figure 3B). Furthermore, *Wolbachia*-

352   infected flies were significantly larger when reared on 100% (p = 0.0401), 12.5% (p = 0.0316),

353   and casein-enriched 12.5% media (p < 0.0001). However, the addition of uridine to the 12.5%

354   media resulted in similarly sized flies regardless of *Wolbachia* infection (p = 0.3685). In summary,

355   *Wolbachia*-free flies were rescued to the same size and level of adult emergence as *Wolbachia*-

356   infected counterparts in uridine (i.e., pyrimidine) supplemented media.
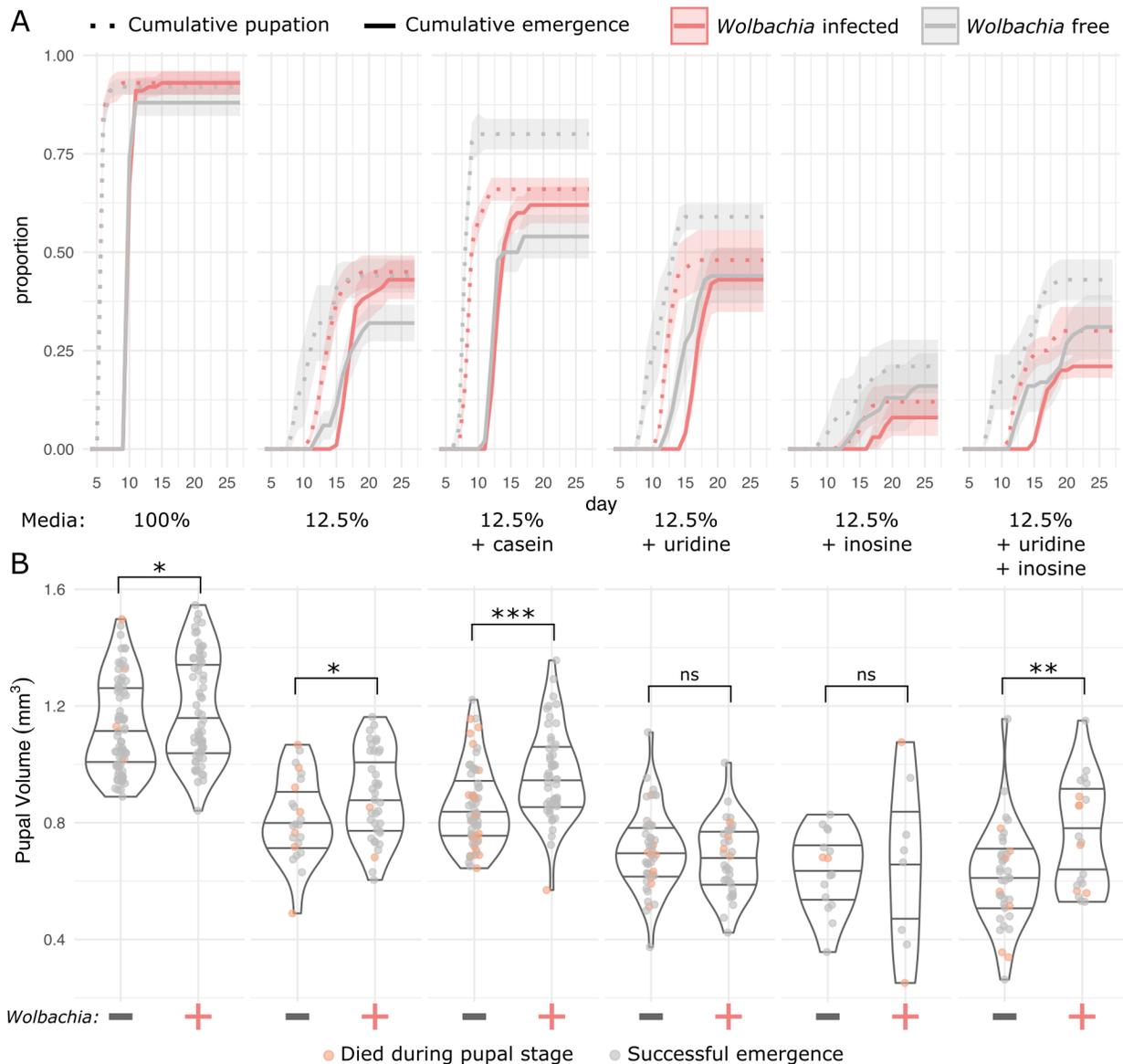
Figure 3. Uridine rescues *Wolbachia*-free flies. Wild type flies were reared on 100% media, 12.5% media, or 12.5% media containing either casein, uridine, inosine, or both inosine and uridine. The media designations in the middle correspond to the figures above and below. Biological replicates included 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

17

366 pupal sizes comparisons are the same regardless of whether flies that died during pupation were

367 included. ***p < 0.001; **p < 0.01; *p < 0.05; ns = not significant.

