Loss of cytoplasmic incompatibility and minimal fecundity effects explain relatively low *Wolbachia* frequencies in *Drosophila mauritiana*

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ABSTRACT

Maternally transmitted *Wolbachia* bacteria infect about half of all insect species. Many *Wolbachia* cause cytoplasmic incompatibility (CI), reduced egg hatch when uninfected females mate with infected males. Although CI produces a frequency-dependent fitness advantage that leads to high equilibrium *Wolbachia* frequencies, it does not aid *Wolbachia* spread from low frequencies. Indeed, the fitness advantages that produce initial *Wolbachia* spread and maintain non-CI *Wolbachia* remain elusive. *w*Mau *Wolbachia* infecting *Drosophila mauritiana* do not cause CI, despite being very similar to CI-causing *w*No from *D. simulans* (0.068% sequence divergence over 682,494 bp), suggesting recent CI loss. Using draft *w*Mau genomes, we identify a deletion in a CI-associated gene, consistent with theory predicting that selection within host lineages does not act to increase or maintain CI. In the laboratory, *w*Mau shows near-perfect maternal transmission; but we find no significant effect on host fecundity, in contrast to published data. Intermediate *w*Mau frequencies on the island Mauritius are consistent with a balance between unidentified small, positive fitness effects and imperfect maternal transmission. Our phylogenomic analyses suggest that group-B *Wolbachia*, including *w*Mau and *w*Pip, diverged from group-A *Wolbachia*, such as *w*Mel and *w*Ri, 6–46 million years ago, more recently than previously estimated.

INTRODUCTION

Maternally transmitted *Wolbachia* infect about half of all species throughout all major insect orders (Werren and Windsor 2000; Zug and Hammerstein 2012; Weinert et al. 2015), as well as other arthropods (Jeyaprakash and Hoy 2000; Hilgenboecker et al. 2008) and nematodes (Taylor et al. 2013). Host species may acquire *Wolbachia* from common ancestors, from sister species via hybridization and introgression, or horizontally (O'Neill et al. 1992; Rousset and Solignac 1995; Huigens et al. 2004; Baldo et al. 2008; Raychoudhury et al. 2009; Gerth and Bleidorn 2016; Schuler et al. 2016; Turelli et al. 2018). *Wolbachia* often manipulate host reproduction, inducing cytoplasmic incompatibility (CI) and male killing in *Drosophila* (Laven 1951; Yen and Barr 1971; Hoffmann et al. 1986; Hoffmann and Turelli 1997; Hurst and Jiggins 2000). CI reduces egg hatch when *Wolbachia*-uninfected females mate with infected males. Three parameters usefully approximate the frequency dynamics and equilibria of CI-causing *Wolbachia* that do not distort sex ratios: the relative hatch rate of uninfected eggs fertilized by infected males (H), the fitness of infected females relative to uninfected females (F), and the proportion of uninfected ova produced by infected females (μ) (Caspari and Watson 1959; Hoffmann et al. 1990). To spread deterministically from low frequencies, *Wolbachia* must produce $F(1 - \mu) > 1$, irrespective of CI. Once they become sufficiently common, CI-causing infections, such as *w*Ri-like *Wolbachia* in *Drosophila simulans* and several other *Drosophila* (Turelli et al. 2018), spread to high equilibrium frequencies, dominated by a balance between CI and imperfect maternal transmission (Turelli and Hoffmann 1995; Kreisner et al. 2016). In contrast, non-CI-causing *Wolbachia*, such as *w*Au in *D. simulans* (Hoffmann et al. 1996), typically persist at lower frequencies, presumably maintained by a balance between positive *Wolbachia* effects on host fitness and imperfect maternal transmission (Hoffmann and Turelli 1997; Kreisner et al. 2013). When $H < F(1 - \mu) < 1$, "bistable" dynamics result, producing stable equilibria at 0 and at a higher frequency denoted p_s, where $0.50 < p_s \leq 1$ (Turelli and Hoffmann 1995). Bistability explains the pattern and (slow) rate of spread of *w*Mel transinfected into *Aedes aegypti* to suppress the spread of dengue, Zika and other human diseases (Hoffmann et al. 2011; Barton and Turelli 2011; Turelli and Barton 2017; Schmidt et al. 2017).

In contrast to the bistability observed with *w*Mel transinfections, natural *Wolbachia* infections seem to spread via "Fisherian" dynamics with $F(1 - \mu) > 1$ (Fisher 1937; Kriesner et al. 2013; Hamm et al. 2014). Several *Wolbachia* effects could generate $F(1 - \mu) > 1$, but we do

31 not yet know which ones actually do. For example, *w*Ri has evolved to increase *D. simulans*
32 fecundity in only a few decades (Weeks et al. 2007), *w*Mel seems to enhance *D. melanogaster*
33 fitness in high and low iron environments (Brownlie et al. 2009), and several *Wolbachia*
34 including *w*Mel protect their *Drosophila* hosts from RNA viruses (Hedges et al. 2008; Teixeira
35 et al. 2008; Martinez et al. 2014). However, it remains unknown which if any these potential
36 fitness benefits underlie *Wolbachia* spread in nature. For instance, *w*Mel seems to have little
37 effect on viral abundance in wild-caught *D. melanogaster* (Webster et al. 2015; Shi et al. 2018).
38 *D. mauritiana, D. simulans* and *D. sechellia* comprise the *D. simulans* clade within the nine-
39 species *D. melanogaster* subgroup of *Drosophila*. The *D. simulans* clade diverged from *D.*
40 *melanogaster* approximately three million years ago (mya), with the island endemics *D. sechellia*
41 (Seychelles archipelago) and *D. mauritiana* (Mauritius) thought to originate in only the last few
42 hundred thousand years (Lachaise et al. 1986; Ballard 2000a; Dean and Ballard 2004;
43 McDermott and Kliman 2008; Garrigan et al. 2012; Brand et al. 2013; Garrigan et al. 2014). *D.*
44 *simulans* is widely distributed around the globe, but has never been collected on Mauritius
45 (David et al. 1989; Legrand et al. 2011). However, evidence of mitochondrial and nuclear
46 introgression supports interisland migration and hybridization between these species (Ballard
47 2000a; Nunes et al. 2010; Garrigan et al. 2012), which could allow introgressive *Wolbachia*
48 transfer (Rousset and Solignac 1995).
49 *D. mauritiana* is infected with *Wolbachia* denoted *w*Mau, likely acquired via introgression
50 from other *D. simulans*-clade hosts (Rousset and Solignac 1995). *Wolbachia* variant *w*Mau may
51 also infect *D. simulans* (denoted *w*Ma in *D. simulans*) in Madagascar and elsewhere in Africa
52 and the South Pacific (Ballard 2000a; Ballard 2004). *w*Mau does not cause CI in *D. mauritiana*
53 or when transinfected into *D. simulans* (Giordano et al. 1995). Yet it is very closely related to
54 *w*No strains that do cause CI in *D. simulans* (Merçot et al. 1995; Rousset and Solignac 1995;
55 James and Ballard 2000, 2002). (Also, *D. simulans* seems to be a "permissive" host for CI, as
56 evidenced by the fact that *w*Mel, which causes little CI in its native host, *D. melanogaster*,
57 causes intense CI in *D. simulans* [Poinsot et al. 1998].) Fast et al. (2011) reported that a *w*Mau
58 variant increased *D. mauritiana* fecundity four-fold. This fecundity effect occurred in concert
59 with *w*Mau-induced alternations of programmed cell death in the germarium and of germline
60 stem cell mitosis, possibly providing insight into the mechanisms underlying increased egg

production (Fast et al. 2011). However, the generality of this finding across *w*Mau variants and host genetic backgrounds remains unknown.

Here, we assess the genetic and phenotypic basis of *w*Mau frequencies in *D. mauritiana* on Mauritius by combining analysis of *w*Mau draft genomes with analysis of *w*Mau transmission in the laboratory and *w*Mau effects on host fecundity and egg hatch. We identify a single mutation that disrupts a locus associated with CI. The loss of CI in *w*Mau is consistent with theory demonstrating that selection within host species does not act to increase or maintain the level of CI (Prout 1994; Turelli 1994; Haygood and Turelli 2009), but instead acts to increase $F(1 - \mu)$, the product of *Wolbachia* effects on host fitness and maternal transmission efficiency (Turelli 1994). The loss of CI helps explain the intermediate *w*Mau frequencies on Mauritius, reported by us and Giordano et al. (1995). We find no *w*Mau effects on host fecundity, and theoretical analyses show that even a two-fold fecundity increase cannot be reconciled with the observed intermediate population frequencies, unless maternal *w*Mau transmission is exceptionally unreliable in the field. Finally, we present theoretical analyses illustrating that the persistence of two distinct classes of mtDNA haplotypes among *Wolbachia*-uninfected *D. mauritiana* is unexpected under a simple null model. Together, our results contribute to understanding the genomic and phenotypic basis of global *Wolbachia* persistence, which is relevant to improving *Wolbachia*-based biocontrol of human diseases (Ritchie 2018).

MATERIALS AND METHODS

Drosophila Husbandry and Stocks

The *D. mauritiana* isofemale lines used in this study ($N = 32$) were sampled from Mauritius in 2006 by Margarita Womack and kindly provided to us by Prof. Daniel Matute from the University of North Carolina, Chapel Hill. We also obtained four *D. simulans* stocks (lines 196, 297, 298, and 299) from the National *Drosophila* Species Stock Center that were sampled from Madagascar. Stocks were maintained on modified version of the standard Bloomington-cornmeal medium (Bloomington Stock Center, Bloomington, IN) and were kept at 25°C, 12 light:12 dark photoperiod prior to the start of our experiments.

92 **Determining *Wolbachia* infection status and comparing infection frequencies**
93 One to two generations prior to our experiments DNA was extracted from each isofemale line
94 using a standard 'squish' buffer protocol (Gloor et al. 1993), and infection status was determined
95 using a polymerase chain reaction (PCR) assay (Simpliamp ThermoCycler, Applied Biosystems,
96 Singapore). We amplified the *Wolbachia*-specific *wsp* gene (Forward: 5'-
97 TGGTCCAATAAGTGATGAAGAAAC-3'; Reverse: 5'-AAAAATTAAACGCTACTCCA-3';
98 Braig et al. 1998) and a nuclear control region of the *2L* chromosome (Forward: 5'-
99 TGCAGCTATGGTCGTTGACA-3'; Reverse: 5'-ACGAGACAATAATATGTGGTGCTG-3';
100 designed here). PCR products were visualized using 1% agarose gels that included a molecular-
101 weight ladder. Assuming a binomial distribution, we estimated exact 95% binomial confidence
102 intervals for the infection frequencies on Mauritius. Using Fisher's Exact Test, we tested for
103 temporal differences in *w*Mau frequencies by comparing our frequency estimate to a previous
104 estimate (Giordano et al. 1995). All analyses were performed using R version 3.5.1 (R Team
105 2015).
106 We used quantitative PCR (qPCR) (MX3000P, Agilent Technologies, Germany) to confirm
107 that tetracycline-treated flies were cleared of *w*Mau. DNA was extracted from *D. mauritiana*
108 flies after four generations of tetracycline treatment (1-2 generations prior to completing our
109 experiments), as described below. Our qPCR used a PowerUp™ SYBR™ Green Master Mix
110 (Applied Biosystems™, California, USA) and amplified *Wolbachia*-specific *wsp* (Forward: 5'-
111 CATTGGTGTTGGTGTTGGTG-3'; Reverse: 5'-ACCGAAATAACGAGCTCCAG-3') and
112 *Rpl32* as a nuclear control (Forward: 5'-CCGCTTCAAGGGACAGTATC-3'; Reverse: 5'-
113 CAATCTCCTTGCGCTTCTTG-3';
114 Newton and Sheehan 2014).
115
116 ***Wolbachia* DNA extraction, library preparation, and sequencing**
117 We sequenced *w*Mau-infected *R9*, *R29*, and *R60 D. mauritiana* genotypes. Tissue samples for
118 genomic DNA were extracted using a modified CTAB Genomic DNA Extraction protocol. DNA
119 quantity was tested on an Implen Nanodrop (Implen, München, Germany) and total DNA was
120 quantified by Qubit Fluorometric Quantitation (Invitrogen, Carlsbad, California, USA). DNA
121 was cleaned using Agencourt AMPure XP beads (Beckman Coulter, Inc., Brea, CA, U.S.A),
122 following manufacturers' instructions, and eluted in 50 µl 1× TE Buffer for shearing. DNA was

