Wolbachia is a nutritional symbiont

Amelia RI Lindsey[1*], Audrey J Parish[2], Irene LG Newton[2], Jason M Tennessen[2], Megan W Jones[1], Nicole Stark[2]

[*]To whom correspondence should be addressed (alindsey@umn.edu)

[1]Department of Entomology, University of Minnesota, St. Paul, Minnesota, 55108

[2]Department of Biology, Indiana University, Bloomington, Indiana, 47405

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.

INTRODUCTION

Maternally transmitted microbes have evolved numerous ways to manipulate host physiology ultimately facilitating their own success. Strategies include supplementation of the host's diet via nutrient provisioning, protection against parasites and infections, and even direct manipulation of the host's reproduction (1). A high percentage of arthropod species are infected with bacteria in the genus *Wolbachia* (Alphaproteobacteria: Rickettsiales), a maternally transmitted infection long considered to be a "reproductive parasite" (2-8). This classification was due to the seemingly selfish ways in which *Wolbachia* alters host reproductive outcomes ultimately facilitating its own spread through the population (1, 9, 10). For example, the conversion to asexual reproduction or the induction of sperm-egg incompatibilities (so-called "cytoplasmic incompatibility") by *Wolbachia* can result in a rapid sweep of *Wolbachia*-infected matrilines across a population (11, 12).

However, the line between "parasite" and "mutualist" is becoming increasingly blurred as *Wolbachia*-mediated benefits for the host are uncovered (13). For example, certain strains of *Wolbachia* are clear nutritional mutualists, such as the bed bug-infecting *Wolbachia* that produce B vitamins to support these obligately hematophagous insects (14). Many other *Wolbachia* strains have been characterized for their ability to protect host insects against secondary infections, especially viruses (15). Indeed, evolutionary theory predicts that vertical transmission will select for mutualism, a process that we have been fortunate to watch happen in real time in some *Wolbachia*-host associations (16). Additionally, there are data indicating that *Wolbachia* can spread through a population even in the absence of so-called "parasitic" reproductive manipulations (17).

We previously identified that *Wolbachia* infection results in a suite of changes to the expression of fly nucleotide metabolism pathways (18). 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 (19). Furthermore, *Wolbachia* encode for an abundance of amino acid importers, which would provide necessary precursors for *Wolbachia*'s *de novo* synthesis of nucleotides (3, 19). We hypothesized that *Wolbachia* plays a supporting role in host nutrition. We show that *Wolbachia* infection is beneficial for flies, especially under nutrient limited conditions, and that *Wolbachia* is responsive to changes in the expression of fly metabolic pathways. These data support the idea that *Wolbachia* may broadly act as a nutritional symbiont, potentially underlying its evolutionary success as a common infection in the most abundant group of eukaryotes on earth.

METHODS

Fly husbandry

Fly stocks were maintained on standard Bloomington cornmeal-agar medium at 25 °C on a 24-hour, 12:12 light:dark cycle under density-controlled conditions and 50% relative humidity. *Wolbachia* colonization status was confirmed with PCR assays using *Wolbachia*-specific 16S primers WspecF and WspecR (20). Genotypes used in nutritional assays (below) included: DGRP-320 (RRID:BDSC_29654), a *Wolbachia*-infected isogenic wild-type strain with genome sequence available which we refer to as "wild type" below (21), and a *Wolbachia*-infected white line, w^{145} (RRID:BDSC_145). *Wolbachia*-cleared counterpart stocks were generated with antibiotics via three generations of tetracycline treatment (20 µg/mL in the fly food for three generations), followed by re-inoculation of the gut microbiome by transfer to bottles that previously harbored male flies from the original stock that had fed and defecated on the media for one week (22). Stocks were allowed to recover from any potential transgenerational effects of the antibiotic treatment for at least an additional ten generations prior to use in any experiment. All other fly stocks (e.g., RNAi lines), are detailed in Supplementary Table S1.