368

369 _Wolbachia_ infection is responsive to pyrimidine biosynthesis gene expression

370 To determine whether _Wolbachia_ infection was sensitive to changes in host metabolism, we

371 knocked down individual genes within the fly's _de novo_ nucleotide synthesis pathways (Figure

372 2A-B) using _Wolbachia_-infected TRiP RNAi fly stocks (Figure 4A). Short-hairpin RNA (shRNA)

373 expression was driven in the entire body of the fly by an Act5C-Gal4 driver. We compared gene

374 expression and _Wolbachia_ titer in knockdown flies to sibling controls (flies not expressing a gene-

375 targeting shRNA, and instead with the TM6B balancer). We successfully knocked down three

376 genes in the pyrimidine synthesis pathway (_su(r)_, _Dhod_, _rudimentary-like_) and four genes in the

377 purine synthesis pathway (_prat2_, _ade2_, _ade5_, _CG11089_) (Figure 4A, Supplemental Figure S2).

378 Several gene knockdowns were lethal to fly development, including _rudimentary_ in the de novo

379 pyrimidine biosynthesis pathway and _CG2246_, _CG6767_, _Prat_, and _AdSL_ in the de novo purine

380 biosynthesis pathway. Broad-spectrum knockdown of _de novo_ purine synthesis had no significant

381 impacts on _Wolbachia_ titer (gene*knockdown: $F_{3,31}$ = 0.652, p = 0.588; gene: $F_{3,31}$ = 0.644, p =

382 0.593; knockdown: $F_{1,31}$ = 0.593, p = 0.447; Supplemental Figure S2). In contrast, knockdown of

383 the _de novo_ pyrimidine synthesis pathway resulted in significant increases in _Wolbachia_ titer ($F_{1,38}$

384 = 10.3916, p = 0.0026; Figure 4B). This pattern was specifically driven by the enzymatic loci

385 (_Dhod_: p = 0.034 and _rudimentary-like_ (_r-l_): p = 0.013). In contrast, knockdown of the negative

386 regulator _sur(r)_ had no effect on _Wolbachia_ titer (p = 0.530), perhaps indicating that _Wolbachia_

387 titers are responsive to low fly pyrimidine synthesis activity, but not elevated levels. To account

388 for any effects of the TM6B balancer on measurements of _Wolbachia_ abundance, we crossed the

389 Act5C-Gal4 driver to a TRiP line expressing shRNA against a foreign gene: _Photinus pyralis_

390 luciferase (UAS-anti-Ppyr\LUC). We found no significant changes in _Wolbachia_ titer, or in

391 nucleotide gene expression that could be driving the results in Figure 4B (Supplemental Figure

18

392   S3). We then asked if the change in *Wolbachia* titer was tissue-specific by measuring the relative

393   *Wolbachia* titers in ovaries and the remaining carcasses after knockdown of *r-l* (Figure 4C). There

394   was no significant interaction of knockdown and tissue ($F_{1,32} = 0.20$, p = 0.658) or of tissue alone

395   ($F_{1,32} = 0.20$, p = 0.658) on *Wolbachia* titers. We again saw a significant effect of *r-l* knockdown

396   on *Wolbachia* titers ($F_{1,32} = 13.07$, p = 0.001) both in ovaries (p = 0.007) and in carcasses (p =

397   0.0127) (Figure 4C). Finally, given these interactions with pyrimidine synthesis, we used flies with

398   a homozygous hypomorphic allele *Dhod²* (RRID:BDSC_1936, Supplemental Table S1) to assess

399   the importance of *Wolbachia* in a pyrimidine synthesis-impaired genetic background. This stock

400   is naturally infected with *Wolbachia*, but removal of the symbiont lead to significant delays and

401   reductions in pupation and adult emergence (Figure 4D; pupae: $F_{1,247} = 11.0058$, p = 0.0009;

402   adults: $F_{1,247} = 12.0550$, p = 0.0057).