123 sheared using a Covaris E220 Focused Ultrasonicator (Covaris Inc., Woburn, MA) to a target
124 size of 400 bp. We prepared libraries using NEBNext® Ultra™ II DNA Library Prep with
125 Sample Purification Beads (New England BioLabs, Ipswich, Massachusetts). Final fragment
126 sizes and concentrations were confirmed using a TapeStation 2200 system (Agilent, Santa Clara,
127 California). We indexed samples using NEBNext® Multiplex Oligos for Illumina® (Index
128 Primers Set 3 & Index Primers Set 4), and 10 µl of each sample was shipped to Novogene
129 (Sacramento, CA) for sequencing using Illumina HiSeq 4000 (San Diego, CA), generating
130 paired-end, 150 bp reads.
131
132 ***Wolbachia* assembly**
133 We obtained published reads ($N = 6$) from Garrigan et al. (2014), and assembled these genomes
134 along with the *R9*, *R29*, and *R60* genomes that we sequenced. Reads were trimmed using Sickle
135 v. 1.33 (Joshi and Fass 2011) and assembled using ABySS v. 2.0.2 (Jackman et al. 2017). *K*
136 values of 41, 51, 61, and 71 were used, and scaffolds with the best nucleotide BLAST matches to
137 known *Wolbachia* sequences with E-values less than 10^{-10} were extracted as the draft *Wolbachia*
138 assemblies. We deemed samples infected if the largest *Wolbachia* assembly was at least 1
139 million bases and uninfected if the largest assembly was fewer than 100,000 bases. No samples
140 produced *Wolbachia* assemblies between 100,000 and 1 million bases. Of the six sets of
141 published reads we analyzed (Garrigan et al. 2014), only lines *R31* and *R41* were *w*Mau-infected.
142 We also screened the living copies of these lines for *wsp* using PCR, and both were infected,
143 supporting reliable *w*Mau transmission in the lab since these lines were sampled in nature.
144 To assess the quality of our draft assemblies, we used BUSCO v. 3.0.0 to search for
145 homologs of the near-universal, single-copy genes in the BUSCO proteobacteria database
146 (Simao et al. 2015). As a control, we performed the same search using the reference genomes for
147 *w*Ri (Klasson et al. 2009), *w*Au (Sutton et al. 2014), *w*Mel (Wu et al. 2004), *w*Ha (Ellegaard et
148 al. 2013), and *w*No (Ellegaard et al. 2013).
149
150 ***Wolbachia* gene extraction and phylogenetics**
151 To determine phylogenetic relationships and estimate divergence times, we obtained the public
152 *Wolbachia* group-B genomes of: *w*AlbB that infects *Aedes albopictus* (Mavingui et al. 2012),
153 *w*Pip_Pel that infects *Culex pipiens* (Klasson et al. 2008), *w*Pip_Mol that infects *Culex molestus*

154 (Pinto et al. 2013), *w*No that infects *Drosophila simulans* (Ellegaard et al. 2013), and *w*VitB that
155 infects *Nasonia vitripennis* (Kent et al. 2011); in addition to group-A genomes of: *w*Mel that
156 infects *D. melanogaster* (Wu et al. 2004), *w*Suz that infects *D. suzukii* (Siozios et al. 2013), four
157 *Wolbachia* that infect *Nomada* bees (*w*NFe, *w*NPa, *w*NLeu, and *w*NFa; Gerth and Bleidorn
158 2016), and three *Wolbachia* that infect *D. simulans* (*w*Ri, *w*Au and *w*Ha; Klasson *et al*. 2009;
159 Sutton *et al*. 2014; Ellegaard *et al*. 2013). The previously published genomes and the five *w*Mau-
160 infected *D. mauritiana* genomes were annotated with Prokka v. 1.11, which identifies homologs
161 to known bacterial genes (Seemann 2014). To avoid pseudogenes and paralogs, we used only
162 genes present in a single copy, and with no alignment gaps, in all of the genome sequences.
163 Genes were identified as single copy if they uniquely matched a bacterial reference gene
164 identified by Prokka v. 1.11. By requiring all homologs to have identical length in all of the draft
165 *Wolbachia* genomes, we removed all loci with indels. 143 genes, a total of 113,943 bp, met these
166 criteria when comparing all of these genomes. However, when our analysis was restricted to the
167 five *w*Mau genomes, our criteria were met by 694 genes, totaling 704,613 bp. Including *w*No
168 with the five *w*Mau genomes reduced our set to 671 genes with 682,494 bp. We calculated the
169 percent differences for the three codon positions within *w*Mau and between *w*Mau and *w*No.
170 We estimated a Bayesian phylogram of the *Wolbachia* sequences with RevBayes 1.0.8
171 under the GTR + Γ model, partitioning by codon position (Höhna et al. 2016). Four independent
172 runs were performed, which all agreed.
173 We estimated a chronogram from the *Wolbachia* sequences using the absolute chronogram
174 procedure implemented in Turelli et al. (2018). Briefly, we generated a relative relaxed-clock
175 chronogram with the GTR + Γ model with the root age fixed to 1 and the data partitioned by
176 codon position. The relaxed clock branch rate prior was Γ(2,2). We used substitution-rate
177 estimates of $\Gamma(7,7) \times 6.87\times10^{-9}$ substitutions/3rd position site/year to transform the relative
178 chronogram into an absolute chronogram. This rate estimate was chosen so that the upper and
179 lower credible intervals matched the posterior distribution estimated by Richardson et al. (2012),
180 assuming 10 generations/year, normalized by their median estimate of 6.87×10^{-9} substitutions/3rd
181 position site/year. Although our relaxed-clock analyses allow for variation in substitution rates
182 across branches, our conversion to absolute time depends on the unverified assumption that the
183 median substitution rate estimated by Richardson et al. (2012) for *w*Mel is relevant across these
184 broadly diverged *Wolbachia*. (To assess the robustness of our conclusions to model assumptions,

185 we also performed a strict-clock analysis and a relaxed-clock analysis with branch-rate prior
186 Γ(7,7).) For each analysis, four independent runs were performed, which all agreed. Our analyses
187 all support *w*No as sister to *w*Mau.
188 We also estimated a relative chronogram for the host species using the procedure
189 implemented in Turelli et al. (2018). Our host phylogeny was based on the same 20 nuclear
190 genes used in Turelli et al. (2018): *aconitase, aldolase, bicoid, ebony, enolase, esc, g6pdh, glyp,*
191 *glys, ninaE, pepck, pgi, pgm, pic, ptc, tpi, transaldolase, white, wingless* and *yellow*.
192
193 **Analysis of *Wolbachia* and mitochondrial genomes**
194 We looked for copy number variation (CNV) between *w*Mau and its closest relative, *w*No across
195 the whole *w*No genome. Reads from the five infected *w*Mau lines were aligned to the *w*No
196 reference (Ellegaard et al. 2013) with bwa 0.7.12 (Li and Durbin 2009). We calculated the
197 normalized read depth for each alignment over sliding 1,000-bp windows by dividing the
198 average depth in the window by the average depth over the entire *w*No genome. The results were
199 plotted and visually inspected for putative copy number variants (CNVs). The locations of CNVs
200 were specifically identified with ControlFREEC v. 11.5 (Boeva et al. 2012), using a ploidy of
201 one and a window size of 1,000. We calculated *P*-values for each identified CNV with the
202 Wilcoxon Rank Sum and the Kolmogorov-Smirnov tests implemented in ControlFREEC.
203 We used BLAST to search for pairs of CI-factor (*cif*) homologs in *w*Mau and *w*No genomes
204 that are associated with CI (Beckmann and Fallon 2013; Beckmann et al. 2017; LePage et al.
205 2017; Lindsey et al. 2018; Beckmann et al. 2019). (We adopt Beckmann et al. (2019)'s
206 nomenclature that assigns names to loci based on their predicted enzymatic function, with
207 superscripts denoting the focal *Wolbachia* strain.) These include predicted CI-inducing
208 deubiquitylase (*cid*) *w*Pip_0282-*w*Pip_0283 ($cidA$-$cidB^{wPip}$) and CI-inducing nuclease (*cin*)
209 wPip_0294-wPip_0295 ($cinA$-$cinB^{wPip}$) pairs that induce toxicity and rescue when expressed/co-
210 expressed in *Saccharomyces cerevisiae* (Beckmann et al. 2017 and Beckmann et al. 2019);
211 WD0631-WD632 ($cidA$-$cidB^{wMel}$) that recapitulate CI when transgenically expressed in *D.*
212 *melanogaster* (LePage *et al*. 2017); and *w*No_RS01055 and *w*No_RS01050 that have been
213 identified as a Type III *cifA*-*cifB* pair in the *w*No genome (LePage et al. 2017; Lindsey et al.
214 2018). *w*No_RS01055 and *w*No_RS01050 are highly diverged from $cidA$-$cidB^{wMel}$ and $cidA$-
215 $cidB^{wPip}$ homologs and from $cinA$-$cinB^{wPip}$; however, this *w*No pair is more similar to *cinA*-

216 *cinB*$^{w\mathrm{Pip}}$ in terms of protein domains, lacking a ubiquitin-like protease domain (Lindsey et al.
217 2018). We refer to these loci as *cinA-cinB*$^{w\mathrm{No}}$.
218 We found only homologs of the *cinA-cinB*$^{w\mathrm{No}}$ pair in *w*Mau genomes, which we extracted
219 from our draft *w*Mau assemblies and aligned with MAFFT v. 7 (Katoh and Standley 2013). We
220 compared *cinA-cinB*$^{w\mathrm{No}}$ to the *w*Mau homologs to identify single nucleotide variants (SNVs)
221 among our *w*Mau assemblies.
222 *D. mauritiana* carry either the *ma*I mitochondrial haplotype, associated with *w*Mau
223 infections, or the *ma*II haplotype (Rousset and Solignac 1995; Ballard 2000a; James and Ballard
224 2000). To determine the mitochondrial haplotype of each *D. mauritiana* line, we assembled the
225 mitochondrial genomes by down-sampling the reads by a factor of 100, then assembling with
226 ABySS 2.0.2 using a *K* value of 71 for our data (150 bp reads) and 35 for the published data (76
227 bp reads) (Garrigan et al. 2014). Down-sampling reads prevents the nuclear genome from
228 assembling but does not inhibit assembly of the mitochondrial genome, which has much higher
229 coverage. We deemed the mitochondrial assembly complete if all 13 protein-coding genes were
230 present on the same contig and in the same order as in *D. melanogaster*. If the first attempt did
231 not produce a complete mitochondrial assembly, we adjusted the down-sampling fraction until a
232 complete assembly was produced for each line.
233 Annotated reference mitochondrial sequences for the *D. mauritiana* mitochondrial
234 haplotypes *ma*I and *ma*II were obtained from Ballard et al. (2000b), and the 13 protein-coding
235 genes were extracted from our assemblies using BLAST and aligned to these references. The
236 *ma*I and *ma*II reference sequences differ at 343 nucleotides over these protein-coding regions.
237 We identified our lines as carrying the *ma*I haplotype if they differed by fewer than five
238 nucleotides from the *ma*I reference and as *ma*II if they differed by fewer than five nucleotides
239 from the *ma*II reference. None of our assemblies differed from both references at five or more
240 nucleotides.
241
242 **_w_Mau phenotypic analyses**
243 Previous analyses have demonstrated that *w*Mau does not cause CI (Giordano et al. 1995). To
244 check the generality of this result, we reciprocally crossed *w*Mau-infected *R31 D. mauritiana*
245 with uninfected *R4* and measured egg hatch. Flies were reared under controlled conditions at
246 25°C for multiple generations leading up to the experiment. We paired 1–2-day-old virgin

247 females with 1–2-day-old males in a vial containing spoons with cornmeal media and yeast
248 paste. After 24 hr, pairs were transferred to new spoons, and this process was repeated for five
249 days. Eggs on each spoon were given 24 hr at 25°C to hatch after flies were removed. To test for
250 CI, we used nonparametric Wilcoxon tests to compare egg hatch between reciprocal crosses that
251 produced at least 10 eggs. All experiments were carried out at 25°C with a 12 light:12 dark
252 photoperiod.