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

130 coloring to facilitate counting. A flame-sterilized probe was used to transfer 20 embryos into each
131 vial of sterile media.
132
133 Nutritional supplementation experiments
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.
140
141 Pupal size measurements
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 [$V = (4/3)\ \pi\ (\text{width}/2)^2\ (\text{length}/2)$] (Supplemental Figure S1) (25, 26).
149
150 Knockdown of fly nucleotide metabolism genes
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^1w^*; 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.
166
167 Real-time quantitative RT-PCR analyses of target gene expression
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 Real-time quantitative PCR analyses of *Wolbachia* titer
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 Nucleotide metabolism biosynthesis and gene expression
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 Statistics and Data Visualization
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

were then assessed with Wilcoxon rank sum tests (function 'wilcox.test'). Gene expression and *Wolbachia* titers from knockdown experiments were assessed with two-way ANOVAs (function 'aov') including delta delta Ct as the response, and target locus, genotype (knockdown versus sibling), and their interaction as fixed effects. *Wolbachia* titers in dissected flies were also assessed with ANOVA, here with genotype, tissue, and their interaction as fixed effects. Pairwise comparisons were performed with t-tests.

RESULTS

Wolbachia is beneficial under nutrient limited conditions

To assess the impact of *Wolbachia* infection on fly development, we reared flies on 100% strength and 25% strength media and quantified pupation and adult emergence. We found a significant interaction between *Wolbachia*, media strength, and time that impacted fly pupation ($F_{1,1063}$ = 24.7147, $p < 0.0001$) and adult emergence ($F_{1,1063}$ = 22.2897, $p < 0.0001$). Additionally, we found a significant interaction between the presence of *Wolbachia* and the media strength (wild type pupae: $F_{1,1063}$ = 0.6517, $p = 0.0063$; wild type adults: $F_{1,1063}$ = 0.1746, $p = 0.0062$; w^{145} pupae: $F_{1,711}$ = 8.8129, $p = 0.0009$; w^{145} adults: $F_{1,711}$ = 19.0998, $p < 0.0001$), along with a significant impact of *Wolbachia* infection alone on fly pupation and adult emergence (wild type pupae: $F_{1,1063}$ = 0.2874, $p < 0.0001$; wild type adults: $F_{1,1063}$ = 0.5375, $p < 0.0001$; w^{145} pupae: $F_{1,711}$ = 10.5483, $p < 0.0001$; w^{145} adults: $F_{1,711}$ = 7.0718, $p < 0.0001$). These effects are driven by the nutrient-limited conditions where *Wolbachia*-infected flies developed faster (1-5 days depending on genotype, Figures 1B and 1E) and a larger percentage of flies reached adulthood. For example, while the wild type flies with and without *Wolbachia* reared on 100% strength media reached adulthood in 82% and 81% of cases, on the 25% strength media these were reduced to 75% and 66% respectively. The *Wolbachia*-mediated advantage was more prominent for a second fly genetic background, w^{145}. Even on 100% strength media, the *Wolbachia*-free w^{145} flies experienced a 5% reduction in adult 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

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).