403

19
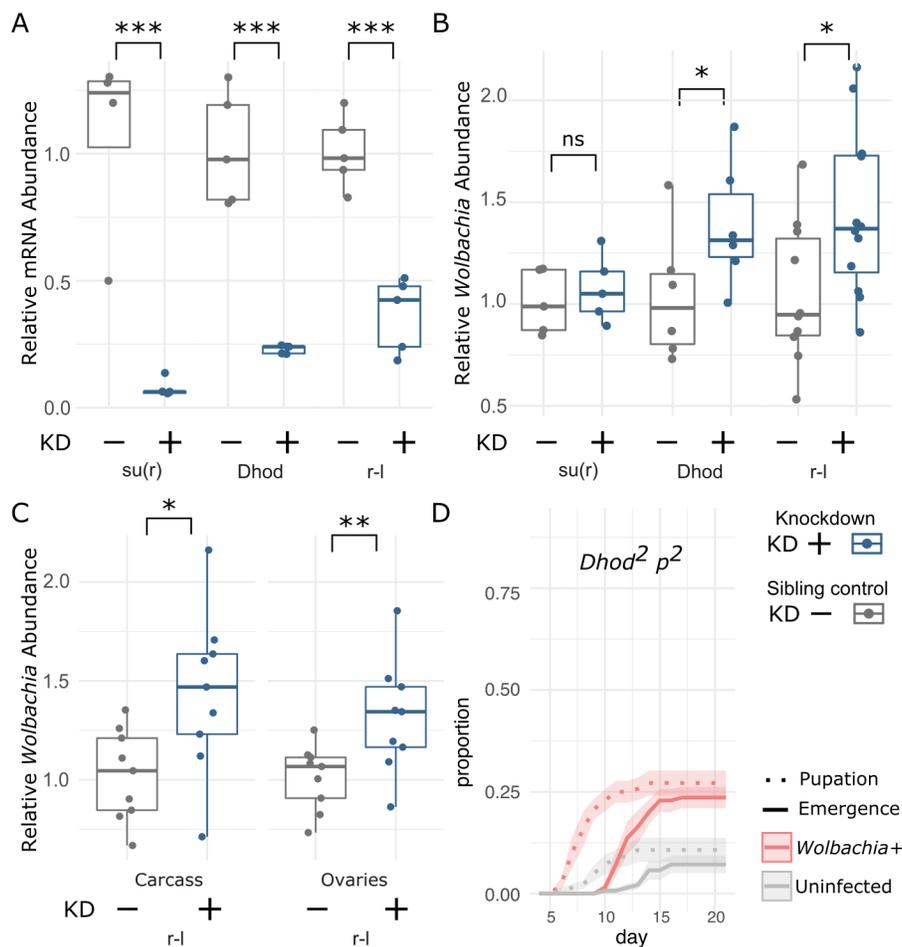
404   **Figure 4. *Wolbachia* titers are responsive to fly pyrimidine synthesis gene expression.**

405   Expression of fly genes in the *de novo* pyrimidine pathway was constitutively knocked down using

406   standard transgenic approaches. **(A)** *Drosophila* gene knockdown was verified with qRT-PCR

407   leveraging gene specific primers and normalization to 18S expression. **(B)** *Wolbachia* titers in

408   whole adult females and **(C)** dissected ovaries and carcasses were quantified with qPCR using

409   *Wolbachia*-specific primers and normalization to fly genome copy number via 18S amplification.

410   Legend: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns p = not significant. **(D)** Development of

411   conventionally reared *Wolbachia*-infected and -uninfected *Dhod²* p^2 flies on 100% strength media.

412   Biological replicates included 20 larvae per each of ten vials. Dotted and solid lines indicate

413   cumulative pupation and adult emergence, respectively, with shaded regions defining standard

414   error. *Wolbachia*-infected flies are in red and *Wolbachia*-uninfected are in grey.

415

416   **DISCUSSION**

417   *Wolbachia* has long been appreciated for its diverse impacts on a range of ecdysozoan hosts,

418   and there has been increasing speculation as to the role *Wolbachia* might play in host metabolism

419   (13, 18, 19). Here, we show that *Wolbachia* significantly impacts fly development, resulting in

420   larger flies more likely to reach adulthood. The strength of this phenotype varied by fly genotype

421   and the makeup of the food: *Wolbachia* infection was highly beneficial under nutrient limited

422   conditions. Removal of the extracellular microbial community indicated that this advantage was

423   directly mediated by *Wolbachia*. In fact, *Wolbachia* significantly buffered flies against the stresses

424   normally associated with removal of the microbiome (36, 37). Furthermore, *Wolbachia* titers were

425   responsive to changes in the expression of fly pyrimidine biosynthesis, and the supplementation

426   of pyrimidines rescued adult emergence of the *Wolbachia*-uninfected flies. We propose that

427   pyrimidines are a significant component of the advantage provided by *Wolbachia* infection.