253 To determine if *w*Mau generally enhances *D. mauritiana* fecundity, we assessed the
254 fecundity of two *w*Mau-infected isofemale lines from Mauritius (*R31* and *R41*); we also
255 reciprocally introgressed *w*Mau from each of these lines to assess host effects. To do this we
256 crossed *R31* females with *R41* males and backcrossed F_1 females to *R41* males—this was
257 repeated for four generations to generate the reciprocally introgressed $R41^{R31}$ genotypes (*w*Mau
258 variant denoted by superscripts). A similar approach was taken to generate $R31^{R41}$ genotypes.
259 This approach has previously revealed *D. teissieri*-host effects on *w*Tei-induced CI (Cooper et al.
260 2017). To assay fecundity, we reciprocally crossed each genotype (*R31, R41,* $R31^{R41}$, $R31^{R41}$) to
261 uninfected line *R4* to generate paired infected- and uninfected-F_1 females with similar genetic
262 backgrounds. The *w*Mau-infected and uninfected F_1 females were collected as virgins and placed
263 in holding vials. We paired 3–7-day-old females individually with an uninfected-*R4* male (to
264 stimulate oviposition) in vials containing a small spoon filled with standard cornmeal medium
265 and coated with a thin layer of yeast paste. We allowed females to lay eggs for 24 hours, after
266 which pairs were transferred to new vials. This was repeated for five days. At the end of each 24-
267 hr period, spoons were frozen until counted. All experiments were carried out at 25°C with a 12
268 light:12 dark photoperiod.

269 We also measured egg lay of *w*Mau-infected (*R31*) and tetracycline-cleared uninfected
270 (*R31-tet*) genotypes over 24 days, on apple-agar plates, to more closely mimic the methods of
271 Fast et al. (2011). We fed flies 0.03% tetracycline concentrated medium for four generations to
272 generate the *R31-tet* genotype. We screened F_1 and F_2 individuals for *w*Mau using PCR, and we
273 then fed flies tetracycline food for two additional generations. In the fourth generation, we
274 assessed *w*Mau titer using qPCR to confirm that each genotype was cleared of *w*Mau infection.
275 We reconstituted the gut microbiome by rearing *R31-tet* flies on food where *R31* males had fed
276 and defecated for 48 hours. Flies were given at least three more generations to avoid detrimental
277 effects of tetracycline treatment on mitochondrial function (Ballard and Melvin 2007). We then

278 paired individual 6–7-day-old virgin *R31* (*N* = 30) and *R31-tet* (*N* = 30) females in bottles on
279 yeasted apple-juice agar plates with an *R4* male to stimulate oviposition. Pairs were placed on
280 new egg-lay plates every 24 hrs. After two weeks, we added one or two additional *R4* males to
281 each bottle to replace any dead males and to ensure that females were not sperm limited as they
282 aged.
283 We used nonparametric Wilcoxon tests to assess *w*Mau effects on host fecundity. We then
284 estimated the fitness parameter *F* in the standard discrete-generation model of CI (Hoffmann et
285 al. 1990; Turelli 1994). We used the '*pwr.t2n.test*' function in the '*pwr*' library in R to assess the
286 power of our data to detect increases to *F*. Pairs that laid fewer than 10 eggs across each
287 experiment were excluded from analyses, but our results are robust to this threshold.
288 To estimate the fidelity of maternal transmission, *R31* and *R41* females were reared at 25°C
289 for several generations prior to our experiment. In the experimental generation, 3-5 day old
290 inseminated females were placed individually in vials that also contained two males. These *R31*
291 (*N* = 17) and *R41* (*N* = 19) sublines were allowed to lay eggs for one week. In the following
292 generation we screened F1 offspring for *w*Mau infection using PCR as described above.
293
294 **RESULTS**
295 ***Wolbachia* infection status**
296 Out of 32 *D. mauritiana* lines that we analyzed, 11 were infected with *w*Mau *Wolbachia*
297 (infection frequency = 0.34; binomial confidence interval: 0.19, 0.53). In contrast, none of the *D.*
298 *simulans* stocks (*N* = 4) sampled from Madagascar were infected, precluding our ability to
299 directly compare *w*Mau and *w*Ma. Our new *w*Mau frequency estimate is not statistically different
300 from a previous estimate (Giordano et al. 1995: infection frequency, 0.46; binomial confidence
301 interval, (0.34, 0.58); Fisher's Exact Test, *P* = 0.293), based largely on assaying a heterogenous
302 collecton of stocks in various laboratories. These relatively low infection frequencies are
303 consistent with theoretical expectations given that *w*Mau does not cause CI (Giordano et al.
304 1995; our data reported below). The intermediate *w*Mau frequencies on Mauritius suggest that
305 *w*Mau persists at a balance between positive effects on host fitness and imperfect maternal
306 transmission. Quantitative predictions, based on the idealized model of Hoffmann and Turelli
307 (1997), are discussed below. The maintenance of *w*Mau is potentially analogous to the
308 persistence of other non-CI-causing *Wolbachia*, specifically *w*Au in some Australian populations

309 of *D. simulans* (Hoffmann et al. 1996; Kriesner et al. 2013) and *w*Suz in *D. suzukii* and *w*Spc in
310 *D. subpulchrella* (Hamm et al. 2014; Conner et al. 2017; Turelli et al. 2018; but see Cattel et al.
311 2018).
312
313 **Draft *w*Mau genome assemblies and comparison to *w*No**
314 The five *w*Mau draft genomes we assembled were of very similar quality (Supplemental Table
315 1). N50 values ranged from 60,027 to 63,676 base pairs, and our assemblies varied in total length
316 from 1,266,004 bases to 1,303,156 bases (Supplemental Table 1). Our BUSCO search found
317 exactly the same genes in each draft assembly, and the presence/absence of genes in our *w*Mau
318 assemblies was comparable to those in the complete genomes used as controls (Supplemental
319 Table 2). In comparing our five *w*Mau draft genomes over 694 single-copy, equal-length loci
320 comprising 704,613 bp, we found only one SNP. Four sequences (*R9*, *R31, R41* and *R60*) are
321 identical at all 704,613 bp. *R29* differs from them at a single nucleotide, a nonsynonymous
322 substitution in a locus which Prokka v. 1.11 annotates as "bifunctional DNA-directed RNA
323 polymerase subunit beta/beta."
324 Comparing these five *w*Mau sequences to the *w*No reference (Ellegaard et al. 2013) over
325 671 genes with 682,494 bp, they differ by 0.068% overall, with equivalent divergence at all three
326 codon positions (0.067%, 0.061%, and 0.076%, respectively).
327
328 ***Wolbachia* phylogenetics**
329 As expected from the sequence comparisons, our group-B phylogram places *w*Mau sister to *w*No
330 (Figure 1A). This is consistent with previous analyses using fewer loci that placed *w*Mau (or
331 *w*Ma in *D. simulans*) sister to *w*No (James and Ballard 2000; Zabalou et al. 2008; Toomey et al.
332 2013). Our chronogram (Figure 1B) estimates the 95% credible interval for the split between the
333 group-B versus group-A *Wolbachia* strains as 6 to 36 mya (point estimate, 16 mya). Reducing
334 the variance on the substitution-rate-variation prior by using Γ(7,7) rather than Γ(2,2), changes
335 the credible interval for the A-B split to 8 to 46 mya (point estimate, 21 mya). In contrast, a strict
336 clock analysis produces a credible interval of 12 to 64 mya (point estimate, 31 mya). These
337 estimates are roughly comparable to an earlier result based on a general approximation for the
338 synonymous substitution rate in bacteria (Ochman and Wilson 1987) and data from only the *ftsZ*
339 locus (59–67 mya, Werren et al. 1995). However, our estimates are much lower than an

alternative estimate based on comparative genomics (217 mya, Gerth and Bleidorn 2016). We discuss this discrepancy below.

The observed divergence between *w*No and *w*Mau is consistent across all three codon positions, similar to other recent *Wolbachia* splits like that between *w*Ri and *w*Suz (Turelli et al. 2018). Conversely, observed divergence at each codon position generally varies across the chronogram, leading to inflation of the *w*No-*w*Mau (181,476 years; credible interval = 27,711 to 701,205 years; Figure 1B) and *w*Ri-*w*Suz (16,214; credible interval = 1,254 to 70,584) divergence point estimates; the latter is about 1.6 times as large as the value in Turelli et al. (2018). (Nevertheless, the confidence intervals of our and Turelli et al. (2018)'s *w*Ri-*w*Suz divergence estimates overlap.) To obtain an alternative estimate of *w*No-*w*Mau divergence, we estimated divergence time using the observed third-position pairwise divergence (0.077%, or 0.039% from tip to MRCA) and Richardson et al. (2012)'s estimate of the "short-term evolutionary rate" of *Wolbachia* third-position divergence within *w*Mel. This approach produces a point estimate of 57,000 years, with a credible interval of 30,000 to 135,000 years for the *w*No-*w*Mau split. In Cooper et al. (2019), we address in detail how a constant substitution-rate ratio among codon positions across the tree, assumed by the model, affects these estimates.