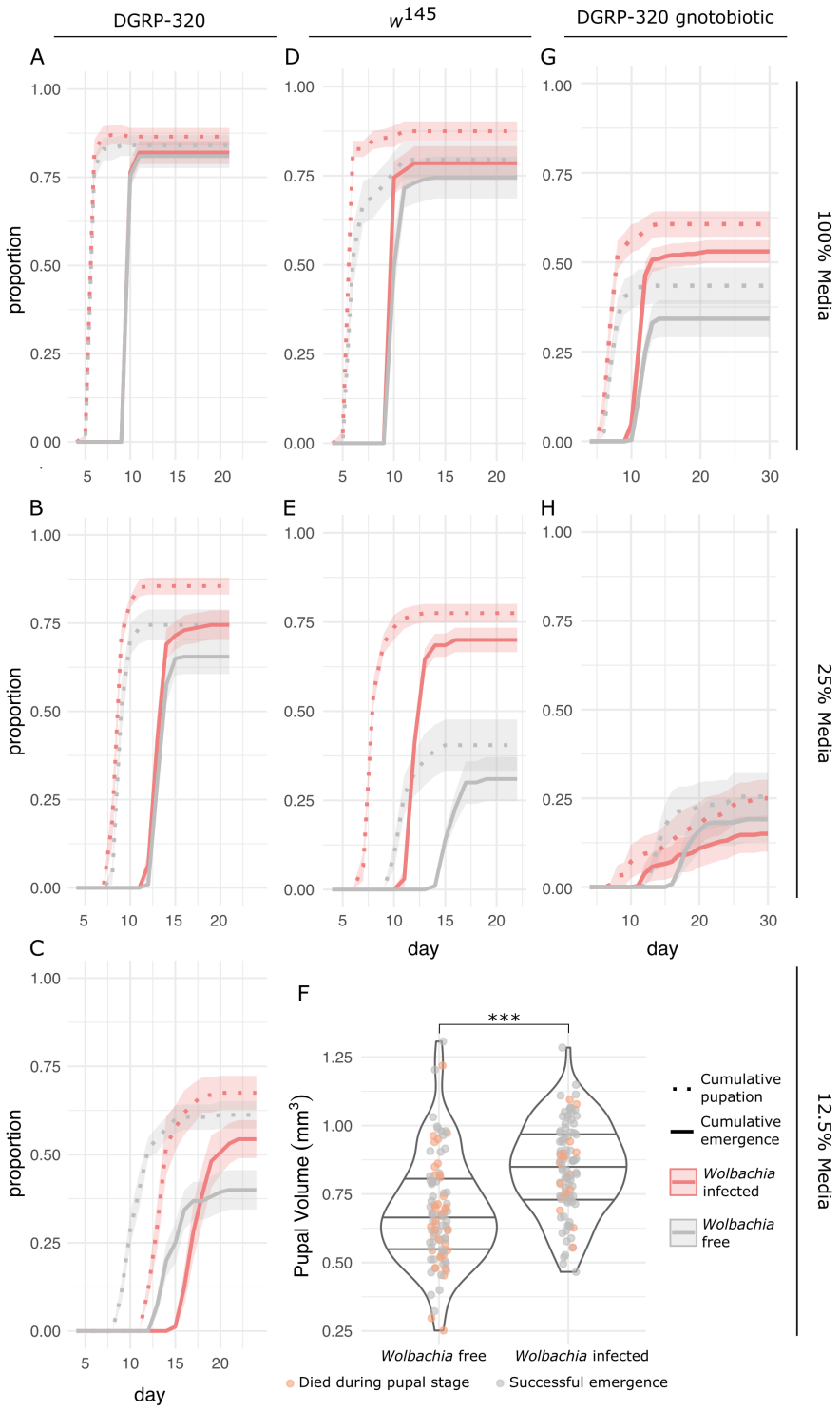