428   Indeed, the patterns of *Wolbachia* gene expression seem to be attuned to fly development which

429   could be a strategy for supporting the exponential growth that occurs during larval stages, and

430　　mitigating fitness impacts of the infection during reproductive stages. Importantly, while the

431　　transcription of *Wolbachia*'s nucleotide metabolism pathways are quite high pre-pupation, the flies

432　　have comparatively low *Wolbachia* titers during that time (30). Genomic data suggest that

433　　*Wolbachia* imports amino acids from the host: while these may be used as precursors for

434　　nucleotide biosynthesis, the depletion of free amino acids could induce host stress responses (15,

435　　38). Changes in *Wolbachia* titer and physiology across development and under different

436　　nutritional scenarios likely reflect such balancing acts.

437

438　　While *Wolbachia* infections have received considerable attention for their impacts on

439　　reproduction, we know much less about how *Wolbachia* interact with their host during juvenile

440　　stages. Importantly, many insects spend the majority of their lifetime as juveniles. Juvenile insect

441　　metabolism is typically quite distinct from adults, and this time period has profound impacts on

442　　metamorphosis, adult physiology, and fitness (39). Critically, all insect growth occurs during the

443　　larval period: in *Drosophila,* larvae increase in body mass by ~200-fold over the course of 4 days

444　　(39). We found that *Wolbachia*-free flies had higher levels of pupal mortality, especially during

445　　nutritional stress, suggesting insufficient metabolic reserves. This effect was particularly

446　　pronounced when flies were reared on an excess of protein (Figure 3). Importantly, amino acids

447　　are a key nutritional cue for determining the timing of pupation (i.e., signaling critical weight) due

448　　to a direct and indirect effects on the timing and intensity of ecdysone pulses (40-43). An influx of

449　　amino acids without other essential nutrients needed for growth might impact nutritional signaling

450　　and thus the timing of critical weight, perhaps triggering flies to pupate early without sufficient

451　　reserves for metamorphosis. Given *Wolbachia* likely imports amino acids, this may reduce the

452　　concentrations of free amino acids and delay signaling critical weight, allowing flies to accumulate

453　　more metabolic reserves before pupation.

454

21

455   During the ~200-fold increase in larval body mass, there is a concomitant 25-fold increase in DNA

456   and RNA content that creates a huge demand for pyrimidines, well exceeding the fly's

457   endogenous biosynthetic capacity (44). Considering that the rate of *Drosophila* development is

458   highly sensitive to *de novo* pyrimidine synthesis (44), bacterially-derived nucleotides could give

459   the host a significant advantage. Pyrimidines are synthesized by a series of metabolic reactions

460   that use ribose sugar and the amino acids glutamine and aspartate to generate uridine-5'-

461   monophosphate, UMP (45): the precursor for uracil, cytosine, and thymine. This pathway is

462   conserved in both *Drosophila* and *Wolbachia*, suggesting that infected flies generate pyrimidines

463   in multiple compartments: the host cytoplasm/mitochondria and *Wolbachia*. *Wolbachia*'s

464   production of pyrimidines could also represent far more than simply additional biosynthesis, as

465   the enzyme dihydroorotate dehydrogenase (*Dhod*) is located in the inner mitochondrial

466   membrane where it couples pyrimidine synthesis to electron transport (46, 47). As such,

467   provisioning of *Wolbachia*-generated pyrimidines could reduce the biosynthetic burden normally

468   imposed upon mitochondria. Additionally, fly enzymes used for pyrimidine synthesis, CAD and

469   UMP synthase, are negatively regulated by UMP (48). Therefore, *Wolbachia* could simultaneously

470   impact free amino acid pools, supplement pyrimidines, reduce mitochondrial burdens, and

471   spatially segregate components of a normally inhibitory interaction.

472

473   While we found that pyrimidines seemed to be important for the fly-*Wolbachia* symbiosis, we did

474   not see any changes in *Wolbachia* titer due to repression of purine synthesis. In fact, the addition

475   of inosine to media negatively impacted flies (Figure 3). Purine nucleotides act as signaling

476   molecules that impact a range of functions. For example, activation of the adenosine receptor

477   globally suppresses fly metabolism (49), which can cause insecticidal effects (50). Another

478   possibility is misincorporation of inosine into RNAs. In mammalian cell lines, this can happen

479   when inosine is in excess, ultimately impacting translation (51). While altering the expression of

480   fly purine metabolic gene expression did not impact *Wolbachia* titers in our experiments, we

22

481   cannot not rule out the potential for other changes in *Wolbachia* gene expression or physiology.