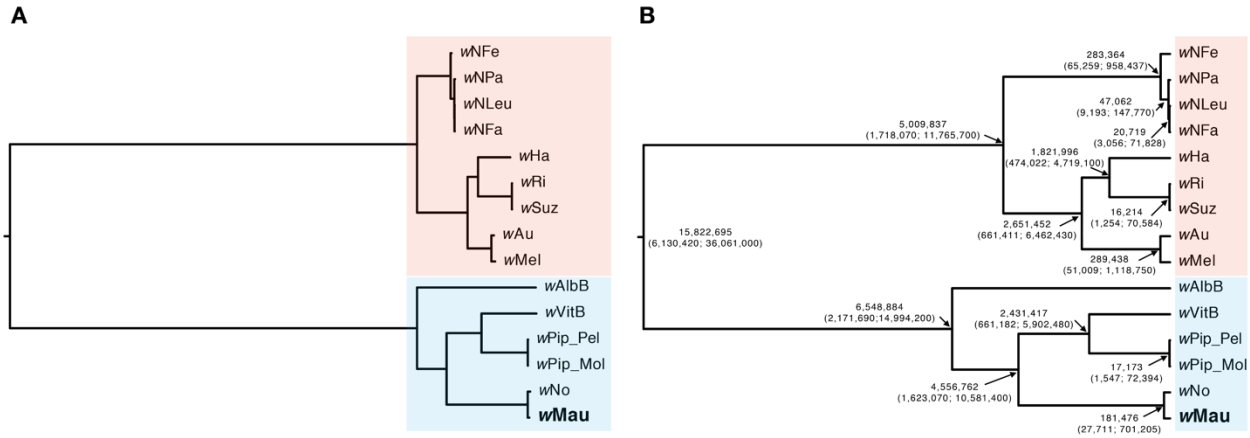
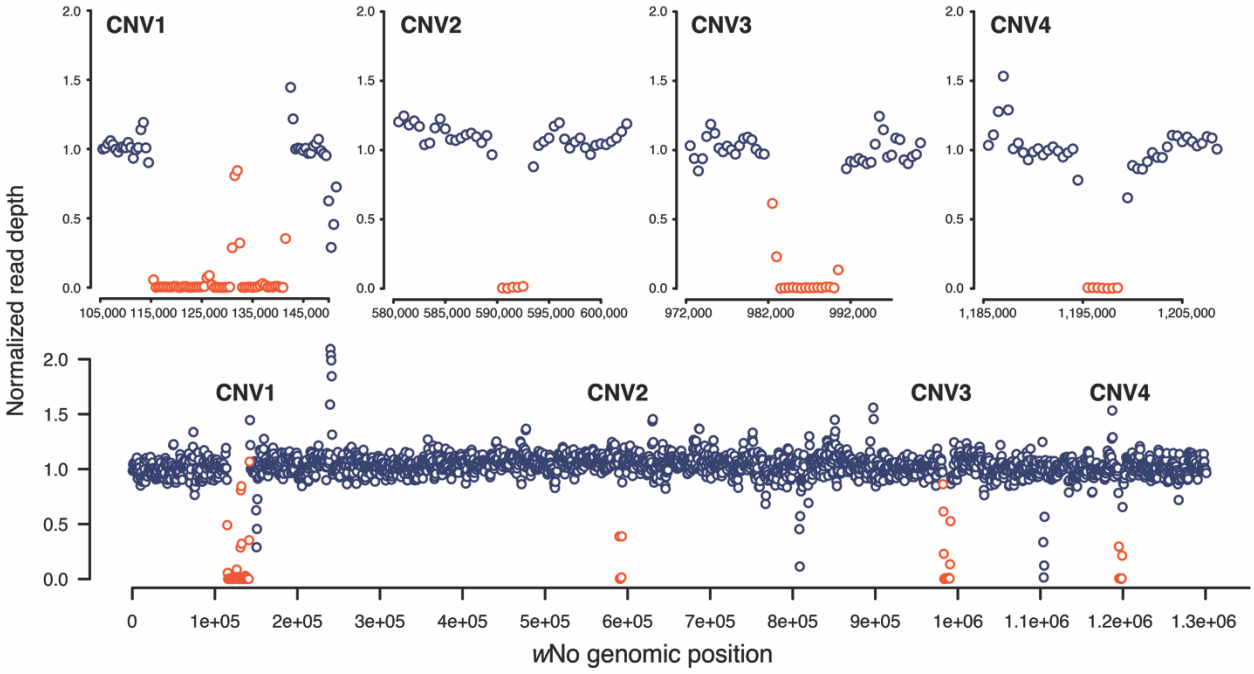


Figure 1. A) An estimated phylogram for various group-A (red) and group-B (blue) *Wolbachia* strains. All nodes have Bayesian posterior probabilities of 1. The phylogram shows significant variation in the substitution rates across branches, with long branches separating the A and B clades. **B)** An estimated chronogram for the same strains, with estimated divergence times and their confidence intervals at each node. To obtain these estimates, we generated a relative relaxed-clock chronogram with the GTR + Γ model with the root age fixed to 1, the data partitioned by codon position, and with a Γ(2,2) branch rate prior. We used substitution-rate estimates of $\Gamma(7,7) \times 6.87\times10^{-9}$ substitutions/3rd position site/year to transform the relative chronogram into an absolute chronogram.

366 **Analysis of *Wolbachia* and mitochondrial genomes**

367 We looked for CNVs in *w*Mau relative to sister *w*No by plotting normalized read depth along the
368 *w*No genome. There were no differences in copy number among the *w*Mau variants, but
369 compared to *w*No, ControlFREEC identified four regions deleted from all *w*Mau that were
370 significant according to the Wilcoxon Rank Sum and Kolmogorov-Smirnov tests (Figure 2 and
371 Supplemental Table 3). These deleted regions of the *w*Mau genomes include many genes,
372 including many phage-related loci, providing interesting candidates for future work (listed in
373 Supplementary Table 4).

374



375

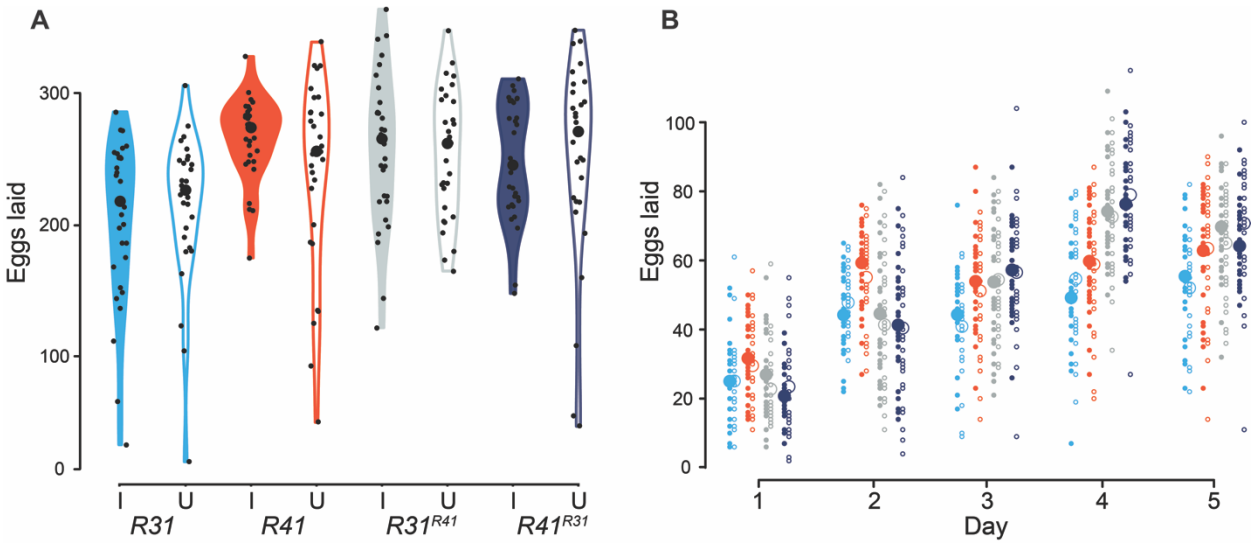
376 **Figure 2.** All *w*Mau variants share four large deletions, relative to sister *w*No. Top panel) The
377 normalized read depth for *w*Mau *R60* plotted across the four focal regions of the *w*No reference
378 genome; 10 kb of sequence surrounding regions are plotted on either side of each region. Bottom
379 panel) The normalized read depth of *w*Mau *R60* plotted across the whole *w*No reference genome.
380 Regions that do not contain statistically significant CNVs are plotted in dark blue, and regions
381 with significant CNVs are plotted in red. All *w*Mau variants share the same CNVs, relative to
382 *w*No.

383

384 To test the hypothesis that *cif* loci are disrupted, we searched for pairs of loci known to be
385 associated with CI and found homologs to the *cinA-cinB*^{wNo} pair in each of our draft assemblies,
386 but we did not find homologs to the *cidA-cidB*^{wMel}, *cidA-cidB*^{wPip}, or to the *cinA-cinB*^{wPip} pairs.
387 There were no variable sites in *cinA-cinB*^{wNo} homologs among our five *w*Mau assemblies.

388 Relative to *w*No, all *w*Mau variants share a one base pair deletion at base 1133 out of 2091
389 (amino acid 378) in the *cinB*^{wNo} homolog. This frameshift introduces over 10 stop codons, with
390 the first at amino acid 388, potentially making this predicted CI-causing-toxin protein
391 nonfunctional. We also identified a nonsynonymous substitution in amino acid 264 of the
392 *cinB*^{wNo} homolog (*w*No codon ACA, Thr; *w*Mau codon AUA, Ile) and two SNVs in the region
393 homologous to *cinA*^{wNo}: a synonymous substitution in amino acid 365 (*w*No codon GUC, *w*Mau
394 codon GUU) and a nonsynonymous substitution in amino acid 397 (*w*No codon GCU, amino
395 acid Ala; *w*Mau codon GAU, amino acid Asp). Disruption of CI is consistent with theoretical
396 analyses showing that selection within a host species does not act directly on the level of CI
397 (Prout 1994; Turelli 1994; Haygood and Turelli 2009). Future functional analyses will determine
398 whether disruption of regions homologous to *cinA*-*cinB*^{wNo} underlie the lack of *w*Mau CI.
399 Of the *D. mauritiana* lines tested (*N* = 9), one line (uninfected-*R44*) carries the *ma*II
400 mitochondrial haplotype, while the other eight carry *ma*I. Rousset and Solignac (1995) reported a
401 similar *ma*II frequency, with 3 of 26 lines sampled in 1985 carrying *ma*II. The *ma*I and *ma*II
402 references differ by 343 SNVs across the proteome, and *R44* differs from the *ma*II reference by 4
403 SNVs in the proteome. Four of our *ma*I lines (*R23*, *R29*, *R32*, and *R39*) are identical to the *ma*I
404 reference, while three (*R31*, *R41*, and *R60*) have one SNV and one (*R9*) has two SNVs relative to
405 *ma*I reference. One SNV is shared between *R9* and *R60*, but the other three SNVs are unique.
406 Our results agree with past analyses that found *w*Mau is perfectly associated with the *ma*I
407 mitochondrial haplotype (Rousset and Solignac 1995; Ballard 2000a; James and Ballard 2000).
408 The presence of *ma*II among the uninfected is interesting. In contrast to *ma*I, which is associated
409 with introgression with *D. simulans* (Ballard 2000a; James and Ballard 2000), *ma*II appears as an
410 outgroup on the mtDNA phylogeny of the *D. simulans* clade and is not associated with
411 *Wolbachia* (Ballard 2000b, Fig. 5; James and Ballard 2000). Whether or not *Wolbachia* cause CI,
412 if they are maintained by selection-imperfect-transmission balance, we expect all uninfected flies
413 to eventually carry the mtDNA associated with infected mothers (Turelli et al. 1992). We present
414 a mathematical analysis of the persistence of *ma*II below.
415
416 **Analysis of *w*Mau phenotypes**
417 In agreement with Giordano et al. (1995), we found no difference between the egg hatch of
418 uninfected females crossed to *w*Mau-infected males (0.34 ± 0.23 SD, *N* = 25) and the reciprocal

419 cross (0.29 ± 0.28 SD, $N = 24$), indicating no CI. In contrast to Fast et al. (2011), we find no
420 evidence that *w*Mau affects *D. mauritiana* fecundity (Supplemental Table 5 and Figure 3),
421 regardless of host genetic backgrounds. Across both experiments assessing *w*Mau fecundity
422 effects in their natural backgrounds (*R31* and *R41*), we counted 27,221 eggs and found no
423 difference in the number of eggs laid by infected (mean = 238.20, SD = 52.35, $N = 60$) versus
424 uninfected (mean = 226.82, SD = 67.21, $N = 57$) females over the five days of egg lay (Wilcoxon
425 test, $W = 1540.5$, $P = 0.357$); and across both experiments that assessed *w*Mau fecundity effects
426 in novel host backgrounds ($R31^{R41}$ and $R41^{R31}$), we counted 30,358 eggs and found no difference
427 in the number of eggs laid by infected (mean = 253.30, SD = 51.99, $N = 60$) versus uninfected
428 (mean = 252.67, SD = 63.53, $N = 60$) females over five days (Wilcoxon test, $W = 1869.5$, $P =$
429 0.719). [The mean number of eggs laid over five days, standard deviation (SD), sample size (N),
430 and P-values from Wilcoxon tests are presented in Supplemental Table 5 for all pairs.]
431



432

433 **Figure 3.** *w*Mau infections do not influence *D. mauritiana* fecundity, regardless of host genomic
434 background. **A)** Violin plots of the number of eggs laid by *D. mauritiana* females over five days
435 when infected with their natural *w*Mau variant (*R31I* and *R41I*), when infected with a novel
436 *w*Mau variant ($R31^{R41}I$ and $R41^{R31}I$), and when uninfected (*R31U*, *R41U*, $R31^{R41}U$, and
437 $R41^{R31}U$). Large black dots are medians, and small black dots are the eggs laid by each replicate
438 over five days. **B)** The daily egg lay of these same infected (solid circles) and uninfected (open
439 circles) *R31* (aqua), *R41* (red), $R31^{R41}$ (gray), and $R41^{R31}$ (dark blue) genotypes is reported. Large
440 circles are means of all replicates, and small circles are the raw data. Only days where females
441 laid at least one egg are plotted. Cytoplasm sources are denoted by superscripts for the
442 reciprocally introgressed strains.
443
444

We sought to determine if *w*Mau fecundity effects depend on host age with a separate experiment that assessed egg lay over 24 days on apple-agar plates, similar to Fast et al. (2011). Across all ages, we counted 9,459 eggs and found no difference in the number of eggs laid by infected (mean = 156.29, SD = 138.04, $N = 28$) versus uninfected (mean = 187.70, SD = 168.28, $N = 27$) females (Wilcoxon test, $W = 409$, $P = 0.608$) (Figure 4). While our point estimates indicate that *w*Mau does not increase host fecundity, egg lay was generally lower and more variable on agar plates relative to our analyses of egg lay on spoons described above.