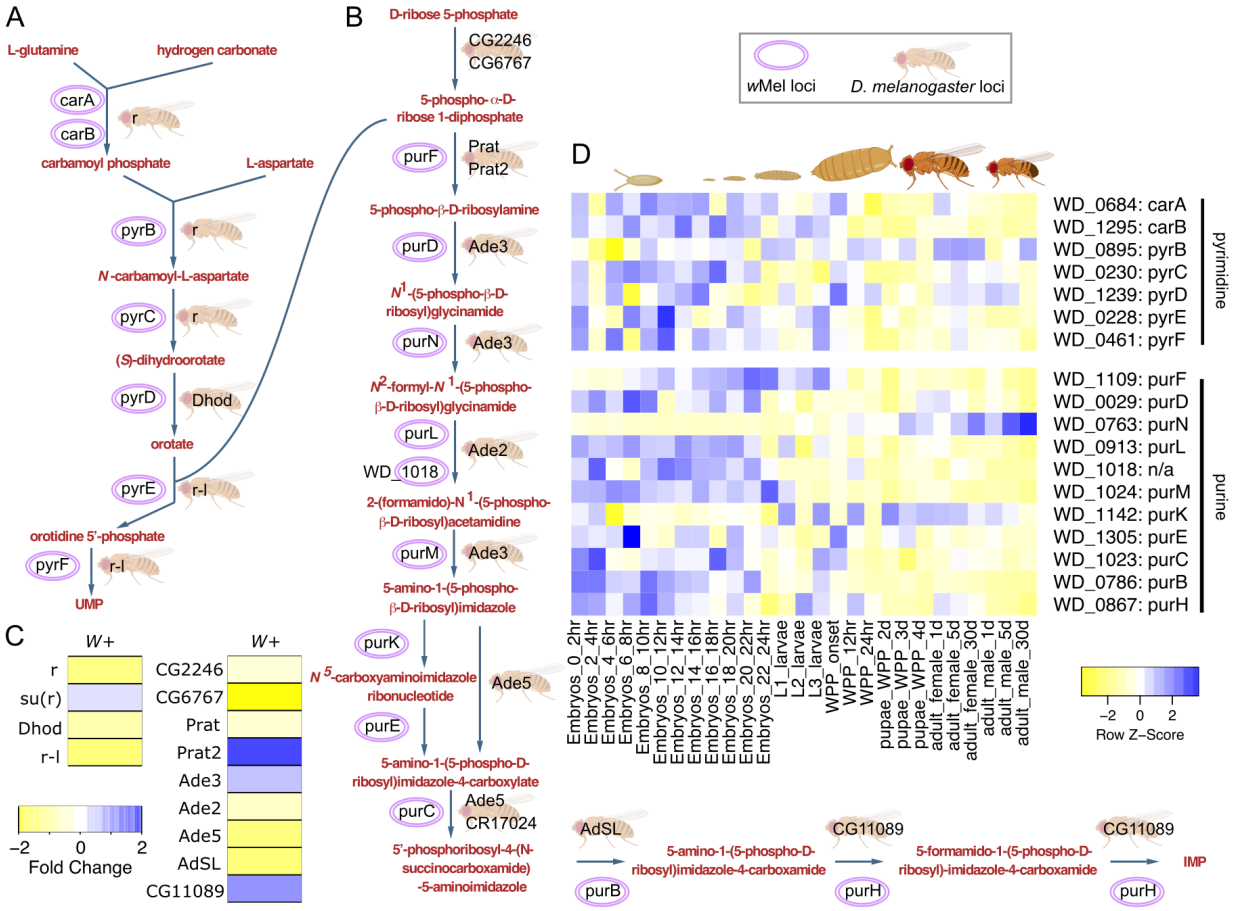