482   Indeed, we previously found an interactive effect of *Wolbachia* infection and purine gene

483   expression on virus replication (18), highlighting both the importance of purines to the symbiosis

484   and the complexity of the metabolic landscape.

485

486   The role of *Wolbachia* as a nutritional symbiont raises numerous questions about the cell biology

487   of *Wolbachia* and how the relationship with the host is regulated. Is *Wolbachia* specifically

488   providing pyrimidines (e.g., via nucleotide transporters)? Or are flies farming and consuming their

489   *Wolbachia* infections (e.g., autophagy) and pyrimidines so happen to be abundant in *Wolbachia*

490   cells and also a limiting metabolite for the fly? As is the case for many symbioses, it is not always

491   clear if a change in the symbiont (e.g., gene expression or titer), is due to the microbe detecting

492   and responding to the host's status, or the host releasing control of the microbe and allowing a

493   change in microbial physiology. *Wolbachia* do encode for a number of proteins that would allow

494   them to detect their environment and regulate gene expression accordingly (52). However, each

495   *Wolbachia* is enclosed by a host-derived vesicle (53, 54), so perhaps the host tightly regulates

496   *Wolbachia*'s "experience".

497

498   While nutritional mutualisms in insects are not rare, most of the systems for which this is described

499   are of a much more binary nature. Insects that exclusively feed on unbalanced diets (e.g., blood,

500   plant sap) have obligate microbial symbionts to synthesize metabolites that the host cannot

501   generate *de novo*: the partners complement each other (55, 56). Here, both flies and *Wolbachia*

502   have their own complete pathways for nucleotide biosynthesis, which perhaps has led us to

503   overlook the relevance of this biosynthetic redundancy. We propose that the metabolic and

504   developmental impacts of *Wolbachia* are adaptive and might explain the broad success and

505   spread of *Wolbachia* even in the absence of direct reproductive manipulations.

506

23

DECLARATIONS

Conflicts of interest

The authors declare that they have no competing interests.

Data Availability

Supplemental Table S1. Fly stocks

Supplemental Table S2. Primers

Supplemental Figure S1. Pupal measurement.

Supplemental Figure S2. Purine knockdowns.

Supplemental Figure S3. Knockdown controls.