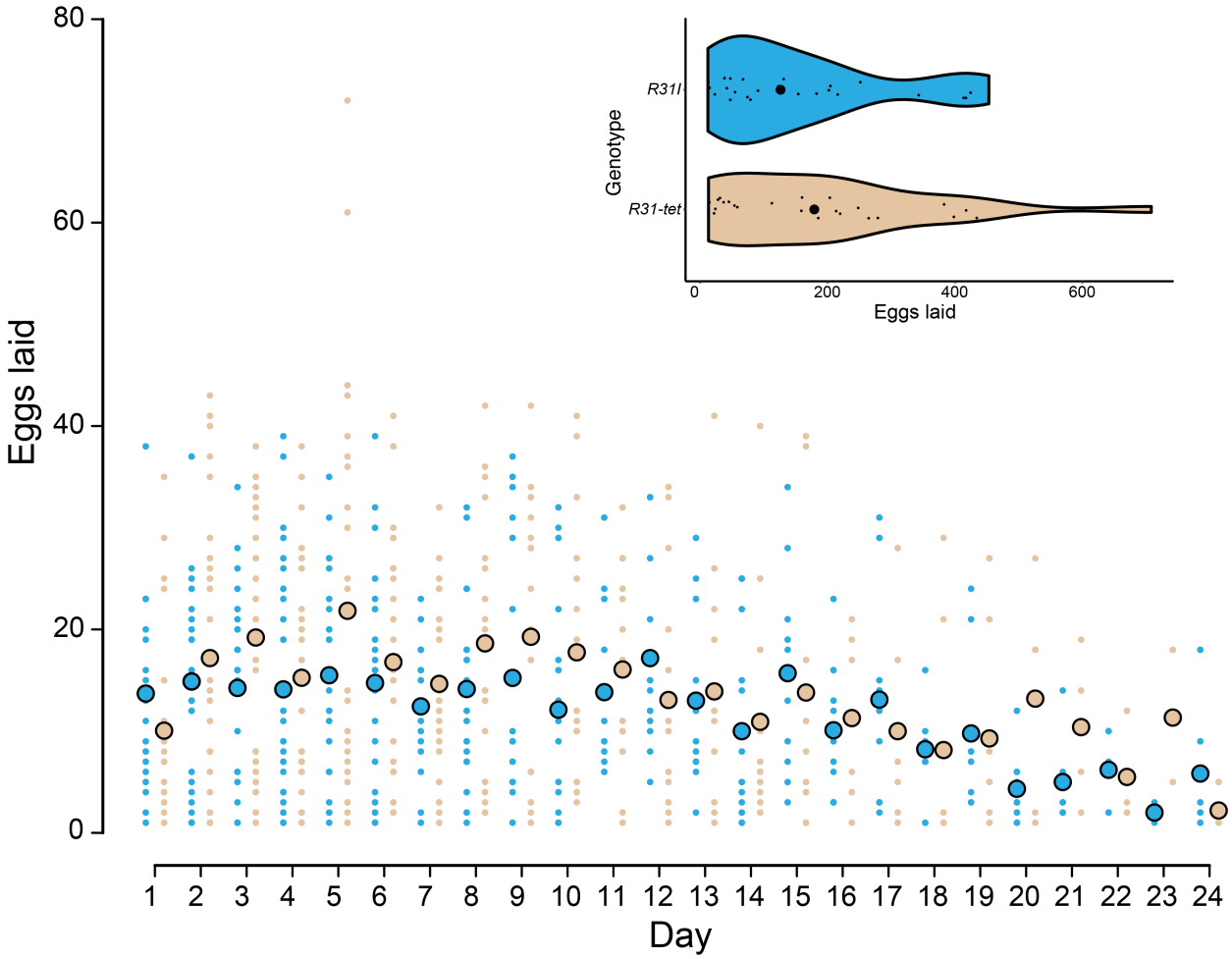


Figure 4. The mean number of eggs laid by infected *R31* (*R31I*, large aqua dots) and uninfected *R31-tet* (large tan dots) genotypes are similar. Egg counts for each replicate are also plotted (small dots). Violin plots show egg lay across all ages for each genotype; large black circles are medians, and small black circles are the number of eggs laid by each replicate.

461 With these data, we estimated the fitness parameter F in the standard discrete-generation
462 model of CI (Hoffmann et al. 1990; Turelli 1994). Taking the ratio of replicate mean fecundity
463 observed for *w*Mau-infected females to the replicate mean fecundity of uninfected females in
464 naturally sampled *R31* and *R41 D. mauritiana* backgrounds, we estimated $F = 1.05$ (95% BC_a
465 interval: 0.96, 1.16). Following reciprocal introgression of *w*Mau and host backgrounds (i.e., the
466 $R31^{R41}$ and $R41^{R31}$ genotypes), we estimated $F = 1.0$ (95% BC_a interval: 0.93, 1.09). Finally,
467 across all 24 days of our age-effects experiment, we estimated $F = 0.83$, 95% BC_a interval: 0.52,
468 1.32) for *R31*, which overlaps with our estimate of F for *R31* in our initial experiment (Table 1).
469 BC_a confidence intervals were calculated using the two-sample acceleration constant given by
470 equation 15.36 of Efron and Tibshirani (1993). (Estimates of F and the associated BC_a
471 confidence intervals are reported in Table 1 for each genotype and condition.) Consistent with
472 our other analyses, we find little evidence that *w*Mau significantly increases fecundity. However,
473 our data do not have much statistical power to detect values of F on the order of 1.05, which may
474 suffice to produce $F(1 - \mu) > 1$ and deterministic spread of *w*Mau from low frequencies. We
475 present our power calculations in Figure 1B of the Supplementary Information.
476

Table 1. Estimates of the relative fitness parameter F indicate that *w*Mau fecundity effects are likely to be minimal.

***w*Mau variant/age class**	***F***	**95% BC_a interval**
R31	0.988	(0.862, 1.137)
R41	1.107	(0.995, 1.265)
$R31^{R41}$	1.012	(0.911, 1.122)
$R41^{R31}$	0.992	(0.884, 1.143)
R31(across age)	0.833	(0.515, 1.323)

477
478
479 Finally, we assessed the fidelity of *w*Mau maternal transmission under standard laboratory
480 conditions. We excluded sublines that produced fewer than 8 F1 offspring. In all cases, *R31* ($N =$
481 17) sublines produced offspring that were all infected, indicating perfect maternal transmission.
482 In contrast, one *R41* subline produced one uninfected individual out of a total of 18 F1 offspring
483 produced; all other *R41* sublines meeting our criteria ($N = 15$) produced only infected F1
484 offspring, resulting in nearly perfect maternal transmission across all *R41* sublines ($\mu = 0.0039$).

485 **Mathematical analyses of *Wolbachia* frequencies and mtDNA polymorphism**

486 If *Wolbachia* do not cause CI (or any other reproductive manipulation), their dynamics can be
487 approximated by a discrete-generation haploid selection model. Following Hoffmann and Turelli
488 (1997), we assume that the relative fecundity of *Wolbachia*-infected females is F, but a fraction
489 μ of their ova do not carry *Wolbachia*. Given our ignorance of the nature of *Wolbachia*'s
490 favorable effects, the F represents an approximation for all fitness benefits. If $F(1 - \mu) > 1$, the
491 equilibrium *Wolbachia* frequency among adults is
492

493 $$\hat{p} = 1 - \frac{\mu F}{F - 1}. \quad (1)$$

494

495 Imperfect maternal transmisson has been documented for field-collected *D. simulans* infected
496 with *w*Ri (Hoffmann and Turelli 1988; Turelli and Hoffmann 1995; Carrington et al. 2011), *D.*
497 *melanogaster* infected with *w*Mel (Hoffmann et al. 1998) and *D. suzukii* infected with *w*Suz
498 (Hamm et al. 2014). The estimates range from about 0.01 to 0.1. Given that we have documented
499 imperfect maternal transmission of *w*Mau in the laboratory, we expect (more) imperfect
500 transmission in nature (Turelli and Hoffmann 1995; Carrington et al. 2011). In order for the
501 equilibrium *Wolbachia* frequency to be below 0.5, approximation (1) requires that the relative
502 fecundity of infected females satisfies
503

504 $$F < \frac{1}{1 - 2\mu}. \quad (2)$$

505

506 Thus, even for μ as large a 0.15, which greatly exceeds our laboratory estimates for *w*Mau and
507 essentially all estimates of maternal transmission failure from nature, *Wolbachia* can increase
508 fitness by at most 43% and produce an equilibrium frequency below 0.5 (Supplemental Figure
509 1A). Conversely, (1) implies that a doubling of relative fecundity by *Wolbachia* would produce
510 an equilibrium frequency $1 - 2\mu$. If $\mu \leq 0.25$, consistent with all available data, the predicted
511 equilibrium implied by a *Wolbachia*-induced fitness doubling significantly exceeds the observed
512 frequency of *w*Mau. Hence, a four-fold fecundity effect, as described by Fast et al. (2011), is
513 inconsistent with the frequency of *w*Mau in natural populations of *D. mauritiana*. Field estimates
514 of μ for *D. mauritiana* will provide better theory-based bounds on *w*Mau fitness effects that