Figure 1. *Wolbachia* infection is beneficial under nutrient limited conditions. Flies (wild type and w^{145}, with and without *Wolbachia*) were reared on different concentrations of media to test the impact of *Wolbachia* infection. Biological replicates included 20 larvae per each of 10 vials.

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 Dynamics of the redundant *de novo* nucleotide biosynthesis pathways
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

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.



307

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

315 from BioRender.com. Abbreviations: UMP: uridine monophosphate; IMP: inosine
316 monophosphate. Fly gene alternate names: Ade2: Pfas; Ade3: Gart; Ade5: Paics.
317
318 Pyrimidines contribute to the *Wolbachia* infection advantage
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 =

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.

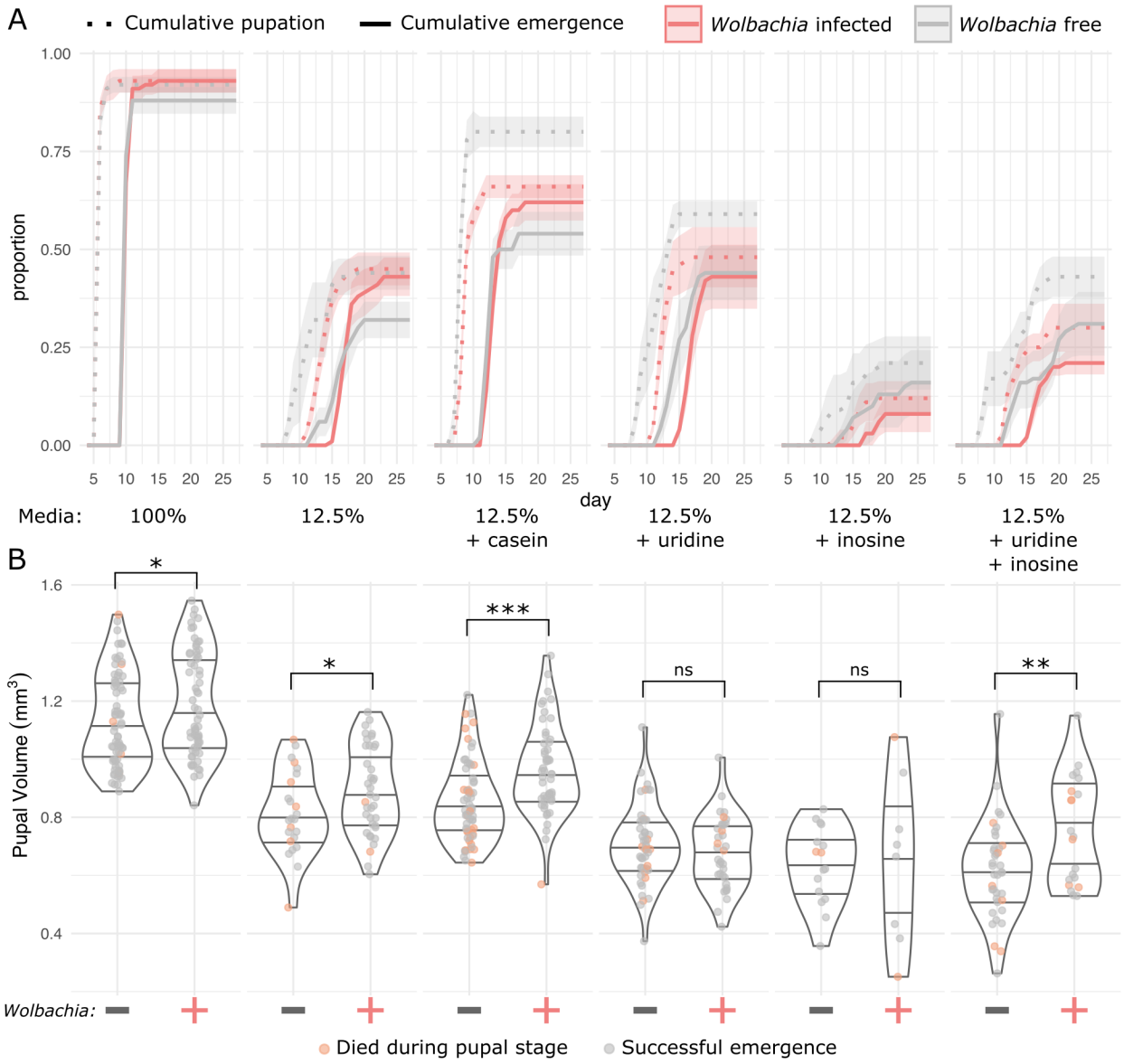
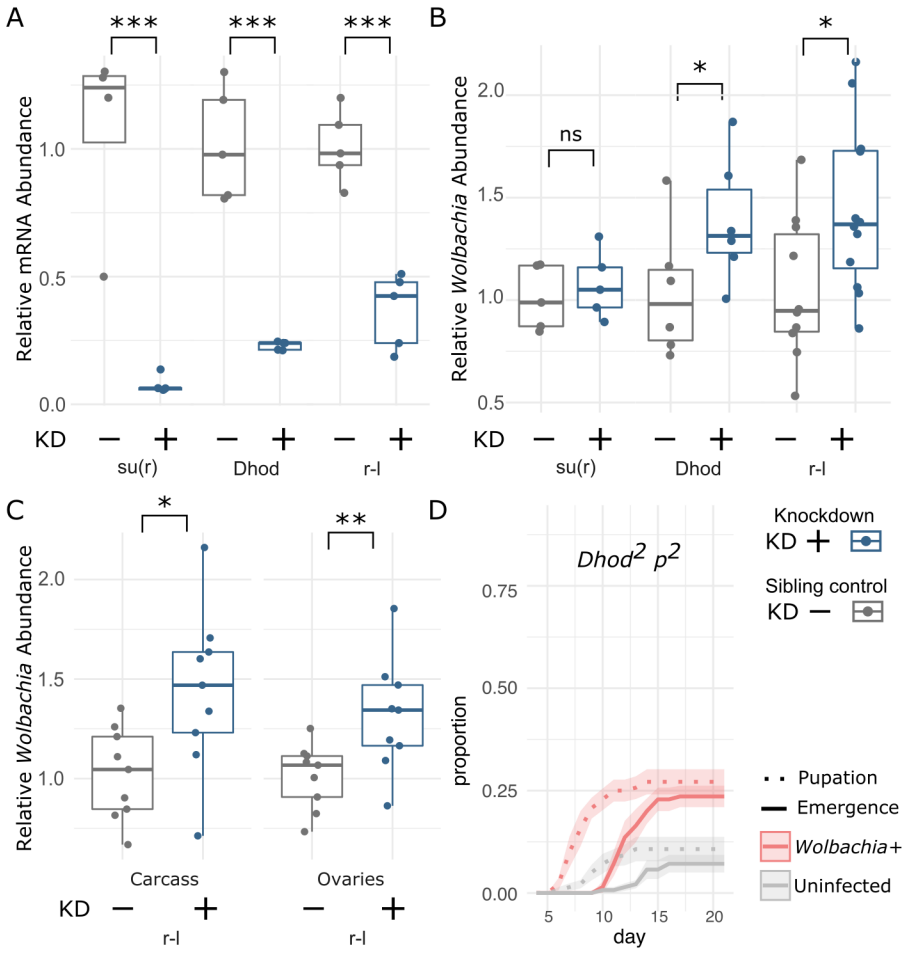