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657

658

659 **Supplemental Tables and Figures**

660

661 **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

662

663

664 **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

665
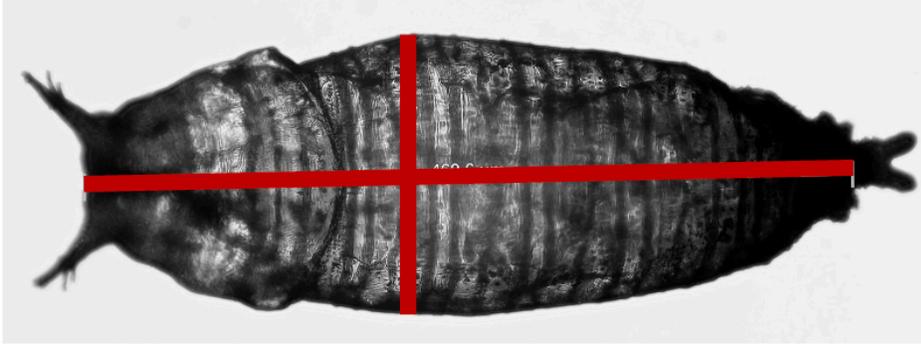
666

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

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

669

670
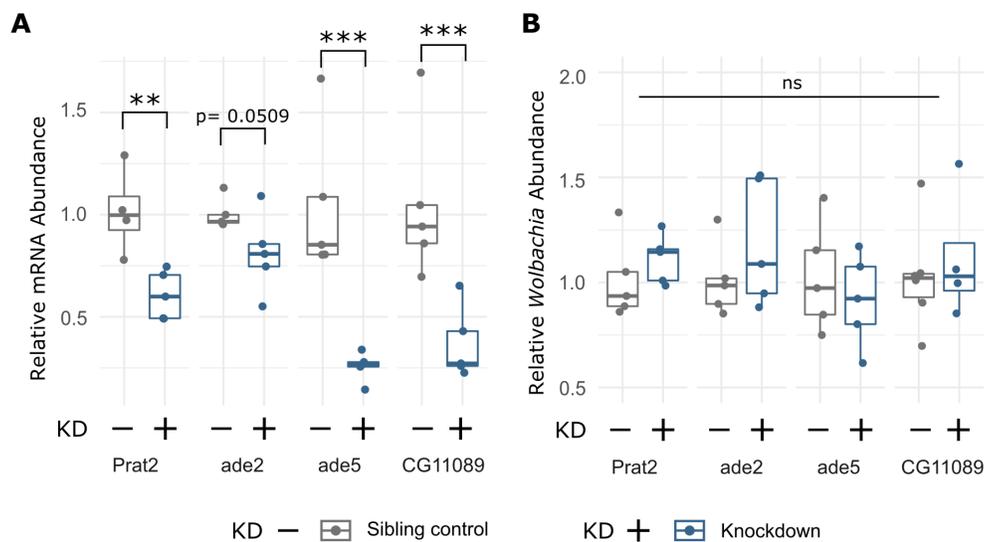
671

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

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

674 <0.001; ** p < 0.01; * p < 0.05; ns p = not significant. **(B)** Knockdown of purine synthesis had no

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

676 0.644, p = 0.593; knockdown: $F_{1,31} = 0.593$, p = 0.447).
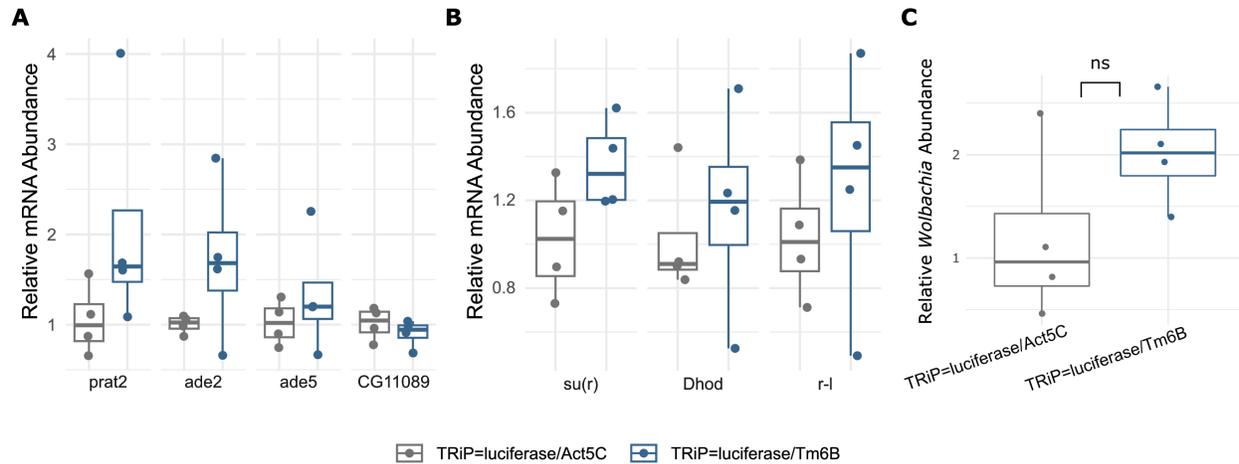
677

678 **Figure S3. Validation of knockdown controls.** Impact of balancer chromosomes on (A) purine

679 gene expression, (B) pyrimidine gene expression, and (C) *Wolbachia* titer. **(A)** The TM6B

680 balancer had no significant impacts on purine gene expression (gene*balancer: $F_{3,24}$ = 1.311, p =

681 0.2939; gene: $F_{3,24}$ = 1.311, p = 0.2939; knockdown: $F_{1,24}$ = 4.146, p = 0.0529). Importantly, while

682 the mean expression of the purine loci may have been marginally higher in the presence of the

683 balancer chromosome, this means that we would underestimate the effects of knockdown due to

684 an even larger difference between the sibling and knockdown flies (see main text). **(B)** The TM6B

685 balancer had no significant impacts on pyrimidine gene expression (gene*balancer: $F_{2,18}$ = 0.227,

686 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

687 significant difference in *Wolbachia* titer due to the balancer chromosome (t-test, t = 1.8404, df =

688 8, p = 0.1153). Again, the trend towards marginally higher *Wolbachia* titer in the presence of the

689 balancer only means that we would underestimate the effects of knockdown.