515 would be consistent with *w*Mau tending to increase when rare on Mauritius, i.e., conditions for
516 $F(1 - \mu) > 1$.
517 Our theoretical analysis, addressing the plausibility of a four-fold fitness increase caused by
518 *w*Mau, assumes that the observed frequency of *w*Mau approximates selection-transmission
519 equilibrium, as described by (1). With only two frequency estimates (one from a heterogeneous
520 collection of laboratory stocks), we do not know that the current low frequency is temporarlly
521 stable. Also, we do not know that the mutations we detect in *cinA*-*cinB*$^{w\mathrm{No}}$ homologs are
522 responsible for the lack of *w*Mau CI. One alternative is that *D. mauritiana* has evolved to
523 suppress CI (for host suppression of male killing, see Hornet et al. 2006 and Vanthournout and
524 Hendrickx 2016). Host suppression of CI is expected (Turelli 1994), and it may explain the low
525 CI caused by *w*Mel in *D. melanogaster* (Hoffmann and Turelli 1997). However, the fact that
526 *w*Mau does not produce CI in *D. simulans*, a host that allows *w*Mel and other strains to induce
527 strong CI even though little CI is produced in their native hosts, argues against host suppression
528 as the explanation for the lack of CI caused by *w*Mau in *D. mauritiana*. Nevertheless, the loss of
529 CI from *w*Mau may be quite recent; and *w*Mau may be on its way to elimination in *D.*
530 *mauritiana*. If so, our equilibrium analysis is irrelevant – but this gradual-loss scenario is equally
531 inconsistent with the four-fold fecundity effect proposed by Fast et al. (2011).
532 As noted by Turelli et al. (1992), if *Wolbachia* is introduced into a population along with a
533 diagnostic mtDNA haplotype that has no effect on fitness, imperfect *Wolbachia* maternal
534 transmission implies that all infected and uninfected individuals will eventually carry the
535 *Wolbachia*-associated mtDNA, because all will have had *Wolbachia*-infected maternal ancestors.
536 We conjectured that a stable mtDNA polymorphism might be maintained if *Wolbachia*-
537 associated mtDNA introduced by introgression is deleterious in its new nuclear background. We
538 refute our conjecture in Appendix 1. We show that the condition for *Wolbachia* to increase when
539 rare, $F(1 - \mu) > 1$, ensures that the native mtDNA will be completely displaced by the
540 *Wolbachia*-associated mtDNA, even if it lowers host fitness once separated from *Wolbachia*.
541 How fast is the mtDNA turnover, among *Wolbachia*-uninfected individuals, as a new
542 *Wolbachia* invades? This is easiest to analyze when the mtDNA introduced with *Wolbachia* has
543 no effect on fitness, so that the relative fitness of *Wolbachia*-infected versus uninfected
544 individuals is F, irrespective of the mtDNA haplotype of the uninfected individuals. As shown in

545 Appendix 1, the frequency of the ancestral mtDNA haplotype among uninfected individuals,
546 denoted r_t, declines as
547
548 $$r_{t+1} = r_t/[F(1 - \mu)]. \quad (3)$$
549
550 Assuming $r_0 = 1$, recursion (3) implies that even if $F(1 - \mu)$ is only 1.01, the frequency of the
551 ancestral mtDNA haplotype should fall below 10^{-4} after 1000 generations. A much more rapid
552 mtDNA turnover was seen as the CI-causing *w*Ri swept northward through California
553 populations of *D. simulans* (Turelli et al. 1992; Turelli and Hoffmann 1995). Thus, it is
554 theoretically unexpected, under this simple model, that mtDNA haplotype *ma*II, which seems to
555 be ancestral in *D. mauritiana* (Rousset and Solignac 1995; Ballard 2000a), persists among
556 *Wolbachia*-uninfected *D. mauritiana*, given that all sampled *Wolbachia*-infected individuals
557 carry *ma*I. However, spatial variation in fitnesses is one possible explanation for this
558 polymorphism (Gliddon and Strobeck 1975), which has persisted since at least 1985.
559
560 **DISCUSSION**
561 ***w*Mau is sister to *w*No and diverged from group-A *Wolbachia* less than 100 mya**
562 Our phylogenetic analyses place *w*Mau sister to *w*No, in agreement with past analyses using
563 fewer data (James and Ballard 2000; Zabalou et al. 2008; Toomey et al. 2013). The relationships
564 we infer agree with those from recently published phylograms (Gerth and Bleidorn 2016;
565 Lindsey et al. 2018) (Figure 1A).
566 Depending on the prior used for substitution-rate variation, we estimate that *w*Mau and other
567 group-B *Wolbachia* diverged from group-A strains about 6–46 mya. This is roughly consistent
568 with a prior estimate using only *ftsZ* (58–67 mya, Werren et al. 1995), but is inconsistent with a
569 recent estimate using 179,763 bases across 252 loci (76–460 mya, Gerth and Bleidorn 2016).
570 There are several reasons why we question the Gerth and Bleidorn (2016) calibration. First,
571 Gerth and Bleidorn (2016)'s chronogram placed *w*No sister to all other group-B *Wolbachia*, in
572 disagreement with their own phylogram (Gerth and Bleidorn 2016, Figure 3). In contrast, our
573 phylogram and that of Lindsey et al. (2018) support *w*AlbB splitting from all other strains at this
574 node. Second, the Gerth and Bleidorn (2016) calibration estimated the split between *w*Ri that
575 infects *D. simulans* and *w*Suz that infects *D. suzukii* at 900,000 years. This estimate is more than

576 an order of magnitude higher than ours (16,214 years) and nearly two orders of magnitude higher
577 than the 11,000 year estimate of Turelli et al. (2018) who found 0.014% third position
578 divergence between *w*Ri and *w*Suz (i.e., 0.007% along each branch) over 506,307 bases.
579 Raychoudhury et al. (2009) and Richardson et al. (2012) both estimated a rate of about 7×10^{-9}
580 substitutions/3rd position site/year between *Wolbachia* in *Nasonia* wasps and within *w*Mel,
581 respectively. An estimate of 900,000 years requires a rate about 100 times slower, 7.8×10^{-11}
582 substitutions/3rd position site/year, which seems implausible. Finally, using data kindly provided
583 by Michael Gerth, additional analyses indicate that the third-position rates required for the
584 *Wolbachia* divergence times estimated by Gerth and Bleidorn (2016) between *Nomada flava* and
585 *N. leucophthalma* (1.72×10^{-10}), *N. flava* and *N. panzeri* (3.78×10^{-10}) (their calibration point),
586 and *N. flava* and *N. ferruginata* (4.14×10^{-10}) are each more than 10 times slower than those
587 estimated by Raychoudhury et al. (2009) and Richardson et al. (2012), which seems unlikely.
588 Our analyses suggest that the A-B group split occurred less than 100 mya.
589
590 **The lack of CI is consistent with intermediate *w*Mau infection frequencies**
591 Across 671 genes (682,494 bases), the *w*Mau genomes were identical and differed from *w*No by
592 only 0.068%. Across the coding regions we analyzed, we found few SNVs and no CNVs among
593 *w*Mau variants. Our analyses did identify four large deletions shared by all *w*Mau genomes,
594 relative to *w*No. Despite the close relationship between *w*Mau and *w*No, *w*No causes CI while
595 *w*Mau does not (Giordano et al. 1995; Merçot et al. 1995; Rousset and Solignac 1995, our data).
596 We searched for all pairs of loci known to cause CI and found only homologs to the *cinA*-
597 $cinB^{wNo}$ pair in *w*Mau genomes. All *w*Mau variants share a one-base-pair deletion in the *w*Mau
598 region homologous to $cinB^{wNo}$. This mutation introduces a frameshift and more than ten stop
599 codons. Future functional work will help determine if disruption of this predicted-toxin locus
600 underlies the lack of CI in *w*Mau. Regardless, the lack of CI is consistent with the prediction that
601 selection within host lineages does not directly act on the intensity of CI (Prout 1994; Turelli
602 1994). We predict that analysis of additional non-CI-causing strains will reveal additional
603 examples of genomic remnants of CI loci. Among non-CI *Wolbachia*, the relative frequency of
604 those with non-functional CI loci, versus no CI loci, is unknown.
605 Irrespective of whether CI was lost or never gained, non-CI *Wolbachia* have lower expected
606 equilibrium infection frequencies than do CI-causing variants (Kriesner et al. 2016). The *w*Mau

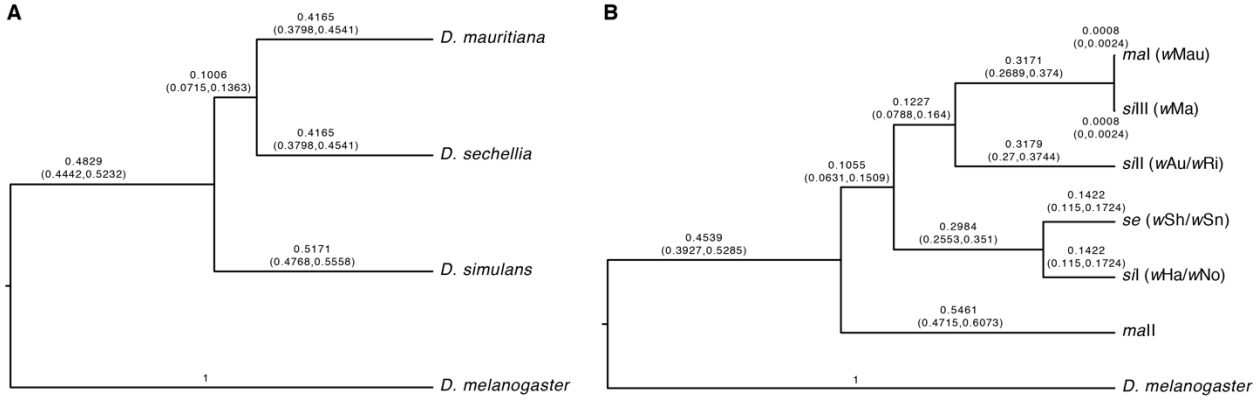
607 infection frequency of approximately 0.34 on Mauritius (Giordano et al. 1995; our data) is
608 consistent with this prediction. Additional sampling of Mauritius, preferably over decades, will
609 determine whether intermediate *w*Mau frequencies are temporally stable. Such temporal stability
610 depends greatly on values of F and μ through time suggesting additional field-based estimates of
611 these parameters will be useful.

612 *w*Mau co-occurs with essentially the same mitochondrial haplotype as *w*Ma that infects *D.*
613 *simulans* on Madagascar and elsewhere in Africa and the South Pacific (Rousset and Solignac
614 1995; Merçot and Poinsot 1998; Ballard 2000a; James and Ballard 2000; James et al. 2002;
615 Ballard 2004), suggesting that *w*Mau and *w*Ma may be the same strain infecting different host
616 species following introgressive *Wolbachia* transfer (see below). *w*Mau and *w*Ma phenotypes are
617 also more similar to one another than to *w*No, with only certain crosses between *w*Ma-infected
618 *D. simulans* males and uninfected *D. simulans* females inducing CI (James and Ballard 2000).
619 Polymorphism in the strength of CI induced by *w*Ma could result from host modification of
620 *Wolbachia*-induced CI (Reynolds and Hoffmann 2002; Cooper et al. 2017), or from *Wolbachia*
621 titer variation that influences the strength of CI and/or the strength of CI rescue by infected
622 females. Alternatively, the single-base-pair deletion in the $cinB^{w\mathrm{No}}$ homolog or other mutations
623 that influence CI strength, could be polymorphic in *w*Ma. *w*Ma infection frequencies in *D.*
624 *simulans* are intermediate on Madagascar (infection frequency = 0.25, binomial confidence
625 intervals: 0.14, 0.40; James and Ballard 2000), consistent with no CI, suggesting replication of
626 rarely observed *w*Ma CI is needed. Including *D. simulans* from the island of Réunion in this
627 infection-frequency further supports the conjecture that *w*Ma causes little or no CI (infection
628 frequency = 0.31, binomial confidence intervals: 0.20, 0.45; James and Ballard 2000).
629 Unfortunately, no Madagascar *D. simulans* stocks available at the National *Drosophila* Species
630 Stock Center were *w*Ma infected, precluding detailed analysis of this strain.