Figure 3. Uridine rescues *Wolbachia*-free flies. Wild type flies were reared on 100% media, 12.5% media, or 12.5% media containing either casein, uridine, inosine, or both inosine and uridine. The media designations in the middle correspond to the figures above and below. Biological replicates included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

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

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 *Dhod*^{2} (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

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 $Dhod^2$ 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

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

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

507 **DECLARATIONS**

508 Acknowledgements

509 This work was supported by UMN AGREETT startup funds to ARIL. ILGN in supported by the
510 National Institute of Allergy and Infectious Diseases of the National Institutes of Health under R01
511 AI144430. JMT is supported by the National Institute of General Medical Sciences of the National
512 Institutes of Health under a R35 Maximizing Investigators' Research Award (MIRA;
513 1R35GM119557). The authors thank Richard Hardy for support and feedback. Research was
514 further made possible by Flybase (NIH 5U41HG000739), and stocks obtained from the
515 Bloomington *Drosophila* Stock Center (NIH P40OD018537).

516

517 Conflicts of interest

518 The authors declare that they have no competing interests.

519

520 Data Availability

521 Supplemental Table S1. Fly stocks

522 Supplemental Table S2. Primers

523 Supplemental Figure S1. Pupal measurement.

524 Supplemental Figure S2. Purine knockdowns.

525 Supplemental Figure S3. Knockdown controls.

526

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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

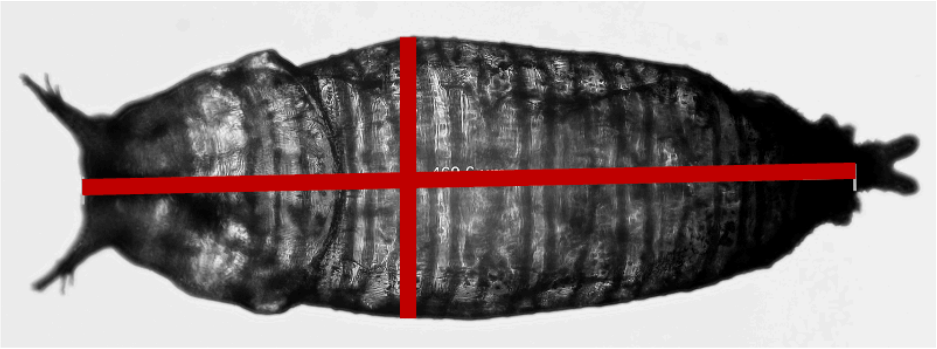

Figure S1. Pupal measurements. Pupal volume was calculated based on length and width (red lines) and assuming a prolate spheroid shape [$V = (4/3)\ \pi\ (\text{width}/2)^2\ (\text{length}/2)$].

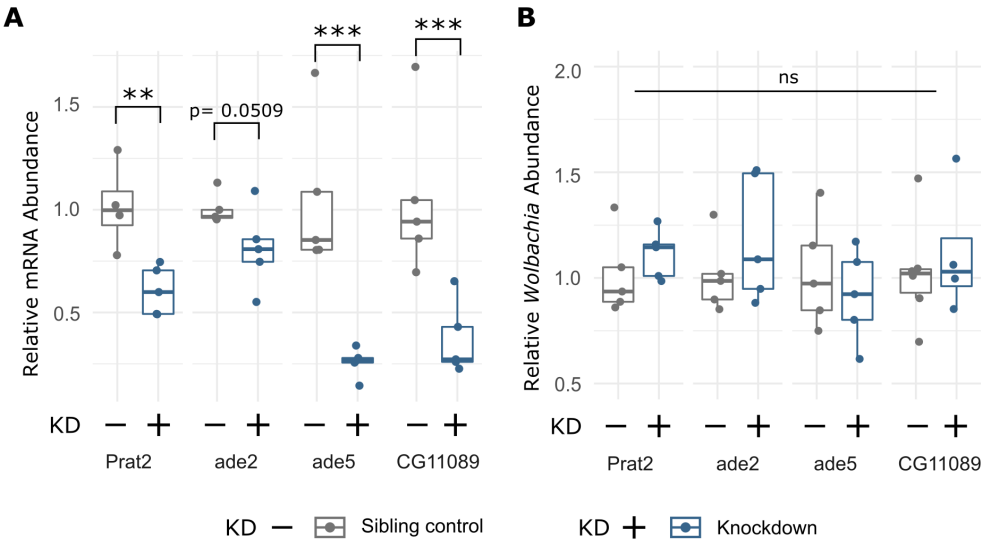

Figure S2. *Wolbachia* titer is not responsive to changes in de novo purine biosynthesis gene expression. (A) qRT-PCR validation of knockdown across four purine loci. Legend: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns p = not significant. **(B)** Knockdown of purine synthesis had no significant impacts on *Wolbachia* titer (gene*knockdown: $F_{3,31} = 0.652$, $p = 0.588$; gene: $F_{3,31} = 0.644$, $p = 0.593$; knockdown: $F_{1,31} = 0.593$, $p = 0.447$).

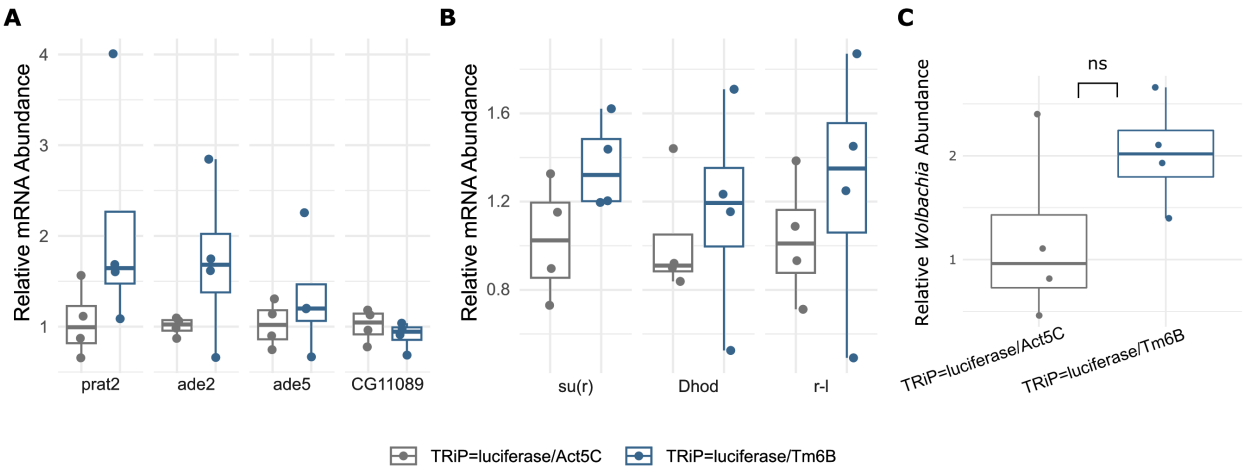

Figure S3. Validation of knockdown controls. Impact of balancer chromosomes on (A) purine gene expression, (B) pyrimidine gene expression, and (C) *Wolbachia* titer. **(A)** The TM6B balancer had no significant impacts on purine gene expression (gene*balancer: $F_{3,24} = 1.311$, $p = 0.2939$; gene: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