631 Our genomic data indicate that *w*Mau may maintain an ability to rescue CI, as the $cinA^{w\mathrm{No}}$
632 homolog is intact in *w*Mau genomes with only one nonsynonymous substitution relative to
633 $cinA^{w\mathrm{No}}$; *cidA* in *w*Mel was recently shown to underlie transgenic-CI rescue (Shropshire et al.
634 2018). *w*Ma seems to sometimes rescue CI, but conflicting patterns have been found, and
635 additional experiments are needed to resolve this (Rousset and Solignac 1995; Bourtzis et al.
636 1998; Merçot and Poinsot 1998; James and Ballard 2000; Merçot and Poinsot 2003; Zabalou et
637 al. 2008). Future work that tests for CI rescue by *w*Mau and *w*Ma-infected females crossed with

638 males infected with *w*No or other CI-causing strains, combined with genomic analysis of CI loci
639 in *w*Ma, will be useful.
640
641 **_w_Mau does not influence _D. mauritiana_ fecundity**
642 While selection does not directly act on the level of CI (Prout 1994; Turelli 1994; Haygood and
643 Turelli 2009), it does act to increase the product *Wolbachia*-infected host fitness and the
644 efficiency of maternal transmission (Turelli 1994). Understanding the *Wolbachia* effects that
645 lead to spread from low frequencies and the persistence of non-CI causing *Wolbachia* at
646 intermediate frequencies is crucial to explaining *Wolbachia* prevalence among insects and other
647 arthropods. The four-fold fecundity effect of *w*Mau reported by Fast et al. (2011) in *D.*
648 *mauritiana* is inconsistent with our experiments and with the intermediate infection frequencies
649 observed in nature. We find no *w*Mau effects on host fecundity, regardless of host background,
650 with our estimates of F having BCa intervals that include 1. Small increases in F could allow the
651 deterministic spread of *w*Mau from low frequencies, although detecting very small increases in F
652 is difficult (Supplemental Figure 1B). Our results are consistent with an earlier analysis that
653 assessed egg lay of a single genotype and found no effect of *w*Mau on host fecundity (Giordano
654 et al. 1995). When combined with the low observed infection frequencies, our fecundity data are
655 also consistent with our mathematical analyses indicating that *Wolbachia* can increase host
656 fitness by at most about 50% for reasonable estimates of μ. Because fecundity is one of many
657 fitness components, analysis of other candidate phenotypes for aiding the spread of low-
658 frequency *Wolbachia* is needed.
659
660 **Introgressive _Wolbachia_ transfer likely predominates in the _D. simulans_ clade**
661 Hybridization and introgression in the *D. simulans* clade may have led to introgressive transfer
662 of *Wolbachia* among host species (Rousset and Solignac 1995). This has been observed in other
663 *Drosophila* (Turelli et al. 2018; Cooper et al. 2019) and *Nasonia* wasps (Raychoudhury et al.
664 2009). The number of *Wolbachia* strains in the *D. simulans* clade, and the diversity of
665 mitochondria they co-occur with, is complex. Figure 5A shows host relationships and Figure 5B
666 shows mitochondrial relationships, with co-occurring *Wolbachia* variants in parentheses. While
667 *D. mauritiana* is singly infected by *w*Mau, *D. simulans* is infected by several strains, including
668 CI-causing *w*Ha and *w*No that often co-occur as double infections within individuals (O'Neill

669 and Karr 1990; Merçot et al. 1995; Rousset and Solignac 1995). *w*Ha and *w*No are similar to
670 *w*Sh and *w*Sn, respectively, that infect *D. sechellia* (Giordano et al. 1995; Rousset and Solignac
671 1995). *w*Ha and *w*Sh also occur as single infections in *D. simulans* and in *D. sechellia*,
672 respectively (Rousset and Solignac 1995). In contrast, *w*No almost always co-occurs with *w*Ha in
673 doubly infected *D. simulans* individuals (James et al. 2002), and *w*Sn seems to occur only with
674 *w*Sh (Rousset and Solignac 1995). *D. simulans* has three distinct mitochondrial haplotypes (*si*I,
675 *si*II, *si*III) associated with *w*Au/*w*Ri (*si*II), *w*Ha/*w*No (*si*I), and *w*Ma (*si*III). The *si*I haplotype is
676 closely related to the *se* haplotype found with *w*Sh and *w*Sn in *D. sechellia* (Ballard 2000b). *w*Ma
677 co-occurs with the *si*III haplotype, which differs over its 13 protein-coding genes by only a
678 single-base pair from the *ma*I mitochondrial haplotype carried by *w*Mau-infected *D. mauritiana*.
679 A second haplotype (*ma*II) is carried by only uninfected *D. mauritiana* (Ballard 2000a; James
680 and Ballard 2000).
681



682

683 **Figure 5. A)** A nuclear relative chronogram. **B)** A mitochondrial relative chronogram with co-
684 occurring *Wolbachia* strains listed in parentheses. See the text for an interpretation of the results,
685 including the artifactual resolution of the phylogeny of the *D. simulans* clade.
686

687

688 The lack of whole *w*Ma genome data precludes us from confidently resolving the mode of
689 *w*Mau acquisition in *D. mauritiana*. However, mitochondrial relationships support the proposal
690 of Ballard (2000b) that *D. mauritiana* acquired *w*Mau and the *ma*I mitochondrial haplotype via
691 introgression from *w*Ma-infected *D. simulans* carrying *si*III. *D. mauritiana* mitochondria are
692 paraphyletic relative to *D. sechellia* and *D. simulans* mitochondria (Solignac and Monnerot
693 1986; Satta and Takahata 1990; Ballard 2000a, 2000b), with *ma*I sister to *si*III and *ma*II outgroup
694 to all other *D. simulans*-clade haplotypes (see Figure 5). Of the nine genomes we assessed, all

695 but one (uninfected-*R44*) carry the *ma*I haplotype, and genotypes carrying *ma*I are both *w*Mau-
696 infected (*N* = 5) and uninfected (*N* = 3). While *w*Ma-infected *D. simulans* carry *si*III, *w*No-
697 infected *D. simulans* carry *si*I. We estimate that *w*Mau and *w*No diverged about 55,000 years
698 ago, with only 0.068% sequence divergence over 682,494 bp. Nevertheless, it seems implausible
699 that *w*No (versus *w*Ma) was transferred directly to *D. mauritiana* as this requires horizontal or
700 paternal transmission of *w*No into a *D. mauritiana* background already carrying the *ma*I
701 mitochondrial haplotype. Although our nuclear result suggests a confident phylogenetic
702 resolution of the *D. simulans* clade (Figure 5A), this is an artifact of the bifurcation structure
703 imposed by the phylogenetic analysis. Population genetic analyses show a complex history of
704 introgression and probable shared ancestral polymorphisms (Kliman et al. 2000) among these
705 three species. Consistent with this, of the 20 nuclear loci we examined, 6 (*aconitase, aldolase,*
706 *bicoid, ebony, enolase, ninaE*) supported *D. mauritiana* as the outgroup within the *D. simulans*
707 clade, 7 (*glyp, pepck, pgm, pic, ptc, transaldolase, wingless*) supported *D. sechellia* as the
708 outgroup, and 7 (*esc, g6pdh, glys, pgi, tpi, white, yellow*) supported *D. simulans*. With successive
709 invasions of the islands and purely allopatric speciation, we expect the outgroup to be the island
710 endemic that diverged first. Figure 5B indicates that the *ma*II haplotype diverged from the other
711 mtDNA haplotypes roughly when the clade diverged, with the other haplotypes subject to a
712 complex history of introgression and *Wolbachia*-associated sweeps, as described by Ballard
713 (2000b).
714 Ballard (2000b) estimated that *si*III-*ma*I diverged about 4,500 years ago, which presumably
715 approximates the date of the acquisition of *w*Mau (and *si*III, which became *ma*I) by *D.*
716 *mauritiana*. This is surely many thousands of generations given previous estimates that consider
717 the temperature dependence of *Drosophila* development (Cooper et al. 2014; Cooper et al. 2018).
718 As shown by our mathematical analyses (Eq. 3), the apparent persistence of the *ma*II mtDNA
719 among *Wolbachia*-uninfected *D. mauritiana*—without its occurrence among infected
720 individuals—is unexpected. More extensive sampling of natural *D. mauritiana* populations is
721 needed to see if this unexpected pattern persists. The persistence of this haplotype is inconsistent
722 with simple null models, possibly indicating interesting fitness effects.
723 While paternal transmission has been observed in *D. simulans* (Hoffmann and Turelli 1988;
724 Turelli and Hoffmann 1995), it seems to be very rare (Richardson et al. 2012; Turelli et al.
725 2018). *w*No almost always occurs in *D. simulans* individuals also infected with *w*Ha,

726 complicating this scenario further. It is possible that horizontal or paternal transmission of *w*Ma
727 or *w*No between *D. simulans* backgrounds carrying different mitochondrial haplotypes underlies
728 the similarities of these strains within *D. simulans*, despite their co-occurrence with distinct
729 mitochondria. Given the diversity of *Wolbachia* that infect *D. simulans*-clade hosts, and known
730 patterns of hybridization and introgression among hosts (Garrigan et al. 2012; Brand et al. 2013;
731 Garrigan et al. 2014; Matute and Ayroles 2014; Schrider et al. 2018), determining relationships
732 among these *Wolbachia* and how *D. mauritiana* acquired *w*Mau will require detailed
733 phylogenomic analysis of nuclear, mitochondrial, and *Wolbachia* genomes in the *D. simulans*
734 clade.
735
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737 MM performed the molecular and phenotypic work, participated in the design of the study, and
738 contributed to the writing; WC performed the phylogenetic and genomic analyses and
739 contributed to the writing; SR contributed to the molecular and phenotypic analyses and to the
740 writing; JB performed the library preparation and contributed to the writing; MT contributed to
741 the analyses, data interpretation, and writing; BSC designed and coordinated the study,
742 contributed to the analyses and data interpretation, and drafted the manuscript. All authors gave
743 final approval for publication.
744
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1112 **Appendix 1. Mathematical analyses of mtDNA and *Wolbachia* dynamics**

1113 Our analysis follows the framework developed in Turelli et al. (1992), but is simplified by the
1114 lack of CI. We suppose that introgression introduces a cytoplasm carrying *Wolbachia* and a
1115 novel mtDNA haplotype, denoted B. Before *Wolbachia* introduction, we assume the population
1116 is monomorphic for mtDNA haplotype A. With imperfect maternal *Wolbachia* transmission,
1117 uninfected individuals will be produced with mtDNA haplotype B. Without horizontal or
1118 paternal transmission (which are very rare, Turelli et al. 2018), all *Wolbachia*-infected
1119 individuals will carry mtDNA haplotype B. Once *Wolbachia* is introduced, uninfected
1120 individuals can have mtDNA haplotype A or B. We assume that these three cytoplasmic types
1121 ("cytotypes") differ only in fecundity, and denote their respective fecundities F_{I}, F_{A} and F_{B}.
1122 Denote the frequencies of the three cytotypes among adults in generation t by $p_{\mathrm{I,t}}$, $p_{\mathrm{A,t}}$ and $p_{\mathrm{B,t}}$,
1123 with $p_{\mathrm{I,t}} + p_{\mathrm{A,t}} + p_{\mathrm{B,t}} = 1$. Without CI, the frequency dynamics are

1124

1125 $$p_{\mathrm{I,t+1}} = \frac{p_{\mathrm{I,t}} F_{\mathrm{I}}(1-\mu)}{\overline{F}},\ p_{\mathrm{A,t+1}} = \frac{p_{\mathrm{A,t}} F_{\mathrm{A}}}{\overline{F}},\ \text{and}\ p_{\mathrm{B,t+1}} = \frac{p_{\mathrm{B,t}} F_{\mathrm{B}} + p_{\mathrm{I,t}} \mu F_{\mathrm{I}}}{\overline{F}},\ \text{with} \qquad \text{(A1)}$$

1126

1127 $$\overline{F} = F_{\mathrm{I}} p_{\mathrm{I,t}} + F_{\mathrm{A}} p_{\mathrm{A,t}} + F_{\mathrm{B}} p_{\mathrm{B,t}}. \qquad \text{(A2)}$$

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1129 If the uninfected population is initially monomorphic for mtDNA haplotype A, the *Wolbachia*
1130 infection frequency will increase when rare if and only if

1131

1132 $$F_{\mathrm{I}}(1-\mu) > F_{\mathrm{A}}. \qquad \text{(A3)}$$

1133

1134 Turelli et al. (1992) showed that if a CI-causing *Wolbachia* is introduced with a cytoplasm
1135 that contains a novel mtDNA haplotype B, which has no effect on fitness, *Wolbachia*-uninfected
1136 individuals will eventually all carry haplotype B. This follows because eventually all uninfected
1137 individuals have *Wolbachia*-infected maternal ancestors. This remains true for non-CI-causing
1138 *Wolbachia* that satisfy (A3). However, we conjectured that if the introduced B mtDNA is
1139 deleterious in the new host nuclear background, i.e., $F_{\mathrm{A}} > F_{\mathrm{B}}$, a stable polymorphism might be
1140 maintained for the alternative mtDNA haplotypes. The motivation was that imperfect maternal
1141 transmission seemed analogous to migration introducing a deleterious allele into an "island" of
1142 uninfected individuals. To refute this conjecture, consider the equilibria of (A1) with

1143

1144 $$F_{\mathrm{I}} > F_{\mathrm{A}} \geq F_{\mathrm{B}}. \quad \text{(A4)}$$

1145

1146 If all three cytotypes are to be stably maintained, we expect each to increase in frequency when
1147 rare. In particular, we expect the fitness-enhancing mtDNA haplotype A to increase when the
1148 population contains only infected individuals and uninfected individuals carrying the deleterious
1149 *Wolbachia*-associated mtDNA haplotype B. From (A1), $p_{\mathrm{A,t}}$ increases when rare if and only if

1150

1151 $$F_{\mathrm{A}} > \overline{F} = F_{\mathrm{I}} p_{\mathrm{I,t}} + F_{\mathrm{B}}(1 - p_{\mathrm{I,t}}). \quad \text{(A5)}$$

1152

1153 In the absence of haplotype A, we expect p_{I} to be at equilibrium between selection and imperfect
1154 maternal transmission, i.e.,

1155

1156 $$p_{\mathrm{I}} = 1 - \frac{\mu F}{F - 1}, \quad \text{(A6)}$$

1157

1158 with $F = F_{\mathrm{I}}/F_{\mathrm{B}}$ (Hoffmann and Turelli 1997). Substituting (A6) into (A5) and simplifying, the
1159 condition for $p_{\mathrm{A,t}}$ to increase when rare is

1160

1161 $$F_{\mathrm{A}}(F_{\mathrm{I}} - F_{\mathrm{B}}) > F_{\mathrm{I}}(1 - \mu)(F_{\mathrm{I}} - F_{\mathrm{B}}). \quad \text{(A7)}$$

1162

1163 By assumption (A4), $F_{\mathrm{I}} > F_{\mathrm{B}}$; hence (A7) contradicts condition (A3), required for initial
1164 *Wolbachia* invasion. Thus, simple selection on *Wolbachia*-uninfected mtDNA haplotypes cannot
1165 stably maintain an mtDNA polymorphism. The "ancestral" mtDNA haplotype A is expected to
1166 be replaced by the less-fit *Wolbachia*-associated haplotype B.

1167 To understand the time scale over which this replacement occurs, let r_{t} denote the frequency
1168 of haplotype A among *Wolbachia*-uninfected individuals, i.e., $r_{\mathrm{t}} = p_{\mathrm{A,t}}/(p_{\mathrm{A,t}} + p_{\mathrm{B,t}})$. From (A1),

1169

1170 $$r_{\mathrm{t+1}} = \frac{r_{\mathrm{t}} F_{\mathrm{A}}}{r_{\mathrm{t}} F_{\mathrm{A}} + (1 - r_{\mathrm{t}}) F_{\mathrm{B}} + \mu F_{\mathrm{I}} [p_{\mathrm{I,t}}/(1 - p_{\mathrm{I,t}})]}. \quad \text{(A8)}$$

1171

1172 If we assume that the mtDNA haplotypes do not affect fitness, i.e., $F_A = F_B$, and that the
1173 *Wolbachia* infection frequency has reached the equilibrium described by (A6), (A8) reduces to
1174
1175 $$r_{t+1} = r_t/[F(1 - \mu)], \quad \text{(A9)}$$
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1177 with $F = F_I/F_B$.
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1203 **SUPPLEMENTAL INFORMATION**

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1205 **Supplemental Tables**

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Supplemental Table 1. *w*Mau assembly statistics.

Genotype	Scaffold count	N50 (bp)	Longest scaffold (bp)	Total length (bp)
R9	36	60,027	169,305	1,266,004
R29	36	61,106	169,295	1,277,467
R31	39	63,676	169,381	1,272,847
R41	42	61,106	170,537	1,303,156
R60	38	63,156	221,751	1,282,564

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Supplemental Table 2. Near-universal, single-copy proteobacteria genes (out of 221) found using BUSCO v. 3.0.0.

Genome	Complete	Duplicated	Fragment	Absent
*w*Ri	179	1	2	39
*w*Mel	179	1	2	39
*w*Au	180	1	2	38
*w*Ha	178	1	3	39
*w*No	180	1	4	36
R9	180	1	4	36
R29	180	1	4	36
R31	180	1	4	36
R41	180	1	4	36
R60	180	1	4	36

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Supplemental Table 3. Copy number variants in *w*Mau relative to sister *w*No. Genomic positions are based on the *w*No reference. There were no CNVs among *w*Mau variants.

Start	End	Change	Wilcoxon Rank Sum Test	Kolmogorov-Smirnov Test
115,000	142,000	1 → 0	< 0.0001	< 0.0001
590,000	593,000	1 → 0	0.0027	0.0050
982,000	991,000	1 → 0	< 0.0001	< 0.0001
1,195,000	1,199,000	1 → 0	0.0005	0.0007

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Supplemental Table 4: Genes present in regions deleted in *w*Mau relative to *w*No. Genes predicted to be pseudogenized in *w*No are shaded grey.

Accession number	Name
Deletion 1 (115,000-142,000):	
wNO_RS00550	Hypothetical protein
wNO_RS06015	Ankyrin repeat domain protein
wNO_RS00560	Pseudo IS256 family transposase, frameshifted
wNO_RS00565	Recombinase family protein
wNO_RS00570	DUF2924 domain-containing protein
wNO_RS00575	Ankyrin repeat domain-containing protein
wNO_RS00580	Ankyrin repeat domain-containing protein
wNO_RS00585	Phage tail protein
wNO_RS00590	Baseplate assembly protein GpJ
wNO_RS00595	Pseudo baseplate assembly protein W, frameshifted
wNO_RS00600	Hypothetical protein
wNO_RS00605	Phage baseplate assembly protein V
wNO_RS00610	Hypothetical protein
wNO_RS00615	Putative minor tail protein Z
wNO_RS00620	Hypothetical protein
wNO_RS00625	Minor capsid protein E
wNO_RS00630	Head decoration protein
wNO_RS00635	S49 family peptidase

wNO_RS00640	Pseudo phage portal protein, frameshifted
wNO_RS00645	Phage head stabilizing protein GpW
wNO_RS00650	Phage terminase large subunit family protein
wNO_RS00655	Ankyrin repeat domain-containing protein
wNO_RS00660	Hypothetical protein
wNO_RS00665	Hypothetical protein
wNO_RS00670	Sigma-70 family RNA polymerase sigma factor
wNO_RS00675	ATP-binding protein
wNO_RS00680	IS110 family transposase
Deletion 2 (590,000-593,000):	
wNO_RS02645	XRE family transcriptional regulator
wNO_RS02650	Hypothetical protein
wNO_RS02655	Hypothetical protein
wNO_RS02660	XRE family transcriptional regulator
Deletion 3 (982,000-991,000):	
wNO_RS04690	Group II intron reverse transcriptase/maturase
wNO_RS06355	Hypothetical protein
wNO_RS04695	Pseudo hypothetical protein, partial
wNO_RS04700	Pseudo cell filamentation protein Fic, partial
wNO_RS04705	Hypothetical protein
wNO_RS04710	DNA methylase
wNO_RS04715	Hypothetical protein
wNO_RS04720	Ankyrin repeat domain-containing protein
wNO_RS04725	Phage terminase large subunit family protein
wNO_RS04730	Phage head stabilizing protein GpW
wNO_RS04735	DUF1016 domain-containing protein
Deletion 4 (1,195,000-1,199,000):	
wNO_RS06095	Ankyrin repeat domain protein
wNO_RS05590	Rpn family recombination-promoting nuclease/putative transposase

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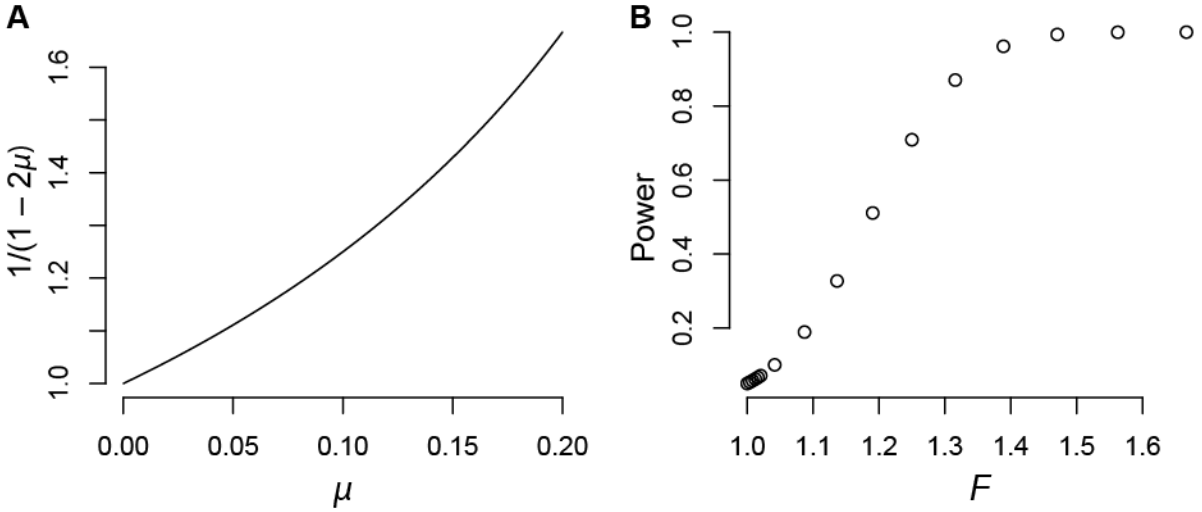
Supplemental Table 5. *w*Mau does not significantly affect *D. mauritiana* fecundity in comparisons of paired infected (I) and uninfected (U) strains sharing host nuclear backgrounds. *N* is the number of females that produced the means and SDs. *P* values are for two-tailed Wilcoxon tests. Cytoplasm sources are denoted with superscripts for introgressed strains.

Strain	Mean eggs laid/5 days	SD	*N*	*P* value
R31I	210.97	55.22	30	0.901
R31U	213.50	58.43	28	
R41I	265.43	31.47	30	0.355
R41U	239.69	73.43	29	
$R31^{R41}I$	257.83	58.15	30	0.762
$R31^{R41}U$	254.67	48.72	30	
$R41^{R31}I$	248.77	45.54	30	0.433
$R41^{R31}U$	250.67	76.35	30	

Supplemental Figures



Supplemental Figure 1. A) Our proposed upper bound, $1/(1 - 2\mu)$, on plausible relative fitness, F, as a function of μ ranging from perfect transmission ($\mu = 0$) to a level of imperfect transmission ($\mu = 0.2$) that has not been observed in nature. B) Our powers to detect values of F with $\alpha = 0.05$.